Cytokines - possible biomarkers for headache syndromes, with special reference to migraine

By

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Front page figure:
Girl looking in the mirror experiencing scotoma during the aura phase of a migraine attack
Front page figure and all figures in chapter 1. Background, are illustrated for this thesis only.
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Odd Bjørn, I love you!

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Abstract

Background: The trigeminovascular theory suggests that neurogenic inflammation and cytokines play an important role in migraine pathophysiology. Previous studies have linked different cytokines to migraine pathogenesis, but the results have been conflicting. Today, no biomarkers are known for primary headaches. The objectives for this thesis were to: 1. Identify cytokines as possible specific biomarkers for migraine, cluster headache and tension-type headache. 2. Define cytokine secretion as possible inflammatory response to pain. 3. Evaluate the use of ELISA assay and ELISpot assay for the purpose of this study. 4. Evaluate strengths and flaws of this pilot study and suggest improvements for future studies.

Methods: Too few tension-type headache patients, and no cluster headache patients, agreed to participate in this study. The only headache group consisted of 11 migraineurs. Control groups consisted of 7 low back pain patients and 21 healthy, pain free individuals. Blood samples were taken both during attack/pain period and attack free/pain free period. Peripheral blood mononuclear cell samples were analyzed using ELISpot assay and serum samples were analyzed using multiplex ELISA assay. Frequencies of cytokine secreting cells and blood cytokine levels were determined, and levels of cytokine production were compared within and between groups.

Results: A higher frequency of TNF-α secreting cells than IL-10 secreting cells was observed in migraineurs. Lower MCP-1 and IL-10 levels and higher TNF-α levels were observed in both migraineurs and low back pain patients compared to healthy, pain free controls.

Conclusions: No cytokines were revealed as possible biomarkers for migraine. A possible upregulation of TNF-α and downregulation of MCP-1 and IL-10 may be of relevance, if not specifically for migraine, at least for pain in general. The results obtained from the ELISpot assay and the ELISA assay were not identical. The two methods provide information about cytokine production from different cell sources. Further investigation of cytokine production and comparison of the two assays are needed.

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Abbreviations

5-HT	Serotonin
CGRP	Calcitonin Gene-Related Peptide
CPT	Cell Preparation Tube
CSD	Cortical Spreading Depression
CSF	Cerebrospinal Fluid
DA	Dopamine
DMSO	DimethylSulfoxide
ELISA	Enzyme-linked Immunosorbent Assay
ELISpot	Enzyme-linked Immunosorbent Spot
FBS	Fetal Bovine Serum
GSP	Great Superficial Petrosal nerve
HRP	Horseradish Peroxidase
ICHD	International Classification of Headache Disorders
IFN	Interferon
IHS	International Headache Society
IL	Interleukin
LC	Locus Coeruleus
MCP	Monocyte Chemotactic Protein
NA	Noradrenalin
NO	Nitric Oxide
NSAD	Non-steroid Anti-inflammatory Drug
PAG	Periaqueductal Gray region
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline with Tween
PHA	Phytohemagglutinin
PVDF	Polyvinylidene Difluoride
rCBF	Regional Cerebral Blood Flow
REK	Regional Committee of Ethics (Norwegian: Regional Etisk Kommitè)
S/P	Streptomycin Penicillin
SCN	Suprachiasmatic Nucleus
SP	Substance P
SPG	Sphenopalatine Ganglion
SSN	Superior Salivatory Nucleus
Strep-RPE	R-Phycoerythrin conjugated Streptavidin
TCC	Trigeminocervical Complex
TG	Trigeminal Ganglion
TGVS	Trigeminovascular System
TMB	Tetramethylbenzidine
TN	Trigeminal Nucleus
TNC	Trigeminal Nucleus Caudalis
TNF	Tumor Necrosis Factor
TTH	Tension-type headache
VIP	Vasoactive Intestinal Peptide
WHO	World Health Organization

1. Introduction

1.1 Burden and prevalence of headache

Headache disorders are considered to be among the most common disorders of the nervous system and in accordance with the World Health Organization's (WHO) ranking of causes of disability, headache is among the ten most disabling disorders for both sexes (1). Chronic or recurring headaches impose a physical, mental and economic burden on sufferers and affect their quality of life. It may also damage family and social life and employment (2). Headache sufferers may have several co-morbidities such as increased incident of depression, anxiety, hypertension and stroke (3). Headache disorders are not only a burden for the sufferers itself, it also affect the people around them and it is becoming a large economic cost for the society (2). A review, written as a preparation for the Eurolight project, estimate that migraine alone costs all of Europe 27 billion euro a year, and other headaches probably as much (2).

Headaches are prevalent in both sexes, but usually predominates in women (4). For cluster headache it is the opposite (5). In 1995-1997 Sjaastad et al. (6) carried out an epidemiological study of headache in Vågå commune, a commune in the mountainous region of southern Norway. A total of 1838 (88,6%) of the available 18-65-year-old inhabitants were interviewed face-to-face, diagnosed following the criteria of the International Headache Society (IHS) and examined personally by Sjaastad (6). Regarding migraine with aura the prevalence was found to be 9.7% with a sex ratio (F/M) of 1.70 (7). The prevalence of migraine without aura was found to be 31% with a sex ratio of 1.69 (8). For tension-type headache a prevalence of 34% was found, with a sex ratio of 1.58 (9). A search was also made for cluster headache and the prevalence was found to be 0.33%, with a sex ratio of 0.20 (5).
In 1989 a similar epidemiological study of headache was carried out in Glostrup, Denmark (10). A total of 740 (75.9%) of the "invited", participated and got examined and diagnosed following the IHS criteria at the Copenhagen County Hospital in Glostrup (10). The lifetime prevalence of migraine was 16% with a sex ratio of about 3, and for tension-type headache it was 78% and the sex ratio 1.25 (10). They only found one case of cluster headache (10).

1.2 Migraine

Migraine is an episodic headache disorder usually divided into two major sub-types, migraine without aura (common migraine) and migraine with aura (classical migraine) (11). Clinical features of migraine are summarised inTable 1. Following the International Classification of Headache Disorders (ICHD; criteria of IHS) (12) both migraineurs with aura and those without aura must have had at least five attacks lasting 4-72 hours, with two or more of these criteria: unilateral location, pulsating quality, moderate or severe pain intensity and/or aggravation by routine physical activity (e.g. walking or climbing stairs). During headache they must have had at least one of the following: nausea and/or vomiting, photophobia and phonophobia. Migraine with aura include recurrent attacks of reversible focal neurological symptoms (the aura phase) that usually develop gradually over 5-20 minutes and last for less than 60 minutes. The aura phase often appear as visual disturbances such as fortification spectra, flickering lights, zigzag patterns and scotomas (11, 12) (see figure on the front cover). Aura can also appear as sensory and/or speech disturbances (12). The pain may be felt deeply behind the eye, but more commonly in the frontotemporal region. The pain may also radiate backward to the occiput and upper neck (11) (Figure 1). A migraine attack is said to be divided into several phases, the premonitory phase being the first, followed by an aura phase (in migraine with aura), a headache phase and then a resolution phase. During 1 to 24 hours preceding the actual headache migraineurs can experience changes in mood, alertness and appetite. These changes can include being irritable, depressed, elated, drowsy or thirsty, or even special food cravings. This period is called the premonitory phase. After the headache phase has passed, a feeling of exhaustion and lethargy may remain for a day or two. The mood can also remain changed under this phase of resolution (11, 12).

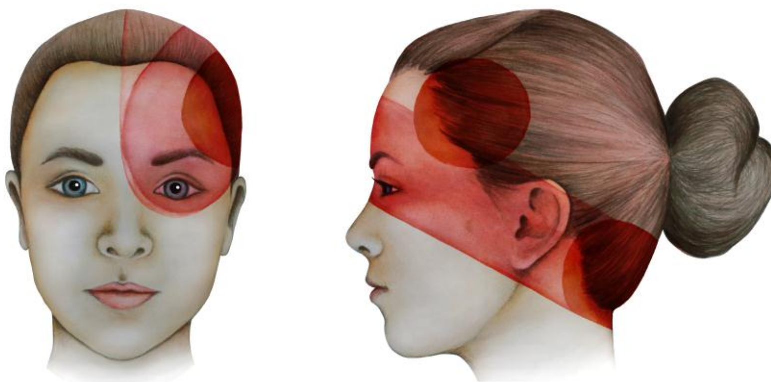

Figure 1: Pain location – Migraine
The pain during a migraine attack appear unilateral and most commonly in the frontotemporal region. It may also appear behind the eye and may radiate backward to the occiput and neck. Here illustrated as red areas.

1.2.1 Migraine pathophysiology

Pathophysiological features of migraine are summarised in Table 2.

Genetic factors

Even though the cause of migraine still is uncertain, it is believed that there is a genetic predisposition for the development of migraine. Several studies clarifies a family history of migraine (most common on the mother's side) (13). Anttila et al. claim to have found the first genetic risk factor for migraine , and a hypothesis is that this 'genetic risk factor' may be involved in regulation of glutamate (14). It is also known that the rare form of migraine, called familial hemiplegic migraine, may be caused by mutations in one of the voltage-gated calcium channel types (15).

Glutamate

Several studies have documented elevated blood glutamate level in migraineurs between attacks and a further increase during attacks (15). It should be mentioned that these findings seem to be stronger in migraineurs with aura than without. Different glutamate receptors have been identified in the trigeminal nucleus caudalis (TNC) of the rat and ionotropic receptor channel blockers seem to be able to block trigeminovascular nociceptive transmission in the trigeminocervical nucleus (TCC). Kainate receptor activation seems important for trigeminocervical transmission after activation of afferents of the dural vasculature, and also dural afferent stimulation after local dural vessel changes (16).

Magnesium deficiency

Magnesium ion is known to gate and block the NMDA glutamate receptor, and therefore play a role in glutamate regulation. The intracellular magnesium ion concentration has been found to be lowered during migraine headache (15). This may indicate a decreased inhibition of glutamate flow through these channels and may be fundamental to an increased cerebral NMDA receptor activity.

Dysfunctional monoaminergic transmission- dopamine and serotonin

The symptoms of the premonitory phase probably arise in the hypothalamus and can be a result of dysfunctional monoaminergic transmission (15). Activation of periaqueductal gray region (PAG), nucleus raphe dorsalis and locus coeruleus (LC) during migraine is consistent with an aminergic dysmodulation in the brainstem (Figure 2).

Migraineurs seem to have both serotonin (5-HT) and dopamine (DA) metabolism disturbances (11, 15). A review article by Peroutka et al. highlights some interesting findings of activation of dopaminergic receptors in migraine (17). Treatment with low doses of the DA agonist, apomorphine, may induce yawning to a higher extent in migraineurs than in healthy

controls. Even higher doses of dopaminergic agonists in animals and humans may induce other premonitory symptoms like irritability, mood fluctuations and sleep disturbances. Treatment with dopaminergic antagonists may reduce migraine-associated nausea and vomiting. These findings suggest a dopamine deficiency in migraine (15, 17).
The 5-HT$_2$ antagonist, pizotyline, may cause euphoria, drowsiness and craving to eat sweet foods, which represent some of the already mentioned premonitory symptoms (11). Injection of reserpine, which depletes the 5-HT depot, have been shown to induce migraine like headaches (15). Serotonine content of blood platelets decrease at the onset of migraine headache and at the same time the urinary secretion of the 5-HT metabolite, 5-IHAA, increase (15). This discharge of platelet serotonine is thought to reflect depletion of serotonine at central synapses, probably in raphe-cortical pathways, and may play a role in inducing migraine headache (15, 18). Different 5-HT receptors that may play a role in migraine headache are 5-HT$_1$ and 5-HT$_2$ sub-types. 5-HT$_{1B}$, 5-HT$_{1D}$ and 5-HT$_{1F}$ receptors are found in the trigeminal system and are thought to have anti-migraine function when activated (15, 16, 19). 5-HT$_{1F}$ receptors are found in the trigeminal nucleus (TN) and the trigeminal ganglion (TG) and 5-HT$_{1D}$ receptors are co-localized with calcitonin gene-related peptide (CGRP) on fibres in the trigeminal tract (15, 16). 5-HT$_{1B}$ and 5-HT$_{1D}$ agonists (triptans) have shown to be effective anti-migraine medications (19). Possibly by mediating vasoactive constriction of dilated cranial vessels and inhibiting vasoactive peptide release in the dura, and thereby neurogenic inflammation. Possibly by inhibiting central pain impulses in the TCC, and also affecting descending impulses from PAG. On the other hand, activation of 5-HT$_{2A}$ receptors on unmyelinated sensory nerve fibres causes pain. 5-HT$_{2A}$ antagonists and medications which downregulate serotonin synthesis in nucleus raphe seem to have anti-migraine effects (19).

The trigeminovascular system – component of migraine pathophysiology?

Nervus Trigeminus is the facial sensory neuron and goes from pons to the trigeminal ganglion (TG), where it divides into three branches: nervus opthalmicus, nervus maxillaris and nervus mandibularis (20). The trigeminovascular system (TGVS) compose of neurons within the trigeminal ganglion, their peripheral projections to the meningeal and cerebral vessels, and their second-order neurons in the trigeminal nucleus caudalis (TNC) in the brainstem (21). It is also speculated that the trigeminal nucleus extend beyond the traditional nucleus caudalis to the dorsal horn at the C_1 and C_2 levels. The entire group of cells is then called the trigeminocervical complex (TCC) (15). For illustration see Figure 2.

