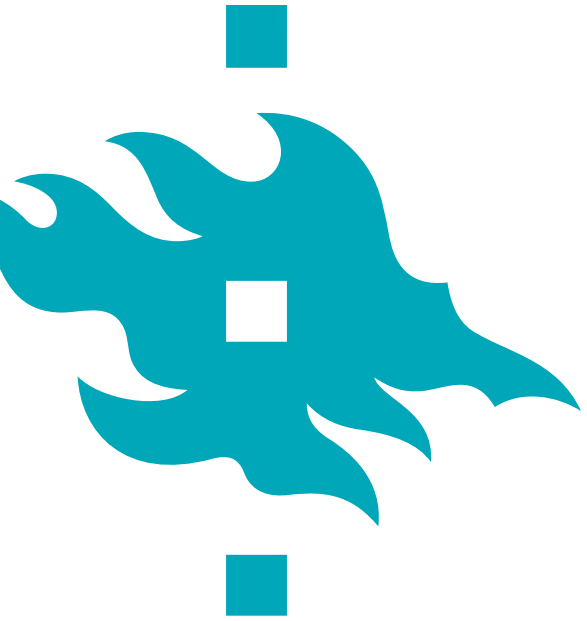

UNIVERSITY OF HELSINKI
FACULTY OF MEDICINE

IDENTIFICATION OF GENETIC SUSCEPTIBILITY LOCI FOR MIGRAINE

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Timo Verneri Anttila

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"If your experiment needs statistics,
you ought to have done a better experiment."
Sir Ernest Rutherford, 1871-1937

To my dear family

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals. In addition, some unpublished data are presented.

I Trait Components Provide Tools to Dissect the Genetic Susceptibility of Migraine. Anttila V, Kallela M, Oswell G, Kaunisto MA, Nyholt DR, Hämäläinen E, Havanka H, Ilmavirta M, Terwilliger J, Sobel E, Peltonen L†, Kaprio J, Färkkilä M, Wessman M, Palotie A. **Am J Hum Genet;79(1):85-99, 2006.**

II Consistently Replicating Locus Linked to Migraine on 10q22-q23. Anttila V*, Nyholt DR*, Kallela M, Artto V, Vepsäläinen S, Jakkula E, Wennerström A, Tikka-Kleemola P, Kaunisto MA, Hämäläinen E, Widén E, Terwilliger J, Merikangas K, Montgomery GW, Martin NG, Daly M, Kaprio J, Peltonen L†, Färkkilä M, Wessman M, Palotie A. **Am J Hum Genet;82(5):1051-63, 2008.**

III A high-density association screen of 155 ion transport genes for involvement with common migraine. Nyholt DR, LaForge KS†, Kallela M, Alakurtti K, Anttila V, Färkkilä M, Hämäläinen E, Kaprio J, Kaunisto MA, Heath AC, Montgomery GW, Göbel H, Todt U, Ferrari MD, Launer LJ, Frants RR, Terwindt GM, de Vries B, Verschuren WMM, Brand J, Freilinger T, Pfaffenrath V, Straube A, Ballinger DG, Zhan Y, Daly MJ, Cox DR, Dichgans M, van den Maagdenberg AMJM, Kubisch C, Martin NG, Wessman M, Peltonen L†, Palotie A. **Hum Mol Genet;17(21):3318-31, 2008.**

IV Genome-wide association study of migraine implicates a common susceptibility variant on 8q22.1. Anttila V, Stefansson H, Kallela M, Todt U, Terwindt GM, Calafato MS, Nyholt DR, Dimas AS, Freilinger T, Müller-Myhsok B, Artto V, Inouye M, Alakurtti K, Kaunisto MA, Hämäläinen E, de Vries B, Stam AH, Weller CM, Heinze A, Heinze-Kuhn K, Goebel I, Borck G, Göbel H, Steinberg S, Wolf C, Björnsson A, Gudmundsson G, Kirchmann M, Hauge A, Werge T, Schoenen J, Eriksson JG, Hagen K, Stovner L, Wichmann HE, Meitinger T, Alexander M, Moebus S, Schreiber S, Aulchenko YS, Breteler MM, Uitterlinden AG, Hofman A, van Duijn CM, Tikka-Kleemola P, Vepsäläinen S, Lucae S, Tozzi F, Muglia P, Barrett J, Kaprio J, Färkkilä M, Peltonen L†, Stefansson K, Zwart JA, Ferrari MD, Olesen J, Daly M, Wessman M, van den Maagdenberg AM, Dichgans M, Kubisch C, Dermitzakis ET, Frants RR and Palotie A, on behalf of the International Headache Genetics Consortium. **Nat Genet;42(10):869-73, 2010**.

* These authors contributed equally to the respective work.
† Deceased
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ABBREVIATIONS

AMD	Age-related macular degeneration
ANOVA	Analysis of variance
ASP	Affected sib pair
bp	Base pair
CADASIL	Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
CAMERA	Cerebral Abnormalities in Migraine, an Epidemiological Risk Analysis
CEU	Central Europeans in Utah
CEPH	Centre d'Etude du Polymorphisme Humain
CHB	Han Chinese in Beijing
cM	CentiMorgan
CMH	Cochran-Mantel-Haenszel
CNS	Central nervous system
CNV	Copy number variant/variation
cRNA	complementary ribonucleic acid
CSD	Cortical spreading depression
DALY	Disease-adjusted life year
DMQ2	deCODE Migraine Questionnaire, 2nd edition
DMQ3	deCODE Migraine Questionnaire, 3rd edition
DNA	Deoxyribonucleic acid
DZ	Dizygotic twin
eQTL	Expression quantitative trait locus
FHM	Familial hemiplegic migraine
FHM1	FHM phenotype caused by mutations in *CACNA1A*
FHM2	FHM phenotype caused by mutations in *ATP1A2*
FHM3	FHM phenotype caused by mutations in *SCN1A*
fMRI	Functional magnetic resonance imaging
$FMSQ_{FS}$	Finnish Migraine-Specific Questionnaire for Family Studies
GABA	Gamma-aminobutyric acid
GWA	Genome-wide association
HDL	High-density lipoprotein
HGP	Human Genome Project
HNR	Heinz-Nixdorf Recall Study
HLOD	Logarithm of odds under heterogeneity
IBD	Identity by descent
IBS	Identity by state
ICD-10	International Statistical Classification of Diseases and Related Health Problems, 10th edition
ICHD-I	International Classification of Headache Disorders, 1st edition
ICHD-II	International Classification of Headache Disorders, 2nd edition
iControlDB	Illumina Control Database, www.illumina.com
IHS	International Headache Society, www.i-h-s.org
JPT	Japanese in Tokyo
kb	Kilobase
KEGG	Kyoto Encyclopedia of Genes and Genomes
KORA	Kooperative Gesundheitsforschung in der Region Augsburg

LCA	Latent class analysis
LCL	Lymphoblastoid cell line
LD	Linkage disequilibrium
LDL	Low-density lipoprotein
LOD	Logarithm of odds
LUMINA	Leiden University Migraine Neuro Analysis
MA	Migraine with aura
MAF	Minor allele frequency
Mb	Megabase
MDS	Multidimensional scaling
MELAS	Mitochondrial Encephalomyopathy, Lactic Acidosis, Stroke-like episodes; a mitochondrial disease
MIM	Mendelian Inheritance in Man
MO	Migraine without aura
mtDNA	Mitochondrial deoxyribonucleic acid
MZ	Monozygotic twin
NCBI	National Center for Biotechnology Information
NHGRI	National Human Genome Research Institute
NMDA	N-methyl-D-aspartic acid
NPL	Non-parametric linkage (analysis)
NPL_{pairs}	Non-parametric linkage (analysis) of IBD shared alleles
NPL_{qtl}	Non-parametric quantitative trait linkage (analysis)
OMIM	Online Mendelian Inheritance in Man
OR	Odds ratio
PAG	Periaqueductal grey
PCR	Polymerase chain reaction
PFO	Patent foramen ovale
RNA	Ribonucleic acid
RVCL	Retinal vasculopathy with cerebral leukodystrophy
SHM	Sporadic hemiplegic migraine
SNP	Single nucleotide polymorphism
TCA	Trait component analysis
UCLA	University of California, Los Angeles
UTR	Untranslated region
VNTR	Variable number tandem repeat
WHO	World Health Organization
WTCCC	Wellcome Trust Case-Control Consortium
YLD	Years lived with disability
YRI	Yoruba in Ibadan, Nigeria

ABSTRACT

Migraine is the most common cause of chronic episodic headache, affecting 12%-15% of the Caucasian population (41 million Europeans and half a million Finns). Migraineurs suffer a considerable loss in quality of life and have increased risk for a wide range of conditions, from depression to stroke. Migraine is characterized by episodic attacks of headache accompanied by sensitivity to external stimuli lasting 4-72 hours, and in a third of cases by neurological aura symptoms, such as loss of vision, speech or muscle function. No biochemical markers identifying migraine have been found and its underlying pathophysiology (including the triggers of migraine onset and individual migraine attacks) is largely unknown. The aim of this study was to identify genetic factors associated with the hereditary susceptibility to migraine in order to gain a better understanding of migraine mechanisms.

We report the first whole genome association study of migraine, as well as genetic linkage and association analyses of patients drawn from a large Finnish migraine patient collection, along with migraineurs from similar collections in Australia, Denmark, Germany, Iceland and the Netherlands. Overall, we studied the genetic information of over 6,500 migraine patients and some 50,000 population-matched controls. We also developed a new migraine analysis method called the trait component analysis, which is based on individual patient responses instead of clinical diagnoses. Using this method, we detected a number of new genetic loci for migraine, including loci on 17p13 (HLOD 4.65) and 10q22-q23* (female-specific HLOD 7.68) showing significant evidence of linkage and five other loci (2p12, 8q12*, 4q28-q31, 18q12-q22*, and Xp22*) having suggestive evidence of linkage (four of which, indicated by asterisks, replicated previous findings). The 10q22-q23 locus was the first genetic locus found to show linkage with migraine in multiple populations and studies and has been consistently detected in six different genome-wide linkage scans.

In a candidate gene study of 155 ion transport genes, we found that common variants played no significant roles in migraine susceptibility. The role of common variants was further examined by the first genome-wide association study in migraine, conducted on 2,748 migraine patients and 10,747 matched controls followed by replication in 3,202 patients and 40,062 controls. In this study, we detected the first common variant associated with migraine, which is carried by approximately 20% of the general population. A follow-up expression quantitative trait study suggested that the detected variant has a functional effect on the transcription of the nearby gene *MTDH/AEG-1*, providing an interesting link to the dysregulation of glutamate clearance from the synaptic cleft.

In summary, in this thesis we found several promising genetic loci for migraine, detected the first gene affecting common migraine susceptibility, through a variant estimated to account for 2.5% of total migraine heritability and 10.7% of the population attributable risk for migraine. We also report a promising hypothesis for a biological mechanism for migraine.

INTRODUCTION

Diseases with complex etiology form the most challenging problems faced by doctors and geneticists as well as patients, and conditions such as high blood pressure, diabetes, depression and migraine are very much part of daily life. Advances in the understanding of the genetics of these so-called "complex diseases" promise major improvements in quality of life for large segments of the population, but have proved to be difficult to study due to complicated interrelationships between environmental and innate factors involved in their pathophysiology. A recent discussion in the British Medical Journal even discussed the validity of the whole field of modern genetics to medicine (Le Fanu, 2010, Weatherall, 2010).

Neuropsychiatric conditions, including migraine (MIM 157300), are the most important cause of disability in all regions of the world, accounting for more than 37 percent of total years lived with disability (YLD) among adults aged 15 years and older. Migraine forms a major part of that burden, ranking 19th in YLD in the general population and 9th among women (Lopez et al., 2006). In Europe, migraine is the most common and costly neurological disease (Andlin-Sobocki et al., 2005). In a large US study, half of migraine patients reported at least one emergency room visit per year due to migraine, while 90% had at least one clinic visit and 15% had done so more than five times in the previous year (Osterhaus et al., 1992).

Migraine is the most common cause of chronic episodic headache. It affects approximately 12%-15% of the population (Hagen et al., 2000). Most of the common migraine spectrum is formed in two subtypes: migraine without aura and migraine with aura (previously known as common migraine and classical migraine, repectively). Both conditions are complex diseases, and so far no genetic variants influencing the susceptibility to either condition have been convincingly identified. There are no quantifiable laboratory measurements or radiological or performance changes for use in the study of migraine, and the migraine diagnosis is based solely on a patient's description of attacks.

In recent years, advances in analysis methods and genotyping technologies have enabled detailed genetic studies in hundreds and thousands of individuals at a time. This is the key to studying diseases with complex inheritance, as the effects of individual variants within the population are small and thus require more samples to reach sufficient statistical power for detection. In this thesis, we introduce a new method of stratifying different types of migraine, which we use to investigate the genetic susceptibility and background of the disease. We also present the first genome-wide association study in migraine.

FINNISH SUMMARY

Migreeni on yleisin kroonisen kohtauksellisen päänsäryn syy ja siitä kärsii 12-15% väestöstä (Hagen et al., 2000). Monitekijäisten kansantautien - kuten migreenin, diabeteksen ja masennuksen - etiologian ymmärtäminen on eräs nykylääketieteen ja -genetiikan vaikeimmista haasteista. Nämä taudit ovat osa päivittäistä elämää niin lääkärin vastaanotolla kuin kotonakin ja niiden tutkimuksen edistysaskeleilla on mahdollisuus parantaa monien potilaiden elämänlaatua. Monitekijäisten tautien tutkimus on kuitenkin osoittautunut hankalaksi moninaisten ympäristö- ja yhteisvaikutusten vuoksi ja tulokset ovat usein jääneet heikoiksi. Tuore keskustelu British Medical Journalissa jopa kyseenalaisti nykygenetiikan arvon lääketieteelle (Le Fanu, 2010, (Weatherall, 2010).

Neuropsykiatriset taudit, johon ryhmään migreenikin (MIM-koodi 157300) kuuluu ovat johtava elämänlaadun laskun syy kaikkialla maailmassa ja ne muodostavat 37 prosenttia toimintakyvyttömyyden kanssa eletyistä elinvuosista (YLD, years lived with disability) yli 15-vuotiailla. Migreeni muodostaa merkittävän osan tästä sairaustaakasta, ja on 19. vakavin elämänlaadun laskija koko väestössä ja yhdeksänneksi vakavin naisten keskuudessa (Lopez et al., 2006). Euroopassa migreeni on eniten kustannuksia ja elämänlaadun laskua aiheuttava neurologinen tauti (Andlin-Sobocki et al., 2005). Eräässä amerikkalaistutkimuksessa todettiin, että puolet migreenipotilaista joutuu käymään sairaalapäivystyksessä kerran vuodessa migreenin vuoksi, 90% kertoi tarvinneensa ainakin yhden terveyskeskuskäynnin viimeisen vuoden aikana aikana sen vuoksi ja 15% tarvitsi vähintään viisi käyntikertaa (Osterhaus et al., 1992).

Yleisellä migreenillä on kaksi päätyyppiä: auraton ja aurallinen migreeni, jossa jälkimmäisessä kohtaukseen liittyy kivun lisäksi erilaisia neurologisia oireita, kuten näkö- ja puhevaikeuksia. Tällä hetkellä käytössä ei ole laboratorio- tai kuvantamistutkimuksia, joilla migreeni voitaisiin osoittaa. Molemmat muodot kuuluvat edellä mainittuun monitekijäisiin tauteihin, eikä ennen tätä tutkimusta yhtään yleiseen migreenialttiuteen vaikuttavaa geneettistä tekijää ole varmuudella tunnistettu. Viime vuosien aikana analyysi- ja genotyypitysteknologian kehitys on ensimmäistä kertaa mahdollistanut satojen ja jopa tuhansien potilaiden geneettisen tiedon tutkimisen yksittäisessä tutkimuksessa. Näin suuret potilasmäärät ovat ehdoton vaatimus monitekijäisten tautien tutkimuksessa, koska yksittäisten muutosten merkitys on vähäinen ja siksi riittävän tilastollisen voiman saavuttaminen vaatii laajojen potilasaineistojen tutkimista. Tässä väitöskirjatutkimuksessa esittelemme uudenlaisen lähestymistavan, oirekomponentti-analyysin, migreenin luokitteluun sekä sovellamme sitä uusien geneettisten alttiusalueiden tunnistamiseen suomalaisessa ja kansainvälisessä potilasaineistoissa. Tätä analyysiä käyttämällä tunnistimme kaksi tärkeää migreenille altistavaa geenialuetta sekä toistimme useita muita. Tärkeimmät genomin ionikanavat kattanut geenitutkimus poissulki näiden roolin yleisessä migreenissä. Suorittamamme ensimmäinen migreenin kokogenomin assosiaatiotutkimus (käsittäen n. 5 700 potilasta ja 50 000 verrokkia) tunnisti ensimmäisen migreenialttiuteen vaikuttavan variantin, jonka osoitimme säätelevän lähellä sijaitsevan geenin ilmaisua. Tämän variantin säätelyvaikutus on ensimmäinen geneettiseen migreenialttiuteen populaatiotasolla ehdotettu mekanismi.

REVIEW OF THE LITERATURE

1 STUDYING THE HUMAN GENOME

Introduction

"Despite the ever-accelerating pace of biomedical research, the root causes of common human diseases remain largely unknown, preventative measures are generally inadequate, and available treatments are seldom curative. Family history is one of the strongest risk factors for nearly all diseases – including cardiovascular disease, cancer, diabetes, autoimmunity, psychiatric illnesses and many others – providing the tantalizing but elusive clue that inherited genetic variation has an important role in pathogenesis on disease". These are the starting lines of the International HapMap Consortium's first paper in 2005 (The International HapMap Consortium, 2005), which marked the beginning of the genome-wide association era.

In the few years since, impressive strides have been made in the genetics of common diseases. Large international consortia, which genotype tens and even hundreds of thousands of patients per study, have discovered numerous disease-associated variants and uncovered many new pathways associated with disease. For some conditions, like Crohn's disease (Barrett et al., 2008) and type II diabetes (Sladek et al., 2007), entirely new mechanisms have been detected. In the age of genome-wide association studies, tens of thousands of individuals have had a portion of their common variants genotyped, thereby forming a treasure trove of genetic information. However, many challenges remain in using the information to shape meaningful biological insights, especially due to the relatively small individual impacts of most detected variants. Therefore, a critical issue is coming up with new geno- and phenotyping methods to improve detection power. Due to the various challenges, most variants and pathways probably still remain to be found. Especially among diseases of the brain, knowledge of the genetic etiologies is weak.

Up until the late 1990's, technological and financial restrictions severely limited the size – and thus the attainable statistical power – of genetic studies. Typical studies used up to ten families and a few dozen affected individuals. This study size provided sufficient statistical power for the study of rare recessive Mendelian diseases – conditions where a mutation in the primary gene is necessary for the condition to occur, although its effects may be affected by one or more modifier genes. Indeed, genes and mechanisms for many such diseases were discovered in the 1990's, a notable example of this being identification of genes for the conditions forming the "Finnish disease heritage" (Norio, 2003a) – a group of roughly 40 genetic diseases more common in Finland than elsewhere in the world (Peltonen et al., 2000).

The completion of the main part of two key projects in early part of the first decade of the 21st century, the Human Genome Project (Lander et al., 2001) (HGP) and the International HapMap Project (The International HapMap Consortium, 2005) (see later in this Chapter) raised great hopes of understanding the basis of common

diseases. Huge amounts of both public and private funding were spent mapping a complete sequence (HGP) and to understand how the sequence behaves in different populations (HapMap), ushering in the era of genome-wide association studies. Now, more than 500 reported genome-wide association studies later (Hindorff et al., 2009), the conclusion appears to be that evolution has been remarkably successful in removing completely or at least limiting the contribution of penetrant mutations with large effects for common diseases (Goldstein, 2009). While this is good news for the species as a whole, it means that more comprehensive approaches such as whole genome and whole exome sequencing are needed, and that a lot of work remains in understanding the genetic background of common diseases.

Historical background

It has long been observed that many discrete characteristics of offspring correspond more closely to those of their parents than to those in the general population; for example, people with blue eyes will have more blue-eyed offspring than average, and offspring of plants with larger fruits are likely to bear larger fruits compared to average. Darwin's formulation of the concepts of natural selection and evolution in 1859 introduced a new theory of inheritance (Darwin, 1859), whereby evolution of species was shown to give rise to completely new features and traits. Mendel showed in 1865 that inheritance patterns in peas followed certain mathematical rules (Mendel, 1866), thus suggesting that small, discrete units of heredity exist. With the biological basis of heredity established, attention turned to its role in human features and disease. Galton had identified the usefulness of twins for genetic studies in 1875, and Garrod identified the first human disease with a Mendelian inheritance pattern, alkaptonuria, in 1902 (Garrod, 1902). The discovery of the structure of DNA in 1953 (Watson and Crick, 1953b) and the resulting implications for understanding the genetic code opened the study of genetics to chemical analysis. Shortly after, the correct number of human chromosomes were identified in 1956 (Harper, 2006, Tjio and Levan, 1956). The first gene sequence was described in 1972 (Min Jou et al., 1972) and the first genome sequenced (a

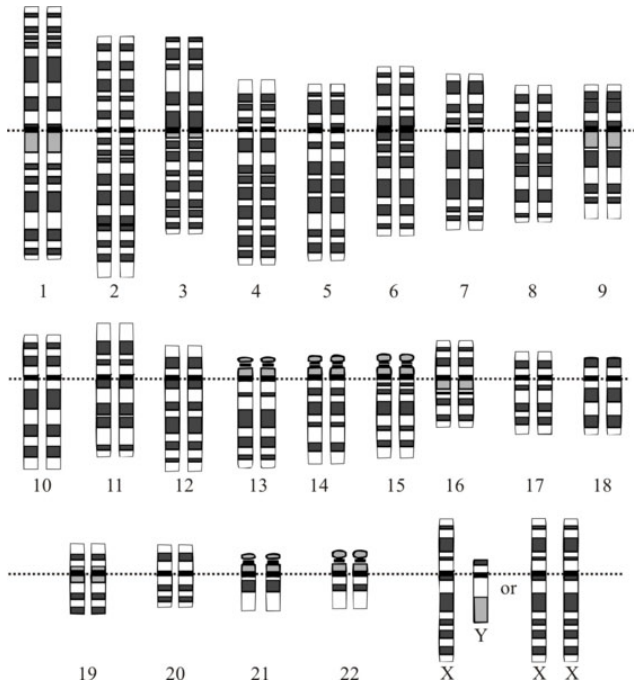

Figure 1. The human karyotype, showing chromosomes aligned along the location of the centromere. Image courtesy of the NHGRI.

bacteriophage) in 1977 through Sanger sequencing (Sanger et al., 1977). However, DNA analysis was painstaking, slow and difficult work, until it was made much easier by polymerase chain reaction (PCR) in 1983, which allowed easy amplification of DNA, necessary for large-scale experiments.

The human genome consists of roughly three billion pairs of nucleotides, divided into 22 pairs of autosomal (i.e. not sex-dependent) chromosomes and one pair of sex chromosomes (X and Y for males, X and X for females) which reside in the cell nucleus. A complete set of chromosomes is called a karyotype **(**Figure 1). Half of the chromosomes, 22 autosomal and one X chromosome are inherited from the maternal parent, and 22 autosomal and either an X or a Y from the paternal parent. In addition, small cell organelles called mitochondria, maternally inherited along with the maternal chromosomes, contain mitochondrial DNA (mtDNA). By comparison, mtDNA is minuscule, at 15,000 to 17,000 bases long.

A chromosome is comprised of one very large DNA molecule as well as DNA-associated proteins. The associated proteins package and organize the DNA into the tight space of the nucleus. The two complementary strands of DNA form a double helix, consisting of a phosphate backbone on the outside of the helix, and pairs formed of four bases on the inside: adenine (A), cytosine (C), guanine (G), and thymine (T). Typically, the most energy-efficient configuration is attained by linking A and T together, and C and G together (Watson and Crick, 1953a).

Genetic variation

The elements making up the variation in the human genome can be divided into different classes that vary in size. There is some overlap among groups due to historical reasons. The classes are listed below and those directly measured in and therefore most relevant to this thesis are 1 and 3b.

1. ***Single nucleotide polymorphism*** (SNP) is a difference in a single base pair and is the most common variation in the human genome. For instance, for an A/C SNP some individuals in the population carry an A-T pair at a given locus while others carry a C-G pair. A variant more frequent than 5% in the population is considered a common variant, while variants less than 0.5% frequent are considered rare variants. Current estimates place the number of SNPs with a population frequency of greater than 1% in the human genome at around 10,000,000 – one variant per every 300 bases, and roughly 1% of these are thought to be of functional importance (The International HapMap Consortium, 2005). It is estimated that the common SNPs are responsible for 90% of all genome variation. SNP data is used for Studies III and IV.
2. ***Insertions*** and ***deletions*** are changes to the length of the sequence due to the addition or removal, respectively, of one or more base pairs. Because of the three-base reading frame of the translation process, changes in length not divisible by three corrupt the reading frame, usually resulting in major changes to the protein, often through a premature termination of the protein chain. Traditionally, changes in size less than 571 bp were referred to as *indels*, but this definition has considerable overlap with the subsequent classes.
3. ***Repeat sequences (interspersed repeats, simple sequence repeats, segmental duplications, tandem repeats and copy number variants)*** are

various forms of sequence that have been copied over and over into the genome, by transposable elements, a group of genomic hitchhikers. Even though the repeats have been historically considered "junk DNA" in terms of translation to proteins, repeating/duplicating/transposing a piece of the genomic sequence is a major evolutionary force (Lander et al., 2001), that facilitates the formation of new genes by recombining the existing sequence in new ways. Similarly,

a. ***Interspersed (or transposon-derived) repeats*** estimated to comprise about 45% of the sequence of the human genome, but they are probably considerably more common. These repeats are a type of genomic parasite, a short piece of sequence which encodes for a few proteins required to bring the code into the cell nucleus and then randomly insert it into the DNA, where it is then ready for a new round of translation and re-entry.

b. ***Simple sequence repeats*** are a class of repeats where one or more nucleotides is repeated over and over (e.g. $[CATG]_n$ for CATGCATGCATG… sequence). They comprise 3% of the human genome and occur about once every two kilobases. Occasionally, mistakes in the DNA copying process (as the copying enzymes are more susceptible to mistakes when copying repeated sequences (Tautz and Schlotterer, 1994)) result in the lengthening of repeats. These differences in length can be used as a distinguishing feature between individuals, as well as being the causative mechanism in various expansion repeat disorders. In these disorders, extension of the repeat past a certain threshold causes disease, in cases where past a certain threshold the structure of the created protein is sufficiently different to alter its behavior in the cell. This group of diseases includes conditions such as the Fragile X syndrome (De Boulle et al., 1993), Huntington's disease (Walker, 2007) and various spinocerebellar ataxias (Orr et al., 1993). These repeats are further divided based on the length of the repeated sequence into *satellite DNA* (>500 bases), *minisatellites* (14-500 bases), and *microsatellites* (1-13 bases). Microsatellite length data of (di-, tri-, and tetranucleotide repeats) is used for Studies I and II.

c. ***Segmental duplications*** are 1-200 kb pieces of sequence that have been transferred in bulk from one location in the genome to another (intra- or interchromosomally), forming an estimated 5% of the genome. Segmental duplications located in close proximity are the basis for contiguous gene syndromes, such as the Smith-Magenis syndrome (Chen et al., 1989) and Charcot-Marie-Tooth syndrome 1A (Reiter et al., 1997). The syndromes involve known nearby duplications on chromosome 17 that align during replication, resulting in loss of the DNA sequence between the duplications. Large parts of certain chromosomes are known to arise from sections created by numerous segmental duplications.

d. ***Copy number variants (CNVs)*** are a special form of repeat sequences, which have become important in recent years as new platforms are capable of interrogating all common large-scale CNVs in a given genome. Through copy number variation, an individual can have multiple copies of a gene or region, because the length of the repeated sequence is long (commonly defined as > 1kb in length) and the copy

number typically ranges from zero to six. There is considerable variation in the possible size of copy number variations, which can extend up to several megabases long (Feuk et al., 2006). Homo- or heterozygous deletions (i.e. having a CNV with zero and one copies, respectively) are more easily interpreted in a biological context (Stefansson et al., 2008), because the loss of sequence at this scale frequently leads to severe phenotypes, such as mental retardation (Webber et al., 2009). However, the relevance of having excess copies of a CNV is not as well understood. Most CNVs have been found to be tagged by one or more common SNPs. Therefore, their roles in common diseases have largely been covered by SNP studies, which have not uncovered variants with high effect sizes, suggesting that the roles of common CNVs in common diseases is minor (Wellcome Trust Case-Control Consortium, 2010). However, much hope is currently placed on rare and/or large-scale CNVs (Walters et al., 2010) that are not yet sufficiently tagged by existing SNP studies.

4. ***Chromosomal abnormalities*** are major changes in the chromosome structure, often involving millions of bases at a time. There are five different classes of such changes. *Deletions* and *duplications* act as their counterparts in CNVs and both usually have severe consequences on the survivability of the organism, but certain whole-chromosome duplications can lead to non-lethal phenotypes such Down syndrome (Roizen and Patterson, 2003) and Klinefelter's syndrome (Klinefelter, 1986). *Inversions* involve the rotation of a segment of DNA from end to end, and if the inversion is not associated with an additional change in sequence length, the inversion does not lead to any pathology. In fact, an inversion on chromosome 8 is highly common among European populations (McEvoy et al., 2009)). *Insertions* and *translocations* involve pieces of a chromosome added or exchanged between chromosomes, which can be asymptomatic, but are more frequently observed in various cancers.

The sequence of any two full human genomes differs from one another by 0.1%, or one change per approximately 1,000 bases (The International HapMap Consortium, 2005). As a practical example of genetic differences between individuals, Levy et al. calculated the difference between two individuals from the same population (the HGP reference sequence, and the Venter genome) to be 12.3 Mb, divided into 3.2 Mb in SNPs (of which 1.3 Mb were novel), and 300,000 heterozygous and 560,000 homozygous indels. The non-SNP variation (i.e. variation due to CNVs, segmental duplications, inversions) was estimated to account for 74% of variant bases, or 4% of the genome (Levy et al., 2007). Further, 17% of the known genes (4,107/23,224) were found to contain a non-synonymous mutation, and a full 44% of known genes were found to have mutations in the UTR or coding regions. A 2003 paper estimated that segmental duplications alone account for 3.5% of total variation (Cheung et al., 2003).

The various elements of the genome have specific mechanisms that cause their occurrence. Concentrating on the elements forming the basis of this thesis, SNPs and microsatellites, the former occur due to *de novo* mutations caused by radiation and chemicals, as well as mistakes made by the enzymes copying DNA. Microsatellite length changes occur at roughly once every 1,000 generations (Weber and Wong, 1993), by slippage of the DNA replication machinery which occurs every now and

then when copying repeated sequence (Kruglyak et al., 1998). The most important part of the genome in terms of human survival is the coding sequence, comprising a few percent of the total sequence. The human genome contains an estimated 20,000 to 26,000 genes, with additional variation provided by differential processing through alternative splicing and transcriptional control, which allows a coding sequence to be transcribed in different ways. Unlike repeated sequence, the coding sequence is highly conserved (Sorek et al., 2004), since most random changes to the coding sequence are likely to have a major effect on the resulting protein.

While the changing nature of the genomic landscape is a tradeoff paid for evolutionary flexibility, it also results in the existence of genetic conditions. While evolutionary pressure keeps truly severe mutations in check, a number of additional factors can partially subvert the process causing particular diseases to become more prevalent. One such subversion is the so-called genetic bottleneck: a situation where a small subsection of the general population is the founder population for a new population, which remains isolated from outside genetic influences. A genetic bottleneck causes unusually high population frequencies of certain genetic markers, and thus using a population that has undergone a genetic bottleneck increases the power of genetic studies (de la Chapelle, 1993). Such bottlenecks usually occur for social or political reasons – for example, in the case of a small tribe that is cast out of a major population group for religious, political, or language reasons – like the Hutterites (Ober et al., 2000) in Canada and Eastern United States, and the Ashkenazi Jews in Israel (Hammer et al., 2000). Another classic example is the extensive use of the population isolate of Northern Finland, and especially the Kuusamo region (Varilo et al., 2000), to map complex diseases, such as asthma (Laitinen et al., 2001). The details of the Finnish genealogical history have been extensively debated elsewhere (Peltonen et al., 1999, Norio, 2003b), and will not be discussed here beyond the fact that the special features of the Finnish population isolates make them useful in disease gene mapping.

Another factor preserving harmful genetic mutations through evolution is the existence of recessive mutations: mutations that need to be present on the haplotype inherited from both parents in order for the corresponding phenotype to manifest. Given a sufficiently rare frequency of recessive mutations in the population, as was shown by G.H. Hardy in 1908 (Hardy, 1908), the frequency of the rare mutation stays largely unchanged in a population since the occurrence of its phenotype is very rare. As a result, the recessive mutation will likely remain in the population forever. The situation is complicated further when the mutation has a beneficial effect in addition to the negative effect; the classical example is the mutation underlying sickle cell anemia, where having a single copy of the mutation is beneficial as it provides resistance for malaria while having two copies results in the manifestation of disease (Kwiatkowski, 2005).

Methods of studying the genetics of human diseases

Twin studies

Twin studies are the classical starting point to finding genetic causes for diseases. Given that monozygotic (MZ) twins share 100% of their genome, dizygotic (DZ) twins 50% and both share the same environmental background, by comparing the

incidence difference of a given condition or trait between the MZ and DZ groups gives a direct estimate of half (100%-50% = 50%) of the genetic load for that phenotype. Through further calculations it is possible to estimate the environmental component (roughly equal to total risk minus genetic risk). From these calculations the amount of heritability associated with a particular phenotype can be determined, a key metric in determining whether genetic studies are warranted. Heritability is a measure of the proportion of phenotypic variation that is attributable to genetic variation, and is equal to the genotype variance divided by the phenotypic variance (H^2, reflecting all possible genetic variance) or the additive variance divided by the phenotypic variance (h^2, reflecting only the additive variance). The latter is used more commonly, as h^2 can be readily estimated from twin studies as twice the difference in correlation between MZ and DZ twins.

Linkage studies in families

The traditional next step in trying to find genetic risk factors is a family-based linkage study. In a linkage study, the segregation of genetic markers located across the genome is compared to the segregation of the study phenotype in the pedigree. The fit of a marker inherited along a phenotype is calculated as a the LOD score (the primary outcome measure in Studies I and II), defined as the base 10 logarithm of the likelihood of the given marker inheritance pattern divided by the random likelihood of the pattern. The chance to detect the haplotype that co-segregates with the disease status (if any) increases by collecting as large families as possible with multiple affected individuals. In practical terms it is observed that every additional informative meiosis increases the attainable LOD score by 0.3. In practice, a linkage analysis tests haplotypes defined by microsatellite markers in order to find single markers (two-point analysis) or multiple markers (multipoint analysis) that associate with the disease status. A limiting step in the success of linkage studies is the “conversion step”, which is the transformation of long-range haplotype segregation information (assumed to tag rare, possibly family-specific mutations) to the identification of the underlying mutations. However, rather than being a problem with the linkage study design, this difficulty is more due to the fact that information on the polymorphisms at a detected locus is only available for the most common polymorphisms, and therefore rare haplotypes causing disease are left unnoticed.

Candidate gene association studies

In the candidate gene approach, a hypothesis-based selection of a limited number of interesting genes is made based on positional information from linkage studies, functional information from pre-existing hypotheses or a combination of the two (Hirschhorn et al., 2002). Subsequently, a selection of genetic markers located in or near the selected genes is genotyped, typically in a case-control design of obtaining a sample of patients and another of healthy controls as identical to the patient sample in every way (except the phenotype studied) as possible. The frequencies of genetic markers in the two groups compared, and markers with clear frequency differences are considered to be associated with the phenotype to a degree of confidence given by the statistical significance of the frequency difference. A clear drawback to this approach is that it requires a priori knowledge of how the pathways or systems work in order to make a valid selection of genes. As a result, this inference is usually made on very limited information (Buckland, 2001). A second problem is that unlike in linkage and genome-wide association studies, only a narrow area covering the gene and its immediate surroundings is genotyped, because it is rarely feasible to cover

much of the intergenic area due to technological constraints. The distribution of eQTLs' (changes to the sequence that affect the expression of a gene) (Nica and Dermitzakis, 2008) distances from the alleles they affects is relatively even until a distance of around 1 Mb (Stranger et al., 2007), so that even if the studied gene is chosen correctly, its contribution to disease susceptibility may be via a long-range modifier or other distant mechanism that fall far outside the studied area.

Genome-wide association studies

The latest addition to the geneticists' arsenal has been the GWA study. In a GWA study, the testing is similar to the candidate gene study in that a pool of affected and control individuals is college, but instead of having to pre-determine the (usually narrow) regions of interest, a large number of markers with roughly equal distribution across the genome are genotyped at once. The GWA approach has some major advantages over others; unlike a candidate gene study it is relatively hypothesis-free with regard to disease mechanisms as every gene in the genome is tested in roughly the same manner. One drawback is that a number of assumptions regarding the frequency of alleles and the underlying LD structure have dictated the array design. In contrast to a linkage study, it directly studies the SNP variation instead of the haplotype structure, so the problematic conversion step required in linkage studies is avoided. However, GWA studies only test common markers as the genotyping platforms and calling algorithms are typically only capable of handling SNPs with a frequency >1%. There is relatively low power to detect rare SNPs that fall between genotyped markers.

