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IDENTIFICATION OF LOCI FOR MIGRAINE WITH AURA

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To my dear family

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List of Original Publications

I P Tikka-Kleemola, MA Kaunisto, E Hämäläinen, U Todt, I Goebel, J Kaprio, C Kubisch, M Färkkilä, A Palotie, M Wessman, M Kallela. Genetic association study of *Endothelin1* and its receptors *EDNRA* and *EDNRB* in migraine with aura. Cephalalgia (2009), in press.

II P Tikka-Kleemola, E Hämäläinen, K Tuomainen, M Suvela, A Artma, O Kahre, M Wessman, A Palotie, K Silander. The enhancement of homogenous mass extension reaction: comparison of two enzymes. Molecular and Cellular Probes (2007) 21:216-221.

III MA Kaunisto*, PJ Tikka*, M Kallela, SM Leal, JC Papp, A Korhonen, E Hämäläinen, H Harno, H Havanka, M Nissilä, E Säkö, M Ilmavirta, J Kaprio, M Färkkilä, RA Ophoff, A Palotie, M Wessman. Chromosome 19p13 loci in Finnish migraine with aura families. American Journal of Medical Genetics, Part B: Neuropsychiatric Genetics (2005) 132B:85-89.

IV P Tikka-Kleemola, V Artto, S Vepsäläinen, E Sobel, S Räty, MA Kaunisto, V Anttila, E Hämäläinen, M-L Sumelahti, M Ilmavirta, M Färkkilä, M Kallela, A Palotie, M Wessman. A visual migraine aura locus maps to occipitotemporal lobe epilepsy locus at 9q21-q22. Submitted.

Study I also appears in the thesis of Mari Kaunisto (2005)

* These authors contributed equally to the respective work.

Abbreviations

ASP	affected sib-pair
bp	base pair
BMI	body mass index
CBF	cerebral blood flow
CEPH	Centre d'Etudes du Polymorphisme Humain
chr	chromosome
cM	centiMorgam
CNV	copy number variation
CSD	cortical spreading depression
DNA	deoxyribonucleic acid
EA	episodic ataxia
ELOD	expected Lod score
EMLOD	expected maximum Lod score
EQV	equivalent migraine
dom	dominant
FHM	familial hemiplegic migraine
GABA	gamma-amino butyric acid
GWAS	genome-wide association study/studies
HA	headache
HVR	hereditary vascular retinopathy
HWE	Hardy-Weinberg equilibrium
IBD	identity be descent
IHS	the International Headache Society
kb	kilobase
LCA	latent class analysis
LD	linkage equilibrium
Lod	Logarithm of odds
LodHet	Lod score under locus heterogeneity
LodHom	Lod score under locus homogeneity
MA	migraine with aura
MAF	minor allele frequency
MALDI-TOF	matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry
Mb	megabase
MD	missing diagnosis
MO	migraine without aura
na	not available
NoHa	no headache
NPL	non-parametric linkage
OR	odds ratio
PCR	polymerase chain reaction
rec	recessive
SNP	single nucleotide polymorphism
tagSNP	tagging SNP
TCA	trait component analysis
UCSC	University California Santa Cruz
WHO	The World Health Organization

Abstract

Common migraine, *i.e.* migraine with (MA) or without aura (MO), is a chronic neurological disorder affecting about 10% of the Caucasian population. In MA, migraine headache is preceded by visual, sensoric and/or dysphasic reversible aura symptoms. Twin and family studies have suggested a multifactorial mode of inheritance for common migraine, and a stronger genetic component for MA than for MO. Since there is no biological or genetic marker to identify common migraine, aura symptoms provide a distinctive character to identify those suspected of suffering from migraine. The aim of this study was to identify MA susceptibility loci in well-phenotyped migraine samples with familial predisposition using different gene mapping methods.

Genes coding for endothelin1 and its receptors EDNRA and ENDRB are potential candidate genes for cortical spreading depression (CSD), which is considered to be the underlying mechanism of migraine aura. The role of these genes in MA was studied in 850 Finnish migraine cases and 890 control individuals. Rare homozygous *EDNRA* SNPs showed nominal association with MA and with the age of onset trait (<20 years). This result was also detected in the pooled analysis on 648 German MA cases and 651 control individuals when the test was adjusted for gender and sample origin. Evaluation of SNP genotyping reactions with two different DNA polymerase enzymes ensured that the genotype quality was high, and thus the discovered associations are considered reliable. The role of the 19p13 region was studied in a linkage analysis of 72 Finnish MA families. This region contains two migraine-associated genes: *CACNA1A*, which is associated with a predisposition to a rare Mendelian form of MA, familial hemiplegic migraine (FHM), and the insulin receptor gene (*INSR*) that is associated with common migraine. No evidence of linkage between the 19p13 and MA was detected.

A novel visual aura locus was mapped to chromosome 9q21–q22 with significant evidence of linkage using a genome-wide linkage approach in 36 Finnish MA families. Five additional, potential loci were also detected. The 9q21–q22 region has previously been linked to occipitotemporal lobe epilepsy and MA, both of which involve prominent visual symptoms. Our result further supports a shared background for these episodic disorders.

1 Introduction

Many chronic diseases with adult onset, such as vascular diseases, obesity, depression and migraine reduce the welfare of diseased individuals and increase the burden on the health care system. Often these diseases show familial aggregation but no single gene is liable for the disease onset. Environmental factors can promote or prevent both the onset of disease and clinical symptoms. When an individual's susceptibility to disease cumulates from both inherited variations and environmental factors, the disease is said to be a "complex disease". The spectrum of non-inherited somatic mutations, viral risk factors, diet and their interaction with genetic and other environmental factors make gene mapping efforts for complex diseases a challenge.

Migraine is a common chronic headache disorder affecting approximately every tenth individual in Western countries. Its human and social burden is often underestimated, although the World Health Organization (WHO) includes migraine as one of the top 20 diseases causing disability (http://www.who.int). The one-year economic burden of migraine is severe, being €276 million in Finland and €27 billion in Europe (Sillanpää *et al*. 2008, Stovner *et al.* 2008). The first descriptions of migraine originate from the Middle East 5,000 years ago, but the complexity of headache disorders started to be understood in the late 19th century (reviewed by Rapoport and Edmeads 2000). The fundamental reason for migraine disorders is not known but the adaptation to repetitive environmental stress is reduced in migraine patients (Coppola *et al.* 2007). Therefore, migraine might be the survival mechanism of nervous system "to rest and retreat from the external stresses" (Pearce 1986).

So far, genetic studies have not identified susceptibility variants for common forms of migraine, *i.e.* migraine with (MA) and without (MO) aura. In this thesis, the genetic susceptibility of MA has been investigated using different gene mapping methods in migraine patients with a familial predisposition. Particular attention was paid to the technical optimization of the genotyping process.

Review of literature

2 Migraine

2.1 Clinical characteristics of migraine

Migraine is an episodic headache disorder that is usually characterized by unilateral, pulsating and numbing pain that is often related to nausea and sensitivity to external stimuli. For diagnosing purposes, the International Headache Society (IHS) has introduced descriptions of migraine symptoms to ease identification of migraines and exclude the possibility of other disorders. The first international classification of headache disorders was introduced by the Headache Classification Committee of IHS in 1998. The updated second version was published in 2004 (Table 1; Headache Subcommittee of the International Headache Society 2004), and it has been widely accepted for use in diagnosis and research.

Table 1. Migraine subtypes according to the International Classification committee of IHS (Headache Subcommittee of the International Headache Society 2004).

Subtype	Subform	Migraine
1.1		**Migraine without aura (MO)**
1.2		**Migraine with aura**
	1.2.1	Typical aura with migraine headache (MA)
	1.2.2	Typical aura with non-migraine headache
	1.2.3	Typical aura without headache
	1.2.4	Familial hemiplegic migraine (FHM)
	1.2.5	Sporadic hemiplegic migraine
	1.2.6	Basilar-type migraine
1.3		**Childhood periodic syndromes that are commonly precursors of migraine**
	1.3.1	Cyclic vomiting
	1.3.2	Abdominal migraine
	1.3.3	Benign paroxysmal vertigo of childhood
1.4		**Retinal migraine**
1.5		**Complications of migraine**
	1.5.1	Chronic migraine
	1.5.2	Status migrainosus
	1.5.3	Persistent aura without infarction
	1.5.4	Migrainous infarction
	1.5.5	Migraine-triggered seizure
1.6		**Probable migraine**
	1.6.1	Probable migraine without aura
	1.6.2	Probable migraine with aura
	1.6.5	Probable chronic migraine

A normal migraine attack lasts 4–72 hours, and a patient must have at least five migraine attacks with two characteristic symptoms and associated consequences of the headache to have

a migraine without aura (MO) diagnosis (Table 2). In about one third of all migraine attacks (Russell *et al.* 1996), migraine headache is preceded by reversible visual, dysphasic or sensory symptoms or combination of them that develop in 5–20 minutes and disappear within 60 minutes. These patients have typical aura with migraine headache (MA). If aura symptoms include any motor symptoms, the patient has a familial or sporadic form of hemiplegic migraine (Table 1). In some cases aura is followed by headache without migraneous features or the headache is absent.

Table 2. Diagnostics criteria for migraine without (MO) and with aura (MA) according to the International Classification committee of IHS (Headache Subcommittee of the International Headache Society 2004).

1.1 Migraine without aura (MO)

A. At least 5 attacks fulfilling criteria B-C
B. Headache attacks lasting 4–72 hours (untreated or unsuccessfully treated)
C. Headache has at least two of the following characteristics
    1. Unilateral location
    2. Pulsating quality
    3. Moderate or severe pain intensity
    4. Aggravation by or causing avoidance of routine physical activity
D. During headache at least one of the following:
    1. Nausea and/or vomiting
    2. Photophobia and phonophobia
E. Not attributed to another disorder

1.2.1 Typical aura with migraine headache (MA)

A. At least 2 attacks fulfilling criteria B-D
B. At least one of the following, but no motor weakness
    1. Fully reversible visual symptoms including positive features (*e.g.* flickering lights, spots or lines) and/or negative features (*e.g.* loss of vision)
    2. Fully reversible sensory symptoms including positive features (*e.g.* pins and needles) and/or negative features (*e.g.* numbness)
    3. Fully reversible dysphasic speech disturbance
C. At least two of the following
    1. Homonymous visual symptoms and/or unilateral sensory symptoms
    2. At least one aura symptom develops gradually over ≥5 minutes and/or different aura symptoms occurs in succession over ≥5 minutes
    3. Each symptoms lasts ≥5 and ≤60 minutes
D. Headache fulfilling criteria B-D for 1.1 migraine without aura begins during the aura or follows aura within 60 minutes
E. Not attributed to another disorder

A migraine attack can be divided into three phases (Figure 1; Headache Subcommittee of the International Headache Society 2004, Linde 2006): In the first premonitory phase the aggravating factors, such as psychosocial stress and alcohol consumption, decrease the threshold for migraine attacks. About 70% of patients experience premonitory symptoms hours or days beforehand. These symptoms include hyperactivity, concentration difficulties, yawning or craving for some specific food (Giffin *et al.* 2003). The final triggering factor can

be some specific food, irritating environmental disturbance or menstruation. In the headache phase about one third of migraine patients experience reversible aura symptoms (Russell *et al.* 1996) followed by the migraine headache occurring in both MA and MO attacks. Some patients also experience a postdromal phase with cognitive difficulties even though the patient is past the headache phase.

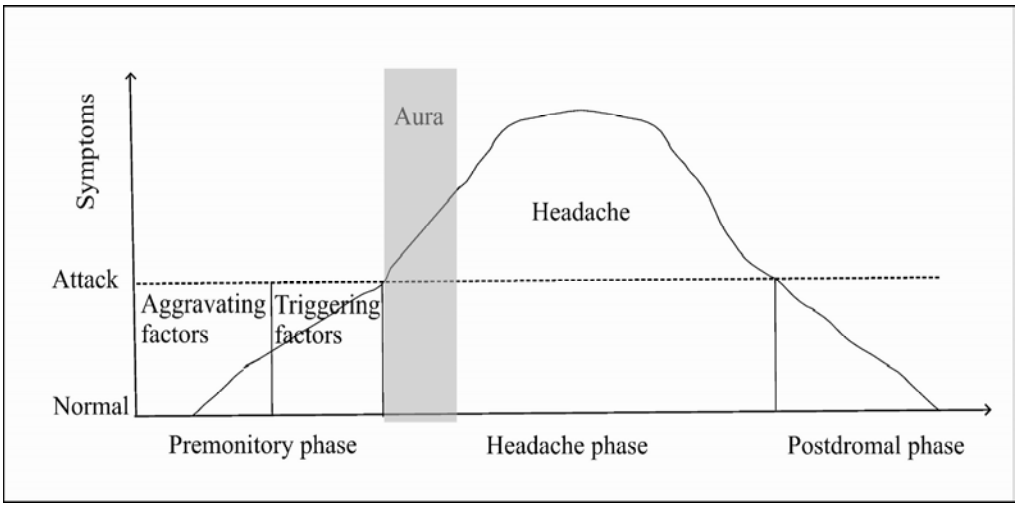

Figure 1. Three phases of migraine attack. The figure modified from Linde (2006).

2.2 Epidemiology

Epidemiological studies examine disease risk factors and differences in disease rates (*i.e.* prevalence) in different populations. Genetic epidemiology determines the genetic component of a particular phenotype and its relation to environmental factors (Morton 1982). The effect by which genetic variation contributes to a phenotypic variation is defined as heritability. Twin studies are often used to estimate heritability by comparing the correlation of a phenotype between monozygotic and dizygotic twins (Medlund *et al.* 1976, Kaprio 2000). If the effect of the genetic components is higher than environmental factors in disease predisposition, it may facilitate the identification of the genetic variants.

2.2.1 Prevalence

Prevalence is the proportion of a population with a disease during a particular period of time, typically either one year or life-time. The prevalence studies on the Danish and Dutch populations have shown a one-year risk of 10–16% and a life-time risk of 16–23% for migraine (Rasmussen *et al.* 1991, Launer *et al.* 1999). Similar figures were summarized in the comprehensive evaluation of headache prevalence in different populations based on the WHO's project "The Global Campaign to Reduce the Burden of Headache Worldwide" (Stovner *et al.* 2007). According to this study, the global current prevalence of the IHS-based migraine is >10% in the adult population when the life-time risk for migraine is about 14%. About two thirds of migraine patients are females in all populations, but before puberty migraine is slightly more common in boys than girls (Abu-Arefeh and Russell 1994). The urban life-style seems to correlate positively with a life-time prevalence of migraine. In Europe the prevalence of migraine is three times higher than in Africa (15% *vs.* 5%; Stovner *et al.* 2007). In Finland the number of migraine patients is estimated to be 440,000 corresponding to a population prevalence of 8% (Sillanpää *et al.* 2008). In contrast to common migraine, sporadic and familial hemiplegic migraine consist of only a fraction of migraine prevalence; according to a Danish study the prevalence of hemiplegic migraine is only 0.01% (Lykke Thomsen *et al.* 2002).

The two main types of migraine, MA and MO, can co-occur but typically the prevalence of MA or MO is evaluated separately. The life-time prevalence of MA does not differentiate largely by gender being 8% for women and 7% for men (Russell *et al.* 2002). For MO the male prevalence (7%) is the same as for MA, but in females the prevalence is 19%. When the co-occurrence of MO and MA was evaluated in the clinical sample, 40% of patients were found to have both forms of attacks (Kallela *et al.* 2001). However, in the population-based twin sample only 6% of males and 7% of females with migraine suffered from both attacks (Ulrich *et al.* 1999). The difference probably originates from a selection bias; those in the clinical sample were likely more severely affected than those in the population-based twin study (Rasmussen and Stewart 2000).

2.2.2 Genetic factors

A positive family history of migraine has been reported in 37–91% of probands with migraine (Russell 1997). The epidemiological studies on twins showed two-time higher concordance rates for monozygotic (32–48%) than dizygotic (12–31%) twins suggesting genetic components in migraine susceptibility (Mulder *et al.* 2003). The genetic component accounts for about half (34–60%) of migraine susceptibility (Honkasalo *et al.* 1995, Larsson *et al.* 1995, Mulder *et al.* 2003), but population-specific variation exists. In MO the additive genetic effect is estimated to be 61% and for MA 65% (Gervil *et al.* 1999, Ulrich *et al.* 1999b).

Segregation studies on families have suggested multifactorial inheritance of both MA and MO (Russell *et al.* 1995), but a monogenic pattern of inheritance may be possible in some families due to a high prevalence of migraine in each generation (Russell 1997). Also, the high number of migraine-affected offspring of migraneous females has suggested a possibility of mitochondrial inheritance. The risk study on families has shown that the first degree relatives of a patient with MO have almost twice the risk of MO and 1.4-times risk for MA (Russell and Olesen 1995). However, the risk for MA has been estimated to be four times higher in the first-degree relatives of a MA patient than in the general population, but no increased risk for MO has been detected. Therefore, the migraine family risk calculations suggest that MA and MO are distinct conditions. However, in some studies no association between the migraine type (*i.e.* MA and MO) of a proband and type of migraine in relatives was found (Stewart *et al.* 1997, Nyholt *et al.* 2004).

2.2.3 Environmental factors

Since migraine is a complex disease, environmental factors have an important role in migraine aetiology. The most common triggering factors in migraine are occasional psychosocial, dietary, physical, environmental and hormonal factors (Scharff *et al.* 1995, Wöber *et al.* 2006), which partially explain the episodic nature of migraine. Also social factors including low education and economical status increase the incidence of migraine (Stewart *et al.* 1992, Lyngberg *et al.* 2005), but in adolescents with a family history of migraine the economical background did not seem to play role in migraine susceptibility (Bigal *et al.* 2007). A Swedish

study on twins showed that twins reared apart before age of three were more similar for migraine than those separated in later age (Svensson *et al.* 2003). Despite the low number of twins they suggested that environmental, not genetic, factors modify the difference between family members.

Between MA and MO the environmental factors seem to differentiate: A spouse of a MO patient has a significantly increased risk for MO but not for MA, but the spouse risk was missing in MA indicating higher environmental risk in MO than in MA (Russell and Olesen 1995). This was also shown in the Danish study that pointed no difference in living conditions or life style between discordant MA twins (Ulrich *et al.* 2000). Furthermore, menstrual migraine is related to MO but not to MA suggesting a greater hormonal background for MO than MA (Headache Subcommittee of the International Headache Society 2004).

2.3. Pathophysiology of migraine

Traditionally migraine was regarded as a cascade of vasoconstriction followed by vasodilatation (Wolff 1953). Currently migraine is considered to be a neuronal disorder having its origin in the central nervous system. Modern imaging techniques have emphasized the primary role of neurons in migraine (Pietrobon and Striessing 2003, Rogawski 2008). Although the role of neurons in migraine is not completely understood, the current knowledge suggests a central role of cortical spreading depression, a state of cortical neurons, in migraine pathophysiology. It is thought to cause the aura phase and eventually trigger migraine headache through the activation of trigeminovascular fibers.

2.3.1 Attack onset

Individuals suffering from migraine are most of the time symptom-free. They experience only episodic attacks. Psychosocial or environmental factors predispose to migraine along with genetic factors, but the pathophysiological mechanisms that trigger the attack are unknown. The primary defect could be the imbalance in brainstem nuclei which regulate pain and vascular system (Weiller *et al.* 1995). Along with a sensitive brainstem, the cortex of migraneours is different compared to controls. Individuals with migraine suffer from reduced habituation to external stimuli, and thus migraine can also be considered a reflection of a dysfunction of cortical information processing due to abnormal cortical excitability (Chronicle

and Mullerners 1996, McColl and Wilkinson 2000, Giffin and Kaube 2002). The basic reason for this is unknown but it may originate from an abnormal release of neurotransmitters or imbalance of ion channel functions (Pietrobon and Striessing 2003).

2.3.2 Migraine aura

Migraine aura is probably the best characterized phase of a migraine attack. Most typically (>92%) aura is visual (Russell and Olesen 1996, Eriksen *et al.* 2004, Kelman 2004). The aura can also be a speech symptom (30–38%) or a sensory (33–44%) disturbance, and very rarely a motor paralysis in hemiplegic migraine. Visual aura typically starts with flickering zigzag lines that are followed by defects in visual fields (*i.e.* scotomas or heminanopia; Russell and Olesen 1996). The gradual spreading of visual symptoms and complete reversibility within 5–60 minutes suggests the episodic involvement of the central nervous system, especially the visual cortex, in the pathophysiology of migraine aura.

In 1944, a Brazilian scientist A. Leão described a neurophysiological phenomenon, cortical spreading depression (CSD), in a rodent brain (Leão 1944). CSD was later suggested to be a correlate of visual migraine aura (Bowyer *et al.* 2001). CSD is a slow propagating wave (2–6 mm/min) of neuronal and glial depolarization that has also been recorded in the cortex, hippocampus, striatum and cerebellum (Davies *et al.* 1995, Moskowitz 2008). CSD induces changes in K^+, Na^+ and Ca^{2+} ions, nitric oxide, arachidonic acid and prostaglandin concentrations (Wei *et al.* 1992, Strassman *et al.* 1996). These changes may theoretically sensitize trigeminovascular afferents to generate migraine pain, and thus form a link between the aura and headache phases of migraine.

