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Aspects of inflammation and nitric oxide in Cluster Headache

Anna Steinberg

From the Department of Clinical Neuroscience,
Karolinska University Hospital,
Karolinska Institutet, Stockholm, Sweden

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Karolinska Institutet

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To my family

CONTENTS

ABSTRACT

Cluster Headache (CH) is an uncommon headache disorder, with severe implications for the individual patient. The headache is excruciating, unilateral and appearing in attacks. It is common that CH patients show ipsilateral associated symptoms, like for example conjunctival injection, lacrimation and nasal congestion. The pathophysiology of CH is still not completely understood. The overall objective of this thesis was to explore if inflammation and nitric oxide participate in the pathophysiology of CH.

Study I

The aim of study I was to identify differentially expressed genes during clinical phases of CH, assuming that changes of pathophysiological importance would also be observed in peripheral venous blood. Blood samples were drawn at 3 consecutive occasions from 3 episodic CH patients: during attacks, between attacks and in remission, and at 1 occasion from 3 matched controls. Global gene expression was analyzed with microarray tehnology using the Affymetrix Human Genome U133 2.0 Plus GeneChip® Set. In addition, quantitative RT-PCR on S100P gene expression was analyzed in 6 patients and 14 controls. Small differences were seen intraindividually and large differences interindividually. Intraindividual comparisons showed upregulation of several S100 calcium binding proteins; S100A8 (calgranulin A), S100A12 (calgranulin C), and S100P during active phase of the disease compared to remission. The S100A8 and S100A12 proteins are considered markers of non-infectious inflammatory disease, while increased levels of S100P have been associated to different forms of cancer. RT-PCR analysis of S100P confirmed the Affymetrix´ results.

Study II

We investigated the cytokine interleukin-2 (IL-2) as a possible marker of immune system involvement in the pathophysiology of CH. Eight episodic CH patients and 16 healthy headache-free control subjects matched for age and gender were studied. Venous blood samples were drawn from the CH patients at three occasions; during active period between headache attacks, during attack and in remission. Venous blood samples were drawn once from each control subject. We analyzed IL-2 gene expression, using quantitative real-time polymerase chain reaction (RT-PCR). Patients with CH had significantly increased relative IL-2 gene expression levels during active period between headache attacks compared to during attacks, remission and controls.

Study III

In this study we have investigated white blood cell accumulation into potential inflammatory areas intracranially in 14 CH patients, both in active period and in remission, and 5 control subjects, with single photon emission computer tomography (WBC-SPECT). To enable precise definition of regions of interest (ROI:s) in the brain, all CH patients and control subjects also underwent magnetic resonance imaging (MRI) of the brain. We found no statistically significant difference in ^{99m}Tc-labeled WBC uptake between CH patients in active period and controls. Furthermore CH patients in active period were not significantly different in uptake compared to CH patients in remission.

Study IV

We investigated the role of nitric oxide (NO) in CH, by measuring its oxidation products, nitrite and nitrate, in the cerebrospinal fluid (CSF). We collected CSF from 14 episodic CH patients. Lumbar puncture was performed at two occasions: in active period between headache attacks, and in remission, not earlier than three weeks after their last headache attack. Eleven healthy volunteers served as controls. To estimate NO production, we determined the levels of NO-oxidation end products (NOx), that is, the sum of nitrite and nitrate, by using capillary electrophoresis (CE). CH patients in active period had significantly increased NOx levels compared with those in remission and control subjects. CH patients had also significantly enhanced NOx levels in remission compared to control subjects.

Keywords: Cluster Headache, gene expression, inflammation, WBC-SPECT, nitric oxide.

LIST OF PUBLICATIONS

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Gene Expression Profiling in Cluster Headache: A Pilot Microarray Study.
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Acta Neurol Scand. 2010 Sep 29. doi: 10.1111/j.1600-0404.2010.01434.x. [Epub ahead of print]

III. Anna Steinberg, Rimma Axelsson, Lars Ideström, Susanne Müller, Ingela Nilsson Remahl.
White blood cell SPECT during active period of Cluster Headache and in remission.
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IV. Anna Steinberg, N Peter Wiklund, Lou Brundin, Ingela Nilsson Remahl.
Levels of nitric oxide metabolites in cerebrospinal fluid in cluster headache.
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ABBREVIATIONS

BBB	Blood brain barrier
CE	Capillary electrophoresis
CGRP	Calcitonin gene related peptide
CH	Cluster Headache
CSF	Cerebrospinal fluid
DBS	Deep brain stimulation
GCA	Giant cell arthritis
GCOS	Gene Chip® Operating Software (Affymetrix)
IL-2	Interleukin-2
IQR	Interquartile range
LP	Lumbar puncture
MM	Mismatch
MRI	Magnetic resonance imaging
MP-RAGE	Magnetization-prepared rapid gradient echo
NO	Nitric oxide
NOS	Nitric oxide synthases
eNOS	Endothelial NOS
iNOS	Inducible NOS
nNOS	Neuronal NOS
NOx	NO-oxidation end products; nitrite and nitrate
ONS	Occipital nerve stimulation
PET	Positron emission tomography
PM	Perfect Match
ROI	Region of interest
Q-RT-PCR	Quantitative Real-time polymerase chain reaction
^{99m}TC-LABELED WBC	^{99m}Tecnetium-labeled white blood cells
TNC	Trigeminal nucleus caudalis
TVS	Trigeminovascular system
WBC-SPECT	White blood cell single photon emission computer tomography
VIP	Vasoactive intestinal polypeptide

INTRODUCTION

Clinical description of cluster headache (CH)

Cluster Headache is an extremely painful headache disorder characterized by periods (clusters) of recurrent, unilateral attacks of excruciating pain with a retro-orbital maximum. The attack duration varies from 15 - 180 minutes[1]. CH usually appears between 20 and 65 years of age[2] and during active phase the attacks occur from once every second day to 8 times a day. Most patients show ipsilateral symptoms; conjunctival injection, lacrimation, nasal congestion, rhinorrhoea, eyelid oedema, forehead/facial sweating, miosis and/or ptosis, indicating ipsilateral autonomic dysfunction. Episodic CH occurs in periods lasting between 7 days to 1 year, separated by pain-free periods, lasting 1 month or longer. About 10 % of the patients develop chronic CH without remission periods[1].

Epidemiology

An established estimation of the prevalence of CH, i.e. the proportion of the population with current CH, is almost 0.1%, (70-90/100.000)[2]. A similar prevalence was reported in a survey of 18-year old Swedish men, 0.09%[3] and in an Italian study, around 0.07% (56-69 per 100.000)[4] .

A *lifetime* prevalence, i.e. the proportion of the population with previous or current CH, of 0.38%, was found in a study from Vågå, Norway, in which the diagnosis was made by face-to-face interviews[5]. These higher prevalence data are supported by the result from the Swedish twin registry, showing a lifetime prevalence of 0.2%[6]. CH is rare in children[2].

CH is more common in men. Earlier studies have shown a male: female ratio 5-6:1[5, 7], but over the last years this dominance has been reported to be less prominent, 2.5-3.5:1[8]. This change may be caused by a higher observance for the disorder in women, life style changes, or unknown factors.

Genetic aspects

Until recently CH was regarded as a sporadic rather than an inherited disorder. Data from family and twin studies have suggested a heritable component, and it has been estimated that first-degree relatives of CH patients have a 5-18 times and second-degree relatives 1-3 times higher risk to develop CH than the general population[8-10]. The variation of the reported risk can partly be explained by methodological differences. Studies from segregation analysis have suggested than an autosomal dominant gene with incomplete penetrance occur in some families and an autosomal recessive inheritance in others[11].

The calcium channel gene (CACNA1A) was in focus for genetic research in neurological diseases around ten years ago, because of mutations in the gene found to be responsible for several episodic neurologic disorders, e.g. familial hemiplegic migraine. The CACNA1A gene has also been studied in CH, but has been judged to be of minor importance[12, 13].

The role of NO in CH pathophysiology has been extensively discussed. In an association study of 91 CH patients and 111 matched controls, polymorphism of three NOS genes (nNOS, iNOS, and eNOS) were studied. No significant genetic differences between patients and controls regarding NOS were found[14].

PET studies have established that hypothalamus has a pivotal role in CH[15]. As a consequence, genes involved in regulation of the circadian rhythm have been of interest. The hypocretin neuropeptides, hypocretin-1(orexin-A) and hypocretin-2 (orexin-B), are expressed in a small population of neurons in the lateral hypothalamus and project into all brain regions. They interact with two receptors; HCRTR1 and HCRTR2[8, 9]. A positive association study was published in 2004, when a highly significant association between the 1246G>A polymorphism of the HCRTR2 gene and CH was found[8, 9]. Thereafter several

genetic studies have been performed regarding the HCRTR2 gene, with divergent results. Two of these studies indicate an association between the HCRTR2 gene and CH[16, 17], while one did not[18].