Targeted and whole genome/exome resequencing

Traditionally, once a sufficiently narrow area within a linkage region, candidate gene or around a common variant has been identified, previously unknown variants (generally novel rare variants or incompletely tagged common ones) in the area are studied by resequencing the region. However, due to the prohibitive cost of resequencing large areas and performing the analysis in many samples, the confidence in both sample and location selection has to be very high in order to avoid the same problems that limit candidate gene analysis. Recently, whole-exome (ie. targeting the sequence of all protein-coding regions) and whole-genome resequencing have become viable alternatives being of reasonable cost for small sample sizes. Capturing rare exome sequence variants is a promising approach to detect rare variants with higher effect sizes explaining a larger portion of the missing heritability in common diseases, as demonstrated by a recent paper (Ng et al., 2009). Future improvements in sequencing technology promise a substantial decrease in the cost of sequencing full genomes, so it is likely that full-genome sequencing – which captures practically all sequence variation and not just common variation - will relatively soon take precedence over GWA studies.

Simple and complex diseases and models of inheritance

Genetic diseases and their studies are divided into several classes based on the estimated complexity of the disease inheritance and the mathematical implications of the inheritance pattern. If a disease directly follows Mendel's laws of segregation in an extended family, the implication is that a single causative gene or locus exists (though the implication is not conclusive). These diseases are therefore referred to as

simple or *Mendelian diseases*. Due to the rarity of such diseases (usually <1/10,000), in almost every case there will only be one gene that segregates with the disease, as the likelihood of carrying two such mutations is vanishingly small. However, different genes may cause the same disease even though the model of inheritance is simple, and different variations within the same genes can exist. For example, three different genes for familial hemiplegic migraine (FHM) are known; *CACNA1A* (Ophoff et al., 1996), *ATP1A2* (De Fusco et al., 2003), and *SCN1A* (Dichgans et al., 2005), and over twenty mutations in *CACNA1A* and over thirty mutations of *ATP1A2* have been reported (de Vries et al., 2009a), with variable migraine phenotypes (Ducros et al., 2001).

In practice, having only one target to study makes the analysis relatively straightforward, with only few confounding factors (such as incomplete penetrance – i.e. that not all individuals with the mutation necessarily exhibit the trait, phenotype or clinical symptoms in question). Examples of such diseases are Marfan's syndrome (Faivre et al., 2007), sickle cell anemia (Kwiatkowski, 2005) and Huntington's disease (The Huntington's Disease Collaborative Research Group, 1993). Perhaps the best known example are the two variants conferring lactose persistence, where the presence of a single SNP is sufficient to confer the phenotype of being able to break down lactose, one in northern Europeans (Enattah et al., 2002) and another in pastoral groups of eastern Africans (Ingram et al., 2007).

However, in most cases the severity of these disease phenotypes results in negative evolutionary pressure (i.e. since offspring carrying a mutation causing a severe disease will have less – or indeed none at all – offspring of its own, the mutation stays rare), these kinds of mutations are rare in the general population. This also means the effect of these conditions on public health even taken together is low, though of course potentially devastating for anyone directly or indirectly affected by them. For common heritable diseases, such as diabetes and depression, the implication is that since evolution keeps mutations with a large contribution to a disease rare, the heritability either represents a sum of a multitude of rare mutations, interplay between common genetic variants with small individual effects, or a combination of the two. In any case, when no easily discernible pattern of inheritance can be observed, the disease is classified as a *complex disease.* For these diseases, detecting an underlying variant or mutation is much more difficult, and generally requires sample sizes in the multiple thousands, due to the inability to tell apart the minute differences between clinical phenotypes caused by the different mutations. For this reason, various studies have explored better phenotyping methods, such as latent classes (Nyholt et al., 2004) (see Chapter 4) and endophenotypes (Paunio et al., 2004). Additional role in these diseases may be played by some unknown and/or poorly understood mechanisms, such as methylation or some other epigenetic mechanism (lit. "above genetics", referring to a hereditary mechanism that is independent of the DNA code, e.g. DNA methylation or chromatin remodeling). In a Swedish study, where the hunger status of grandparents was found to associate with metabolic syndrome phenotype presentation in the grandchildren (Kaati et al., 2002).

How the presence of a mutation or variant affects the phenotype, whether for a simple or complex disease, is determined by the model of inheritance. The first difference is whether the mutation behaves in a dominant (the effect of the mutation is strong enough to cause disease when carrying only a single damaged allele) or recessive

(when the mutated allele has to be inherited from both parents). Second, the mutation can be autosomal (reside in the gender-independent chromosomes), or be either X- or Y- or mitochondrially linked. In X-linked diseases, such as hemophilia A (Rosendaal et al., 1990), a recessive form of a disease will be rare in females, but more common in males – and only women can transfer affected alleles to male children. In Y-linked diseases, only males can be affected. Mitochondrial diseases (such as the MELAS syndrome (Goto et al., 1990)) are inherited from the mother, but as only a proportion of the inherited mitochondria may be affected (and thus the effects may be limited to any one or any combination of tissues), the disease can have a number of different phenotypic presentations.

Phenotyping approaches

The phenotypes used to compare frequencies of any genetic variant are divided into two groups; *dichotomous traits* and *quantitative traits*. For a dichotomic trait, the phenotype is binary in nature – affected vs healthy, presence vs absence of an event such as myocardial infarction, or a biomarker that is within vs outside normal parameters. Dichotomous traits are more commonly used in genetic studies, as they can be readily used to estimate odds ratios (OR) in a case-control design (Pearce, 1993). To a certain extent, making such a division is always arbitrary in nature: for example, the migraine diagnostic criterion for pain considers moderate, severe, or unbearable intensity as “intense pain”, and mild pain as “no intense pain”. Not only is this definition highly subjective, but it also assumes a major difference to exist between the border categories (mild and moderate) (Donner and Eliasziw, 1994). Further discussion on the matter can be found in Chapter 4.

Normally distributed phenotypes where a numerical value can be assigned to samples in the analysis (e.g. height, LDL cholesterol level, concentration of an enzyme) are called quantitative traits. This kind of phenotype is in general terms more suitable for general features and measures, such as the examples mentioned above. However, the analysis of quantitative traits has been adapted to disease studies through use of endophenotypes such as C-reactive protein concentration (Elliott et al., 2009), the carotid artery wall thickness in cardiovascular disease (Duggirala et al., 1996, Gerdes et al., 2002) and the timing of the cognitive decline in Alzheimer’s disease (Martins et al., 2005). Quantitative phenotypes also contain larger amounts of information in comparison to a dichotomous trait, and thus the statistical analysis tools are somewhat more sophisticated for this kind of analysis. The challenges for a quantitative trait are mostly related to measurement accuracy, representativeness of a measurement (e.g. for hormone levels that vary by the time of day), and the underlying model of inheritance. The first two of these challenges can, to a certain extent, be addressed by having multiple measurements available. However, the question of the underlying model of inheritance is a more complex problem; genetics of stature are a good example of a highly heritable and easily measurable trait, where success in determining the genetic background has long eluded researchers (Visscher, 2008), as discussed in the previous chapter.

One approach to address this problem has been the concept of extreme phenotypes (Allison et al., 1998). In this type of analysis (see Figure 2), only the far ends of the phenotype distribution are considered, effectively turning a quantitative trait into a dichotomous trait (in the sense that now a major, non-linear difference between the two groups exist). However, the quantitative measurements available for all samples allows for the use of more powerful statistical methods. Another assumption in this approach is that by using the extremes, the genotype distribution is more akin to that found in a Mendelian disease. For example, this approach has been used to study people with extreme HDL cholesterol levels in plasma (Cohen et al., 2004). The underlying assumption is that an individual with measurement value in the top percentiles for a given trait would likely carry many of the "cholesterol-increasing" variants, and that a person in the bottom percentiles would lack many or most of them.

Methods of correction

Methodical error correction is paramount given the large amounts of data involved in a typical genetic association study and the issues involved in measuring any biological data. Standard correction methods for genome-wide data include testing for Hardy-Weinberg equilibrium (Hardy, 1908) (the frequencies of genotypes are within the ranges that can occur in nature), minor allele frequency, genotyping success rates, heterozygosity and gender as well as accounting for population stratification.

The vast amount of genetic information available in the current GWA chip technology (up to 1 million markers per chip at the time of writing) allows a robust correction for geographic stratification, unknown relatedness between samples and genetic outliers. This is a considerable strength of the GWA approach, and while it is possible to achieve the same effect with an extremely strict study design, such a study design is very difficult to implement in practice.

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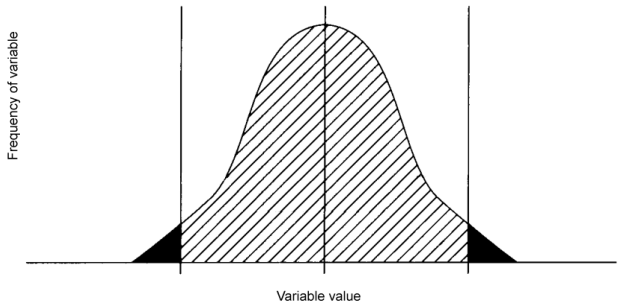

Figure 2. The concept of extreme phenotypes: considering only the values in the black areas converts a quantitative measure to an extreme dichotomous one.

One of the major contributions from the HapMap project was the

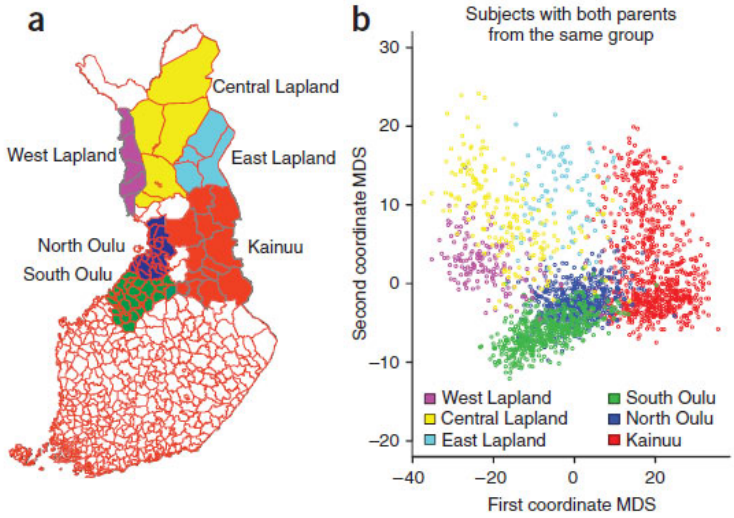

Figure 3. Comparison of a) the region of origin of individuals from Lapland in northern Finland with b) their multidimensional scaling (MDS) analysis results. The results clearly indicate the correlation between geographic and genetic identity, down to a county level. From Sabatti et al. (2009), used with permission.

identification of how population stratification affects the distribution of common variants in the genome sequence. Based on pattern analysis (such as multidimensional scaling), the amount of available SNP information from a GWA array can be used to distinguish the genetic origin of an individual (or, more correctly, the identity of the haplotypes that an individual has inherited), down to a scale of a few hundred kilometers in Europe (Lao et al., 2008), or down to the level of individual counties (see Figure 3) within countries (Sabatti et al., 2009). In GWA studies, this information is used to exclude population outliers from the study sample, which for its part increases the power of the study as non-representative samples are removed. Similarly, the SNP information is used to accurately determine cryptic relatedness (unexpected long distance relatedness between the samples). For non-GWA data, detecting these types of errors is next to impossible; the minimum amount of information considered sufficient for a population stratification analysis, for example, is around 10,000 markers located across the genome (Purcell et al., 2007).

The Human Genome Project

The basis for the modern genetic studies was laid in the study of the first haploid genome by the Human Genome Project (HGP), which compiled a single consensus sequence of DNA by studying the DNA of two anonymous males and two anonymous females - though most of the information came from one of the males due to quality considerations (Osoegawa et al., 2001). The first working draft was released in 2001(Lander, Linton et al. 2001; Venter, Adams et al. 2001) and the main project was completed in 2004 (International Human Genome Sequencing Consortium, 2004). The project established a number of genetic measurements for the first time, such as

the number of genes in the human genome and allowed standardized approaches to mapping the genome, forming the basis for the whole-genome approaches. This was the first project to provide a near-complete sequence for a vertebrate genome. The HGP also provided the first comprehensive look at the make-up of the genome. The amount of coding sequence (genetic code which can be translated into working proteins) was measured at less than 1-2%, while various repeat sequences account for more than 50%, the meaning of which will be discussed in Chapter 2. Key to this thesis, the HGP laid the basis for a structured approach to genome-wide analysis beyond the resolution of linkage studies that rely on the comprehensive genetic maps created in the earlier phases of HGP and allowed for the standardized mapping of sequence variants directly.

The International HapMap Project

To continue the work of the HGP, the HapMap project sought to develop a haplotype map of the human genome by sampling a small number of individuals from distinct population groups from different parts of the world. In order to construct a haplotype map, a combination of existing SNP data from the dbSNP database (www.ncbi.nih.gov/projects/SNP/), validated ancestral alleles found by comparison of HGP information and that of the Chimpanzee Genome Sequencing Project (The Chimpanzee Sequencing and Analysis Consortium, 2005) and previously identified SNPs from commercial sources (Matsuzaki et al., 2004) were used to identify variants across the genome every 5 kb apart. A haplotype map reveals which markers are inherited together and was considered as a useful tool in interpreting the data from the Human Genome Project, because it would show how sequences differ between individuals and populations. The map gave the first glimpse of how evolutionary selection works on a genomic level. The HapMap project also mapped the positions of common single nucleotide polymorphisms (SNPs) and provided a publicly available resource for data verification, interpretation and imputation for future projects. Given the prohibitive cost of full genome sequencing, assaying common variants was meant to provide a shortcut to uncovering variants with effects on common diseases.

Three populations (CEU – Central Europeans in Utah, representing a Caucasian population; CHB+JPT – Han Chinese in Beijing and Japanese in Tokyo, as an Asian population; YRI – Yorubans in Ibadan, Nigeria, as an African population) were selected for study. 30 full trios (parents and single offspring, a total of 90 individuals) were selected from the CEU and YRI populations, and 45 and 44 unrelated individuals from the CHB and JPT populations, respectively. The project was executed in different phases. In Phase I, a single SNP with minor allele frequency greater than five percent was genotyped at every 5 kilobases for a total of 1,007,329 SNPs (The International HapMap Consortium, 2005). In Phase II, the amount of SNPs was increased to 3.1 million (Frazer et al., 2007). In Phase III (in press at the time of writing), the number of individuals has been increased to 1,184 and the number of populations to 11 (The International HapMap 3 Consortium, 2010). Through the extensive analysis of these individuals, the HapMap project created vast amounts of information on common human SNP variation, just as the HGP did for linkage information. The HapMap data paved the way for the creation of genome-wide association (GWA) arrays that concurrently genotype a portion of the common SNP variation in the genome.

The genome-wide association era

The genetic map made available by the Human Genome Project combined with the information on common genetic variation from the HapMap project made it possible to design and mass produce chip arrays that simultaneously genotype a large number of common variants across the human genome. The *candidate gene approach*, preceding the GWA era, was burdened by the need to correctly guess the targets, the lack of a true way to assess stratification and case/control-matching among other things (Hirschhorn et al., 2002). The GWA study is limited in its targeting of a particular variant frequency spectrum and, as practice has shown, a particular effect size range (see Figure 4; (Manolio et al., 2009). The first such study was conducted on age-related macular degeneration (AMD), and a variant of *rs11200638* was detected to confer risk for AMD by affecting the promoter of gene *HTRA1*, a serine protease (Dewan et al., 2006, (Yang et al., 2006). The first large-scale study on a common disease was on type II diabetes, which confirmed the previously known association to the *TCF7L2* gene and detected three other associating SNPs (Sladek et al., 2007). A key study, published in 2007, was the Wellcome Trust Case-Control Consortium (WTCCC) study (Wellcome Trust Case-Control Consortium, 2007). For this paper, 14,000 patient samples representing seven diseases (bipolar disorder, coronary artery disease, Crohn's disease, hypertension, rheumatoid arthritis, type I and II diabetes) were analyzed separately against 3,000 shared controls. Genes were identified for every one of the studied disorders except hypertension, and several were identified for most diseases including nine for Crohn's disease. The WTCCC study addressing bipolar disorder was the first GWA study of a neuropsychiatric disorder, and resulted in the identification of a single significant SNP. Since then, over 500 GWA studies have been conducted in human diseases (see Figure 5).

However, the detected variants have all had small effect sizes and thus account for only a small proportion of the estimated heritability. For Crohn's disease, one of the early success stories of GWA studies, the 32 known loci account for roughly 20% of the total heritability (Barrett et al., 2008). For height, one of most genetically determined human features (80-90% estimated heritability), 40 known loci explain only 5% of the variance (Visscher, 2008). Possible causes for this lack of explained heritability have been a heated topic of discussion of late, and a recent review outlined the possible culprits (Maher, 2008). First, the inherent limitations of the GWA approach may be to blame: only common variation for a given sample is studied, which may dilute signals from indirectly tagged variants; future resequencing efforts should be able to solve this problem. Second, low penetration might be to blame; an association test expects every allele to be similar, and if the effect of the variant is modified by some unknown factor, the association will be considerably reduced; this problem will be difficult to solve, and likely involves massively greater sample sizes. Third, previously undetected copy number variation may be to blame, for which resequencing and improved CNV detection techniques should help. Fourth, the problem might not be in the detection techniques but rather in inadequate understanding of either the true phenotypes (an idea that plays a major role in this thesis) or the causative biological networks. In both of these cases, the needed improvements and solutions require a better understanding of the biology behind the phenotypes. Finally, the least favorable possibility is that the heritability estimates for common diseases are strongly inflated, and that no large effects have been found simply because they do not exist.

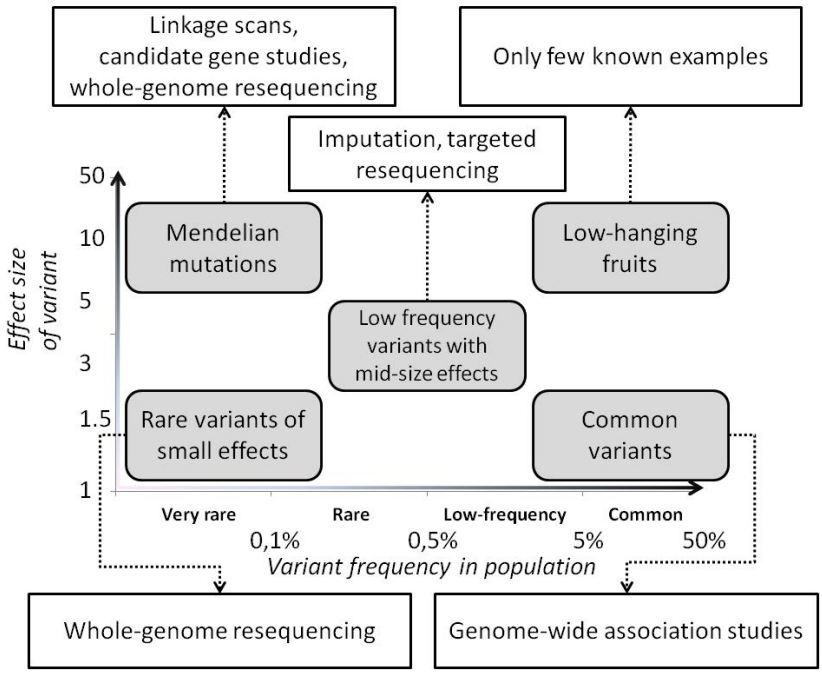

Figure 4. Setting the stage: the different categories of genetic variation based on the frequency and effect size of a variant. For rare variants with high effect sizes, the traditional approaches of linkage scans and candidate gene studies were relatively successful (see Hirschhorn et al. 2002), and variants of this category involved in migraine are studied in Study I and II. Currently these variants are beginning to be tackled with whole-genome sequencing approaches (discussed later in this Chapter). For the low-hanging fruits, evolution has effectively removed these from the gene pool, and only isolated examples remain (such as the lactase gene mutation, as discussed earlier). Rare variants of small effects are currently outside of reach, and will require considerable additional whole-genome resequencing efforts to be found – and without new understanding of biological networks etc. these will likely have little meaning. The middle group of low frequency variants will be the interesting territory for the next few years, as data from the 1000 Genomes project and the various re-sequencing efforts becomes available. Considerable inroads into the common variant category have been made with GWA studies in the recent years, and the study of variants in this category form the basis of Studies III and IV. Adapted from Manolio et al., 2009.

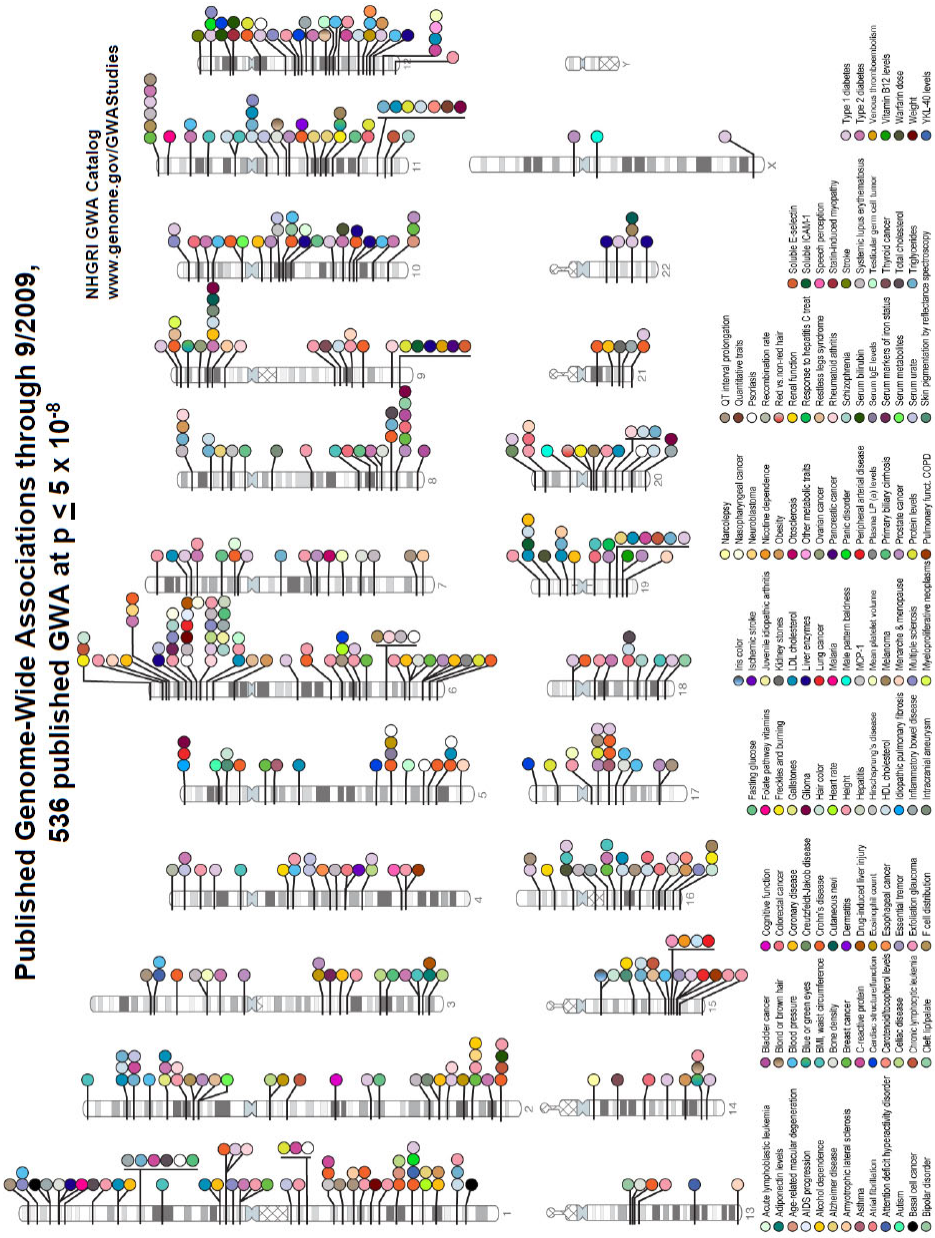

Figure 5. Published Genome-wide Associations as of September 2009 (Hindorff et al., 2009).

2 HEADACHE DISORDERS AND CHANNELOPATHIES

Neuropsychiatric disorders and relevant diagnostic divisions

Disorders of the brain are among the leading cases of disease and disability. "Neuropsychiatric disorders" is a WHO term for the group of disorders covering all of these disorders, covering ICD-10 code groups F and G. These groups contain both severe, often eventually fatal diseases like amyotrophic lateral sclerosis and Huntington's disease, and quality-of-life conditions like depression, Alzheimer's disease and migraine. Diseases from either group can have a serious effect on the quality of life of any sufferer. In terms of disability-adjusted life years (DALY), a measurement which takes into account not only years of life lost due to deaths caused by the disease but also the amount of disability caused and the number of years lived with the disability (YLD), diseases of this group are by far the most severe burden among the working age (15-59 years; see Table 1) (Murray et al., 2002). In this range, neuropsychiatric disorders account for 35% of the total disease burden in Europe, although they account for only 11% of estimated mortality (V. Anttila, unpublished data based on WHO measures).

Table 1. Disability-adjusted life years lost to the major disease groups per 100,000 inhabitants in Finland and in the EU in 2004 among 15-59-year-olds, according to WHO Global Burden of Disease.

Condition	Finland	EU average
Neuropsychiatric conditions	4 336	3721
Unintentional injuries	1 444	959
Cardiovascular diseases	1 432	1105
Malignant neoplasms	1 075	1334
Sense organ diseases	873	854
Digestive diseases	804	529
Intentional injuries	737	441
Musculoskeletal diseases	492	493
Respiratory diseases	452	625
Diabetes mellitus	244	225
Infectious and parasitic diseases	113	230
Maternal conditions	104	74
Endocrine disorders	79	157
Nutritional deficiencies	74	73
Oral conditions	72	74
Respiratory infections	59	62
Congenital anomalies	53	33
Genitourinary diseases	38	57
Other neoplasms	12	18
Skin diseases	9	13
Perinatal conditions	2	2

A 2004 epidemiological study places the number of people in Europe (28 countries; EU member countries after May 1st 2004 plus Iceland, Norway and Switzerland) affected with a neurological disorder at 127 million (out of 466 million; 27.3%) (Andlin-Sobocki et al., 2005). Within the spectrum of neurological disorders, various forms of headache, formally headache disorders, are one of the most common disease classes affecting population worldwide, affecting 46% of the adult population (Stovner et al., 2007). Large-scale epidemiological studies confirm relatively stable prevalence on all continents (see Figure 6), across ethnic groups and living conditions. Headache disorders affect both sexes, and all ranges of age and socio-economic conditions (Lipton et al., 2001); and detailed reports of their presence exists from 2,000 years ago (Sacks, 1995).

In approaching headache disorders, a number of further divisions can be made. Traditionally neuropsychiatric disorders have been divided according to medical specialties into neurology, neurosurgery and psychiatry (Price et al., 2000). Of these categories, headache disorders fall under the first, though a psychological component has long been suspected and should not be discounted. In neurology, diseases of the nervous system are primarily divided into those affecting the central nervous system (CNS; the parts of the nervous system enclosed in meninges; the brain and the spinal cord) and the peripheral nervous system (the rest of the nervous system). The central nervous system is isolated from the rest of body by the blood-brain barrier, which separates the blood in the circulatory system from the cerebrospinal fluid. Maintenance of this barrier is a crucial for brain function, and failure to uphold the barrier has been implicated as a mechanism in epilepsy (Oby and Janigro, 2006, Uva et al., 2008, van Vliet et al., 2007) and suspected to play a part in migraine as well. Most headache disorders are thought to happen within the CNS, though improper modulation of signals from the peripheries is an important feature. Within CNS disorders, headache disorders are categorized as episodic and paroxysmal disorders. This last category includes, in addition to headache disorders, epilepsy, cerebrovascular disorders, sleep disorders and certain movement disorders.

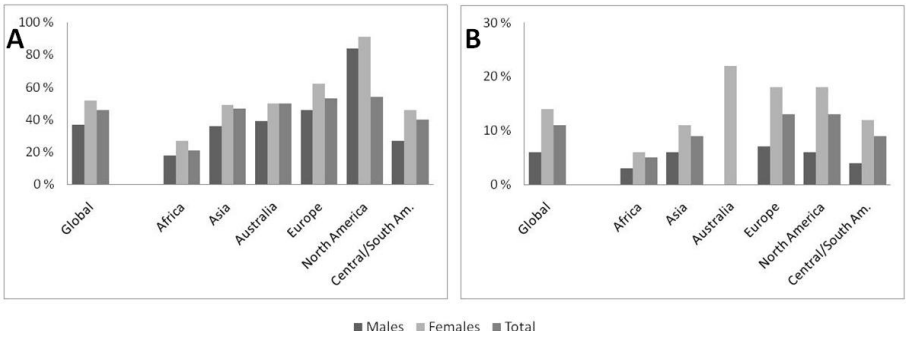

Figure 6. Current headache (A) and current migraine (B) prevalence of headache, globally and divided by continent. Data from Stovner et al. 2007. Data for total and male migraine prevalence for Australia is not available. Please note that for current headache prevalence in North America, the authors note that the the gender-specific rates are based on a single study, whereas the total prevalence is based on a meta-analysis, and is therefore likely to be more accurate. Am. – America.

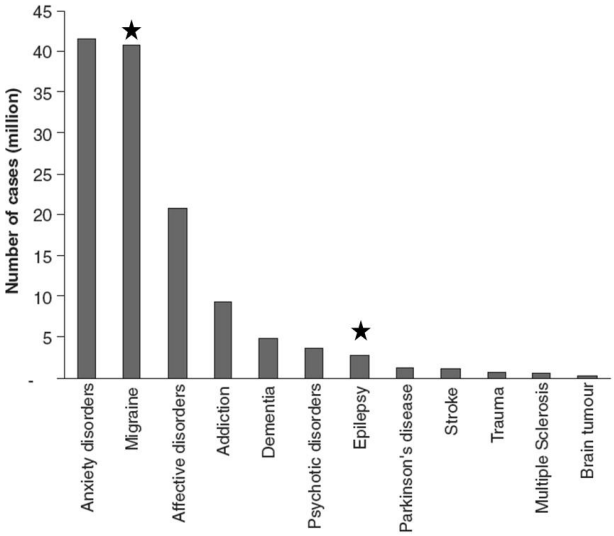

Figure 7. Total number of cases of the major neurological disorders in Europe. The two major diseases where channelopathies are thought to play a major role (migraine and epilepsy) in the list are denoted by a star. Adapted from Andlin-Sobocki et al., 2005, used with permission.

Episodic diseases of the brain and their comorbidity

The diseases making up the group of episodic and paroxysmal disorders fall essentially into two categories; headache disorders are a group where relatively little is known of the underlying pathophysiology (outside of a few specific examples, such as FHM, discussed further below). Within the headache disorders, only broad, descriptive distinctions can be made, such as the difference between tension-type headache and migraine, and which can be largely theoretical, such as the difference between MA (migraine with aura) and MO (migraine without aura). The second category, covering cerebrovascular and sleep disorders, as well as epilepsy, consists of a large group of disorders where the mechanisms are much more understood, with many known causes (for example, dozens of causes for insomnia are known, from fluoroquinolone to fatal familial insomnia). A large number of similarities exist between the two categories. Perhaps the best known overlap is between two of the more common representatives of the two categories, migraine and epilepsy (see Figure 7). For example, the diagnostic criteria for migraine recognize class 1.5.5. Migraine-triggered seizure, defined as an epileptic seizure triggered by migraine aura, as well as coining the term *migralepsy* for the pathognomonic overlap (International Headache Society, 2004). Significant comorbidity exists as well; a 1994 study found that the frequency of migraine in patients suffering from epilepsy was 24%, compared to 12% in non-epileptic relatives (Ottman and Lipton, 1994). An Icelandic study reported an 8.1-fold increase in the risk of developing epilepsy for children with

migraine with aura (Ludvigsson et al., 2006), and an Italian study in a pediatric headache center reported that children with migraine have a 3.2 times higher risk of developing epilepsy compared to patients with tension-type headache, and children with epilepsy having a 4.5-fold increased risk of developing migraine (Toldo et al., 2010). In a Finnish study it was found that for males with MA, having a family member with migraine significantly increased the risk of epilepsy (Artto et al., 2006). Epileptic attacks are at least occasionally accompanied by migraine-like headache, and certain medications (such as valproate and topiramate (Goadsby et al., 2002), and certain other antiepileptics currently in development (Bialer and White, 2010)) work for both conditions. The characteristic Jacksonian march of symptoms in certain forms of epilepsy (i.e. the progression of motor symptoms through different parts of the body in order of the representation of those symptoms on the motor cortex as the seizure progresses) is very similar to cortical spreading depression passing through the visual cortex in migraine aura. Further, a number of rare disorders, including mutations in the $Na_V1.7$ ion channel (*SCN9A*) which cause primary erythromelalgia (Yang et al., 2004), $Na_V1.1$ ion channel (*SCN1A*) which causes familial hemiplegic migraine and epilepsy (Castro et al., 2009), and EAAT1 glutamate transporter (*SLC1A3*) causing episodic ataxia and hemiplegia (de Vries et al., 2009b) have clinical presentations characterized with both epileptic seizures and migraine-like pain. Most mutations known in episodic disorders are of the former type, that is, mutations in ion channel genes (with the last being in a gene encoding a protein that is part of the same synaptic space, as discussed in the discussion). Hence genetically many of the diseases of the second category are classified as *channelopathies*, and based on the clinical overlap it is hoped that lessons learned from this group could help in deciphering mechanisms for headache disorders as well.

Channelopathies

Channelopathies are a group of diseases where a defect in an ion channel protein is causative (see Table 2 (Graves and Hanna, 2005)). Ion channels play a critically important role in maintaining homeostasis as well as cellular and neuronal signaling, and are found in virtually all human cells, as well as being highly conserved throughout evolution (Graves et al., 2005). Ion channels consist of an alpha subunit, a pore-forming structure that allows the movement of ions between the two sides of the cell's plasma membrane. This structure is typically not open in "resting state", but is induced into the open form by a change in voltage on the plasma membrane, or the binding of a specific ligand. In addition, most channels contain additional subunits, which modulate the alpha unit. Most channelopathies are congenital Mendelian diseases, while a few are acquired (usually as a result of an autoimmune reaction). These diseases affect mostly the brain and the muscles (including the heart), as the basic function of these tissues (muscle contraction, transmitting neuronal signals) is highly dependent on ion channel function, though anumber of autoimmune diseases, such as myasthenia gravis (Vincent, 2002) and the Lambert-Eaton myasthenic syndrome (Takamori et al., 2000) also possess an ion channel component, due to an autoimmune response against an ion channel.

Table 2. CNS and muscle channelopathies according to the affected channel. Adapted from Neurological channelopathies, Graves and Hanna, 2005.

Channel type	Affected gene	Phenotype	CNS	Muscle
Sodium channel	*SCN1A*	Familial hemiplegic migraine	x	
	SCN1A	Generalised epilepsy with febrile seizures plus syndrome (GEFS+)	x	
	SCN1B		x	
	SCN2A	Severe myoclonic epilepsy of infancy	x	
	SCN4A	Hypokalemic periodic paralysis		x
	SCN4A	Hyperkalemic periodic paralysis		x
	SCN4A	Paramyotonia congenita		x
	SCN4A	Potassium aggravated myotonia		x
Chloride channel	*CLCN1*	Myotonia congenita (Thomsen's, Becker's)		x
Calcium channel	*CACNA1A*	Familial hemiplegic migraine	x	
	CACNA1A	Episodic ataxia type 2	x	
	CACNA1H	Childhood absence epilepsy	x	
	CACNA1S	Hypokalemic periodic paralysis		x
	CACNA1S	Malignant hyperthermia		x
	CACNL2A	Malignant hyperthermia		x
Potassium channel	*KCNA1*	Episodic ataxia type 1	x	
	KCNE3	Hypokalemic periodic paralysis		x
	KCNE3	Hyperkalemic periodic paralysis		x
	KCNJ2	Andersen's syndrome		x
	KCNQ2	Benign familial neonatal convulsions	x	
	KCNQ3	Benign familial neonatal convulsions	x	

Primary and secondary headaches

As headache is a natural response to various noxious stimuli, headache disorders are divided into two main groups in the International Classification of Headache Disorders (International Headache Society, 2004) (see next Chapter) based on the cause of the pain. The first and more common group, ***primary headaches***, consists of conditions where some unknown cause is affecting the cranial pain pathways themselves (see Table 3). In this group, the cause and mechanisms are poorly understood, in contrast to the ***secondary headaches***, where a definite cause can be identified. The cause is generally some other condition and headache is part of the typical pathophysiology of that disease, or a direct response to it (e.g. pain caused by release of markers of cell damage from the brain tissue, due to damage by physical force, or chemical damage). A third group, ***neuralgias and other headaches***, are a small group of conditions where a secondary reason (such as neural inflammation in post-herpetic neuralgia) is affecting some part of the cranial pain pathways, making it in theory both a primary and secondary headache.

Table 3. Primary and secondary headaches, adapted from International Classification of Headache Disorders, second edition, 2004.