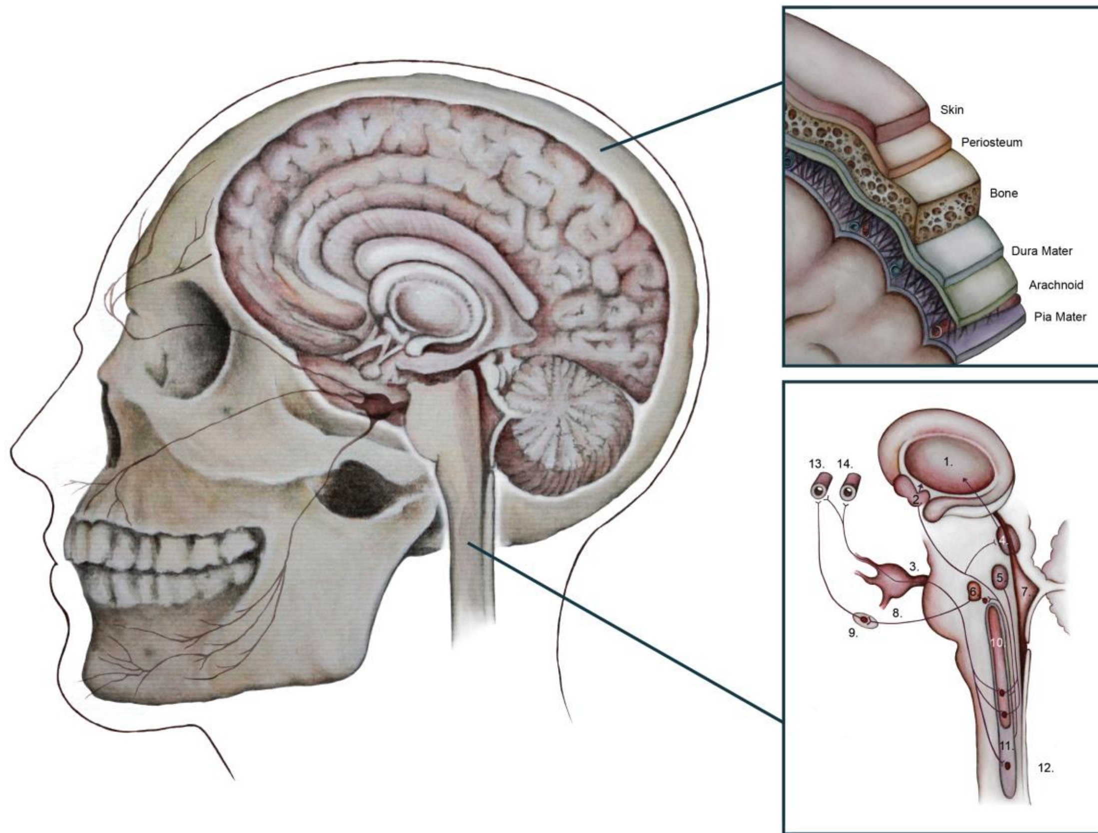

Figure 2: The Trigeminovascular system
This figure illustrates the Trigeminovascular System (TGVS); the Trigeminal Ganglion with its three nerve branches and its connections to different nuclei in the brain stem, to the meningeal vessels and other brain areas. 1. Thalamus. 2. Hypothalamus. 3. Trigeminal ganglion, TG. 4. Periaqueductal gray region, PAG. 5. Locus coeruleus, LC. 6. Superior salivatory nucleus, SSN. 7. The forth ventricle. 8. Greater superficial petrosal nerve, GSP. 9. Sphenopalatine ganglion, SPG. 10. Trigeminal nucleus caudalis, TNC. 11. Trigeminocervical complex, TCC. 12. C_1 and C_2 level of the cervical spine. 13. Dural vessels. 14. Pial vessels.

The trigeminovascular theory concerns the large cerebral vessels, the pial and dural vessels and the large venous sinuses. It also concern the nociceptive afferent fibres (C—fibres and Aδ-fibres) from the ophthalmic division of the trigeminal ganglion, which surrounds the mentioned structures, and their second-order neurons in the brainstem (15, 16), see Figure 2. It is believed that the afferent fibres surrounding the cerebral and meningeal vasculature get activated and sensitized (peripheral sensitization) during migraine attacks (22). Activation of these primary afferents leads to activation and sensitization of second-order afferents in the TCC (central sensitization) and impulses are carried to brain structures involved in pain perception, including thalamus and PAG (15, 22). PAG send both ascending projections to thalamus and descending projections to the synapse between primary and second-order afferents in the dorsal horn (where it modulate nociceptive afferent information) (21). From

thalamus the nociceptive impulses goes to the somatosensory cortex, gyrus cinguli and insula (19).

Calcitonin gene-related peptide

Stimulation of the trigeminal ganglion has shown to lead to a rise in cranial venous levels of calcitonin gene-related peptide (CGRP) and substans P (SP) (23). During migraine, CGRP, but not SP, is elevated in the external jugular vein blood (15, 16, 21). CGRP is a vasoactive neuropeptide synthesized in dorsal root ganglion neurons and is released from peripheral nerve endings in the brainstem and in the meninges (22, 24). As mentioned under section "*Dysfunctional monoaminergic transmission- dopamine and serotonin"* 5-HT_{1D} receptors are co-localized with CGRP on fibres in the trigeminal tract. It is therefore possible that 5-HT in some way is involved in the release of vasoactive substances, among them CGRP (15). Released CGRP from the trigeminal ganglion produce vasodilation of the meningeal vessels and also neurogenic inflammation, as a result of proinflammatory substance secretion in the dura by activated mast cells and other local mononuclear cells (19, 22).

Neurogenic inflammation

Neurogenic inflammation results from activation of primary sensory neurons and their release of inflammatory mediators such as SP and CGRP (25). CGRP receptors relevant to possible neurogenic inflammation in migraineurs, is found in dural mast cells and other mononuclear cells, in the trigeminal ganglion, in second order sensory neurons within the trigeminal nucleus and in arterial smooth muscle cells. Activation of CGRP receptors in arterial smooth muscle cells leads to vasodilation of cerebral and extracerebral arteries (26). Activation of CGRP receptors in dural mast cells result in the release of cytokines and other inflammatory agents (eg. IL-6, TNF-α and NO) (27). In the trigeminal ganglion, CGRP increases its own synthesis and stimulates release of both nitric oxide (NO) and inflammatory cytokines (eg. IL-6, IL-10, IL-1β) from glial cells, but it also seem to decrease the levels of some cytokines (e.g. TNF-α and MCP-1) (23). It is suggested that elevated neuronal CGRP receptors in the trigeminal ganglia may sensitize the trigeminal ganglia when activated by CGRP. That the increased CGRP synthesis and following neurogenic inflammation possibly may promote, sustain and intensify the nociceptive activity during migraine by mediating peripheral sensitization (23, 26).

For a short introduction to inflammation in general and some inflammatory factors, see appendix A and Table 9.

Triggers

Environmental influences are also thought to be involved in determining the susceptibility to migraine. Such environmental influences may be "triggers" like stress, too much or too little sleep, food, hormones and even afferent stimulation like glare, flickering light, noise and smells (13, 28).

Pathophysiology of aura

The origin of visual aura symptoms is the visual cortex and not the retina (11). It is thought that the migraineous aura may be a result of a phenomenon called cortical spreading depression (CSD), where waves of depolarization spread from the occipital lobe and forward over the cortex, at a speed of 2-3 mm/min, immediately followed by prolonged nerve cell depression (15, 29). A brief excitation followed by the depression is thought to be the neurophysiological basis of the sensory symptoms experienced during migraineous aura, and may be a result of enhanced activity in raphe-cortical pathways (29). CSD may be triggered by glutamate, especially by NMDA receptor agonists (29). It may also be triggered by descending afferents from the cerebral cortex (activated by emotion or stress), from thalamus (activated by excessive afferent stimulation such as glare, flickering lights and/or sound) or from hypothalamus (as a response to changes of the body's internal environment) (15). Studies of acute migraine attacks have revealed regional cerebral blood flow (rCBF) reduction, which spread following the cortical surface, from the posterior to the parietal and temporal lobes, at the beginning of the attack (29). The aura symptoms seem to appear during the early phase of the spreading oligemia and cease while the hypoperfusion continue to spread and the headache develops (29). The spreading oligemia lasts for several hours and is then followed by a delayed hyperemia. It does not seem likely that the vascular changes during CSD causes the migraine headache, because the headache phase usually begins while the cerebral blood flow still is diminished and CSD is not associated with migraine without aura (15). It is more likely that these changes may be secondary to neural changes in the brainstem. It is demonstrated that activation of the trigeminal nerve can mediate cerebral, dural and extracranial blood flow to increase, either by directly release of vasodilator peptides or via parasympathetic outflow trough the GSP nerve (Figure 2). Stimulation of LC can induce both cerebral vasoconstriction and extracranial vasodilation, and the nucleus raphe dorsalis can dilate both circulations. The vascular changes may possibly then aggravate the headache by further stimulating the vascular afferents (15).

1.3 Tension-type headache

Patients with tension-type headache (TTH) may have a headache of 30 minutes duration only recurring every few months, to a continuous, unremitting ache present "all day and every day" (30). The headache develops during or after stress (30). Following ICHD, both episodic and chronic TTH must have at least two of the following features: bilateral location, a pressing or tight (non-pulsating) quality, mild or moderate intensity and/or not aggravated by physical activity (12). For pain location, see Figure 3. The headache is not accompanied by nausea and vomiting like migraine, but photophobia or phonophobia may be present (12). The definition chronic headache is when the headache recurs 15 or more days per month (31). Clinical features of TTH are summarised inTable 1.

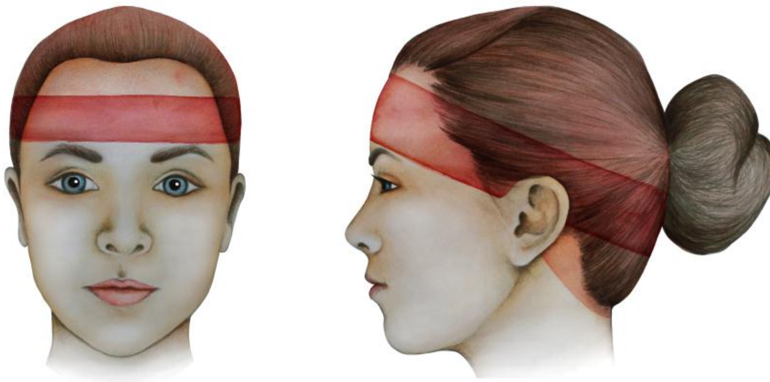

Figure 3: Pain location – Tension-type headache
The pain during a TTH attack appear as a bilateral, pressing or tight bond around the head and neck. Here illustrated as red areas.

1.3.1 Tension-type headache pathophysiology

The ethology behind TTH still seems to be unclear, but it is thought that psychological factors like stress, depression and anxiety may be risk factors for the development (30) (Table 2). As for genetic factors, a Danish family study done on patients with chronic TTH show an increased risk among first- and second-degree relatives (32). Another Danish study done on a population of twins with episodic TTH conclude that the primary cause is environmental, not genetic (33). Stress and anxiety may contribute to increased excitability in central nociceptive pathways as a result of an dysfunctional limbic control of the descending (inhibitory) pain control system (34). It also seem that excessive contraction of neck, forehead and jaw muscles, in addition to a lowered pain threshold in pericranial sites (frontal, temporal and

occipital region), which indicate a central deficit of pain control by the brain's endogenous pain-control system, is of great importance (30). Some studies, summarized by Ashina et al. 2005, indicate presence of sensitization at the level of the spinal dorsal horn and the trigeminal nucleus (central sensitization) in patients with chronic TTH, but not in episodic TTH (34). It is suggested that sustained muscle activity may be sufficient to induce peripheral sensitization of muscular nociceptors in patients with TTH (34). One hypothesis, which has been challenged, is that release of inflammatory mediators from muscle afferents induces the peripheral sensitization (34, 35). According to Lance et al. 2005 it does not exist any evidence that sustained vasoconstriction may cause tension-type headache nor aggravate the headache like it possible may for migraine headache (30). In contrast with migraine, plasma levels of CGRP and glutamate have been reported to be normal in patients with TTH (34).

1.4 Cluster headache

Cluster headache is defined as a severe piercing and strictly unilateral head or facial pain (36). The pain has a tendency to appear in bouts, usually recurring from once every other day to 8 times a day for a period of weeks or months, separated by intervals of complete freedom for months or even years (12, 36). The attacks usually appear at a precise time of day or night (36). Following ICHD, a patient with cluster headache must have at least 5 attacks fulfilling these criteria: severe or very severe unilateral orbital, supraorbital, and/or temporal pain, lasting 15-180 minutes (12). The headache must also be accompanied by at least one of the following: ipsilateral conjunctival injection and/or lacrimation, ipsilateral nasal congestion and/or rhinorrhoea, ipsilateral eyelid oedema, ipsilateral forehead and facial sweating, ipsilateral miosis and/or ptosis, and/or a sense of restlessness or agitation (12). Contrary to migraine most of the cluster headache sufferers are men and the pain may be relieved by pacing up and down (36). See Figure 4 for pain location and Table 1for clinical features of cluster headache.

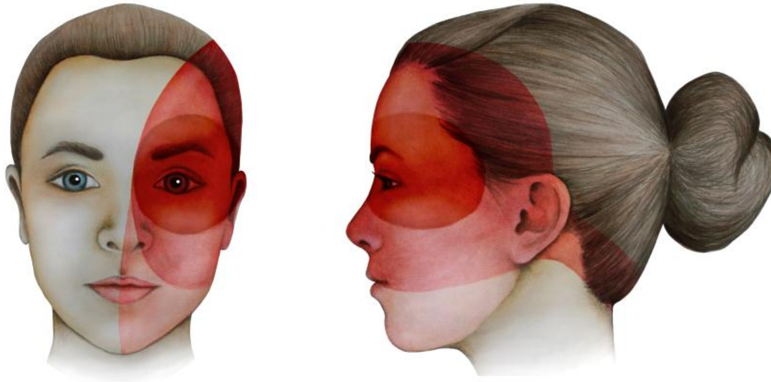

Figure 4: Pain location – Cluster headache
The pain during a cluster headache attack appear unilateral and in the orbital, supraorbital, and/or temporal region. The pain may also spread back to the neck. Here illustrated as red areas.

1.4.1 Cluster headache pathophysiology

There may be a genetic predisposition for the development of cluster headache, but more research is needed on this topic (37). Cluster headache pathophysiology is believed to consist of three major features: 1. Episodic pattern of the attacks; 2. Cranial autonomic features; and 3. Trigeminal distribution of the pain (36). Lance and Goadsby (2005) give us a review over articles and their general conclusion on this topic (pathophysiological features of cluster headache are summarised in Table 2). The main conclusions is that the ipsilateral hypothalamic gray matter is likely to be the primary driving area for cluster headache and that the pain and the autonomic phenomena may be mediated by suddenly discharge of central trigeminal, parasympathetic and sympathetic pathways (36). The daily rhythm of the attacks, activation in the ipsilateral hypothalamus during attacks and an increase in hypothalamic gray matter in patients with cluster headache suggest the involvement of the hypothalamus and the suprachiasmatic nucleus (SCN, which control the circadian rhythm) (36). Hormonal changes such as lowering levels of melatonin and testosterone, and rising levels of cortisol, prolactin, platelet serotonin and blood histamine are also indicators of hypothalamus involvement in cluster headache (36). Several aspects indicate a postganglionic sympathetic lesion and a resulting denervation super sensitivity of receptors in patients with cluster headache (36). Examples are: 1. Partial Horner's syndrome, like ptosis and miosis. 2. Deficient pupillary dilation in response to tyramine (releases noradrenalin (NA) from nerve terminals). 3. Excessive pupil dilation in response to phenylephrine (sympathomimetic agent). 4. Lack of

sweating on the medial aspect of the forehead (mediated by sympathetic fibers derived from the internal carotid artery. All are ipsilateral to the affected side.
The partial Horner's syndrome may be explained by edema of the wall of the internal carotid artery comprising postganglionic sympathetic neurons in the perivascular plexus (36). It has been demonstrated narrowing of the lumen of the internal carotid artery, just beyond its entry to the skull, at the height of an attack of cluster headache and the effect persisted after the pain had ceased. At the same time the ophthalmic artery was dilated, which suggest that the distension of the arterial wall may be caused by vasodilator peptides released from the internal carotid ganglion cells, activated by discharge along the GSP nerve (Figure 2), causing lacrimation and conjunctival, nasal and cutaneous vasodilation (36). Vasoactive intestinal peptide (VIP), a marker of cranial parasympathetic activation, is elevated during attacks (36). Other disorders involving the internal carotid artery are characterized by pain localized to the eye, which also is the case for cluster headache (36). An edema of the wall of the internal carotid artery may be a plausible cause of the partial Horner's syndrome, though maybe not of the pain itself since the effect observed by Ekbom and Greitz (1970) persisted after the pain had ceased (36). The ophthalmic division of the trigeminal system dilates the internal carotid artery during trigeminal pain (36). The vasodilation observed during cluster headache may therefore be a secondary phenomenon, a vasodilator reflex mediated by the trigeminal nerve as the afferent limb and the GSP nerve as the efferent one, which also may be the case for migraine (36). Cranial levels of CGRP, a marker for trigeminal activation, are elevated during cluster attacks (36). Findings that compression of the upper cervical spine, at the level of C_1 and C_2, causes episodic pain with lacrimation and redness of the eye suggest that afferents in upper cervical roots also may play a part in the pain generation during cluster headache attacks (36).