Pathophysiology

The pathophysiology of CH is largely unknown, but the discussion has mainly been focused around six mechanisms; vasodilation[19, 20], inflammation [21, 22], hypothalamic dysfunction[15, 23], trigeminovascular system activation[24], autonomic system imbalance[24, 25] and increase of nitric oxide levels[26, 27].

Vasodilation

The ipsilateral ophthalmic artery is shown to be markedly dilated during CH attacks[20], although dilation of cranial vessels is regarded to be unspecific and observed in many different headache syndromes[19]. Pain inducing capsaicin injection into the forehead, in headache-free volunteers, caused dilation of cranial vessels, probably mediated by the trigeminoparasympathetic reflex[19].

Early observations of precipitation of CH by vasodilators such as alcohol, histamine[28-30] and nitroglycerine[31] suggested that vasodilation may be essential for the generation of the pain during attacks. Subsequently, cerebral blood flow studies[32, 33] were unable to support a primary role of vasodilation in CH[19], although vasodilation may be involved as a pathophysiological step towards generation of pain. Critical steps may be activation of the trigeminal vascular system leading to vasodilation, protein extravasation, neurogenic inflammation and sensitizing of meningeal receptors[24, 34].

Inflammation

Inflammation is characterized by five cardinal signs; redness, heat, swelling, pain and loss of function. Marked vascular changes, including vasodilation and increased permeability, which are induced by the actions of various inflammatory mediators, make it possible for WBC to migrate out into the tissue[35].

In the clinical routine care corticosteroids usually have a positive effect when CH patients suffer from frequent CH attacks, suggesting a role for immunological mechanisms in the disease. Earlier studies on inflammatory mechanisms have shown partially conflicting results. Transient recurrent venous vasculitis in the cavernous sinus has been suggested by some authors [22,36]. One study of episodic CH patients showed neither clinical symptoms nor changes in routine laboratory tests in support of a systematic inflammatory disorder[37]. Another study showed a trend of an immune response that reacted differently than healthy volunteers and patients with active biopsy-verified giant cell arthritis (GCA). Pro-inflammatory adhesion molecules appeared down-regulated during active CH period and up-regulated during remission, while a reverse pattern was observed among patients with GCA[38]. Several other studies have also shown changes in immune competent factors[21, 39, 40].

Potential inflammatory involvement in CH has been further supported by imaging studies. Gallium-67 citrate is picked up in areas of inflammation, and Gallium SPECT has shown a lesion in the region of the cavernous sinus, which fades as the patient moves out of cluster[41]. Another Gallium SPECT study, however, has shown that increased parasellar activity on Gallium SPECT was neither specific for CH, nor for the active period of episodic CH[42].

Inflammation is normally accompanied by the extravasation of plasma proteins into the surrounding tissue. The hypothesis of a sterile inflammation in the cavernous sinus of CH patients was first tested in year 2000 with radiolabelled human serum albumin (^{99m}Tc-HSA) uptake and SPECT/MRI by Goebel et al [43] who reported albumin leakage into the cavernous sinus ipsilaterally to the pain in CH patients during active CH period. A subsequent study by Schuh-Hofer et al in 2006, however, using the same technique, were

unable to confirm these findings, since no statistically significant difference was seen between the ^{99m}Tc-HSA uptake in the ipsilateral cavernous sinus before and after induction of a CH attack and there was no evidence of ^{99m}Tc-HSA extravasation in the cavernous sinus in active period compared with remission phase [44].

The role of neurogenic inflammation by release of vasoactive peptides, such as calcitonin gene related peptide (CGRP) and substance P, from trigeminal fibers has received increased attention over the years[24, 25, 45].

Hypothalamic activation

CH attacks tend to occur with circadian rhythmicity; consequently hypothalamus is thought to be involved in CH pathophysiology, being regarded as the place of the “biological clock”. Hormonal studies have shown alterations in plasma levels of melatonin, cortisol, testosterone, prolactine, growth hormone, gonadotrophins and thyrotropins in CH patients compared with controls, indicating that hypothalamus is involved in CH[23, 46-48]. PET-studies have shown activation of hypothalamic ipsilateral grey matter in patients who had acute attacks, which has been interpreted as a dysfunction in hypothalamus in CH patients [15]. Morphometric studies using functional MRI have also observed an increased volume of the grey matter of the corresponding area in hypothalamus[49].

Trigeminovascular system activation

The trigeminovascular system (TVS) includes neurons located within the trigeminal ganglion that projects both peripherally and centrally. At the peripheral level, the trigeminal nerve innervates pain-sensitive supratentorial structures such as main arteries and dura mater. Centrally projecting fibers synapse in the brain stem, principally in the trigeminal nucleus caudalis (TNC). In migraine it is hypothesized that dysfunction in the brainstem may cause dilation of arteries

innervated by the trigeminal nerve. This might lead to plasma protein extravasation, neurogenic inflammation and sensitizing of meningeal receptors. Subsequently, a trigeminal sensory pain response will be activated, in which signals are transferred to the brainstem, and further on to thalamus and the cortical areas, resulting in the experience of pain. A release of vasoactive peptides, such as calcitonin gene related peptide (CGRP) and substance P, from trigeminal fibers accelerates the neurogenic inflammation and vasodilation [50, 51]. For CH the principal TVS mechanism might be similar to that in migraine, with the exception that the "start focus" probably is situated in the hypothalamus. CGRP is a marker of the TVS and increased levels have been found in blood from the external jugular vein during CH attacks, which the authors conclude as an evidence for activation of the TVS [24].

Autonomic nervous system imbalance

During CH attacks most patients suffer from autonomic symptoms such as ipsilateral lacrimation, conjunctival injection, nasal congestion, rhinorrhoea, miosis and ptosis.

Ipsilateral lacrimation, conjunctival injection, nasal congestion and rhinorrhoea are caused by activation of the cranial parasympathetic outflow from the seventh cranial nerve[25]. There is also evidence that the severity of pain is correlated with the autonomic symptoms and the pain itself may trigger these symptoms[52]. Markedly raised levels of vasoactive intestinal polypeptide (VIP) have been measured during spontaneous CH attacks[24]. These findings were considered as evidence of involvement of the cranial parasympathetic nervous system. Sympathetic impairment with ipsilateral miosis and ptosis are also common, and believed to be due to transient injury of the postganglionic fibres[25]. Some authors have suggested that the sympathetic symptoms may be caused by periorbital venous vasculitis, compromising the carotid canal and consequently the transversing sympathetic fibers[22, 36]. The autonomic dysregulation in CH might also originate centrally in association with a hypothalamic disturbance[53, 54].

Increase of Nitric oxide levels

The role of nitric oxide (NO) in CH has been discussed over the latest years. NO is synthesized from L-arginine by a family of enzymes, the nitric oxide synthases (NOS). NO diffuses freely across membranes[26] and has multiple functions in the body. For example NO prevent platelet activation, inhibit monocyte adhesion and leucocyte function. Further on it is an important neurotransmitter and the L-arginine NO-pathway regulates the vascular tone acting as a potent vasodilator[55]. NO diffuses into vascular smooth muscle cells and causes vasodilation[56]. Apart from regulating blood flow, NO is thought to play a role in processing sensory information[57, 58] and pain sensitisation[59]. In the trigeminal ganglion, CGRP and nitric oxide synthases (NOS) co-localize in many neurons[60]. It has been suggested that NO causes release of CGRP[61], but other studies have not confirmed this result[62, 63]. It is also possible that CGRP is causing release of NO. In the human forearm, CGRP-induced dilation was partly inhibited by a NOS inhibitor[64]. Increased NO levels may also be an expression of enhanced inflammatory activity[65-67].

D'Amico et al. demonstrated enhanced nitrite-levels in plasma among CH patients, both in active period and in remission[26]. The authors suggested that a basal hyperfunction of the L-arginine-NO pathway may occur in both phases of CH, while a later study was unable to confirm those results[56].

TREATMENT

Since the CH attacks are extremely painful there is usually a need for both acute and prophylactic medication. The utmost aim is to get the patient attack-free during a bout of cluster attacks by prophylactic medication. This is difficult, since there is no one solution that fits all. Some patients even choose to be without prophylactic medication, if the CH attacks are sparse, or because of side effects.

Acute treatment

Because of the rapid onset, the severe intensity of the CH attacks and often short attack duration, fast acting acute therapy is needed. The acute treatment includes primarily sumatriptan injection, triptan nasal sprays or 100 % oxygen inhalation. The most effective acute treatments are sumatriptan injection [68]. In a double-blind, placebo-controlled trial, the 5-$HT_{1B/1D}$ receptor agonist sumatriptan injected subcutaneously was effective in about 75 % of all CH patients, i.e. patients were pain-free within 20 minutes[69, 70].

Triptan nasal sprays have a slower time of onset, than sumatriptan injection. Nevertheless many CH patients use triptan nasal sprays since they wish to avoid injections. Orally administered acute medications tend in most cases to be ineffective, since they do not give effect fast enough. The mechanism of action for triptans has been related to inhibition of CGRP release from trigeminal neurons in vitro[62, 71] and inhibition of CGRP induced vasodilation in vivo[74]. Contraindications to sumatriptan and other triptans are cardio- and cerebrovascular disorders and untreated arterial hypertension.