2.3.2.1 Cortical spreading depression in migraine aura

The triggering mechanisms for spontaneous CSD are unknown, but brain injury in human, potassium, pin brick, glutamate or electrical stimuli in rodents can trigger it (reviewed by Sanchez-del-Rio and Reuter 2004). Among the various substances that have been shown to trigger CSD, endothelin1 (EDN1) is especially interesting since, as a potent vasoconstrictor, it has been shown to induce CSD in rats (Dreier *et al.* 2002, Kleeberg *et al.* 2004). Furthermore, elevated plasma EDN1 levels have been measured in migraine attacks (Färkkilä *et al.* 1992, Gallai *et al.* 1994, Kallela *et al.* 1998, Hasselblatt *et al.* 1999), but contradictory result has also been presented (Nattero *et al.* 1996).

Modern neuroimaging techniques have contributed substantially to a better understanding of the vascular and neuronal changes that occur during the aura. In the scintillating scotoma type of aura, a regional increase in cerebral blood flow (CBF) has been recorded during scintillations that is followed by long lasting regional hypoperfusion during scotoma in the occipital lobe (Welch *et al.* 1998, Hadjikhani *et al.* 2001). A similar cascade is considered to happen in CSD in which activation is followed by depression. This may explain why typical (positive) visual aura symptoms like, scintillations, during migraine aura are followed by defects in visual field (*e.g.* scotomas; Smith *et al.* 2006, personnal communication by doc. Mikko Kallela). Similarly, sensory aura is considered a positive symptom while both motor and dysphasic auras have been considered as negative symptoms.

A recent study in mice describes the increase in CBF on a molecular level. CSD causes an increase in the concentration of extracellular potassium. In order to compensate the ionic imbalance, neurons adjacent to the vessels consume free O_2 at the expense of more distant tissues, thereby producing anoxic depolarization (Takano *et al.* 2007). In other words, excitable neurons cause the oxygen deprivation of more distant neurons. However, a study by Brennan and colleagues (2007) showed that vascular changes can precede CSD. This may suggest that vascular changes are not only a passive response to metabolic demands. These studies do not explain the triggering factors for CSD, however, they do give an intriguing insight into the propagation of CSD.

Auras with motor or sensory symptoms have been suggested to originate from events similar to those in CSD (Pietrobon and Striessing 2003), because the spreading of these symptoms occurs at a similar rate as visual aura. Most migraine attacks are without aura and thus the spreading depression has been proposed to occur on clinically silent areas of cerebral cortex (Goadsby 2001). For example, hippocampal spreading depression could be clinically silent and has been reported to activate the trigeminal fibers that may further trigger the migraine headache (Kunkler and Kraig 2003). However, a role of the visual cortex in MO is unlikely since the visual cortex of MO patients is similar to healthy individuals, whereas MA patients lack the inhibitory activity of visual cortex. This may suggest a greater cortical excitability in MA patients, who are more prone to CSD in occipital cortex than MO patients (Palmer *et al.* 2000). Alternatively, it has also been proposed that aura and headache may be parallel processes of episodic dysfunction in brainstem nuclei, because an increase of CBF has been

detected in several areas of brainstem in MO patients (Weiller et al. 1995, Pietrobon and Striessing 2003).

2.3.2.2 Role of ion channel genes in migraine aura

So far, no undisputed predisposing genes have been identified for common migraine. However, for a rare monogenic subtype of MA, familial hemiplegic migraine (FHM), three genes has have been identified (Table 3).

Table 3. Genes predisposing to familial hemiplegic migraine (FHM).

FHM type	Chr	Gene	Protein	Function	Reference
FHM1	19p13	*CACNA1A*	α2A subunit of voltage gated Ca^{2+} channel	Mediates the entry of Ca^{2+} ions into excitable cells	Ophoff *et al.* 1996
FHM2	1q23	*ATP1A2*	Na^+/K^+-ATPase α2 subunit	Establishes and maintains the electrochemical gradients of Na^+ and K^+ ions across the plasma membrane	De Fusco *et al.* 2003
FHM3	2q24	*SCN1A*	Voltage-gated sodium channel α subunit	Generation and propagation of action potentials in nerve and muscle	Dichgans *et al.* 2005

Although the main symptoms of aura and headache of FHM are very similar to MA, the distinctive characteristic of FHM is motor aura that mainly consists of unilateral motor weakness or paralysis that may last to several days (Thomsen *et al.* 2002). At the worst, some FHM patients can suffer from disturbance of consciousness, fever and permanent cerebellar symptoms and progressive ataxia and/or nystagmus (Pietrobon and Striessing 2003, Headache Subcommittee of the International Headache Society 2004).

Due to similarities and co-occurance between common migraine and FHM, dysfunction of neuronal ion transportation can provide a model for predisposition for common forms of migraine. Mutations in genes encoding ion channels disturb the rhythmic function of exposed tissue that may also explain the episodic nature of migraine (Gargus 2006, Bernad and Shevell 2008). The common feature in FHM mutations seems to be the increased extracellular glutamate and potassium concentrations that increase susceptibility to CSD, especially with the FHM1 mutation (van den Maagdenberg *et al.* 2004, 2007).

2.3.3 Migraine headache

Pain sensation in the skull is primarily restricted to the meningneal blood vessels that are innervated by fibers of trigeminal nerve. The fundamental reason for causing the fibers to activate in migraine pain is unknown. However, dysfunction of brain stem nuclei, spreading depression or changes in CBF have been suggested to be the underlying causes (Figure 2; reviewed by Goadsby 2001, Pietrobon and Striessing 2003 and Dalkara *et al.* 2006). Two theories, neurogenic inflammation and sensitization of trigeminal nerves, try to explain the pathophysiology of migraine pain.

According to the neurogenic inflammation theory, trigeminocervical nerve endings release pro-inflammatory neuropeptides such as nitric oxide, calcitonin gene-related peptide, endothelin3 and substance P that induce vasodilatation and changes in vascular permeability that allow plasma proteins in dura mater during neurogenic inflammation (Peroutka 2005, Dalkara *et al.* 2006, Durham 2006). CSD is suggested to alter the integrity of the blood-brain barrier by activating metalloproteasis leading to trigeminovascular activation due to protein leakage and oedema (Gursoy-Ozdemir *et al.* 2004). However, the drugs that block vascular permeability have failed prevent migraine headache (Peroutka 2005)

The sensitization theory suggests the activation of three pain mechanisms and merges the neurogenic inflammation as a part of pathophysiological process (reviewed by Schürks and Diener 2008). First the pain receptors are activated, then trigeminal neurons release vasoactive and pro-inflammatory neuropeptides, and finally a pain synapse from the trigeminal nerve triggers the release of neurotransmitters from meningneal blood vessels into the dura. The released transmitters induce increased blood flow and plasma extravasation that cause perivascular inflammation, which is thought to trigger sensitization in intracranial neurons (Burstein 2001). This can make the headache more severe and decrease tolerance to environmental stimuli (Hargreaves and Shepheard 1999). In 79% of migraine patients, sensitization is experienced as allodynia that is abnormal sensation *e.g.* to cold or warm during

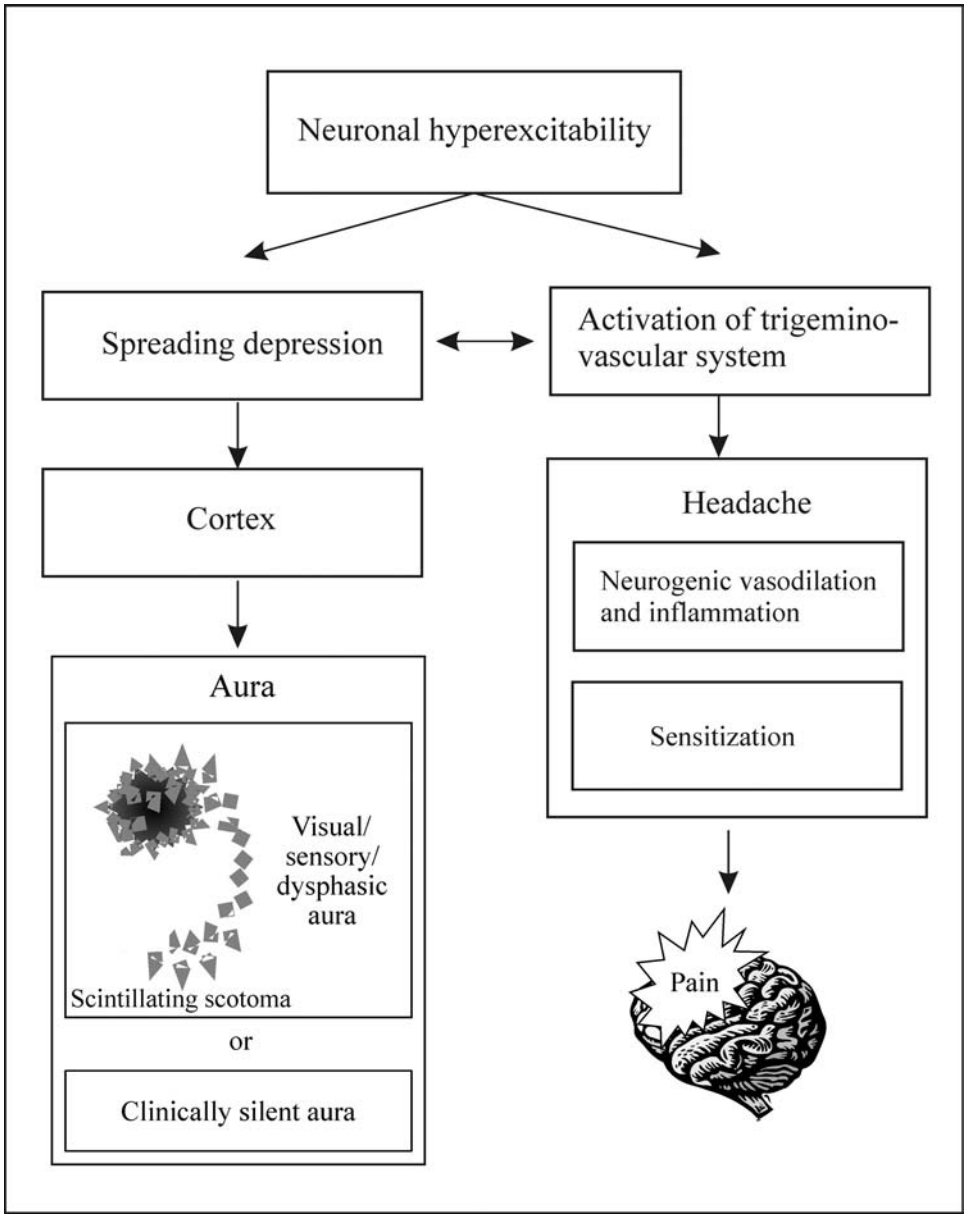

Figure 2. Overview of pathophysiological theories on common migraine.

migraine attacks that is normally ignored due to habituation (Burstein *et al.* 2000). The most effective antimigraine drugs are triptans, serotonin receptor agonists. They inhibit the effect of activated nociceptive trigeminal afferents, especially in the beginning of migraine headache before sensitization has taken place (Goadsby *et al.* 2002).

2.3.4 Comorbidity of migraine

Comorbidity is defined as greater than coincidental association of two conditions in the same individual (Feinstein 1970). In migraine the comorbidity with other neurological disorders or associated conditions may help to reveal the pathophysiological pathways leading to disease susceptibility. Common migraine can co-occur with stroke, psychiatric disorders, asthma, obesity and epilepsy (Scher *et al.* 2005). Table 4 summarizes some of the disorders or conditions that show comorbidity with migraine in Odds ratio (OR). OR defines the probability of relationship between the affected and control populations.

Among the comorbid disorders or conditions, the episodic nature of epilepsy highlights the possibility of shared background for both migraine and epilepsy. Epilepsy is frequently associated with increased risk of migraine before and after an epileptic seizure (Lipton *et al.* 1994). When about 0.5–1% of the population has epilepsy, about 6% of migraine patients suffer from epilepsy and 8–15% of epilepsy patients have migraine (Andermann and Andermann 1987). According to another study by Ottman and Lipton (1994) epilepsy patients have 2.4 times higher risk of migraine compared to relatives without epilepsy. In a Finnish study by Artto *et al.* (2006) association between familial migraine with and without aura and epilepsy showed significant comorbidity (OR=6.8) in men. In children between 5–15 years the risk of epilepsy is 3.7 times higher in migraine patients than in control population (Ludvigsson *et al.* 2006). Interestingly, presence of MA doubles the risk (OR=8.2). Despite comorbidity, epilepsy and migraine have clinical differences (De Simone *et al.* 2007): In epilepsy no female-prominent prevalence is detected and the age at onset in epilepsy can often be in the extremes of a lifespan. Clinical symptoms in an epileptic attack can also be more drastic, resembling sometimes the most severe symptoms of FHM. Furthermore, anatomical abnormalities predispose more often to epilepsy than migraine although migraine patients do not routinely undergo neuroimaging studies.

Table 4. Summary of the conditions associated with migraine or its subtypes.

Migraine type	Associated condition	OR (95% CI)	Ratio [1]	No of participants	Sample type	Reference
Migraine MA MO	Major depression	3.5 (2.6-4.6) 4.9 (3.3-7.2) 3.0 (2.2-4.1)	218/536 78/158 140/378	1,696	Population, USA	Breslau *et al.* 2000
Migraine	Anxiety	3.9 (2.5-6.0)	31/340	1,843	Population, USA	McWilliams *et al.* 2004
Severe headache/ migraine	Respitory disease [2]	1.7 (1.5-2.0)	708/3,045	15,330	Population, USA	Kalaydjian and Merikangas 2008
Chronic migraine	Obesity	1.7 (1.2-2.3)	6/401	30,849	Population, USA	Bigal and Lipton 2006
MA [3]	Ischemic stroke [4] *low risk group* *high risk group*	 3.9 (1.9-8.1) 1.0 (0.2-4.2)	 9/1418 2/1418	27,519	Prospective cohort study on women, USA	Kurth *et al.* 2008
MA	Myocardial infarction [4] *low risk group* *high risk group*	 1.3 (0.4-4.2) 3.3 (1.5-7.5)	 3/1418 7/1418	27,519	Prospective cohort study on women, USA	Kurth *et al.* 2008
MA	Patent foramen ovale (PFO)	4.6 (2.0-10.6)	44/93	186	Clinic sample, Schwitzerland	Schwerzmann *et al.* 2005
Migraine	White matter abnormalities	4.1 (2.1-8.4)	71/312	629	Meta-analysis of clinical-based studies	Swarz and Kern 2004
Migraine MA MO	Epilepsy	3.7 (1.6-8.3) 8.2 (2.3-28.9) 1.4 (0.5-4.0)	19/94 13/19 6/19	282	Medical record based study on adolescents, Iceland	Ludvigsson *et al.* 2006

OR, Odds ratio; CI, Confidence interval
1) Ratio of comorbid migraine patients of all migraine patients, 2) Includes asthma, chronic bronchitis and emphysema, 3) No risk in MO, 4) Participants were stratified in risk score groups based on the Framingham risk score profiles (http://www.framinghamheartstudy.org/risk/)

Regardless of some differences, the reduced threshold for neuronal sensibility proposes of shared background for migraine and epilepsy (reviewed by Bigal *et al.* 2003, Haut *et al.* 2006, De Simone *et al.* 2007). In migraine the periodic cortical hypersensitivity, which is probably needed for CSD to evolve, induces an increase of extracellular K^+. The elevated K^+ level has been proposed to sensitize the human epileptic neocortex *in vitro* (Koch *et al.* 2005). Interestingly, epilepsy drugs that suppress ion signalling, including topiramat and valproate, are also effective migraine drugs (Rogawski 2008). In particular, the epilepsy drug lamotrigine that inhibits Na^{2+} channel-mediated glutamate release is shown to reduce the frequency of migraine aura, but its effect on migraine headache is contradictory (Lampl *et al.* 2005,

Mulleners and Chronicle 2008). Mutations in the FHM genes are known to increase the predisposition to some rare monogenic and polygenic forms of epilepsies, which further indicates that ionic imbalance may trigger epileptic seizures (Haan *et al.* 2008, Helbig *et al.* 2008, Weber and Lerche 2008). Epileptic seizures are also observed in FHM1 mutant mice and rats (Haan *et al.* 2008). However, the majority of patients with FHM do not manifest epilepsy suggesting the involvement of other genetic and environmental factors in epilepsy.

3 Gene mapping of complex traits

By March 2009 the molecular basis of 2,492 phenotypes had been identified (http://www.ncbi.nlm.nih.gov/Omim/mimstats.html). These phenotypes are either caused by mutation(s) in a single gene that are inherited following Mendel's laws, or by multiple genetic and environmental factors interacting to produce a complex trait. The methods to identify a gene for Duchenne muscular dystrophy, a severe disorder related to muscular weakness, was inspirational for the gene mapping efforts of the early 1980's (Worton and Thomson 1988, Strachan and Read 2004). Both cytogenetic and molecular genetic studies were used in the gene mapping of this disease. A great improvement in gene mapping was the first human marker map based on restriction fragment length polymorphisms introduced by Botstein and co-workers 1980. Since then, linkage mapping has been especially successful in the search for genes coding for rare Mendelian diseases in isolated populations (de la Chapelle 1993, Peltonen *et al.* 1999, Norio 2003). In the early 1990's, mapping disease-predisposing loci using a family-based sample and genetic markers was defined as positional cloning (Collins 1992). In a study using the positional cloning strategy, around 1,000 genetic markers are usually genotyped in families with affected individuals to identify the predisposing locus with linkage analyses. The linked region is then searched for associated variants. Unfortunately, applying positional cloning to complex disease research has been challenging, most probably due to the role of common low risk variants in common disease susceptibility (*e.g.* Newton-Cheh and Hirschhorn 2005, Altshuler *et al.* 2008).

3.1 Theories on the genetic contribution in complex diseases

Three theories try to explain the role of genetic variants in complex diseases, although no single model provides a complete explanation of the onset of disease (Gibson 2009). According to the "common disease–common variant" model, disease susceptibility is thought to originate from several genetic variants with moderate effects that are cumulated in affected individuals. The second theory, the "rare alleles of major effect" model, emphasizes that some complex diseases originate from rare variants. Required incidence in a population is gained when diseased individuals are homozygous for hundreds of rare variants occurring at the frequency of 1/10,000. The third recently introduced model called the "infinitesimal theory" suggests that the clinical manifestation of complex diseases originates from hundreds or thousands of common and rare variants with low relative risk that explain only a fraction of disease liability. According to this theory, the genetic contribution to a symptom can be even greater than to the disease itself (Gibson 2009).

Models for analyzing genetic contributions in complex phenotypes and traits take into account the effect sizes of the variants being investigated. For example, hypertriglyceridaemia can be modelled as a mosaic of contributions from rare and common variants with varying effect sizes (Hegele 2009; Figure 3). However, in some common diseases mutations or variants in a single gene have a significant role in disease predisposition, for example 80% of patients with venous thrombosis have a rare mutation (allelic frequency of ~2% in the Dutch population) in the *Factor V* gene (Bertina *et al.* 1994). In Alzheimer's disease, on the other hand, a dose-dependent common variant of the Apolipoprotein E gene (epsilon type 4 allele) predisposes to Alzheimer's disease with almost total penetrance by the age of 80 in the homozygous genotype (Corder *et al.* 1993). For example, in Eastern Finland 17% of the population and 36% of Alzheimer's disease patients have the epsilon type 4 allele (Kuusisto *et al.* 1994). However, only 2% of height variation in a population is explained by 12 low risk common variants (OR≤1.2; Lettre *et al.* 2008), thus genetic basis for height can be as heterogenic as in hypertriglyseridaemia. Based on this small number of examples, the genetic contribution in common phenotypes cannot be simply explained accurately with a single model.

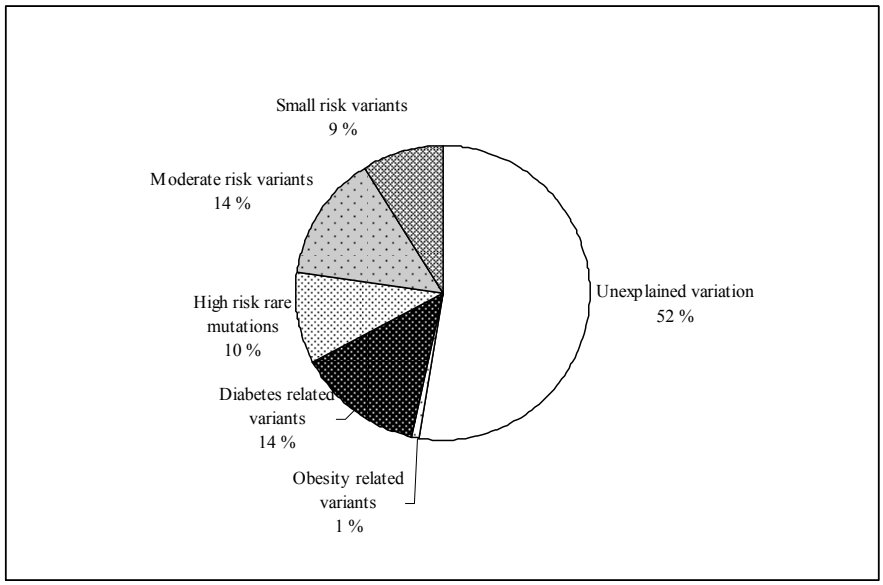

Figure 3. Pie diagram of the relative contributions of rare and common genetic variants in severe hypertriglyceridaemia. Modified from a figure by Hegele (2009).