Table 1: Clinical features of primary headaches

	Migraine	**Cluster headache**	**Tension-type headache**
Genetic background	Yes	Unknown	Yes/No (chronic/ episodic)
Sex ratio (F/M)	1,70	0,20	1,58
Location	Normally unilateral, frontotemporal	Unilateral, orbital and/or temporal	Bilateral (like a bond around the head)
Quality	Pulsating	Piercing	Pressing or tight (non-pulsating)
Pain intensity	Moderate or severe	Severe or very severe	Mild or moderate
Precipitating/risk factors	Stress, unregular sleep, food, hormones, glare, flickering lights, noise, smells	Alcohol	Stress, depression, anxiety, excessive muscle contraction in neck, forehead and jaw
Frequency	From 1 attack in a lifetime to 1 attack almost every day	Bouts of 1 attack every other day to 8 attacks a day	From 1 attack every few months to almost every day
Duration	4-72 hours/attack	15-180 min/attack	30 min/attack to a continuous headache
Symptoms accompanying attack	Nausea/vomiting, photophobia, phonophobia	Conjunctival injection, lacrimation, nasal congestion, rhinorrhoea, eyelid oedema, forehead and facial swetting, miosis, ptosis	
Aura/visual disturbances	~ 10%	No	No
Physical activity	Aggravate pain	Relieve pain	Do not aggravate pain

Table 2: Patophysiological features of primary headaches

	Migraine	Cluster headache	Tension-type headache
Hypothalamus involvement	Yes	Yes	No
Involvement of upper cervical spine (C1 and C2 level)	Possible	Possible	Yes
Vascular component	Yes	Yes	No
Parasympathetic involvement	Probably	Yes	No
Sympathetic involvement	No	Yes	No
Central sensitization	Yes	No	Yes
Peripheral sensitization	Yes	No	Possible
Hormonal imbalance	↓ Dopamine	↓ Melatonine and Testosterone ↑ Cortisol, Prolactin and Histamine	No
Serotonine	↓	↑	Normal
CGRP	↑	↑	Normal
Glutamate	↑	Normal	Normal
Inflammatory component	Probably	Probably	Unknown

1.5 Headache, inflammation and cytokines

Today there are no biomarkers for differentiation of headache syndromes. Diagnosis is made by clinical examination, excluding organic causes of headache and following clinical criteria. Basing the diagnosis on just clinical symptoms can make it difficult to distinguish between the different headache syndromes, specially between migraine and TTH. If it is possible to find specific biomarkers for the different headache syndromes it may help distinguish them from each other and hence, make it easier to give a proper diagnosis and targeted treatment.

There are reasons to believe that there is an inflammatory response during migraine and cluster headache attacks. Both of them are associated with blood serotonin and CGRP changes and both of them have a vascular component. On the other hand there are no reasons to expect an inflammatory response of significance during tension-type headache attacks. This type of headache is believed to only consist of a neurologic component and is not associated with blood serotonin and CGRP changes. See Table 2 for some of the pathophysiological features of the three mentioned headache syndromes.

Cytokines possess multiple biologic activities that promote cell-cell interaction and play an important role in immunology, pain and inflammation (24) (see Table 9, appendix A). Several studies have linked different cytokines to migraine pathogenesis (Table 3), but the results have been conflicting. In addition, other authors indicate a possible contribution of TNF-α and IL-1β gene polymorphism to migraine headache (38).

Cytokines are of interest for this study as possible biomarkers that can help differentiate the three mentioned headache syndromes. Studies have shown that some cytokines, such as IL-6, TNF-α and IL-1, induce dose-dependent hyperalgesia, and may therefore be considered as pain mediators in neurogenic inflammation (39, 40). IL-10 has been shown to inhibit hyperalgesic responses to TNF-α, IL-1β and IL-6, whereas IL-4 only to TNF-α (41, 42).

It is of interest to compare samples from the different headache groups with low back pain patients and healthy, pain free individuals. Lumbago, or low back pain, defined as back pain located in the lumbar region (between the bottom of the ribcage and above the legs), is considered to be a very painful condition (43). By comparing samples from headache and low

back pain patients, it may be possible to define cytokines as an inflammatory response to pain in general or to the different painful conditions.

Table 3: Cytokines and migraine

This table lists some of the migraine studies that have covered the topic “cytokines and migraine”. Control subjects are healthy, pain free individuals, if not otherwise noted. *Control subjects are patients with TTH, **During attack vs. outside attack. (24, 44-49)

Authors	Cytokines	Headache syndrome	Samples
Munno et al. 1998	Elevated IL-4 and IL-5, no significant difference of IL-10 and IFN-γ	Migraine without aura	Plasma, outside attack
Fidan et al. 2006	Elevated IL-10 and IL-6, no significant difference of IL-1β, IL-2, TNF-α and IFN-γ	Migraine with and without aura	Serum, during attack
Sarchielli et al. 2006	Elevated TNF-α, IL-6 and IL-1β, decreased IL-4	Migraine without aura	Plasma, during attack
Bockowski et al. 2009	No significant difference, but a tendency of elevated IL-1α and TNF-α*	Migraine with and without aura (children)	Plasma, outside attack
Perini et al. 2005	Elevated IL-10, TNF-α and IL-1β, no significant difference of IL-6, IL-4 and IL-2**	Migraine with and without aura	Plasma, during and outside attack
Munno et al. 2001	Elevated IL-10, undetectable levels of IL-4, IL-5 and IFN-γ	Migraine without aura	Plasma, during attack
Empl et al. 2003	No significant difference of TNF-α and IL-6	Migraine with and without aura	Serum, during attack

1.6 Enzyme-linked immunoassay techniques

For studying cytokines, enzyme-linked immunoassay techniques, such as ELIspot assay and ELISA multiplex cytokine assay, will be used. Both assays are solid phase protein assays using membrane or beads as the solid support. They are based on enzyme-linked immunoassay techniques using primary protein-specific capture antibodies that bind to the protein of interest (cytokines in our case) and secondary biotinylated detector antibodies, which also bind to the protein of interest, making a complex. Streptavidin, conjugated to an enzyme or a fluorescent protein, which bind to the biotinylated detector antibody, is also used

for ELISpot and multiplex ELISA respectively (Figure 5 A.). A substrate solution for development is also needed using the ELISpot assay (Figure 5 B.). A development step is not needed for ELISA; the fluorophore quantity is measured directly. (50, 51).

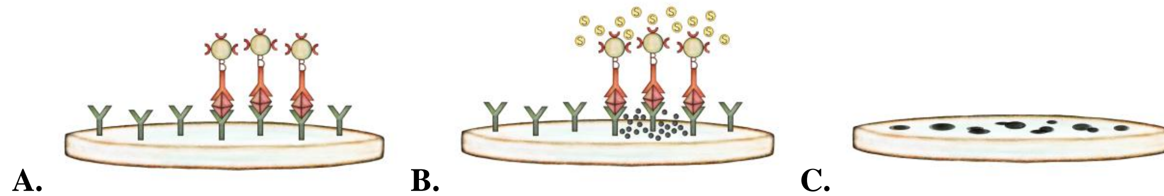

Figure 5: Enzyme-linked immunoassay techniques
A. The ELISpot assay uses a membrane as the solid support. Bound protein-specific capture antibodies (green Y) make a complex with cytokines (red), biotinylated detector antibodies (orange Y + B) and enzyme conjugated Streptavidine (circle with red Cs on). **B.** For development, a substrate solution is added (S). The enzyme convert the substrate to a colored precipitate (dark blue). **C.** Dark spots emerge on the membrane. Each spot represent a cytokine secreting cell.

1.7 Objectives

This thesis is considered to be a pilot study and the objectives are:

1. Identify cytokines as possible specific biomarkers for migraine, cluster headache and tension-type headache
2. Define cytokine secretion as possible inflammatory response to pain
3. Evaluate the use of ELISA assay and ELISpot assay for this purpose
4. Evaluate strengths and flaws of this pilot study and suggest improvements for future studies

We do hope this study, and further studies on this topic, will contribute to increased understanding and knowledge about the physiological processes involved in the development of headache, and valuable knowledge about headache that later can turn out to be useful regarding treatment

2. Methods and materials

2.1 Study preparations

2.1.1 Applications

This master's thesis project in Human Physiology was approved by the Program Committee, Institute of Biomedicine, Faculty of Medicine and Odontology, University of Bergen autum 2009. Project application was accepted by the Regional Committee of Ethics (REK). A research protocol describing the project title, introduction, aims, patient selection and methods was included.

2.1.2 Patient selection

The original plan was to include 20 patients with migraine, cluster headache and tension-type headache respectively, and two control groups consisting of 20 low back pain patients and 20 healthy, pain free individuals.

All patients with a severe acute headache or low back pain, hospitalized at the Department of Neurology, Haukeland University Hospital, during February 2010 to February 2011, were asked to participate.

Due to the limited timespan of the study, only 15 patients with headache and 7 with low back pain agreed to participate and gave their written consents.

21 male divers at the age of 20-25 from the Norwegian Navy, already participating in another ongoing study at Haukeland University Hospital, were included in the healthy, pain free group. The group was not age and gender matched. Previous studies have not found any correlation between age, gender and cytokine production in migraineurs (24, 48, 52).

2.1.3 Examination, diagnose and exclusion

Clinical examination and diagnosis were made by neurologists at the Department of Neurology, Haukeland University Hospital. Headache patients were personally interviewed about the on-going headache attack and their case history using questionnaires based on the

diagnostic criteria from the International Classification of Headache Disorders (ICHD), 2nd edition (12) (for questionnaires see appendix B and C). Diagnoses were confirmed and clinical data, such as history of other diseases and the use of medications, were known using these questionnaires. Patients were excluded from the study if they had other inflammatory diseases, if they had low back pain and headache, combined headache, or if they had other underlying organic causes of headache than migraine, cluster headache and tension type headache. A different questionnaire was used for the low back pain patients to rule out any history of headache or other inflammatory diseases. Medical screening of the healthy pain free individuals was already done by the Norwegian Navy prior to the study and questionnaires about their medical condition were filled out. There were no reasons not to include them in the healthy pain free control group.

Of the 15 headache patients, 11 were diagnosed with migraine. Patients excluded from the study were two with tension type headache, one with combined headache (tension type headache + migraine) and one with brain hemorrhage. No patients with cluster headache were hospitalized during the period of patient recruitment. Some of the clinical data regarding the migraine and low back pain patients are listed in Table 4.

Table 4: Clinical data – migraineurs and low back pain patients
This table lists some of the clinical data from the questionnaires. *Medications taken were triptans, opioids and NSAIDs.

Diagnosis	N	Sex (F/M)	Age, min – max (median)	History of headache (%)	Family history of headache N (%)	Frequency of headache attacks (days per month)	Medications* taken prior blood sampling N (%)
Migraine	11	10/1	16-66 (43)	100	7 (64)	2-15	6 (55)
Low back pain	7	5/2	32-58 (53)	0	5 (71)	0	7 (100)

2.2 Sample preparations

Blood samples were collected by bioengineers at the Laboratory for Clinical Biochemistry and serum was isolated and stored at the Laboratory for Neurological Research, Department of Neurology, Haukeland University Hospital, if not otherwise noted.

2.2.1 Blood sampling

Blood samples were collected from patients at different periods of time. The first blood samples were collected at the time of hospitalization while patients still were in intense pain. The second blood samples were collected 1- 2 months after hospitalization, during a pain free period. The patients had been pain free for at least two days. Blood samples from the healthy, pain free divers were collected, and serum isolated and stored, prior to this study. The divers had not been training or diving, and had no complaints of headache or pain for at least two days before sampling.

2.2.2 Serum isolation and storage

For each patient 2 x serum separator gel tubes (catalog no. 367957, BD Biosciences, Trondheim, Norway) were filled with blood (Table 5). These tubes contain clot activator (silica particles that coat the walls) and serum separator gel (53). In order to allow coagulation the samples were stored upright at room temperature (20˚C) for 30 minutes. The samples were then centrifuged at 3000 *rpm* for 10 minutes. The serum separator gel isolate serum from blood cells by forming a physical barrier between the two during centrifugation (53). After centrifugation, serum (the upper layer) was transferred into cryotubes and stored at -80°C until analysis.

Table 5: Serum samples
Serum samples were isolated for all participating patients, but not all of the patients gave blood both during attack/pain period and attack free/pain free period.

Group	Attack/pain period	Attack free/pain free period	Both periods
Migraine	1	2	8
Low back pain	3		4
Healthy, pain free		21	

2.2.3 Peripheral blood mononuclear cell isolation

Isolation of peripheral blood mononuclear cells (PBMCs) was performed according to the manufacturers description (54).

For each patient 3 x cell preparation tubes (CPTs) (catalog no. 362780, Puls AS, Oslo, Norway) were filled with blood (Table 6). These tubes contain sodium heparin anticoagulant and blood separation media. The blood samples were stored upright at room temperature (20˚C) until centrifugation. PBMCs were isolated by centrifugation at 1650 *g* for 20 minutes at 20°C. During centrifugation the gel portion of the media moves and separates PBMCs and platelets from the denser blood components below (Figure 6) (54). Blood plasma (upper yellow solution) was transferred to a 15 ml centrifuge tube and stored at - 20˚C. PBMCs (whitish layer) were transferred to a 15 ml centrifuge tube, washed and cryopreserved at - 80°C. The PBMCs were washed twice with sterile phosphate buffered saline (PBS) (the Production Laboratory, Haukeland University Hospital), and centrifuged at 300 *g* for 10 minutes at 4°C, to reduce the quantity of platelets present, resulting in a suspension of concentrated mononuclear cells (54).