In CH, oxygen treatment has an obvious role, since it can safely be used in patients with cardio- and cerebrovascular disorders. Oxygen inhalation has no known side-effects. Disadvantage of oxygen treatment, however, are that the effect is moderate particularly for severe attacks and patients must have access

to the oxygen cylinder and regulator, which is often impractical. In a smaller study, CH patients were treated with 100% oxygen and were released from cluster pain in 75% within 15 minutes[72]. The results were later confirmed in a double-blind, randomized, placebo-controlled crossover trial[73]. The mechanism of oxygen treatment is regarded as a vasoconstrictor effect[74, 75].

Prophylactic treatment

Among several options for prophylactic treatment verapamil is generally accepted as the first choice[76]. Around 70% of CH patients have satisfactory effect of verapamil, with an individual improvement of more than 75% [77]. CH patients often need high verapamil doses compared to other indications, probably because the cardiovascular effects are directly related to blood levels of verapamil, whereas the effect on CH takes place across the blood brain barrier (BBB)[78]. Verapamil easily crosses the BBB, although a P-glycoprotein restricts the brain uptake of verapamil by transporting it out of the brain[78]. An ECG is required before start of treatment, since there is a risk for development of atrioventricular block and symptomatic bradycardia in susceptible patients[79]. Verapamil is a calcium channel blocker, but the mechanism of action in CH is not clarified. A calcium channelopathy has been proposed to be a part of CH pathophysiology. Although verapamil is described as an L-type calcium blocker, the drug has possible effects on a range of other calcium channels, probably present in hypothalamus, the supposed centre of origin of CH pathophysiology[78]. Of the three isoforms of NO-synthases (NOS), nNOS, eNOS and iNOS, the activities of the first two are regulated by calcium-dependent influx into the cells[80]. In contrast, iNOS is calcium-independent, and the enzyme is not expressed unless the cells have been stimulated for example by certain cytokines like interferon-gamma and interleukin-1[81]. A potential mechanism of action of verapamil would be inhibition of the calcium-dependent isoforms nNOS and eNOS which would support a pathophysiological role of nitric oxide in CH.

When the CH attacks appear extremely often (5-8 times/day), the pain situation is intolerable. In this situation steroid medication is normally preferred, in order to achieve a rapid control of attacks. Usually 500 mg metylprednisone is

given intravenously over 3-5 days, followed by oral prednisone during 2-3 weeks. It is necessary to keep the steroid treatment short to avoid side-effects [68].

Lithium carbonate is effective as a prophylactic drug, particularly for chronic cases, but has side-effects as hypothyroidism, tremor and renal dysfunction. Therefore, close control is needed to measure plasma lithium, renal and thyroid function[68].

Other prophylactic drugs are pizotifen, methyserigide and anti-epileptic drugs for example topiramate and valproate.

Surgical treatment

Surgical treatment should be considered with caution, because no long-term observational data exist yet. For intractable CH when all medical therapy has failed, surgery might be an alternative. In some cases, blockade of the occipital nerve is effective and occipital nerve stimulation (ONS) could be a choice. The mechanisms of action for ONS are not clear, but PET studies have shown effects on central pain-modulating centres [82]. ONS may also have direct impact on nociceptive neuronal activity in the trigeminal nucleus caudalis [83]. Deep brain stimulation (DBS) of the posterior inferior hypothalamus has also been shown to be effective in nearly 60% of medically refractory chronic cluster headache patients[84]

AIMS

The overall objective of this thesis was to explore the participation of inflammation and nitric oxide in the pathophysiology of CH.

Based on this, the specific objectives were:

- To identify differentially expressed genes during different clinical phases of CH.
- To investigate cytokine interleukin-2 (IL-2) as a possible marker of immune system involvement in the pathophysiology of CH.
- To investigate if CH patients show signs of intracranial inflammation, with recruitment of white blood cells from the peripheral blood, when using white blood cell single photon emission computer tomography (WBC-SPECT).
- To investigate nitric oxide (NO) in CH by measuring its stable oxidation end products (NOx), nitrite and nitrate, in the cerebrospinal fluid (CSF).

MATERIALS AND METHODS

Patients and controls

Only patients with episodic CH according to the IHS criteria[1] in active period were included in the studies. Episodic CH is defined as attacks occurring in periods lasting 7 days to 1 year separated by pain-free periods lasting 1 month or longer. Chronic CH is defined as CH attacks occurring for more than 1 year without remission or with remissions lasting less than 1 month [1]. All patients were followed with several phone interviews during the studies to check their CH "status". This was important to enable separation of active period from remission, and to exclude patients who developed chronic CH. The phone calls were also a tool to prevent drop outs.

Patients were defined in all studies as in active phase if they had repeated attacks during the last 4 days, and as in remission if no attacks had occurred within at least three weeks, except for Study III where absence of attacks within at least two weeks were regarded as in remission. The reason for the shorter time frame in Study III was logistical difficulties in the planning of repeated SPECT examinations and the need to avoid drop outs. To be included in all studies the patients had to be free from prophylactic treatment for at least three days before the study started. Patients started to scale down the medication a week before study start.

All four studies had a longitudinal design, where the patients were examined in different phases of CH. Healthy controls were mainly members of the staff or other colleagues in study I, II and IV. In study III control subjects were recruited in connection with a routine SPECT examination, since the local Radiation Safety Committee would not admit healthy controls. The number of control subjects in study III was restricted due to the fact that almost all patients who came for routine SPECT had anti-inflammatory medication or antibiotics.

All studies were approved by the regional Ethics Committee and all patients provided their informed consent.

The patient characteristics are listed in Figure 1.

Patient No	Gender	Age at inclusion	Prophylaxis before inclusion	Included in Study No
1	Male	46	No	I,II
2	Male	40	No	I,II
3	Male	38	Verapamil	I,II
4	Male	39	No	II,III,IV
5	Male	63	No	II
6	Female	54	No	II,IV
7	Male	39	No	II
8	Female	54	No	II
9	Female	33	Verapamil	III,IV
10	Male	50	No	III,IV
11	Male	48	No	III
12	Male	31	Pizotifen	III
13	Female	32	No	III
14	Male	68	Verapamil	III
15	Male	40	Verapamil	III
16	Male	63	No	III
17	Male	26	No	III
18	Male	26	No	III
19	Male	37	No	III
20	Male	37	Topiramate	III
21	Male	67	Verapamil	III
22	Male	51	No	IV
23	Male	56	No	IV
24	Female	37	Verapamil	IV
25	Female	53	Verapamil	IV
26	Male	46	No	IV
27	Male	38	No	IV
28	Male	49	No	IV
29	Male	46	No	IV
30	Male	42	Verapamil	IV
31	Male	63	No	IV

Figure 1: Patient characteristics

Inclusion criteria in Study I-IV

1. Episodic CH
2. Active CH period during inclusion
3. No prophylactic treatment for at least three days before study start
4. Compliance regarding treatment
5. Able to understand and follow instructions
6. Willing to attend complementary examinations during remission
7. Able to speak Swedish or English

Patients had to be excluded for the following reasons: demanding workload in combination with active, excruciating CH; inadequate compliance regarding medication or logistical requirements; or symptoms in spontaneous regress before study start. Other patients who were included could not bear to be without prophylactic treatment. It is notable that the attack frequency rose markedly when patients during active bouts were withdrawn from prophylactic treatment. Retired patients, patients who were on sick leave and patient with irregular working hours were easier to include than others. For inclusion in the studies, an active phase was required, although the number of attacks could vary. An important factor was logistic demands and the life around patients. It is notable that the dropout frequency was rather high (Study II: 4 patients. Study III: 11patients. Study IV: 8 patients). In study I there were no drop outs, but two patients were withdrawn due to poor RNA quality. The reasons for the dropouts varied, but a main problem was that patients did not shift into remission during the study time. Four patients in study IV, who developed postpunctional headache, did not want to go through a second LP in remission.

CH phases studied

1. Active phase between attacks - Study I-IV
2. Active phase during attacks - Study I – II
3. Remission - Study I-IV

Methods

1. *Study I:* Global gene expression in venous blood by use of PAXgene Blood RNA system, microarray and quantitative real-time polymerase chain reaction (RT-PCR) technologies.
2. *Study II:* Gene expression of Interleukin-2 (IL-2) in venous blood by use of PAXgene Blood RNA system and RT-PCR technology.
3. *Study III:* White blood cell uptake in defined regions of interest in the brain by the use of single photon emission computer tomography (WBC-SPECT) and neuro-navigation with MRI.
4. *Study IV:* Nitric oxide levels in cerebrospinal fluid by use of capillary electrophoresis.