3.2 Study sample in genetic studies

3.2.1 Sampling

When epidemiological studies have confirmed the role of genetic factors in disease susceptibility, the next step is to collect an appropriate cohort of related and/or unrelated individuals. Genetic studies are performed on large families, parents–affected child trios, affected sib-pairs or samples of affected cases and control individuals. Family-based studies are considered to be able to identify high-risk variants enriched in families and case–control samples are able to identify variants that are shared among large groups of affecteds. However, both study approaches have strengths and weaknesses that are reviewed by Newton-Cheh and Hirschhorn (2005), Hirschhorn and Daly (2005), Laird and Lange (2006) and Rodriguez-Murillo and Greenberg (2008). For instance, individuals for a case-control population study are relatively easy to collect, especially for diseases with a late onset. However, families are valuable for studies where only a few patients are available as adequately powered genome-wide association studies (GWAS) require thousands of cases and controls. A family sample does not suffer from population stratification, *i.e.* differences in allele frequencies between subpopulations in a population due to different ancestries that may seriously bias the association in a case–control sample. Furthermore, families have the

advantage of phase information of transmitted alleles that can be followed in the linkage and association analyses. Pedigree structure also provides information on heritability and shared environmental factors. Even if a family-based study is not conducted, information on familial predisposition among cases shows significantly higher power to detect genetic variants than cases where familial background is unknown (Amos 2007).

Regardless of the use of either a family or a case–control sample, the origin of the sample from either a population- or a clinic-based cohort can have an effect on the end result. Patients that are recruited through clinics can be more severely affected than patients from population-based sample, thereby possibly causing a bias to a specific symptom (Holt and Weiss 2000). A population-based study enables the exploration of the entire spectrum of a disease. However, in all cases the selection and phenotyping of a representative control sample is essential.

3.2.2 Isolated populations

Complex disease studies can be complicated by the susceptibility to false positives due to population heterogeneity. To increase the homogeneity isolated populations, *e.g.* those from Finland, Iceland, Sardinia (Italy) and Jewish communities (Ashkenazis), have been used in gene mapping studies for many diseases, including combined familial hyperlipidemia, type 2 diabetes or height (Pajukanta *et al.* 2004, Grant *et al.* 2006, Lettre *et al.* 2008, Barroso *et al.* 2008). However, recently regional stratification has been noticed in the ~1100 year-old Icelandic population that has been considered homogenous (Helgason *et al.* 2005) and similar results have been obtained from Finland (Salmela *et al.* 2008, Jakkula *et al.* 2008). Stratification can be further evaded by focusing genetic studies on regional isolates of 10–20 generations, such as the Kainuu isolate originating from a few founders that underwent a rapid population expansion and thus show a low number of founder alleles (Peltonen *et al.* 2000, Service *et al.* 2006). Regional isolates have been useful in identifying susceptibility genes; for example, the isolates of Kainuu and Quebec (Canada) were used in an asthma study (Laitinen *et al.* 2004), and Botnia in studies on multiple sclerosis and type 2 diabetes (Saarela *et al.* 2006, Diabetes Genetics Initiative of Broad Institute of Harvard and MIT *et al.* 2007). Achievements in studies on the Icelandic and Finnish population may also lie in the nation-wide healthcare registries, standardized phenotyping, positive attitude towards disease research and easily accessible genealogical records (Peltonen *et al.* 2000, Varilo and Peltonen 2004).

3.2.3 Phenotyping strategies for gene mapping studies

In genetic studies the accurately defined clinical phenotype assures the reproducibility and validity of genetic findings. A representative example is the study searching for variants for type 2 diabetes: The strongest signal was identified for the body mass index (BMI), the major risk factor for type 2 diabetes, since the controls were not adjusted for their BMI (Frayling *et al*. 2007, Attia *et al.* 2009). Thus analyses may reveal a locus predisposing to a risk factor instead of the clinical end-diagnosis if samples are not controlled over all predisposing factors. Adjustment for BMI and blood lipid measurements is often used to remove the effect of predisposing variants for obesity and coronary heart diseases (Thorleifsson *et al.* 2009, Aulchenko *et al.* 2009). In neuropsychiatric diseases, like in schizophrenia, autism and migraine, there are no good biological markers to confirm the diagnosis. In these diseases, clinical subtyping based on trait symptoms, endophenotyping or latent class analysis (LCA) are used to identify the genetic components in disease susceptibility (Rindskopf and Rindskopf 1986, Schulze and McMahon 2004). In trait component analysis (TCA), clinical symptoms such as pulsating headache are used to identify the susceptibility loci for migraine (Anttila *et al.* 2006). Endophenotypes are clinical entities associated with a disease such as quantitative cognitive traits in schizophrenia (Paunio *et al.* 2004) that may have an even stronger genetic factor than disease itself (Gibson 2009). LCA models association between observed variables that predicts a nonobservable (latent) variable. This method is used in several genetic studies of neuropsychiatric disorders such as alcoholism, migraine and schizophrenia (Korczak *et al.* 1999, Nyholt *et al.* 2004, Boks *et al.* 2008). The advantage in both TCA- and LCA-based methods is their ability to increase statistical power by enabling analysis on more individuals as affecteds than an analysis based on the end-diagnosis.

3.2.4 Sample size and power

Statistical power calculations are important to perform as a part of the study design to give a reference of the required sample size in the context of the realistic genetic effect, even though the true genetic model can be unknown. Ignorance of the genetic effect, population stratification and environmental differences increase the possibility of a false negative finding (type II error; Göring *et al.* 2001, Lohmueller *et al.* 2003, Evans and Cardon 2006). In genetic studies power of 80% is considered adequate to either reject or accept the null hypothesis of

no finding. This is justified by the study of Wacholder and co-workers (2004) showing that with a statistical power of >80%, the probability of false positives (type I error) is acceptable. If the power is high, the sample size is adequately large to be sensitive to background noise if the genetic effect of a predisposing variant is estimated to be realistic. For common polymorphisms the effect size (*i.e.* OR) is expected to be in the range of 1.1–1.5 (Zondervan and Cordon 2007). This creates great demands on the sample size. In their classic study, Risch and Merikangas (1996) showed that a sample size of 2,500 affected sib pairs is required to detect variant with the relative risk of ≤2. For studies where the effect size of an associated variant is 1.2, the required sample size is 6 times higher than that for studies where the effect size has been estimated to be 2.0 (Figure 4). High risk allele frequencies and disease prevalence further increase the power of a sample to detect predisposing variants. Thus, when several variants with different effect sizes are tested, large samples are required.

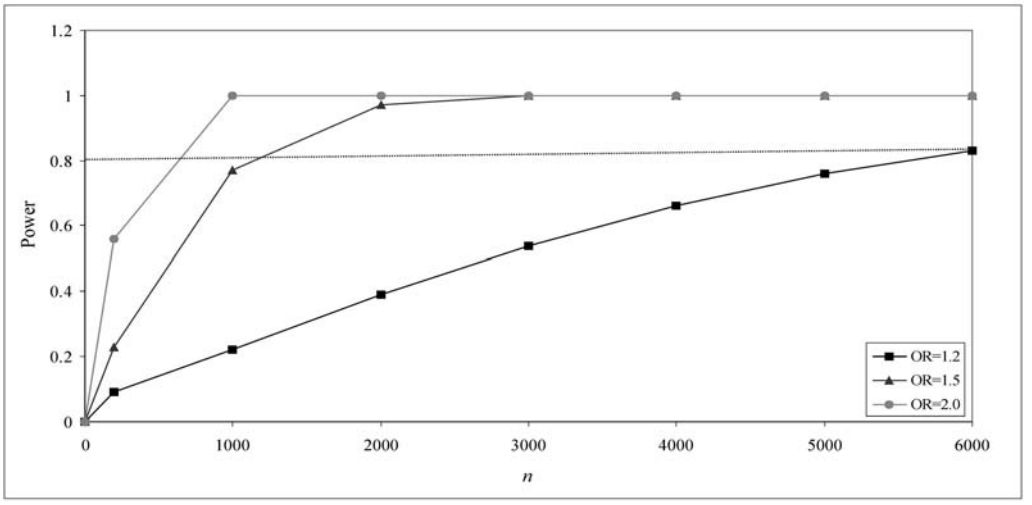

Figure 4. Effect of OR to power (*y*-axis) in the association test when disease prevalence is 1% and minor allele frequency (MAF) is 10% in a study population with an equal number of cases and controls (*x*-axis; when *n*=1000 there are 500 cases and 500 controls). Power estimates are derived from the Genetic power calculator program (Purcell *et al.* 2003). The dotted horizontal line shows the threshold for statistical power at 80%.

3.3 Typing of genetic markers

3.3.1 Variation in the human genome

The genetic difference between two randomly selected individuals is at least 0.2% due to variations in the genome (Sebat 2007). The traditional definition of "variation", or "polymorphism", is the difference between any two deoxyribonucleic acid (DNA) strains at the same locus with a frequency ≥1%. Variation may range from microscopically visible chromosome anomalies to submicroscopic variations from 1 bp to about 3 Mb (Feuk *et al.* 2006). Table 5 lists the types of the submicroscopic genetic variation in the human genome.

Table 5. Overview on the submicroscopic variation in the human genome.

Variation	Size	Variability	Total estimated number	Number in an individual human [1]	Reference
Single nucleotide polymorphisms	1 bp	Bi/tri-allelic	~9-10 million (frequency ≥5%)	3,213,401	International HapMap Project 2007
Microsatellite	1-6 bp <200 bp in length	Multiallelic	~1 million	447,165	Ellegren 2004
Insertion/deletion variation	1 bp >1 kb	Multiallelic	~1 million (biallelic)	918,996	Dawson *et al.* 2001, Weber *et al.* 2002
Minisatellite	6-100 bp in 20-50 copies	Multiallelic	150,000	93,568	Näslund *et al.* 2005
Alu elements	<500 bp	Biallelic	~1,000,000	1,738,571	Batzer and Deininger 2002
Copy number variation	1 kb ~ (3 Mb)	Multiallelic	12% of the genome	62	Redon *et al.* 2006
Inversions	200 kb ~ 10Mb	Multiallelic	176	90	Bansal *et al.* 2007

1) Levy *et al.* 2007

3.3.2 Genetic marker maps

Many of the human variation types are equally distributed all over the genome and follow Mendelian inheritance. Microsatellite and single nucleotide polymorphisms (SNPs) are typically used in genetic marker maps. Genetic mapping is based on the existence of linkage disequilibrium (LD). LD defines the co-inheritance of alleles of two loci. In other words, if

two loci reside in proximity to each other the probability of recombinations in meiosis is low and LD is high reflecting the common ancestry. Usually D' or r^2 figures are used to measure LD between two markers: r^2 equals 1 when the marker loci have identical allele frequencies and every occurrence of an allele of a marker predicts an allele at the other locus (Zondervan and Cardon 2004). D' is 1 if recombination has not occurred between the alleles.

Due to the heterozygosity and ubiquitous occurrence of microsatellites, short tandem repeats of mono-, di-, tri- and tetranucleotides (*e.g.* $[CA]_n$), have been the most widely used markers in the genetic mapping of Mendelian and complex diseases from the late 1980s to early 2000s. Usually a map of about 400 evenly spaced microsatellite markers is considered to provide adequate information to identify the predisposing locus. Recombination maps of human genome have been published to ease the gene mapping efforts by recording heterozygosity ratios and a linkage distance between the markers (Weissenbach *et al.* 1992, Kong *et al.* 2002).

Single nucleotide polymorphisms (SNPs) are presently the most used genetic markers. A SNP is an allelic variation, insertion or deletion of one nucleotide in the genome. The total number of common SNPs (MAF≥5%) is estimated to be 9–10 million, which constitutes 90% of the variation in the human population (International HapMap Project 2007). Each individual has approximately one SNP per thousand nucleotides (Levy *et al.* 2007). The International HapMap Project was officially launched in 2002 to determine the common patterns of the DNA sequence variation in the human genome, by characterizing sequence variants, their frequencies and correlations between them in DNA samples from populations with ancestry from Africa, Asia and Europe (The International HapMap Consortium 2003). The project is based on the estimation that 90% of SNP variation is common which arise from a single historical mutation (Gabriel *et al.* 2002). Since the mutation rate is low (1.8×10^{-8} mutations per site per generation, Kondrashov 2003) nearby SNPs are usually in high LD. These SNPs form a haploblock which is delimited either by mutation or recombination. In many haploblocks there are only a few haplotypes which represent most of the variation among a population. One or more SNPs that are called tagging SNPs (tagSNPs) are sufficient to identify the common haplotypes.

The high-throughput genotyping of SNP markers has enabled also the identification of copy-number variants (CNVs) that are polymorphic deletions, duplications or insertions that range from 1 kb to several megabases (Sebat *et al.* 2004, Conrad *et al.* 2006). In the SNP genotyping

process CNVs violate the expected statistical pattering of genotype data (McCarroll 2008). CNVs are considered to have an effect on the gene load that can cause predisposition to disease. Therefore, information of CNV regions has been integrated to commercial SNP marker sets.

3.3.3 Quality of genotyping

3.3.3.1 Genotyping errors

Genotyping is defined as the process where biochemical assays are used to determine the genotype of an individual. In genotyping several methods and techniques are used (Shi 2001, Gupta *et al.* 2008). Knowledge of the genotyping method and control of the genotyping process are essential in rejecting false genotypes that bias data analyses. Douglas and co-workers (2000) defined genotyping error as the misinterpretation of observed genotype from the true underlying genotype. Negligence to genotyping errors may lead to reduced power and consequently to type I or II errors, false positive or false negative results, respectively. Genotype error might originate from quality or genetic variation of a DNA sample, equipment precision, the amplification reaction or the human factor (Ewen *et al.* 2000, Pompanon *et al.* 2005, Kirsten *et al.* 2007). Table 6 summarizes different types of genotyping errors and their consequences.

Table 6. Summary of genotyping errors.

Origin of genotyping error	**Consequence**
DNA	
Low quantity/quality	Erroneous allele call
	Allelic dropout
	Short allele dominance
High CG content	Short allele dominance
Mutation/variation in the priming site	Null allele
Copy number variation	Allelic dropout
	Change in allelic ratios
	Dominance of heterozygotes
Insertion/deletion	A new allele
	Homoplasy [1]
Genotyping equipments and reagents	Allelic dropout
	Erroneous allele call
Human factor	
Sample swap	Mistaken allele
Cross-contamination	Mistaken allele
Oversight	Mistaken allele

1) Analogous fragments of different alleles

Both Bonin *et al.* (2004) and Pompanon *et al.* (2005) stated that good theoretical and practical skills are principal factors to avoid genotyping errors. Only clean, high quality reagents should be used, and genotyping should be performed in a controlled and monitored environment with proper dilutions (Fernando *et al.* 2001; Bonin *et al.* 2004; Pompanon *et al*. 2005). All steps in a genotyping process must be controlled with both positive and negative controls with a sufficient amount of replication. Additionally, the sample handling, processing and data management should be automated to the highest extent possible to prevent human errors.

3.3.3.2 Controlling the genotyping errors

Checking for the Mendelian inheritance of genotyped pedigree members is a rational and a standard procedure to track genotyping errors. Inheritance checking of commercial marker maps estimated a total error rate of 0.25% for over 100,000 microsatellite marker genotypes and an error rate of 1.37% for over 22,000 genotypes when custom-tailored markers were used (Ewen *et al.* 2000). Removing genotyping errors is important since error rate of 1% in a sib-pair data may cause 21-58% loss in linkage information (Douglas *et al.* 2000). However, the amount of unidentified genotyping may not be perceived, especially in the case of biallelic markers: The probability of undetectable errors is 66% in a four-person nuclear family (Douglas *et al.* 2002). If the same nuclear family is genotyped with four-allele marker, the probability of an undetectable error is 41%. To solve the problem of undetectable errors in pedigrees, a model has been created to estimate a posterior probability of mistyping error at each observed genotype based on map density, prior error rate, marker position and allele frequency in pedigrees with the inheritance phase information (Sobel and Lange 1996, Sobel *et al.* 2002). These mistypings may originate, for instance, from double recombination events which expand the genetic map compared to the expected map distances. The use of the mistyping model may halve the amount of undetectable errors (Douglas *et al.* 2000).

In a case-control sample every 1% increase in the sum of genotyping errors elevates the required sample size by 2–8% (Gordon *et al.* 2002). Therefore, pedigree-independent quality control procedures (the allele size, intensity and morphology of the amplified allele, the confidence score to the observed genotype) can be used to identify genotyping errors (Ewen *et al.* 2000, Sobel *et al.* 2002). Checking whether the alleles in question are in Hardy-Weinberg equilibrium can be used to check the quality of the data (Gomes *et al.* 1999). According to the Hardy-Weinberg principle, alleles from the outbred control population should have allele

frequencies p and q such that $p^2+2pq+q^2=1$. However, inbreeding, migration, mutation, a rapid population diminution (genetic bottleneck) and a small population may bias the equilibrium.

3.4 Methods to identify predisposing loci

3.4.1 Linkage analyses

In positional cloning linkage analyses are used to identify the region that carries the variant(s) predisposing to disease. Then predisposing variants are identified by LD-based association mapping and/or sequencing. Even tough positional cloning has not been successful in identifying genes to many common diseases, some success stories are listed in Table 7.

Table 7. Predisposing variants for complex diseases identified by positional cloning.

Disease	Locus	Gene	OR [1]	Risk variant
Crohn´s disease	16q12 Hugot *et al.* 2001	NOD2, Hugot *et al.* 2001, Ogura *et al.* 2001	3	Several coding variants
Schizophrenia	8p11-p21 Stefansson *et al.* 2002	NRG1 Stefansson *et al.* 2002	NA	Seven-marker haplotype
Asthma	7p15 Laitinen *et al.* 2001	*GPRA* Laitinen *et al.* 2004	1.4	Haplotype tagging SNP
Familial combined hyperlipidemia	1q21-q23 Pajukanta *et al.* 1998	*USF1* Pajukanta *et al.* 2004	NA	Two-SNP haplotype
Multiple sclerosis	17q24 Kuokkanen *et al.* 1997	*PRKCA* Saarela *et al.* 2006	1.64	Two-SNP haplotype
Type 2 diabetes	10q25 Duggirala *et al.* 1999, Reynisdottir *et al.* 2003	*TCF7L2* Grant *et al.* 2006	1.41	Allele of microsatellite marker
Genetic susceptibility to leprosy	6q25 Mira *et al.* 2003	*LTA* Alcaïs *et al.* 2007	5.63 (2.11) [2]	5'-UTR-SNP

OR = odds ratio, UTR = untranslated region, NA = data not available
1) Risk for heterozygous carrier if specifically given, 2) The most significant OR of subsample is given and a total sample OR is in parenthesis

Linkage analysis is based on the chromosomal regions (haplotypes) that are transmitted to diseased offspring more often than expected. When linkage is detected the recombination in meiosis has occurred between the studied marker and the predisposing locus with a probability of <50% (Morton 1955, Teare and Barrett 2005). The recombination probability between two loci is called a recombination fraction (θ). In other words, if two loci reside in different chromosomes they are inherited independently and θ equals 0.5. If two loci are linked, they are not inherited independently and are in linkage disequilibrium (LD), and thus, θ approaches 0.

Results of the linkage analysis are based on likelihood ratios and they are often reported as logarithm of the odds (Lod; logs to the base 10; Morton 1955) scores. A Lod score is a ratio of a likelihood of two loci being linked at a given θ ($\theta<0.5$) and a likelihood of no linkage ($\theta=0.5$). The Lod score of 3.3 ($p=4.9\times10^{-5}$) is regarded as a threshold for a genome-wide significance (Lander and Kruglyak 1995). When the Lod score is ≤-2 the linkage is considered to be excluded.

In Mendelian diseases the inheritance model can be estimated by studying the disease segregation in families. The model includes estimations of a mode of inheritance that is conventionally either the dominant or recessive model in which, respectively, one or two predisposing alleles are required for disease susceptibility. A disease allele frequency is estimated in a population and penetrance figures are assessed as to whether one or two alleles of a locus are predisposing to disease either with full or reduced effects. The phenocopy number estimates individuals affected without genetic predisposition. However, for the complex diseases the onset is the sum of interacting genetic and environmental factors, and thus, assumptions of the inheritance model used in parametric analyses are often hard to define (Göring and Terwilliger 2000b).