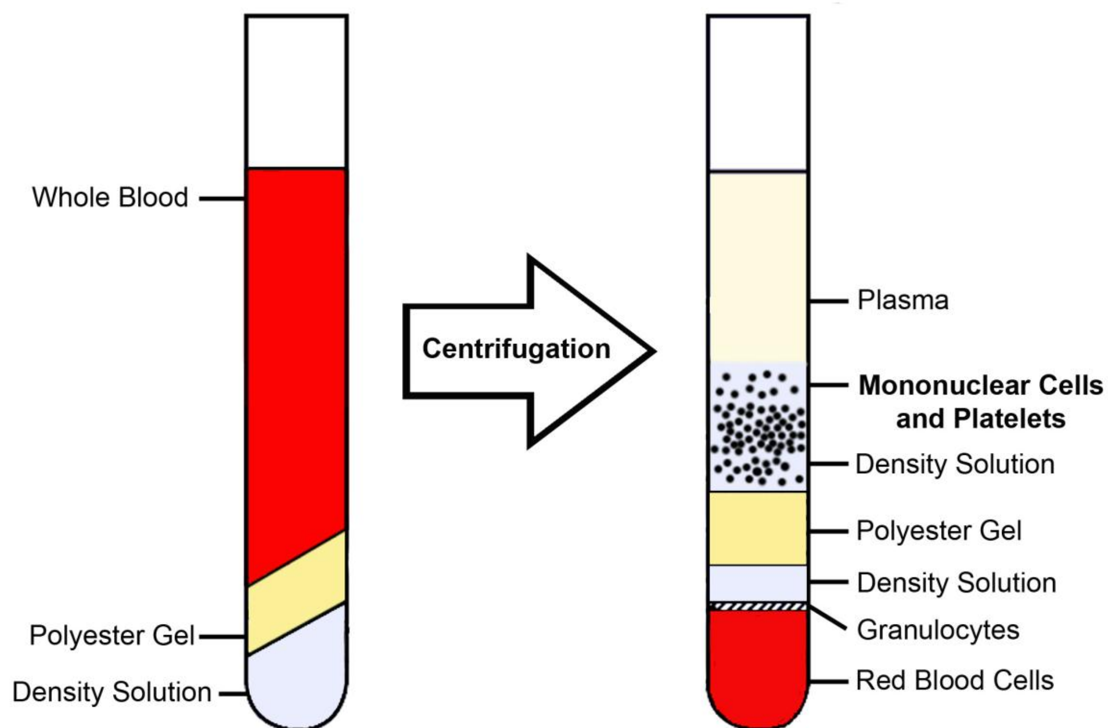

Figure 6: Isolation of peripheral blood mononuclear cells

Table 6: PBMC samples
PBMC samples were only isolated for five migraine patients. One of the patients did not give blood during both the attack and the attack free period.

Group	Attack/pain period	Attack free/pain free period
Migraine	4	5

2.2.4 Cryopreservation and storage of PBMCs

The isolated PBMC samples were transferred to cryotubes and freezing media containing 90% (900µl) Fetal Bovine Serum (FBS) (catalog no. A15-152, PAA The Cell Culture Company, Cölbe, Germany) and 10% (100µl) cryoprotectant, DimethylSulfoxide (DMSO) (catalog no. 1.02931.0500, VWR, Oslo, Norway), was added. Addition of freezing media is to avoid damage of cells due to lethal ice crystal formation and osmotic injury during freezing (55). In order to avoid intracellular freezing, the PBMC samples were gradually brought to lower temperatures (~ 1°C/min) by using an alcohol-free cool cell box (catalog no. 479-0492, VWR, Oslo, Norway) the first 24 hours at -80˚C (55). The tubes were transferred to a cryobox (catalog no. 479-0111, VWR, Oslo, Norway) for further storage (1-2 weeks) at -80˚C.

2.2.5 Thawing of cryopreserved PBMCs

It is important to thaw cryopreserved PBMC samples rapidly to minimize osmotic variations due to slow thawing and also to avoid the toxic effect of DMSO (55). The PBMC samples were thawed by adding room tempered cell media (RPMI 1640 supplemented with Glutamine (catalog no. 12-702F, Lonza, Walkersville, USA), containing 10 % heat inactivated FBS (catalog no. A15-152, PAA The Cell Culture Company, Cölbe, Germany) and 1 % Streptomycin Penicillin (S/P) antibiotics (catalog no. P4333, Sigma-Aldrich, Steinheim, Germany)). Thawed PBMC samples were transferred to 15 ml centrifuge tubes, washed in cell media and centrifuged at 300 *g* for 15 minutes at 4˚C. After centrifugation, supernatant was removed (not entirely to avoid drying out the cells) and the pellet re-suspended in 2 ml cell media. Depending on pellet size after isolation, 1.25-10 µl of PBMCs, suspended in cell media, was diluted (1:8000 – 1:1000) in 10 ml of filter sterilized CASYton (catalog no. 05651808001, Roche Applied Science, Oslo, Norway). Viable PBMCs were counted using a

CASY cell counter and analyzer (Roche Diagnostics, Oslo, Norway). The rest of the PBMC samples were washed once more and the pellet re-suspended in 2 ml cell media.

2.3 Analysis

In order to look for different inflammatory markers for migraine, the frequency of cytokine producing cells and blood cytokine levels were determined by analysing peripheral blood mononuclear cell (PBMC) samples and serum samples. Analyses were done by using human ELISpot assay kits (catalog no. 3430-2HW-Plus, 3520-2HW-Plus and 3510-2HW-Plus, Mabtech, Nacka Strand, Sweden) and human cytokine 25-plex assay kit (catalog no. LHC0009, Invitrogen, Paisley, UK). The ELISpot assays were scanned using an automated ImmunoScan Plate Reader (CTL-Europe GmbH, Bonn, Germany) and the frequency of cytokine producing cells determined by using ImmunoSpot Analysis software (CTL-Europe GmbH, Bonn, Germany). The cytokine 25-plex ELISA assay was measured on a Luminex® 100™ system (Luminex, Austin, TX) and the cytokine levels determined by using StarStation software (Applied Cytometry Systems, Dinnington, Sheffield, UK).

As a "cytokine fishing expedition" all serum samples (Table 5) were analysed using the ELISA 25-plex cytokine assay kit. Mean cytokine levels were compared within and between the different groups; migraine, low back pain and healthy, pain free. Due to the limited time span and the low budget of this study, only samples from five headache patients were analysed using the ELISpot assay (Table 4). The frequency of IL-17 and TNF-α secreting cells was compared within the group, attack vs. attack free.

2.3.1 ELISpot assay

ELISpot assays for human interleukin-10 (IL-10), interleukin-17 (IL-17) and tumor necrosis factor α (TNF-α) were performed according to the manufacturer's descriptions, with minor modifications (56-58). Instead of using the PVDF-based membrane plates from the kits, MultiScreen HTS HA plates (catalog no. MSHAN4550, Millipore, Oslo, Norway) were used. This was done to avoid the critical activation step needed for the PVDF-based membrane plates. A mock experiment was carried out to compare the different plates.
The assay protocols for the different cytokines were virtually identical, with minor differences in recommended cell number per well and use of polyclonal activators for positive assay controls. Negative assay controls (wells without coating and wells without cells) were also

applied for each sample. Layouts (not shown) and all dilutions for the IL-10, IL-17 and TNF-α assays were made according to the manufacturer's descriptions and personal recommendations from Gun Kesa, PhD Research Scientist at Mabtech AB (56-58).

DAY 1 - Coating

100 µL/well of coating antibody solution was added in appropriate wells and incubated overnight (14-24 hours) at 4˚C. Coating antibodies were diluted in sterile PBS and calculations of volume PBS and antibody needed were done according to equation 1 and 2.

Equation 1: Total volume PBS/media needed for all wells

Total volume PBS/media needed for all wells = (number of wells) x (µL/well)

Equation 2: Volume antibody/stimulant needed

$$C_1 \times V_1 = C_2 \times V_2$$

C_1 = Stock concentration (µg/ml)

V_1 = Volume antibody/stimulant needed (ml)

C_2 = Needed concentration (µg/ml)

V_2 = Total volume PBS/media needed for all wells (ml)

DAY 2 - Plating

The plates were blocked with 250 µL/well of cell media for 2 hours at 37°C in a 5% CO_2 incubator (Binder, Tuttlingen, Germany). Cell suspensions of different concentrations (cells/mL) (Table 7) were made by diluting the isolated PBMC sample in cell media. Calculations of volume isolated PBMC sample needed were done according to equation 1, 3 and 4.

Equation 3: Number cells needed per mL

Needed number cells/mL = Number cells/well x Number wells/mL

Equation 4: Total volume isolated PBMC sample needed for all wells

Volume isolated PBMC sample needed (mL) = (total volume needed (mL) * number cells needed/mL) / (counted number viable cells/mL)

Table 7: Cell suspensions

Concentration of the different cell suspensions added in each well is expressed as cells/ml. A concentration of $1x10^6$ cells/ml gives $1x10^5$ cells/well.

	TNF-α (cells/ml)	IL-10 (cells/ml)	IL-17 (cells/ml)
Non stimulated	2.5×10^6	2.5×10^6	5×10^6
	1.25×10^6	1.25×10^6	2.5×10^6
Stimulated	5×10^5	1×10^6	2×10^6
	2.5×10^5	5×10^5	1×10^6

After blocking, 100 µl/well of diluted cell suspension (Table 7) was added to the appropriate wells. For the positive assay control (stimulated) wells only 50 µl/well of cell suspension, double the wanted concentration (cells/mL), was added. Then 50 µl/well of stimulant diluted in cell media, double the wanted concentration, was added in the same wells giving a total of 100 µl/well. For IL-10 and IL-17 a dilution of 1:1000 (1µl/ml) of the polyclonal activator anti-CD3 mAb was used. For TNF-α, the polyclonal activator phytohemagglutinin (PHA), with a concentration of 5 µg/ml, was used. Calculation for the PHA dilution was made according to equation 1 and 2. The plates were then incubated overnight (14-24 hours) at 37˚C in a 5% CO_2 incubator.

DAY 3 - Development

The plates were washed with sterile PBS followed by 5 washes with PBS containing 0.05% Tween 20 (PBS-T). For the last 2 washes the plates were soaked for 2-5 minutes and filtered using an Empore™ 96-well Vacuum Manifold (catalog no. 66879-U, Sigma-Aldrich, Steinheim, Germany). PBS-T was made by dissolving one PBS tablet (catalog no. 09-9400-100, Medicago AB, Uppsala, Sweden) per litre sterile water, and adding 0.05% Tween20 (catalog no. 8.22184.500, VWR, Oslo, Norway).

After washing, the plates were incubated for 2 hours at room temperature (20˚C) with 100 µl/well of detection antibody solution. To avoid non-specific spot formation, due to protein aggregates, during development, the detection antibody solution was filtered through a 0,2µm filter (catalog no. 514-4126, VWR, Oslo, Norway) (59). The detection antibody solution was made by diluting the antibody in PBS containing 0.5% FBS. Calculations of volume detection antibody needed were made according to equation 1 and 2.
After incubation, the plates were washed and 100 µL/well of Streptavidine-HRP diluted in PBS-0.5% FBS was added. The plates were incubated for 1 hour at room temperature (20˚C).
After incubation, the plates were washed and 100 µl/well of the ready-to-use tetramethylbenzidine (TMB) substrate solution was added. Development time, time until distinct spots emerged, differed for the different cytokines (usually between 10 to 40 minutes). TNF-α was the fastest to develop.
The development process was stopped by rinsing the plates under running water. The plates were tapped dry on paper in between and rinsing was repeated until no more soap remained. The plates were allowed to dry in the dark for 2 days at room temperature (20˚C).

DAY 5 - Counting
The fifth day, after punching out membranes on clear sealing tape (catalog no. 95.1994 and 82.1586, Sarstedt, Ski, Norway), membranes were scanned and spots were counted. Each spot represented a cytokine secreting cell. To avoid fading after scanning, the membranes were covered with aluminium foil and stored at room temperature (20°C) in the dark.

2.3.2 Cytokine 25-plex ELISA assay

Cytokine 25-plex ELISA assay was performed according to the manufacturer’s descriptions (51). All headache samples and low back pain samples were analyzed in one experiment. The healthy pain free samples were analyzed in a separate experiment together with other samples from the other ongoing study at Haukeland University Hospital. All standards were run as duplicates and samples as singles, allowing 80 samples per plate.

All samples were allowed to thaw and all reagents to warm to room temperature (20°C) for approximately 30 minutes. Dilutions were made according to the manufacturer’s descriptions (51).

1x working wash solution was prepared by diluting concentrated wash solution with deionized water (1:20) and mixed until completely dissolved. To avoid unnecessary clotting of wells, assay diluent was centrifuged at 3000 *min-1* for 10 min and the pellet (salt precipitate) was discarded. One 16-plex and one 14-plex standard vial were reconstituted by adding 0.5 mL assay diluent in each vial. The solutions were then combined, making standard 8 (1:1). Serial dilutions were made by diluting 300 µL of standard 8 in 600 µL assay diluent, making standard 7 (1:3). 300 µL of standard 7 was then diluted in additional 600 µL assay diluent, making standard 6 (1:9) and so on (making 8 different standard dilutions, with standard 1 diluted 1:2187).

All 96 wells were pre-wet by adding 200 µL of working wash solution. The antibody bead solution was mixed by gently vortexing and then diluted 1:4 in working wash solution and sonicated for 30 sec. Dilution was done to easier distribute the beads more equal in each well.

After each incubation, the solution added was aspirated off from the bottom of the plate by using a multiscreen vacuum manifold (catalogue no. MSVMHTS00, Millipore, Oslo, Norway), and the beads washed. After each washing step, the bottom of the plate was carefully blotted on clean paper towels until completely dry, to avoid leakage when adding new solutions.

100 µL of the diluted bead solution was added into each well and washed twice with 200 µL/well of working wash solution. To protect the beads from light, a black plate cover was used. 50 µL/well of incubation buffer was added into each well and 100 µL/well of standard dilutions were added into appropriate wells. 50 µL/well of assay diluent, followed by 50 µL/well of sample, was added to appropriate wells. The plate was then incubated in the dark at room temperature (20°C) on an orbital shaker for 2 hours. Lipemic samples (whitish colour) were centrifuged at 2000 *g* for a couple of minutes in a micro-centrifuge (Galaxy Mini, 521-2812, VWR, Oslo, Norway) and the lipid layer discarded. Standard dilutions were stored at -20°C overnight for the next experiment.

1 mL of 10x biotinylated antibody concentrate was diluted in 10 mL of biotin diluent (1:11). After two washing steps, 100 µL/well of the diluted biotinylated anibody solution was added. The plate was incubated for 1 hour.