The PAXgene Blood RNA system

Gene expression was studied in the first two publications. The physiological RNA instability results in rapid RNA degradation in blood samples[85, 86]. The PAXgene Blood RNA system is a method that preserves the RNA expression profile during and immediately after blood is drawn. The method is used for isolation of cellular RNA from whole blood. The stabilisation reagent contains a cationic detergent that is known to penetrate blood cells and interact with intracellular anionic species, including nucleic acids. Once blood is drawn into the PAXgene tube, the detergent rapidly lyses red blood cells and enters white blood cells and forms stable complexes. RNA is there for extracted from total

cells (i.e. both RBC and WBC)[87]. The cellular RNA profile may remain stable for up to 5 days at room temperature[86].

The influence of time on the quantity of complementary DNA (cDNA) using the PAXgene collection tubes was tested. Four blood samples from one single, healthy donor were collected into PAXgene tubes, and then left in room temperature for 2-12 hours before they were stored at 8°C until further processed. RNA was extracted according to the manufacturer's recommendations day 1, day 2, day 4 and day 5 after sampling. The extracted RNA from all samples was stored at - 70°C. The concentration of RNA was determined by ultraviolet absorbance at 260 nm. RNA was converted to cDNA using reverse transcriptase, since cDNA is more stable than RNA[88]. The quantity of mRNA expression was measured with real time quantitative polymerase chain reaction (RT-PCR) technique[89]. The test showed that the quantity of cDNA was maintained at an approximately constant level during 5 days following the blood sampling.

Microarray technology

This method has the ability to measure the level of expression for thousands of genes in one sample. The approach is screening rather than hypothesis driven. We have used Affymetrix-chip in study I (Affymetrix Human Genome U133 2.0 Plus GeneChip®). An Affymetrix-chip consist of 1.3 million[90] minimal squares, shared over an area of about 1 cm^2. Every square consist of different sets of probes. (A probe is a labeled bit of DNA/ RNA used to find its complementary sequence). The Affymetrix-chip is constructed of single-stranded 25 base pairs oligonucleotides synthesized for hybridization to complementary single-stranded cDNA, from the target tissue. While using extremely large amounts of identical probes in each square on the chip, the probability increases that complementary cDNA (the sample) hybridizates. The cDNA incorporates a detection reagent, a fluorescent dye. After hybridization the chip is scanned by a laser. The level of the hybridized probe will correspond to the amount of the cDNA. The oligonucleotides are synthesized in situ and located on the chip with extreme precision. The method is light-directed and is named photolithography[91]. A gene is represented on the chip by 11-20 unique probe pairs of 25 base pairs oligonucleotides. The probe pairs are designed as a Perfect Match (PM) and Mismatch (MM). The MM probes serve as control for hybridization[91].

The advantages with the Microarray technology are obvious. With this method it is possible to receive information from a small amount of sample, regarding gene expression, about many thousands of genes. Due to the large amounts of results, this method can have a screening approach i.e. work as a generator of hypotheses.

Disadvantages with the method are that high quality RNA is needed for reliable results and the high risk of false positive results when performing multiple testing. It might also be that not all observed intensities are associated with expressed genes, it could e.g. be noise due to non-specific binding or cross-hybridization[92].

Before statistical analyses starts, the data have to go through several steps of quality control:

Quality control 1: Total RNA quantity was determined by ultraviolet absorbance at 260 nm. The purity of the isolated RNA was determined by measuring the ratio of absorbance at 260 and 280 nm.

Quality control 2: The signal intensity of all probes on each chip was scaled to a single value, 100, to allow comparisons between samples.

Quality control 3: A quality control of reversed transcriptase. After hybridization the ratio between probe sets located in the 3' and 5' end, are counted for the control genes β-actin and GAPDH.

Quality control 4: The PM/MM system. For each gene a set of paired oligonucleotide probes are designed. Each pair contains both a perfect match probe of the gene (a canonical sequence), and a mismatch probe (a mutation). The mismatch probe measures the degree of cross hybridization, i.e. false positive signals.

Quantitative Real-time polymerase chain reaction (Q-RT-PCR)

Q-RT-PCR is a well-established and powerful method to quantify gene expression. With this method very small changes/differences in PCR products can be quantified. The method can be used to quantify DNA levels of for example viral infections and for gene expression analyses. To measure gene expression, mRNA is isolated and converted to cDNA before analysis. There are two different method of Q-RT-PCR; absolute and relative quantification[89].

When using absolute quantification the input transcript is determined with the use of a standard curve. In contrast, when using relative quantification the change in gene expression is compared to a reference gene (endogenous control). By using an endogenous control as a reference, quantification of mRNA can be normalized for differences in the amount of total RNA added to each reaction. Results are given as a dimensionless normalized ratio.

To preserve the instant level of mRNA blood can be drawn with the PAXgene system described above, which includes RNA stabilizing buffer. mRNA is then converted to cDNA by the enzyme reverse transcriptase. The cDNA is subsequently amplified in the exponential PCR process, where the amplified product is recorded at each PCR cycle. Normally a high number (often 40) of repeated PCR are used in the Q-RT-PCR[93].

A PCR cycle contains a raise of the temperature to melt the double stranded cDNA or DNA, creating single stranded DNA that are used as new templates. The temperature of the reaction is then lowered, to enable the oligonucleotide primers to anneal to the new single stranded DNA. (primer=nucleic acid strand that serves as a starting point for DNA replication) The enzyme DNA polymerase will then initiate elongation of DNA by adding single complementary nucleotides, whereby a new double stranded DNA is created[94].

The final step is detection and quantification and there are several different methods for this. One example is when a DNA-based probe, complementary to the target nucleic acid molecule, is used. The probe is supplied with a fluorescent marker in one end and a quencher at the other end. When the DNA polymerase reaches the probe during the annealing stage, the fluorophore and quencher are

separated, resulting in a light of fluorescence, which is detected in every PCR cycle[95].

Q-RT-PCR is a stable method, but of course there are limitations, e.g. RNA is extremely labile and care has to be taken to avoid contamination with nucleases.

White blood cell single photon emission computer tomography (WBC-SPECT)

Tomographic techniques are giving cross-section images of the studied subject. When using SPECT the emitted gamma emission from the patient is counted in several well-known three-dimensional directions. For the purpose of studying white blood cell distribution in the body, these can be labelled with ^{111}Indium or ^{99m}Tecnetium. The latter is more preferable because of shorter investigation time and lower radiation burden to the patient. The SPECT images are usually acquired over 180° or 360 ° in small angular steps. Thereafter the concentration of activity can be calculated[96].

A balance must always be kept between resolution and sensitivity. Those qualities are competitive, and both are influenced by the choice of collimator. The collimator is often produced by lead and consists of several openings. Photons which are sent from the radionuclide have the same probability for all emission directions. A gamma camera without collimator cannot produce images, since the incoming directions of photons are not known. A collimator filters incoming photons and creates information about incoming directions [96]. The construction of the collimator influences the resolution and sensitivity of the gamma camera, so that small openings in the collimator generate higher resolution and lower sensitivity. The distance between the gamma camera and the patient also influences the resolution of the images; the shorter the distance, the better the resolution[96].

The following is a schedule for a WBC-SPECT:

- The patient leaves a blood sample
- White blood cells are separated and labeled with ^{99m}Tecnetium.
- The blood sample with the ^{99m}Tecnetium-labeled WBC (^{99m}Tc-labeled WBC) are re-infused to the patient
- WBC migrates, besides the sites of physiological accumulation, to regions of inflammation
- A gamma camera register the radiation from the ^{99m}Tecnetium-labeled WBC in the brain after 2 hours

The ^{99m}Tecnetium-labeled re-injected WBC behaves like the native WBC and is distributed into a bound and a circulating part. Around 45 minutes after reinjection, balance has been reached and 40% of the injected activity is in the circulating blood and the rest of activity is equally distributed to the liver, spleen, lungs and bone marrow. Uptake in inflammatory regions is fast[97].

All CH patients and control patients in study III underwent an MRI of the brain to enable precisely anatomically defined regions of interest (ROI:s). All WBC-SPECT-studies were fused with the corresponding MRI using Hermes software (Hermes/NUD, Stockholm, Sweden). ROI:s were manually drawn on the MRI-images using anatomical borders without visual access to the WBC-SPECT-images. SPECT images only are not sufficient for defining ROI:s due to the lack of anatomical landmarks and absence of high specific binding.

Capillary electrophoresis

In study IV we intended to investigate the role of NO in CH, by measuring its oxidation products (NOx); nitrite and nitrate, in the CSF. The reason for not measuring NO directly was that NO has a very short half-life, just 3-5 seconds[81]. To estimate NO production, we determined the levels of NOx by using capillary electrophoresis (CE). An advanced technique for the analysis of ions[98, 99]. The ions are separated on the bases of their charge ratio and allow individual detection of nitrite and titrate. CE has been shown to quantify nitrite and nitrate in biological fluids close to the millimolar (mM) range[100]. The main components in CE are sample vial, source and destination vials, a capillary, electrodes, a high voltage power supply, a detector and computer, see Figure 2. The source vial, destination vial and capillary are filled with an electrolyte. The migration of the ions starts when the electric field is applied between the source and destination vials. A small volume of sample is injected at the positive end of the capillary and separated ions are detected near the negative end of the capillary. The output of the detector is sent to a computer[98] (Figure 2).