Primary headaches	***Secondary headaches***
1. Migraine 2. Tension-type headache 3. Cluster headache 4. Other primary headaches	Headache attributed to: 5. … head and/or neck trauma 6. … cranial or cervical vascular disorder 7. … non-vascular intracranial disorder 8. … a substance or its withdrawal 9. … infection 10. … disorder of homeostasis 11. … disorder of cranium, neck, eyes, ears, nose, sinuses, teeth, mouth or other facial or cranial structures 12. … to a psychiatric disorder
Neuralgias and other headaches	
13. Cranial neuralgias, central and primary facial pain and other headaches 14. Other headache, cranial neuralgia, central or primary facial pain	

3 MIGRAINE

Introduction

Migraine is a complex neurological syndrome, with two main forms (migraine without aura, MO, and migraine with aura, MA). It is typically characterised by severe, neurogenic pain and neurological symptoms varying from mild tiredness to paralysis, lasting from days to weeks. Migraine has been a recognized disease since ancient Greece, where Hippocrates and Aretaeus wrote extensively about *hemicrania*, a condition of the nerves (Sacks, 1995), with the latter also describing aura and recognizing its connection to some but not all migraine. As detailed in the previous chapter, migraine has a roughly equal prevalence across the globe, and thus as far as can be determined, a similar presentation across both time and place. Despite its common nature and the extent to which it burdens the healthcare system, the importance of migraine as a disease entity was realized late, with the first specialist institution founded in 1970 (Sacks, 1995) and the first specific clinical criteria set in 1988 (International Headache Society, 1988). Considerable underreporting and lack of awareness of migraine has been widely reported (as reviewed by Buse et al. (Buse et al., 2009)) as well as under-treatment - a US study in 2007 found that 43% of migraine patients had never used migraine preventative medication (Lipton et al., 2007). A study on headache specialists (though positive selection may play a role) estimated the lifetime prevalence of migraine to be as high as 71.9% for men and 81.5% for women (Evans et al., 2003).

Prevalence, incidence and effect on public health

Migraine is roughly three times more common among women than in men. A clear majority of the difference between migraine incidence between the sexes occurs at the time of puberty, and is virtually removed after menopause (as seen in Figure 8 (Stewart et al., 1991)), suggesting a link between hormonal balance and migraine.

The overall prevalence of migraine and the incidence peak during working age makes migraine a somewhat unusual neurological disease. Estimates of the one-year prevalence range from 10% (Rasmussen et al., 1991) to 15% (O'Brien et al., 1994),

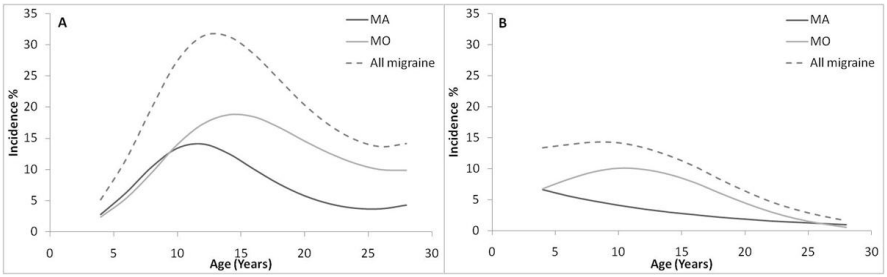

Figure 8. Sex-specific incidence rates of migraine with and without aura for A) females and B) males per 1000 person-years based on 10,131 survey respondents in Washington County, Maryland, USA. Adapted from Stewart et al., 1991.

with the largest study of 51,383 subjects reporting 12% (Hagen et al., 2000). Lifetime prevalence estimates between 13-18% in Europe (Stovner et al., 2006), it is by far the most common neurological condition in this age group. Lifetime prevalence of MA alone has been estimated to be 7% (Ulrich et al., 1999). According to WHO statistics, migraine ranks as the 19th most severe disease according to years lived with disability (YLD) in the global population, and the 9th among women (Leonardi and Mathers, 2003).

In terms of disability-adjusted life years (DALY), the WHO 2004 estimate (World Health Organization, 2008) of loss due to migraine is 177 years per 100,000 Finns (2004 average for EU member states: 176), making it the 4th most severe neuropsychiatric condition overall (after unipolar depressive disorders, alcohol use disorders and dementias including Alzheimer's disease; V. Anttila, unpublished data based on WHO measures; see Figure 9 (Lokal_profil, 2009)). Among the 15-59 age group, migraine is the third most severe, with dementia understandably much rarer in this age group. A 1991 study in the US estimated the number of people suffering moderate to severe disability to be 11.3 million (8.7 million females, 2.6 million males), with 4.5 million people (3.4 million females, 1.1 million males) suffering from such attacks monthly (Stewart et al., 1992). The same study found that in the US migraine correlates strongly with economic status, with a 60% higher prevalence of migraine in the poorest income group when compared to the highest income groups. The effects on quality of life are considerable (Solomon et al., 1993).

In Europe, a 2004 study estimated the total cost of migraine to be €27 billion in the EU, accounting for roughly a third of all costs due to neurological diseases – most in indirect costs (Andlin-Sobocki et al., 2005) (see Table 4). A US study in 2008 found the average direct cost of migraine to be $2,571 per patient (significantly higher than a matched migraine-free cohort) (Hawkins et al., 2008), with most of the costs arising from out-patient care and prescriptions; in-patient and emergency department care, though common in this group, accounted for only $1.25 billion (or ~10%) of direct costs as procedures or advanced interventions are rarely needed. An epidemiological study in 1999 calculated the average number of bed rest days due to migraine in the US to be 3.8 for men and 5.6 for women per year, for a total of 112 million bedridden days (Hu et al., 1999) – or approx. 1.8% of total workdays lost due to migraine.

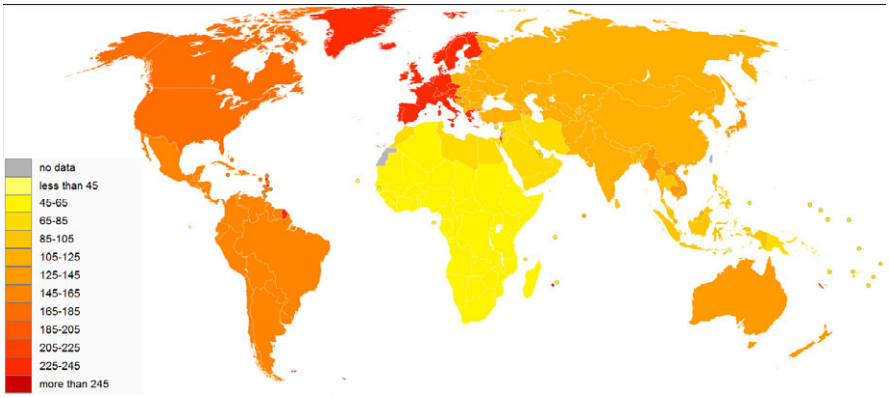

Figure 9. Disease-adjusted life years lost due to migraine in 2002 per 100,000 inhabitants, according to WHO data. For reference, see text. No data was available for Morocco.

Table 4. Costs due to brain disorders in Europe in 2004, organized by disease category. All costs in millions of euros, adjusted for purchasing power parity. From Andlin-Sobocki et al., 2005. Used with permission.

€ million in costs	Healthcare	Direct non-medical	Indirect	Total
Neurosurgical diseases	**4 099**	**269**	**3155**	**7523**
Brain tumour	1 162	269	3155	4586
Trauma	2 937			2937
Neurological diseases	**21 286**	**20259**	**42389**	**83934**
Epilepsy	2 752	4240	8554	15546
Migraine and other headaches	1 495		25507	27002
Multiple sclerosis	2 194	3977	2598	8769
Parkinson's disease	4 582	6140		10722
Stroke	10 263	5901	5730	21895
Neurological/mental disorders	**12 840**	**42337**		**55176**
Dementia	12 840	42337		55176
Mental disorders	**97 221**	**9336**	**132985**	**239542**
Addiction	16 655	3962	36657	57274
Affective disorders	28 639		77027	105666
Anxiety disorders	22 072		19301	41373
Psychotic disorders	29 855	5374		35229
All brain disorders	**135 445**	**72200**	**178530**	**386175**

Migraine attack

A migraine attack typically consists of four phases, any of which may be absent from an attack. These phases are the premonitory phase, the aura phase, the headache phase, and the postdromal phase (see Figure 10). The first (premonitory) and last (postdromal) phases are characterized by either positive or negative symptoms. The positive symptoms are roughly similar to an episode of hypomania, including hyperactivity, cravings and elevated and optimistic mood. More common are the

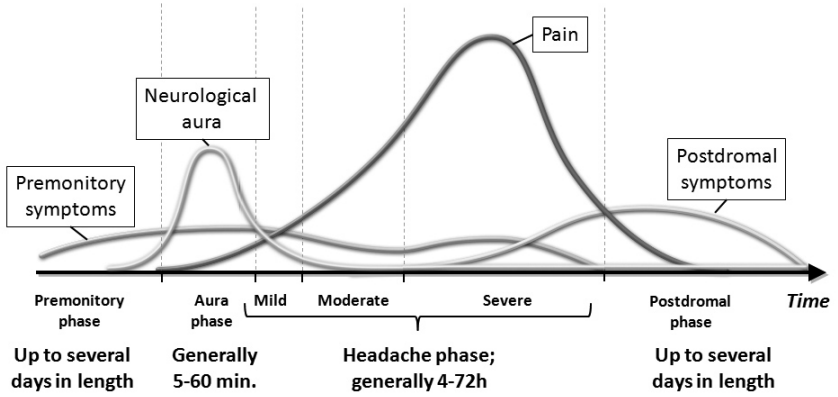

Figure 10. The typical procession of a migraine attack. Considerable variation exists – for example, the pain can immediately progress into the severe phase, or alternate between mild and severe pain.

negative symptoms, similar to a depressive episode, including sadness, hopelessness, hypoactivity, excessive sleep and lack of motivation (International Headache Society, 2004).

The aura phase, present in MA patients and absent in MO patients, is detailed below. The headache phase of a migraine attack typically lasts 4-72 hours. The pain has a number of typical features, which include a combination of some of the following: 1) a pulsating nature, that is, the intensity of the pain intensifies and de-intensifies with the heartbeat, 2) it is aggravated by physical activity, both suggesting a vascular link, 3) unilateral location, suggesting a central origin and the involvement of only one brain hemisphere, 4) to be of moderate or severe intensity, and 5) to be associated with nausea and/or vomiting and/or sensitivity to light or sounds, characterizing a general oversensitivity of the sensory system. Osmophobia (the fear of smells or in this case oversensitivity to smells) is, although not an official part of the criteria, considered a part of the clinical picture as well (International Headache Society, 2004).

Migraine aura and the cortical spreading depression

The migraine aura, presence of which differentiates the two main forms of common migraine, refers to gradually developing neurological symptoms (ranging from auditory hallucinations to full paralysis, and covering most neurological symptoms possible), lasting at least several minutes and less than one hour, although in reality the variation is greater and aura may, in rare cases, last even for several weeks. The symptoms are fully reversible, though they may be followed by a post-ictal phase after the attack, and the symptoms are typically only one-sided (International Headache Society, 2004).

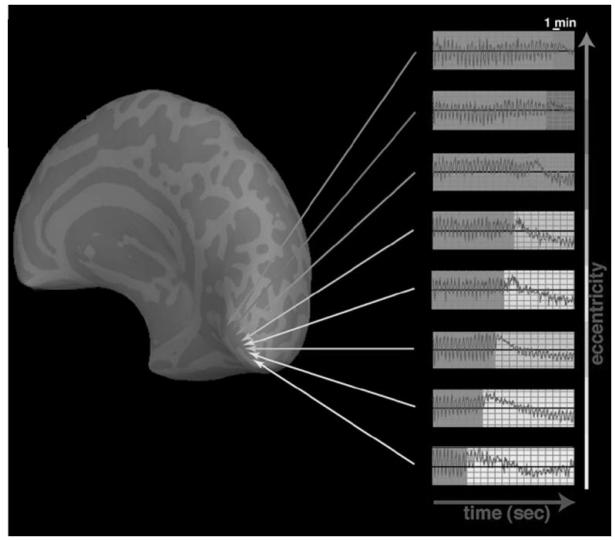

Figure 11. Time series of fMRI imaging results showing a wave of cortical spreading depression, as it begins to spread from the occipital lobe of the brain of a patient having a migraine attack. Different measurement points along the cortex reveal that the depolarization wave is spreading across the visual cortex. Adapted from Hadjikhani et al., 2001. Used with permission.

The aura is thought to be the outcome of a slowly spreading wave of depolarization in the brain, called cortical spreading depression (CSD), first detected in 1944 by Leao et al. (Leao, 1944). It is a wave of increased neuronal activity and cerebral blood flow, which moves at 2-3 mm / minute (Pietrobon, 2005). After the wave has passed, a recovery period

occurs within the neurons and cerebral blood flow is reduced. In imaging studies (see Figure 11), the spread of the CSD correlates with symptoms on the affected cortical region – for example, as the wave spreads across the visual cortex, the patient can observe a corresponding pattern in the visual field (Hadjikhani et al., 2001). Typically, the migraine aura includes a combination of visual, sensory, and speech disturbances. These are divided into positive and negative symptoms, depending on whether the disturbance is adding sensory (non-existing) information or removing it. Examples of positive symptoms include scintillating scotoma (visual cortex), where objects "gain" jagged edges, or the sensory symptom of "pins and needles", a pain sensation without an external cause (sensory cortex). Negative symptoms can include partial blindness (visual cortex) or numbness (sensory cortex).

International Classification of Headache Disorders

The first version of the headache classification, ICHD-I (Headache Classification Committee of the International Headache Society, 1988), was published in 1988 by the International Headache Society (see Table 5). It was the first time a hierarchical classification of all headache-related disorders was published, and represented a major step in reproducibility for both research and clinical practice for disorders which are, after all, largely description-based. It is part of the WHO International Classification of Diseases (ICD-10). It was last updated in 2004 with the introduction of the 2nd edition (see Table 6), ICHD-II (International Headache Society, 2004).

Table 5. Diagnostic criteria for Migraine without Aura, 1988 (Headache Classification Committee of the International Headache Society, 1988) and 2004 (International Headache Society, 2004). Criteria have remained unchanged between the editions.

1.1 Migraine without aura
A. At least five attacks fulfilling criteria B–D
B. Headache attacks lasting 4–72 h (untreated or unsuccessfully treated)
C. Headache has at least two of the following characteristics: 1. Unilateral location 2. Pulsating quality 3. Moderate or severe intensity (inhibits or prohibits daily activities) 4. Aggravation by walking stairs or similar routine physical activity
D. During headache, at least one of the following: 1. Nausea and/or vomiting 2. Photophobia and phonophobia
E. At least one of the following: 1. History, physical - and neurological examinations do not suggest secondary cause of headache 2. History, physical - and neurological examinations do suggest such disorder, but it is ruled out by appropriate investigations 3. Such disorder is present, but migraine attacks do not occur for the first time in close temporal relation to the disorder

Table 6. Diagnostic criteria for migraine with aura, according to the 1988 IHS classification (1.2 Migraine with Aura) (Headache Classification Committee of the International Headache Society, 1988) and the 2004 classification (1.2.1 Typical Aura with Migraine Headache) (International Headache Society, 2004).

1.2 Migraine with aura (1988)

A. At least two attacks fulfilling criterion B

B. At least three of the following four characteristics:

1. One or more fully reversible aura symptoms indicating focal cerebral cortical and/or brain stem dysfunction
2. At least one aura symptom develops gradually over >4 min, or >= 2 symptoms occur in succession
3. No aura symptom lasts >60 min. If more than one aura symptom is present, accepted duration is proportionally increased
4. Headache follows aura with a free interval of >60 min (it may also begin before or simultaneously with the aura)

C. At least one of the following:

1. History, physical - and neurological examinations do not suggest secondary cause of headache
2. History, physical - and neurological examinations do suggest such disorder, but it is ruled out by appropriate investigations
3. Such disorder is present, but migraine attacks do not occur for the first time in close temporal relation to the disorder

1.2.1 Typical aura with migraine headache (2004)

A. At least two attacks fulfilling criteria B–D

B. Aura consisting of 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 (i.e., loss of vision)
2. Fully reversible sensory symptoms including positive features (i.e., pins and needles) and/or negative features (i.e., numbness)
3. Fully reversible dysphasic speech disturbance

C. At least two of the following:

1. Homonymous visual symptoms (note: additional loss or blurring of central vision may occur) and/or unilateral sensory symptoms
2. At least one aura symptom develops gradually over >=5 min, and/or different aura symptoms occur in succession over >= 5 min
3. Each symptom lasts >= 5 and <= 60 min

D. Headache fulfilling criteria B–D for 1.1 Migraine without aura begins during the aura or follows aura within 60 min

E. Not attributed to another disorder (note: history and physical - and neurological examinations do not suggest any of the disorders listed in groups 5–12 [see Table 3], or history and/or physical and/or neurological examinations do suggest such disorder but it is ruled out by appropriate investigations, or such disorder is present but attacks do not occur for the first time in close temporal relation to the disorder)

Migraine pathophysiology: neuronal versus vascular theory

Relatively little is known of the pathophysiology of migraine and discussion has been dominated by two theories: the neuronal and the vascular theories of migraine. The former is currently favored (Dodick and Silberstein, 2006). The consensus is that some triggering event, with or without genetic or environmental predisposing factors, sets up a state of cortical neuronal hyperexcitability (Pietrobon and Striessnig, 2003). This puts the brain in a state that allows the propagation of a strong polarizing wavefront. Various mechanisms have been implicated to explain why the brain is susceptible to such waveforms, like excess glutamate in the brain, but no consensus on the matter exists yet. In contrast, the propagation of brain waves is more strictly controlled in the normal state brain (Lopes da Silva, 1991). CSD and its progression across different regions of the cortex are considered to be responsible for the various aura symptoms. The intense neuronal activity associated with the passing wave and the reduced blood flow following it are thought to trigger the pain sensation of migraine. The likely mechanism for the pain is thought to be a combination of neurogenic sterile inflammation and central sensitization (i.e. incorrect pain handling). This central sensitization is believed to arise from brainstem dysfunction causing impaired nociception, especially in the region of periaqueductal grey (PAG), various

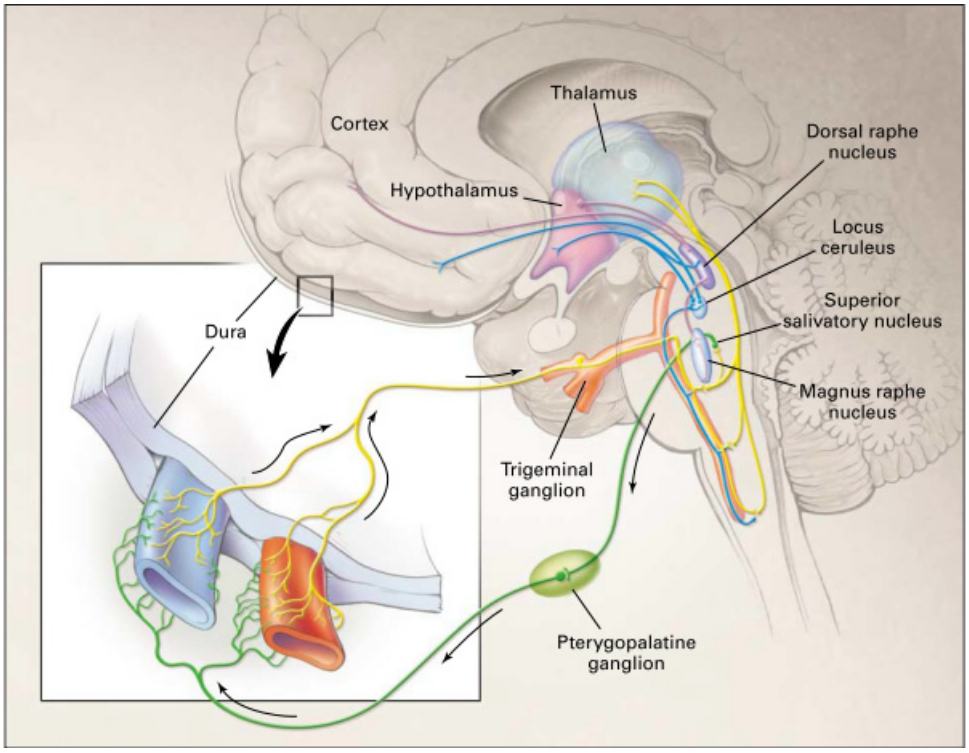

Figure 12. Illustration of the relevant anatomical structures involved in migraine pathophysiology, from Goadsby et al, 2002. Copyright © [2002] Massachusetts Medical Society. All rights reserved. (reprinted with a permission from New England Journal of Medicine).

brainstem nuclei and the trigeminal ganglion (see Figure 12). A generalized decreased inhibition in this region is thought to underlie both the pain perception, the sensitivity to sensory stimuli (Pietrobon et al., 2003), and the response to stress (Domingues et al., 2009), which is one of the key triggers of migraine. Other observations supporting the role of a neuronal mechanism in migraine include a recent imaging study that pointed to the role of hypothalamic activation in migraine (Denuelle et al., 2007) and several studies that have pointed to abnormal response to stimuli and signal processing in migraineurs between attacks. Abnormal habituation is seen to characterize the response to both visual and auditory signals in MA patients (Afra et al., 2000) and both MA and MO patients (Wang et al., 1996), suggesting a migraine brain potentiates certain types of abnormal repeated stimuli. Abnormal habituation suggests an ongoing interictal habituation dysfunction among migraineurs. A number of studies have linked such habituation defects to glutamate in *Aplysia* (Ezzeddine and Glanzman, 2003) and mice (Bespalov et al., 2007). In rats, blockage of metabotropic glutamate receptors were found to influence short-term habituation to olfactory stimuli (Best et al., 2005, (Yadon and Wilson, 2005). In summary, the neuronal theory of migraine postulates that this neurogenic activity is sufficient to cause migraine, and that the vascular aspects of migraine are caused by CSD or other neuronal phenomena (Dodick et al., 2006).

The vascular theory of migraine is based largely on the observation that a number of vasodilatory substances can induce migraine in humans (Schytz et al., 2009) and that migraine medications, like ergotamine (Tunis and Wolff, 1953) and triptans (Humphrey and Goadsby, 1994), produced strong vasoconstriction. Vascular smooth muscle dysfunction is also often suggested to play a role in migraine pathophysiology (Tietjen, 2009). The vascular theory is also supported by a number of findings in monogenic syndromes, as discussed in chapter 5. However, current understanding heavily favors the neuronal hypothesis, which accounts for the vascular effects as incidental or unrelated (Goadsby, 2009). For instance, a recent imaging study showed the lack of cerebral vasodilation during migraine attacks (Schoonman et al., 2008).

Regardless of the underlying theory, a number of observations point to a potentially pivotal role of enhanced brain glutamate levels behind the hyperexcitability of a migrainous brain, as well as in the triggering of migraine attacks (Goadsby et al., 2006). The main findings supporting a key role for glutamate in migraine pathophysiology include: (i) glutamate receptor antagonists may have acute anti-migraine activity (Andreou and Goadsby, 2009); (ii) the great majority of preventive migraine agents, despite belonging to widely different pharmacological classes, share the ability to block CSD in experimental animal models (Ayata et al., 2006) and may modify the glutamate-mediated trigeminal pain pathway (Shields and Goadsby, 2005, (Storer and Goadsby, 1999); (iii) the increased cortical release of glutamate fully explains the dramatically increased susceptibility to CSD seen in transgenic mouse models of FHM1 (Tottene et al., 2009, (van den Maagdenberg et al., 2010); (iv) noxious dural stimulation, as an experimental animal model for acute migraine, increases glutamate release from trigeminal ganglion neurons (Goadsby and Classey, 2000); (v) plasma (Ferrari et al., 1990) and cerebrospinal fluid (Martinez et al., 1993) levels of glutamate are increased in migraineurs with and without aura in between attacks, further rising during attacks; (vi) a recent magnetic resonance spectroscopy study suggested that migraineurs have different glutamate-to-glutamine ratios when compared to controls, suggesting malfunction of excitatory amino acid transporters in

deep brain structures of migraineurs (Prescot et al., 2009); (vii) in a patient with a severe phenotype of migraine-like headaches and additional neurological episodic features, a missense mutation in the EAAT1 glutamate transporter gene was associated with severely reduced glial uptake of glutamate (Jen et al., 2005); and (viii) glutamate-mediated thalamocortical transmission is crucial for head pain (Storer et al., 1999). A recent study suggested that in trigeminal neurons, glutamate release (as well as the release of calcitonin-gene related peptide, another neurotransmitter intricately linked with migraine) is controlled by calcium channels (Xiao et al., 2008), providing a possible link between glutamate and the known familial hemiplegic migraine genes (see Chapter 5).

Are common forms of migraine distinct or part of the same spectrum?

One of the key open questions in migraine research is whether the two main types of common migraine are separate entities or simply slightly weaker/stronger versions of the same condition. The main arguments in favor of the "distinct disorders" hypothesis are that: the affected-sibling risk ratios are considerably different: 1.9 for MO, 3.8 for MA (Russell and Olesen, 1995); a majority of patients never suffer from attacks with aura; the existence of a form of migraine where aura is present without headache (equivalent migraine); and that these forms are traditionally clearly dichotomized in diagnostic criteria ((International Headache Society, 1988)). The "distinct disorders" hypothesis is also supported by several population-based surveys (Russell et al., 1995, Russell et al., 1996, Russell et al., 2002) and it should be noted that a majority of the comorbidity studies in migraine (see below) have found positive correlations specifically with MA and not migraine in general. One example of this is a recent study which showed that the risk of developing epilepsy was considerably increased in MA patients, but not in MO patients (Ludvigsson et al., 2006). Furthermore, the study by Kruit et al., 2004 showed that the increased risk for brain lesions was only found among MA patients.

Counterarguments in favor of the "variations on a spectrum" hypothesis also exist (e.g. Kallela et al., 2001b) and are supported by genetic studies and a latent class analysis of migraine (Ligthart et al., 2006, Nyholt et al., 2004). Arguments in favor of this view include that most MA patients suffer from both types of attacks with varying frequencies and that patients suffering attacks where solely aura is present being a rarity, as well as that affected-sibling risk ratios are higher across the diseases - that is, the likelihood for a second sibling to develop MA when the first sibling has MO is greater than the general risk (Ulrich et al., 1999), and vice versa. Indeed, most MA patients suffer from attacks with and without aura, and thus fall on a frequency (or severity, depending on the viewpoint) gradient between pure MA and pure MO. Pure MA, referring to only having attacks that are associated with aura, is very rare. The gradient can be viewed as a frequency/severity continuum where position depends on the proportion of attacks aura is present. This view is reflected on our interpretation of the results of Study IV, where our findings seem to support the "variations on a spectrum" hypothesis (see Study IV discussion for further details). The continuum is dichotomized for clinical purposes into one extreme, where no aura is ever present (MO) and everyone else (MA) regardless of the ratio of MA and MO attacks. It could be argued that the second edition of the diagnostic criteria has moved from the earlier complete dichotomy towards a more flexible view. The introduction of new

subcategories for MA (1.2.1 Typical aura with migraine headache and 1.2.2 Typical aura with non-migraine headache) separates the aura and headache parts and the new 1.5.1 Chronic migraine category introduced a formal frequency cut-off for the number of migraine attacks per month. It is therefore quite possible that a future version of the criteria will more finely assess the headache and aura aspects of migraine. Thus far, there is not much robust genetic or biological evidence to conclusively support either the "variations on spectrum" or the "distinct disease" hypothesis apart from a few interesting twin studies (Ligthart et al., 2006, Nyholt et al., 2004) and Study IV.

Major comorbid disorders

Migraine is comorbid with a large number of other neuropsychiatric and immunological conditions, suggesting that migraine itself is a common reaction to a broad array of disturbances in the brain. It is present more frequently than expected in patients with depression, anxiety disorders, various pain disorders and clinical and sub-clinical brain lesions. The classical comorbid disorder is tension-type headache, though several epidemiological studies have found no comorbidity between it and migraine (Rasmussen et al., 1992, Ulrich et al., 1996); however, it has been shown that in migraine triggering factors (such as alcohol and chocolate) may worsen tension-type headache as well (Ulrich et al., 1996). Migraine has been reported to be associated with increased lower back pain with an OR 2.1 in patients with less than 30 pain days per year and 3.4 in patients having more than 30 days with back pain (Hestbaek et al., 2004). A Norwegian study found that migraine patients were almost twice as likely to report musculoskeletal symptoms (OR 1.9), with headache frequency being a strong predictor (OR 5.3 for female chronic headache patients, 3.6 for males) (Hagen et al., 2002). For psychiatric comorbidity, the connection between depression and migraine has been reported in both directions; depression increases the risk of migraine (RR 3.4), while migraine increases the risk of depression (RR 5.8) (Breslau et al., 2003). Another large Norwegian study found that migraine patients were more likely to suffer from depression (OR 2.7) as well as anxiety disorders (OR 3.2) (Zwart et al., 2003). Bipolar disorder (BPD) is another psychiatric phenotype that has been extensively linked to migraine: a 2003 study found migraine to be twice as common among BPD patients as controls (Hirschfeld et al., 2003), and a 2006 study estimated the increase in prevalence to be 2.5-fold (McIntyre et al., 2006). A range of sleep disorders, from obstructive sleep apnea to hypersomnia, have been reported to be comorbid with migraine (Rains and Poceta, 2006). Finally, a Finnish study which included samples also used in this thesis, reported an increased risk for psychiatric disorders (OR 4.09), as well as allergies (1.83) and hypotension (1.43), as well as for stroke and epilepsy (Artto et al., 2006) (comorbidity with latter is discussed in previous chapter).

Interesting aspect of the clinical background of migraine are the links between patent foramen ovale (PFO), a congenital weakness in the atrial heart wall, stroke and migraine with aura. PFO is a relatively symptomless condition present in 15-25% of the population (Di Tullio et al., 2007), and is thought to provoke migraine aura through an unknown mechanism. A 2005 study found PFO to be much more common in migraine patients than migraine-free controls (47% vs. 17%) (Schwerzmann et al., 2005), and presence of more than small right-to-left shunt increased the risk of migraine 7.78-fold. PFO has been found to mostly associate with MA, while the risk for MO patients was much closer to the controls (29% for MA, 4% for MO, 2% for

migraine-free controls) (Carerj et al., 2003). PFO closure trials for migraine relief have largely provided inconclusive results (Schwedt et al., 2008), though a number of anecdotal positive studies exist (Post et al., 2004). Only one large, randomly controlled trial has been reported so far (Dowson et al., 2008), in which no significant benefit from closure could be demonstrated, although the high prevalence of PFOs in MA patients was confirmed.

Both PFO and migraine with aura increase the risk for cryptogenic strokes (Lamy et al., 2002). A number of reasons have been suggested involving blood flow through the right-to-left shunt and passage of small emboli through PFO (Anzola et al., 1999) to serotonin-activated platelets (Buzzi and Moskowitz, 2005). Our group has also reported a case study of a young female patient with a large shunt who suffered a stroke at a young age (Artto et al., 2008). MA is strongly linked with an increased risk of ischemic stroke. Interest was sparked by the CAMERA study, a large-scale imaging project on migraine patients (Kruit et al., 2004), that suggested that patients with migraine with aura in particular have a high risk of subclinical white matter brain lesions with an OR of 13.7 for posterior circulation territory lesions. A recent meta-analysis estimated the risk of ischemic stroke to be roughly two-fold (Schurks et al., 2009a).

4 THE SEARCH FOR VARIANTS PREDISPOSING TO MIGRAINE

Heritability of migraine

The heritability estimates For migraine are fairly high. A 1995 Finnish study estimated the heritability to be between 34% and 51% (Honkasalo et al., 1995), depending on the type of migraine. Interestingly, the this study also calculated the heritabilities for the various migraine symptoms; the highest estimate was for unilaterality (56%) and the lowest for nausea and vomiting (45%). A large-scale 2003 study placed the range of migraine heritability in six countries between 34% to 57% (Mulder et al., 2003). A Danish study estimated that the difference in migraine rates between monozygotic (MZ) and dizygotic (DZ) twins is significantly higher in MA (concordance rate 34% for MZ vs. 12% for DZ twins) (Ulrich et al., 1999). For MO, the difference in concordance rates was also significant but not as large, 43% for MZ vs. 31% for DZ (Gervil et al., 1999).

Having a first-degree relative with migraine has been shown to increase risk for any migraine (Russell and Olesen, 1993). Compared with the general population, first-degree relatives of MO patients have 1.9 times higher risk for MO, and 1.4 times higher risk for MA (Russell et al., 1995). However, for relatives of MA patients, the risk of MA was four-fold, while risk of MO did not differ from the general population, suggesting genetic distinctness of these forms of migraine. Further, affected-sibling risk ratios were found to differ, 1.9 for MO and 3.8 for MA.

Familial hemiplegic migraine and other monogenic syndromes

The first genetic clues on what causes CSD in humans were obtained from familial hemiplegic migraine (FHM), a very severe Mendelian form of migraine with aura. FHM is considered a useful analog of migraine with aura, as the aura in FHM is very similar to that of MA apart from the hemiplegia (Thomsen et al., 2002). For this reason, FHM is considered an extreme form of MA. It is characterized by aura attacks where, at least occasionally, unilateral motor weakness is present, which suggests the involvement of the motor cortex. The unilateral motor weakness is fully reversible and is associated with positive or negative symptoms of the visual or sensory cortex.

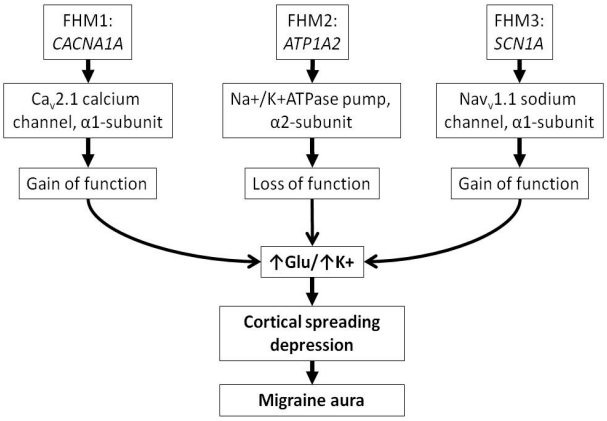

Figure 13. Known genes in familial hemiplegic migraine (FHM) and their proposed effects on susceptibility to cortical spreading depression. Glu – glutamate, K^+ - potassium. Gain or loss of function refers to the effect of the causative mutations on the function of the channel named. Adapted from Sanchez del-Rio et al., 2006.

In 1993, a French study in two families revealed a locus on chromosome 19 for FHM (Joutel et al., 1993). This was followed by a number of studies, including one in Finland (Hovatta et al., 1994). Finally, in 1996 the first gene for FHM (*CACNA1A)* was identified (Ophoff et al., 1996). FHM is an autosomal dominant disorder, and today three known mutations in ion channel genes are known (FHM1: *CACNA1A* (Ophoff et al., 1996), FHM2: *ATP1A2* (De Fusco et al., 2003), FHM3: *SCN1A* (Dichgans et al., 2005)). Due to the autosomal dominant nature, FHM diagnosis requires at least one first- or second-degree relative with the same kind of attacks; without an affected relative, the diagnosis is sporadic hemiplegic migraine (SHM). *CACNA1A* encodes a subunit of a neuronal P/Q-type $Ca_V2.1$ channel, mutations in which have also been reported to be associated with episodic ataxia type 2 and spinocerebellar ataxia type 6 (Jodice et al., 1997) as well as epilepsy (Chioza et al., 2001). So far, more than 20 mutations in *CACNA1A* have been reported, and interestingly they result in a range of different neurological phenotypes. *ATP1A2* encodes an α2-subunit of a sodium / potassium pump, in which mutations have also been associated with mental retardation and epilepsy (Jurkat-Rott et al., 2004), cerebellar problems (Spadaro et al., 2004), and benign childhood convulsions (Vanmolkot et al., 2003). *SCN1A* encodes a sodium channel which has a well known role in epilepsy (Graves et al., 2005, (Meisler and Kearney, 2005). Figure 13 shows the proposed biological effects of these mutations (Sanchez-Del-Rio et al., 2006), and detailed review of these mutations can be found in a recent review (de Vries et al., 2009a). A sporadic form of hemiplegic migraine (SHM) also exists (Thomsen et al., 2003a), which has been shown to confer increased familial risk to MA but not MO (Thomsen et al., 2003b).

Migraine headache also presents as a symptom in a number of monogenic conditions, including cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL). CADASIL is a disease caused by mutations in the *NOTCH3* gene, which plays a role in smooth muscle function in the brain vasculature (Joutel et al., 1996). Another example of a mutation related to the vascular system is the *TREX1* gene mutationthat causes retinal vasculopathy with cerebral leukodystrophy (RVCL), a progressive condition involving blindness due to vascular retinopathy, brain infarcts and vascular dementia (Richards et al., 2007). RVCL and its comorbidities with migraine and stroke have led to suggestions of the significance

of vascular dysfunction in migraine, (Tietjen, 2009, Vanmolkot et al., 2008), and recently support by a small Italian empirical study (Napoli et al., 2009).

Genetic studies in common migraine

Several candidate genes have been tested for association with the risk of migraine in case-control studies, with the most cited example being the C677T variant of the methylenetetrahydrofolate reductase (*MTHFR*) gene (Kowa et al., 2000). This mutation is quite interesting as it has been reported to be associated with, in addition to migraine, acute lymphoblastoid leukemia (Wang et al., 2010), abdominal aortic aneurysms (McColgan et al., 2009), ischemic stroke (Bentley et al., 2010), breast cancer susceptibility (Zhang et al., 2010), reduction in the risk for colorectal cancer (Taioli et al., 2009), and a large number of other phenotypes. This variant has been thoroughly studied in migraine, including several studies with sufficient sample sizes (Lea et al., 2004, Scher et al., 2006, Todt et al., 2006b, Kaunisto et al., 2006, Schurks et al., 2008 and Rubino et al., 2009a). Two recent meta-analyses have reported very slight association between the C677T variant and migraine with aura (e.g. p=0.03 for association to migraine in Schurks et al.) (Rubino et al., 2009a, Schurks et al., 2009b), mainly due to the two largest studies to date showing negative results (Todt et al., 2006b) and (Kaunisto et al., 2006). A recent meta-analysis suggested that the association can only be detected in non-Caucasian samples (Schurks et al., 2009b). If this kind of stratification truly exists in the data, it could explain the large number of associations to this variant in candidate gene studies, where stratification is hard or impossible to detect (see Chapter 2).