Since common diseases do not follow the Mendelian laws of inheritance, non-parametric, also known as model-free, statistical analysis methods have been created (*e.g.* the SimWalk, Sobel and Lange 1996; the Genehunter, Kruglyak *et al.* 1996; and the Merlin, Abecasis *et al.* 2002, programs). Model-free methods are based on the excess of shared segments that are identical by descent (IBD) between affected individuals. However, the absence of parental data and an insufficient number of polymorphic markers may weaken the identification of maternal or

paternal transmitted alleles that are IBD. Göring and Terwilliger (2000c) considered that the distinction between model-based and model-free methods is contrivent when complicated and heterogeneous data is analyzed: in a large data set, the number of independent observations (degrees of freedom) becomes high, which is solved by some simplifying probability (*e.g.* dominant or recessive) model leading to equivalent statistical tests. Furthermore, Wacholder *et al.* (2004) proposed that when a studied marker has an unknown function in complex disease predisposition, the dominant model may be the most reasonable choice since the difference in the effect of one or two alleles of a marker is probably lower than the effect between zero or one allele.

Linkage studies are optimal for a search of rare variants for diseases with a high effect in families (Risch 2000). However, susceptibility to common disease is suggested to rise from common variants with a low effect. A common variant (>10% frequency) increases parental homozygosity at the disease locus, thereby reducing linkage information. Therefore, an association study using affected and non-affected individuals may be a convenient method to detect common variants with a relatively low effect (Risch and Merikangas 1996).

3.4.2 Association studies

Traditionally genetic association mapping is used to fine-map the linked region or in candidate gene studies. However, the present knowledge of human variation based on the HapMap data has shifted the focus of gene mapping studies from positional cloning to association-based LD mapping. According to the HapMap project, GWAS performed with ≥552,000 SNPs are sufficient to cover the common variation in the Caucasian population ($r^2 \geq 0.8$) and ≥1,090,000 SNPs in the African population (The International HapMap Consortium 2007). The high-throughput, micro-array based genotyping technologies have reduced genotyping costs making GWAS feasible (Hirschhorn and Daly 2005, McKinney and Merriman 2007). In addition, large sample collections have provided adequately sized samples for association testing. The major advantage in LD mapping is, however, that the use of dense marker maps can identify a predisposing locus within a few kb, whereas the linked region can be the size of 10 cM corresponding to about 10 Mb (Hirschhorn and Daly 2005, Cheung *et al.* 2005, Bourgain *et al.* 2007).

Association studies are usually performed on population samples, but population admixture and stratification can cause fluctuations in allele frequencies that may lead to false positive findings. To avoid type I errors, tests for family-based association have also been created. In families, affected individuals have a higher expected frequency of shared susceptibility alleles than population cases, and it is possible to test both linkage and association simultaneously (Laird and Lange 2006). Family-based association analyses have been used in candidate gene studies for conditions such as schizophrenia and obesity (Hennah *et al.* 2003, Zhao *et al.* 2006), and in many GWAS (see Table 8).

By March 2009, 273 GWAS have been published showing an association p-value of $<1.0x10^{-5}$ when more than 100,000 SNPs have been tested (http://www.genome.gov/gwastudies/). The first studies were using samples of about 1,000 cases and a corresponding amount of controls with about 300,000 SNPs genotyped. However, the present common practice in a GWAS is to use over 2,000 cases and 2,000 controls with more than 500,000 SNPs genotyped to reveal the effect size of common variants (MAF~10%; Table 8). A study design of this setup seems to have adequate power to detect variant(s) with a p-value of $\sim 5\times10^{-8}$ for genome-wide significance (Attia *et al.* 2009, Gibson 2009).

The ability to identify common variants with a small effect (OR<2) requires large samples (Table 8). An exception to low OR-values is the study by Stefansson *et al.* (2008) where they could associate a rare CNV to schizophrenia with an OR as high as 16. But because the frequency of the CNV microdeletion was low (0.17% in cases and 0.02% in controls) large population samples were required.

When the sample size is sufficient, the second issue is whether the studied markers adequately cover the genetic variation in a sample. Imputation methods are introduced to infer genotypes of untyped markers based on the genotype data of resequenced and/or densely genotyped individuals (McCarthy *et al.* 2008). For example, a total of over 1.8 million variants were studied with the imputation method identifying several new loci for bipolar disorder and type 2 diabetes (Table 8, Ferreira *et al.* 2008, Zeggini *et al.* 2008). However, although the imputation method has made it considerably easier to study the association of as many as 3 million SNPs based on the HapMap data (International HapMap Consortium 2007, McCarthy

and Hirschhorn 2008) considered that for identification of distinct causal variants, resequencing of the LD regions is needed.

Table 8. Predisposing loci identified in complex diseases by GWAS.

Trait	Initial sample cases/controls	Method	Replication sample (cases/controls)	Number of studied SNPs [1]	No of significant loci [2]	OR	Previously identified by linkage	Reference
Obesity	694 from 288 families	Multi-stage	3,445/6,426	86,604	1	1.2	no	Herbert *et al.* 2006
Type 2 diabetes	1,380/1,323	Two-stage	2,617/2,894	392,935	*TCF7L2*, *SLC30A8*, *HHEX*	1.2-1.7	yes/no	Sladek *et al.* 2007
Joint GWAS on common diseases	~2,000/ ~3,000	Genome-wide genotype database	-	469,557	24 loci for 7 traits	1.1-5.5	yes/no	WTCCC 2007
Bipolar disease [3]	1,098/1,267	Multi-population study	4,387/6,206	1.8×10^6	*ANK3* *CACNA1C*	1.4-1.5 1.2	no	Ferreira *et al.* 2008
Type 2 diabetes [3]	4,549/5,579	Meta-analysis	24,194/55,598	2.2×10^6	10 new loci	1.1-1.4	no	Zeggini *et al.* 2008
Type 1 diabetes	3,561/4,646	Meta-analysis	6,225/6,946 3,064 trios	335,565	4 new loci	1.1	no	Cooper *et al.* 2008
Ischemic stroke	1,661/10,815	Multi-population study	4,576/19,343	310,881	4q25	1.3	no	Gretarsdottir *et al.* 2008
Schizophrenia	2,160 trios, 5,558 parent-offspring pairs	CNV	1,433/33,250 3,285/7,951	>300,000	Deletions on 1q21, 15q11 and 15q13	2.2-16.5	yes/no	Stefansson *et al.* 2008

GWAS, genome-wide association study; OR, Odds ratio; WTCCC, The Wellcome Trust Case Control Consortium; CNV, copy number variation

1) After quality checking, 2) Associated gene is given if informed, 3) Study involves imputated SNPs

All identified associated variants in Table 8 for single diseases are validated in other independent populations. A replication sample reduces the possibility of false positive findings due to unknown population structure. However, the replication of the association signal may be complicated by population heterogeneity, in which case protective alleles may nullify the effect of causative variants, or genotypic and environmental interactions restraining the onset of disease (Gibson 2009).

Due to methodological differences, loci identified by linkage analysis do not necessarily overlap with associated loci in GWAS. However, in a joint cohort study on seven common diseases, many novel susceptibility loci were observed but several loci for coronary artery disease, Crohn's disease, rheumatoid arthritis and type 1 and 2 diabetes were already identified using linkage studies (Table 8, The Wellcome Trust Case Control Consortium 2007, Mathew 2007). Also comparison between GWAS and linkage analysis studies on human *cis*-acting regions pointed out that 15 of 27 loci were identified by linkage analysis (Cheung *et al.* 2005). The advantage of GWAS is their capability to identify common variants with fine resolution and thus increasing the screening speed, but naturally an association on the linked locus can be considered further evidence of true association and linkage.

3.4.3 Methods to evaluate the significance of genetic findings

In genetic studies multiple nominally significant results are expected due to the stochastic variation of genome or genotype data and the large number of performed tests (Dudbridge and Koeleman 2004). Correction for multiple testing is performed for a number of tested variants, hypothesis or genetic models. A p-value of 0.05 (95% confidence interval) after the correction for multiple independent tests is often considered an indication of a significant result. However, a more stringent p-value of 0.01–0.001 may be preferred to ensure the significance of the result (Doerge and Churchill 1996). In GWAS the number of statistical tests is high, especially when multiple traits are tested. In linkage analysis a p-value of 5×10^{-5} is considered a threshold for significance (Lander and Kruglyak *et al.* 1995), and in GWAS a p-value of 5×10^{-8} is suggested to be a conservative level for genome-wide significance (Attia *et al.* 2009).

Many alternative methods have been developed to define the significance of statistical tests and some of them are listed in Table 9. Frequentist methods, such as the Bonferroni correction and permutation testing, take all tested hypothesis into account. One can also use the Bayesian

method, like the "False positive report probability", where association is corrected based on the observed p-value and the probability of the association being true. The Bonferroni correction is a standard method to correct the bias originating from multiple testing. However, because it assumes independence between the tests, the correlation among genetic variants and phenotypes is ignored. Therefore, the permutation test which retains the correlation in the original data provides a less conservative method to test the significance.

Table 9. Some methods used in the correction for multiple testing.

Correction method	Description	Notice	Reference
Family-wise error rate	Recommendation for a significance thresholds of human genome-wide linkage scan	Suggestive and significant evidence of linkage with a Lod score of 1.9 and 3.3, respectively	Lander and Kruglyak 1995
Bonferroni (Šidák) correction	Multiplying the p-value by the number of hypotheses	Assumes independence between the tests	Šidák 1967
Permutation testing	Random shuffling of phenotypes	Computationally time-consuming on a large scale	Churchill and Doerge 1994
False discovery rate	Controlling the expected proportion of falsely rejected hypothesis	Applicability to quantify vast amount of false positives is unknown	Benjamini and Hochberg 1995
False positive report probability	Calculates posterior probability of the validity of an association	Bayesian method, needs estimates of prior probabilities and risk ratios	Wacholder *et al.* 2004

3.5 Genetic factors complicating complex disease studies

The GWAS in Table 8 show promising steps in the identification of genetic variants for complex diseases. However, even a dozen of variants do not necessarily explain the whole genetic picture of a disease. Locus heterogeneity (distinct loci that are associated with the same trait) is evident for many complex diseases and obviously complicates the identification of genetic interactions. Also not all diseased individuals in a sample share the same susceptibility to the identified locus or variant which reduces the power of the study. Heritable epigenetic factors (DNA methylation, histone modifications, imprinting, untranslated RNAs), and gene-gene interactions (epistasis and long distance chromatin interactions) complicate the identification of genetic variants even further (reviewed by Jirtle and Skinner 2007, Bartkuhn and Renkawitz 2008, McCarthy and Hirschhorn 2008). Thus more advanced studies

combining both expression and genetic variation data will give a better understanding of complex disease genetics.

3.6 Genetic studies on migraine

3.6.1 Migraine loci idenfied by genome-wide linkage studies

By February 2009, eleven genome-wide linkage studies have been published identifying 16 suggestive or significant migraine loci based on the migraine end-diagnosis, TCA or LCA (Table 10). In four of them significant linkage between the end-diagnosis of MA or MO and loci at 4q21–q24, 6p12–p21, 11q24 and 14q21–q22 has been identified (Wessman *et al.* 2002, Björnsson *et al.* 2003, Carlsson *et al.* 2003, Cader *et al.* 2003, Soragna *et al.* 2003). The more recent studies have used different phenotyping strategies, such as TCA and LCA, to identify loci for different migraine trait components or symptom groups. In TCA the IHS based symptoms are used as traits (Anttila *et al.* 2006), and in LCA individuals are divided in four groups based on the severity of migraine symptoms (Nyholt *et al.* 2004). These studies have identified significant loci at 4q24, 5q21, 10q22–q23, 17p13 and several other suggestive loci (Nyholt *et al.* 2005, Lea *et al.* 2005, Anttila *et al.* 2006 and 2008, Ligthart *et al.* 2008).

So far, only in two studies have fine-mapping results for the linked regions at 6p12–p21 and 10q22–q23 been reported (Norberg *et al.* 2006, Anttila *et al.* 2008), but both of them failed to show significant association in the studied regions. However, the emerging consistency between linkage results and with both the increasing number of studies and phenotyping strategies has strengthened the confidence that the majority of significant linkage findings are true positives. It is especially encouraging when samples with different nationalities have been used. The advantage of using different phenotyping strategies has been obvious when these methods were compared in a study by Anttila and co-workers 2008 on a sample of 1,675 individuals from 210 migraine families. They found that the most heavily replicated locus on 10q22–q23 would have been identified only in one study instead of four studies (Nyholt *et al.* 2005, Anttila *et al.* 2006, 2008, Ligthart *et al.* 2008) if only the MA end diagnosis based phenotype was used.

Linkage studies on migraine have also shown that gender-specific genetic factors have a role in migraine pathophysiology as has been suggested by epidemiological studies. Female-

specific contribution has been detected in two populations: at loci 10q22-q23 and 18q12 in Finns, and at 4q21 in Icelanders (Björnsson *et al.* 2003, Anttila *et al.* 2006, 2008).

Table 10. The loci showing best evidence for linkage in the genome wide linkage studies on migraine. Loci showing significant, suggestive or replicated evidence of linkage are bolded, underlined or italicized, respectively.

Reference	Trait [1]	No of families	No of affecteds [2]	Nationality	Locus
Wessman *et al.* 2002	MA	50	252	Finnish	**4q24**
Carlsson *et al.* 2003	MA/MO	1	17	Swedish	**6p12–p21**
Björnsson *et al.* 2003	MO	103	289	Icelandic	**4q21** [3]
Soragna *et al.* 2003	MO	1	22	Italian	**14q21–q22**
Cader *et al.* 2003	MA	43	248	Canadian	**11q24**
Nyholt *et al.* 2005	LCA	756	556	Australian	**5q21**, 8q21, 10q22, 13q21, *6p12–p21, 1q21–q23*
Lea *et al.* 2005	LCA	92	380	Australian	18p11, 3q29
Anttila *et al.* 2006	TCA	50 [4]	225	Finnish	
	pulsation				**17p13**
	phonophobia and age at onset				**4q24**
	MA and/or MO (full criteria)				18q12
Anttila *et al.* 2008	LCA/TCA	210	621	Finnish and Australian	
	pulsation			Finnish and Australian	**10q22–q23**
	unilaterality			Finnish	**10q22–q23**
	pulsation			Australian	**10q22–q23**
	pulsation			Finnish	2p12
	pain intensity			Australian	8q12
	pulsation			Finnish	Xp22
	attack length			Finnish	*18q12*
	pain intensity			Australian	*14q21*
Ligthart *et al.* 2008	LCA/TCA	105	234	Dutch	
	LCA				1q23, 13q21, 20p
	photo/phonophobia				5q22
	pain intensity				10q22
	pulsation				13q21
Jonker *et al.* 2009	age at onset	258	na	Dutch	19q [5]

No, number; MA, migraine with aura; MO, migraine without aura; LCA, latent class analysis; TCA, trait component analysis; na, not available

1) In TCA the trait that showed the best linkage signal on a locus is given, 2) Number of affected individuals that has been used as primary selection criterion for the study, 3) Slightly relaxed criteria of MO, 4) The same families as in Wessman *et al.* 2002, 5) The significance of this locus was not defined.

An eye-catching feature in replicated migraine loci on 1q21–q23, 4q21–q24, 5q21–q22, 6p12–p21, 10q22–q23, 13q21, 14q21–q22 and 18q12 (Table 10) is the phenotypic heterogeneity in the linked traits. This inconsistency between traits may be due to susceptibility variant(s) that in these loci predispose to several symptom-specific processes (Anttila *et al.* 2008). For example, the first MA locus on 4q was replicated in the Icelandic MO sample based on the overlapping NPL results on 4q21–q24 (Wessman *et al.* 2002, Björnsson *et al.* 2003). Although the analyzed end-diagnosis was different, MA *versus* MO, the TCA analyses on the same Finnish families showed linkage to 4q21–q24 with age at onset, photophobia, phonophobia and unilaterilaty traits (Anttila *et al.* 2006) that are shared in migraine headache of both MA and MO. Furthermore, both the Icelandic and Finnish study also showed linkage on the 18q12 region that was further confirmed and replicated in the Finnish TCA study on individuals affected with MA and/or MO with normal attack length of 4–72 h (Anttila *et al.* 2006, 2008). The use of the LCA method has shown overlapping results at 1q21–1q23 and 13q21 (Nyholt *et al.* 2005, Ligthart *et al.* 2008), but the specific characteristics of each of the latent groups have not been reported, and thus, the predisposing trait components cannot be determined.

It is interesting that the pulsating migraine headache trait has been the most successful symptom for a locus identification on 2p12, 10q22–q23, 13q21, 17p13 and Xp22 (Anttila *et al.* 2008) even though the trait has not shown consistency between the studies. It can be an easily identified clinical symptom, but the pulsating migraine pain may indicate a role of neurovascular mechanisms in migraine. Another interesting feature in the genome-wide linkage studies on migraine is the almost total lack of overlapping results among common migraine, especially MA, and FHM loci. Suggestive evidence of linkage has been identified in only two LCA studies for the FHM2 region (Table 10, Nyholt *et al.* 2005, Ligthart *et al.* 2008), but the role of aura in these loci was not established. The Danish linkage study on the FHM families suggested a possibility of locus heterogeneity in FHM (Thomsen *et al.* 2007) and interestingly one of their nominal linkage signals is located in the proximity of the 10q21–q22 locus that has been linked to several common migraine traits (Anttila *et al.* 2008). The recent linkage study on a Spanish FHM family affected also with MA and MO showed significant linkage on 14q32 (Cuenca-León *et al.* 2009). Further studies will show whether the additional FHM loci predispose also to common migraine.

3.6.2 Candidate gene and locus studies on common migraine

Susceptibility of several candidate genes or loci has been studied on common migraine in samples of cases and controls, and families. Unfortunately, none of these studies have lead to the identification of susceptibility variants. Certainly, the variability of migraine symptoms along with multiple environmental and genetic factors complicates the genetic studies. Table 11 summarizes some of the candidate gene or locus studies on common migraine that have also been studied in another independent sample.

3.6.2.1 Candidate gene and locus studies on the FHM loci

Due to many similar characteristics between FHM attacks and common migraine, the role of FHM loci has been studied in common migraine (Table 11). Three studies have reported suggestive evidence of linkage between the FHM1 locus at 19p13 and common migraine (May *et al.* 1995, Nyholt *et al.* 1998, Terwindt *et al.* 2001), but five studies have failed to identify linkage to the FHM1 locus (Hovatta *et al.* 1994, Lea *et al.* 2001, Noble-Topham *et al.* 2002, Kaunisto *et al.* 2005, Kirchmann *et al.* 2006). Sequencing studies covering exons and selected areas of the *CACNA1A* gene have not identified associated variants (Lea *et al.* 2001, Brugnoni *et al.* 2002, Wieser *et al.* 2003, Kirchmann *et al.* 2006). The role of the FHM2 gene, *ATP1A2,* in common migraine is not solved since contradictory results in family and case–control samples have been published (Jen *et al.* 2004, Todt *et al.* 2005, Kirchmann *et al.* 2006, Netzer *et al.* 2006). So far, no studies have been reported concerning the role of the FHM3 gene in common migraine.

Since mutations in the three FHM genes disturb the neuronal ion balance, a similar pathophysiological imbalance in ion signalling may provide a pathway to common migraine. The association between 155 ion channel genes and MA was studied in an extensive multi-cohort study of the European-origin samples (Table 11, Nyholt *et al.* 2008). After correcting for multiple testing, none of the tested common SNPs showed significant association between 841 Finnish MA cases and 884 unrelated non-migraine controls. However, a significant epistatic interaction was identified between the potassium (*KCNB*2) and calcium (*CACNB2*) channel genes in the Finnish population. Interestingly, the potassium channel gene on 8q13 is

located in the proximity of the suggestive linkage signal of the pain intensity trait identified in an Australian sample at 8q12 (Anttila *et al.* 2008).

Table 11. Candidate genes or loci studied in common migraine.