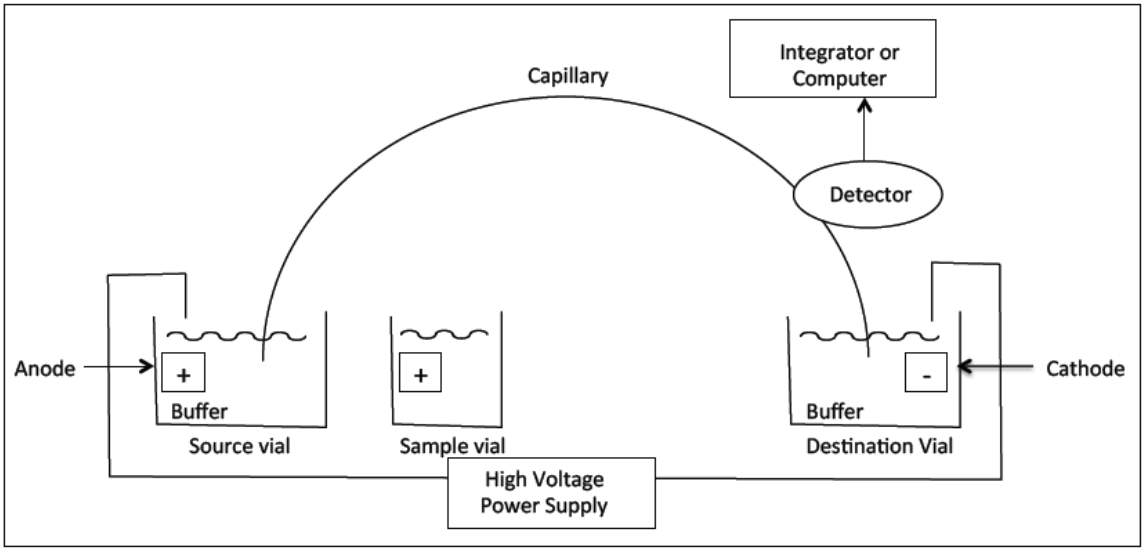

Figure 2: Diagram of CE system. (From Capillary Electrophoresis reference[101])

Statistics

Study I

The Affymetrix GeneChip® Operating Software (GCOS) version 1.0 was used for initial raw data processing. For comparison of different normalisation methods we used the Robust Multichip Average method[102] implemented in the module Affy of the BioConductor microarray analysis software. For the relative gene expression levels, the GCOS Expression algorithm was used for transcript quantification and microarray data comparison.

To enable identification of up- and downregulated genes between chip pairs, pairwise comparisons on signal values from different disease stages (period between attack, attack, remission) and controls were performed. Totally 6 comparative analyses were performed. All samples were analysed in triplicates. A transcript had to be replicated as either increased or as decreased in all three samples to be regarded as a signal change.

All probe IDs were checked against Affymetrix annotations, Netaffx analysis and annotation center at www.affymetrix.com/analysis/netaffx.

Fischer´s exact test was used for comparisons of selected genes within a group of genes, upregulated S100 genes, compared to all genes represented on the chip.

Statistical analysis on gene expression from the RT-PCR were performed between the disease stages (period between attack, attack, remission) by paired ANOVA, followed by Tukey-Kramer post test for multiple comparisons. $P<0.05$ was chosen to indicate statistical significance.

Study II

Comparisons of relative IL-2 gene expression levels were performed by the use of Friedman´s ANOVA. Wilcoxon matched pairs tests were performed for dependent samples and Mann-Whitney U test for independent samples. $P<0.05$ was chosen to indicate statistical significance.

Study III

A Mann-Whitney U test was used to investigate the difference in uptake between CH and control subjects. A Wilcoxon Matched Pairs test was used to compare the uptake in CH patients in active period and in remission and at pain side and non-pain side of cavernous sinuses. Both tests were implemented in Statistica 9.0 (StatSoft. Inc, Tulsa, Oklahoma, USA). Data were analyzed on the 2 h post injection acquisitions. The significance level for statistical testing was taken $p<0.05$.

Study IV

Student´s t-test was used as we regarded the data to be normally distributed. A p-value of 0.05 was considered significant. Non-parametric test were also performed.

RESULTS

Study I

Altogether small differences were seen intraindividually and large differences interindividually. Pairwise comparisons of signal values from different disease phases and controls, comparisons between different phases in the same individuals and between patients and matched controls, generated evidence of relatively few regulated genes. Results are presented as fold change in a log2 ratio. The results are shown in Table 2, in study I.

All raw data are available at http://www.ebi.ac.uk/arrayexpress/browse.html?keywords=human+cluster+headache&expandefo=on .

The most apparent finding was the upregulation of several calcium binding S100 genes; S100A8, S100A12, and S100P; during active period and attacks compared to remission (except for S100A12, which did not show upregulation during attacks). The S100A8 and S100A12 are considered markers of non-infectious inflammatory disease, while the function of S100P has been connected to different forms of cancer[103] .

Moreover, Annexin 3 (also S100 protein like) was upregulated during attacks compared to remission. So were also ICAM3 and TNFRSF10C. The latter, TNFRSF10C is a member of the tumour necrosis family of genes, where all regulate cytokines. ICAM3 is an adhesion molecule, which is also regarded to be involved in the immune system.

Fisher´s exact test was used for comparison of the S100 genes compared to all genes on the chip (~22 000 genes) which revealed an upregulation for period versus remission ($p < 0.0001$), and for attack versus remission ($p = 0.00037$). There were no significant differences for this transcript in relation to controls.

The upregulation of S100P could be confirmed with RT-PCR. The test showed significant differences between attacks and remission (p=0.048). Two patients had a higher expression level during period compared to attacks, but still both were higher compared to remission. Comparisons of all groups revealed significant differences between attacks and remission (p=0.025), see Figure 3. Patients in period showed no significant difference compared to remission and to controls (p=0.664 and p= 0.0146, respectively). Comparison of means between attack and controls was considered non-significant (p= 0.089.) The comparison between remission and controls was also considered not significant (p=0.15). The results were corresponding with the result from the microarray analysis, i.e. changes in S100P expression were seen within the same patients but not significantly in relation to controls, both in the microarray analysis and in the quantitative RT-PCR analysis.

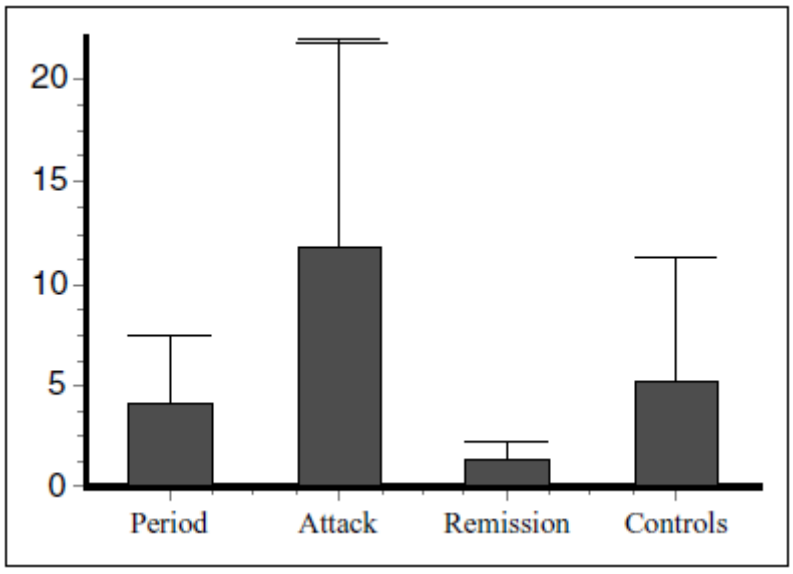

Figure 3: S100P relative gene expression levels. (Relative levels to GAPDH). Comparisons of all groups showed significant differences between attacks and remission (p=0.025).

Study II

The relative gene expression of the cytokine IL-2 was studied in eight episodic CH patients and 16 healthy headache-free control subjects matched for age and gender. Venous blood samples were drawn from the CH patients on three occasions; during active period between headache attacks, during an attack and in remission. Venous blood samples were drawn once from each control subject. We analyzed IL-2 gene expression using RT-PCR.