Other genes with reported association to migraine include the dopamine beta-hydroxylase gene (*DBH*), for which two protective variants have been reported (*rs1611115* (Fernandez et al., 2009) and *rs2097629* (Todt et al., 2009)), the insulin receptor (*INSR)* (McCarthy et al., 2001, (Netzer et al., 2008), estrogen receptor 1 (*ESR1*) (Colson et al., 2004, (Oterino et al., 2008) and tumor necrosis factor alpha (*TNF-alpha*) (Yilmaz et al., 2010). However, the same problem as for *MTHFR* exists for these studies as well; sample sizes have generally been relatively small, and larger follow-ups, e.g. for *MTHFR* and *ESR1* (Kaunisto et al., 2006), have failed to replicate the findings. In addition, considerable publication bias is likely to be involved (personal communication with multiple groups) and would skew the meta-analysis results.

A number of targeted linkage scans in samples of one or few families preceded the genome-wide linkage scans. A targeted Australian study detected significant linkage to the 19p13 locus (Nyholt et al., 1998b). Another locus on 19p13, distinct from the FHM1 locus was reported in 2001 in a North American family (Jones et al., 2001). Two further Australian studies concentrated on the X chromosome, finding a locus (Nyholt et al., 1998a) and detecting significant association to a locus on Xq24-

Table 7. Reported genome-wide linkage scans in large migraine family samples, and significant loci reported according to the Lander-Kruglyak significance definition (Lander and Kruglyak, 1995).

Study	Nationality	Families	Significant loci	Ref
Wessman et al., 2002	Finnish	50	4q24	1
Cader et al., 2003	Canadian	42	11q24	2
Björnsson et al., 2003	Icelandic	103	4q21	3
Nyholt et al., 2005	Australian	*	5q21	4
Russo et al., 2005	Italian	10	15q11-q13	5
Lea et al., 2005	Australian	92		6
Anttila et al., 2006	Finnish	50	4q24, 17p13	7
Anttila et al., 2008	Finnish, Australian	210	10q22-q23	8
Ligthart et al., 2008	Dutch	105	-	9
Tikka-Kleemola et al., 2010	Finnish	36	9q31	10
Oedegaard et al., 2010	American	31	-	11

Footnote: References are 1 (Wessman et al., 2002), 2 (Cader et al., 2003), 3 (Björnsson et al., 2003), 4 (Nyholt et al., 2005), 5 (Russo et al., 2005), 6 (Lea et al., 2005), 7 (Anttila et al., 2006), 8 (Anttila et al., 2008), 9 (Ligthart et al., 2008), 10 (Tikka-Kleemola et al., 2010), 11 (Oedegaard et al., 2010). Study 4 marked with an asterisk used 790 sib pairs instead of families.

q28 (Nyholt et al., 2000). A 2002 Swedish genome-wide scan reported a locus on 6p12-p21 (Carlsson et al., 2002), as did an Italian study for a locus on 14q21-q22 (Soragna et al., 2003). The first genome-wide linkage scan in a large family sample was conducted in 2002 (Wessman et al., 2002). Table 7 lists the genome-wide linkage scans published in migraine so far.

A number of loci with genome-wide significant evidence of linkage have been reported, but despite a number of studies replications have been sparse (with notable exceptions 4q24, 10q23 and 18q12). More importantly, the linkage loci have not yielded any genetic variants for migraine, though a number of candidate genes within the best linkage regions have been studied for association. Examples of candidate gene studies based on the linkage information include the promising candidate genes such as the GABA gene cluster on 15q12 (Oswell et al., 2008) and *AQP4* on 18q12 (Rubino et al., 2009b). This dearth of results in a highly heritable disorder suggests possible heterogeneity in the phenotypes, which has served as an impetus to develop alternative phenotyping methods.

Alternate migraine phenotyping methods

The standard approach to migraine phenotyping involves the end diagnosis, formed on symptom traits based on a patient's description of attacks. In order for a headache to be considered migraine, it has to fulfill a number of symptom criteria, listed in the ICHD-II (International Headache Society, 2004). While this works well in clinical practice, given that many kinds of headache can fulfill the criteria it is likely that for research purposes this introduces heterogeneity to the end diagnosis. A related issue is the nature of the MA and MO diagnoses. An MA attack also needs to fulfill MO criteria for pain, which suggests it would make sense to analyze both groups together. The first genome-wide linkage screen (Wessman et al., 2002) attempted to combine MA and MO groups into a "general migraine" group, but all of the detected linkage signals were reduced. However, if the theory regarding the interrelatedness of MA and MO (as discussed in Chapter 4) is correct, the ability to combine samples from both groups could increase statistical power for future samples.

Another potential source of heterogeneity is the aura outlier group. When looking at the patient information in the Finnish pedigrees in detail, there is a clear outlier group which we refer to as "unclassified migraine with aura". These patients are in reality most likely aura patients, but their aura presents in a form not be recognized by the current IHS criteria. Examples of nonconforming aura are atypical visual symptoms, motor symptoms without accompanying sensory symptoms and aura of atypical length. This phenomenon has been noticed at the other International Headache Genetics Consortium sites as well (G. Terwindt, personal communication, U. Todt, personal communication, and T. Freilinger, personal communication). Given that this group does present with pain fulfilling the MO criteria, this group likely represents a source of heterogeneity within the MO diagnosis group.

For these reasons, alternate phenotyping methods in migraine have been proposed. The first of these was the latent class analysis by Nyholt et al. (Nyholt et al., 2004). The latent class analysis is a subset of structural equation modeling. It is used to find subtypes of related cases (i.e. latent classes) from multivariate categorical data in the absence of direct knowledge of class membership (Goodman, 1974). Is has been widely employed in disease epidemiology (Kaldor and Clayton, 1985) for diseases such as multiple sclerosis (Zwemmer et al., 2006) and rheumatoid arthritis (Schumacher and Kraft, 2007). The analysis provides a maximum likelihood estimate of the classes that fulfill the observed symptom distribution. Nyholt et al. (2004) estimated the number of latent classes in migraine to be four, with the first class lacking migraine (the healthy individuals in families), the second with mostly mild headache, and the two migraine groups denoted as CL2 and CL3 that roughly correspond to MO and MA, respectively. The use of these latent classes instead end diagnoses has repeatedly shown to improve migraine linkage signals (Anttila et al., 2008, Goodman, 1974, Nyholt et al., 2005).

Another alternate method, the trait component analysis, is discussed under Study I and II in the Results part of this thesis.

AIMS OF THE STUDY

The purpose of this thesis was to identify genetic loci and variants influencing the susceptibility to migraine, first within the larger context of the Finnish Migraine Genetics Project, which aims to study the clinical characteristics of migraine patients and identify migraine predisposing genes in the Finnish population. In later parts of the thesis, our scope was expanded to cover data from collaborators in the International Headache Genetics Consortium, a joint venture of migraine research groups from a number of countries. The main approaches used in this thesis were demonstrating linkage through a genome-wide linkage study and demonstrating SNP association through either a candidate gene or a genome-wide association study.

The specific aims of this thesis were:

1. To develop an alternative migraine phenotyping method to improve the statistical power for genetic studies
2. To apply the method developed in Aim 1 to identify genetic factors influencing susceptibility to migraine, specifically by studying the contribution of
    a. rare variants by means of genome-wide linkage scans
    b. common variants by a candidate gene approach and a genome-wide association study

STUDY DESIGN, SUBJECTS AND METHODOLOGY

Study design

To address Aim 1, each of the studies in this thesis employed a new phenotyping method, the trait component analysis, to the various datasets. For Aim 2a, Studies I-II used microsatellite marker data from three independent genome-wide linkage scans (two Finnish and one Australian) to investigate rare haplotypes affecting migraine susceptibility. For Aim 2b, Studies III-IV used SNP data from a number of multinational samples to investigate the role of common variants in migraine susceptibility. In addition, to satisfy Aim 1, in each study alternate phenotyping methods were used to further dissect the genetic background of migraine.

In Study I, we re-examined data genotyped for an earlier study (Wessman et al., 2002) of 438 Finnish subjects (296 with migraine) from 50 independent, multigenerational families (see Table 8) using the novel phenotyping approach of trait component analysis (TCA). Additional genotyping for finemapping was performed on the same set of subjects based on the initial results. The subjects were selected from a clinic-based patient collection of roughly 7,000 individuals and 1,400 families, based on the severity of their migraine symptoms. Selection favored the most disabling cases. In Study II, 454 Finnish migraine patients and 241 unaffected family members from 31 independent, multigenerational families were selected from the same collection. In addition, 269 Australian migraine patients and 387 unaffected family members within 152 independent nuclear families selected from two Australian twin cohorts were studied. Microsatellite markers were genotyped, and additional finemapping performed on the same samples based on initial results. To serve as a replication sample, an additional set of 192 migraine patients and 132 unaffected family members from 27 independent, multigenerational Finnish families were studied.

In Study III, a candidate gene hypothesis based on the channelopathy aspect of rare forms of migraine was used as the basis of a SNP association study, conducted in a Finnish study sample of 841 unrelated MA cases and 884 unrelated migraine-free controls were used for a candidate gene study of 155 ion transporter genes. In Study IV, the genome-wide association study approach was used to address the role of common genetic variants in migraine, through a genome-wide association analysis on 2,748 migraine with aura cases and 10,747 controls, followed by a replication in four populations with MA or MO patients.

Table 8. The numbers of families and samples genotyped in each study. Numbers in parentheses indicate the corresponding numbers in the replication set of the study.

	Families	Family members with migraine	Family members without migraine	Unrelated individuals with migraine	Control individuals
I	50	296	142	-	-
II	183 (27)	723 (192)	628 (132)	256	230
III	-	-	-	841 (2,835)	884 (2,740)
IV	-	-	-	2,748 (3,202)	10,747 (40,062)

Study subjects

The study designs of Study I and the parts of Studies II-IV relating to Finnish patients were approved by the Helsinki University Central Hospital Ethics Committee (approval #622/E0/02). The Queensland Institute for Medical Research Human Research Ethics Committee and the Australian Twin Registry approved the parts relating to Australian patients. Studies III and IVwere approved by (in addition to the previous) by the ethics committees of the University of Kiel, Univeristy of Cologne, Ludwig-Maximilians-Universität in Munich, Leiden University Medical Centre and the Danish Research Ethics Committee. Informed consent was obtained from all subjects.

i. Finland

Since 1992, a group of neurologists under Mikko Kallela and Markus Färkkilä (major participants of the group include Ville Artto, Hanna Harno, Hannele Havanka, Matti Ilmavirta, Salli Vepsäläinen, Markku Nissilä, Erkki Säkö and Marja-Liisa Sumelahti) have been collecting a Finnish migraine family database from headache clinics around Finland. Primary collection points were headache clinics in Helsinki, Turku, Jyväskylä, Tampere and Kemi. All participants were asked to fulfill the validated Finnish Migraine Specific Questionnaire for Family Studies ($FMSQ_{FS}$ (Kallela et al., 2001a)) and to provide a blood sample. The criterion for inclusion in the database was that at least three members of the family (defined as grandparents, parents, parents' siblings siblings and own children) have migraine. Based on the questionnaire responses, over 200 variables were recorded that included information on the IHS symptoms, typical attack features, age of onset, other diseases, place of birth, etc. Mikko Kallela diagnosed all patients based on the questionnaire data. A neurologist (Mikko Kallela, Markus Färkkilä or Ville Artto for the majority of patients) performed a physical examination of the index patient in each family and sometimes other family members as well. At the time of writing, the collection consists of over 7,000 blood samples and questionnaire responses from roughly 1,400 families.

ii. Australia

Subjects from two Australian twin cohorts were used in Study II: one of twins born 1902-1964 (Heath et al., 1997) and another of twins born 1964-1971 (Heath et al., 2001). IHS symptom data (Headache Classification Committee of the International Headache Society, 1988) was gathered using an extensive semi-structured telephone interview that included diagnostic questions for migraine.

iii. Germany

For studies III and IV, two sets of German migraine with aura subjects were studied. Patients in the first set were recruited at a headache center in Kiel for a patient collection maintained at the University of Cologne. All patients were diagnosed as having MA by experienced neurologists with a specialization in headache disorders, from either a face-to-face interview or a detailed telephone interview. Interviews were standardized by using a comprehensive migraine questionnaire (Todt et al., 2006a). The second set was recruited at the Department of Neurology at the Klinikum Großhadern of the Ludwig-Maximilians-Universität in Munich. All were diagnosed as MA patients in a face-to-face interview by an

experienced headache specialist, using a German translation of the Finnish $FMSQ_{FS}$ questionnaire (Kallela et al., 2001a), accompanied by a follow-up telephone interview when necessary.

iv. The Netherlands

The Dutch sample consisted of MA patients recruited through a web page or at an outpatient clinic at the Leiden University Medical Centre, and selected to take part in the clinic-based Leiden University Migraine Neuro Analysis (LUMINA) study. For the patients recruited through the web page, diagnoses were assigned based on data from an extended questionnaire, followed by a telephone interview. For the patients recruited through the outpatient clinic, diagnoses were assigned directly by a physician experienced in diagnosing migraine patients.

v. Iceland

The majority of individuals suffering from migraine headache were recruited based on responses to a screening questionnaire mailed to a random sample of 20,000 residents of the Reykjavik area aged 18–50 years. Additional patients were recruited through a list of patients provided by two neurologists or through responses to an advertisement in the Icelandic Migraine Society newsletter. All recruits were asked to answer the DMQ2 or DMQ3 questionnaires (Kirchmann et al., 2006). A validation follow-up was conducted by an experienced physician.

vi. Denmark

A computer search of the Danish National Patient Register of all hospitalized patients in Denmark was used as the basis for recruiting MA patients with a family history of MA. A screening telephone interview was conducted for all eligible patients. If a patient was confirmed to suffer from MA in the screening interview, any relatives with migraine-like headache were also diagnosed according IHS criteria (International Headache Society, 2004) in an extensive validated semi-structured telephone interview, performed by trained physicians.

Control samples

For Studies I and II, unaffected individuals (coded as having “unknown” phenotype due to the chosen linkage analysis approach; see below) from each family were used to estimate founder haplotype frequencies. For the discovery cohort of Study III, 884 unrelated control samples with no personal or family history of migraine were obtained from the Finnish Twin Cohort, based on age- and sex-matching to the cases in that study. For the replication phase of Study III, matched controls were obtained at each study site from individuals participating in other studies who screened negative for migraine. For the initial phase of Study IV, population-matched controls were obtained from the Finnish Health2000 study, the Finnish Helsinki Birth Cohort study (Barker et al., 2005), the German KORA S4/F4 study (Wichmann et al., 2005), the German Heinz-Nixdorf Recall study (Schmermund et al., 2002) , the German PopGen cohort (Krawczak et al., 2006), the Illumina iControlDB database (consisting of caucasians matched to the other German controls), and the Dutch Rotterdam Study I (Hofman et al., 2007). For the replication phase, additional controls were obtained from deCODE Genetics to represent Icelandic and Danish controls, and from the Max Planck Institute study (Heck et al., 2009) and the GlaxoSmithKline study (Muglia et al., 2008) for German controls. For an overview of the control samples used for each population, see Table 9.

Table 9. Methods used to obtain diagnostic information in each study sample of Studies I-IV. Asterisk indicates that for the German study samples, Munich and Cologne samples were pooled and analyzed against the same control set.

Study	Sample set	Sample nationality	Recruitment location	Recruitment method	Collection method Primary	Collection method Secondary	Migraine patients	% with MA	Controls
I	Finnish MA #1	Finnish	Helsinki	Tertiary clinic	D.v.q.	T.f., p.e.	296	83 %	-
II	Finnish MA #2	Finnish	Helsinki	Tertiary clinic	D.v.q.	T.f., p.e.	368	81 %	-
	Australian twins	Australian	Brisbane	Population-based	B.q.	-	269	71 %	-
	Finnish MA repl.	Finnish	Helsinki	Tertiary clinic	D.v.q.	T.f., p.e.	155	81 %	-
III	Finnish c-c	Finnish	Helsinki	Tertiary clinic	D.v.q.	T.f., p.e.	841	100 %	884
	Leiden repl.	Dutch	Leiden	Pre-existing cohort	D.q.	T.i.	800	34 %	946
	Cologne repl.	German	Cologne	Tertiary clinic	T.i.	D.q.	601	100 %	651
	Munich repl.	German	Munich	Tertiary clinic	P.e.	D.v.q.	288	100 %	*
	Brisbane repl.	Australian	Brisbane	Population-based	B.q.	-	1,146	85 %	1,143
IV	Finnish GWA	Finnish	Helsinki	Tertiary clinic	D.v.q.	T.f., p.e.	1,064	100 %	3,513
	Cologne GWA	German	Kiel	Tertiary clinic	T.i.	D.q.	994	100 %	2,317
	Munich GWA	German	Munich	Tertiary clinic	P.e.	D.v.q.	282	100 %	*
	Leiden GWA	Dutch	Leiden	Web or tertiary clinic	D.q.	T.f.	879	100 %	4,917
	Icelandic repl.	Icelandic	Reykjavik	Population cohort	D.v.q.	-	900	37 %	35,221
	Danish repl.	Danish	Glostrup	Registry search	R.s.	T.f.	1,116	70 %	1,342
	Dutch repl.	Dutch	Leiden	Web or tertiary clinic	D.q.	T.f.	349	100 %	2,082
	Munich repl.	German	Munich	Tertiary clinic	P.e.	D.v.q.	837	0 %	1,406

Footnote: b.q. – brief questionnaire, c-c – case-control, GWA – genome-wide association study, MA – migraine with aura, p.e. – physical examination, d.q. – detailed questionnaire, d.v.q – detailed, validated questionnaire, repl. – replication, r.s. – registry search, t.f. – telephone follow-up, t.i. – telephone interview.

Phenotyping methodology

In each study sample, diagnostic questions regarding various headache features were asked based on ICHD-I (International Headache Society, 1988) or ICHD-II (International Headache Society, 2004) as detailed in Table 9. Answers to these questions and a number of additional questions were used to determine MA or MO diagnosis in accordance to the IHS criteria (see Chapter 3). In the case of the Australian questionnaire, a single question regarding the presence of visual aura was used to determine the difference between MA and MO. In cases where the diagnosis assignment was ambivalent due to insufficient or conflicting information, telephone follow-ups were conducted to ascertain the correct diagnosis. For linkage analysis in Studies I and II, the direct answers to the diagnostic questions were used as additional phenotypes in TCA and for Study II statistically estimated latent classes (based on previous empirical estimation for models of symptom profiles) were used in LCA.

Genotyping methods

a. DNA extraction

DNA was extracted from peripheral blood lymphocytes from patient blood samples using standard methods.

b. Microsatellite genotyping

For Study I, we analyzed microsatellite data generated previously at UCLA (Wessman et al., 2002). In that study, 350 polymorphic microsatellite markers covering the autosomes and the X chromosome from the Human MapPairs Genome-Wide Screening Set (Broman et al., 1998) were genotyped. Extracted DNA was amplified by multiplex PCR assays with fluorescent primers designed to detect the microsatellite loci. Pipetting of the reactions was performed by a Hydra Microdispenser (Robbins Scientific). The amplification reactions were run in microtiter 96-well plates, by Tetrad thermal cyclers (MJ Research). The resulting PCR fragments, along with a size-standard ladder and fragments from known control individuals from Fondation Jean Dausset CEPH, were separated on 6% acrylamide gels by electrophoresis using a LI-COR DNA 4200 Genetic Analyzer (LI-COR). The bands on the gels were automatically interpreted into genotypes by the Saga1.0 software package (University of Washington and LI-COR). Allele sizes were standardized to those of CEPH control individuals. Markers that failed genotyping were replaced by microsatellite markers from the Genome Database and from the Marshfield genome database. All genotypes were verified by human inspection. In the finemapping phase, 14 additional finemapping markers were genotyped. Capillary electrophoresis, as employed by the MegaBACE 1000 DNA Sequencing System (GE Healthcare Bio-Sciences), was used to separate DNA fragments. Alleles were called by the MegaBACE Genetic Profiler 1.5 software (GE Healthcare Bio-Sciences).

For Study II, 387 markers were genotyped at the Finnish Genome Center, using standard methods either on the ABI or the MegaBACE genotyping systems. Genotyping was based on the LMS-MD10 microsatellite marker set (Applied Biosystems, Foster City, CA, USA). For the ABI system, genotyping was performed with the ABI 3730 capillary sequencing instrument, and PCR products were resolved with the ABI 3730 data collection software and sized with the

Genemapper software package from Applied Biosystems. For the MegaBACE system, capillary electrophoresis employed by the MegaBACE 1000 DNA Sequencing System (GE Healthcare Bio-Sciences, Piscataway, NJ, USA), was used for separating DNA fragments. Alleles for this system were called by the MegaBACE Genetic Profiler 1.5 software.

c. Illumina Golden Gate assay

In Study II, a custom-made Illumina Golden Gate assay (Illumina, San Diego, CA, USA) was used to genotype 1,536 SNPs at the Broad Institute in Boston, MA, USA. SNPS were chosen from within the region identified in the previous parts of the study (chr10, 78.233–88.884 Mb, NCBI build 35). The tagSNPs on chromosome 10 were selected using Haploview's Tagger option with the CEU population in the HapMap SNP set as the reference population. (v21). All tagSNPs had minor allele frequencies >10% and r^2 thresholds of >0.8. The Illumina BeadStudio software version 3.1.0.0 (Illumina) was used for calling SNP genotypes. Quality control cut-offs were >97% for sample-specific call rates and >95% for SNP-specific call rates. In total, 1,323 SNPs passed quality control.

d. Perlegen SNP genotyping

In Study III, a custom-made ion-transport SNP array was designed by Perlegen Sciences Inc. (Mountain View, CA, USA) based on a SNP list provided by us. Our list contained 5,975 tagSNPs with MAF >10% that targeted 155 ion channel genes. The array was manufactured by Affymetrix Inc. (Santa Clara, CA, USA) and quality control was performed by Perlegen.

e. Illumina HumanHap GWA assays

Genome-wide association (GWA) genotyping for Study IV was performed at the Wellcome Trust Sanger Institute using either the Illumina HumanHap 610k or 550k SNP microarrays following the manufacturer's Infinium II protocol (Illumina Inc.). Automated genotype calling was performed using the Illuminus software (Teo et al., 2007), which was followed by manual inspection of cluster quality for any SNP with a p-value $<1x10^{-4}$. Strict quality control criteria (>95% for sample-specific call rate, >97% for SNP-specific call rate, Hardy-Weinberg equilibrium p-value $>10^{-6}$ and >1% MAF) were applied. To identify contaminations and other error sources, overall sample heterozygosity proportions were calculated and outliers eliminated. To exclude related individuals a strict pi-hat (measure of IBD proportion, defined as [proportion of SNPs at IBD=2] + [proportion of SNPs at IBD=1]/2) cut-off of 12.5% was used. Due to the combined analysis in three populations, each SNP was required to have been successfully genotyped in each of the three study samples. In total, 429,912 markers passed all quality control criteria.

f. eQTL study

For Study IV, an eQTL study was performed on 75 samples of umbilical tissue (primary fibroblasts, lymphoblastoid cell lines and primary T-cells) obtained from the GenCord resource. Umbilical cord was chosen because it is readily available and allows the acquisition of multiple cell types for each individual. Sample collection was performed systematically on full term or near full term pregnancies to ensure homogeneity for sample age. Umbilical cords were collected from 75 newborns of Western European origin born at the maternity ward of the

University of Geneva Hospital and from each umbilical cord three cell types were derived: 1) primary fibroblasts, 2) LCLs and 3) primary T-cells (for detailed Methods see Dimas et al. 2009). Total RNA was extracted from these three cell types and 1.5 μg of cRNA was hybridized to Illumina's WG-6 v3 Expression BeadChip array to quantify transcript abundance. Intensity values were log2 transformed and normalized independently for each cell type using quantile normalization for sample replicates, and median normalization across all individuals. Each cell type was renormalized using the mean of the medians of each cell type expression values. For the genotypes of the expression study, DNA samples were extracted from umbilical cord tissue LCLs with the Puregene cell kit (Gentra-Qiagen, Venlo, the Netherlands) and genotyping was performed using the Illumina 550K SNP array (Illumina Inc., San Diego, USA).

Statistical methods

a. Two-point linkage analysis of microsatellite

In Study I, two-point linkage analysis of microsatellite markers were employed using an affecteds-only strategy (i.e., all individuals not classified as affected were considered to have an ''unknown'' phenotype) to allow for reduced penetrance, lack of environmental exposure, etc. In Study I, parametric and nonparametric LOD scores were calculated for the 350 markers genotyped both under locus homogeneity and under locus heterogeneity using the AUTOGSCAN (Hiekkalinna et al., 2005) program, which employs the LINKAGE (Lathrop and Lalouel, 1984) and HOMOG (Ott, 1983) analysis softwares. In parametric analysis a dominant mode of inheritance and locus heterogeneity was assumed. Each of the IHS traits and trait groups were analyzed, in turn, as the phenotype. All analyses were performed with the disease-gene frequency set at 0.001 under the assumption of autosomal dominant inheritance and a phenocopy proportion of 2.4%, which reflects the frequency of MA in the population. The strategy is in accordance with Göring and Terwilliger (Goring and Terwilliger, 2000) and in line with our previous research (Wessman et al., 2002).

b. Multi-point linkage analysis for microsatellite markers

For Study I, Multipoint parametric and nonparametric analyses were performed for regions showing evidence of linkage in the parametric two-point analysis by use of the program GeneHunter, version 2.1_r5beta (Kruglyak et al., 1996a). Parametric linkage analysis was performed using the model presented under two-point linkage analysis, while allowing for locus heterogeneity. For Study II, we used the nonparametric MERLIN NPLpairs Z-score statistic (Weeks and Lange, 1988) to test for increased allele sharing among affected individuals (Kruglyak et al., 1996a). To avoid biasing our results on possible overrepresented rare variants in a few large families, we also analyzed the Finnish genome-wide sample as a set of nuclear families. For consistency with the previous Australian genome-wide linkage scan and in order to use the information from unaffected individuals, we used a nonparametric quantitative trait linkage (NPL_{qtl} Z-score) statistic for the analyses of the Australian families in order to obtain additional linkage information from unaffected individuals. In this analysis, affected individuals were coded as "1", unaffected individuals were coded as "0", and those with missing phenotypes were coded as "x". Multipoint nonparametric linkage analysis was performed with the MERLIN computer program. The MERLIN NPL_{pairs} and

NPL_{qtl} Z-score statistics were implemented in the general Whittemore and Halpern framework (Whittemore and Halpern, 1994). Based on the Z-scores, a likelihood ratio test for linkage performed by MERLIN was used to estimate the LOD score statistic using the exponential modeling procedure of Kong and Cox (Kong and Cox, 1997).

c. SNP association analyses

In Study III, single marker basic allelic association tests were performed using PLINK v1.00 (Purcell et al., 2007). Significance of the test results was evaluated through the max(T) permutation procedure in PLINK.

In Study IV, the primary analysis method was the assessment of genotype differences for genome-wide SNP data between cases and controls using two-tailed Cochran-Mantel-Haenszel (CMH) test for 2x2xK stratified data (K = 3), as implemented in PLINK software version 1.06 (Purcell et al., 2007), with a nominal variable used to code for population identity. For population-specific results, the CMH test was used with K=1. For the meta-analysis of the top marker, CMH test with K=7 was implemented in manner similar to the primary analysis, but for a single marker. Significance of the test results was evaluated by comparing them to the generally accepted 5×10^{-8} (Altshuler et al., 2008), and a genomic inflation factor (estimated from median of all χ^2 values) was calculated to verify correct case-control set matching. To evaluate statistical power of the SNP association analyses, the limiting minor allele frequencies necessary for 80% and 50% statistical power for various odds ratios (OR) can be seen from Figure 14. For these calculations, a multiplicative model and a disease prevalence of 15% were assumed. For each study, the study-specific significance limit was used (3.8×10^{-5} for Study II, 1.9×10^{-5} for Study III, and 5.0×10^{-8} for Study IV).

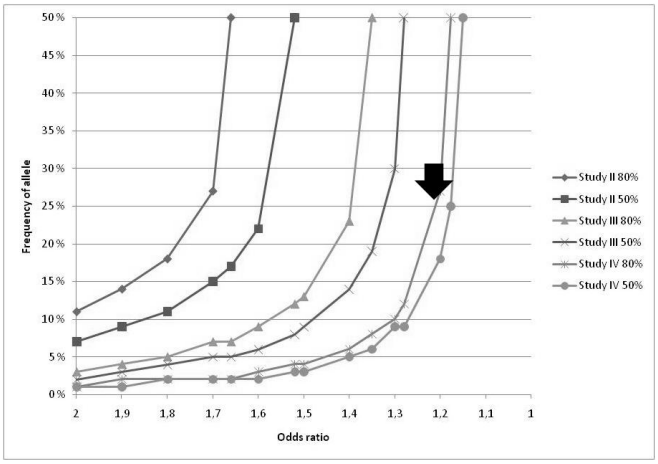

Figure 14. The limits of statistical power for the SNP association analyses in Studies II-IV. The black arrow indicates the position of the variant identified in Study IV.

d. Additional quality control measures for GWA data

i. Elimination of population stratification

To eliminate population outlier samples based on SNP data pattern analysis, IBS distances for each sample pair in the same population were estimated using the "genome" option of PLINK software version 1.06 (Purcell et al., 2007). The resulting IBS distance matrix data was

subjected to multidimensional scaling analysis, which identifies the eigenvectors within the data in decreasing order of magnitude. In accordance with standard practice, the first 20 eigenvector dimensions were examined for each population sample, and outlier samples based on a visual examination were excluded from the analysis.

ii. Verification of case-control matching
To ascertain that the control sets properly matched the case sets and to check that the population stratification analysis had worked properly, genomic inflation factors were estimated from the association analysis results. The inflation factor was calculated by finding the median χ^2 value from a 1-d.f. allelic χ^2 test. For each of the study populations, the median value was less than 1.1, generally accepted as sufficient matching.

e. eQTL study

The expression level values from the Illumina expression array for each individual were analysed for correlation with the common variant genotypes of the same individual using the Spearman rank correlation test. The same method was used in a previous study in 2007 by Stranger et al., which identified around 1,500 genes with either *trans*- or *cis*-acting association with a common sequence variant (Stranger et al., 2007).

f. Transcription binding site analysis

Known human transcription factor binding site motifs (456 in total) were extracted from the TRANSFAC 12.2 database (Matys et al., 2006). Sequence region +/- 1400 bases from *rs1835740* was retrieved from the human NCBI37 genome assembly using the Ensembl API (Hubbard et al., 2009). The positions retrieved were chr8:98,165,513-98,168,313. Transcription factor motif hits within the retrieved sequence were computed using Nmscan software, distributed as part of the NestedMICA suite (Down and Hubbard, 2005). The sequence background model required for significance cut-off calculation was trained on 1,000 randomly chosen human intergenic sequences (protein coding sequence was excluded). A bootstrapping procedure was used to assess the significance of the maximum bit scores of the motifs within the region of interest: the nucleotide sequence was shuffled 10,000 times retaining the nucleotide content, and scored against the same binding site motifs, each time recording the maximum bit score hit, and matches that were found to be below expectation of 0.01 were retained. After scanning the genomic region close to *rs1835740*, we retrieved the 31-way Eutherian mammal multiple alignment from 98,166,803 bp to 98,166,938 bp using Ensembl Compara to display the nearest statistically significant matches found for the motif scanning.

RESULTS AND DISCUSSION

1. Introduction of an Alternative Phenotyping Method, the Trait Component Analysis, for Family-based Linkage Studies in Migraine

The key motivation for the studies in this thesis was to find a better method of studying the genetics of migraine, which would improve the understanding of the condition's causative pathophysiology – largely unknown at the start of the thesis. Factors, such as only having a descriptive diagnosis instead of quantifiable laboratory markers create innate difficulties for the study of neuropsychiatric conditions. Migraine is not exempt of these difficulties. Due to the lack of biomarkers, the migraine diagnosis is based on particular diagnostic questions (the IHS diagnostic criteria; see Chapter 3 for more details), which represent the current idea of the condition's underlying pathophysiology. However, given our limited knowledge, the diagnostic method is not necessarily a refrelction of the true biology, which is an additional confounding factor for downstream analyses.

To try to address some of this phenotypic heterogeneity, more quantifiable phenotypes were explored. A new phenotyping approach called the trait component analysis (TCA) was developed in a bid to increase the statistical power of genetic studies. This approach was made possible by the introduction of the 2nd edition of the criteria in 2004 (International Headache Society, 2004), which for the first time outlines the attack symptoms of both migraine with (MA) and without aura (MO) in a similar manner. This consistency allowed us to consider a better way of dealing with the common situation among Finnish migraine family pedigrees of having both MA and MO cases. Finnish migraine families were gathered following the ascertainment of a MA index case and MO is often seen in the same pedigrees. Furthermore, we often observed situations where the parents of the index case both had MO or where a grandparent's MA would manifest itself again in a grandchild while the parent between the two suffers from MO. Initial attempts to consider both MA and MO as affected at the same time led to a loss of known linkage signals (Wessman et al., 2002), suggesting that some stratification was necessary.

In practice, TCA forms multiple phenotypes directly from the diagnostic questions of migraine. TCA studies aim to study the diagnostic questions' biological relevance in addition to finding new genetic loci relating to migraine. Instead of the traditional diagnosis approach, TCA directly uses patient responses as phenotypes. In this way the usual process of combining questions' answers to determine a migraine diagnosis is bypassed.Instead of considering, for instance, the presence of two vascular symptoms (pulsation, pain intensity, unilaterality and aggravation by physical exercise or prevention of normal activity) as "migraine", patients with any given vascular symptom, like pulsating pain, are considered to be a group by themselves. According to the IHS criteria, any combination of two or more of those four symptoms leads the same outcome, which leads to considerable heterogeneity in the group. For example, diffuse pain of moderate intensity covering the whole head that prevents working is considered the same as unbearably intense pulsating pain on one temple that does not prevent one from working. In TCA, each person gets one

phenotype value for each symptom, and is analysed several times (once per phenotype). The extra burden of proof due to the increased number of tests is corrected for by having a higher significance limit that is determined by the number of tests. Another important feature of TCA is that it allows the study of the two main forms of migraine, MA and MO, in the same phenotypic context. In addition to the reported co-segregation of the two diseases discussed in Chapter 3, we have also made the empirical observation in the families we have studied that a parent with either migraine diagnosis often has a child with the other type of migraine (i.e. if a parent has MA, the offspring has MO, or vice versa). A diagnosis-based linkage analysis would consider such case as negative transmission, and thus the offspring's diagnosis as a sporadic occurrence. However, if the parent and child share similar symptoms, such as pulsating pain, TCA would acknowledge the successful transmission. Being able to account for these situations increases the number of relevant meioses considered in the analysis, which results in higher detection power.

In Study I, we re-analyzed data from a 2002 genome-wide linkage scan of 50 migraine with aura families (Wessman et al., 2002) using the trait component approach. In a two-point linkage analysis assuming locus heterogeneity, significant evidence of linkage was found with two loci: *D17S945* (17p13, HLOD score 4.65) and *D4S1647* (4q24, HLOD score 4.53). The locus on 4q24 had been previously identified in 2002 (Wessman et al., 2002), but the locus on 17p13 had previoulsy only shown nominal evidence of linkage in the same study. A further six markers showed suggestive evidence of linkage (HLOD score > 2.60, the limit of suggestive evidence after correction for the number of phenotypes tested): *D4S2380* (4q22), *D4S2394* (4q28), *D4S1520* (4q31), *D18S877* (18q12), *D18S862* (18q21), *D18S1364* (18q22). The suggestive markers defines two new loci: 4q28-q31 and 18q12. Ther latter, 18q12, replicates a loci previously identified in the Icelandic population (Björnsson et al., 2003).

1.a. Improved linkage to the previously detected locus on 4q24

The first point of comparison between the diagnosis-based and TCA approaches is the primary finding of the original 2002 study, the locus on 4q24. The original study used the presence of migraine aura as the main phenotype. Comparing the linkage results (Figure 15) based on migraine aura (the MA diagnosis) and migraine pain (the MO diagnosis) shows that the 4q24 locus is linked to the former, which suggests that the IHS symptom combination does not have sufficient resolution at this locus in these samples. However, comparison to the results of the TCA analysis (Figure 16) suggests that the inheritance may be more complicated than this; the main peak at 105 cM is identified by most of the traits and is improved by some. The LOD score for marker *D4S1647* using TCA was 4.53 (p-value 2.47 x 10^{-6}) and was previously at 4.20 (p-value 5.46 x 10^{-6} for MA). More importantly, clearer results are observed in the areas surrounding the peak. For example, TCA shows a much stronger signal at the previously nominal peak at 141 cM (4q28-q31), the LOD score increased from 1.55 to 2.99.

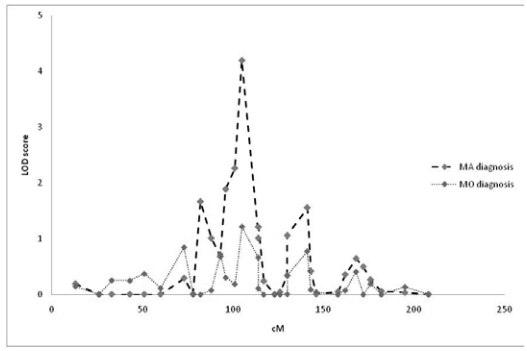

Figure 15. Two-point linkage analysis results from chromosome 4 data in Study I using MA and MO diagnoses. Dotted lines connecting the points are for illustration purposes. V. Anttila, unpublished data.