Linkage studies

Locus	Families	Nationality	Phenotype	Method	Result	Reference
Chromosome 19						
FHM1	4	Finnish	MA/MO	Parametric	Negative	Hovatta *et al.* 1994
FHM1	28	German	MA/MO	ASP	Positive	May *et al.* 1995
FHM1	4	Australian	MA/MO	Parametric, NPL	Positive	Nyholt *et al.* 1998
FHM1	82	Australian	MA/MO	NPL, association	Negative	Lea *et al.* 2001
FHM1	36	Dutch	MA	ASP	Positive	Terwindt *et al.* 2001
19p13 (*INSR*)	16	North American (Caucasian)	MA	Parametric, NPL	Positive	Jones *et al.* 2001
19p13 (FHM1)	64	Canadian	MA	Linkage, TDT	Negative	Noble-Topham *et al.* 2002
19p13 (FHM1, *INSR*)	72	Finnish	MA	Parametric, NPL	Negative	Kaunisto *et al.* 2005
FHM1	34	Danish	MA	Parametric, NPL, sequencing	Negative	Kirchmann *et al.* 2006
Chromosome 1						
1q21-q31 (FHM2)	10		MA		Negative	Russo *et al.* 2005
1q31-q42	3	Australian Caucasian	MA/MO	NPL	Significant	Lea *et al.* 2002
replication sample	83		MA/MO	TDT	Nominal	
FHM2	34	Danish	MA	Parametric, NPL, sequencing	Negative	Kirchmann *et al.* 2006
Other loci						
X chromosome	3	Australian	MA/MO	Parametric, NPL	Significant	Nyholt *et al.* 1998
4q24, 14q24, 15q11-q13	10	na	MA	Parametric, sequencing	Significant for 15q11-q13	Russo *et al.* 2005

Association studies

Candidate region/gene	Sample (cases/controls)	Nationality	No of tested variants	Significantly associated variants	Tested/ associated phenotype	Reference
Locus identified using linkage analyses on candidate loci						
19p13	825/765	North American	13	3	MA	McCarthy *et al.* 2001
INSR/GTF2F1	825/765	North American	16	5	MA/MO	
INSR/GTF2F1	275/275	Australian Caucasian	2	1 (*INSR*)	MA/MO	
1q23-q31	243/243	Australian Caucasian	6	No	MA/MO	Fernandez *et al.* 2007
GABA-A receptors (15q11-q13)	270/273	Northern European	56	No	MA	Netzer *et al.* 2008
replication sample	379/379	Northern European	4	No	MA	
GABA-A receptors	898/900	Finnish	34	No	MA	Oswell *et al.* 2008
Candidate gene studies						
155 ion channel genes	841/884	Finnish	5257	No	MA	Nyholt *et al.* 2008
replication sample	2835/2740	Caucasian	66	No	MA	
ESR1	484/484	Caucasian	1	1	MA/MO	Colson *et al.* 2004
MTHFR	652/269	Caucasian	1	1	MA	Lea *et al.* 2004
MTHFR, ESR1	898/900	Finnish	32	No	MA	Kaunisto *et al.* 2006

MA, migraine with aura; MO, migraine without aura, FHM, familial hemiplegic migraine; NPL, non-parametric linkage; ASP, affected sib-pair test; TDT, transmission disequilibrium test; No, number

3.6.2.2 Other susceptibility loci

Often migraine loci or genes that show association or linkage in one population are not replicated in other independent samples. The *5',10'-methylenetetrahydrofolate reductase* (*MTHFR*) gene that may alter the threshold for migraine attack and the *oestrogen receptor* (*ESR1*) that may mediate menstrual migraine are the most intensively studied candidate genes for migraine. Although they have shown association in several small migraine samples, they have failed to show significant association in large and comprehensive case–control studies (Kaunisto *et al.* 2006, Todt *et al.* 2006). Such contradictory results are often based on low and heterogeneous sample sizes or differences in phenotype criterion (Wessman *et al.* 2007).

Other potential candidate genes for migraine are the *GABA-A* receptor genes that mediate the function of a neuronal suppressor, gamma-amino butyric acid. A linkage study on candidate regions proposed the *GABA-A* receptors as candidate genes for MA (Russo *et al.* 2005), but studies in Finnish and Caucasian populations failed to show association to these genes (Oswell *et al.* 2008, Netzer *et al.* 2008). Studies on variants of the insulin receptor gene (*INSR*) located in the proximity of FHM1 have also shown contradictory results in samples of European origin (McCarthy *et al.* 2001, Kaunisto *et al.* 2005, Netzer *et al.* 2008b).

3.6.3 Genetic studies on comorbid families

Although the co-occurrence of common migraine with epilepsy and stroke is well established, no comprehensive genome-wide analyses have been performed on large samples of comorbid families or individuals. An encouraging linkage finding was identified at 9q22 in a single large Belgian family with occipitotemporal lobe epilepsy and MA (Deprez *et al.* 2007). Characteristics for these disorders are visual symptoms that suggest a possibility of a shared genetic background for epilepsy and migraine. Further evidence of this shared aetiology has been shown in another Belgian study where a FHM2 mutation was associated with occipitotemporal epilepsy and migraine (Deprez *et al.* 2008).

Hereditary vascular retinopathy (HVR), a neurovascular disorder characterized by a progressive loss of vision, is another disorder where the genome-wide linkage analysis has been performed on a family comorbid with migraine (Ophoff *et al.* 2001). HVR shows

autosomal dominant inheritance and it is associated with an increased risk of stroke and the Raynaud syndrome that are both also associated with migraine. A genome-wide linkage analysis revealed a locus at 3p21 that may also predispose to migraine (Hottenga *et al.* 2005). The identified gene for HVR encodes for mammalian *3′-5′ DNA exonuclease* but its role in migraine needs to be further examined (Richards *et al.* 2007, Stam *et al.* 2008).

4 Aims of the present study

The aim of this work was to identify susceptibility loci for migraine with aura (MA) and its traits using well-phenotyped samples and state of art gene mapping methods. The specific aims of this thesis were:

1. To study the role of vasoactive genes *EDN1*, *EDNRA* and *EDNRB* in MA susceptibility using a case–control study setting.

2. To improve the Sequenom genotyping method for cost efficiency and accurate fine-mapping.

3. To study the contribution of both the Mendelian FHM1 and common migraine locus at 19p13 in MA families.

4. To identify loci predisposing to migraine aura in families

5 Materials and Methods

5.1 Study subjects

The ethics committee of the Helsinki University Central Hospital approved these studies (approval no. 622/E0/02) and an informed consent was obtained from all individuals. For study III an approval was also gained from the ethics committee of the University of California, Los Angeles. For the German sample in study I an approval was obtained from the Bonn University Hospital Ethics Committee (approval no. 184/00)

5.1.1 Migraine families

Altogether 108 families were genotyped for studies III and IV. The families were obtained from the Finnish Migraine Gene Project, in which over 1,400 families with a total of 5,000 samples have been collected nationwide since 1996. The migraine patients have been recruited from headache clinics in Helsinki, Turku, Jyväskylä and Kemi and using advertisements in the newsletter of the Finnish Migraine Society. When a member, *i.e.* an index case, of a family has been clinically diagnosed with migraine, he/she has been asked to contact relatives who have been believed to suffer from migraine. If the relatives were willing to participate in the study, the validated Finnish Migraine Specific Questionnaire for Family Studies was mailed to first-degree relatives along with a request for a donation of a blood sample (Kallela *et al*. 2001). The diagnoses have been made according to the diagnostic IHS criteria by experienced neurologists (Headache Classification Committee of the International Headache Society 1988, Headache Classification Subcommittee of the International Headache Society 2004). In the case of incomplete or contradictory answers telephone interviews have been used to confirm the diagnosis.

For study III the MA families (n=72) were selected based on a seemingly autosomal dominant mode of inheritance within the families. The end diagnosis distributions of 762 genotyped individuals are shown in Table 12. On average each pedigree had five affecteds and three generations.

Table 12. End diagnosis distributions among genotyped individuals in families (studies III and IV).

		Study III [1]		Study IV [2]	
End-diagnosis	**IHS code [3]**	***n***	**% female**	***n***	**% female**
MA	1.2.1	417	72.2	160	79.3
Typical aura with non-migraine HA	1.2.2	na	-	8	25.0
Typical aura without HA, EQV	1.2.3	8	62.5	4	25.0
Probable MA	1.6.2	na	-	47 [4]	83.8
MO	1.1	91	61.5	35	60.0
Probable MO	1.6.1	na	-	28	42.9
HA	2	23	43.5	12	58.3
NoHA	-	170	30.0	48	37.5
MD	-	48	47.9	9	55.6
Total	-	757	58.9	351	63.8

IHS, The International Headache Society; MA, migraine with aura; HA, headache; EQV, equivalent migraine; MO, migraine without aura; HA, headache; NoHA, no headache; MD, missing diagnosis; na, not available

1) Diagnoses are based on the first diagnostic criteria of IHS (Headache Classification Committee of the International Headache Society 1988), 2) Diagnoses are based on the second diagnostic criteria of IHS (Headache Classification Subcommittee of the International Headache Society 2004), 3) Codes are according to the second diagnostic criteria of IHS, 4) Number of patients that are suffering from aural features that do not fulfil all IHS criteria for migraine aura. Furthermore, migraine headache does not necessarily fulfill IHS's criteria.

In study IV the target phenotype was as homogenous visual migraine aura in a family as possible, regardless of the associated headache characteristics. From 36 families we were able to identify 185 aura patients of which 84% had scintillating scotoma type of aura. The mean number of aura affecteds was five in a family with an average of three generations. In total we genotyped 351 individuals whose IHS-based diagnoses are described in Table 12.

5.1.2 Unrelated migraine cases

In study I, the initial case sample consisted of 898 migraine cases (Kaunisto *et al*. 2006). The majority of cases (*n*=613) were selected from the cohort of Finnish MA families. The cases were mainly index-cases of the families but also affected spouses were included. One third (*n*=285) of the cases originated from a Finnish cohort of same sex twin pairs born before 1958

with a family history of migraine (Kaprio *et al*. 1978). However, after the re-evaluation of the re-filled questionnaires (*n*=37) or identification of spurious family relationships (*n*=11), 48 migraine cases were rejected from analyses. Thus, the final case sample consisted of 850 migraine patients. Table 13 describes the gender specific diagnosis and trait distributions of the study sample. In study I the visual aura trait included 142 migraine patients that according to the IHS criteria should be classified to the category of probable migraine with aura (the IHS code 1.6.2). They were included in the trait analyses if some migraine related symptoms in their attacks share the same underlying susceptibility variants than MA symptoms.

The replication sample in the study I was obtained from Germany. The MA diagnosis of 648 Caucasian origin patients was made according to the IHS criteria (Table 13; Netzer *et al*. 2008). All patients were interviewed personally or by telephone and a questionnaire was filled out for each of them (Todt *et al*. 2005).

Table 13. Number of subjects in the MA, trait and control groups by sex and country used in study I.

Trait	Finnish sample			German sample			All
	n	$n_{females}$ (%)	n_{males}	*n*	$n_{females}$ (%)	n_{males}	*n* (females %)
MA	708	559 (79)	149	648	499 (77)	149	1356 (78)
Visual aura	850	673 (79)	177	607	461 (76)	146	1457 (78)
Phonophobia	601	526 (84)	99	568	449 (79)	119	1169 (83)
Photo- and phonophobia	462	403 (84)	79	548	433 (79)	115	1010 (83)
Age of onset (<20 years)	403	347 (82)	75	377	283 (75)	94	780 (81)
Controls	890	685 (77)	149	651	495 (76)	156	1541 (77)

5.1.3 Control samples

The Finnish control sample in study I consisted of 890 controls from the Finnish twin-cohort of opposite-sex pairs born 1939–1957 with no first-degree relatives with migraine. The German controls used as a replication sample included 651 individuals of Caucasian descent and they completed a migraine-specific questionnaire enabling the identification of migraine-suspicious individuals (Todt *et al.* 2005).

A sample set of 31 anonymous Finnish blood donors and one negative water control sample was used to optimize and evaluate the success of the SNP genotyping reactions in studies I and II.

5.2 Methods

5.2.1 DNA extraction

Either the standard phenol-chloroform extraction procedure (Blin and Stafford 1976) or the Autopure LS automated DNA purification instrument (Gentra Systems, Minneapolis, USA) was used to extract DNA from peripheral blood. The German DNA samples were extracted by salting out procedures.

5.2.2 Genotyping

5.2.2.1 Microsatellite markers

In study III the 72 families were genotyped using eight microsatellite markers surrounding the migraine candidate genes *INSR* and *CACNA1A* on 19p13. The markers covered a region of 11.4 Mb with a mean distance between markers of 1.6 Mb. The marker and gene positions based on the UCSC database (http://genome.ucsc.edu; Karolchik *et al*. 2003) are shown in Figure 5.

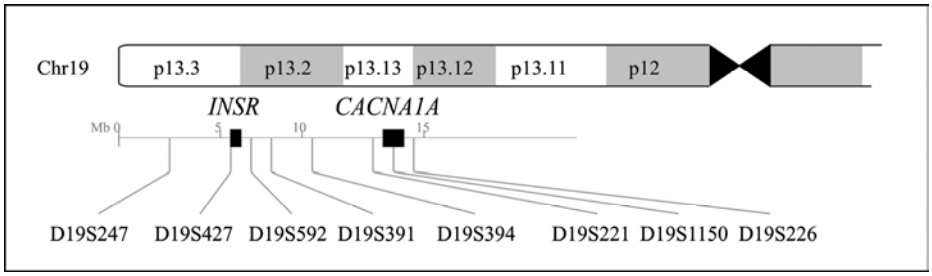

Figure 5. Locations of the eight genotyped microsatellite markers surrounding the *INSR* and *CACNA1A* genes on chromosome 19p13. (Chr, chromosome; Mb, megabase)

The genome-wide linkage analysis on the 36 families (study IV) was performed with the ABI Prism® Linkage Mapping Set v2.5 MD consisting of 400 microsatellite markers in a map with a resolution of approximately 10 cM (Applied Biosystems, Foster City, CA, USA). The

genetic map positions were based on the recombination rates derived from the Icelandic population (Kong *et al*. 2002).

5.2.2.2 Microsatellite genotyping

The repeat sequences of di- tri- or tetranucleotides were amplified by PCR (polymerase chain reaction, Mullis and Faloona 1987) using fluorescence-labelled oligos according to the manufacturer's protocol. In study III DNA fragments were separated by capillary electrophoresis using the ABI3700 DNA analyzer (Applied Biosystems, Foster City, CA, USA), called with GeneScan Software (Applied Biosystems) and analyzed with Genotyper Software (Applied Biosystems). In study IV the ABI3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA) was used to separate the PCR fragments that were called with the ABI3730 data collection software and analyzed with the ABI Genemapper software package (Applied Biosystems). Genotypes of the CEPH (Centre d'Etudes du Polymorphisme Humain) individuals were used as controls to evaluate the performance of genotyping reactions and to standardize the allele calling.

All genotypes were verified by human inspection. Incompatibilities in Mendelian inheritance were controlled using the PedCheck program (O'Connell and Weeks, 1998). Genotype mistyping analysis was performed with the SimWalk2 program to reveal inconsistencies in genotypes based on allele frequencies, marker order map, phenotype model and pedigree structures (Sobel and Lange, 1996; Sobel *et al*. 2002). In mistyping analysis an overall error rate of 2.5% was used and all genotypes indicated with a probability of mistyping were rejected.

5.2.2.3 SNP markers

In study I a total of 33 SNPs were genotyped covering both the gene and flanking regions of the *endothelin1* and its *receptor A* and *B* genes. The Haploview program (Barrett *et al*. 2005) was used to identify the haplotype tagging SNPs based on genotypes of Caucasian individuals in the public database of the International HapMap Project (http://www.hapmap.org/; The International HapMap Consortium 2003). If the designing of primers for tagging SNP failed, a

nearby SNP in as high LD (r^2) as possible was selected to compensate for the original tagging SNP. The additional synonymous or non-synonymous SNPs from gene coding regions and template sequences were obtained from the UCSC Genome Browser (http://genome.ucsc.edu/, Karolchik *et al.* 2003). The Positions and LD structure of analyzed SNPs are shown in Figure 1 (study I).

For study II 96 SNPs were genotyped including the 32 SNPs from study I. The rest of the SNPs were selected either from the public databases mentioned above or from the Seattle SNP database (http://pga.gs.washington.edu/).

5.2.2.4 SNP genotyping

The SNP genotyping (studies I and II) was performed with the homogenous Mass Extension Mass ARRAY genotyping system (Sequenom®, San Diego, CA, USA). The allele identification is based on the mass differences between two alleles of a SNP (Leushner *et al.* 2000). Figure 6 shows an overview of the primer detection reaction for the A/T SNP polymorphism: A known region of genomic DNA is amplified and then used as a template for a primer extension reaction. A detection primer is designed to anneal adjacent to a SNP site (A/T). The DNA polymerase enzyme mediates the extension of the detection primer with a single thymidine dideoxynucleotide triphosphate (ddTTP). To gain at least 50 Da difference in mass of extension products a primer for allele T is firstly extended with an adenosine deoxynucleotide (dATP) and then terminated by adding a guanine dideoxynucleotide triphosphate (ddGTP).

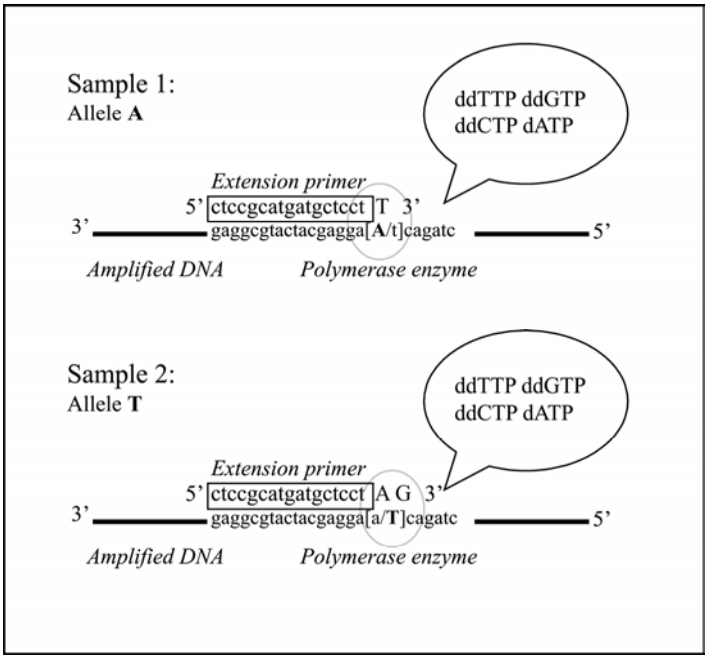

Figure 6. Overview on the SNP identification by using the homogenous Mass Extension Mass ARRAY.

For the Sequenom genotyping system the AssayDesign 2.0.7.0 software (Sequenom) was used to design multiplexes of 4–6 SNPs for the PCR and extension reactions. PCR and extension primers were purchased from Proligo France SAS (Paris, France) and Metabion International AG (Martinsried, Germany), respectively. The extension reaction was performed with 0.6 U/reaction using either ThermoSequenase® (GE Healthcare, Chalfont St. Giles, UK) or TERMIPol® DNA polymerase (Solis Biodyne OÜ, Tartu, Estonia). Genotyping of each SNP was first evaluated in a single-plex reaction for optimal primer concentrations in the sample set of 31 anonymous blood donors. In the second optimization step SNPs were genotyped in a multi-plex reaction of 4–6 SNPs to detect possible primer-primer interactions and assess the optimal primer concentrations between multiple primer pairs before sample genotyping. Aside from optimization modifications, the multiplex PCR reactions were performed according to the manufacturer's instructions.

Extension products spotted on microchips were detected with a real-time matrix-assisted laser-desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry analysis. The genotypes were called by the SpectroCaller software (Sequenom). All genotypes were verified by human inspection with the Typer 3.3 software (Sequenom). The quality of genotypes was

inspected by the KariOTyper in-house web interface (Study II). The SPSS (version 12.0.1) software was used to calculate the numeric parameters in order to compare the performance of two DNA polymerases in study II. In study I genotype distributions in the whole sample and for cases and controls were tested separately for the Hardy-Weinberg equilibrium (HWE) by the χ^2 method implemented in the PLINK 1.0 program in order to reject the suspicious markers (Purcell *et al*. 2007). In the HWE test a p-value <0.001 was used as an exclusion threshold for SNPs (http://www.hapmap.org/downloads/data-handling_protocols.html).

5.3 Statistical methods

5.3.1 Simulations and power calculations

In study III genotype data was simulated to estimate the power of a 72 family-study to detect linkage. The trait was assumed to be inherited by the autosomal dominant mode with a reduced penetrance of 90%, the disease allele frequency was 0.1% and a proportion of phenocopy was 2.4% (Hovatta *et al*. 1994, Wessman *et al*. 2002). The simulated marker with a heterozygosity ratio of 0.8 had 5 alleles of equal frequencies. The SLINK package was used to conduct a simulation on studied families (Ott 1989; Weeks *et al*. 1990): Genotypes of each pedigree were replicated 500 times with the SLINK program and the expected Lod score (ELOD), expected maximum Lod score (EMLOD) and power were obtained using the program MSIM (Lathrop *et al*. 1984). The ELODHET program was used to analyze the data allowing for linkage admixture (Ott 1989; Weeks *et al*. 1990). Power was defined as the proportion of replicates where the Lod score was ≥3 under locus homogeneity and ≥3.3 under locus heterogeneity (Terwilliger and Ott, 1994).

In study IV the simulation was performed in a condition of no linkage between disease locus and the marker loci using the program SIMULATE (Ott 1989). The best linked marker (D9S1690) was simulated in 1,000 replicates of the pedigree set based on detected allele frequencies assuming autosomal dominant inheritance.

In study I the power estimation was performed with the Genetic Power Calculator for discrete traits (Purcell *et al*., 2003; http://pngu.mgh.harvard.edu/~purcell/gpc/). The disease allele was estimated to have a major dominant effect of 1.5 to disease onset with a frequency of 0.2.

Prevalence of MA was assessed to be 5%. In addition, the studied variant was allowed to be in LD of 0.8 (D' prime) with the predisposing allele with an allele frequency of 0.20.

5.3.2 Linkage analyses

In studies III and IV, analyses were performed with the affecteds-only method, and thus, the individuals without a studied end-diagnosis or a trait were treated as unknowns. By this method the possibility of reduced penetrance or lack of environmental exposure was recognized (Anttila *et al.* 2008). In study III in families where both parents were affected the descendents were rejected from analysis to avoid problems caused by bilinearity. Alternatively, in the case of multiple affected relatives of a married-in member, they were used to form an independent family. All parametric analyses were performed by assuming a dominant inheritance with a disease allele frequency of 0.1%, a penetrance of 90% and a phenocopy rate of 2.4% (Hovatta *et al*. 1994; Wessman *et al*. 2002). The allele frequencies for markers were calculated from all genotyped individuals.