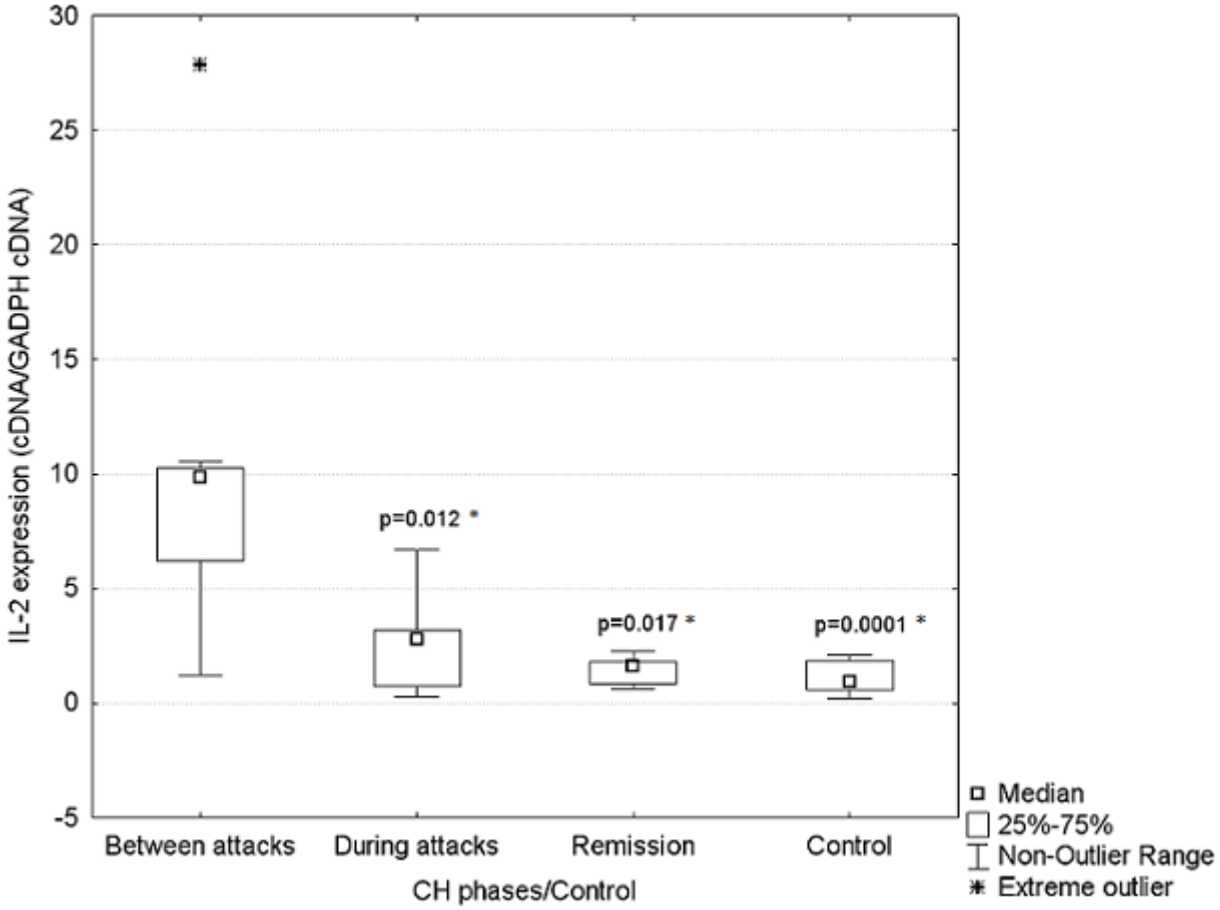

Figure 4: Relative IL-2 Expression in different phases of Cluster Headache.(Relative levels to GAPDH). * The p-values are compared to Between attacks.

Friedman's ANOVA rejected the null hypothesis of no difference between the three phases (p=0.008). In pairwise comparisons, CH patients had significantly increased relative IL-2 gene expression values during active period between attacks (median 9.9, IQR 6.2-10.3) compared to in remission (median 1.6, IQR 0.9-1.8, p=0.017) and control subjects (median 0.9, IQR 0.6-1.9, p=0.0001). The relative IL-2 gene expression values during the active period between attacks were also significantly higher than during attacks (median 2.8, IQR 0.7-3.2, p=0.012).

A trend to higher relative IL-2 gene expression level was also noted during attacks in relation to controls and remission, although not statistically significant.

The relative IL-2 expression value was deviating in one patient. The statistical analysis was repeated after exclusion of this patient, assuming that the value was an outlier; the difference between active period, remission and controls remained statistically significant.

Relative IL-2 gene expression values among CH patients during different phases of the disorder and among healthy headache-free control subjects are presented in Fig 4.

Study III

14 CH patients and 5 control subjects were included in the study. The control subjects were all recruited in connection with a routine SPECT examination. Reasons for examination were suspected osteomyelitis in a knee or leg (2), infection around hip prostheses (1) and fever of unknown cause (2). None had signs of intracranial accumulation of white blood cells.

A first visual evaluation of the MRI fused WBC-SPECT images revealed an intensive 99mTc-labeled WBC uptake in clivus in CH patients, independently of CH period, and also in controls. By contrast a low ^{99m}Tc-labeled WBC uptake was seen in the cavernous sinuses in CH patients, independently of CH period, and also in controls.

The average labelling efficiency of WBC was 45% (range 17-72 %).

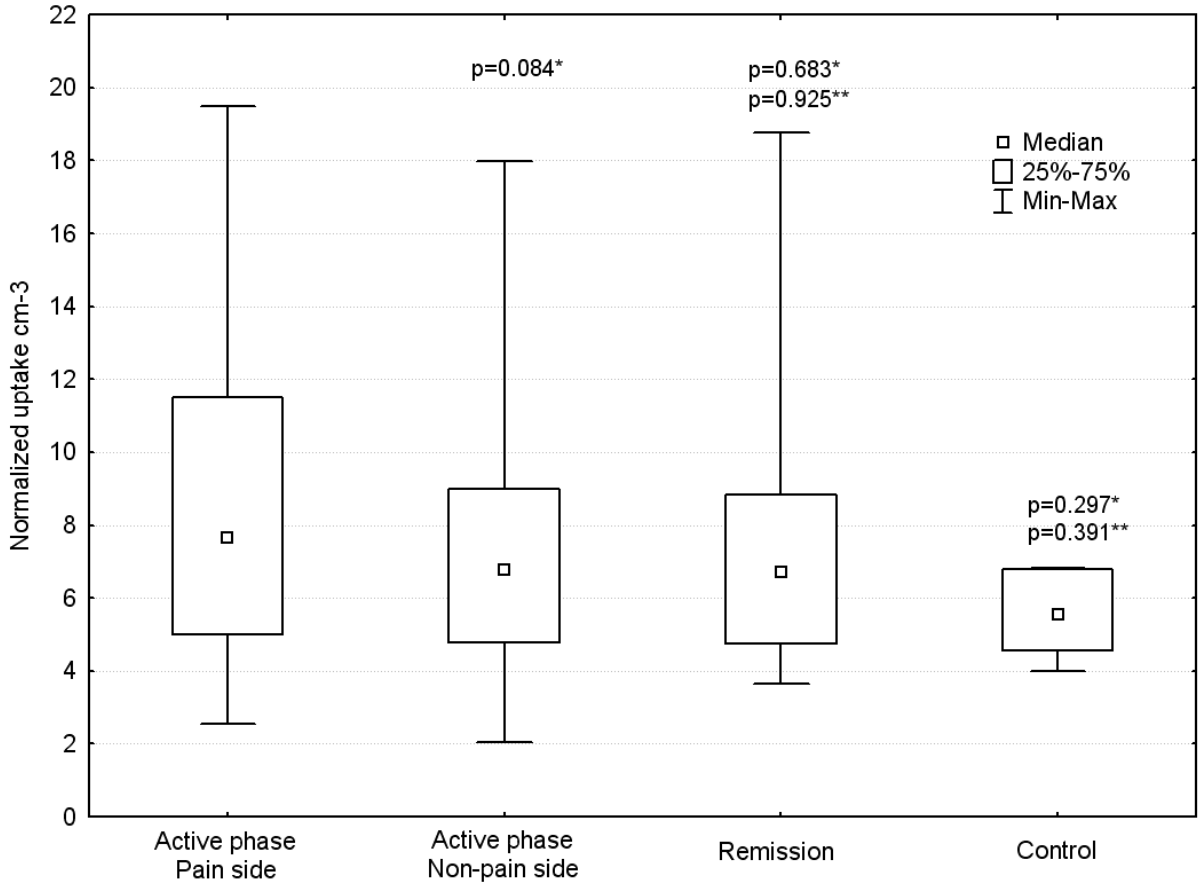

Figure 5: The ^{99m}Tc-labeled WBC 2 h post injection uptake in cavernous sinus in CH patients, active phase and remission, compared to controls. Pain side indicates the side of the cavernous sinus ipsilateral to the CH attacks. Remission and controls include both sides of cavernous sinus. *p-values for comparison with pain side. **p-values for comparison with non-pain side.

The following normalised uptake was obtained for CH vs. controls, presented as median and interquartile range (cm^{-3}; IQR): Cavernous sinus: 7.1 (5.0-10.3) vs. 5.6 (4.6-6.8; p=0.34), sagittal sinus: 12.4 (11.3-14.2) vs. 10.5 (10.5-11.3; p=0.09), clivus: 23.0 (14.5-30.5) vs. 24.2 (19.6-31.4; p=0.69.)

No significant differences in normalized uptake were found in any of the regions listed above, when comparing CH patients in active period and remission.