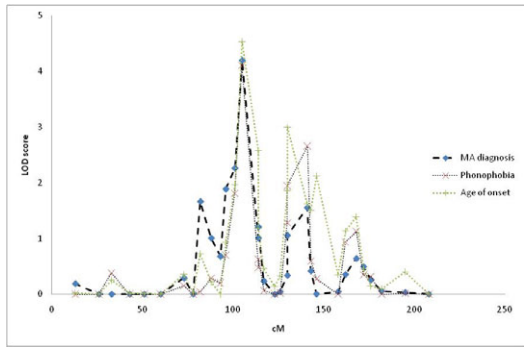

Figure 16. Two-point linkage analysis results from chromosome 4 data in Study I, comparing the results from the MA diagnosis, phonophobia trait phenotype, and the age of onset <20. Dotted lines between points are for illustration purposes. V. Anttila, unpublished data.

The consistency in detecting this locus was considered a good indicator for the reliability of TCA and of the locus itself. The linkage of many of the migraine traits to this locus suggests a robust link between this locus and migraine susceptibility. This locus was the first locus to show significant evidence of linkage to migraine in a large multigenerational family sample (Wessman et al., 2002) and was also the first one to be replicated (Björnsson et al., 2003). A 2004 study in schizophrenia (Paunio et al., 2004) used a similar endophenotype-based approach. The study utilized diagnostic features as quantitative traits (such as measures scoring verbal memory and visual working memory) and showed improved linkage scores and new loci in comparison to the end diagnosis approach. Interestingly, several traits (such as measures of executive function, delayed memory and verbal learning) showed evidence of linkage to a locus on 4q24 that overlapped and encompassed the migraine peak. Recently, a study in

families with both bipolar disorder and migraine (Oedegaard et al., 2010) (comorbid disorders, as discussed in Chapter 4) showed suggestive evidence of linkage to 4q24 that even centred on the same marker found in the 2002 Wessman et al. study and Study I. Given that this signal is stronger when concentrating on the migraine diagnosis only, limited conclusions regarding the role of this locus in the pathophysiology of bipolar disorder can be drawn. However, the presence of a moderately strong signal in families where migraine and bipolar disorder are comorbid, combined with the close phenotypic relationship between schizophrenia and bipolar disorder, suggests that this locus may indeed play a key role in processes behind the pathogenesis of several brain disorders.

1.b. A new locus on 17p13

The primary finding in Study I was the identification of the new 17p13 locus (though this locus has shown nominal evidence in a previous Australian scan (Lea et al., 2005)). This locus had shown nominal evidence of linkage in the original 2002 study (see Figure 17). Significant evidence of linkage was found to marker *D17S945* (LOD score under locus homogeneity 4.65, p-value = 1.85×10^{-6}, LOD score under locus heterogeneity 4.65, p-value = 1.1×10^{-5}, multipoint LOD score 3.94 at 22.0 cM), with an ASP LOD score of only 0.82 (p-value 0.026), suggesting a dominant effect at this locus. A number of adjacent markers also showed evidence of linkage, though the signal was clearly concentrated around marker *D17S945*. Multipoint analysis placed the top signals at 19.4 cM (GeneHunter parametric analysis (Kruglyak et al., 1996a)) and 22.0 cM (GeneHunter nonparametric analysis).

It is quite intriguing that the use of a trait that still correlates with the diagnosis itself illuminates a new locus. Using the MA diagnosis as phenotype finds only a relatively

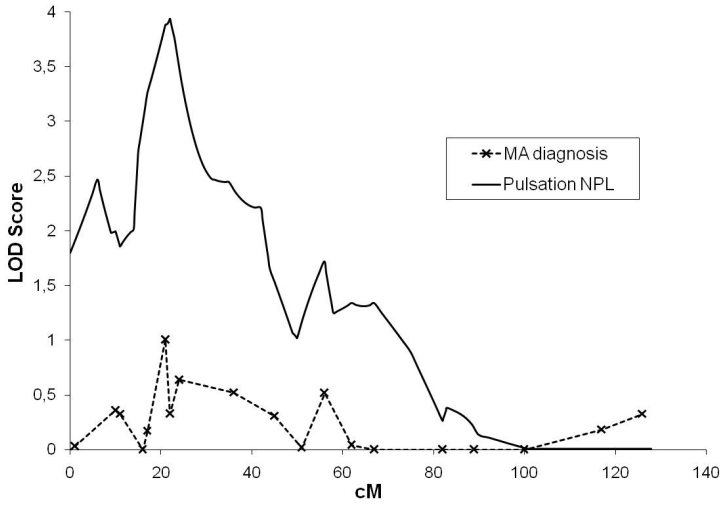

Figure 17. Chromosome 17 linkage results, showing the multipoint linkage analysis results using the pulsation phenotype, and the two-point linkage results from Wessman et al. 2002 using MA end diagnosis for comparison. Dotted line connecting MA results is for illustration purposes. NPL – non-parametric linkage.

low evidence of linkage (LOD score 1.01, p-value 0.016). There is a considerable difference between the individuals in each group: 51 (out of 252) of the individuals considered as affected in the original 2002 study were now coded as unknown (i.e. they are MA patients without the pulsating pain phenotype), and 66 (out of 235) individuals previously considered unknown were now coded as affected (i.e. they are MO patients with the pulsating pain phenotype). In total, these two groups together represent almost 50% of the affected individuals in the study sample, so the considerable change in LOD score (from nominal to significant) is plausible in that sense. A family-specific analysis showed that roughly a third of the families in the study showed a high degree of linkage to this locus, suggesting that this signal may be due to a relatively high-impact variant of either lower penetrance or lower allele frequency. Another possible explanation is that pulsation is a useful measure in distinguishing and separating some underlying subtype of migraine out of the whole spectrum of the disorder. For example, if sporadic forms of migraine were to have a lower incidence of pulsating pain due to missing or having some pathophysiological component associated with the pulsating sensation, this could explain the behavior of the linkage signal at the 17p13 locus.

1.c. Additional new loci detected

In addition to the 17p13 locus, two loci with suggestive evidence of linkage were detected. On 18q12, a previously identified locus in the 2002 scan as well as in the Icelandic population (Björnsson et al., 2003) was detected with nearly every analyzed trait. The traits showing linkage to this locus are those more clearly associated with the headache component of migraine, especially since the top associating traits is, with the highest score found with the strict application of the IHS criteria for MO, and especially the “vascular” criteria of pain (pain intensity, unilaterality, aggravation, and pulsation). Considering the phenotypes as well as the high ASP scores at this locus that suggest a recessive effect, it is not surprising that this locus displayed only nominal evidence of linkage in the original 2002 MA scan. However, if the underlying genetic locus is recessive in effect, the sample size needed to better describe it is probably considerably higher.

On 4q28-q31 a third new locus was detected by various traits, with the age of onset under 20 years showing the strongest evidence of linkage. We believed that limiting the age of onset of migraine might allow us to concentrate on the most severe migraine cases by biasing the case distribution in favor of the genetic cases and away from the sporadic cases that make up the migraine spectrum. Our success in doing so is a matter for debate, but the strong increase in linkage signal observed at this locus suggests that this approach would be useful in future studies. However, more work is required to validate this method of alternate migraine phenotyping.

1.d. Conclusions

The somewhat unexpected results of this study, such as the large significance gains for the 17p13 locus, suggest that the trait component analysis is useful in uncovering previously hidden factors determining migraine susceptibility. While the approach in this study was used in a largely hypothesis-generating fashion, the results raise several intriguing possibilities: (1) the presence of subgroups with the two diagnosis groups that are genetically considerably more homogeneous, or conversely a more heterogeneous group with unusual symptom groupings; (2) a potentially useful role

for pulsation as a key trait in distinguishing cases with a higher genetic load; and (3) the existence of several new loci affecting migraine susceptibility.

The trait component analysis (TCA) is based on the idea that individual clinical symptoms might provide a better stratification method than latent classes or end diagnoses. It is important to note that all three of the main loci detected by TCA are also clearly present in the diagnosis-based results. However, only nominal evidence of linkage is observed using the diagnoses and the considerable improvement of the detected signals strongly suggests two possible explanations: (1) the existence of some confounding subgroup present in the diagnosis phenotype analyses and not in TCA; or (2) the individual traits better reflect the underlying biology of these loci. Therefore, based on these successful results the approach was applied as the primary analysis method in Study II, the subsequent Finnish migraine scan.

2. Genome-wide Linkage Scan Using Multiple Populations

In Study II, we collaborated with a migraine research group from the Queensland Institute for Medical Research in Brisbane, Australia to analyze 210 families consisting of 1,675 individuals suffering from MA and MO. The primary study sample consisted of two independent scans from Finland (58 multigenerational families) and Australia (125 nuclear families). The replication set came from Finland (27 multigenerational families that were not related to the primary Finnish study sample). The purpose of this study was to expand our efforts in alternate migraine phenotyping and to attempt the replication of a locus in a population different from that of the initial discovery sample. Replication of a locus in two populations as genetically diverse as the Finns and Australians would provide strong evidence for a shared migraine pathway or mechanism. In this study, we applied the three different migraine phenotyping methods used at the time: the end diagnosis, LCA, and TCA. The high number of families and individuals in this study gave us the opportunity to make a good comparison of the different phenotyping methods. Significant evidence of linkage was observed at a locus on chromosome 10q22-q23 (LOD score 5.50 in Finns, 3.50 in Australians, 2.41 in an independent Finnish replication study). In addition, four previously reported loci - 8q21 from (Nyholt et al., 2005), 14q21 (Soragna et al., 2003) as well as 18q12 and Xp21 (Wessman et al., 2002) were replicated successfully.

2.a. Robust detection of a new locus on 10q22-q23

The primary finding in this study was the identification and replication of the 10q22-q23 locus in all three study samples using multiple markers. In both the Finnish and Australian primary scans, significant evidence of linkage was observed with markers around 100 cM on chromosome 10. The 95% confidence intervals placed the top of the peaks between 99-114 cM among Finns and 94-115 cM among Australians with the highest multipoint LOD scores of 4.91 and 3.42, respectively. An overlapping signal was also observed in the replication sample, which had only been genotyped for markers finemapping the 10q22-q23 locus by applying the Lander-Kruglyak replication threshold of 1.8 from (Lander et al., 1995). A combined analysis of the Finnish and Australian scans using the pulsation phenotype resulted in a multipoint LOD score of 5.24 at 102 cM. A comparison between the different phenotyping methods in the combined study sample showed that TCA performed consistently better at detecting this locus than either of the other methods (the highest LOD score was 5.24 with TCA, 3.37 with LCA and 1.89 with diagnosis-based analysis). The difference was especially apparent in the Australian sample, in which only nominal evidence of linkage was observed using the other phenotyping methods. Interestingly, comparison with the results from the previous Finnish and Australian scans showed that this locus has been previously observed. However,only by using TCA has this locus been revealed in the Finns of the 2002 study by Wessman et al. (see Figure 18); not even nominal evidence of linkage was observed using the MA diagnosis-based analysis. At the time, this was the first locus in migraine for which multiple replications had been observed, including the replication of a locus in the same population as the original finding.

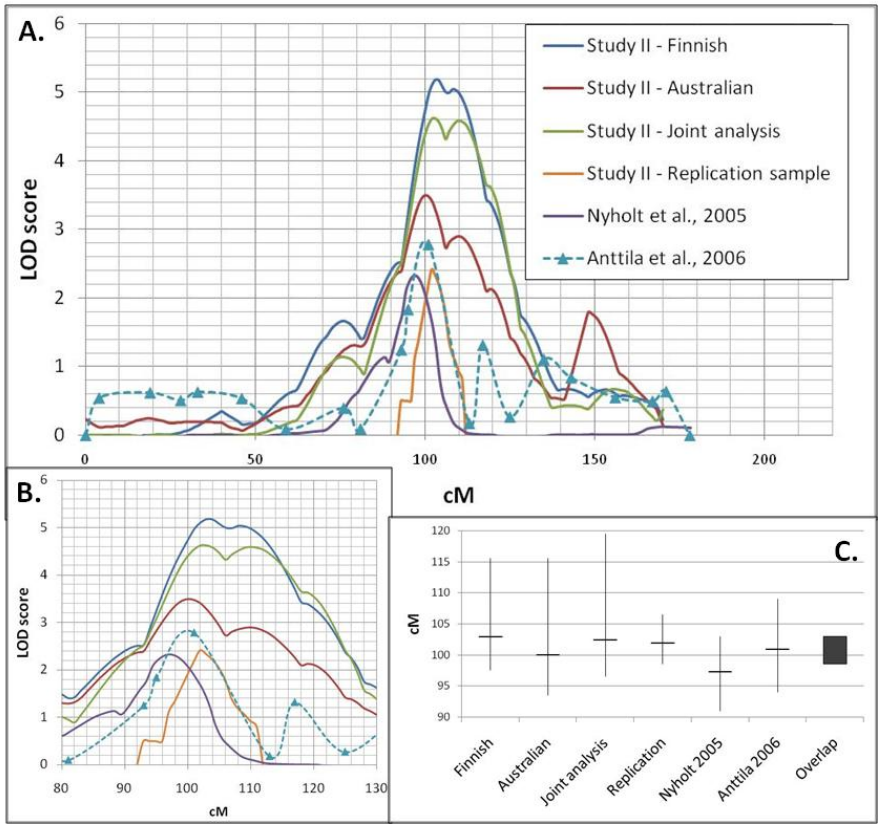

Figure 18. Linkage analysis results at the 10q22-q23 locus. A. Non-parametric multipoint linkage analysis results across chromosome 10 from Study II, combined with results from a previous study by Nyholt et al. (2005), and two-point results from Study I. B. Close-up of the 10q22-q23 peak, between 80 cM and 130 cM. C. Comparison of the localization of the top linkage signal from each study. The horizontal line in each case indicates the location of the highest linkage signal in that study sample, and the vertical line indicates its 95% confidence interval. Final category shows the overlap between the studies.

Further improvement on the linkage signal was observed in a female-only analysis, which had been employed in a previous study (Björnsson et al., 2003). In the Finnish sample, highly significant evidence of linkage was observed this way (HLOD score 7.68, p-value 1.68×10^{-9}). This finding suggests either that there is a higher degree of heterogeneity among males at this locus, or that some female-specific (for example, a hormone-related) mechanism underlies the signal at this locus.

2.b. No association to common SNPs targeting 10q22-q23

Given the significance of the linkage signal at this locus, a SNP-based follow-up study was conducted in order to detect any common variants underlying the linkage signal. Even though the area of maximal allele sharing in the linkage analysis was fairly narrow (just a few megabases in size), due to the ideas proposed by Kruglyak and Lander (Kruglyak and Lander, 1996b), we decided to expand the area for a SNP association follow-up study to between 78.3 Mb and 88.8 Mb, so as to cover functionally interesting genes at both ends. This region was then genotyped using an Illumina Golden Gate assay, covering 1,537 SNPs within this interval, targeting 40 trios and 324 cases and 214 unrelated controls from the linked families optimized based on the haplotype information. However, none of the SNPs tested showed significant association to the migraine or TCA phenotypes. Given the relatively low number of cases and controls tested, and that only common variants were interrogated, the result is perhaps not surprising.

2.c. Reproducibility of trait component analysis and detected loci

In both Study I and Study II TCA clearly improves the linkage results more than would be expected by random chance – that is, the increase in linkage scores overcomes the increased significance limits calculated by taking into account the extra testing. This extra burden was estimated to correspond to testing five (Study I sample) or six (Study II sample) independent phenotypes (the equivalent independent number of traits, after the correlation between them has been accounted for using the matSpD software (Nyholt, 2004)), consequently resulting in LOD score significance limits of 4.00 (Study I; increase of 0.7) and 3.83 (Study II, increase of 0.78). It should be noted that though the latter used a larger number of effective tests (hence the larger increase), the uncorrected estimate for the original significant limit was less conservative in Study II. This difference was due to a new method of estimating that limit; in Study I, we applied Lander and Kruglyak's approach (Lander et al., 1995) which estimates the significance limit using limit theory for the absolute significance limit for a linkage study with infinite markers, while in Study II we applied a correction which takes into account the lack of complete inheritance information in a linkage study, as described previously (Nyholt et al., 2005).

Prior to these studies, two loci had been replicated in migraine; 4q21-4q24 and 18q12, both reported in Finns (Wessman et al., 2002) and Icelanders (Björnsson et al., 2003). In this study, the improvement in existing peaks and the appearance of new ones happens in a consistent and reproducible manner (i.e. the new loci show a high degree of replication; see Table 10).

Table 10. Genome-wide linkage scans in migraine and loci with significant (shaded, cross) or suggestive (cross) evidence of linkage reported.

Study	1p31-p12	1q23-q31	2q33	3q29	4p16	4q21-q24	4q28-q31	5q13-q21	6p12-p21	6q12-q22	6q25	7q31	8q21	9q31	10q22-q23	11q24	12p13	12q21	13q14-q33	14q21-q22	15q14-q23	16p12	17p13	18p11	18q12	20q11-q13	Xp21	Note
1						x																						
2									x																			a
3	x									x		x				x			x			x						
4																				x								a
5					x	x																			x	x	x	b
6		x	x	x		x															x		x	x	x			c
7		x						x	x	x			x		x				x	x								c
8*						x	x								x			x			x		x		x		x	c
9*													x		x					x					x		x	c
10		x						x							x				x							x		d
11								x			x			x		x			x									
12						x																						
Repl.		+				+		+	+	+			+		+				+	+	+		+	+	+	+	+	

Footnote: Study references are 1 (Wessman et al., 2002), 2 (Carlsson et al., 2002), 3 (Cader et al., 2003), 4 (Soragna et al., 2003), 5 (Björnsson et al., 2003), 6 (Lea et al., 2005), 7 (Nyholt et al., 2005), 8 (Anttila et al., 2006), 9 (Anttila et al., 2008), 10 (Ligthart et al., 2006), 11 (Oedegaard et al., 2010). The notes in the last column refer to: [a] Single family study, [b] Study used a relaxed definition of migraine diagnosis, [c] Study used endophenotypes, [d] Study concentrated on a special subtype of migraine with aura. Asterisks indicate studies that are a part of this thesis. Repl - replication.

Out of the 27 loci reported in migraine, 15 have been replicated, and most of them multiple times; the top loci based on the current evidence are 10q22-q23 (six scans – Anttila et al. 2008 reported three, with all showing linkage to this locus), 4q21-q24 (five scans), 18q12 and 13q14-q33 (four scans), 5q13-5q21, 14q21-q22 and 1q23-q31 (three scans). Out of the two trait component studies, out of ten reported loci, eight have been replicated since or have themselves replicated previous studies. Compared to bipolar disorder, for example, where reproducibility has been a long-standing problem (Segurado et al., 2003) and schizophrenia, where similar problems have been observed (Badner and Gershon, 2002), the results in migraine are fairly consistent – even though a formal linkage scan meta-analysis has not been performed. However, it is interesting that a number of the best linkage regions in migraine show considerable overlap with the top regions in schizophrenia (1q22, 13q32-34) and bipolar disorder (4q24, 10q22, 14q24-q32, 18q12) (Craddock et al., 2005, Segurado et al., 2003), especially in the light of a recent linkage scan of co-occurring migraine and bipolar disorder (Oedegaard et al., 2010).

The consistency of migraine findings is demonstrated by the locus on 17p13, which was undetectable with the end diagnosis (Wessman et al., 2002). However, a follow-up finemapping study conducted after significant evidence of linkage was detected with the pulsation trait in Study I showed linkage of a number of SNPs genotyped at this locus with the MA end-diagnosis. This suggests that TCA had greater sensitivity to detect this signal. Furthermore, the follow-up of this finding in Study IV provides support to the usefulness of the TCA approach, because the association to this locus seen with end diagnosis is improved by TCA (V. Anttila, unpublished data). Similarly, the 10q22-q23 locus detected in Study II showed consistent and robust linkage signals across studies only when TCA phenotypes were applied. Since

publication, this has been observed in an additional scan (Ligthart et al., 2006). It remains to be seen what genetic variants can be discovered at this locus.

2.d. Comparison of the different phenotyping approaches

The reproducibility of TCA results raises the possibility of two intriguing explanations; first, while finding a genetic cause to a symptom-related mechanism does not necessarily reveal the causes for the onset of a migraine attack, it would in all likelihood allow new insights into the biological processes of a migraine attack. For example, identifying the particular features of migraine showing strongest linkage to the 4q24 locus could reveal additional information involved in what happens on the visual and auditory cortices during photo- and phonophobia (possibly even outside migraine), and could possess application in e.g. epistatic approaches. This highlights a crucial difference in the relationship to the "clinical background" of migraine between the different phenotyping approaches: LCA creates fixed combinations of clinical features, in effect creating a new diagnostic classification, based on symptom structure typical to each class based on heritability estimates, while trait component analysis is based on the hypothesis that the information contained in each response itself is closer to the underlying biology. Based on the traditional assumptions in genetics, where estimates of heritability and affected-sibling risk ratios from twin studies are used as the guideline for all subsequent work, LCA as a methodology should in theory generate superior results. The observations from these two linkage scans indicate that this may not be the case in migraine (see Figure 19), suggesting that some additional confounding factor or factors may be involved. There are at least two possible explanations to the observed results: 1) the existence of a confounding

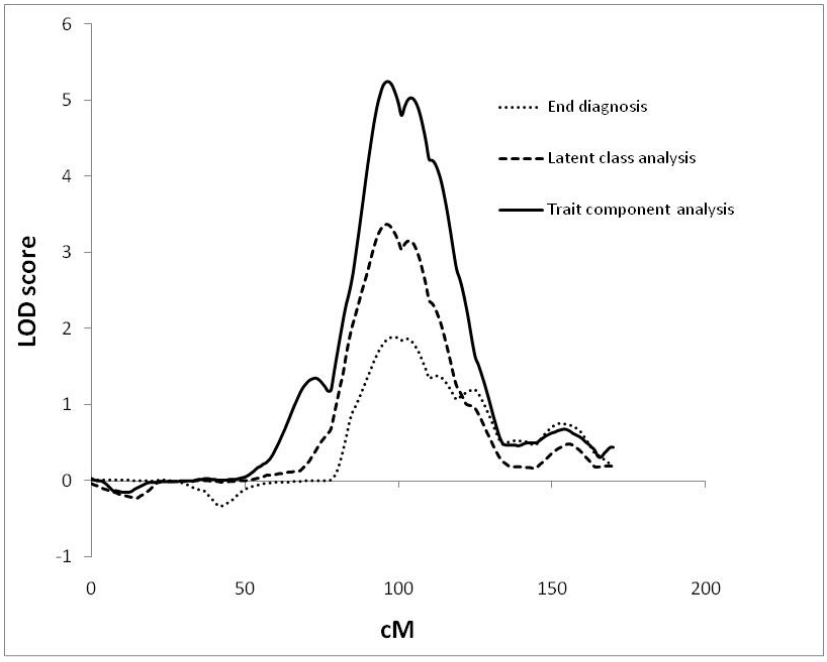

Figure 19. Comparison of the linkage results on chromosome 10 produced by the different phenotyping approaches in the joint Finnish-Australian study sample in Study II.

subgroup (e.g. sporadic migraine due to non-genetic factors), which increases diagnostic heterogeneity, thus influencing both the end diagnosis and the original LCA profile estimation; 2) that the underlying assumption of both LCA and TCA is true – that the symptom questions can be used to distinguish "real" (or "genetic") migraine from something else mimicking itself as migraine (likely some condition related to altered pain processing, possibly with a strong psychological component). This latter possibility has the implication that some combination of traits or traits and heredity profiles (likely with the vascular criteria, especially pulsation, playing a major role) could be used to improve homogeneity in treatment trials, epidemiological studies etc.

One important feature of Study II is the ability to compare a highly enriched family sample (i.e. the Finns) with a typical population-based sample (the Australians), and to show that the method applies in both contexts. In Study I, a possible explanation to the new peaks would have been the presence of very rare variants and haplotypes, with large effect sizes, now present in detectable amounts due to the extreme enriching, but with little relevance on the population level or for practical neurology. However, the pattern of improved linkage signals persists in the Australian population-based set, providing further evidence of the relevance of the TCA approach.

2.e. Conclusions

The detection and replication of the 10q22-q23 locus is the first time when a single locus has been linked to migraine susceptibility in multiple populations within a single paper, and was the first study in which the same markers showed significant evidence of linkage to migraine. The considerably high LOD score in the female group especially provides strong evidence that some key part of the susceptibility resides at this locus. Furthermore, the results of this study confirm the usefulness of trait component analysis in migraine genetics. In comparison between the different phenotyping approaches, it performed consistently better than latent class analysis, and considerably better than the diagnosis-based approach. Encouragingly, the trait component analysis was better or at least equally good at detecting every locus in both Study I and II, suggesting that the gain in power through traits is consistent across different underlying inheritance models and haplotype frequencies.

In these first two studies, we were able to address Aim 1 (the development of a new migraine phenotyping method) quite well with the application of TCA to several clinic-based samples and one population-based sample (the Australian linkage scan). As a result, we provided valuable new insights into the practice of migraine linkage scans. For Aim 2a (studying rare variants through linkage scans), we succeeded reasonably well by identifying and replicating of a number of new loci, including the strongly linked locus on 10q22-q23. However, we have been unable to find the responsible genes at the locus. So Aim 2a has not been an unqualified success and work on the 10q22-q23 locus continues.

3. Candidate Gene Study of 155 Ion Transport Genes

In Study III, we employed a custom-made Perlegen genotyping assay covering 5,257 SNPs targeting 155 ion transport genes. These genes were selected from publicly available databases based on function as ion channels, following on the channelopathy hypothesis (see Figure 20). An initial scan of 841 unrelated migraine samples and 884 unrelated controls failed to detect any significant associations. However, given the relatively low power to detect common variants with this sample size, we selected all SNPs with an allelic p-value < 0.005 (n=66) for follow-up study. No SNPs showed significant association to migraine, and thus we conclude that common variants of moderate effect size in ion transport genes do not play a major role in susceptibility to common migraine within these European populations. Similarly, the three known FHM genes showed no association in the screen, which is in line with previous research by our group (Kaunisto et al., 2005).

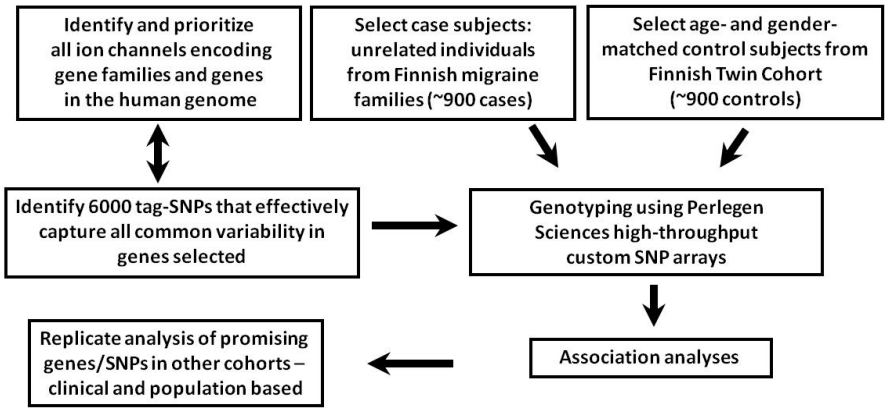

Figure 20. The research plan for Study III. SNP – single nucleotide polymorphism.

3.a. Target selection

The "common variant – common disease" hypothesis proposes that common diseases rise from the genetic effects of variants of relatively high frequency in the general population, which explains why certain diseases are common. From an evolutionary point of view, these common variants would possess only minimal effect on reproductive capability, involving largely diseases appearing after fertility period (neurodegenerative diseases, metabolic syndrome etc.), and thus escape evolutionary pressures.

Before it was possible to use GWA data, which at the time was prohibitively expensive, educated guesses had to be made in the selection of target genes in order to study the role of common variants in ion channel genes. Based on the fact that FHM studies successfully identified causative genes that are all ion channels, we targeted all available ion channel genes (including the three FHM genes) based on the data-mining of the available biological databases (such as the Kyoto Encyclopedia of

Table 11. Categories of ion channels and pumps represented in the array.

Category	Genes on array
Voltage-gated calcium channels	26
Voltage-gated potassium channels	74
Voltage-gated sodium channels	14
Chloride channels	21
ATPase ion transporters	20
Total	155

Genes and Genomes [KEGG] at genome.jp/kegg) for known ion channel genes. An initial list of 768 partially overlapping genes from the various databases was eventually reduced to 155 based on the reliability of their detection and the quality of available data (see Table 11). Regions covering the genes with an additional 20 kb at the 5' end and 10 kb at the 3' end of each gene were included the study. TagSNPs were then selected to cover all common variants with MAF $\geq$ 10% in the study regions with an $r^2 \geq 0.8$. The resulting total was 5,269 SNPs. These markers were first studied in a Finnish sample of 841 unrelated migraine cases and 884 population-based controls with SNPs showing p-values <0.005 selected for replication in multinational samples from Germany, the Netherlands and Australia.

3.b. No association to common variants either with diagnosis or trait component analysis

In the initial Finnish study sample, no significant association to migraine was observed. The SNP with the lowest p-value was *rs13276133* (p-value 0.00041); however, after multiple testing correction through permutation, the empirical p-value was only 0.7713. A total of 66 SNPs in nine genes (*ATP2C2*, *CACNA1E*, *CACNB2*, *KCNE2*, *KCNK12*, *KCNK2*, *KCNS3*, *SCN5A* and *SCN9A*) were selected for replication.

In the replication analysis, none of the 66 SNPs chosen for replication showed consistent evidence of association between cohorts. Given that the estimated power to replicate an association at a p<0.05 level from the initial study was over 70% for each of the top SNPs, we can be relatively confident that the original associations were spurious in nature, especially as even the lowest replication p-value was relatively high (*rs400922* on chromosome 16; empirical p-value 0.15). The use of TCA did not considerably improve results (highest TCA signal was seen with marker *rs12996816* on chromosome 2, which had an allelic p-value of 0.001 using the MA diagnosis, and 6.32×10^{-5} using the pulsation trait; Bonferroni-corrected significance limit for the study was 1.9×10^{-5}). For the trait component analysis part of the study, we used the traits photo- and phonophobia in addition to pulsation, as they were considered to be the best traits based on the 2006 study (Anttila et al., 2006). The top results for the other traits were with markers *rs13276133* (chr 8q21.11; p-value 0.00021, photophobia) and *rs12054449* (chr 3p21.1; p-value 0.00031; phonophobia).

3.c. Possible signs of epistasis between ion channel genes

As many of the ion channels are hetero- or multidimers and thus act together in cells, a SNP x SNP epistasis analysis was conducted among the studied SNPs using the epistasis analysis option in PLINK (Purcell et al., 2007). Given the large number of SNPs available, we followed the recommendation by Blangero et al. (Blangero et al., 2000) to restrict the analysis to those SNPs that have been chosen for replication for showing nominal association ($p < 0.005$) to migraine. In this analysis, uncorrelated ($r^2 = 0.0009$) SNPs in genes *KCNB2* (*rs1431656*) and *CACNB2* (*rs7076100*) were found to have an interaction p-value (pointwise p-value 1.99×10^{-5}, 0.022 after correction) that remained significant after Bonferroni correction adjusted for independence of tests using SNPSpD (Nyholt, 2004). The interaction estimated from the SNP data would confer an OR of 1.64 for migraine. These genes are located on two different chromosomes (*KCNB2* on 8q13 and *CACNB2* on 10p12). However, it should be noted that the interaction was only found in the Finnish cohort. It almost replicated in the Australian cohort (p=0.057), but no interaction was present in the Leiden cohort (both SNPs were successfully genotyped in these three populations only). The *CACNB2* SNP is unfortunately not included in the GWA platform used in Study IV, and so we could not replicate the interaction in the larger study. No previously reported connection was found between the two by a PubMed search or in any of the relevant databases (such as the Kyoto Encyclopedia of Genes and Genomes).

3.d. Conclusions

The results of this study were fairly straightforwardly negative, both with the diagnosis-based approach and the TCA approach. Given that the most likely reason for this, not tagging enough of the variation surrounding these genes, was mostly excluded as a cause in Study IV, and therefore we concluded that common variants of moderate to high effect sizes in these genes do not play a role in migraine susceptibility. Untagged rare variants may of course still play a role in these genes, and given the known FHM pathology, this may even be likely. However, the answer to the role of rare variants in ion channel genes can only be sufficiently answered through resequencing.

4. Genome-wide Association Study in Migraine

In Study IV, we performed a genome-wide association study on 2,748 Finnish, German and Dutch migraine with aura patients and 10,747 population-matched controls. The discovery sample was recruited on the basis of the presence of migraine aura at four sites: two German sites, Cologne and Munich, in addition to the Finnish and Dutch sites – Helsinki and Leiden, respectively. Genotyping was performed using the Illumina HumanHap 610k and 550k arrays at the Sanger Institute with controls obtained from existing studies. Initial analysis in this sample set was followed by a replication study in four populations (Icelandic, Danish, German and Dutch) and a meta-analysis.

4.a. Significant association to marker *rs1835740* on 8q22.1

In the genome-wide association analysis, we detected a single marker with association to migraine that surpasses the threshold for genome-wide significance, 5 x 10^{-8}. The

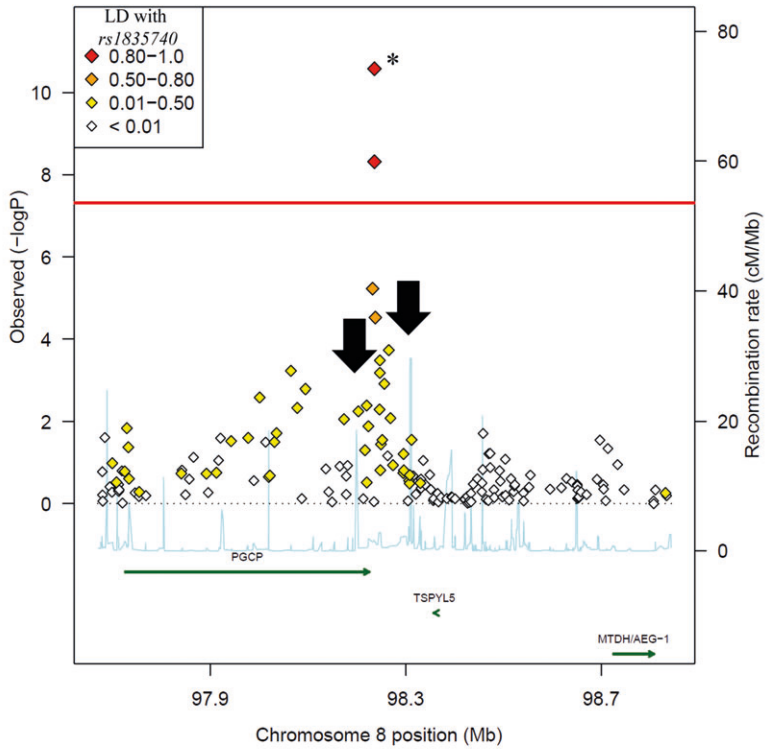

Figure 21. Cochran-Mantel-Haenszel test results for SNP association to migraine phenotype around rs1835740 in Study IV. Red line indicates level of genome-wide significance (5 x 10^{-8}). Black arrows denote recombination hotspots surrounding the identified marker. Asterisk indicates the association result of the marker in meta-analysis.

marker, *rs1835740*, is located at 98.2 Mb on chromosome 8q22.1 and showed an association p-value of 5.12 x 10^{-9} to the minor allele using the Cochran-Mantel-Haenszel test (see Figure 21). This SNP has a roughly 21% minor allele frequency in the general population, with a considerable geographic gradient towards Asia (where the reported risk allele frequency can be as high as 65%). No association was observed to any of the previously detected FHM genes or any of the candidate genes listed in Chapter 4 of the Review of the literature. Haplotype and conditional analyses failed to increase the association signal and no long-range LD was detected, most likely due to two recombination hotspots which surround the associating marker (denoted by black arrows in Figure 21), suggesting that the causative variant underlying the signal is likely to be in close proximity to the detected marker itself. A replication study in Danish, Icelandic and German migraine study samples (overall 2,853 cases and 37,980 controls) replicated the signal on *rs1835740*. Interestingly, the replication was successful not only in migraine with aura samples, as in the primary analysis, but also in migraine without aura samples (see Figure 22) although the relatively low number of cases in this diagnosis group limits the ability to make definitive conclusions for this group, and in a population-based study set (Icelandic set) as well as clinic-based sets (German and Danish set).