In studies III and IV parametric two-point linkage analyses were performed with the LINKAGE package (Lathrop *et al*. 1984) including the MSIM program to calculate Lod scores at the recombination fractions between 0 and 0.5 and the HOMOG program to calculate the homogeneity of pedigrees (Ott 1991). Additionally, Lod scores of affected sib-pairs (ASP; Suarez *et al*. 1978) analysis were calculated to investigate the possibility that a predisposing migraine locus acts in a recessive fashion. The ANALYZE utility program was used to conduct these analyses (Göring and Terwilliger 2000b) and in studies III and IV the software tools AUTOSCAN (Hiekkalinna and Peltonen 1999) and AUTOGSCAN (Hiekkalinna *et al*. 2005) were used to automate the linkage analyses, respectively.

In parametric linkage analysis a Lod score of 3.3 was considered as significant evidence of linkage under locus heterogeneity and the thresholds for suggestive and nominal evidence of linkage were 1.9 and 0.59, respectively (Terwilliger and Ott 1994, Lander and Kruglyak 1995). In the ASP analysis of study IV the thresholds of 3.05 and 1.74 were used to show significant and suggestive evidence of linkage, respectively (Anttila *et al*. 2008).

The non-parametric two-point analysis based on identity by descent (IBD) measurements at the marker loci was performed either with the SimWalk2 v2.82 (study III) or the SimWalk2 v2.91 program without subdividing large families (study IV; Sobel and Lange, 1996). When modelling dominant (p_{dom}) inheritance the largest number of affecteds inheriting an allele from one founder allele is estimated. In the additive inheritance statistics (NPL_{all}) it is estimated whether a few founder-alleles are overly represented in affecteds. In the SimWalk analysis p-values of ≤0.01 and ≤0.03 were considered as significant and suggestive evidence of linkage, respectively (personal communication by Dr. E. Sobel).

5.3.3 Haplotyping of families

In studies III and IV the haplotype analysis was performed to restrict the segment that was shared among families showing linkage to the best linked region based on the SimWalk2 statistics. The haplotypes of all family members were constructed using both the SimWalk2 v2.91 haplotype option (Sobel and Lange 1996) and the GENEHUNTER v2.1_r6 program (Kruglyak *et al*. 1996). Haplotypes transmitted in a family were manually compared among all family members to identify the most prevalent haplotype among affecteds.

5.3.4 Association analyses

In study I, the Haploview 4.0 program (Barrett *et al*. 2005) was used to identify the LD structure and tagging SNPs which define the haploblock structures with the confidence interval method (Gabriel *et al*. 2002). The PLINK 1.0 program was used to calculate allele and genotype frequency based association statistics (Purcell *et al*. 2007). As the exclusion threshold, individuals and SNPs missing >10% of genotypes were excluded from the analyses. For testing allelic association, the Cochran-Armitage (trend) test was used. In both allelic and genotypic tests an uncorrected p-value of ≤0.05 were considered as an indication of possible association and for those SNPs the recessive and dominant gene action tests of the PLINK program for the minor alleles were applied. In the recessive test the genotypes homozygous for the minor allele are tested against all other genotypes between cases and controls, and in the dominant test association genotypes with the minor allele are tested against the homozygous major allele genotypes. The haplotype association analysis and epistasis analysis between studied loci were also performed using the PLINK program. The permutation procedure

implemented in the PLINK program was used to elucidate the significance of the overall test statistics with 1,000 replications (Churchill and Doerge 1994, Purcell *et al*. 2007).

The logistic regression model was used to test the effect of the risk genotype both in the Finnish and German samples when sample origin was an interaction term. The similar effect size enabled the analysis of the pooled sample adjusted for the sample origin and gender using the software SPSS (version 16) and Stata (version 9.2).

6 Results and discussion

This study gives an overview of traditional linkage and association methods to study susceptibility to MA. The major advantage of this study was the large study sample of the Finnish Migraine Gene Project. Careful phenotyping and the use of the diagnostic criteria introduced by the IHS have made this patient collection valuable for the genetic studies of migraine. Furthermore, the use of well-established laboratory and statistical methods facilitated analyses and evaluation of the findings.

6.1 Mass spectrometry-based genotyping method for fine-mapping studies

The modern chip-based genotyping platforms, such as the Affymetrix Inc. GeneChip array (Chee *et al.* 1996) and Illumina Inc. BeadArray technology (Oliphant *et al.* 2002), can efficiently handle a large number of tested variants, although so far in many candidate gene studies, like those on migraine, less than 50 markers are usually studied (Table 11). Therefore, flexible and economical methods for genotyping small number of variants are needed. In our laboratory, the Finnish Genome Center, the MassARRAY® (Sequenom; Leushner *et al.* 2000) system is regularly used for fine-mapping candidate genes and loci. The Sequenom system is specially designed for high-throughput, medium-sized projects, providing a medium-sized multiplexing capacity with high accuracy and sensitivity (Isler *et al.* 2007). In study I the Sequenom genotyping system was used to genotype the variants of the candidate genes. In study II the quality of the Sequenom genotype data was evaluated by comparing the performance of two DNA polymerases.

6.1.1 Role of Endothelin1 and its receptors A and B in MA susceptibility

Our (Färkkilä *et al.* 1992, Kallela *et al.* 1998) and other (Gallai *et al.* 1994, Hasselblatt *et al.* 1999) studies have indicated nominally or significantly elevated levels of EDN1 in blood circulation during migraine attacks, although a contradictory result exists (Nattero *et al.* 1996). The primary receptor for EDN1, *EDNRA*, was previously associated with migraine in a French study (Tzourio *et al.* 2001). Interestingly, Finnish migraine families have shown linkage to the 4q28–q31 region adjacent to *ENDRA* (Anttila *et al.* 2006). Therefore, EDN1 with its receptors may be a link among the neural, vascular and pain related aspects of migraine. In this study we

aimed to analyze *EDN1* and its receptors *EDNRA* and *EDNRB* as candidate genes for MA using a dense set of SNPs.

Association between MA and endothelin1 (*EDN1*) and its two receptors (*EDNRA* and *EDNRB*) were studied in 850 migraine cases and 890 control individuals of Finnish origin. The majority of cases with a family history of migraine were carefully selected from our Finnish migraine family sample of 1,400 families and one third of the cases were from a Finnish cohort of the same-sex twin pairs born before 1958.

6.1.1.1 Overview of the SNP genotype data

Our sample had a statistical power of 91% at the p-value of 0.05. We genotyped altogether 33 SNPs of which one *EDN1* SNP was monomorphic and thus excluded from the analyses. Of the remaining 32 SNPs, 19 were tagging SNPs that defined the LD block. The mean distance between the analyzed 32 SNPs was 7.4 kb. The average success rate of the markers was 99%. All the SNPs were in HWE (with a p-value of >0.001). The frequencies of the minor alleles and minor homozygous genotypes varied between 0.02–0.29 and 0.001–0.25, respectively. Heterozygosity ratios varied from 5% to 49%, the average being 31%. The heterozygosity ratios of the *EDN1* and *EDNRB* genes were similar to the heterozygosity ratios provided by the UCSC database from multiple information sources (htpp//:genome.ucsc.edu). However, the majority of the intragenic SNPs in the *EDNRA* gene demonstrated reduced (3–24%-unit) heterozygosity in both cases and controls compared to the database information (data not shown).

6.1.1.2 Results based on the MA end-diagnosis

We analyzed allelic- and genotype-based association between MA cases and control individuals. Summary of the p-values of the 19 tagging SNPs for the Finnish sample and females are shown in Table 14. When patients with the MA end diagnosis were designated as affecteds (*n*=708), two adjacent *EDNRA* SNPs rs2048894 and rs5334 provided uncorrected genotype-based p-values of 0.046 and 0.028. The *EDNRA* SNPs are in high LD (r^2~1) with each other and the slight difference in p-values is due to a difference in the genotyping success rates (98.8% *vs.* 98.5%). The functional significance of these SNPs is currently unknown, rs2048892 being intronic and rs5334 a synonymous exonic SNP. However, based on the

HapMap Caucasian population data they are not in LD (r^2=0.08) with the *EDNRA* variant that was reported to be associated with migraine in a French study on 140 migraine cases and 1039 controls (Tzourio *et al.* 2001). In gender-specific analyses only the intronic *EDNRB* SNP rs2329047 showed improved allelic association (p=0.035, OR=1.2, 95% CI 1.01–1.42) in the female sample. When we expanded our sample with 142 unrelated migraine patients whose visual aura symptoms did not fulfil all criteria of typical visual aura according to the IHS criteria, the nominal association signals were diminished. However, three *EDNRB* SNPs showed allelic associations of 0.019–0.021 (minor allele frequency (MAF) in cases 12% and 18% in controls) in the male sample. However, the number of males studied is too low (*n*=177) for a statistically significant result.

Table 14. Allele and genotype frequencies of the studied tagging SNPs with p-values and position information when association between MA cases and controls was studied.

SNP	SNP location	MAF (%) cases	MAF (%) controls	p	OR	Genotype freq. (%) cases (11/12/22)	Genotype freq. (%) controls (11/12/22)	p
EDN1 (chr 6p24):								
rs2248580	intron	49	49	0.99	1.00	24/50/25	25/49/26	0.95
rs5369	Exon (s)	10	8	0.16	1.20	1/18/81	1/15/84	0.32
rs5370	Exon (n-s)	19	20	0.41	0.93	3/32/65	5/31/64	0.36
EDNRA (chr 4q31):								
rs6842241	upstream	14	13	0.70	1.04	2/23/75	3/22/76	0.70
rs984457	intron	23	22	0.35	1.09	6/35/59	5/33/62	0.63
rs7655670	intron	26	25	0.59	1.05	7/38/56	7/35/58	0.60
rs1568136	intron	21	22	0.76	0.97	4/34/61	5/33/61	0.50
rs1878404	intron	19	20	0.88	0.99	4/32/65	5/30/65	0.47
rs2048894	intron	18	17	0.30	1.11	5/27/68	3/28/69	0.046
rs5343	3'-utr	28	26	0.27	1.09	7/42/51	8/3755	0.11
rs1517137	intergenic	29	29	1.00	1.00	8/42/49	10/39/51	0.32
EDNRB (chr 13q22):								
rs11149079	intergenic	51	48	0.11	1.12	26/49/25	24/47/28	0.23
rs1924936	intergenic	19	17	0.13	1.16	3/31/66	2/29/59	0.30
rs7982910	intron	38	41	0.06	0.87	15/46/39	18/45/36	0.13
rs2329047	intergenic	39	36	0.07	1.15	16/46/38	13/45/42	0.20
rs9318502	intergenic	40	37	0.12	1.12	17/46/37	14/45/40	0.30
rs1924914	intergenic	29	27	0.18	1.11	9/38/52	6/42/52	0.12
rs4884076	intergenic	12	10	0.12	1.03	.1/21/78	.2/19/81	NA
rs9544662	intergenic	35	32	0.16	1.12	13/43/43	1/44/45	0.19

SNP, single nucleotide polymorphism; MAF, minor allele frequency; OR, odds ratio; freq, frequency; chr, chromosome; s, synonymous; n-s, non-synonymous

When the nominal associations of the *EDNRA* SNP rs2048894 and *EDNRB* SNP rs2329047 were studied more carefully the minor homozygous genotype of rs2048894 was more prevalent in affecteds (5%) than in control individuals (3%). Correspondingly, the

homozygous minor genotype of rs2329047 was more prevalent in females with MA than in female controls (16% *vs.* 12%). The association analysis of haplotypes of three SNPs was performed using a sliding window approach for the best associated SNPs. Haplotypes with both the *EDNRA* SNPs rs2048893 and rs5334 were not showing a p-value of <0.05 in the whole or female sample. However, the best associated haplotype with the *ENDRB* SNP rs2329047 showed an uncorrected p-value of 0.040 in the female sample (50% in cases *vs.* 45% in controls). This result is analogous to the allelic and genotype p-value.

The studied variants of *EDN1* and its receptor genes did not show significant association to MA. However, since both EDNRA and EDNRB act as distinct receptors for EDN1, genetic epistasis (SNP×SNP) was tested in the MA sample. In the test for epistasis two SNPs are assumed to be in linkage equilibrium in the sample population. Only one border-line interesting p-value of 0.05 (OR=1.38) was detected between the *EDN1* SNP rs5370 and the *EDNRA* SNP rs1878404. Thus interchromosomal interactions between the studied variants unlikely cause a predisposition to MA.

6.1.1.3 Association between EDNRA and the traits phonophobia, photo- and phonophobia and age of onset <20 years

Because the previous genome-wide linkage study using TCA showed suggestive evidence of linkage in the proximity of the *EDNRA* gene with the traits phonophobia and photo- and phonophobia and age of onset <20 years (Anttila *et al.* 2006), association between *EDNRA* SNPs and these traits was studied. The same two *EDNRA* SNPs rs2048894 and rs5334 that were showing association to MA also showed nominal association to the age of onset <20 years trait (p-values of 0.020 and 0.012, respectively). Due to the high LD between these SNPs, analyses were continued with rs2048895 because of its better success rate compared to rs5334. When only females were analyzed, a slightly improved association to the age of onset was seen with the tagging SNP rs2048894 (p=0.0081). This result was mainly based on a homozygous minor genotype (6% in cases and 3% in controls). The female-specific analysis also showed association to the *EDNRA* SNP rs7655670 (genotypic p-value of 0.028) but the neither the dominant nor recessive test statistics provided a p-value of <0.05.

6.1.1.4 Analysis of the best associated SNPs in the pooled Finnish and German sample

Although none of our tests showed a p-value <0.05 after permutation procedures, the nominally interesting results of the *EDNRA* and *EDNRB* SNPs rs2048894 and rs2329047 for the MA end diagnosis and age of onset <20 years trait encouraged us to study these variants in a German sample of 648 MA cases and 651 controls. None of the replicated SNPs showed association (-log p>1.3) with MA when the whole German sample or gender-specific analyses were performed (Figure 7). However, since the similar overrepresentation of homozygous minor allele genotypes of rs2048894 and rs2329047 was seen in cases compared to controls in the German as well as in the Finnish sample (Figure 7), an association analysis was performed in the joint sample. First, the effect of the risk genotypes for both samples was tested. Since the gene effect appeared to be similar in both samples, the association analysis was performed in the joint sample using country and gender as covariates. The homozygous minor genotype of rs2048894 showed associations with increased risk of MA (p-value of 0.010, OR=1.61) and the age of onset <20 years (p-value of 0.011, OR=1.69), but no association was detected for the *EDNRB* SNP in the pooled sample.

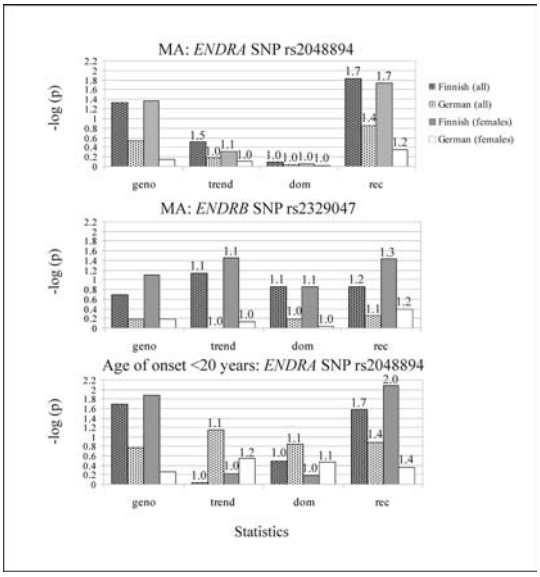

Figure 7. Results (-log(p)) for the *EDNRA* and *EDNRB* SNPs using the MA end-diagnosis and the trait age of onset <20 years, analyzed both in the whole sample and in the female sample originating either from Finland or from Germany. Figures above the bars show the ratio of minor allele or minor genotypes between cases and controls for statistics where the degree of freedom was 1. (Geno, genotypic association test; trend, allelic association based on the Cochran-Armitage test; dom, dominant gene action test; rec, recessive gene action test.)

6.1.1.5 Aspects in sample selection in studies on END1 and its receptors A and B

END1 is considered to be able to trigger CSD that is thought be the underlying mechanism of migraine and especially for visual aura (Kleeberg *et al*. 2004), therefore, an evident aim is to study the role of endothelin genes in patients with MA. However, only in a study by Gallai *et al.* (1994), where the sample of MA patients was low (*n=20)* but of adequately power, were increased EDN1 levels in MA patients detected. The genetic association of the *EDNRA* SNP was also detected in MO patients (*n*=113) but the number of MA patients (*n*=27) was too low for a conclusive result (Tzourio *et al.* 2001). A reason for the low number of patients (*n*=7–50) in these clinical studies is, naturally, the lack of volunteers due to the requirements that the patient take no medication and has to be in a hospital during the migraine attack.

Although the total number of migraine patients was low (*n*=140) in the genetic study by Tzourio and co-workers (2001), their carefully selected sample and a large number of controls (*n*=1,039) were its strengths. The majority of migraine patients had a self-reported family history of migraine that enhanced the power to detect genetic variants (Amos 2007). Also, the life-style and conditions related to EDN1 actions, such as hypertension (Cardillo *et al.* 2002), were controlled to be similar in cases and controls. Furthermore, population stratification may have been avoided by selecting patients born between 1922 and 1932 from a certain region of France. In our sample we were also using a homogenous sample, but in addition, we had a considerably larger group of carefully phenotyped migraine patients (*n*=850) than the French study. Furthermore, our study was the first genetic study exploring the role of *EDN1* variants and its receptors in patients prominently affected with visual aura.

6.1.1.6 Role of EDNRA in MA susceptibility needs further study

Our study revealed no significant association between MA or the studied traits and *EDN1* and its receptors. However, both the MA end diagnosis and the trait age of onset <20 years showed nominal association to rare homogenous genotypes of *EDNRA* SNPs rs2048894 and rs5334. The finding that rare homozygous genotype frequencies (5% *vs.* 3% in the Finnish sample and 7% *vs.* 5% in the German sample) of the *EDNRA* SNP rs2048894 between cases and controls were similar in the Finnish and German samples suggesting that the variant may have an impact on the susceptibility to migraine. The nominal association in the pooled sample with an

OR of 1.6 further strengthened our confidence. In addition, when individuals not having the IHS migraine aura were added to the analysis of MA patients the nominal associations of the two *EDNRA* SNPs were diminished. This indicates that *EDNRA* may mediate central characteristics of migraine aura. Also the previous association study in the French population showed the highest evidence of association to a rare homozygous genotype (Tzourio *et al*. 2001). However, the associated variant in their study was not in LD with our SNP rs2048894 based on the HapMap data. Now evidence from two studies indicate that even larger samples of migraine patients, both with and without aura, with measured plasma EDN1 levels should be studied to clarify the role of *EDNRA* in migraine, as no single variant or haplotype was associated with high significance.

6.1.2 Evaluation of the SNP genotyping reaction

Accurate performance of genotyping reactions diminishes the possibility of type I and II errors. In study II the quality and expenses of the homogenous mass extension reactions (hME) were compared between two commercial DNA polymerases, ThemoSequenase® and TermiPol® in the Sequenom genotyping platform. A total of 96 polymorphic SNPs were evaluated including all the 32 SNPs analyzed in Study I. Both polymerase enzymes have been designed to incorporate dideoxynucleotides (ddNTPs) in the extension reactions that are usually discriminated against by ordinary DNA polymerases. In ThermoSequenase® the active site of a gene encoding the Taq DNA polymerase of the thermophile *Thermoplasma acidophilum* has been modified to enhance the interaction of the DNA polymerase with ddNTPs (Tabor and Richardsson 1995, http://www4.gelifesciences.com). The TERMIPol enzyme originates from a modified gene of *Thermus aquaticus* (http://www.sbd.ee).

6.1.2.1 Comparison between the qualifying parameters of the SNP extension reactions

We selected 96 SNPs that performed well in single-plex reactions for the enzyme comparison. Based on the parameters obtained from the Sequenom Typer 3.3 software, four qualifying parameters were calculated for each SNP genotype (Table 1, Study II). The success rates for the extension reactions performed with TERMIPol® were significantly better ($p=4.8\times10^{-6}$) than with ThermoSequenase®, although a median success rate of 100% was the same for both reactions. Both the extension reaction efficiency and the higher mass extension efficiency

were also significantly better when using TERMIPol® rather than ThermoSequenase®. Both of these parameters describe the completeness of the extension reaction, the first taking into account all extended peak heights in comparison to the unextended primer and pausing peak, and the latter defining the success of the extension reaction of the higher mass allele. For both parameters TERMIPol® showed lower variation in the qualifying parameters, thus demonstrating better enzymatic efficiency than ThermoSequenase® in the same reaction conditions. As was suspected from previous parameters, the bias between the heights of the two alleles of heterozygotes was higher for ThermoSequenase® than TERMIPol®. In a few cases TERMIPol® extended more efficiently the higher mass allele than the lower mass allele, but in four of seven SNPs the same phenomenon was detected also with ThermoSequenase®. Regardless of the better performance of the TERMIPol® enzyme, in four water control samples extension products were detected compared to only one when ThermoSequenase® was used. In these cases DNA cross-contamination was unlikely since only one SNP reaction per multi-plex was showing non-templated extension products in each case. In many cases, the non-templated extension reaction was avoided by using the HotStart TERMIPol® enzyme that needs incubation at 95–97°C for activation (data not shown).