No conclusive increase of ^{99m}Tc WBC uptake at the CH pain side of the cavernous sinus compared to the non-pain side was seen. Data are presented as: pain side median uptake (cm^{-3}; IQR) vs. non pain side median uptake (cm^{-3}; IQR): 7.7 (5.0-11.5) vs. 6.8 (4.8-9.0), (p=0.08). The results for the cavernous sinus, comparing CH pain side versus non pain side, CH in remission and controls, are shown as a box and whisker plot in Figure 5.

In conclusion we found no significant difference in ^{99m}Tc-labeled WBC uptake at the CH pain side of the cavernous sinus compared to the non-pain side, although a trend to a higher uptake at the pain side was noted. Furthermore we found no significant difference in ^{99m}Tc-labeled WBC uptake between CH patients in active period and controls. CH patients in active period were not significantly different in uptake compared to CH patients in remission.

Study IV

In study IV we included twenty-two CH patients during active period. Throughout the study, four patients developed chronic CH and four other patients discontinued due to postpunctional headache. The final study group consisted therefore of 14 CH patients. Eleven healthy volunteers served as control subjects.

CH patients in the active period had significantly increased NOx levels (mean 9.3, 95% CI 8.5–10.1) compared with in remission (mean 7.6, 95% CI 6.9–8.2; p<0.001) and control subjects (mean 6.2, 95% CI 4.9–7.5; p<0.001). (Figure 6). A significant difference was also present between CH patients' NOx levels in remission and control subjects (p=0.034), where CH patients in remission had higher NOx levels.

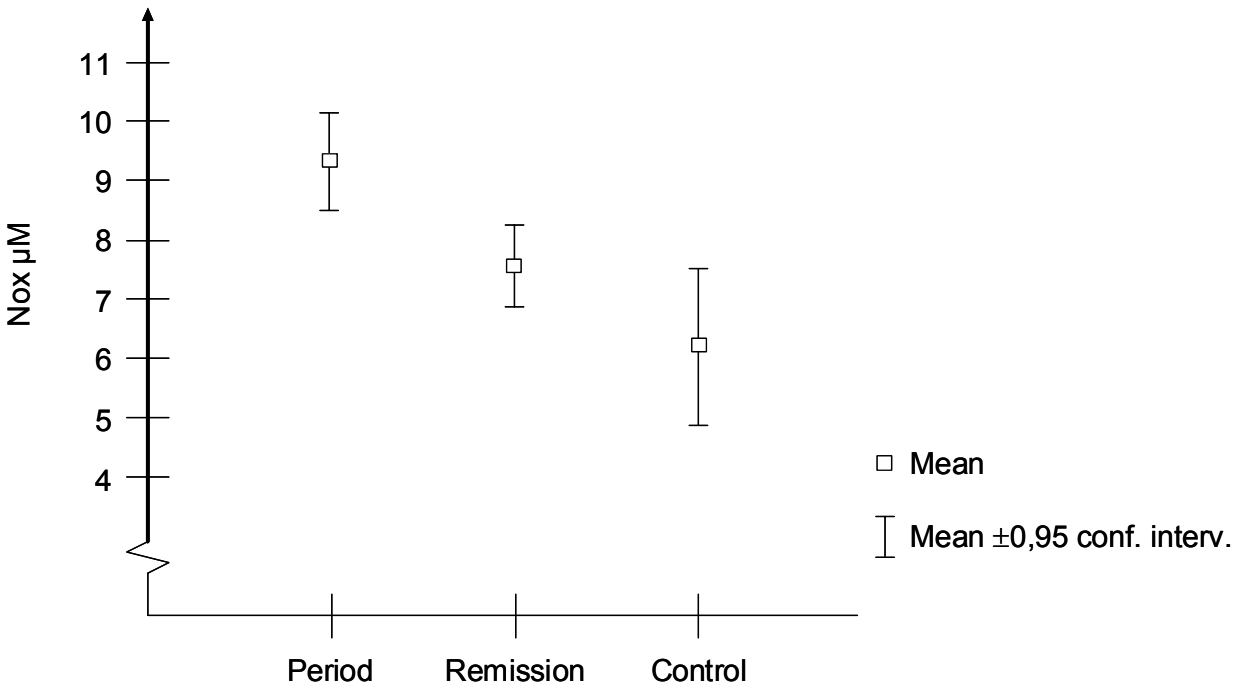

Figure 6: Mean levels of nitric oxide products (NOx) in cerebrospinal fluid from episodic cluster headache patients during both phases of the disease, and also from controls

Thirteen of 14 CH patients showed higher NOx levels in the active period compared with remission.

Two of the patients had a CH attack which started as the LP was performed. Their NOx levels were 9.6 μM and 10.3 μM. Two other CH patients had their last attacks 24 hours before the LP, and their NOx levels were 11.3 μM and 10.1 μM. All four patients showed deceased NOx levels in remission.

Five patients had a nocturnal CH attack the night before the LP. The mean NOx levels were similar in this group compared with others (9.0 μM vs. 9.7 μM, NS).

Clinical routine laboratory analyses of CSF showed normal levels with regard to protein content and inflammatory parameters in eight of 14 patients. A minor albumin increase in CSF was seen in five patients, for two of them both in the active phase and in remission. Two patients showed a modest albumin increase only in the active phase and one patient only in remission. A slight pleocytosis was seen in one patient in both phases of the disease. One patient showed solitary oligoclonal bands in CSF and plasma in remission. Patients with abnormalities in CSF parameters had slightly higher mean NOx levels than other patients (10.0 vs. 8.8 μM), although this difference was not statistically significant. None of the patients with changes in routine laboratory analyses presented any clinical symptoms of concomitant disease.

Seven patients, i.e. about 50% (7 of 14), developed postpunctional headache. All seven of these patients developed postpunctional headache in the active period and three of them (three of 14) also developed postpunctional headache during remission. One of these patients also showed a minor albumin increase in CSF. If the four episodic CH patients who discontinued the study due to the development of postpunctional headache are also included, the percentage of CH patients who developed postpunctional headache during the active period increases to 61%.

Finally, it was explored whether heterogeneity was present between the NO oxidation products, nitrite and nitrate, during the active period and in remission.

Nitrite and nitrate levels were compared to the total NOx levels and found to be of similar distribution.

DISCUSSION

The main objective of this thesis was to explore evidence of inflammatory involvement and the role of nitric oxide in the pathophysiology of CH. Inflammatory involvement was the focus in paper I-III and nitric oxide in paper IV.

Inflammatory involvement in CH

There are at least two major findings in this thesis which speak in favour of inflammatory involvement in CH. The first of these is the observation of upregulation of calcium binding S100 genes, such as S100A8 and S100A12 during active period and attacks, compared to in remission. These two calcium binding genes are considered markers of non-infectious inflammatory diseases. The other is the increased relative IL-2 gene expression level during active period between headache attacks compared to during attacks, remission and controls. Both these findings provide support of a non-infectious inflammatory involvement in CH pathophysiology. Our observation of an upregulation of non-infectious inflammatory genes during active period and attacks compared to remission, are in agreement with earlier studies[21, 22, 36-41, 43], e.g., Empl et al.[21] who measured serum levels of soluble IL-2 receptors in 18 patients with CH and found significantly increased sIL-2R levels compared to headache-free control subjects.

The exact mechanism of a non-infectious inflammation in CH is not known, but there are several potential modes of action that may contribute. Over recent years an increased understanding has developed on the role of a neurogenic inflammation closely related to the activation of the trigeminovascular system in CH and migraine. Release of CGRP and substance P from trigeminal fibers may initiate an inflammatory reaction with plasma protein release and vasodilation[24, 34, 104]. Involvements of white blood cells have been suggested[105].

Interestingly, IL-2 is vital for determining the magnitude and duration of primary and secondary immune responses, and moreover, one of its most important functions is to downregulate immune responses to protect against

autoimmunity[106]. Empl et al have shown that CH patients have significantly increased soluble IL-2 receptors in serum compared with controls[21]. Several earlier studies have shown a change in immunologically competent cells[21, 38-40]. Our finding of an increased IL-2 expression during active periods compared to remission and controls supports the concept of inflammatory involvement in CH. We also showed that IL-2 expression was reduced during attacks compared to active periods between attacks. It can be speculated that pain attacks are related to failure of protection against autoimmunity, but an alternate possibility is that the pain itself can cause a reduced IL-2 production[107, 108] and this issue has to be resolved by further research. In this context it is interesting that intrathecal delivery of a plasmid harbouring the IL-2 gene in rats produced a marked antinociceptive effect, which was maintained up to 6 days[109]. Moreover, IL-2 has been reported to activate the hypothalamus and stimulate the release of corticotropin-releasing factor (CRF)[21, 110-113].

Accumulation of white blood cells is a pivotal part of an immunological response and the documentation of white blood cell migration into the area of interest was in focus of the third paper. We decided to use a novel method in CH research with ^{99m}Tecnetium labeled white blood cells identified with SPECT in which the emitted gamma emission from the patient is counted in several predefined three-dimensional directions. MRI of the brain was used to enable precisely anatomically defined regions of interest (ROI:s).