1 mL of 10x R-Phycoerythrin conjugated Streptavidin (Strep-RPE) concentrate was diluted in 10 mL Strep-RPE diluent (1:11). After two washing steps, 100 µL/well of the diluted Strep-RPE was added. The plate was incubated for 30 minutes.

After the last incubation, the beads were washed three times and re-suspended in 100 µL/well of working wash solution. The samples were then analysed with a Luminex® 100™ instrument (Luminex, Austin, TX) and the concentration of the samples (pg/mL) were determined from the standard curve using StarStation 2.3 software (Applied Cytometry Systems, Dinnington, Sheffield, UK).

2.4 Statistics

Statistical analyses were performed using GraphPad Prism 5 Software, demo version (http://www.graphpad.com/demos/).

Changes in frequency of cytokine secreting cells, during migraine attack and attack free periods, were analyzed using the Wilcoxon matched-pairs signed rank test, and between cytokine groups (TNF-α, IL-17 and IL-10) using the Kruskal Wallis test. Dunn's Multiple Comparison test was used as post hoc analysis.

Cytokine levels in serum were compared, between migraine, low back pain and healthy, pain free groups, using the one-way analysis of variance (ANOVA). Changes in cytokine levels, during migraine attack and attack free periods, and during low back pain and pain free periods, were analyzed with the two-tailed paired Student's t-test. The Tukey Multiple Comparison test was used as post hoc analysis. Differences were accepted as statistically significant at $p<0.05$.

3. Results

3.1 ELISpot assay

PBMC samples were isolated from five migraine patients (Table 6). The samples were analyzed for TNF-α, IL-10 and IL-17 secreting cells using ELISpot assay kits. Results are illustrated in Figure 8. Mean frequency of TNF-α and IL-17 secreting cells were compared within the migraine group, during attack and attack free periods respectively.

3.1.1 PVDF-based membrane plate vs. HTS HA plate

Figure 7 shows differences seen comparing the use of HA plate with the use of PVDF-based membrane plate. Both plates were coated and plated following the same layout.

The PVDF-based membrane plate developed a much darker background color during development (Figure 7A-C) than the HA-plate (Figure 7 D-F). For IL-10 and IL-17 there were no big differences in mean cytokine secreting cells for the two plates (Figure 7, B vs. E and C vs. F). Even though a big difference in mean TNF-α secreting cells detected was observed for the two plates (Figure 7A vs. D), the mean detected using the HA plate was still high (Figure 7 D).

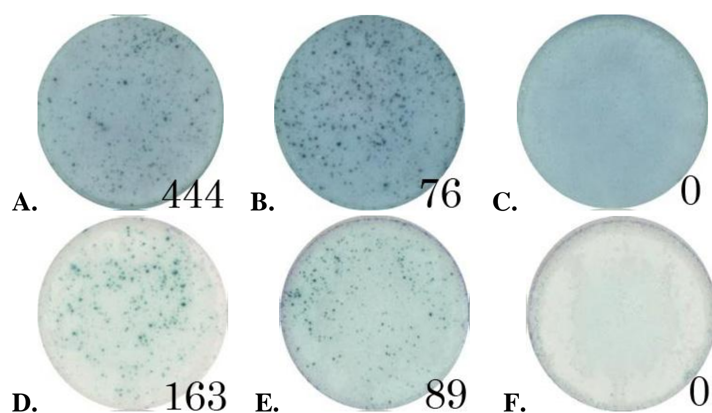

Figure 7: PVDF-based membrane plate vs. HTS HA plate
A-C. PVDF-based membrane plate. **A**. TNF-α. **B**. IL-10. **C.** IL-17. **D-F**. HTS HA plate. **D.** TNF-α. **E**. IL-10. **F.** IL-17. All figures are marked with their mean cytokine secreting cell number per 100.000 cells.

3.1.2 Positive controls

Results for the positive control wells are listed in Table 8.

For TNF-α and IL-17, the test system functioned properly for 8 of 9 samples (positive mean).

For IL-10, the test system functioned properly for 5 of 9 samples (positive mean).

Table 8: Positive controls

Each patient sample was stimulated with a polyclonal activator as a control of the test system's functionality. A positive mean of cytokine secreting cells per 100.000 cells in the control wells, indicated a functional test system.

Patient	TNF-α	IL-10	IL-17
p.1 (attack)	positive	positive	positive
p.1 (attack free)	positive	negative	positive
p.6 (attack)	positive	positive	positive
p.6 (attack free)	negative	negative	negative
p.7 (attack)	positive	positive	positive
p.7 (attack free)	positive	negative	positive
p.8 (attack)	positive	positive	positive
p.8 (attack free)	positive	negative	positive
p.9 (attack free)	positive	positive	positive

3.1.3 Cytokine secreting cells in migraine patients

Mean of cytokine secreting cells (unstimulated) per 100.000 cells were calculated for those PBMC samples analyzed where the test system functioned properly.

Results for the migraine patients are presented in Figure 8.

Mean frequency of TNF-α secreting cells were significantly higher than mean frequency of IL-17 secreting cells ($P = 0.0047$). No secreting cells were found for IL-17. No differences were found, between migraine attack and attack free periods. There was a high distribution of frequencies of TNF-α and IL-10 secreting cells (Figure 8 A). Changes observed, during attack compared to attack free period, were not identical. Two of the patient means were elevated during attack, while one was decreased (Figure 8 B).

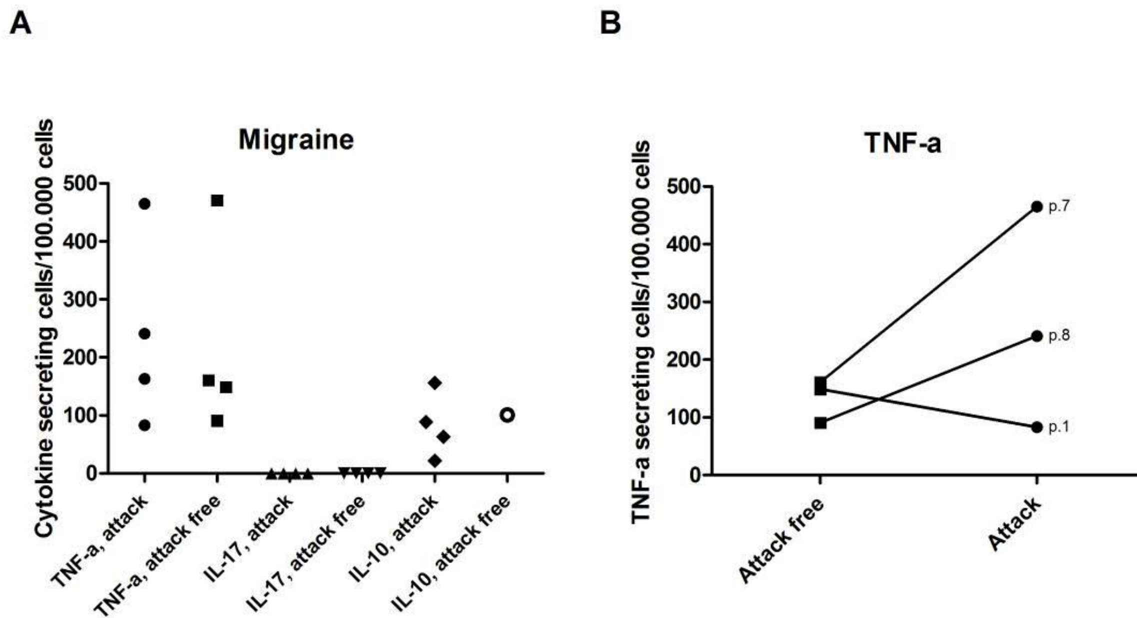

Figure 8: Cytokine secreting cells in migraine patients
The frequency of cytokine secreting cells is expressed as mean secreting cells per 100.000 cells. Each spot represent one patient. **A.** Changes in frequency of cytokine secreting cells, during migraine attack and attack free periods, were compared for TNF-α and IL-17 using the Wilcoxon matched-pairs signed rank test. Comparison between the different cytokines was done using the Kruskal Wallis test, with Dunn's Multiple Comparison test as post hoc analysis. Differences were accepted as statistically significant at $p<0.05$. **B.** Changes of individual mean TNF-α levels, during attack compared to attack free period. Each patient is marked with an individual patient number (eg. p.1).

3.2 ELISA assay

Serum samples (Table 5) were analyzed for 25 different cytokines using a 25-plex ELISA assay kit. These cytokines were MCP-1, TNF-α, IL-10, IL-1β, IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-12 (p40/p70), IL-13, IL-15, IL-17, IFN-α, IL-1ra, IL-2r, IP-10, MIG, MIP-1α, MIP-1β, GM-CSF and RANTES. Figure 9 to Figure 17 show the results for MCP-1, TNF-α, IL-10, IL-1β and IL-17. Mean cytokine levels (pg/ml) were compared between and within groups.

3.2.1 Cytokine levels in migraine and low back pain patients

MCP-1 result is shown in Figure 9 and Figure 10.

Mean MCP-1 levels were high (min. 210 pg/ml). A high distribution of results was observed within each group (Figure 9). Mean MCP-1 levels were significantly higher (P = 0.0223, one-way ANOVA) in the healthy, pain free group than in the migraine, attack free group. A tendency also observed for all the other patient subgroups. In the migraine group, 5 of 8 of the

individual mean MCP-1 levels were decreased during attack (Figure 10 A). 3 of 4 of the individual mean levels in the low back pain group were elevated during pain (Figure 10 B).

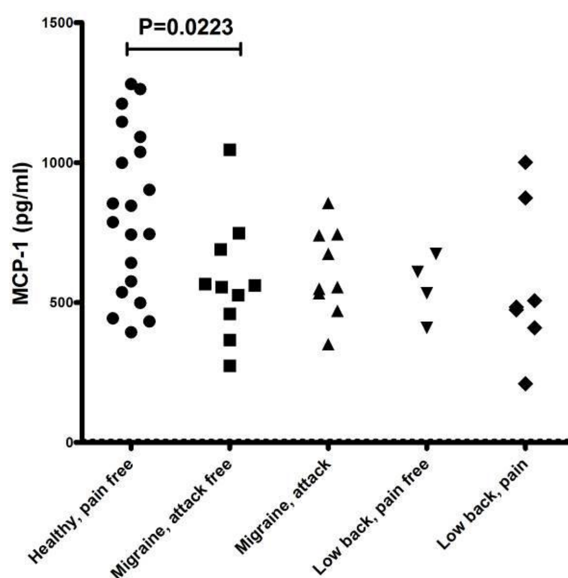

Figure 9: MCP-1 levels
Individual MCP-1 levels are expressed as mean (pg/ml). Each spot represent the mean (pg/ml) for one patient. MCP-1 levels were compared between each group by using one-way ANOVA (P-value is highlighted by capped line). The Dunnett's multiple comparison test was used as post hoc analysis. Differences were accepted as statistically significant at p<0.05. The assay sensitivity for MCP-1 (8 pg/ml) is shown with a broken line.

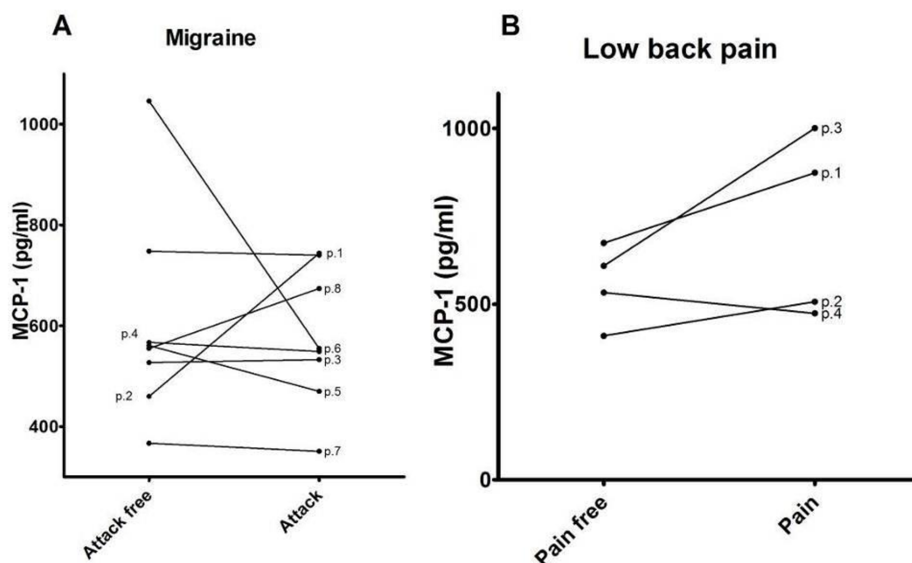

Figure 10: MCP-1 levels in migraineurs and low back pain patients
Serum MCP-1 level for each patient is expressed as mean (pg/ml). Each spot in each group represent the mean (pg/ml) for one patient. P.1 in **A.** and in **B.** is NOT the same patient. Changes in mean MCP-1 level, **A.** during migraine attack and attack free periods, and **B.** during low back pain and pain free periods, were analyzed using the two-tailed paired Student's t-test. Differences were accepted as statistically significant at p<0.05.

TNF-α result is shown in Figure 11and Figure 12.

Interestingly, 33 % of the healthy, pain free individuals had a mean below the assay sensitivity (5 pg/ml), while all patients were above this limit (Figure 11). Changes of the individual TNF-α levels, during an attack compared to attack free period, were not identical (Figure 12 A). This observation was also true for low back pain patients (Figure 12 B).

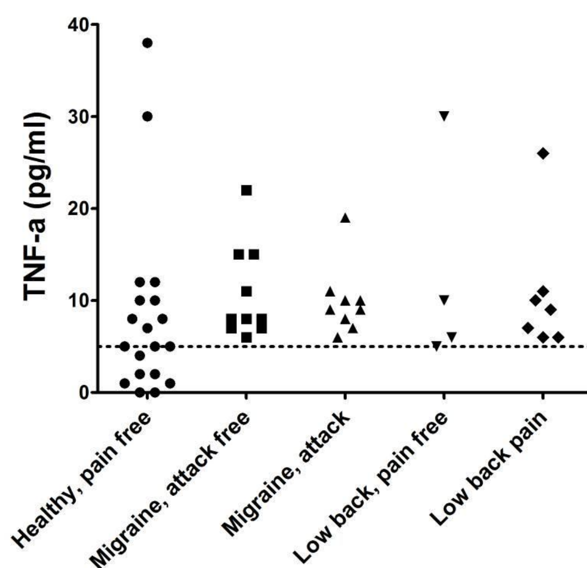

Figure 11: TNF-a levels

Serum TNF-α level for each patient is expressed as mean (pg/ml). Each spot represent one patient. TNF-α levels were compared between each group using one-way ANOVA. The Tukey multiple comparison test was used as post hoc analysis. Differences were accepted as statistically significant at $p<0.05$. The assay sensitivity for TNF-α (5 pg/ml) is shown with a broken line.