6.1.2.2 Inter-variation test

To be certain that the differences in the extension reactions were not based on sporadic environmental factors during laboratory work, an inter-assay variation test was done for two well-performing multiplexes of 5 and 6 SNPs five times. The success rates of SNPs were high, the mean success rate being 99% for ThermoSequenase® and 100% for TERMIPol®, but the coefficent variation was higher for ThermoSequenase® than for TERMIPol®. Similarly the reaction efficiency rates and the allele specific bias values were closer to optimum for reactions performed with TERMIPol®. In the inter-assay variation test, the higher mass allele was not amplified in one assay performed with ThermoSequenase® resulting in homoplasy, which was also detected in three of the original 96 SNP assays. Homoplasy could have been predicted already from higher unextended primer peaks and the existence of minor pausing peaks (Figure 8).

6.1.2.3 Expenses of the Sequenom SNP genotyping reaction

The DNA polymerase enzyme is one of the major determining factors of the cost of genotyping. However, it should be noted that good enzyme performance reduces costs by

decreasing the need for re-runs of genotyping reactions. In 2006 the total genotype cost for a single reaction in our laboratory was $0.79 with ThermoSequenase® and $0.68–0.70 with TERMIPol® using a 384-plate. A noticeable reduction in genotyping costs is achieved by designing multiplexes with large numbers of SNPs, since multiplying the number of genotyped SNPs in one multi-plex reduces the enzyme cost per SNP in the same proportion.

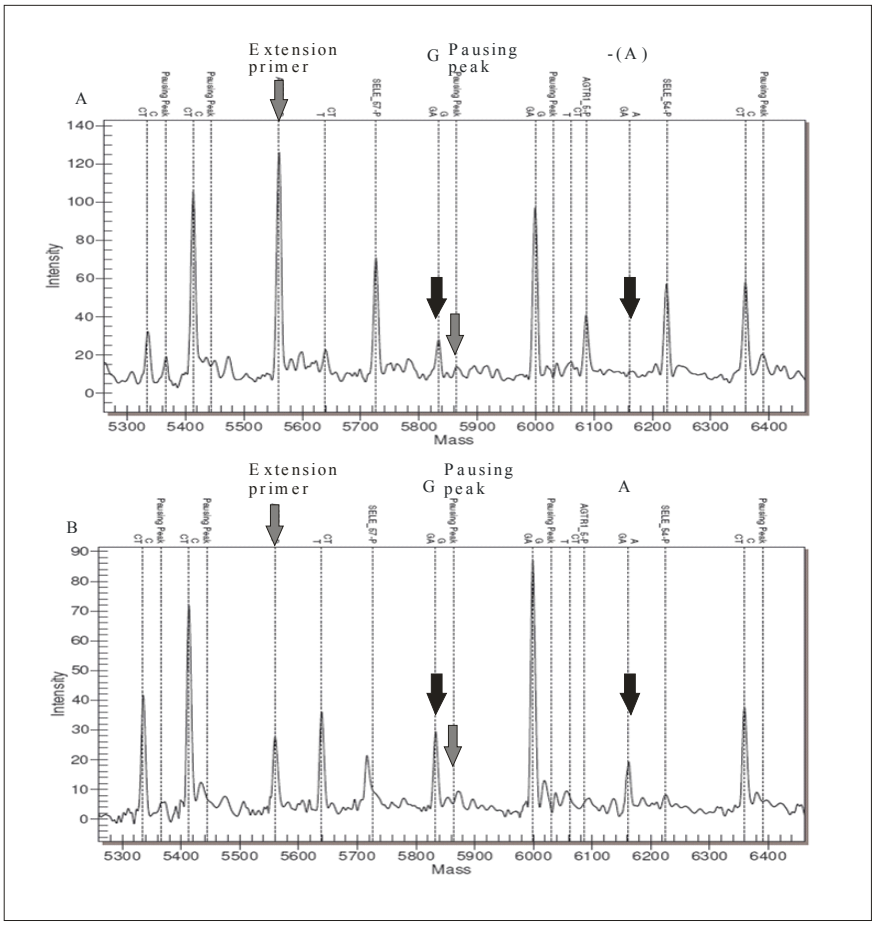

Figure 8. Comparison between the mass spectrograph profiles of discrepant genotypes in a multiplex assay format. (A) ThermoSequenase® amplifies the lower mass allele G and a minor pausing peak for the sample. (B) The same sample amplified with TERMIPol® produces heterozygous genotype G/A.

6.1.2.4 Optimization of genotyping reaction enhances the quality of the SNP genotype data

Based on our results, TERMIPol® advanced the hMEs reactions with higher precision and efficiency at a lower cost than ThermoSequenase®. A drawback, however, is that TERMIPol is prone to non-templated extension. Similar results were also observed in a study by Lovmar *et al.* (2005) where they studied the quality of different DNA polymerases in their four-color fluorescence minisequencing Tag-microarray system (Lindroos *et al.* 2002). In this method, the identification of alleles is based on fluorescently labelled ddNTPs that are incorporated to extension primers while in the MassArray® system. The alleles are subsequently separated based on known mass differences between alleles. In both genotyping systems the optimization of extension reactions has enabled the designing of cost-efficient and quality reactions. However, "in house" optimization of extension reactions of the MassARRAY® platform has been complicated by the use of the iPLEX® genotyping system in which "ready-to-use" reaction mixtures are used (http://128.135.75.36/iPLEXAppNote.pdf).

Based on the optimization results presented here, the extension reactions for study I were performed using the TERMIPol® polymerase providing a high mean success rate of 99% for genotypes. None of the 32 SNPs used in Study I showed a dropout of the higher mass allele in heterozygotes in Study II. Furthermore the manual checking of the genotypes along with the automated genotype control program (KariOTyper) and the HWE calculations ensured (Gomes *et al.* 1999) that the quality of genotypes in Study I was high. The best associated *EDNRA* SNP rs2048894 was also included in the inter-array test of two multi-plexes. Both enzymes worked well in the extension reaction (the mean success rate was 100% for both enzymes). However, in one of five reaction sets a success rate of 80% was detected for the ThermoSequenase® reactions (Table 15). In the worst case scenario the missing one fifth of genotypes would have substantially biased our end-result, since already the 0.3 unit lower success rate of rs5334 reduced the p-value by 1.7 times compared to its neighbouring marker rs2048894 in high LD in study I. Therefore, in association studies where family information is missing, the rejection of false genotypes is essential. For example, in the Wellcome Trust Case Control Consortium project GWAS of seven complex diseases 6.2% of SNPs were rejected after quality control procedures because of missing data, genome-wide heterozygosity, population stratification and allele distribution testing (The Wellcome Trust Case Control

Consortium 2007). It has been estimated that a genotyping error rate of 3% may reduce LD measures (both D' and r^2) by 35% (Akey *et al.* 2001). These facts demonstrate that lack of quality checking procedures and optimization of the reaction conditions, especially when the associated variants are rare, may seriously bias results.

Table 15. The summary of the inter-assay variation test for the *EDNRA* SNP rs2048894.

Parameter	ThermoSequenase®				TERMIPol®			
	Extension reaction efficiency	Higher mass allele extension efficiency	Allele-specific bias	Success rate (%)	Extension reaction efficiency	Higher mass allele extension efficiency	Allele-specific bias	Success rate (%)
Mean	0.27	0.54	1.73	99	0.64	0.90	1.28	100
SD	0.068	0.044	0.29	5.0	0.10	0.020	0.23	0.0
CV (%)	25	8.2	17	5.1	15	2.2	18	0.0
Median	0.25	0.54	na	100	0.61	0.90	na	100
Min	0.19	0.45	na	80	0.48	0.85	na	100
Max	0.50	0.62	na	100	0.83	0.95	na	100

CV, coefficient of variation; Min, minimum; Max, maximum; na = not available

6.2. Identification of MA susceptibility loci using the linkage approach

6.2.1 The role of the FHM1 and INSR loci in Finnish MA families

In study III we analyzed the role of the 19p13 locus for MA in 72 Finnish families. This region contains two migraine associated genes *INSR* and the FHM1 gene *CACNA1A* (Ophoff *et al.* 1996, McCarthy *et al.* 2001). Our previous genome-wide linkage analysis performed on 50 Finnish MA families indicated nominal linkage to the D19S427 marker in the proximity of the *INSR* gene. The intragenic marker of the *CACNA1A* gene, D19S1150, on the other hand, showed no linkage (Wessman *et al.* 2002). Because of the nominal linkage finding we wanted to analyze the 19p13 region more closely with eight microsatellite markers surrounding both candidate genes (Figure 5). The 50 families that were used in the initial genome-wide linkage study were included in this study with 22 additional MA families totalling 72 families with 757 genotyped individuals.

6.2.1.1 Analysis results of the family sample

We performed a simulation analysis under a hypothesis of no linkage to assess the power of our study sample. Only 35% of our families were required to reach the power 80% with a

corresponding expected maximum Lod (EMLOD) score of 6. Regardless of the significant statistical power none of the studied markers showed linkage to the MA at 19p13. The highest LodHet score of 0.16 (p=0.35) was detected for the marker D19S394 located between the *INSR* and the *CACNA1A* genes. NPL analyses supported the results of the parametric analysis. When individuals with MO (*n*=91) were included in the analysis, the linkage signal did not improve.

The marker D19S427, which in our previous study had indicated nominal linkage to MA, showed a LodHet score of 0.07 (p=0.43) in study III. The plausible explanation for the different linkage results of D19S427 may be the use of different genotyping platforms; LI-COR DNA 4200 Genetic Analyzer (LI-COR BioSciences, Lincoln, NA, USA) was used in the initial linkage scan and the ABI3700 DNA analyzer was used in this study. When the genotypes of D19S427 run with the ABI platform were re-analyzed in the 50 families from our previous study (Wessman *et al.* 2002), a LodHet score of 0.28 was acquired and not the LodHet score of 1.7 that was detected in the initial analysis of the LI-COR genotypes (unpublished data).

6.2.1.2 Susceptibility of single families to 19p13

Despite the lack of evidence of linkage in all families, we studied whether any of the families contributed to the 19p13 region by ranking the families according to their Lod scores. Figure 9 shows the family based distributions of the Lod scores for the four markers (D19S427, D19S592, D19S394 and D19S1150) selected based on location and the highest detected Lod score (θ=0). Only five families showed a Lod score >0.59 for these markers, and thus, the role of the 19p13 region in the majority of the 72 families was improbable. However, ten families showed nominal evidence of linkage if a recombination fraction (θ=0–0.5) was taken into account, but no adjacent markers showed a Lod score above 0.59 (Table 16). When the highest detected Lod score was compared to the simulated maximum Lod score, three families (66, 86, 95) showed equal values. In each of these families a major haplotype covering the best linked marker distinguished the affected family members. On the other hand, all three families showed linkage to a different marker. This may be an indication of locus heterogeneity or a false positive linkage signal in these families.

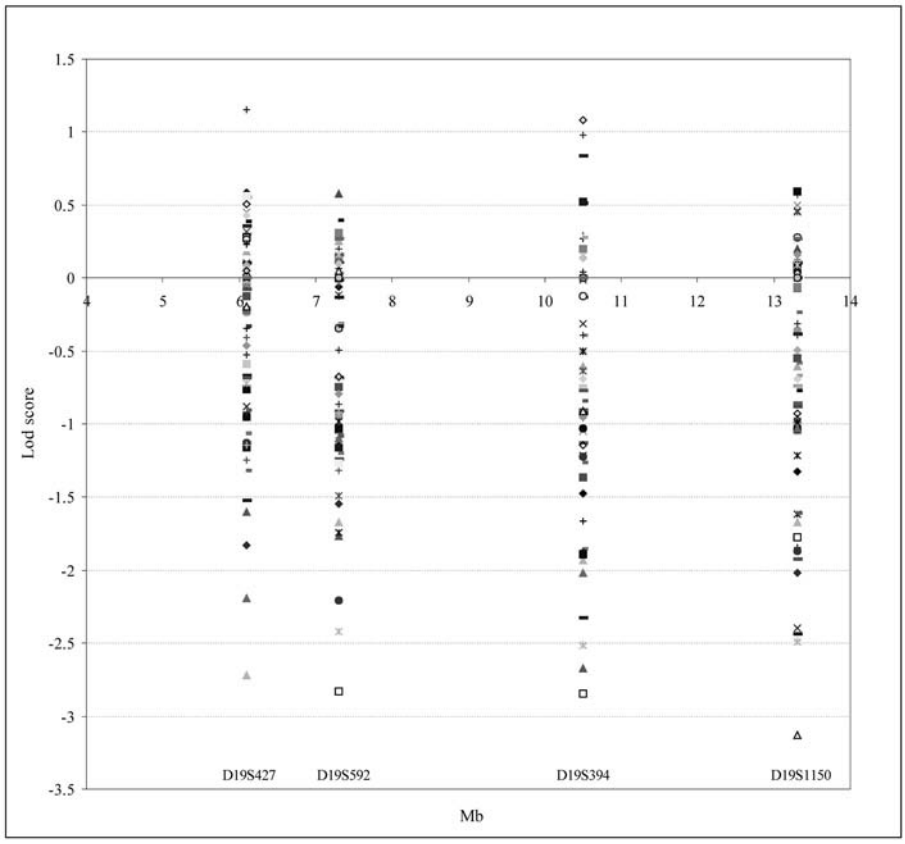

Figure 9. The distribution of individual family Lod scores at θ=0.0 for four microsatellite markers D19S427, D19S592, D19S394 and D19S1150. Each symbol represents one family.

Table 16. Simulated and maximum two-point Lod scores for individual families. Lod scores ≥0.59 for any of the studied marker are shown.

	Fam 86		**Fam 95**		**Fam 103**		**Fam 66**		**Fam**		**Fam 16**		**Fam**		**Fam 83**		**Fam 46**		**Fam 2**	
	Lod	θ	Lod	θ	**Lod**	θ	Lod	θ	**Lod**	θ	**Lod**	θ	**Lod**	θ	**Lod**	θ	**Lod**	θ	**Lod**	θ
Simulated results																				
EMLOD	.71	0	.68	0	1.34	0	.48	0	1.62	0	.85	0	.73	0	.99	0	.22	0	.85	0
Max lod	1.15		1.14		2.16		.84		4.36		1.42		1.04		1.43		.64		1.42	
Marker																				
D19S247	.30	0	.01	.3	.003	.4	.28	0	0	.5	.06	0	0	.5	.85	0	.59	0	.56	0
D19S427	1.15	0	.19	.3	.51	0	.35	0	0	.4	0	.5	0	.5	0	.5	.09	0	.59	0
D19S592	.20	0	.03	.3	.30	.3	0	.5	0	.5	0	.5	.04	.2	0	.5	.13	0	0	.5
D19S391	.30	0	0	.5	.04	.2	.28	0	0	.5	0	.5	.21	0	0	.5	.17	0	0	.5
D19S394	.09	.2	.06	.3	1.30	.05	.84	0	0	.5	0	.5	.98	0	0	.5	0	.5	0	.5
D19S221	.09	.2	.08	0	.01	.4	.55	0	0	.5	.96	0	.04	.2	0	.5	.17	0	0	.5
D19S 1150	.09	.2	.57	0	.32	.1	0	.5	.59	0	0	.5	.17	.2	0	.5	0	.5	0	.5
D19S226	.09	.2	1.14	0	0	.5	.55	0	0	.5	0	.5	.18	.2	0	.5	0	.5	0	.4

Fam, family; Lod, logarithm of odds; EMLOD, expected maximum Lod score; max, maximum

6.2.1.3 Role of the FHM1 locus in the Finnish MA families

The characteristic aura symptoms are considered to be the main consequence of FHM gene mutations, and therefore, the role of the FHM1 locus was examined in Study IV. The genome-wide microsatellite marker map used included two markers D19S221 and D19S226 surrounding the *CACNA1A* gene that were also analyzed in Study III. In addition, we genotyped the *CACNA1A* intragenic marker D19S1150 in Study IV. These markers did not show linkage between the visual aura phenotype and the *CACNA1A* locus. When all three markers were analyzed in the joint sample set of study III and IV totalling 108 families, no evidence of linkage between the FHM1 locus and MA was detected (LodHet ~0.0; unpublished data). Therefore, the role of the FHM1 locus in the Finnish migraine families with total of 1,108 genotyped individuals was not found to be significant.

6.2.1.4 19p13 is not linked to MA in Finnish families

Our results indicate that the 19p13 region in not the major contributor to MA susceptibility in the Finnish families. This finding further confirms results from five other linkage studies on families with Caucasian origin (Hovatta *et al.* 1994, Monari *et al.* 1997, Lea *et al.* 2001, Noble-Topham *et al.* 2002, Kirchmann *et al.* 2006, see also Table 11). However, in three studies evidence of linkage has been detected between the FHM1 locus and MA/MO and MA families (May *et al.* 1995, Nyholt *et al.* 1998, Terwindt *et al.* 2001). The role of common variants of ion transport genes, including the *CACNA1A* gene, has been extensively studied in a Finnish case–control sample of 841 MA cases and 884 controls (Nyholt *et al.* 2008). The results showed no evidence of association between *CACNA1A* and MA. However, the possibility of rare variants contributing to common migraine susceptibility was not disregarded. On the other hand, genome-wide linkage analyses that are thought to identify rare variants enriched in families have not shown linkage to the 19p13 locus. Sequencing studies have also been unable to identify rare or common variants predisposing to common migraine (Lea *et al.* 2001, Brugnoni *et al.* 2002, Wieser *et al.* 2003, Kirchmann *et al.* 2006), however, due to the large size of the *CACNA1A* gene (0.3 Mb) sequencing studies have primarily concentrated on exons so the majority of intronic variants remains uncharacterized.

Efforts to study the susceptibility of *INSR* to common migraine have been minor compared to the numerous studies on *CACNA1A*. The *INSR* was first shown to associate with common migraine in a study of 827 Caucasian migraine cases and 764 control individuals (McCarthy *et al.* 2002). Previously, variants of the *INSR* gene were studied in a German case–control sample that showed association with migraine only when the German sample was combined with the original association sample of McCarthy's group (Netzer *et al.* 2008b). Our study and the previous linkage and sequencing studies, on the other hand, have failed to show relation between migraine and the *INSR* gene (Monari *et al.* 1997, Kaunisto *et al.* 2005, Curtain *et al.* 2006). Although genetic studies have not confirmed the role of *INSR* in migraine susceptibility, both migraine and diabetes share pathophysiological abnormalities related to vascular and nerve reactivity, and interestingly, patients with diabetes mellitus have less migraine than the control population (Aamodt *et al.* 2007). Thus, even more extensive association or sequencing studies are needed to confirm or exclude the role of the 19p13 region in common migraine susceptibility.

6.2.2 The genome-wide linkage search for visual migraine aura loci

In Study IV susceptibility loci for visual aura were searched for using genome-wide linkage analysis on 36 Finnish MA families. The initiative for this study was the knowledge that previous genome-wide linkage analyses have identified several loci for the end-diagnosis based phenotypes, latent groups of migraine and trait components of migraine headache (see Table 10). Thus, distinctive loci for migraine aura or its trait components, *i.e.* visual, sensory or dysphasic symptoms, have not yet been established.

6.2.2.1 Several loci identified for visual migraine aura

From our cohort of over 1,400 migraine families we identified 36 families primarily affected with the scintillating scotoma type of aura (84% of aura patients). When all individuals affected with migraine aura (n=185) were analyzed assuming autosomal dominant inheritance, two-point linkage analyses pinpointed highly significant evidence of linkage to marker D9S1690 at 105 cM on 9q31 (LodHet=4.7, θ=0.12, Fig 1A in Study IV) with no sex-specific predisposition. The simulation analysis using the allele frequencies of marker D9S1690 and the dominant inheritance model showed that a Lod score of 3.3 would have appeared zero

times by chance with this family sample. The ASP analyses showed suggestive evidence of linkage to several neighbouring markers at 81–113 cM on 9q21–q31. Furthermore, markers on 5q13 and 12p13 showed suggestive and near suggestive evidence of linkage, respectively. Genome-wide NPL analyses were performed in order to avoid possible bias based on the assumption the dominant inheritance model used in the parametric two-point analysis. The SimWalk dominant and NPL_{all} statistics confirmed the significant linkage to the 9q22–q31 region. Furthermore, we also identified significant linkage to 13q14 and suggestive evidence of linkage to four loci on 6q25, 9p24, 11q12 and 12p13 (Figure 1B in Study IV).

6.2.2.2 Significant evidence of linkage to 9q21–q31

The locus at 9q31 was identified using the linkage information of all individuals with visual migraine aura. To distinguish whether the linkage was based on migraine aura or migraine headache, we analyzed families by treating all individuals with migraine headache as affecteds. This analysis revealed only suggestive evidence of linkage for D9S1690 even though the group of affecteds was increased by 19%. The analysis on patients with MA (*n*=160) provided significant but reduced evidence of linkage (LodHet=4.3) compared to analysis with aura affected individuals (*n*=185).