Our analyses of white blood cells within the central nervous system did not reveal findings of statistical significance. There was a trend to a higher uptake in the part of the cavernous sinus that was ipsilateral to the pain, but this observation is not conclusive. This is also true for another trend to higher uptake in the sagittal sinus. There is a possibility that these observations occurred by chance, but also that the method we used may have been too unspecific or that the study sample size too small. Although caution has to be applied in the interpretation of findings by multiple testing of white blood cell uptake at different locations, it may be of interest for further research.

It may be interesting to note that our combination of SPECT with MRI enabled identification of uptake in clivus, which may otherwise have been

interpreted as increased uptake in the cavernous sinus. The findings of Gawel et al[41] who performed gallium-SPECT and found increased uptake of gallium in the region of cavernous sinus during active period could be supportive of our trend finding of increased WBC uptake in cavernous sinus, but the lack of neuroradiological navigation in their study also opened for the possibility of an influence from unspecific uptake in clivus.

Nitric oxide in CH

It is well known that administration an NO donor, such as nitroglycerin or histamine which causes release of NO from vascular sites, may induce CH-like attacks during active CH phase but not during remission[56]. NO has a key role in regulating physiological functions, among these vasomotor regulation[81]. The isoform nNOS is expressed in most regions of the central nervous system, including the cerebellum, the cerebral cortex and various ganglion cells of the autonomic nervous system. Large numbers of nNOS-containing neurons have also been found in the hypothalamus[111].

Activation of the trigeminovascular system is considered an important step in the development of CH attacks[24]. CGRP is a marker of this activation, and increased levels have been found in blood from the external jugular vein during spontaneous CH attacks[24], as well as attacks provoked by NO donors[24, 45]. D'Amico et al. have shown increased nitrite-levels in plasma among CH patients, both in active period and in remission [26]. These findings, all together, indicate that there could be a link between NO, the trigeminovascular system and CH pathophysiology. We intended to evaluate the role of NO in episodic CH, by analysing NOx in CSF, closer to the supposedly affected area in the hypothalamus. To our knowledge, a similar study has not been performed earlier.

We were able to show that CH patients in active period had significantly increased NOx levels in CSF compared with those in remission and control

subjects. CH patients also had significantly enhanced NOx levels in remission compared to control subjects.

Our results are in agreement with D'Amico et al.[26], who found generally raised nitrite levels in plasma among CH patients, and adds support to the observation of a basal hyperactivity of the L-arginine-NO pathway.

In another study by Costa et al., nitrite and L-citrulline (a stoichiometric metabolite, resulting from the conversion of L-arginine to NO, and considered as a reliable index of NOS activity) were measured in plasma in CH patients and controls after administration of nitroglycerin. The authors found no difference between the plasma concentrations of nitrite and L-citrulline between the two groups. They concluded that the result did not support the presence of a basal hyperactivity of the L-arginine-NO pathway in CH patients[56]. The discrepancy between our results and those of Costa et al. may be explained by differences in methodology. It is notable that the study of Costa et al. was performed on plasma rather than CSF, i.e. on a more remote substrate in relation to the brain. Furthermore Costa et al. studied induced attacks, while we studied spontaneous attacks.

The level of NO production has been shown to correlate with disease activity in inflammatory disorders, such as multiple sclerosis, cystitis, cerebral systemic lupus erythematosus and inflammatory bowel disease[65-67, 114, 115]. The NOx levels in our study were higher during active period compared to remission, which correlates with our findings of increased expression of calcium binding S100 genes and IL-2 genes. This may suggest that increased NOx levels may be an expression of enhanced inflammatory activity in CH as well as in other inflammatory conditions.

CH patients might be sensitized for CH attacks by a mechanism related to high NO-levels, and at a certain threshold, the trigeminovascular system is activated. High NO-levels may contribute to and maintain a central hyperalgesia[59, 116-118]. Possibly such a mechanism, in connection with activation of the trigeminovascular system, could be a source of the pain attacks. Interestingly, we found that headache occurring as a reaction to lumbar puncture

was overrepresented in CH and it can be speculated whether this is caused by sensitization secondary to enhanced NOx levels.

In the trigeminal ganglion, CGRP and NOS co-localize in many neurons[60]. It has been suggested that NO causes release of CGRP[61, 119, 120], although there are studies with conflicting results[62, 63]. Treatment of rat trigeminal ganglia cultures with NO donors caused a greater than four-fold increase in CGRP release compared with unstimulated cultures[119].

Inflammation and nitric oxide – an integrated discussion

Several mechanisms participate in the pathophysiology of CH. The clinical picture with frequent CH attacks, the diurnal pattern of attacks and hormonal alterations in CH patients have pointed to a potential defect in the suprachiasmatic nucleus within the hypothalamus[15, 23] as the center of the origin of CH. In hypothalamus large numbers of nNOS containing neurons have been found[111]. One hypothesis is that hypothalamus is responsible for producing boosts of NO, initiated by the suprachiasmatic nucleus. The NO-elevation might trigger the trigeminovascular system.

One of the other cornerstones in CH pathophysiology is NO. We have shown generally raised NO levels in CSF in CH patients compared to healthy controls. The trigeminovascular system is activated in CH[24], and one of the consequences may be neurogenic inflammation within its distribution[34, 104]. As mentioned above, CH patients might be sensitized for CH attacks by a mechanism related to high NO-levels, and at a certain threshold, the trigeminovascular system may be further activated. This may initiate a vicious circle, where both high NO-levels and neuropeptides, such as substance P and CGRP, contribute to a neurogenic inflammation.

The excruciating pain in CH might be related to high NO-levels and release of neuropeptides, giving rise to a neurogenic inflammation with sensitization of vessels and meninges, and inducing vasodilation. CH attacks are often successfully treated with sumatriptan or oxygen[72] both of which are thought to induce their effect by vasoconstriction, indicating that vasodilation is involved in the generation of pain.

Corticosteroids have positive therapeutic effect in CH[25, 68]. The isoform nNOS is expressed in most regions of the central nervous system and in hypothalamus[111]. The cytokine IL-2 can activate the hypothalamus and stimulate the release of corticotropin-releasing factor (CRF)[21, 110-113], but the stimulatory effect of IL-2 on CRH release is inhibited by the corticosteroid dexamethasone[111, 113, 121]. In addition, treatment with dexamethasone in mouse inhibits the nNOS activity[121]. If these observations can be translated into the human brain, dexamethasone may inhibit the generation of a vicious circle by reducing the production of NO. Subsequently, this would reduce the inflammatory activity and the activation of the trigeminal system. Many factors influence the immune system, complicating the interpretations of different interactions, such as pain, stress and other environmental factors[107, 108].

Limitations

In all studies there are relatively small study populations. The main reason for this is that CH is a rare episodic disorder and that patients had to be in active period for inclusion. Several patients could not be included since they would not accept to be without prophylactic treatment. There were also a number of logistical demands for the patients which made it difficult to motivate patients to join the studies.

Unfortunately in paper I two out of five patients were withdrawn from the study, due to poor RNA quality. If it had been possible to get better RNA quality in the beginning, this could of course be avoided. When we started this project RNA

extraction with PAXgene tubes was a new technique. Today the method has been evaluated further and there are reports how to receive better RNA quality, by using modified protocols[122].

In paper III measurements on three different SPECT cameras were performed due to the fact that the first camera was broken. We then had logistic problems to get time for the CH patients in the SPECT camera when they competed with clinical routine patients.

CONCLUSIONS

Based on our studies comprising in total 31 patients and 35 controls, the following conclusions can be drawn.

1. Our finding of an upregulation of S100A8 and S100A12 genes in CH patients during active period may indicate an inflammatory involvement in CH, since both S100A8 and S100A12 are considered markers of non-infectious inflammatory disease.

2. CH patients had significantly increased relative IL-2 gene expression between headache attacks during active CH period, compared to during attacks, remission and controls. This finding adds further support on involvement of inflammation in CH. Since a prominent function of IL-2 is defense against autoimmunity, our observation also may strengthen evidence that the inflammatory involvement is of noninfectious origin.

3. The WBC-SPECT study did not provide statistically significant results. This might be due to the possibility that the method we used for this purpose is too unspecific, or the fact that WBC might not have a prominent role in CH. An important conclusion from the WBC-SPECT study is that MRI mapping is important to avoid misinterpretation.

4. We have shown that CH patients in active period had significantly increased NOx levels in CSF compared with those in remission and with control subjects. CH patients also had significantly enhanced NOx levels in remission compared to control subjects. The level of NO production has been shown to correlate with disease activity in several inflammatory disorders. This suggests that increased NOx levels in CH patients might be an expression of enhanced inflammatory activity as well as in other inflammatory conditions. A connection between those two possible

5. We hypothesize that CH patients might be sensitized for CH attacks by a mechanism related to high NO-levels, and at a certain threshold, the trigeminovascular system is activated. The high NO-levels may also contribute to a central hyperalgesia.

ACKNOWLEDGEMENTS

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