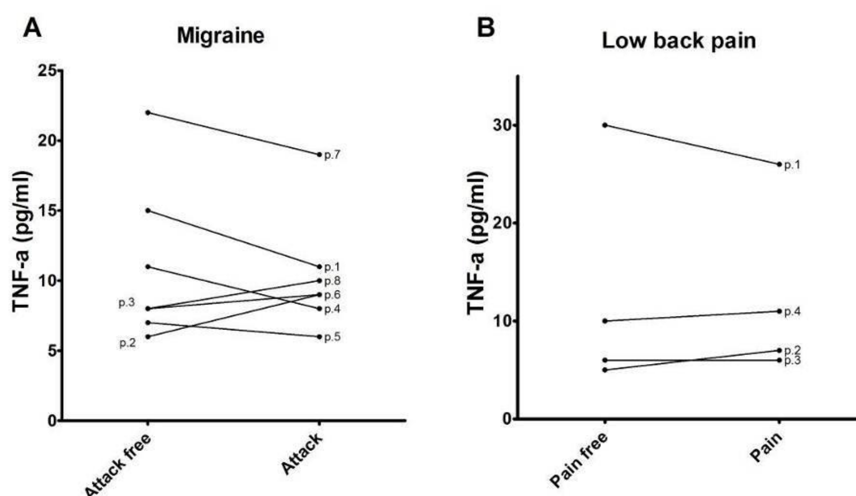

Figure 12: TNF-a levels in migraineurs and low back pain patients

Serum TNF-α levels for each patient are expressed as mean (pg/ml). Changes of mean TNF-α levels for each patient are demonstrated with a line for **A.** migraine and **B.** low back pain patients. P.1 in **A.** and in **B.** is NOT the same patient. Mean TNF-α levels were compared within each patient group using the two-tailed paired Student's t-test. Differences were accepted as statistically significant at $p<0.05$.

IL-10 result is shown in Figure 13.

A tendency of lower mean IL-10 levels in the patient groups than in the healthy, pain free group was observed. 52 % of the healthy, pain free individuals had a mean above the assay sensitivity (3 pg/ml), while 78 % of all patients (73% of the migraine patients and 86% of the low back patients) were below this limit.

Figure 13: IL-10 levels

Serum IL-10 level for each patient is expressed as mean (pg/ml). Each spot in each group represent the mean (pg/ml) for one patient. IL-10 levels were compared between each group by using one-way ANOVA. The Tukey multiple comparison test was used as post hoc analysis. Differences were accepted as statistically significant at $p<0.05$. The assay sensitivity for IL-10 (3 pg/ml) is shown with a broken line.

IL-1β result is shown in Figure 14 and Figure 15.

Within the healthy, pain free group, the distribution of IL-1β levels was high (max. level of 170 pg/ml, not shown in figure). Approximately 50% of the individual means in each group were below the assay sensitivity (15 pg/ml) (Figure 14). For 3 of 4 of those migraine patients with a mean over the detection limit, the mean IL-1β level decreased during attack (Figure 15 A). Both low back pain patients with a mean over the detection limit had an elevated mean IL-1β level during pain (Figure 15 B).

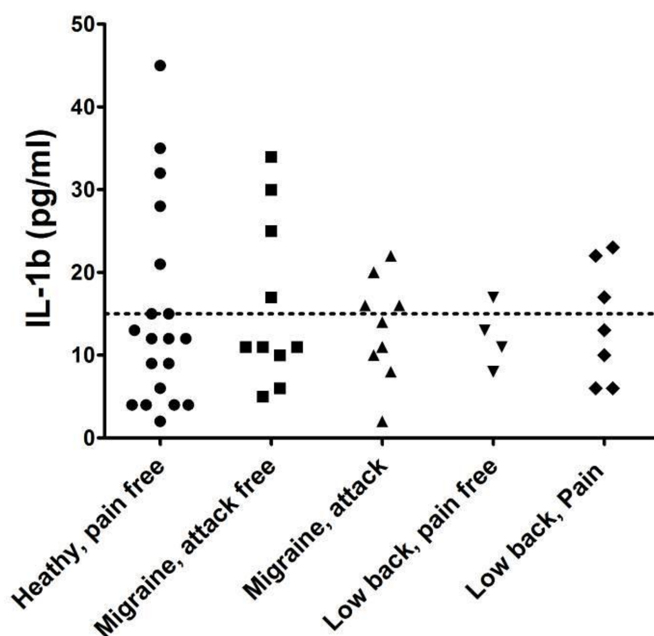

Figure 14: IL-1β levels
Serum IL-1β level for each patient is expressed as mean (pg/ml). Each spot in each group represent the mean (pg/ml) for one patient. IL-1β levels were compared between each group by using one-way ANOVA. The Tukey multiple comparison test was used as post hoc analysis. Differences were accepted as statistically significant at p<0.05. The assay sensitivity for IL-1β (15 pg/ml) is shown with a broken line.

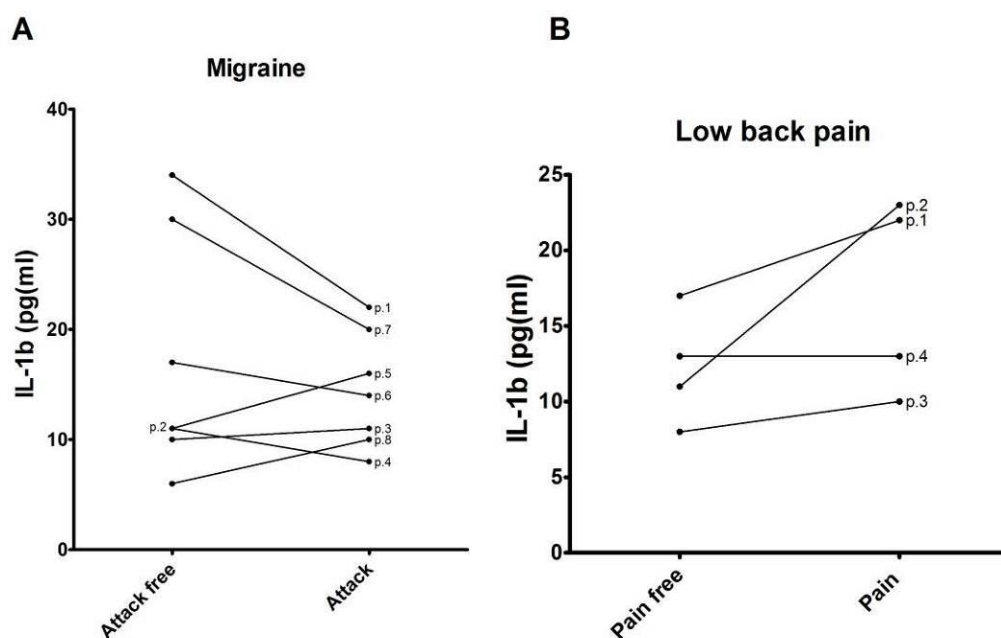

Figure 15: IL-1β levels in migraineurs and low back pain patients
Serum IL-1β level for each patient is expressed as mean (pg/ml). Each spot in each group represent the mean (pg/ml) for one patient and are marked with an individual patient number (eg. p.1). P.1 in **A.** and in **B.** is NOT the same patient. Changes in mean IL-1β level, **A.** during migraine attack and attack free periods, and **B.** during low back pain and pain free periods, were analyzed using the two-tailed paired Student's t-test. Differences were accepted as statistically significant at p<0.05.

IL-17 result is shown in Figure 16 and Figure 17.

The distribution of IL-17 levels was high within the healthy, pain free group and the migraine group (Figure 16). 52 %, 73 % and 43 % of the individual means, for the healthy, pain free individuals, migraine and low back pain patients respectively, were above the assay sensitivity (20 pg/ml). Changes of the individual IL-17 levels, during attack compared to attack free period, were not identical (Figure 17).

Figure 16: IL-17 levels

Serum IL-17 level for each patient is expressed as mean (pg/ml). Each spot in each group represent the mean (pg/ml) for one patient. IL-17 levels were compared between each group using one-way ANOVA. The Tukey multiple comparison test was used as post hoc analysis. Differences were accepted as statistically significant at p<0.05. The assay sensitivity for IL-17 (20 pg/ml) is shown with a broken line.

Figure 17: IL-17 levels in migraineurs

Serum IL-17 levels for each patient (marked with individual numbers) are expressed as mean (pg/ml). Changes of mean TNF-α level for each migraine patient are demonstrated with a line. Mean IL-17 levels were compared within the group using the two-tailed paired Student's t-test. Differences were accepted as statistically significant at $p<0.05$.

3.3 ELISpot vs. ELISA

Both the ELISpot and the ELISA results showed individual changes of the frequency of cytokine producing cells and the cytokine levels, during migraine attack compared to attack free period. Due to this, it became of interest to compare the ELISpot results and the ELISA results for the same patients. This comparison is shown for TNF-α in Figure 18.

Both p.1 (blue) and p.8 (black) showed the same change, of mean frequency of cytokine producing cells (Figure 18 A) and mean cytokine levels (Figure 18 B), during attack. This was not the case for p.7 (red). The mean frequency of cytokine producing cells was elevated during migraine attack, while the mean cytokine level decreased.

IL-17 results from the two assays were also interesting. No IL-17 producing cells were detected for any of the patients using the ELISpot assay (Figure 8). For all, except three migraine patients, changes in mean cytokine levels were detected (Figure 17).

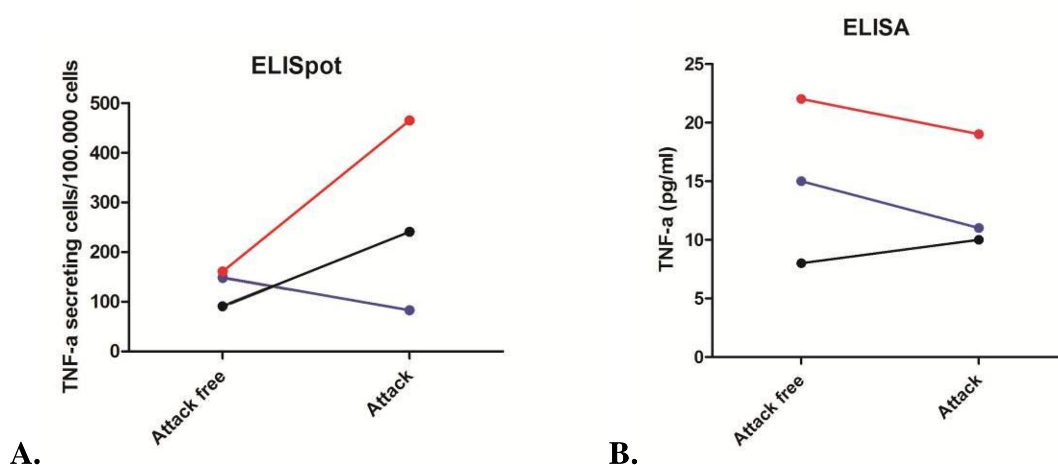

Figure 18: ELISpot vs. ELISA – changes of TNF-α production
This figure shows changes of TNF-α production for three different migraine patients. Both **A.** ELISpot and **B.** ELISA results are shown. P.1 has the color blue, p.7 red and p.8 black. **A.** mean frequency of TNF-α secreting cells (per 100.000 cells). **B.** mean cytokine levels (pg/ml)

4. Discussion

The first objective shifted to only concern migraine: to identify cytokines as possible specific biomarkers. The three other objectives remained unchanged: to define cytokine secretion as a possible inflammatory response to pain, to evaluate the use of ELISA assay and ELISpot assay, and to suggest improvements for future studies.
These aspects are discussed in this chapter, starting with a general discussion of results, continuing with methods and then drawing conclusions. Improvements and future studies are addressed in the final section.

The general discussion of results is primarily based on tendencies seen in graphs. Statistical analyses were performed, but to accurately assess the distribution within the different groups was challenging. This was mainly due to the small and unequal group sizes.

4.1 Results

4.1.1 Blood cytokine secreting cells in migraineurs

A significantly higher frequency of TNF-α secreting cells than of IL-17 secreting cells ($P = 0.0047$) was found (Figure 8). No IL-17 secreting cells were detected at all in migraineurs, despite that IL-17 is believed to have inflammation promoting functions. IL-17 is thought to induce pro-inflammatory cytokines and chemokines (Table 9, Appendix A), and has been shown to cooperate with TNF-α to enhance the inflammatory capacity of innate cells (60). It was expected to see a high frequency of IL-17 secreting cells in addition to TNF-α secreting cells in migraineurs. The results found in our study for IL-17 secreting mononuclear cells indicate that these cells are not a part of neurogenic inflammation in migraineurs. To the best of our knowledge no previous studies have linked IL-17 to migraine before.

A tendency of lower frequency of IL-10 secreting cells than of TNF-α was observed (22-156 vs. 83-456 secreting cells/100.000 cells). This is very interesting since TNF-α is considered to be a pain mediator in neurogenic inflammation and IL-10 has been shown to inhibit the hyperalgesic responses to TNF-α (39, 40, 42).

The ELISpot results do not indicate much regarding the relevance of cytokine producing cells in migraineurs. It would have been interesting to compare migraineurs with other headache patients, patients with other painful conditions and healthy, pain free individuals. Unfortunately this comparison was not done for the ELISpot analysis because of the limited timespan and budget of this study. A comparison of migraineurs with healthy, pain free individuals and low back pain patients was done for the ELISA assay (see section 4.1.2 Serum cytokine levels in migraineurs). To the best of our knowledge, no previous studies have investigated cytokine secreting peripheral blood mononuclear cells in migraineurs before.

4.1.2 Serum cytokine levels in migraineurs

We observed lower levels of MCP-1 in migraineurs and in low back pain patients, both during the pain period and the pain free period, compared to healthy, pain free controls (Figure 9). A significant difference was observed between the healthy, pain free group and the migraine, attack free group ($P = 0.0223$, one-way ANOVA). We suggest that MCP-1 may be of interest as a general pain marker, downregulated as a result of pain signals over time. The high distribution of mean MCP-1 levels seen within the groups made it of interest to investigate the individual mean changes during attack/pain in the migraineurs and low back pain patients. It may be a difference between the two painful conditions; 63% (5/8) of the migraine patients mean decreased during attack, while 75% (3/4) of the low back pain patients mean increased during pain (Figure 10). Previous studies have demonstrated that MCP-1 may be relevant for tension-type headache, but not for migraine (61, 62).