Given the rarity of migraine patients whose attacks are accompanied by aura in 100% of attacks ("MA only"), only 589 were found even with this concerted effort; the remaining 2,142 patients suffer from attacks with and without aura ("Both MA, MO), with varying proportions of attacks with aura. Interestingly, the MA only group showed a fairly uniformly higher association and effect sizes than the Both MA, MO group. In the initial GWA, the effect is less marked (OR 1.33 vs. 1.21), suggesting a

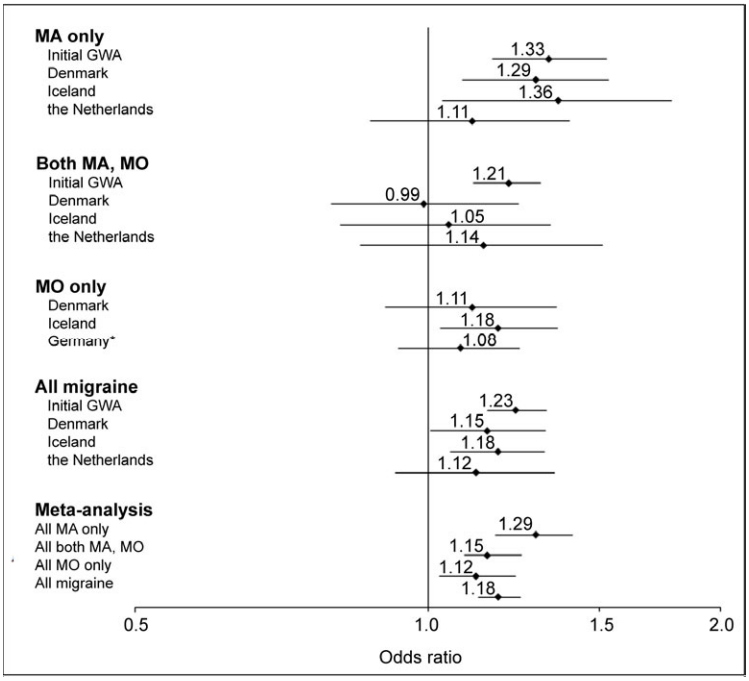

Figure 22. Odds ratios for rs1835740 *in each study sample in Study IV organized by diagnosis. Asterisk indicates German study sample where an outlier control group has been excluded.*

possible explanation could be found in the small sizes of the replication sets in this group (n=293 for Denmark, n=196 for Iceland). However, an ever smaller sample of MA only samples in Iceland (n=137) is still showing an effect size in line with the larger MA only samples.

One thing which cannot be clearly stated from this study, however, is whether this SNP would be associated with aura or pain, since even though the MO only samples show some association (albeit with the small sample sizes in this group, it has to be admitted that only limited amount of the conclusions can be drawn for this group), it may be argued that this group would contain a number of patients with either lower penetrance of the full phenotype (thus limiting the clinical presentation to only migraine without aura) or patients where the aura is yet to manifest itself for some reason (admittedly an unusual progression, given that aura typically manifests itself early in life). However, given that though the sample size of 1,744 MO only patients may be relatively small in the normal GWA context, it is still a relatively decent size for sub-class analysis. Another possible explanation to the difference in OR between the groups - is that because of the differences in the severity of the phenotype, one could speculate that the most severe phenotype, MA only, contains the smallest proportion of sporadic cases, typically considered more likely to have a severe phenotype. In this sense, the results for the SNP *rs1835740* conform nicely with the view of migraine presented in Study I. In Study I (see Figure 23), we illustrated the relationships between the diagnosis groups with partially overlapping concentric circles, reflecting the overlapping nature of the migraine diagnoses. The arrows indicate the patients groups corresponding to the aura groups in Study IV. When comparing this with Figure 24, one can see how the effect size of the identified variant increase towards the more severe end of the spectrum (towards the left edge in Figure 23, towards the right edge in Figure 24). The approach in Study I, where the trait component approach was used to try to identify more severe cases of migraine, in the sense that cases with more symptoms will be more represented in the trait phenotypes, and the "severity of aura" scale reflected in the results

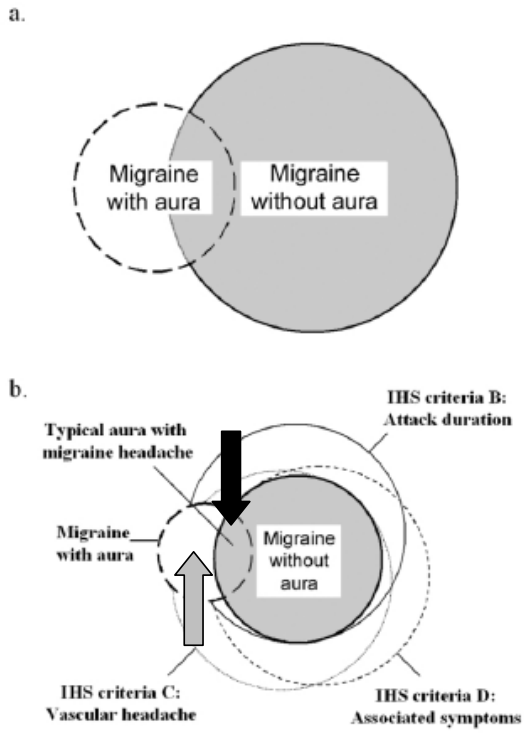

Figure 23. Illustration of the theoretical view of the migraine diagnoses and symptoms underlying the trait component analysis. Black arrow denotes the "Both MA, MO" group in Study IV, grey arrow denotes the "MA only" group. Adapted from Anttila et al. 2006. Used with permission.

of Study IV, show that this kind of stratification can be used to add power in migraine studies. This approach could, after a fashion, be considered an analog to the extremes-only analysis (discussed in Chapter 1) in migraine. However, a more elegant approach, perhaps by using the traits in a principal components or a multivariate adaptive regression analysis could yield further increases in power.

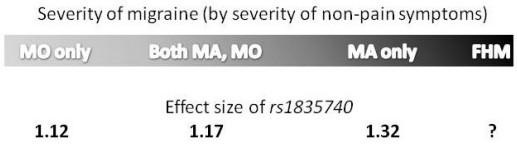

Figure 24. Migraine severity spectrum plotted against the rs1835740 effect size. MO – migraine without aura; MA – migraine with aura; FHM – familial hemiplegic migraine.

4.b. An eQTL Study of *rs1835740*

The associating marker *rs1835740* is located between several functionally interesting genes on chromosome 8q22.1, and therefore an eQTL study (an analysis of transcript expression levels of each gene in a tissue sample compared to common sequence variants) was conducted. Umbilical cord cells from the GenCord resource were

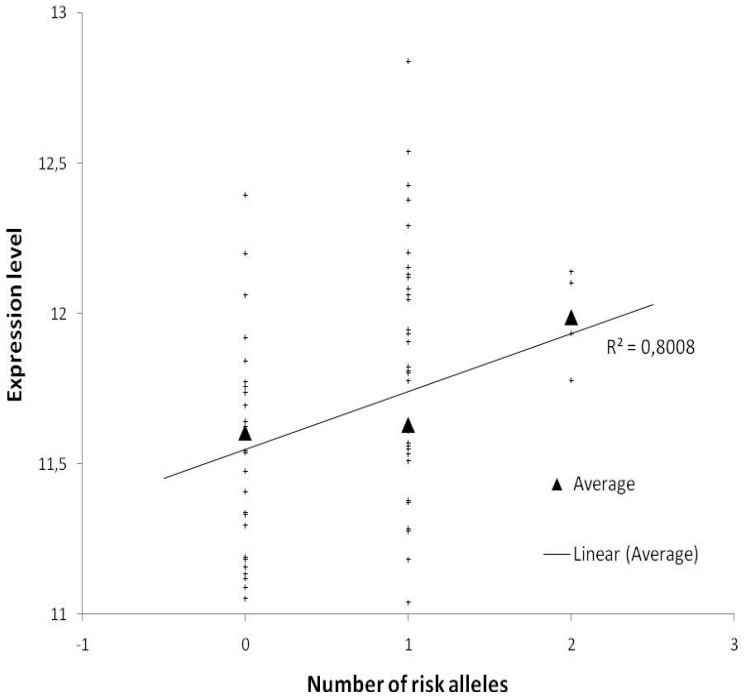

Figure 25. Expression level of the probe targeting MTDH/AEG-1 *in lymphoblastoid cell lines, partitioned by* rs1835740 *genotype of the individual. Expression level scale is arbitrary and conditional on the normalization of the entire dataset. Black pyramids indicate average value in each genotype group, and the solid line indicates the linear trend line for the group average. Box plot of the same data can be seen in Study IV.*

Figure 26. Results of the TRANSFAC binding site analysis (M.Piipari, unpublished data) showing a) the conservation of the sequence around rs1835740 between 98,166,853-98,166,938 bp across 14 species. Bases in grey highlight the nkx3-1 binding motif in the sequence; the base in black is variant rs1835740. b) the nkx3-1 binding motif itself.

genotyped using a GWA study platform and their expression levels were quantified using the Illumina WG-6 array using previously described methods (Stranger et al., 2007). Of the three types of cells tested, only lymphoblastoid cell lines showed that the genotype of this marker strongly correlates with the transcript levels of a nearby downstream gene, *MTDH/AEG-1* (metadherin/astrocyte-elevated gene 1; see Figure 25).

The eQTL link between the detected variant and *MTDH/AEG-1* provides an interesting potential functional effect for the detected association. However, there are a number of potential weaknesses to consider in the eQTL approach, requiring further study. First, even though the detected eQTL is the only significant one present in LCLs for SNP *rs1835740*, this does not mean the same eQTL is present (or the only one) in neural tissue. The two other analyzed tissues, fibroblasts and primary T-cells, did not show a significant eQTL effect for this SNP with any genes in the region. A genome-wide analysis of eQTLs in these three tissues showed that on average, only 30% of detected significant signals in one tissue were present in another tissue and that most eQTLs are specific to a single tissue (Stranger et al., 2007).

Second, even though the significant correlation between *rs1835740* and the transcript levels is promising, it does not necessarily mean *rs1835740* is the causative variant. It is possible (perhaps even likely, given the current opinion on effects of rare and common variants) that the common SNP is reflecting the effect of a nearby rare variant which is also present in the individuals of the eQTL study. Suggestion of this type of effect was present in a binding site analysis we conducted (M. Piipari, unpublished data, see Figure 26,), where a potential nkx3-1 factor binding site was located only 44 base pairs from the SNP (see Figure 26). However, recent studies have suggested this kind of modulation through eQTL variants may underlie most GWA findings, and perhaps goes a way towards explaining the missing heritability (Nica et al., 2010).

4.c. Role of *MTDH/AEG-1* in neurological diseases

The role and function of *MTDH/AEG-1* has generally been studied more in cancer (Sarkar et al., 2009), where the gene has been shown to play a role in tumor cell proliferation (Li et al., 2009) and angiogenesis (Emdad et al., 2009). *MTDH/AEG-1*) has been shown to be involved in a number of biological pathways, such as the TNF-alpha (Boycott et al., 2008)/NF-kB (Sarkar et al., 2008) pathway (tumor necrosis factor alpha/nuclear factor kappa beta) which is involved in various responses to stress and hypoxia (see Figure 27) in brain cells (Dallas et al., 2007).

With clearer links to known migraine physiology, a number of the studied functions of *MTDH/AEG-1* relate to neuropsychological phenotypes; 1) the FOXO1 transcriptional factor (Li et al., 2009), and its effect on the PI3K/Akt pathway (Sarkar et al., 2009), has been shown to play a direct role in regulating epileptiform activity (Shanley et al., 2002) and neuroprotection after epileptic seizures (Shinoda et al., 2004), and a paper implicating the pathway in autism spectrum disorders (Kwon et al., 2006) showed that abnormal activation of the pathway results in exaggerated responses to sensory stimuli reminiscent of migraine. On a more fundamental level, activation of this pathway in *Drosophila melanogaster* by the analog of the FOXO1 transcriptional factor, which is regulated by *MTDH/AEG-1*, has been shown to play a role in modulation of neuronal excitability and survival via the PI3K kinase (Al-Mubarak et al., 2009); 2) *MTDH/AEG-1* directly participates in the regulation of EAAT2/GLT1 levels (excitatory amino acid transporter 2/glutamate transporter 1). EAAT2/GLT1 is the primary glutamate transporter in the brain (Kang et al., 2005) - see Figure 28 (Machado-Vieira et al., 2009). The transporter is responsible for clearing glutamate from the synaptic cleft. The down-regulation of this transporter is

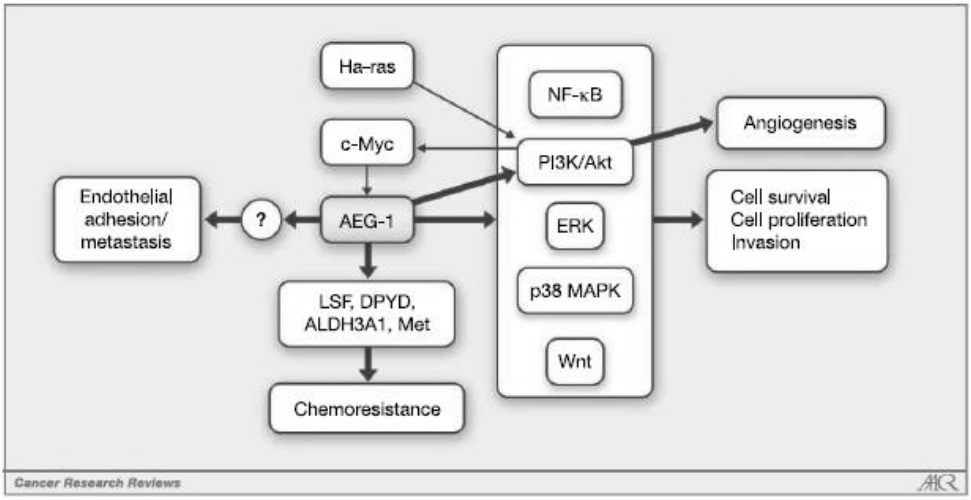

Figure 27. Signal pathway involving MTDH/AEG-1 *proposed in Sarkar et al. 2009. Ha-ras – retrovirus-associated DNA sequence, Harvey type, c-Myc – general transcription factor, LSF – Late SV40 transcription factor, DPYD – dihydropyrimidine dehydrogenase, ALDH3A1 – aldehyde dehydrogenase 3 family member A1, Met – hepatocyte growth factor receptor, NF-kB – nuclear factor-kappaB, PI3K/Akt – intracellular signal pathway, ERK and p38 MAPK – MAP kinase pathways, Wnt – signaling pathway. Used with permission.*

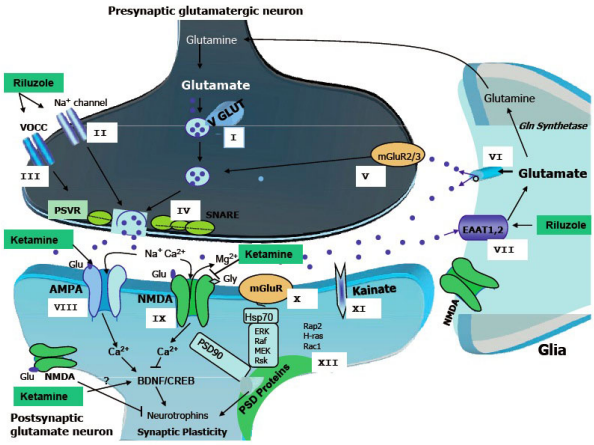

Figure 28. Therapeutic targets for pharmacological compounds targeting glutaminergic neurotransmission (indicated by roman numerals). Numeral VII indicates the glial transporters EAAT1 and EAAT2. From Machado-Vieira et al., 2009. Used with permission.

hypothesized to cause increased ambient glutamate levels. Even though Study III showed there is no association of common variants in ion channel genes with migraine, glutamate homeostasis is indirectly related to ion channel function via the complex interplay between ion channels and glutamate. Glutamate receptors directly influence ion channel activity via regulation of the intracellular concentration of Ca^{2+} in neurons (Fagni et al., 2000). Furthermore, PI3K influences the levels of ion channels that are trafficked into the cell surface (Hou et al., 2008, (Viard et al., 2004). In mice brains, the deletion of the FHM gene *CACNA1A* has a direct effect on glutamate release (Lonchamp et al., 2009) and the regulation of glutamate-dependent NMDA receptor signalling (Mela et al., 2006). Glutamate receptors are targets for anti-epileptic medications (Alexander and Godwin, 2006), anxiety and stress disorders (Swanson et al., 2005) and schizophrenia (Patil et al., 2007). In summary, there is a considerable amount of evidence showing that the functions of *MTDH/AEG-1* (see Figure 27) extend beyond cancer, and that through regulation of EAAT2/GLT1 it is a strong candidate for migraine.

4.d. Population-based results show considerable overlap with linkage findings

Interestingly, in Study IV the genome-wide association analysis show several promising association peaks in the previously identified linkage regions. For example, in the Finnish study sample, the highest association result (SNP *rs16940918*; p-value 6.9×10^{-8}, roughly comparable to LOD score of 6.34 based on the formulae by Nyholt et al. (Nyholt, 2000); V. Anttila, unpublished data) is located close to the marker *D17S945* (LOD score 4.65) reported in Study I (see Figure 29). In the linkage study, this peak was observed primarily with the pulsation trait, and in the GWA study the best trait was aggravation by physical exercise. Interestingly, these two traits are considered to best reflect the peripheral sensitization component of migraine, which

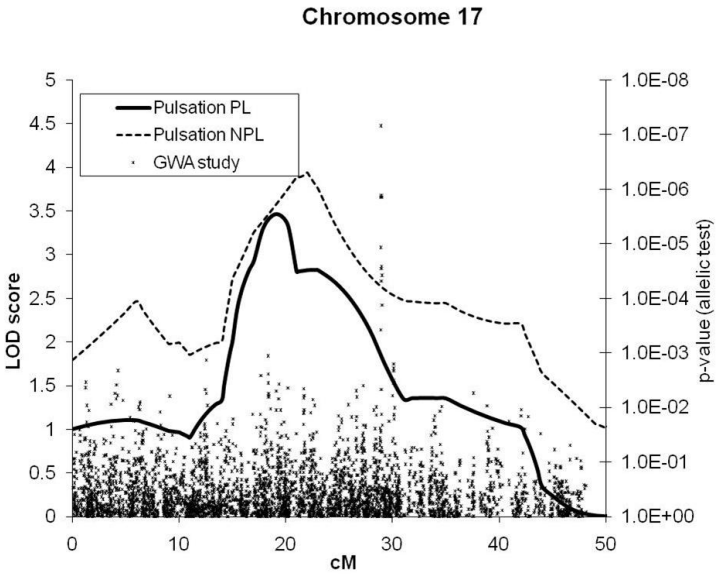

Figure 29. Plot showing linkage scan results from Study I (solid and dashed lines show parametric (PL) and non-parametric (NPL) multipoint linkage results. Individual dots show SNP marker association results from the Finnish study sample in Study IV. Note: the two y-axes are not directly comparable; see text.

drives the central sensitization and contributes to the neurogenic inflammation (Pietrobon et al., 2003). Using trait component analysis, the top p-value rises to 2.6 x 10^{-9} (see Figure 30). The combination of these results suggests that this locus could harbor a Finnish-specific migraine gene.

Similar overlap between population-specific GWA findings and linkage findings was observed in the 4q24 locus (observed in the Dutch population), 10q23 (German populations) and 18q12 (Finns, Dutch). However, all three of these findings in linkage regions are not found in the all samples and thus require futher replication before further hypotheses

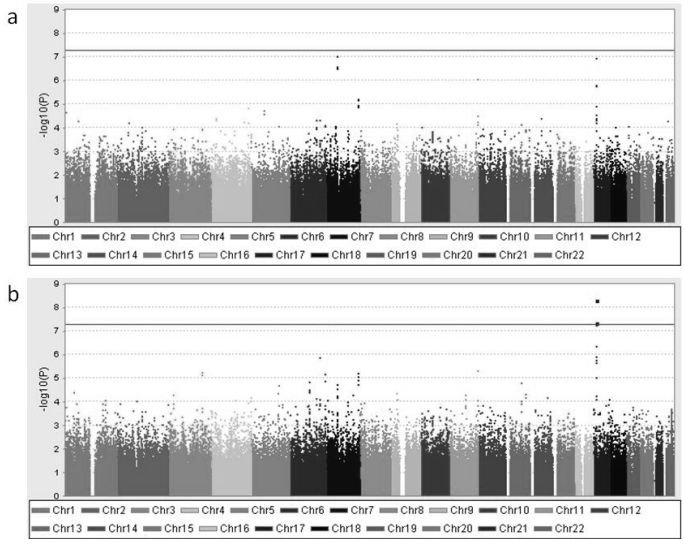

Figure 30. Results of the Finnish GWA association study sample, using a) migraine diagnosis and b) aggravation trait component.

can be inferred from them. One interesting finding is the two associations to 18q12 in the region immediately surrounding the *DCC* gene (deleted in colorectal carcinoma), which encodes the netrin 1 receptor that guides axonal growth cones of neurons and is a promising functional candidate gene.

4.e. Conclusions

In Study IV, we were able to detect and convincingly replicate the first SNP association in migraine. The identification of this variant, along with the eQTL link to a potentially interesting biological mechanism, is a promising finding. Given that the identification was made in migraine patients that are very strongly ascertained for severe migraine at headache clinics, and that the signal is strongest in the pure MA group, which is quite rare – perhaps 0.5% in the population, raises the possibility that this variant will only be useful in the specialist clinic setting and not on the population level. Initial results from the Health2000 control population used in the study, where we were able to gain self-reported headache phenotypes, the variant frequency did not differ between those reporting headache (for any cause) and those without; frequencies were 22.14% and 21.96%, respectively, compared to around 25% in the pure MA cases.

An interesting future study will be to see whether the trait component analysis can be used to increase the detection resolution further in GWA studies as well. If this turns out to be the case, the increase in power due to being able to stratify MO patients into more and less severe cases should be of special significance in GWA studies of MO, where a clear diagnostic marker like aura is not present to help in distinguishing correct phenotypes.

With the current sample size, we are largely able to exclude the role of common high-impact variants in migraine. Further, the lack of results to any of the previously reported candidate genes in any of the study populations suggests that the considerable heterogeneity in migraine may at least partially explain those reports. Upcoming migraine GWA scans will be able to answer this question more thoroughly, especially regarding MO. In the meantime, the MO association observed in this study is not significant enough to draw strong conclusions, as the numbers of samples are fairly low. However, the MAF excess of 3% between the MO only cases (23.2%; n = 1,744) and controls (20.3%; n = 37,980) represents some 55 extra individuals carrying the risk allele, which is a fairly large number of samples to attribute to low penetrance or unusual symptom progression. The results thus suggest that the most likely explanation for the variable amount of association in the Both MA, MO group is the small individual replication sample size, given that the meta-analysis results are in line with the current migraine spectrum theory (see Chapter 3).

For Aim 2b (studying the role of common variants in migraine through a GWA study), we report success. We identified the first SNP, *rs1835740*, with genome-wide significant association with migraine. We were also able to propose a functional mechanism for it, which brings forward the study of migraine genetics. The trait component analysis of GWA data is still ongoing, but it appears to be useful in improving association signals.

CONCLUDING REMARKS AND FUTURE PROSPECTS

Despite the monumental efforts of the last several years, understanding the genetic basis of common diseases remains an elusive goal. A large number of positive associations have been reported, but the small effect sizes across the board suggest either that the detection is incomplete (Maher, 2008). Possible explanations for this may be that the common markers detected are tagging still unknown rare variants, in which case the estimates for effect size as well as the proportion of genetic variance explained are incorrect, or that some unknown mechanism (such as genotype-dependent alternate splicing) is acting between the variants and biology (Nica et al., 2010), or that we simply do not understand genetics to a sufficient depth. How to uncover the hidden heritability remains one of the most important open questions in the field. However, promise of a better understanding of common variants was provided by a recent study in schizophrenia and bipolar disorder reporting strong evidence that the heritability in those disorders is due to at least hundreds of common variants (Purcell et al., 2009).

As discussed in Chapter 4, the findings of published linkage studies in migraine heavily suggest that at least ten loci may play a role in migraine. The first GWA results suggest that the story of migraine genetics is likely similar to those of schizophrenia and bipolar disorder. Even though the results of the candidate gene approach in migraine have been mixed at best and some of our existing data not yet published (e.g. an upcoming Norwegian MO study, V. Anttila, unpublished data) show little or no role of the previously identified candidate genes, the linkage scan results in migraine are encouraging. Published linkage scans in migraine, including those in this thesis, show a considerable amount of overlap with each other, which is to an even larger degree than the findings in bipolar disorder or schizophrenia (Oedegaard et al., 2010). The overlap suggests that there might be common mutation targets in elements of the migraine pathogenesis. Even though overlapping linkage findings do not necessarily indicate the existence of the same causative variant or mutation, they do support the idea that migraine-related mutations concentrate in the same genes. Therefore loci like 4q24, 10q22-q23 and 18q12 provide promising targets for future resequencing and epistasis studies.

One important problem in the studies of the genetic background of migraine is the dynamic nature of the disease. We aimed to address this issue in our data collection. The questionnaire asks about the presence of symptoms at any time and for a self-reported estimation of the number of lifetime migraine attacks (the number of both all attacks and severe attacks). However, current collection methods may be inadequate to reflect the difference between, for example, constant rates of attacks over several decades versus brief but active attack periods. Brief but active attack periods may result from a combination of unusual psychological and environmental exposures rather than genetic predisposition. The nature of migraine is a challenge for the practice of strict diagnostic dichotomy. Many patients develop and lose symptoms of migraine over time. Therefore, the inclusion of longitudinal information would be an interesting approach for genetic studies, which has not been studied thus far. It will be very interesting to see what advances in expression studies and proteomics will offer for migraine diagnostics in the future.

It is entirely possible that the susceptibility to particular symptoms has a distinct biological background and that their appearance and disappearance over time is modulated by other unknown factors (including normal physiological changes, like those in the hormone balance). Even though finding the genetic cause of a particular symptom does not necessarily reveal the causes for the onset of a migraine attack, it provides new insights into the processes that shape the course of a migraine attack. For example, the finding that more linkage information is captured by considering only individuals with sensory symptoms (photo- and phonophobia) and not all MA patients suggests that variations within the locus on 4q24 may reveal something of the pathophysiological processes involved in the triggering of visual and auditory symptoms specific to the corresponding cortices (or their modulation by other regions of the brain). Due to the nature of complex disorders, pathways and mechanisms found in this way would probably not be specific to migraine, but to overall brain function. However, proper understanding of complex network effects of this type calls for a new kind of integration of proteomic, genomic and systems biology approaches.

Regardless of these limitations, sufficiently large sample sets have yielded a number of interesting insights into the underlying pathophysiology of a number of common diseases. The insights show promise for future personalized medicine and basic research. The main achievement of this study is the several new targets identified for future studies in migraine, which remains a relatively poorly understood disorder both in terms of genetics and pathophysiology. The complexity of the comorbidities, difficulty of the phenotype and genetic heterogeneity of such a common disorder remain major challenges and perhaps explain the relative dearth of migraine research groups. However, the first GWA will likely be a good stepping-stone to encourage further interest in the disease. The potential of the glutamate hypothesis will hopefully stimulate further research. Several genome-wide studies of the genetics of an increasing number of migraine patients are being conducte as well as first large-scale migraine meta-analysis. The improved imputation prospects arising from the 1000 Genomes project along with the huge amount of genetic information on migraine patients should make the next few years very exciting for migraine research.

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June 1st, 2010
In Helsinki

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I

Trait Components Provide Tools to Dissect the Genetic Susceptibility of Migraine

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The commonly used "end diagnosis" phenotype that is adopted in linkage and association studies of complex traits is likely to represent an oversimplified model of the genetic background of a disease. This is also likely to be the case for common types of migraine, for which no convincingly associated genetic variants have been reported. In headache disorders, most genetic studies have used end diagnoses of the International Headache Society (IHS) classification as phenotypes. Here, we introduce an alternative strategy; we use trait components—individual clinical symptoms of migraine—to determine affection status in genomewide linkage analyses of migraine-affected families. We identified linkage between several traits and markers on chromosome 4q24 (highest LOD score under locus heterogeneity [HLOD] 4.52), a locus we previously reported to be linked to the end diagnosis migraine with aura. The pulsation trait identified a novel locus on 17p13 (HLOD 4.65). Additionally, a trait combination phenotype (IHS full criteria) revealed a locus on 18q12 (HLOD 3.29), and the age at onset trait revealed a locus on 4q28 (HLOD 2.99). Furthermore, suggestive or nearly suggestive evidence of linkage to four additional loci was observed with the traits phonophobia (10q22) and aggravation by physical exercise (12q21, 15q14, and Xp21), and, interestingly, these loci have been linked to migraine in previous studies. Our findings suggest that the use of symptom components of migraine instead of the end diagnosis provides a useful tool in stratifying the sample for genetic studies.

Migraine (MIM 157300) is the most common cause of chronic episodic severe headache, affecting 10%–12% of the adult population.[1] The genetic component of migraine has been well established in family and twin studies.[2–6] Several loci linked to common forms of migraine with significant evidence of linkage have been reported,[7–15] yet, to date, no genes responsible for susceptibility to the common types of migraine, migraine without aura (MO) and migraine with aura (MA), have been described. Thus far, three genes, *CACNA1A* (MIM 601011), *ATP1A2* (MIM 182340), and *SCN1A* (MIM 182389), which contribute to familial hemiplegic migraine (FHM1–3 [MIMs 141500, 602481, and 609634, respectively), a rare Mendelian subtype of MA, remain the only genes found in headache disorders.[16–18]

Most studies aiming to unravel the genetic basis of headache disorders use the end diagnoses of the International Classification of Headache Disorders (later referred to as "International Headache Society [IHS] classification") as the phenotype when monitoring for linkage or association[19,20] (appendix A). Although diagnosis-based phenotypes have been successfully used to identify predisposing loci and even genes in some complex traits,[21–23] it is likely that they represent an oversimplified model of the genetic background of a disease, which in reality is complex and polygenic. In some complex diseases, the use of clinical traits (referred to as "quantitative traits," "endophenotypes," or "trait components")—for example, lipid values, components of language deficits, or allergy-related phenotypes—provides an opportunity for a more refined analysis.[24–28] These traits probably reflect features that are closer to the molecular background of the disorder than the clinical classification, which represents a consensus among clinicians. The development of alternative phenotypes and/or endophenotypes would be especially useful for headache disorders, for which diagnosis is typically based on the patient's description of attacks and no laboratory, radiological, or any other objective, quantitative findings are used for diagnostics.

The diagnosis and classification of migraine and its subtypes are based on the fulfillment of symptomatic criteria, summarized in the IHS classification (appendixes B and C). Since the migraine diagnosis consists of a combination of traits and/or symptoms, a single affected individual has to present with several traits simultaneously; furthermore, the individual traits are not completely independent of each other (fig. 1). The clinical traits and trait groups forming the basis of the IHS classification provide a natural

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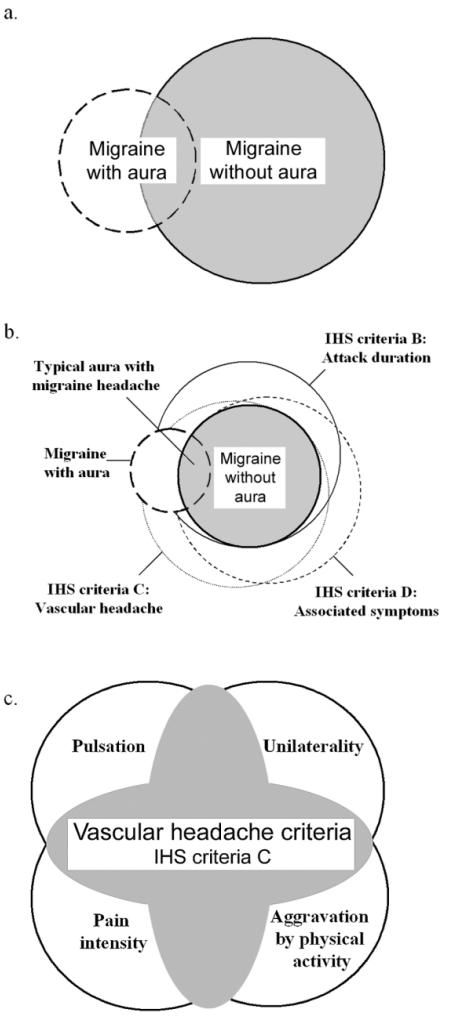


Figure 1. Schematic illustration of some relationships between diagnoses and traits in the 2004 IHS classification. Overlapping circles highlight the fact that each patient can have a unique combination of symptoms, and not all symptoms are needed to fulfill the criteria of the end diagnosis migraine. The circles and their overlapping surface areas do not represent population prevalence; the figure serves purely as an illustration. *a,* Relationship between MA and MO diagnostic groups indicating that some patients may be categorized as having purely MA type or purely MO type of attacks, but a considerable number of patients have both types. *b,* Relationships between traits in the IHS criteria, illustrating that, although criteria B–D are not shared by all patients with MA, those with typical aura with migraine headache have to fulfill the MO criteria as well. *c,* Breakdown of the vascular headache trait group. The shaded area indicates patients who fulfill IHS criteria C; at least two of the individual traits have to cooccur (e.g., pulsation and unilaterality).

and clinically plausible way to monitor for possible trait correlations. Although these criteria work well in clinical settings, they still define a very broad syndrome. For example, the criteria classify both a severe, one-sided, and pulsating headache and a moderate, bilateral, and dull headache as migraine, if the other IHS-defined features are present. Allowance for variability is clearly needed for clinical purposes, but a more narrow definition, especially one that uses individual traits that can be quantified, might serve better in dissecting the underlying pathophysiological mechanisms of migraine. Since the use of one specific trait, motor weakness, led to the discovery of genes causing FHM,[18] a similar approach might be useful in more common forms of migraine. A similar division of the features of migrainous headache could contribute to the selection of a more homogeneous study sample.

In summary, since migraine is a syndrome instead of a clearly differentiated disease, we hypothesized that individual clinical components of migraine (i.e., traits such as pulsating pain and photophobia, among others) might represent reflections of specific rather than shared loci and thus independently contribute to susceptibility to migraine. To test this hypothesis, the individual traits and trait groups of the IHS criteria were used as phenotypes for genomewide linkage analyses. These analyses were performed using the same data set of 50 families as was used in our previous study, in which evidence of linkage to chromosome 4q24 was detected using end point diagnosis MA as the trait.[7] By use of the trait-based approach introduced here, individuals from both the MA and the MO diagnosis groups could potentially be scored as affected. This reflects the sharing of the studied traits by migraine diagnoses 1.1 (migraine without aura [appendix B]) and 1.2.1 (typical aura with migraine headaches [appendix C]). Our hypothesis was that the shared components of the two IHS diagnoses might result in migraine phenotypes more reflective of the underlying biological processes than would a strict adherence to the end diagnoses. Here, the aim was to analyze which IHS traits provide best evidence of linkage to the 4q24 locus and whether additional susceptibility loci could be localized.

Material and Methods

Diagnoses

The study sample consisted of 438 genotyped individuals (186 men and 252 women) in 50 independent, multigenerational Finnish families and was the same set that was used in our previous genomewide screen.[7] The ethics committee of Helsinki University Central Hospital approved the study protocol. The inclusion criterion for the families selected for the previous genomewide screen was a high prevalence of MA within the family. Various migraine features concerning the aura, headache, provoking factors, and prodromal symptoms were recorded by one neurologist (M.A.K.) on the basis of the validated Finnish Migraine Specific Questionnaire for Family Studies.[29] Detailed selection and case-definition procedures have been reported elsewhere.[7] In the previous study, 246 individuals received a diagnosis of MA, and 50 individuals received a diagnosis of MO. In the

present study, the updated IHS classification (2004) was used. Under the new classification, 225 individuals fulfilled the IHS criteria shared between MO (1.1 in the IHS diagnoses) and typical aura with migraine headaches (1.2.1). This constitutes 49% of the study sample.

Formation of Phenotypes from the Individual Traits and Trait Groups

Altogether, nine individual traits (attack length, pulsation, unilaterality, aggravation by physical exercise, intensity of pain, photophobia, phonophobia, nausea, and vomiting) and five trait groups (IHS full criteria, pain criteria, associated symptoms, nausea and/or vomiting, and photo- and phonophobia) were analyzed independently as phenotypes in the linkage analysis. Table 1 summarizes the relative frequencies of the features of migrainous headache in the study sample as reported by the patients, defined by the IHS criteria, and used as traits in the analyses. The important distinction between the individual traits and the trait groups is that the individual traits reflect a distinct migraine feature, whereas the trait groups reflect a clinical consensus.

Since all individuals with vomiting also experienced nausea, the nausea and the nausea and/or vomiting categories were equal, and thus the nausea trait was not analyzed. In addition to the IHS traits, we also used the age at onset of migraine symptoms as a trait. Age was dichotomized to those individuals with the onset of migraine symptoms before age 20 years (211 individuals) and those with a later onset (64 individuals).

Genotyping

The process of the genomewide screen has been described in detail elsewhere.[7] Briefly, this screen covered all autosomes and the X chromosome and used 350 polymorphic microsatellite markers, with an average of 11 cM between loci. In the fine-mapping phase, 14 new microsatellite markers were analyzed (9 on chromosome 17 and 5 on chromosome 18). For fine mapping, capillary electrophoresis, as employed by the MegaBACE 1000 DNA Sequencing System (GE Healthcare Bio-Sciences), was used to separate DNA fragments. Alleles were called by the MegaBACE Genetic Profiler 1.5 software (GE Healthcare Bio-Sciences).