Since results of the NPL analysis supported the overlap of the 9q22–q31 region with the recently identified locus at 9q22 in a single Belgian family affected with occipitotemporal lobe epilepsy and MA, which both have visual manifestation (Deprez *et al.* 2007), we fine-mapped the 9q21–q31 region with 11 additional markers. Analysis on all markers pinpointed the highest NPL_{dom} score of 3.7 to reside at 91 cM on 9q22. The results of the two-point and multi-point analyses are shown in Figure 10. We also genotyped marker D9S257 at 92 cM that was showing significant evidence of linkage in the Belgian study. Regardless of several attempts we were not able to have a success rate greater than 60% for this marker and thus it was excluded from analyses. In addition, we extended our fine-mapping to the 9q33 region (D9S1811 at 124 cM) since this region was previously linked to idiopathic epilepsy in an inbred Turkish family (Baykan *et al.* 2004). Patients in this family were also suffering from MO but their analysis on MO patients and our analysis between aura affecteds and D9S1811 did not show evidence of linkage to migraine. However, a proximal marker at 122 cM showed suggestive evidence of linkage, and when the IHS migraine patients (*n*=225) were treated as

affecteds a nominal linkage signal of LodHet (1.1 θ=0.26; unpublished data) was detected for D9S1811. Therefore, we can hypothesize that the 9q33 region may carry variants predisposing both to migraine and epilepsy attacks.

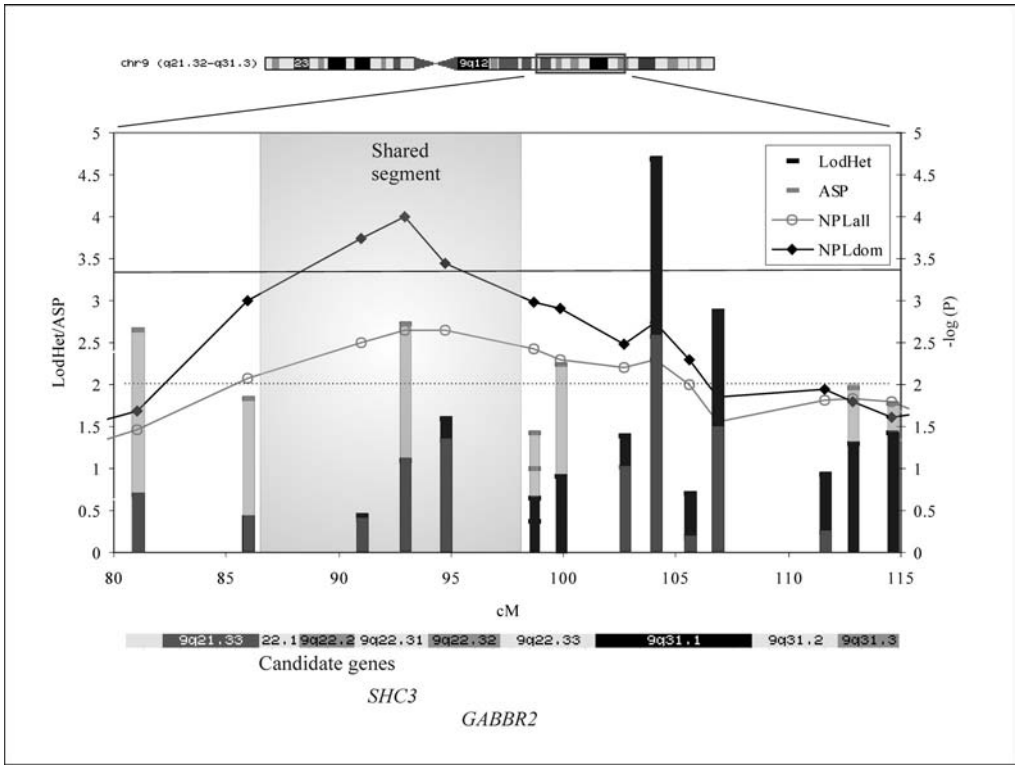

Figure 10. Summary plots of parametric and non-parametric linkage analyses on the 9q21–q31 region showing the shared segment on 9q21–q22 among linked pedigrees and locations of potential candidate genes. Thresholds for significant linkage findings for both parametric (a LodHet score of 3.3) and non-parametric (-log (p) -value of 2) analyses are shown with solid and dotted horizontal lines, respectively.

To define the linked region with more detail the haplotypes for 22 families, showing linkage (p_{NPLdom}>0.5) to the region between 86 and 115 cM based on the SimWalk NPL_{dom} statistics, were estimated. In seventeen families a family-specific shared segment of about 12 cM covering three markers that show allele heterogeneity was detected on 9q21-q22 (Figure 10). The region contains more than 30 known genes and none of them code for proteins related to ion transport. The best potential candidate gene is *SHC3*, which encodes a transforming protein involved in neurotrophin signalling. It is highly expressed in the occipital and temporal lobes and it regulates the adaptive response of neurons against environmental stress (Troglio *et al.* 2004). Because migraine patients suffer from reduced habituation to external stimuli

(Chronicle and Mullerners 1996, Giffin and Kaube 2002), the *SHC3* gene is a good candidate gene for migraine. Another interesting candidate gene, although it is not located in the shared segment of haplotypes, is *GABBR2* that encodes a B-type receptor for gamma-aminobutyric acid (GABA). It activates potassium channels and inactivates calcium-channels (Jones *et al.* 1998), whose functions are considered to be central in the development of CSD (Moskowitz *et al.* 2004, van den Maagdenberg *et al.* 2004). Even more interestingly, increased GABBR2 expression has been detected in patients with temporal lobe epilepsy (Princiville *et al.* 2003), and thus, the role of the *GABBR2* gene in the pathophysiology of migraine aura may be plausible.

Our linkage study on the Finnish MA families shows an overlap with the linkage results of the Belgian family with prominent visual symptoms in both occipitotemporal lobe epilepsy and MA. Alongside the shared genetic background between some rare epilepsies and FHM (Weber *et al.* 2008), this study and the Belgian study may suggest a shared background in MA and epilepsy (Deprez *et al.* 2007). In the Belgian family with occipitotemporal epilepsy and MA, ten individuals had epilepsy and five of them also had MA. When they included an MA patient with one isolated seizure in the analysis, a Lod score of 3.3 was gained. Since Deprez and co-workers (2007) published haplotypes segregating in the Belgian family, we were able to calculate Lod scores for their best linked marker D9S257. Based on the Lod scores shown in Table 17, exclusion of a single MA patient from analysis reduced the linkage signal by 0.3 unit to 3.0 and analysis of six MA patients showed a suggestive Lod score of 2.1.

Table 17. Lod scores calculated for different traits in the Belgian family. The dominant inheritance model with a penetrance of 75% and disease allele frequency of 0.1% was used (Deprez *et al.* 2007)

Trait	***n*** **(affected)**	**Lod (θ=0)**
Occipitotemporal lobe epilepsy or MA	11	3.3
Occipitotemporal lobe epilepsy	10	3.0
MA	6	2.1

MA, migraine with aura

The pathophysiological connection between migraine and epilepsy can be the episodic nature of both disorders that is associated with cortical hyperexcitabilty (reviewed by Haan *et al.* 2008). Also epidemiological studies support a more than co-incidental relationship between

these disorders, and in patients with MA the risk for epilepsy is eight times higher than in normal population (Ludvigsson *et al.* 2008). In our family sample there were only five epilepsy patients originating from three families and of those only three were comorbid migraine–epilepsy patients. The only family with more than one epilepsy patients (*n*=3) showed a Lod score of 0.29 at 81 cM, which is more than a 10 cM distance from the best linked marker in the Belgian study (unpublished data). To get a more conclusive picture of the role of the 9q21–q31 locus in migraine and epilepsy, performing a genetic study on comorbid families and/or patients would be useful.

6.2.2.3 Locus on 12p13

We fine-mapped the region on 12p13 since marker D12S99, in the intron of *neurotrophin3* gene (*NTF3*), showed suggestive evidence of linkage. It has also shown association with two neurophychiatric diseases, Alzheimer's disease and schizophrenia (Nanko *et al.* 1994, Kunugi *et al.* 1998), but samples in these studies were small for conclusive results. Interestingly the protein encoded by the *SHC3* gene on 9q22 is involved in neurotrophin signalling and is known to interact with NTF3 in a rat retina (Nakazawa *et al.* 2002). Thus, epistasis analysis between these genes would be intriguing to perform.

The NPL-analysis with two additional markers improved the linkage to 12p13 significantly (Figure 11). Interestingly, the linked region also contains three potassium voltage-gated channel subunits, *KCNA6*, *KCNA1* and *KCNA5*, that did not show association in the study between 155 ion transporting genes and migraine (Nyholt *et al.* 2008). On the other hand, *KCNA1* has been associated to episodic ataxia type 1 (EA1; Browne *et al.* 1994) that is an autosomal dominant condition characterized by short episodes of ataxia with interictal myokymia. Mutations in *KCNA1* are predicted to increase neuronal excitability by disturbing potassium channel activity (Smart *et al.* 1998). EA1 is occasionally associated to epilepsy (Zuberi *et al.* 1999) but not to migraine, although other episodic ataxias are often related to migraine and the more severe EA2 is caused by mutations in the *CACNA1A* gene (Ophoff *et al.* 1996, Jen 2008).

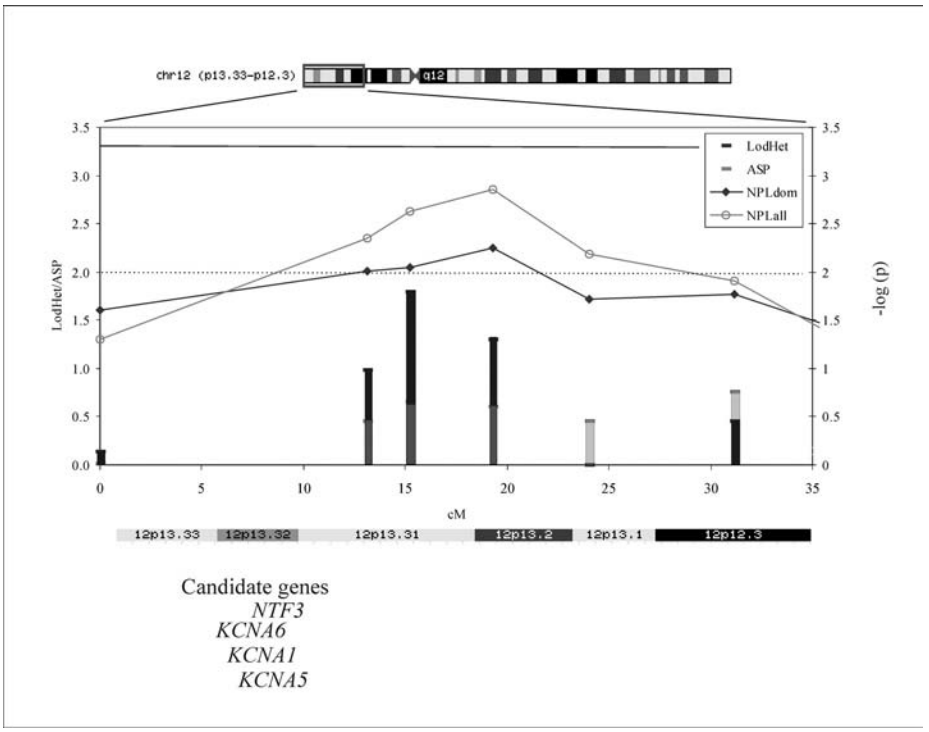

Figure 11. Summary plots of parametric and non-parametric linkage analyses on 12p13–p12 region showing locations of the best potential candidate genes. Thresholds for significant linkage findings for both parametric (a LodHet score of 3.3) and non-parametric (-log (p) -value of 2) analyses are shown with solid and dotted horizontal lines, respectively.

6.2.2.4 Other loci

Our study showed evidence of linkage to loci on 5q13 and 13q14 that reside in the proximity of previously reported migraine loci (Nyholt *et al.* 2005, Ligthart *et al.* 2008, Table 18). The locus on 5q13 locates 26 cM from the best linked marker identified on 5q22 in an Australian study using a LCA based phenotype (Nyholt *et al.* 2005) and in a Dutch study using the migraine symptoms photo- and phonophobia as a trait (Ligthart *et al.* 2008). Even though the distance between our locus and the previously identified locus is considerably large (33.9 Mb), similar distances have been detected between replicated linkage signals and associated variants identified by positional cloning in schizophrenia (<45.2 Mb; Lewis *et al.* 2003, Allen *et al.* 2008). The potential candidate gene on 5q22 is the *KCNN2* gene encoding a small conductance calcium-activated potassium channel. The 5q22 region has also been indicated in ten families with epileptic absence seizures that have not reported migraine (Durner *et al.*

2001). This indicates that the 5q region may also contain variant(s) predisposing to both migraine and epilepsy.

The locus on 13q14 resides 13 cM from the marker D13S1807 at 13q21 reported in the Australian and Dutch studies (Nyholt *et al.* 2005, Ligthart *et al.* 2008). There are several genes that might be relevant in migraine pathophysiology in the 13q14–q21 region: *5-hydroxytryptamine* (*serotonin*) *receptor 2A* (*HTR2A*) has been previously associated with migraine aura in a small sample of Turkish migraine patients (*n*=37; Erdal *et al.* 2001), but this finding has not been replicated in a larger study on Hungarian female migraine patients (*n*=126; Juhasz *et al.* 2003). Other relevant candidate genes in this region are *KCNRG*, *ATP7B*, *TRPC4* and *KCTD4* involved in ion transport (Table 18). So far, three studies in three different populations with different phenotyping strategies report linkage to 5q and 13q regions. This proposes a possibility that different variants on 5q13 and 13q14 predispose to multiple migraine symptoms.

Table 18. Potential candidate genes for MA loci showing evidence of linkage for visual aura trait.[1]

Linked locus				Candidate genes		
Chr	**Marker**	**cM**	**Mb**	**Genes**	**Pos (Mb)**	**Function**
5q13	D5S424	90.9	76.19	*KCNN2*	113.79-113.86	Small conductance Ca^{2+} activated K^+-channel
6q25	D6S441	160.6	153.86	*ESR1*	152.05-152.46	Oestrogen receptor
9p24	D9S286	17.8	8.04	*PTPRD*	8.30-10.60	Tyrosine phosphatase receptor
11q12	D11S4191	65.0	59.76	*KCNK4*	11.81-63.82	K^+-channel protein containing two pore-forming P domains
				KCNE3	73.84-73.84	K^+-voltage gated channel
13q14	D13S263	43.2	40.98	*TRPC4*	37.10-37.34	Potential cation channel
				KCTD4	44.66-44.66	K^+-channel tetramerisation domain
				HTR2A	46.30-46.36	Seretonin receptor
				KCNRG	49.48-49.49	K^+-channel regulator
				ATP7B	51.40-51.48	P-type cation transport ATPase

1) Information on candidate genes is based on the UCSC database (http://ucsc.genome.edu)
Chr, chromosome; cM, centiMorgam; Mb, megabase; Pos, position

Multi-point analysis also detected suggestive evidence of linkage to loci on 6q25, 9p24 and 11q12 (Table 18). The best linkage signal on 6q25 at marker D6S441 is located in the proximity of the *oestrogen receptor* (*ESR1*) that has been associated with menstrual migraine.

However, in our previous study on the Finnish MA patients association with *ESR1* failed after correction for multiple testing (Kaunisto *et al.* 2006). The other linkage signal on 9p24 is located near a potential candidate gene *PTPRD* coding for a protein that regulates neuronal axon guidance. The third region on 11q12 does not overlap with the reported migraine locus on 11q24 (Cader *et al.* 2003). Potential candidate genes for family-based association studies in this region are ion-channel genes, *KCNK4* and *KCNE3*.

Of note is that the FHM genes seemed not to have a central role in migraine aura families. As was previously mentioned, markers surrounding the FHM1 gene did not show linkage in aura families. Correspondingly, the markers adjacent to the FHM2 (1q23) and FHM3 (2q24) loci did not show evidence of linkage to the aura phenotype. On the other hand, in the genome-wide scan we used a 10 cM microsatellite marker map whose resolution may not be sufficient for conclusive results concerning the role of the FHM2 and FHM3 loci in the Finnish migraine aura families. Interestingly, however, in another Belgian study (Deprez *et al.* 2008) a few families with a combination of occipitotemporal lobe epilepsy and common migraine were associated to the FHM2 locus.

6.2.2.5 Genome-wide linkage analysis revealed several loci for visual migraine aura

The strength in our study was the careful selection of migraine families based on their aura phenotype. We chose families primarily affected with the scintillating scotoma type of aura and we were able to identify two significantly linked loci on 9q21−q22 and 12p13 that contain several potential migraine susceptibility genes for further studies. The previous linkage studies on migraine families have not identified the loci on 9q and 12p. In our study the focus has been on as homogenous visual aura symptoms as possible when previous studies have likely pinpointed loci either for the end-diagnosis based phenotype or migraine headache characteristics. For example, when families that were used to identify the MA locus on 4q24 were re-analyzed using TCA, several headache symptoms showed linkage to this locus (Anttila *et al.* 2006). However, aura components were not tested in TCA so the role of aura on the 4q24 locus can not be ignored.

Of note is that visual aura is consisted of different trait components (*e.g.* scintillations, hemianopia, blurring of vision, duration of aura, frequency of aura) and each of them may

have its own genetic background. Visual symptoms are the most frequent characteristic of aura (99% of MA patients; Eriksen *et al.* 2004), and thus, a more challenging but potentially rewarding study could be to collect and genetically analyze families primarily suffering from dysphasic or sensory aura symptoms. Further studies may also benefit if the headache characteristics of aura families are studied. This may reveal specific headache symptoms that are related to migraine aura.

6.3 Concluding notes and future prospects

Although the molecular genetics of migraine has been studied for two decades, no susceptibility variants have been identified for common forms of migraine. It is possible that the complex phenotype of a migraine patient is the major problem. During the last five years the new analysis methods LCA and TCA have provided new tools to unravel this problem. Migraine aura is the best characterized phase of migraine and facilitates the diagnosing of patients and may provide pathophysiological clues to the origin of migraine.

In this thesis the most interesting results were gained when families were selected based on their aura symptoms in order to have as homogeneous group of patients as possible for the TCA study. The significant visual migraine aura locus was identified on chromosome 9q21–q22. Our genome-wide linkage scan was the first study utilizing visual aura as a trait. Results of our study became even more interesting when we found that the locus overlaps with the occipitotemporal lobe epilepsy locus at 9q22. The episodic nature of both disorders probably originates from neuronal excitability and should motivate comprehensive genetic studies on comorbid patients.

Moreover, it is interesting that the potential candidate genes at 5q13, 9q21–q31, 11q12, 12p13 and 13q14 for visual migraine aura families are involved in functions of potassium channels. Potassium channels, like other ion channels, are responsible for selective ion permeability of the cell membranes (Ackerman and Clapham 1997). They have a central role in cellular signalling processes in both excitable and nonexcitable cells. Mutations in potassium channels are known to cause some generalized idiopathic epilepsies by accelerating neuronal hyperexcitability, and thus, their role in other epilepsies is also suggested (Stoffel and Jan 1998, Helbig *et al.* 2008). Although common variants of potassium channels have been

excluded as susceptibility genes in Finnish MA cases (Nyholt *et al.* 2008), the role of rare enriched variants in migraine aura families may be worth investigating.

In this thesis the role of the 19p13 locus and three vasoactive candidate genes were also studied in MA patients. In both studies the pathophysiological background of MA was taken into account when designing the study. The FHM loci have been considered to have a role in migraine aura and EDN1 may be involved in the initiation of migraine aura. Our and many previous studies on the 19p13 locus, using different study approaches, indicate that the FHM1 locus does not have a major role in MA susceptibility. In addition, the nominal association between *ENDRA* and MA, the primary receptor for EDN1, needs to be studied in an even larger sample for a conclusive result of its role in migraine aura pathophysiology. It is probable that only comprehensive resequencing studies will provide adequate information about the role of these loci in migraine susceptibility.

Since the linkage based studies have not led to the identification of predisposing variants in migraine susceptibility, there are great expectations for a GWAS. The International Migraine Genetics consortium has launched a genome-wide study on multiple populations of European origin. The Finnish sample consists of approximately 1,000 cases and 2,000 controls genotyped with over 600,000 SNPs. The study will examine the role of common variants, both SNPs and CNVs, in migraine. The first genome-wide CNV study on the Spanish migraine patients and controls identified eight susceptibility loci but presently no detailed information on the associated loci have been provided (an abstract by Armengol *et al.* 2008). Nevertheless, not until the gene expression data is combined with the results from linkage and/or association studies (Gilad *et al.* 2008) can the genetic background of migraine be considered elucidated.

Although the number of variants identified by GWAS of complex diseases is impressive, our genome-wide scan showed that a linkage study can still be an efficient and economical method, especially when a family predisposition to a specific trait is studied. The use of family material has the advantage of reducing bias due to population stratification and genotyping errors. Moreover, because the methods to detect rare, high-effect variants are still developing and are expensive (Käller *et al.* 2007), the loci identified by linkage analyses will provide valuable candidate regions for the expression analyses of quantitative trait loci of complex diseases such as migraine.

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