A tendency of higher TNF-α levels in patients than in healthy, pain free individuals was observed (Figure 11). No difference was observed between migraineurs and low back pain patients. We suggest that TNF-α may be a general pain marker, upregulated in response to pain. It cannot be dismissed that an overexpression of TNF-α may contribute to the nociceptive activity during migraine. In 2010 Yilmaz et al. observed that the TNF-α -308 A allele was overrepresented in migraineurs compared to healthy controls. They also observed a significant increase of IL-1β +3953 T allele in migraineurs. They suggested that an overexpression of TNF-α and IL-1β in migraineurs would be expected (38). Our observation of TNF-α is in agreement with this suggestion.

A tendency of lower IL-10 levels in patients compared to healthy, pain free controls was observed (the majority of patients had undetectable levels, Figure 13). A trend also observed in CSF (62). This tendency is interesting because IL-10 has been shown to inhibit hyperalgesic responses to TNF-α (Table 9). As also observed for TNF-α and IL-10 secreting cells, IL-10 levels seem to be low when TNF-α levels are high (Figure 8, Figure 11 and Figure 13). Since no difference was observed between the migraine and the low back pain patients this may indicate that IL-10 is downregulated, and TNF-α upregulated, in response to pain in general. Previous studies have observed higher or similar IL-10 levels during migraine compared to healthy controls (24, 44, 45, 48). Fidan et al. (2006) observed an increase during attack compared to attack free period (45). This comparison could not be done in our study because the majority of the patient results were below the detection level. Undetectable IL-10 levels seem to be a recurrent phenomenon (44, 52).

No difference in IL-1β levels was observed in patients compared with healthy individuals (Figure 14). Approximately 50% of all individual means in all groups were undetectable. IL-1β was rarely detected and at low levels in CSF as well (62). In contrast with our findings, TNF-α is thought to induce hyperalgesia mediated through the release of IL-1 and an overexpression of IL-1β would be expected in migraineurs (38, 40).

For the rest of the 21 cytokines tested for, no differences were detected between or within the different groups (results not shown).

A high distribution of results within the groups was observed for the majority of cytokines (eg. Figure 9 and Figure 16), as were undetectable levels (eg. Figure 13, Figure 14 and Figure 16). Both elevated and decreased levels of cytokine production, during attack compared to attack free period, were observed within groups (eg. Figure 8, Figure 10 and Figure 12), indicating that changes of cytokine production may be patient dependent. One may speculate that the unequal distribution of age and gender within groups, the use of medications (triptans, NSAIDs, opoids) prior to blood sampling (Table 4), the possible transitory variation of cytokine levels and the heterogeneity of migraine may be reasons for the high distribution of results and the patient dependency observed.

A previous study found aging associated with significant changes in cytokine production. For some of these cytokines the changes were also gender specific (63). However, several studies have not found any correlation between age, gender and cytokine production in migraineurs (24, 48, 52).

It is possible that the use of medication prior to blood sampling may have influenced the cytokine production. Triptans act like 5-HT agonists and both inhibit TG activation and block transmission in the TGVS. NSAIDs reduce perivascular neurogenic inflammation by affecting T-cells, neutrophils and macrophages. And opoids work as analgesics by inhibiting ascending pathways that carry pain information from primary sensory neurons and by activating descending pain control systems (64). In fact, Munno et al (2001) demonstrated an effect of sumatriptan on IL-10, IL-4 and IL-5 levels in migraineurs (48).

It has been demonstrated a transitory variation of TNF-α levels at different times after migraine onset, where the highest levels were found within the first two hours (24, 46). Regarding IL-1β, IL-6 and IL-4, the results have been conflicting. The different individual cytokine levels observed in this present study may have been a result of blood samples not being collected at specific times.

The high extent of undetectable levels among the 25 cytokines studied may be due to the fact that most cytokines are secreted and act at local sites and only enter the circulation when secreted in large amounts (65). Other possible reasons for the concentrations being too low for detection may be because cytokines have a short half-time, because of dilution in serum or because of consumption after secretion (45, 66).

Despite having large sample sizes and following strict criteria for patient selection and sampling conditions, Bockowski et al. (2010) also demonstrated a high extent of undetectable levels and data not being normally distributed. Statistical analysis and finding significant differences was not feasible (52).

The many conflicting results regarding cytokine levels in migraineurs seen throughout the last couple of years may be due to several reasons. The use of assay kits with different sensitivity levels, different criteria for patient selection, exclusion and sampling conditions, and due to different group sizes studied.

4.2 Methods

4.2.1 Patient recruitment

The exact number of headache patients admitted to Haukeland University Hospital in one year was not known prior to this study. Relatively few headache patients were hospitalized with acute headache this year. One year of patient recruitment was not sufficient to get groups consisting of 20 individuals.

There may be several reasons for the low number of participants. Not all headache patients were sent to the Department of Neurology. Several patients were admitted during evening and nighttime, while the inclusion of patients could be administered only during daytime. Only one neurologist had the responsibility of identifying suitable patients for this study. The personal interviews and the laboratory work were carried out by one person only. Few tension-type patients were admitted to the hospital, probably because their headache is less severe and not disabling. Cluster headache is a rare condition and patients are rarely hospitalized because of relatively short lasting attacks (15-180min).

In order to increase the number of participants the recruitment period could be expanded and the Department of Neurosurgery included. By having one person with the responsibility for personal interviews and one for the laboratory work, possible participants would not have been left out of the study as a consequence of no one being there to recruit them. Inclusion both day and night could also been an alternative.

4.2.2 Sampling criteria

Blood samples, during attack and pain period, were collected the first morning after hospitalization. For how long each patient had been in pain varied (from one hour to several days). The majority had taken medication (opioids, triptans and/or NSAIDs) prior to blood sampling (Table 4). As discussed earlier, different time of blood sampling and the use of medication may have influenced the blood cytokine levels.

It could be ideal to exclude these variables in future studies by setting criteria for the time of sampling and avoid the use of medication. This could have been done in a prospective study with patients being recruited, informed and diagnosed prior to the study. On the other hand, this could make it even more difficult to recruit patients during attack and be guaranteed severe pain intensity at the time of sampling.

Criteria for the pain free samples were set prior to sampling. Samples were taken 1-2 months after hospitalization, during a pain free period, from both migraineurs and low back pain patients. Patients had been pain free for at least two days and the divers had not been training or diving, or had any complaints of pain, during the last two days before sampling. Not all patients met for control sampling (pain free samples) (Table 5). Not all became pain free during the sampling period and for some it was difficult to meet for personal reasons.

4.2.3 PBMC sample storage

It is not recommended to store PBMC samples over a longer period of time (> 1 week) at -80°C due to the possibility of impaired PBMC recovery and viability upon thawing (55). Instead it is recommended to transfer the samples into a liquid nitrogen tank (≤ -132°C) after 24-72 hours and not to store them for more than 6 months (55). Unfortunately this was not an option during the storage of patient PBMC samples because liquid nitrogen tanks were not available. Storage at -80°C for 1-2 weeks has been successful previously at the laboratory. Viable cells were counted after thawing. Each sample had a concentration of about 20 million cells/ml. Storage at -80°C for 1-2 weeks did not impair PBMC recovery and viability of importance for these experiments.

4.2.4 ELISpot assay

The experiments were performed according to the manufacturer’s descriptions, with minor modifications. HTS HA plates were used instead of the PVDF-based membrane plates from the kits because it was easier to use. The PVDF-based membrane plates needed to be activated with alcohol, a critical step which made it difficult to coat several plates at the same time. This step was not needed for the HA plates. During development the PVDF-based membrane plates developed a darker background color than the HTS HA plates, which made it difficult to see when spots emerged and when to abort the development process (Figure 7). The number of spots that developed on the PVDF-based membrane plates was very high, making it difficult for the software to distinguish spots from each other and give an accurate count of cytokine secreting cells.

According to recommendation from MabTech, different concentrations (cells/ml) of cell suspensions were tested for the different cytokines (results not shown). Concentrations used in

the experiments are listed in Table 7. Concentrations above these resulted in overcrowded wells and concentrations below these resulted in almost empty wells, making counting difficult. Two different concentrations were used in order to get a more representative mean of the frequency of cytokine secreting cells.

During rinsing of the HA plates, 0.05% PBS-T and a vacuum manifold were used. This is a well-established and tested procedure at the Broegelmann laboratory when using HA plates. Millipore does not recommend exceeding 0.01% PBS-T, to prevent the possibility of leakage, or using a vacuum manifold since the plates does not require filtration (59, 67). No leakage of importance has been experienced at the lab using 0.05% PBS-T and vacuum manifold. The filtration step can be skipped in future experiments, making the rinsing step more convenient.

After development the plates were left to dry for two days. When punching out the membranes, breakage was experienced around some of the edges. Two days may have been too long and resulted in too dry and crispy membranes. The software counted dark areas (broken bits) as spots, which resulted in unnecessary amount of work during quality control. For future experiments, leaving the plates to dry for one day is considered to be adequate.

Each PBMC sample was stimulated with polyclonal activators to make sure that the test system functioned properly and to test that viable cytokine secreting cells were present. As shown in Table 8 the attack free samples from p.1, p.7 and p.8, plated on the same plate, were negative in the positive control wells for IL-10. Cells and the development process do not seem to have been the problem. The same samples were analyzed for TNF-α and IL-17, using the same enzyme conjugated streptavidin and development substrate, and they were positive. The only step that may have been the reason for the negative results is the coating. The coating antibody solutions used for the three different cytokines were not the same and may explain why these samples were positive for TNF-α and IL-17, but not for IL-10.
The attack free sample from p.6 was negative in the positive control wells for all three cytokines, but the other samples on the same plate (p.1, attack and p.9, attack free) were positive. A possible reason for this may be that this particular sample needed longer development time. This was therefore a negative consequence of plating different samples on the same plate.
For a total of four of five attack free samples, analyzed for IL-10, the test system did not function properly. No comparison of the frequency of IL-10 secreting cells during migraine

attacks and attack free periods could be done. This highlights the importance of having sample aliquots; if there were more samples, new experiments could have been done.

4.2.5 ELISA assay

The experiments were performed according to the manufacturer's descriptions without any modifications. Data was obtained for all samples and for all cytokines tested for. Some of the results are illustrated in Figure 9 to Figure 17 in section 3.2.1 Cytokine levels in migraine and low back pain patients, and discussed in section 4.1.2 Serum cytokine levels in migraineurs.

4.2.6 ELISA vs. ELISpot

The ELISpot assay is considered to be more sensitive than the ELISA assay; it detects cytokine secretion at single cell levels and immediate capture cytokines upon secretion. ELISA may fail to detect soluble cytokines accumulated in serum due to dilution, consumption or degradation of secreted cytokines (66). This is in agreement with our IL-10 results. Mean frequencies of 22-156 secreting cells per 100.000 cells were detected, but a soluble IL-10 level (30 pg/ml) was only detected for one of the same migraine patients (Figure 8 and Figure 13).
The ELISpot assay failed to detect any IL-17 secreting cells, while mean soluble IL-17 levels (19-71 pg/ml) were detected for all of the same migraine patients using the ELISA assay (Figure 8 and Figure 16). This suggests that low frequencies of cytokine secreting cells do not necessarily indicate low levels of soluble cytokines, and vice versa.
In this context it is important to mention that peripheral blood mononuclear cells, such as lymphocytes, macrophages and monocytes, were the only cells tested for cytokine secretion using the ELISpot assay. This may be considered as a limitation of this method when looking at cytokines as possible biomarkers in blood for migraine. Several other cells, in addition to mononuclear cells, like mast cells in the dura, glia cells in the TG and arterial smooth muscle cells, are believed to contribute to the cytokine secretion and neurogenic inflammation in migraineurs. With the ELISA assay, serum concentrations of soluble cytokines were measured, including cytokines secreted from several cell sources (assuming the cells secreted enough cytokines to enter the circulation).
TNF-α results (Figure 8 and Figure 11) did not clearly show a difference between frequency of cytokine secreting cells and levels of soluble cytokines within the migraine group. But the individual data revealed a difference (Figure 18, see p.7 (red)). An elevated frequency of

cytokine secreting cells was detected during attack, but a decreased level of soluble cytokines was detected.
Despite the conflicting results observed for the two methods, they also showed some of the same tendencies for TNF-α; it was no difference between the attack and the attack free period and TNF-α production was higher than IL-10 production.

4.3 Conclusions

4.3.1 Cytokines as biomarkers for migraine

This study did not reveal any distinct cytokines to be considered as specific biomarkers for migraine. Some interesting tendencies for MCP-1, TNF-α and IL-10 were seen though. A possible upregulation of TNF-α and downregulation of MCP-1 and IL-10 seem to be of relevance, if not specifically for migraine, at least for painful conditions in general. Further investigation of these cytokines and a comparison between migraineurs and patients with tension-type headache and cluster headache is of interest.

4.3.2 ELISpot vs. ELISA

It is difficult to tell which method that was best for studying cytokine production in migraineurs. The results obtained were not identical. The two methods provided information about cytokine production from different cell sources. The ELISpot assay revealed cytokine production from peripheral blood mononuclear cells. The ELISA assay revealed cytokine production from other additional cells, believed to be relevant for the neurogenic inflammation in migraineurs, by measuring cytokine levels in serum. Both methods had their advantages and disadvantages. Where one method failed to detect cytokine production the other one did not. Further comparisons have to be done.

4.4 Improvements and future perspectives

It would have been better with larger groups (≥20), equal in size, in order to accurately assess the distribution within the different groups and to determine which statistical analyses to employ. Suggestions how to increase the number of participants could be to: 1. Extend the recruitment period (> 1 year). 2. Include patients both day and night. 3. Include several departments at the hospital. Another suggestion could be to do a multicenter study, including several hospitals and/or general physicians. Even though, this could compromise the homogeneity of sampling and analysis.

Other suggestions for improvements could be to 1. Avoid variables that may affect cytokine levels, by setting criteria for sampling time and the use of medication. 2. Avoid a possible relation between age, gender and cytokine production, by having age and gender matched subgroups.

In this study, comparison of cytokine levels in migraineurs with cluster headache patients and TTH patients was not accomplished. No cluster headache patients and only two tension-type headache patients agreed to participate. Future investigations of differences between the three headache syndromes and other painful conditions, such as low back pain, are of interest.
To our knowledge few have done this comparison before. In 2008 Bø et al. compared CSF cytokine levels between migraineurs and episodic TTH patients. They suggested that MCP-1 along with IL-10 assist in restoring homeostasis in TTH patients, but not in migraineurs (62). In 2009 Bockowski et al. suggested that there may be a difference between TTH patients and migraineurs regarding blood TNF-α levels. In 2010 Bockowski et al. did the same comparison for IL-4, IL-13 and IL-10, without definite conclusions (52).