Linkage Analyses

Parametric and nonparametric LOD scores were calculated using an affecteds-only strategy (i.e., all individuals not scored as affected were considered as unknown) for the 350 markers genotyped. For parametric analysis, a dominant mode of inheritance and locus heterogeneity was assumed. Each of the IHS traits and trait groups were analyzed, in turn, as the phenotype. All analyses were performed with the disease-gene frequency set at 0.001, under the assumption of autosomal dominant inheritance and a phenocopy proportion of 2.4%, reflecting the frequency of MA in the population,[7,30] in accordance with the strategy proposed by Göring and Terwilliger[31] and in line with our previous research. Allele frequencies were calculated from the genotypes of all individuals. The mistyping option of the SimWalk2 program[32] was used to detect genotyping errors. In case of bilineal family structure, the affected married-in spouse, as well as the offspring, were treated as unknown. The initial linkage analysis was performed using a two-point approach—that is, by using a single marker and the trait. The components of the IHS migraine criteria were used as the phenotype. Two-point parametric linkage analysis was performed both under locus homogeneity and under locus heterogeneity by the computer programs LINKAGE[33] and HOMOG.[34] To lessen the dependence of our results on the assumption of a dominance model, we also completed an affected sib pair (ASP) analysis. The identity-by-descent (IBD) status of each ASP was estimated using the program Sibpair, which favors a recessive mode of inheritance. The ANALYZE utility program[31] was used to conduct these analyses. Multipoint parametric and nonparametric analyses were performed for regions showing evidence of linkage in the parametric two-point analysis by use of the program GENEHUNTER,[35] version 2.1_r5beta. Parametric linkage analysis was performed using the model presented above, while allowing for locus heterogeneity. The nonparametric statistic

Table 1. Frequencies of Individual Trait Components and Trait Groups and the Sex Proportions of Those Affected, According to IHS Criteria,[20] in the Study Sample

Trait or Trait Group Corresponding to IHS Criteria	*n*	Percentage of Total	Percentage Male	Percentage Female
Full criteria[a]:	225	52.3	26.2	73.8
Attack length	241	56.0	29.5	70.5
Vascular headache criteria[a]:	322	74.9	33.9	66.1
Unilateral headache	327	76.0	34.3	65.7
Pulsating headache	235	54.7	27.2	72.8
Intensity, moderate and/or severe:	315	73.3	32.4	67.6
Unbearable	109	31.6	27.5	72.5
Severe	136	31.6	28.7	71.3
Moderate	70	16.3	47.1	52.9
Aggravated by physical activity	239	55.6	29.7	70.3
Associated symptoms[a]:	298	69.3	30.5	69.5
Nausea and/or vomiting[a]:	266	61.9	30.1	69.9
Nausea	266	61.9	30.1	69.9
Vomiting	173	40.2	31.8	68.2
Photo- and phonophobia[a]:	229	53.3	25.8	74.2
Photophobia	265	61.6	30.6	69.4
Phonophobia	234	54.4	26.5	73.5

[a] A trait group.

NPL_{all}, which estimates the statistical significance of alleles shared IBD between all affected family members, was calculated also.

To address the multiple-testing issue and to determine the significance of the results, we applied the equation introduced by Kidd and Ott.[36] They suggest that the lower limit of a significant result in a genomewide scan is 3 plus the base 10 logarithm of the number of independent tests performed. An addition of 0.3 to the significance limits is necessary to take into account the extra df from the heterogeneity LOD (HLOD) calculation. Since the traits we studied are not independent of each other, a correlation matrix of the phenotypes was constructed (table 2), and the software matSPD (see Web Resources) was used to calculate the overall correlation coefficient to determine the equivalent number of independent traits.[37–39] This analysis showed that the original 14 traits (where the one duplicate trait, nausea, is eliminated by the analysis) and trait combinations correspond to just five independent variables; thus, the lower limit of significant evidence of linkage is 4.00. That is, because of the high correlation between the 14 traits, they behave statistically like five effectively independent traits. Similarly, the lower limit of suggestive evidence of linkage is 1.6 plus the base 10 logarithm of the number of tests plus 0.3, which equals 2.60. These limits are only slightly less conservative than the ones obtained using the most conservative correction based on the number of tests. Finally, to further determine the significance of the observed LOD scores, we simulated the marker with the best observed LOD score (*D17S945*) in 10,000 replicates of the pedigree set, assuming no linkage and using the program SIMULATE.[40] The tested limits were the best observed LOD score (4.65) and the approximations of the significance limits (4.00 and 2.60). These three limits were reached zero, zero, and five times by chance in the simulated data sets, with the highest result 3.19, which is less conservative than our significance limit obtained from the trait correlation matrix.

Results

Linkage to 13 migraine-related traits was analyzed in a genomewide scan of 438 individuals from 50 migraine-affected families enriched for MA. Significant evidence of linkage was observed with the individual traits pulsation (17p13), photophobia (4q24), phonophobia (4q24), and age at onset (4q24) (fig. 2 and table 3). With the trait combinations, suggestive evidence of linkage was observed with photo- and phonophobia (4q24) and IHS full criteria (18q12), a combination of the shared traits of diagnoses 1.1 and 1.2.1 of the IHS 2004 classification that includes individuals classified with MA and/or MO as their end diagnosis. Table 3 shows the significant and suggestive two-point LOD scores found.

MA Locus on 4q24

Our previously reported and replicated MA locus on 4q24[7,15] showed significant evidence of linkage to photophobia (two-point HLOD 4.39) and phonophobia (4.10) and suggestive evidence of linkage to pain intensity (3.71), unilaterality (3.25), and pulsation (3.14) as well as the photo- and phonophobia trait group (3.97) and the associated symptom trait group (2.98) (fig. 3*a*) with marker *D4S1647*. With the trait photophobia, GeneHunter multipoint parametric (HLOD 2.05) and nonparametric (NPL_{all} 4.13) analyses positioned the peak at 103 cM and 92 cM, respectively (fig. 4*a*).

New Locus on 17p13.1

Significant evidence of linkage was found between the pulsation trait and marker *D17S945,* where a two-point HLOD score of 4.65 was detected (fig. 3*b*). This encouraging result triggered us to fine map the region with nine additional markers, providing a map interval of 1.7 cM. These markers supported the existence of a susceptibility locus in this region. GeneHunter multipoint parametric (HLOD 3.46) and nonparametric (NPL_{all} 3.94) tests positioned the peak at 19.4 and 22.0 cM for the pulsation trait (fig. 4*b*). Other IHS traits showed, at best, nearly suggestive evidence of linkage to this locus, the highest of which was

Table 2. Correlation Matrix of the Analyzed Traits and Trait Groups

Trait	a	b	c	d	e	f	g	h	i	j	k	l	m	n
a	1.000	.944	.710	.623	.698	.639	.707	.773	.685	.685	.476	.658	.674	.663
b	.944	1.000	.753	.620	.739	.649	.736	.706	.625	.625	.434	.604	.657	.623
c	.710	.753	1.000	.737	.975	.747	.954	.884	.794	.794	.573	.706	.798	.720
d	.623	.620	.737	1.000	.716	.720	.705	.716	.661	.661	.511	.646	.679	.643
e	.698	.739	.975	.716	1.000	.734	.930	.882	.787	.787	.556	.701	.791	.714
f	.639	.649	.747	.720	.734	1.000	.723	.700	.668	.668	.493	.596	.657	.601
g	.707	.736	.954	.705	.930	.723	1.000	.879	.787	.787	.565	.710	.791	.724
h	.773	.706	.884	.716	.882	.700	.879	1.000	.894	.894	.632	.784	.814	.784
i	.685	.625	.794	.661	.787	.668	.787	.894	1.000	1.000	.707	.648	.695	.651
j	.685	.625	.794	.661	.787	.668	.787	.894	1.000	1.000	.707	.648	.695	.651
k	.476	.434	.573	.511	.556	.493	.565	.632	.707	.707	1.000	.481	.458	.490
l	.658	.604	.706	.646	.701	.596	.710	.784	.648	.648	.481	1.000	.880	.982
m	.674	.657	.798	.679	.791	.657	.791	.814	.695	.695	.458	.880	1.000	.860
n	.663	.623	.720	.643	.714	.601	.724	.784	.651	.651	.490	.982	.860	1.000

NOTE.—Correlations were determined across individuals and are not dependent on relatedness of cases. Traits are as follows: a = full criteria; b = attack length; c = vascular headache criteria; d = unilaterality; e = pulsation; f = pain intensity; g = aggravation by physical exercise; h = associated symptoms; i = nausea and/or vomiting; j = nausea; k = vomiting; l = photo- and phonophobia; m = photophobia; n = phonophobia.

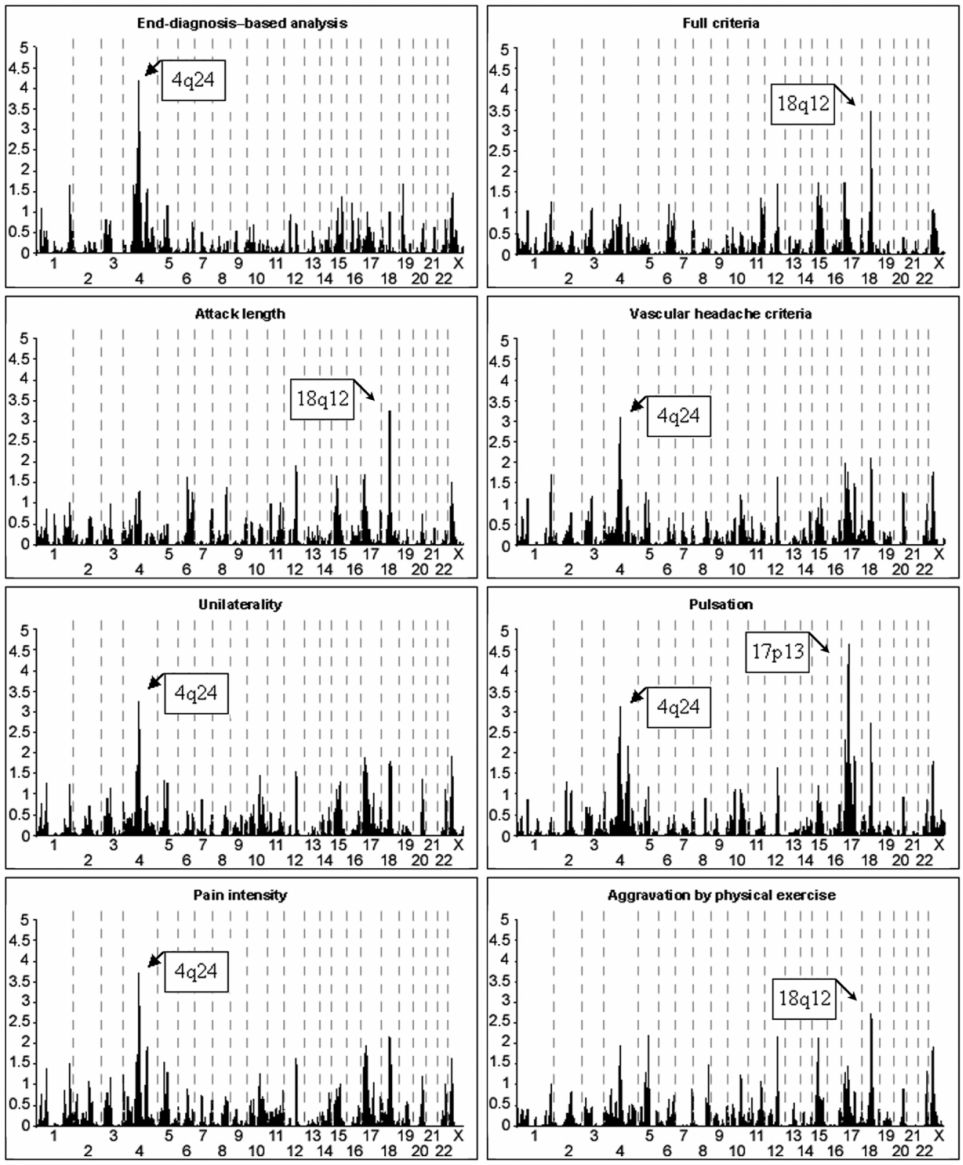
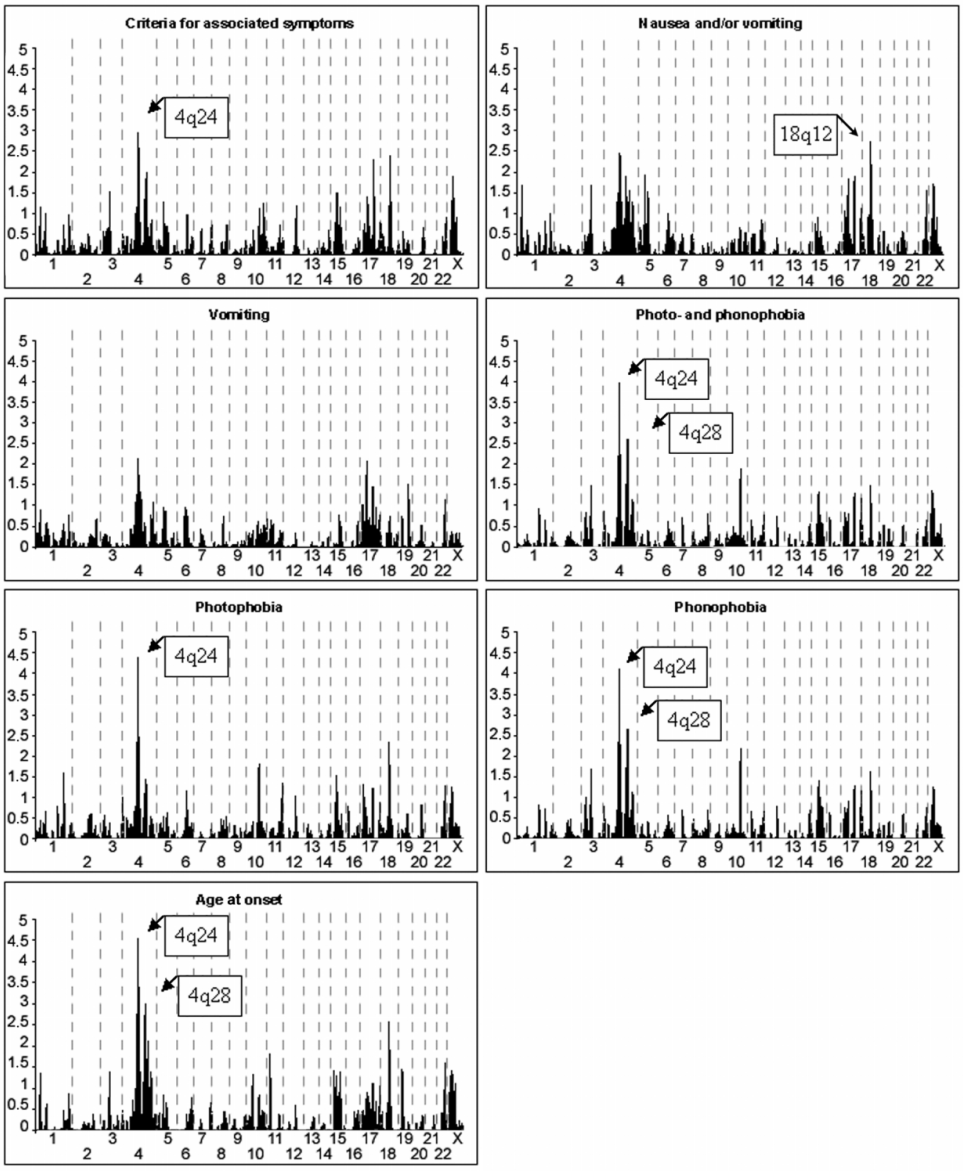


Figure 2. Results of the parametric two-point linkage analysis under heterogeneity for genomewide screen with the end diagnosis MA, the trait components and trait groups, and age at onset used to determine affection status. The lower significance limits are 4.00 for significant evidence and 2.60 for suggestive evidence of linkage. Arrows denote markers with significant or suggestive results.

between the trait vomiting and marker *D17S945* (HLOD of 2.07).

Loci Providing Suggestive Evidence of Linkage

The second new locus was detected on 18q12.1 with marker *D18S877* (fig. 3*c*) and the IHS full criteria (two-point HLOD 3.29). With this marker, individual traits showed suggestive evidence of linkage, with vascular criteria (3.14), attack length (3.00), and aggravation by physical exercise (2.77) showing the highest LOD scores. In addition, vomiting and nausea showed nearly suggestive evidence of linkage at this locus (HLOD 2.66). Neither of



the adjacent markers of the 10-cM marker set (*D18S453*, 12 cM proximal, and *D18S535*, 11.5 cM distal) provided supportive evidence of linkage with any trait. In spite of this, we decided to fine map this locus with five additional markers, providing a map interval of 3.2 cM. Two markers supported a locus in this region, with suggestive (*D18S56* and aggravation by physical exercise) and nominal (*D18S1107* and attack length) evidence of linkage. GeneHunter multipoint parametric analysis positioned the peak at 50.0 cM, with an HLOD of 1.53 (fig. 4*c*) with the IHS full criteria. At this position, however, the NPL_{all} score was 3.40 (at 51.0 cM), suggesting that perhaps the parametric dominant model was not a good fit for this trait. By use of the ASP method and the IHS full criteria, marker

Table 3. Locations and Two-Point LOD Scores for Traits or Trait Groups Providing a LOD Score >2.60 in the Genomewide Screen in at Least One Analysis

Marker (Location) and Trait or Trait Group	Results under Locus Homogeneity		Results under Locus Heterogeneity		ASP Analysis
	LOD Score	*P*	LOD Score	*P*	LOD Score
D4S2380 (4q22):					
Age at onset	1.86	.001713	1.96	.005482	2.93
D4S1647 (4q24):					
Age at onset	4.53	.000002	4.53	.000015	4.02
Photophobia	4.39	.000003	4.39	.000020	2.20
Phonophobia	4.10	.000007	4.10	.000040	2.81
Photo- and phonophobia[a]	3.97	.000010	3.97	.000054	2.76
Intensity	3.71	.000018	3.71	.000097	2.60
Unilaterality	3.25	.000055	3.25	.000281	2.88
Pulsation	3.13	.000073	3.14	.000362	3.49
Vascular headache[a]	3.09	.000081	3.09	.000406	2.30
Associated symptoms[a]	2.98	.000106	2.98	.000524	2.28
Nausea	2.46	.000382	2.46	.001734	3.28
Vomiting and/or nausea[a]	2.46	.000382	2.46	.001734	3.28
Attack length	1.18	.009874	1.24	.028772	2.61
D4S2394 (4q28):					
Age at onset	2.99	.000233	2.99	.001094	.25
D4S1520 (4q31):					
Phonophobia	2.66	.000257	2.66	.001199	.82
Photo- and phonophobia[a]	2.62	.000257	2.62	.001199	1.06
D17S945 (17p13):					
Pulsation	4.65	.000002	4.65	.000011	.82
D18S877 (18q12):					
IHS full criteria[a]	1.06	.013573	3.29	.000256	.26
Vascular headache[a]	.92	.019779	3.14	.000362	.42
Attack length	.95	.018236	3.00	.000500	.09
Aggravation by physical exercise	1.24	.008432	2.77	.000849	.03
D18S862 (18q21):					
Aggravation by physical exercise	1.75	.002264	2.69	.001021	1.21
D18S1364 (18q22):					
IHS full criteria[a]	.03	.364661	.17	.338041	3.04
Attack length	.18	.181291	.29	.256431	2.71

[a] A trait group.

D18S1102, 8 cM distal of the parametric two-point HLOD peak, provided an ASP LOD score of 3.04. Adjacent markers *D18S56* and *D18S535* provided ASP LOD scores of 1.72 and 2.20, respectively.

In addition to the locus on 4q24, a second region on 4q, 4q28-q31, indicated suggestive evidence of linkage with multiple traits and age at onset. Among the traits, the highest evidence of linkage was observed between marker *D4S1520* and the traits phonophobia (HLOD 2.66) and photo- and phonophobia (HLOD 2.62). Furthermore, several markers around marker *D4S1520* showed nearly suggestive evidence of linkage with the same traits.

Loci Providing Nominal Evidence of Linkage

In addition to the chromosomal loci on 4q24, 4q28, 17p13, and 18q12, four other loci with nearly suggestive evidence of linkage were observed on 10q22, 12q21, 15q14, and Xp21. Marker *D10S2327* produced a two-point HLOD score of 2.27 with phonophobia. On chromosome 12q21, marker *D12S1064* produced an HLOD score of 2.17 with the aggravation by physical exercise trait. However, none of the surrounding markers supported linkage to these loci. On 15q14, aggravation by physical exercise produced a two-point HLOD score of 2.14 with marker ACTC. On Xp21, aggravation by physical exercise produced an HLOD of 1.92, associated symptoms an HLOD of 1.91, and unilaterality an HLOD of 1.90 with marker *DXS9896*. At these two loci, a number of adjacent markers also provided nominal evidence of linkage to several traits (three for 15q14 and seven for Xp21).

Age at Onset

In addition to the IHS traits, we analyzed the age at onset of migraine as a trait. Age was dichotomized to consider those patients whose migraine symptoms began before age 20 years as affected and the others as unknown. The analysis showed a significant two-point HLOD score of 4.52 at 4q24 (*D4S1647*). One other marker (*D4S2394*) with

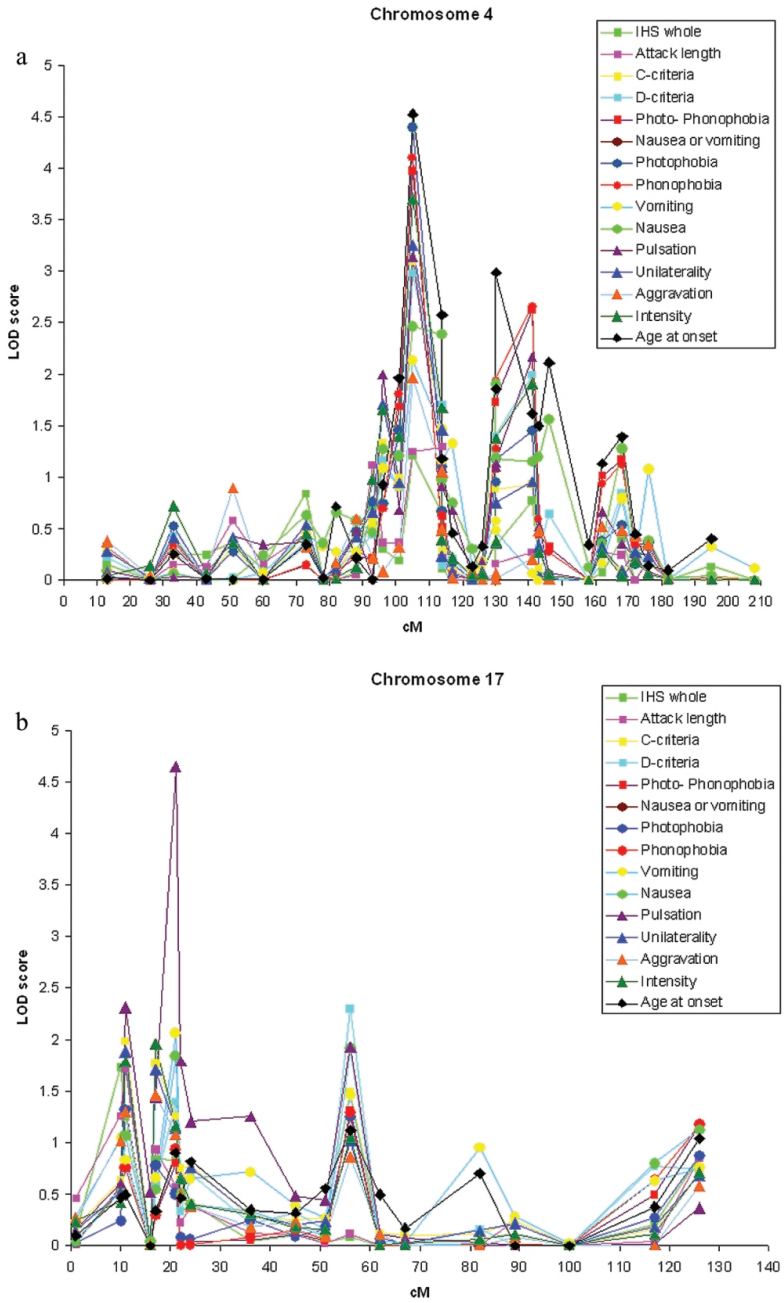
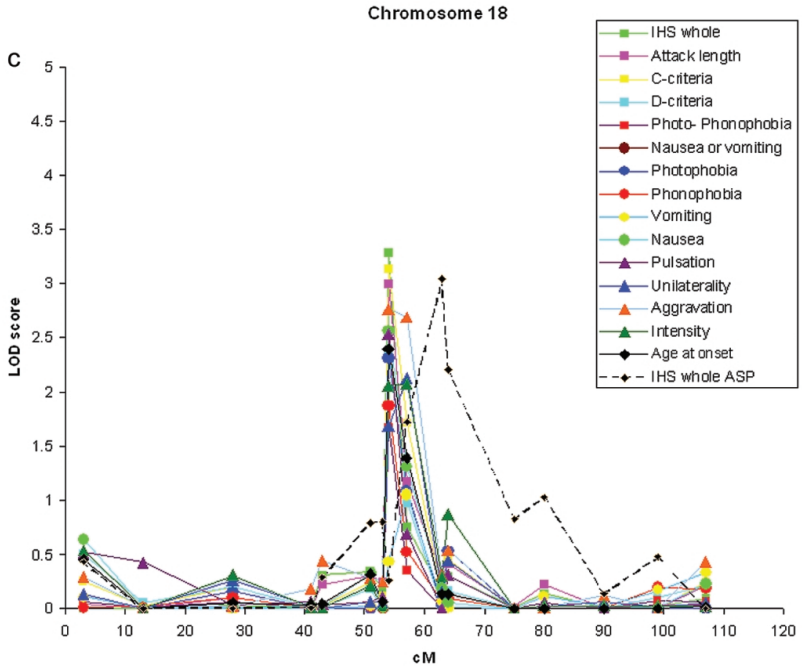


Figure 3. Parametric two-point HLOD scores for chromosome 4 (*a*), chromosome 17 (*b*), and chromosome 18 (*c*) for all trait components and age at onset.



suggestive evidence of linkage was found, on 4q28 (HLOD 2.99), with adjacent markers providing nearly suggestive evidence of linkage, corroborating the second locus on chromosome 4. In analyzing for late onset (at age >20 years), no markers showed evidence of linkage reaching the suggestive level.

Stratification by Sex

In a study of migraine in the Icelandic population,[15] linkage results were improved considerably when the sexes were analyzed separately. Thus, we analyzed the data by treating all females and males, in turn, as unknown for all traits for the chromosomes yielding suggestive or significant results. On the 4q24 locus, no significant differences between the sexes were found. At the 17p13 locus, the pulsation trait showed a two-point HLOD score of 2.02 for females ($n = 174$) and 2.72 for males ($n = 67$). At this locus, no other trait exceeded that score in males, but the intensity trait reached a LOD score of 2.34 in females. On the 18q12 locus, the IHS full criteria produced a nearly significant two-point LOD score of 3.79 for females ($n = 167$) but a LOD of only 1.95 for males ($n = 63$). At this locus, several other traits produced suggestive results (in males, 3.41 for the pain criteria, 3.11 for nausea, and 2.92 for intensity; in females, 2.76 for photophobia), but, with all traits, the sex difference was smaller than the one observed with IHS full criteria. In addition, attack length produced a LOD score of 3.35 in females and 3.23 in males. No other significant differences between the sex-specific phenotypes were found for the two loci.

Discussion

Although introduction of the IHS criteria has done much to ease the diagnostics of migraine disorders and has had a major impact on clinical trials and epidemiological research in this field, none of the features occur in all patients who meet a strict definition of IHS migraine, and no single symptom is required for diagnosis. In other words, although there is considerable correlation between individual symptoms (table 2), migraine is a symptom complex with variable symptom profiles, and individuals presenting with dissimilar symptoms can equally satisfy the same diagnosis (appendixes B and C). In fact, controversy exists about whether MO and MA are actually two separate entities.[41,42] Furthermore, some individuals may not completely satisfy the IHS criteria but are nonetheless considered affected in a clinical setting. We therefore hypothesized that examination of individual symptoms and their subgroupings (i.e., trait components) would provide considerably greater power than use of the IHS end diagnosis to identify genes underlying migraine susceptibility.

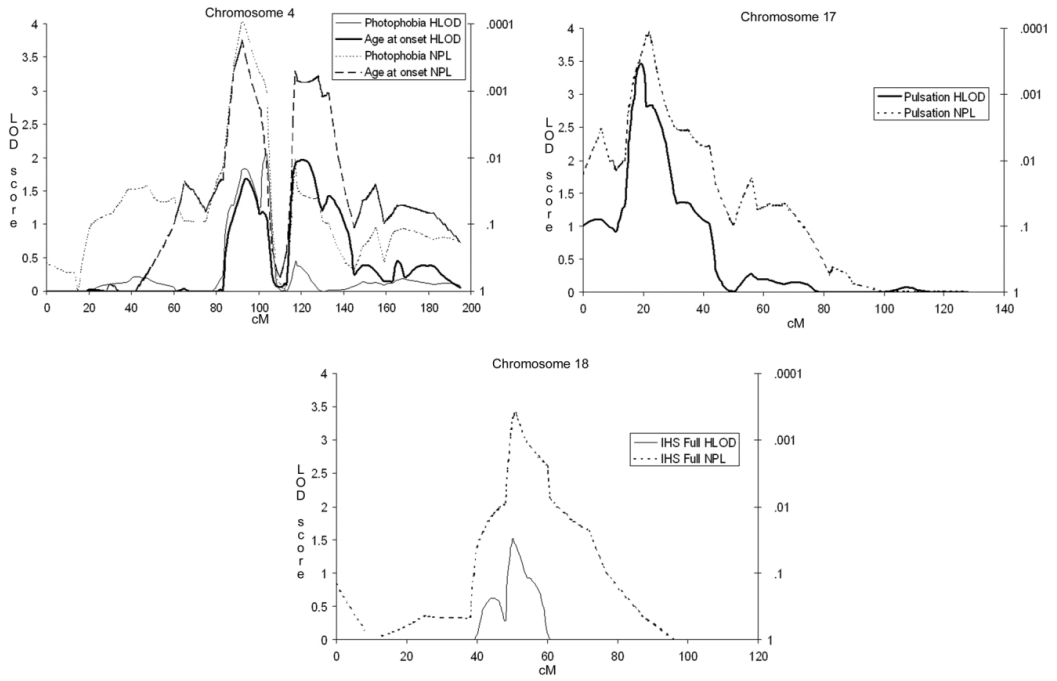


Figure 4. Multipoint parametric HLOD scores (*solid lines*) and nonparametric NPL_{all} scores (*dotted lines*) for chromosome 4 and phonophobia (*a*), chromosome 17 and pulsation (*b*), and chromosome 18 and IHS full criteria (*c*).

The trait-component analysis identified several traits that also show linkage to the previously reported MA locus on chromosome 4q24. Interestingly, the restratification revealed one novel locus (17p13) with significant evidence of linkage to the pulsation trait and two other loci with suggestive evidence of linkage to the traits age at onset (4q28) and IHS full criteria (18q12).

These findings suggest that dissecting the end diagnosis into intermediate phenotypes and/or traits might help to dissect the genetic basis of headache disorders and thus help to stratify study samples into less heterogeneous groups. Here, we were able to identify new loci to which only a subset of migraine-associated symptoms were linked. This supports the well-accepted hypothesis in complex traits that specific gene variants in different loci contribute in different combinations to the individual susceptibility.[43,44] However, this can be verified and further studied only after specific alleles within these loci have been identified. Trait-based approaches have been used in several complex traits, such as asthma,[45] schizophrenia,[46] autism,[26] and hyperlipidemias.[27] The migraine end diagnosis is composed of a number of possibly biologically diverse components; thus, it is plausible to hypothesize different biological processes behind these components. Also, migraine has a distinct set of diagnostic criteria that provide a good starting point for this type of analysis.[19,20] In addition, some biochemical evidence for such an approach has been found in another common primary headache disorder. In tension-type headache, patients with the pulsating subtype seem to have higher calcitonin gene–related peptide levels than those of patients with nonpulsating headache.[47]

Prompted by the differences in the results of the linkage analysis between individual traits and the end diagnosis MA,[7] we analyzed the differences between the affection groups contributing to the peaks at 17p13 and 18q12. Of the individuals in the set pointing to the locus at 17p13, there were 66 individuals with pulsating headache who were coded as unknown in the original study, either because of their lack of aura symptoms or because the migraine was inherited in a bilineal fashion. Conversely, there were 51 individuals with nonpulsating headache who were coded as affected in the original study. Combined, this means a total difference of 49.7% (117 of 235) in the pulsation group classified as affected in this study compared with our previous genomewide scan. Similarly, there were 62 affected individuals in the set pointing to the 18q12 locus who were coded as unaffected in the pre-

vious study because of a non-MA diagnosis or bilineal family structure and, conversely, 61 individuals who did not fulfill the full shared IHS criteria who were coded as affected in the original study. This resulted in a 52% (123 of 225) combined difference between the group showing the 18q12 locus and the end diagnosis group.[7] Most of the difference between these groups is the result of the addition of patients with MO fulfilling the IHS full criteria. In contrast, from the total of 246 individuals with MA, 62 had an attack length of <4 or >72 h. This causes them to fall outside of the 1.2.1 (typical aura with migraine headache) diagnostic group, which is new to the IHS 2004 classification. One could speculate that the change in the classification eliminated excess heterogeneity from the MA group and thus revealed the 18q12 locus.

It is likely that analyzing the trait components of headache is feasible only in highly selected samples—for example, in the present study, in which families with a high incidence of IHS migraine were considered. For instance, if a gene for pulsating headache exists, it will most likely be a small component of the total genetic load that predisposes a patient to the migraine syndrome. In the general population, the coexistence of this pulsating factor and other contributing factors is likely to be rare, but, in study samples already enriched for the presence of migraine, both elements may have a chance to co-occur and predispose the patients in the family to a migraine headache that pulsates ("beats with the heart"). Thus, as for other complex traits, the highly selected families with migraine may be better for identifying some migraine susceptibility genes and thus make identification of those genes a realistic goal. This, in the end, could even help to validate the clinical decisions that form the basis of the current migraine criteria.

It is interesting that, even though several traits provided evidence of linkage at the same locus, individual traits within the main IHS categories provided evidence of linkage at different loci. For example, the traits in the associated-symptoms category (nausea and/or vomiting and photo- and phonophobia) provided evidence of linkage at different loci; vomiting and nausea showed nearly suggestive evidence of linkage on chromosome 18q12, and photo- and phonophobia on chromosome 4q24. Similarly, intensity and pulsation, both traits of the vascular headache criteria, provided evidence of linkage on different chromosomes: 4q24 and 17p13, respectively. This could suggest that, even if the grouping of IHS symptoms is logical and practical in clinical practice, it may not reflect the genetic background of the susceptibility of individual traits. This is not very surprising in light of what is known about the heterogeneity of the clinical findings in Mendelian forms of migraine. In FHM-affected families, family members with the same underlying mutation can have different phenotypes, ranging from severe hemiplegic symptoms to MO to having no migraine at all.[18] In other complex traits, the idea of using subgroups of clinical classifications (e.g., DSM-IV in schizophrenia) is under active study, even though the approach has yet to provide support for stratifying the sample for susceptibility classification. However, much of the trait relationship between susceptibility loci remains speculative as long as the underlying allelic variants are not identified; because the linkage information is provided by relatively few meioses, there is a possibility that some of these trait differences weaken or disappear once association analyses are performed.

In further analyses, it was found that, at the 18q12 locus, a few large families provided clearly negative LOD scores (three families had LOD scores ranging from −1.65 to −2.17), likely contributing to the large difference between the LOD scores under locus homo- and heterogeneity. By analyzing the data without those families, the difference between the scores decreases considerably, with the homogeneity LOD score rising closer to the level of the HLOD score. We also observed a large difference between the sex-specific LOD scores, with a considerably higher female score. The reason for this difference is not clear, as it might reflect a true sex difference at this locus or merely follow from the smaller sample size of males. The sex-specific analysis is a relevant option in migraine because of the high difference in prevalence between the sexes.[1]

At present, several loci, 4q21-q24,[7,15] 5q21,[11] 6p12.2-p21.1,[14] 11q24,[8] 14q21.2-q22.3,[13] and 15q11-q13,[9] with significant evidence of linkage to common forms of migraine have been reported, with only the chromosome 4q21-q24 region detected in two independent studies. Interestingly, the most significant new locus identified in this study, on 17p13, has shown nominal evidence of linkage both in our previous study[7] and in an Australian study.[10] It remains to be seen whether this locus harbors a specific variant(s) contributing to the sensation of pulsating pain. Our results also seem to corroborate several other previous findings. The locus on 18q12 has been implicated in an Icelandic study,[15] in which the same marker (*D18S877*) gave a LOD score of 1.50. That study was conducted using 103 families with MO-affected members, which ties well with our approach and results. Also, a marker 13 cM away (*D18S53*) gave a LOD score of 2.30 in an Australian study.[10] The general observation in migraine, for which different studies provide different loci, is typical for complex traits for which finding a causative variation has proven difficult, such as hypertension[25] and schizophrenia.[48]

Furthermore, it is interesting to note that several of our findings agree with those from an empirical clustering approach called "latent class analysis" (LCA), introduced in a recent article by Nyholt et al.[49] and applied to migraine symptom data in genome scans in two separate collections of migraine-affected Australian families.[10,11] LCA is a statistical method, closely analogous to cluster analysis, for finding subtypes of related individuals (latent classes) from multivariate categorical data.[50] Briefly, a latent class cluster model describes the relationship between a set of observed variables and an unobserved, latent variable. The

Table 4. Results of Previous Genomewide Scans in Migraine with Suggestive or Significant LOD Scores and the Corresponding Results of the Present Study

Chromosomal Arm	Study Reference	Studied Phenotype	Marker	LOD Score	LOD Score in This Study	Trait in This Study
3q	10	LCA-severe[a]	*D3S1311*	2.28[b]	1.23	Intensity
4q	15	Loose MO[c]	*D4S1534*	2.87[b]	2.00	Pulsation
4q	15	Females-only loose MO[c]	*D4S2409*	4.08[b]	2.00	Pulsation
4q	7	MA	*D4S1647*	4.20[d]	4.53	Age at onset
5q	11	LCA-severe[a]	*D5S2501*	3.03[b]	.03	Intensity
6p	14	MA and MO	*D6S452*	5.41[d]	.83	Vomiting
10q	11	LCA-severe[a]	*D10S2327*	2.32[b]	2.27	Phonophobia
11q	8	MA	*D11S4464*	4.24[d]	.53	Nausea and/or vomiting
14q	13	MO	*D14S978*	3.70[d]	.18	Attack length
18p	10	LCA-severe[a]	*D18S53*	2.30[b]	.15	Aggravation by physical exercise
18q	…[e]	MA and MO	*D18S877*	…	3.43	IHS full criteria

[a] LCA refers to latent class analysis, as presented elsewhere.[49]

[b] Nonparametric multipoint results.