According to our findings, we suggest that TNF-α, MCP-1 and IL-10 are the most interesting cytokines to focus on in future studies, as biomarkers for migraine and as general pain markers. IL-1β and IL-17 may also be of interest. The high distribution of cytokine levels and the probable patient dependency seen in migraineurs, probably due to the heterogeneity of the disease and the fact that cytokine levels may be transitory, makes further investigation of interest. A suggestion is to investigate changes of cytokine levels during a longer period of time; before, during and after attack. A prospective study could help answering whether or not

each patient has his/her own cytokine pattern and if it is individual what kind of cytokine change that can be associated with migraine. This requires a more advanced study design with patients hospitalized over a longer period of time and with attacks being induced.

If the heterogeneity of migraine is relevant for cytokine levels, it would be interesting to investigate differences of cytokine levels in migraineurs who have an effect of symptomatic drugs, like triptans, and in migraineurs who do not. This could be done with a prospective study, determining cytokine levels before, during, and after attack, both when using symptomatic drugs and when not. As mentioned earlier Munno et al (2001) have demonstrated an effect of sumatriptan on IL-10, IL-4 and IL-5 levels in migraineurs (48)

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6. Appendix

Appendix A

Inflammation

Inflammation is a complex reaction of vascularized tissue, which involves extravascular accumulation of plasma proteins and leukocytes, that serves a protective function in controlling infections and promoting tissue repair (68). Macrophages already present in the tissues, like microglia in the brain, immediately begin to phagocytize invading pathogens and release molecular mediators (69, 70). These mediators contribute to the inflammatory response and the recruitment and activation of effector cells (70). Cytokines, regulatory proteins that play major roles in regulating the development and behavior of immune effector cells, are some of the mediators which are released (69, 70). They, in addition to other chemoattractants, promote the recruitment and increased production of other leukocytes, such as neutrophils and monocytes, and thereby further phagocytosis (70). Macrophages partially digest the phagocytized pathogens and induce the antigen products to T-lymphocytes and thereby activate them (71). They also secrete IL-1, which promotes further growth and reproduction of the specific lymphocytes. The activated T-cells proliferate and release active and specifically reacting T cells; memory T cells, helper T cells, cytotoxic T cells and suppressor T cells. Helper T cells form several protein mediators, cytokines, which act on other immune cells and bone marrow cell (71). Table 9 present some factors believed to play important roles during inflammation.

Table 9: Inflammatory factors
This table presents some factors believed to play important roles during inflammation (39-42, 65, 71, 72).

Cytokine	Secreted from	Effect
IFN-γ	T cells	B-cell stimulation; T cell differentiation; Macrophage activation
TNF-α	Macrophages, T cells	Induction of fever, IL-1, IL-6 and chemokines (e.g. IL-8); Synthesis of acute-phase proteins by the liver; Increased vascular permeability and adhesion molecules on vascular endothelium (inflammation); Fibroblast proliferation; Activation of T-cells, B-cells and neutrophils; May induce dose-dependent hyperalgesia mediated by IL-1β release.
GM-CSF	Macrophages, T cells	Increase of monocyte and granulocyte production by the bone marrow
IL-1	Macrophages	Induction of fever, chemokines (e.g. IL-8) and IL-6; Synthesis of acute-phase proteins by the liver; Increased vascular permeability and adhesion molecules on vascular endothelium (inflammation); Fibroblast proliferation; Activation of T-cells and B-cells; Platelet production; May induce dose-dependent hyperalgesia.
IL-2	T cells	Stimulation of growth and proliferation of cytotoxic and suppressor T cells and activation of T helper cells; Proliferation of B cells and antibody synthesis
IL-3	T cells	Lymphocyte growth factor
IL-4	T cells	Stimulation of B-cells and immunoglobulin production; Inhibition of IFN-γ-mediated macrophage activation; May limit production of IL-1β, IL-6, IL-8 and TNF-α; May inhibit TNF-α induced hyperalgesia.
IL-5	T cells	Stimulation of B-cells and immunoglobulin production; Activation and increased production of eosinophils
IL-6	Macrophages, T cells	Induction of fever; Synthesis of acute-phase proteins by the liver; Increased vascular permeability; Platelet production; T cell and B cell activation; Increased immunoglobulin synthesis. May induce dose-dependent hyperalgesia.
IL-10	Macrophages, T cells	Inhibition of IL-12 production and expression of costimulators; May inhibit production of IL-1, IL-6, IL-8, GM-CSF and TNF-α; May inhibit hyperalgesic responses to TNF-α, IL-1β and IL-6.
IL-12	Macrophages	T cell differentiation; Stimulation of IFN-γ synthesis
IL-13	T cells	Stimulation of B-cells and immunoglobulin production
IL-15	Macrophages	T cell proliferation
IL-17	T cells	Increased chemokine production from endothelial cells and macrophages; Increased cytokine production from macrophages; GM-CSF and G-CSF production from epithelial cells

Appendix B

Anamnese (pasientens sykehistorie)

Navn:	**Fødselsnr:**

20	**Dato:**	**Sted:**

Nummer/adresse:	**Deltakernr:**

1) Kvinne ☐ Mann ☐

2.a) Har andre i familien migrene? Ja ☐ Nei ☐ Hvem?

2.b) Har andre i familien spenningshodepine? Ja ☐ Nei ☐ Hvem?

2.c) Har andre i familien klasehodepine? Ja ☐ Nei ☐ Hvem?

2.d) Har andre i familien annen hodepine? Ja ☐ Nei ☐ Hvem?

Type hodepine

3.a) Hvor gammel var du da du for første gang ble plaget med hodepine (av den art som førte til innleggelse)?

År

3.b) Hvor mange hodepineanfall har du i måneden?

ganger i måneden

3.c) Hvor mange timer/dager pleier hodepinen vare om gangen?

timer dager

3.d) Mener du det er noe som utløser hodepinen (som mat, drikke, stress, menstruasjon, fysiske anstrengelser, nakkebevegelser)?

Ja ☐ Nei ☐

Hvis ja, hva?

Har du andre sykdommer? Ja ☐ Nei ☐

Hvis ja, hvilke?

3.e) Hvilke lindrende, ikke-medikamentelle faktorer har vist seg å fungere for deg (eks. frisk luft, stille rom, fysisk aktivitet osv.) ?

4) Hvilke tidligere medisinske undersøkelser (som f.eks MR, CT) har blitt utført i forbindelse med hodepineplagene og hva var resultatene?

Undersøkelse 1: **Resultat 1:** **Undersøkelse 2:** **Resultat 2:** **Undersøkelse 3:** **Resultat 3:** **Undersøkelse 4:** **Resultat 4:**

5.a) Bruker du smertestillende ved anfall? Ja ☐ Nei ☐

Navn på legemidler	**Mengde (mg)**

5.b) Hvor mye bedre blir du etter å ha tatt smertestillende?

Litt bedre ☐ (ikke i stand til å utføre daglige gjøremål, må fortsatt holde sengen)

Bedre ☐ (evnen til å utføre daglige gjøremål er delvis svekket)

Mye bedre ☐ (fungerer normalt)

6.a) Bruker du forebyggende medisiner for hodepinen? Ja ☐ Nei ☐

Hvis ja, fyll ut:

Navn på legemidler **Daglig mengde (mg)** **Fom. dato (dd.mm.år)**

6.b) Har antall hodepineanfall blitt redusert etter du begynte med nåværende forebyggende medisiner?

Ja ☐ Nei ☐ Usikkert ☐

Hvis ja,

Fra ☐ anfall i måneden

Til ☐ anfall i måneden

6.c) Har hodepinen endret karakter etter du begynte med nåværende forebyggende medisiner (som redusert varighet og/eller styrke)?

Ja ☐ Nei ☐ Usikkert ☐

Hvis ja, hvordan?

6.d) Hvis du har brukt andre forebyggende medikamenter før, angis de her:

Navn på legemidler	Daglig mengde (mg)	Fom. – tom. dato(dd.mm.år)

6.e) Hvorfor byttet du eventuelt forebyggende medisin?

Ingen effekt ☐

Plagsomme/alvorlige bivirkninger?

I så fall, hvilke?

Annen årsak:

7.a) Har du andre sykdommer? Ja ☐ Nei ☐

Hvis ja, hvilke?

7.b) Bruker du forebyggende medisiner for denne/disse sykdommen(e)? Ja ☐ Nei ☐

Navn på legemidler	**Daglig mengde (mg)**	**Fom. dato (dd.mm.år)**

8) Røyker du? Ja ☐ Nei ☐

9) Bruker du p-piller eller annen hormonell prevensjon? Ja ☐ Nei ☐

Hvis ja, hvilken?

Appendix C

Spørreskjema for registrering av akutt hodepine

Navn:	**Fødselsnr:**

20	**Dato:**	**Sted:**

Nummer/adresse:	**Deltakernr:**

1.a) Når startet hodepinen som forårsaket innleggelse?

Dato (dd.mm.år)	Klokkeslett (time.min)

1.b) Når ble du lagt inn pga denne hodepinen?

Dato (dd.mm.år)	Klokkeslett (time.min)

1.c) Når var hodepinen borte?

Dato (dd.mm.år)	Klokkeslett (time.min)

2) Kontaktet du fastlege og/ eller legevakten på grunn av denne hodepinen? Ja ☐ Nei ☐

3.a) Har du (hyppig) hodepine også utenom den som var årsak til innleggelse? Ja☐ Nei ☐

3.b) Hvis ja, er hodepinen som førte til innleggelse annerledes enn din vanlige hodepine?

Ja☐ Nei ☐

Hvis ja, på hvilken måte?

Hvis ja,

Navn på legemidler	**Mengde (mg)**	**Når tok du medisinene (kl)**

4.b) Hvor kraftig var hodepinen da du tok dine vanlige medisiner hjemme?

Sett inn tall fra 0 til 10 ☐

4.c) Hadde medisinene du tok hjemme noen effekt? Ja ☐ Nei ☐

5.a) Fikk du medisiner for hodepinen av din fastlege eller på legevakten? Ja ☐ Nei ☐

Navn på legemidler	**Mengde (mg)**	**Når tok du medisinene (kl)**

5.b) Hvor kraftig var hodepinen da du var hos lege/legevakt?

Sett inn tall fra 0 til 10 ☐

5.c) Hadde medisinene du fikk av fastlege/legevakt noen effekt? Ja ☐ Nei ☐

6.a) Fikk du noen medisiner for hodepinen ved/etter innleggelse? Ja ☐ Nei ☐

Navn på legemidler	**Mengde (mg)**	**Når tok du medisinene (kl)**

6.b) Hvor kraftig var hodepinen da du fikk medisiner ved/etter innleggelse?

Sett inn tall fra 0 til 10 ☐

6.c) Hadde medisinene du fikk på sykehuset noen effekt? Ja ☐ Nei ☐

7.a) Merket du i forbindelse med hodepineanfallet som medførte innleggelse (kryss av for det du merket):

Forstyrrelser av syn ☐

Nummenhet i armer, kropp eller ansikt ☐

Forstyrrelser av tale ☐

Svimmelhet ☐

Lyd- og lysskyhet ☐

Kvalme og/eller oppkast ☐

Hode- og nakkestivhet ☐

Konjunktival injeksjon (rødt øye) ☐

Tåreflod ☐

Tett nese ☐

Rennende nese ☐

Svette i panne og/eller ansikt ☐

Miose (sammentrekning av pupill) ☐

Ptose (hengende øyelokk) ☐

Øyelokksødem ☐

Forverring ved moderat fysisk aktivitet ☐

Annet [ ]

7.b) Har du nakkesmerter i forbindelse med hodepinen? ☐ Ja ☐ Nei

7.c) Hvor i hodet var det vondt?

Høyre side ☐

Venstre side ☐

Begge sider ☐

Panne ☐

Bak øynene ☐

Tinningen ☐

7.d) Skiftet smerten side under anfallet? Ja ☐ Nei ☐

7.e) Hvordan var smerten?

Pulserende (dunkende) ☐

Pressende/strammende ☐

Svært intens ☐

7.f) Forandret hodepinen karakter under anfallet? Ja ☐ Nei ☐

Hvis ja, forklar hvordan:

(Fylles ut av helsepersonell)

Medikamenter brukt under det aktuelle hodepine anfallet, fyll ut mengde (mg)

Hjemme:

Triptaner
Imigran
Maxalt
Naramig
Zomig
Relpax

Opioider
Paralgin Forte
Petidin
Morfin
Paracet
Kodein
Subutex

NSAIDS
Ibux
Naproxen
Cataflam
Voltaren

Annet
Nozinam

Ved innleggelse:

Triptaner
Imigran
Maxalt
Naramig
Zomig
Relpax

Opioider
Paralgin Forte
Petidin
Morfin
Paracet
Kodein
Subutex

NSAIDS
Ibux

Naproxen
Cataflam
Voltaren

Annet
Nozinam

Hvilke undersøkelser ble utført ved innleggelse av pasient?

MR ☐ Resultat

CT ☐ Resultat

Annet ☐

Hvis annet, hvilke(n) undersøkelse(r)

Resultat(er)

Kryss av for hvilken hodepine som passer for pasienten og dens symptomer:

Spenningshodepine ☐
Pressende/strammende hodepine som varer fra 30 min til 7 dager
Mild eller moderat intensitet (vil hemme, men ikke forhindre normal daglig aktivitet)
Forverres ikke av moderat fysisk aktivitet
Bilateral lokalisasjon (kjennes på begge sider av hodet)
Tidligere undersøkelser tyder ikke på en underliggende årsak til hodepinen

Migrene med aura ☐
Synsforstyrrelser i form av mørke/blinde prikker, flekker eller annet forstyrrende mønster i synsfeltet (aura)
Hodepinen følger auraen med et fritt intervall på mindre enn 60 min (hodepinen kan også begynne før eller under auraen)
Pulserende hodepine med ensidig eller bilateral lokalisasjon
Moderat eller betydelig intensitet (hemmer eller forhindrer normal daglig aktivitet)
Forverres av moderat fysisk aktivitet
Under hodepinen foreligger kvalme og/eller oppkast, og/eller lyd- og lysskyhet
Hodepinen varer mellom 4 og 72 timer
Tidligere undersøkelser tyder ikke på en underliggende årsak til hodepinen

Migrene uten aura ☐
Pulserende hodepine med ensidig eller bilateral lokalisasjon
Moderat eller betydelig intensitet (hemmer eller forhindrer normal daglig aktivitet)
Forverres av moderat fysisk aktivitet
Under hodepinen foreligger kvalme og/eller oppkast, og/eller lyd- og lysskyhet
Hodepinen varer mellom 4 og 72 timer
Tidligere undersøkelser tyder ikke på en underliggende årsak til hodepinen

Klasehodepine ☐
Svært intens ensidig smerte lokalisert orbitalt eller supraorbitalt og/eller i tinningen
Varer mellom 15 og 180 minutter ubehandlet
Hodepine ledsages av minst en av de følgende tegn (må være på samme side som smerten)

- konjunktival injeksjon
- tåreflod
- tetthet i nesen
- renning fra nesen
- svetting i panne og ansikt
- miose
- ptose
- øyelokksødem

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Sted	**Dato**	**Signatur**