[c] Refers to a reduced end diagnosis; in the study,[15] it included patients who did not fulfill either vascular headache criteria or associated symptom criteria of the IHS classification.

[d] Parametric two-point results.

[e] A marker in the present study, with a distance of 13 cM to the *D18S53* marker in the study by Lea et al.[10]

categories of this latent variable are called "latent classes," or clusters. Therefore, LCA groupings consist of individuals with different numbers and combinations of symptoms and thus reflect a measure of severity, different from the traditional end point diagnosis based on strict IHS diagnostic criteria and also different from the individual-trait analysis used here. The LCA approaches migraine from a different viewpoint than does our trait-component analysis, as the LCA classes are, in effect, new classifications combining features from both end diagnosis and trait components, formed on the basis of heritability estimates. The important point, however, with respect to our approach, is that the LCA analysis[49] named a typical trait most correlated with each class in a manner approaching the present analysis, and it is interesting to note that several results from these two analyses agreed with each other.

Interestingly, the Australian LCA study of twins[11] implicated a locus on 10q22 (nonparametric multipoint LOD score of 2.13), which was attained with marker *D10S2327,* which also gave a two-point LOD score of 2.78 in this study. They also reported this marker to be "most associated with phonophobia and photophobia,"[11(p.500)] and it is intriguing that the same marker and same clinical trait produce suggestive results in two different populations. Our 12q21 peak marker *D12S1064* had a nominal LOD score of 0.74 in our previous study,[7] which also reported the Xp21 peak marker *DXS9896* (LOD 1.08). Marker ACTC on chromosome 15 lies next to a genomic region containing three GABA receptor genes that were linked to migraine in an Italian study of 10 families with MA.[9] As can be expected, many loci reported in other studies (table 4) did not show evidence of linkage beyond the nominal level. Similarly, no linkage to the FHM1,[18] FHM2,[16] and FHM3[17] loci on chromosomes 19p13, 1q23, and 2q24 was found in this study. Traits with the highest evidence of linkage at these loci were age at onset (LOD score 0.53), photo- and phonophobia (0.93), and photophobia (0.62), for the FHM1, FHM2, and FHM3 loci, respectively. In our earlier 2002 study, none of the three loci showed evidence of linkage.[7] The newly identified regions contain several counterparts to the known FHM genes. Other interesting candidates for additional research include genes participating in energy metabolism and neurotransmitter release.

Further studies performed with these traits are needed to confirm whether trait analysis can reclassify patients into more-homogeneous groups. The findings here suggest that trait information collected to establish the IHS-based diagnosis can provide an additional tool for stratifying a migraine study sample. This should be applicable not only to family studies but also to case-control studies. It is hoped that subsequent studies will confirm the validity of this approach.

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Appendix A

Migraine Subtypes According to the International Classification of Headache Disorders, 2nd Edition, 2004[20]

1.1 Migraine without aura
1.2 Migraine with aura
- 1.2.1 Typical aura with migraine headache
- 1.2.2 Typical aura with nonmigraine headache
- 1.2.3 Typical aura without headache
- 1.2.4 Familial hemiplegic migraine
- 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 Cyclical 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
- 1.6.3 Probable chronic migraine

Appendix B

IHS Criteria for Migraine without Aura, 1988 and 2004[19,20]

1.1 Migraine without aura
- A. At least five attacks fulfilling criteria B–D
- B. Headache attacks lasting 4–72 h (untreated or unsuccessfully treated)
- C. Headache has at least two of the following characteristics:
  1. Unilateral location
  2. Pulsating quality
  3. Moderate or severe intensity (inhibits or prohibits daily activities)
  4. Aggravation by walking stairs or similar routine physical activity
- D. During headache, at least one of the following:
  1. Nausea and/or vomiting
  2. Photophobia and phonophobia
- E. At least one of the following:
  1. History, physical- and neurological examinations do not suggest secondary cause of headache
  2. History, physical- and neurological examinations do suggest such disorder, but it is ruled out by appropriate investigations
  3. Such disorder is present, but migraine attacks do not occur for the first time in close temporal relation to the disorder

Appendix C

Comparison of the Criteria for IHS Diagnoses 1.2 Migraine with Aura of the 1988 Classification and the New 1.2.1 Typical Aura with Migraine Headache of the 2004 Classification

1.2 Migraine with aura (1988)
- A. At least two attacks fulfilling criterion B
- B. At least three of the following four characteristics:
  1. One or more fully reversible aura symptoms indicating focal cerebral cortical and/or brain stem dysfunction
  2. At least one aura symptom develops gradually over >4 min, or ⩾2 symptoms occur in succession
  3. No aura symptom lasts >60 min. If more than one aura symptom is present, accepted duration is proportionally increased
  4. Headache follows aura with a free interval of <60 min (it may also begin before or simultaneously with the aura)
- C. At least one of the following:
  1. History, physical- and neurological examinations do not suggest secondary cause of headache
  2. History, physical- and neurological examinations do suggest such disorder, but it is ruled out by appropriate investigations
  3. Such disorder is present, but migraine attacks do not occur for the first time in close temporal relation to the disorder

1.2.1 Typical aura with migraine headache (2004)
- A. At least two attacks fulfilling criteria B–D
- B. Aura consisting of 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 (i.e., loss of vision)
  2. Fully reversible sensory symptoms including positive features (i.e., pins and needles) and/or negative features (i.e., numbness)
  3. Fully reversible dysphasic speech disturbance
- C. At least two of the following:
  1. Homonymous visual symptoms (note: additional loss or blurring of central vision may occur) and/or unilateral sensory symptoms
  2. At least one aura symptom develops gradually over ⩾5 min, and/or different aura symptoms occur in succession over ⩾5 min
  3. Each symptom lasts ⩾5 and ⩽60 min
- D. Headache fulfilling criteria B–D for 1.1 Migraine without aura begins during the aura or follows aura within 60 min
- E. Not attributed to another disorder (note: history and physical and neurological examinations do not sug-

gest any of the disorders listed in groups 5–12, or history and/or physical and/or neurological examinations do suggest such disorder but it is ruled out by appropriate investigations, or such disorder is present but attacks do not occur for the first time in close temporal relation to the disorder)

Web Resources

The URLs for data presented herein are as follows:

matSpD, http://genepi.qimr.edu.au/general/daleN/matSpD/
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for migraine, *CACNA1A, ATP1A2, SCN1A,* FHM1, FHM2, and FHM3)

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II

Consistently Replicating Locus Linked to Migraine on 10q22-q23

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Here, we present the results of two genome-wide scans in two diverse populations in which a consistent use of recently introduced migraine-phenotyping methods detects and replicates a locus on 10q22-q23, with an additional independent replication. No genetic variants have been convincingly established in migraine, and although several loci have been reported, none of them has been consistently replicated. We employed the three known migraine-phenotyping methods (clinical end diagnosis, latent-class analysis, and trait-component analysis) with robust multiple testing correction in a large sample set of 1675 individuals from 210 migraine families from Finland and Australia. Genome-wide multipoint linkage analysis that used the Kong and Cox exponential model in Finns detected a locus on 10q22-q23 with highly significant evidence of linkage (LOD 7.68 at 103 cM in female-specific analysis). The Australian sample showed a LOD score of 3.50 at the same locus (100 cM), as did the independent Finnish replication study (LOD score 2.41, at 102 cM). In addition, four previously reported loci on 8q21, 14q21, 18q12, and Xp21 were also replicated. A shared-segment analysis of 10q22-q23 linked Finnish families identified a 1.6-9.5 cM segment, centered on 101 cM, which shows in-family homology in 95% of affected Finns. This region was further studied with 1323 SNPs. Although no significant association was observed, four regions warranting follow-up studies were identified. These results support the use of symptomology-based phenotyping in migraine and suggest that the 10q22-q23 locus probably contains one or more migraine susceptibility variants.

Introduction

Migraine (MIM 157300) is the most common cause of chronic episodic severe headache. It affects some 15% of the adult population and has a well-established genetic component[1–4] on the basis of family and twin studies. It is more prevalent among women, with a ratio of roughly one male to every three female migraineurs.[1] Migraine is the most common neurological cause of a doctor visit and places a heavy financial, social, and psychological burden on a significant part of the general population. The estimated annual cost of migraine in Europe is €27 billion.[5]

Although evidence from family studies and twin studies have demonstrated the contribution of genetic factors to migraine susceptibility,[3,6,7] identification of specific genetic variants for common forms of migraine has not been forthcoming. No variants predisposing to common forms of migraine have been convincingly established, and no whole-genome association (WGA) studies have been reported for any headache disorders to date. Genome-wide linkage studies have pointed to several loci in both migraine with and without aura.[8–15] Unfortunately, so far there has been little concordance between linkage reports because most studies have identified a locus or two, which have not been convincingly replicated in other studies. Applying findings from other complex disorders suggests that the lack of progress in gene identification may be attributable to etiologic or phenotypic heterogeneity, gene-environment interaction, or epistasis. Another possible reason is genetic (locus) heterogeneity, in which only a subset of pedigrees segregates markers linked to a particular risk locus. Then, even if the study sample consists of a large number of families, individual large families within the sample carrying rare, relatively high-impact gene variations predisposing to migraine can be overly represented in the linkage signal. This would explain some of the difficulties with replication, and better understanding of how to account for these factors would help in targeting future studies as well as help in interpreting results from whole-genome association studies. Finally, we hypothesize that one of the reasons behind this inconsistency might be related to the difficulty of phenotyping headache disorders, causing heterogeneity in sample ascertainment.

One of the major impediments to gene identification of migraine is the lack of valid biological markers with which

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Table 1. Diagnostic Criteria for Migraine without Aura and for the Headache Associated with Typical Aura with Migraine Headache According to the International Classification of Headache Disorders, Second Edition

1.1. Migraine without Aura[a]
A. At least five attacks fulfilling criteria B–D
B. Headache attacks lasting 4–72 hr (untreated or unsuccessfully treated)
C. Headache has at least two of the following characteristics:
1. Unilateral location
2. Pulsating quality
3. Moderate or severe intensity (inhibits or prohibits daily activities)
4. Aggravation by walking stairs or similar routine physical activity
D. During headache, at least one of the following:
1. Nausea and / or vomiting
2. Photophobia and phonophobia
E. [Exclusion of secondary causes of headache]

[a] For typical aura with migraine headache (1.2.1): "Headache fulfilling criteria B–D for 1.1 Migraine without aura begins during the aura or follows aura within 60 min."

a presumptive diagnosis of migraine can be made. A migraine diagnosis is based on fulfillment of symptom criteria formulated by the International Headache Society (IHS).[16,17] The criteria define two main subtypes of migraine, migraine with aura (MA) and migraine without aura (MO), which together account for a majority of all migraine. Most studies performed so far have used the migraine end diagnosis as the primary phenotype, i.e., by considering only patients with either MA or MO diagnosis as affected in analysis. Although the IHS classification works well and is fundamental in clinical practice, it may not be an optimal strategy for uncovering underlying genetic mechanisms and pathways contributing to the disease. The second edition of the IHS classification[17] introduced the same basic symptom criteria (see Table 1) for MO and typical aura with migraine headache (a major subgroup of MA). This, combined with studies suggesting migraine with and without aura are manifestations of the same underlying disorder,[18,19] have led to joint genetic analysis of patients from both diagnosis groups. This, in turn, gave rise to the idea of concentrating on one or few cardinal migraine symptoms, which might better reflect the underlying pathophysiology.

Two alternative analytic strategies, one utilizing latent classes,[18] the other examining trait components,[11] have recently been developed for use in genetic studies of migraine. In the latent-class analysis (LCA) approach, individuals are classified into empirically derived groups on the basis of patterns of IHS symptom clustering observed in a large Australian twin sample.[18] Although considerably more individuals were classified as being affected with "migrainous headache" via LCA (prevalence 36% versus 15% for clinically determined migraine), additional studies in Australian[18] and Dutch[20] twin populations have shown that the LCA classification is able to demonstrate linkage to loci undetectable with only the end diagnosis. An alternate strategy is the trait-component analysis (TCA) approach, which takes direct advantage of the available clinical information in the IHS symptom data in order to classify the patients into groups. This approach has the advantage of reflecting known variables obtained directly from patients with no intervening hypotheses about latent structure and relationships of the traits. It is also simple to implement from patient questionnaires or interviews and has proved to be successful in demonstrating linkage to loci undetectable with traditional methods in a previous Finnish study.[11] Encouraged by our previous results with these alternative phenotyping strategies and their potential to facilitate data integration from different phenotyping schemes, we genotyped and analyzed two new, independent genome-wide linkage scans from Finland and Australia. The samples are of roughly equal size but have differences in their ascertainment strategies and pedigree structures, allowing us to test the phenotyping methods in a variety of conditions. Further, the special population history of Finns provides an advantage to potential restriction of any linked locus through extended haplotype sharing.

Material and Methods

Patients

The Finnish study sample for the genome-wide scan consisted of 690 migraine patients and their relatives (407 women and 283 men) in 58 independent, multigenerational families. The Australian sample consisted of 661 individuals (420 women and 241 men) in 125 independent nuclear families. The Finnish replication sample consisted of 324 migraine patients (202 women and 122 men) in 27 independent, multigenerational families. In total, we studied 1675 individuals from 210 independent families. All participants gave informed consent, and approval to conduct the research was obtained from the Helsinki University Central Hospital Ethics Committee for the Finnish study and from the Queensland Institute of Medical Research (QIMR) Human Research Ethics Committee and the Australian Twin Registry for the Australian study. For the follow-up association study, two study samples from the Finnish population were used. The first study sample consisted of 39 unrelated trios with discordant parents selected so that both the affected parent and an affected offspring carry the family-specific segregating "risk haplotype" and that the unaffected parent did not. The case-control set contained 256 unrelated MA cases selected from the Finnish patient collection and 230 controls from a Helsinki-based-population control sample.

Diagnoses and Phenotypes

The Finnish families were selected from a large Finnish migraine patient collection, ascertained from neurology clinics nation wide during the last 15 years. The patients have been collected from families with three or more affected members fulfilling migraine criteria upon admission. Data on IHS attack symptoms as well as other clinical features were collected with the validated Finnish Migraine Specific Questionnaire for Family Studies ($FMSQ_{FS}$)[21] and by a neurologist's examination of index patients. The same neurologist (M.K.) diagnosed all Finnish patients. The replication sample consists of large families selected from the same patient collection, with a preference for more severe migraine patients, including those with hemiparesis symptoms, because of findings in the Finnish genome-wide sample.

Table 2. Distribution of Migraine Diagnoses within the Finnish Study Samples

	Finns				Australians	
	Genome-wide Sample		Replication Sample		Genome-wide Sample	
Diagnosis	n	of Total	n	of Total	n	of Total
Pure MA[a]	169	24%	44	14%	191	24%
Pure MO	79	11%	35	11%	78	10%
Unclassified MA[b]	89	13%	33	10%		
Mixed migraine[c]	110	16%	78	24%		
Equivalent migraine	7	1%	2	1%		
Headache	26	4%	11	3%		
No headache	169	24%	61	19%	230	28%
Possible migraine[d]	27	4%	18	6%		
Unknown	19	3%	42	13%	305	38%
MA end diagnosis	368	53%	155	48%	191	24%
Total	690	100%	324	100%	804	100%

Note the Australian symptom data do not allow the strict separation of migraine with aura patients into unclassified MA, mixed migraine, and pure migraine with aura subgroups.

[a] Pure MA refers to patients with all attacks fulfilling IHS criteria for migraine with aura.

[b] Unclassified MA refers to an additional, non-IHS diagnosis group for patients that cannot be grouped into any of the defined IHS categories. Patients in this category suffer from attacks in which clearly aural features are present but not in a form recognized by the current diagnostic criteria.

[c] Mixed migraine refers to a patient group in which attacks both with and without aura are commonly present.

[d] Possible migraine refers to a patient group with episodic headache with some migrainous features, who may or may not fulfill one of the probable migraine (1.6) diagnoses of the IHS criteria but miss required aspects of migraine with or without aura.

The Australian families were selected from two population-based twin cohorts, one of nuclear families of twins born between 1902 and 1964[22] and one of twins born between 1964 and 1971,[23] with an overall prevalence of 15.3% of IHS migraine without aura. The included pedigrees were selected on the basis of having at least one pair of siblings affected for the common LCA-derived "migrainous headache" phenotype (prevalence of 36%[18]) and then prioritized on the maximum number of available siblings, irrespective of affection status. Data on IHS attack symptoms[16,17] were gathered with an extensive semistructured telephone interview that included diagnostic questions for migraine (Australian questionnaires for the older and younger cohorts, see Web Resources), developed by an experienced migraine researcher (K.R.M.).[24] Using a similar screening approach, Stewart et al.[25] obtained a 92.6% positive predictive value of their telephone interview diagnosis compared with their clinical examination. For the younger cohort, data for two IHS diagnostic variables (Table 1), nausea and vomiting (ICHD-II code: 1.1.D.1), were recorded together. For the older cohort, data on three variables, pain intensity (1.1.C.3), typical attack length (1.1.B), and whether patients have had at least five attacks during lifetime (1.1.A), were unavailable, but symptom patterns of the younger cohort were used to extrapolate those phenotypes for the older cohort. Data on whether an individual's headache was aggravated by walking stairs or similar routine physical activity (1.1.C.4) were missing for both cohorts, and thus that trait was excluded from the study. We used an answer to a visual aura-specific question to determine the MA end diagnosis.

Table 3. Number of Affecteds, Frequencies of Individual Trait Components, and the Gender Proportions of Those Affected for All Traits and Trait Groups within the Study Samples

Phenotype (n)	of Total	Finns	Australians	Males	Females
Total subjects (1675)	-	61%	39%	39%	61%
MA end diagnosis (621)	37%	41%	31%	21%	49%
Latent class CL23[a] (790)	47%	48%	45%	29%	60%
Latent class CL3 (599)	36%	36%	36%	15%	49%
Attack length (781)	47%	45%	49%	35%	60%
Unilaterality (727)	43%	48%	36%	30%	52%
Pulsation (778)	46%	49%	42%	35%	54%
Intensity (1033)	62%	67%	53%	46%	71%
Nausea/vomiting (870)	52%	55%	48%	34%	63%
Photophobia (918)	55%	59%	47%	37%	66%
Phonophobia (826)	49%	50%	47%	31%	61%

[a] Refers to a combination of latent classes CL2 and CL3.

Three different phenotype groups were prepared. "MA end diagnosis" covers all migraine with aura patients and includes individuals from diagnosis groups "pure MA," "unclassified MA," and "mixed migraine" as affected (see Table 2 for definitions). Table 2 details the diagnosis distribution within the study samples, including a detailed diagnosis breakdown for the two Finnish study samples, in which the larger amount of available clinical information and expertise allows for a higher diagnostic specificity for the clinical diagnosis. The Australian study questionnaire has fewer migraine-specific questions and is designed to identify migraine with high sensitivity but does not allow for distinguishing between different subtypes of MA. The latent-class definitions were estimated from each patients' symptom distribution with the same algorithm as in the original LCA study.[18] In brief, of the four latent cluster groups in LCA (termed CL0, CL1, CL2, and CL3), all individuals satisfying the IHS MA or MO diagnostic criteria are encompassed by groups CL2 and CL3, and the combination of these two groups will be referred to as "LCA migrainous headache." Group CL3, which has the majority of MA patients, is referred to as "LCA severe migraine." Trait-component phenotypes were recorded directly from the questionnaire data of all patients fulfilling any migraine diagnosis. Table 3 summarizes the proportions of the different phenotypes.

Genotyping

All genotyping was performed in the Finnish Genome Center, with the same equipment and conditions. The genotyping procedure was conducted with standard methods on the ABI or the MegaBACE genotyping systems. Genotyping was based on the LMS-MD10 microsatellite marker set (Applied Biosystems, Foster City, CA, USA). The marker set uses 387 markers for a 9.5 cM average intermarker distance and covered all autosomes and the X chromosome. For the ABI system, genotyping was performed with the ABI 3730 capillary sequencing instrument, and PCR products were resolved with the ABI 3730 data collection software and sized with the Genemapper software package from Applied Biosystems. For the MegaBACE system, capillary electrophoresis employed by the MegaBACE 1000 DNA Sequencing System (GE Healthcare Bio-Sciences, Piscataway, NJ, USA), was used for separating DNA fragments. Alleles for this system were called by the MegaBACE Genetic Profiler 1.5 software. In addition, seven more markers were genotyped at chromosome 10q22-q23, resulting in a coverage of 2.21 cM average intermarker distance from marker

D10S218 to D10S2470. The Finnish replication sample was genotyped only for these markers. All genotypes were verified by human inspection, and the PedCheck1.1[26] computer program was used for detecting genotyping errors.

For the follow-up association study, an Illumina Golden Gate assay (Illumina, San Diego, CA, USA) was used to genotype 1536 single-nucleotide polymorphisms (SNPs) in altogether 564 individuals across the region defined by the shared haplotype (chr10, 78.233–88.884 Mb, NCBI build 35) at the Broad Institute. These 1536 SNPs on chromosome 10 (build 35, 78.233−88.884 Mb) were selected as tag-SNPs with Haploview's Tagger-option with CEU population in the HapMap SNP set (v21), and we selected to tag SNPs with minor allele frequency $\geq$10% and r2 threshold of $\geq$0.8. The selected 1536 tag SNPs tagged 94% of the 8290 SNPs (MAF $\geq$0.10) with r2 $\geq$0.8 and 99% of the SNPs with r2 $\geq$0.5. The Illumina BeadStudio software version 3.1.0.0 (Illumina) was used for calling the SNP genotypes, and each SNP was evaluated for quality of the genotypes. Only samples that had success rate of $\geq$97% and SNPs with 95% were considered in the statistical analyses of the SNP data, and thus of the 1536 original SNPs, 1323 passed our rigorous quality control. Because of the difficulty involved in genotyping the region around the known CNV at ~81.3 Mb, there were no successfully genotyped SNPs between 81,058,202 and 81,674,055 base pairs, resulting in a 615 kilobase gap in the assay coverage.

Linkage and Association Analysis

For the genome-wide analyses, multipoint nonparametric linkage analysis was performed with the MERLIN computer program.[27] The MERLIN NPL_{pairs} and NPL_{qtl} Z score statistics are implemented in the general framework of Whittemore and Halpern.[28] These Z scores are used by MERLIN to construct a likelihood ratio test for linkage and define a LOD score statistic with the exponential modeling procedure of Kong and Cox.[29]

For the Finnish families, in line with our previous research,[8,11] we employed an affecteds-only strategy (i.e., all individuals not classified as affected were considered to have an "unknown" phenotype) to allow for reduced penetrance, lack of environmental exposure, etc. We used the nonparametric MERLIN NPL_{pairs} Z score statistic[30] to test for increased allele sharing among affected individuals. To avoid biasing our results on possible overrepresented rare variants in a few large families, we also analyzed the Finnish genome-wide sample as nuclear families. For consistency with the previous Australian genome-wide linkage scan,[8] in order to use the information from unaffected individuals, we used a nonparametric quantitative trait linkage (NPL_{qtl} Z-score) statistic for the analyses of the Australian families in order to obtain additional linkage information from unaffected individuals. In this analysis, affected individuals were coded as "1," unaffected individuals were coded as "0," and those with missing phenotypes were coded as "x." The validity of this, as well as the original regression Haseman-Elston approach[31] for binary traits, has been proven consistently.[32] For the combined genome-wide analysis of Finnish and Australian pedigrees, we used nuclear families to avoid biasing the signal because of the larger Finnish families, and the NPL_{pairs} Z-score statistic was used with the usual "affection" phenotype coding of 0, 1, and 2 to represent unknown/missing, unaffected, and affected individuals, respectively. In addition, we performed a sex-specific analysis by alternatively considering only the affected females or males as "true" affecteds and treating the affecteds of the other gender as having an "unknown" phenotype. In addition, a haplotype shared-segment analysis was performed in the Finnish families. The GENEHUNTER software,[33] version 2.1_r5beta, was used for construction of pedigrees showing the paternal and maternal haplotypes for the additional markers at this locus for the families showing a family-specific NPL_{all} score greater than 1.00 at the location of the highest LOD score.

For the follow-up association study, PLINK software version 1.00[34] was used for all analyses. We employed the DFAM analysis (−dfam) to detect association in the combined set of trios and the case-control subjects. Results were corrected through adaptive permutation (−perm) with PLINK default settings.

Significance Limits

To account for all the phenotypes tested, we needed to apply robust correction for multiple testing. To start, rather than to use the significance thresholds of Lander-Kruglyak (L-K),[35] conservative for microsatellite-based linkage scans due to the unrealistic assumption of having complete (100%) inheritance information, we estimated the significance thresholds for affected sibpair analysis of 400 markers by using the formulae presented by Feingold et al.[36] The L-K threshold for significant evidence of linkage ($p = 0.000022$, corresponding to a standard LOD score of 3.63) is decreased to $p = 0.00009$ (corresponding to a LOD score of 3.05). Similarly, the threshold for suggestive linkage is reduced from $p = 0.00074$ (LOD score of 2.19) to $p = 0.0023$ (LOD score of 1.74).[37] These theoretically derived thresholds are consistent with those obtained via simulation by ourselves[8] and others.[38–41] To correct for the multiple phenotypes (including the sex-specific analyses) used in this study, we applied the program matSpD (see Web Resources) to estimate the equivalent total number of independent tests performed (six), resulting in robust Bonferroni-corrected significance thresholds of 6.18 [$5.40 + \log_{10}(6)$] for highly significant evidence of linkage, 3.83 [$3.05 + \log_{10}(6)$] for significant evidence of linkage, and 2.52 [$1.74 + \log_{10}(6)$] for suggestive evidence. For the replication set, we applied the L-K replication threshold of LOD 1.8 (nominal evidence of linkage, $p = 0.01$, for five independent tests), equal to fine mapping a 10 cM area.[35] For the follow-up association study, we used the snpSpD program (see Web Resources) to estimate the number of independent SNP tests after accounting for LD (761.7), resulting in Bonferroni-corrected significance threshold of 6.73×10^{-5}.

Results

Genome-wide multipoint linkage analysis of 387 microsatellite markers was performed in two independent study samples; this was followed by an analysis of a locus-specific Finnish replication sample. All samples were analyzed separately as well as jointly. A locus on 10q22-q23 showed significant evidence of linkage in Finns as well as in the joint analysis and suggestive evidence of linkage in the Australian study. A sex-specific analysis, considering only females as affected, improved the linkage signal to the level of highly significant evidence of linkage. No other loci showed linkage in both samples. Population-specific loci on 2p12, 8q12, and Xp22 showed suggestive evidence of linkage.

Genome-wide Population-Specific Linkage Analysis

We first wanted to identify regions linked to any of the migraine traits in the individual study populations. In

Table 4. Phenotypes Showing Genome-wide Significant LOD Scores at the 10q22-q23 Locus and Their LOD Scores in Each Sample

Phenotype	Finnish, NPL_{pairs}, 103 cM	Australian, NPL_{qtl}, 106 cM	Joint, NPL_{pairs}, 102 cM
MA end diagnosis	*4.65*	0.00	1.58
LCA migrainous headache	*4.81*	0.91	3.00
TCA unilaterality	*5.18*	0.00	0.62
TCA pulsation	*4.24*	3.50	*4.62*
TCA pain intensity	*5.03*	1.32	*3.75*
TCA nausea/vomiting	*3.90*	0.25	2.88
TCA photophobia	*4.22*	0.11	2.40
TCA phonophobia	*5.03*	0.00	1.63

Note that numbers in italics represent genome-wide significant evidence of linkage (LOD > 3.83).

the Finnish study sample, the MERLIN NPL_{pairs} analysis showed significant evidence of linkage to a locus on 10q22-q23. The highest LOD score (5.50) was observed at 103 cM with the TCA unilaterality phenotype, with the 95% CI placing the locus between 99 cM and 114 cM. Significant evidence of linkage at this locus was also shown by the MA end diagnosis, LCA migrainous headache, and five additional TCA phenotypes (see Table 4). In the Finnish study sample, no other chromosomal region showed significant evidence of linkage, and only two regions showed suggestive evidence of linkage (on 2p12, NPL_{pairs} LOD score 2.60 at 100 cM with TCA pulsation phenotype; 1.93 for MA end diagnosis, and 1.74 for LCA migraine and on Xp22, NPL_{pairs} LOD score 2.96 at 39 cM with TCA pulsation phenotype, 1.19 for MA end diagnosis, and 1.72 for LCA severe migraine), although a previously detected locus on 18q12[9–12] showed sufficient evidence for replication (NPL_{pairs} LOD 2.46 at 86 cM with TCA attack-length phenotype, 0.21 for MA end diagnosis, and 0.41 for LCA migrainous headache). Encouragingly, the 10q22-q23 locus is robustly replicated in the Australian study sample with a highly suggestive NPL_{qtl} score of 3.50 at 100 cM with the TCA pulsation trait, with the 95% CI located between 94 cM and 115 cM. Other phenotyping methods provided modest signals in the Australian study sample at the 10q22-q23 locus. In the Australian sample, suggestive evidence of linkage was found to a region on 8q12 (NPL_{qtl} LOD of 2.63 at 86 cM with the TCA pain intensity phenotype, 0.29 for MA end diagnosis, and 1.27 for LCA migrainous headache), and a previously detected locus on 14q21 was replicated (NPL_{qtl} LOD 2.23 at 26 cM with TCA pain intensity phenotype, 0.24 for MA end diagnosis, and 1.68 for LCA migrainous headache). The genome-wide results for all traits are shown in Figure 1.

Genome-wide Joint Analysis of Australian and Finnish Study Samples

Results of a joint MERLIN NPL_{pairs} analysis yielded significant evidence of linkage to the same region as in the

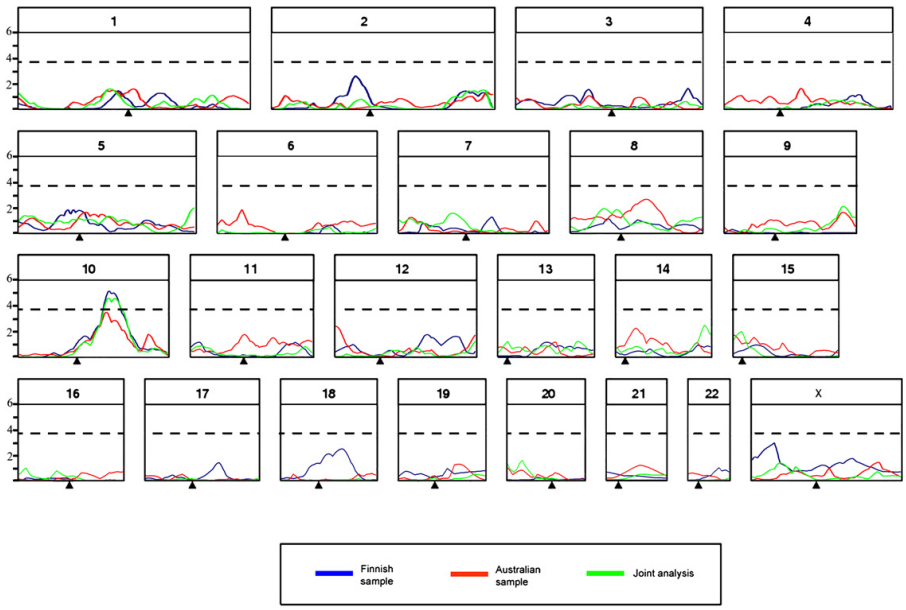

Figure 1. Maximum LOD Scores in the Genome-wide Screen

The graphs show values across all phenotypes and phenotyping methods for the Finnish study sample in the NPL_{pairs} analysis, the Australian study sample in the NPL_{qtl} analysis, and the NPL_{pairs} analysis performed on both study samples together. The dotted line denotes the level of significant evidence of linkage (LOD > 3.83).

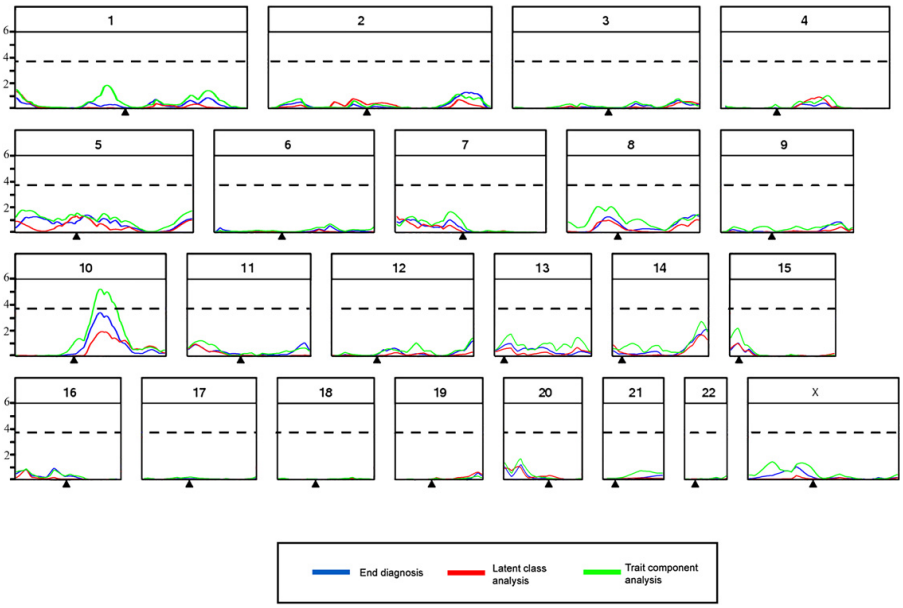

Figure 2. Genome-wide Comparison of the Three Genotyping Methods in the Combined Study Sample
The graphs show the highest LOD score detected with each phenotyping method in the joint analysis of the two genome-wide screens in the NPL_{pairs} analysis performed with both study samples together. The horizontal lines and boxes indicate the maximum LOD scores at 10q22-q23 for each method. The dotted line denotes the level of significant evidence of linkage (LOD $>$ 3.83).

individual analysis, between 98 cM and 117 cM at 10q22-q23. The highest LOD score (4.62) was found at 102 cM with the TCA pulsation phenotype, and significant evidence of linkage was also detected with TCA pain-intensity phenotypes (see Table 4). Neither of the regions showing suggestive linkage in only one sample showed evidence of linkage above nominal level (2p12 highest NPL_{pairs} LOD score 0.57, for 8q12, 1.01; and for Xp22, 1.36) in the joint analysis. Comparison of results from each of the three phenotyping methods in the joint analysis is presented in Figure 2.

Fine Mapping the Locus on 10q22-q23

Seven additional markers were genotyped in both initial study samples to increase the available linkage information across the implicated 10q22-q23 region. When including those markers in the joint-linkage analysis, the highest peak was found at 106 cM (NPL_{pairs} LOD score of 4.11 with the TCA pulsation phenotype, 1.28 for MA end diagnosis, and 2.16 for LCA migrainous headache). These results are detailed in Figure 3.

Finnish Replication Study

We genotyped an independent Finnish replication sample of 27 families for the seven additional microsatellite markers at the 10q22-q23 locus to further strengthen the evidence of linkage. Because the families providing most of the linkage signals to the 10q22-q23 locus in the genome-wide study were found to suffer from a severe form of migraine that included some hemiparesis symptoms (although not severe enough to qualify as familial or sporadic hemiplegic migraine), this clinical phenotype was used as the basis of selecting the families for the replication study (see Table 5). In the linkage analysis, the highest peak was found at 102 cM with the TCA pulsation

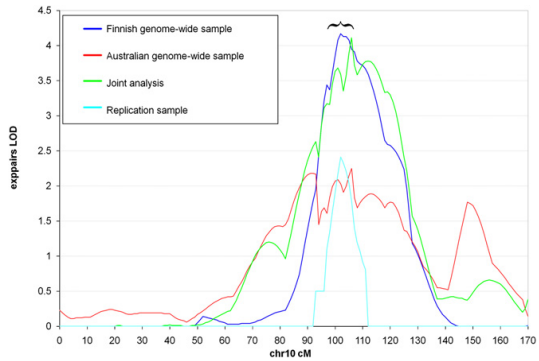

Figure 3. Positioning the Linkage Peaks on Chromosome 10
The graphs show maximum attained LOD scores in each study sample in the Merlin multipoint analyses, including the seven additional microsatellite markers. The bracket denotes the area covered by the family-specific haplotype segregating with the affection status.

Table 5. The Number and Proportion of Migraineurs with Hemiparesis and Hemisensory Symptoms in the Finnish Study Samples

	Previous Genome-wide Sample[11]	Current Genome-wide Sample	Replication Sample
n in total sample	441 (-)	690 (-)	324 (-)
with hemiparesis symptoms	42 (9.5%)	67 (9.7%)	51 (15.7%)
with hemisensory symptoms	87 (19.7%)	117 (17.0%)	70 (21.6%)

phenotype (NPL_{pairs} LOD score of 2.41), sufficient for replication (LOD > 1.8).

Sex-Specific Findings

In line with previous studies[11,12] that have suggested sex-specific effects at linked loci, we performed a sex-specific analysis for chromosome 10. In the Australian study, sex-specific analyses yielded no improvement in the linkage signal. However, in the Finnish study sample, considering only affected females yielded a considerable increase in the LOD score, resulting in highly significant evidence of linkage with a number of phenotypes, including the MA end diagnosis. The highest NPL_{pairs} LOD score was 7.68 (at 103 cM, TCA phonophobia phenotype, 4.37 for MA end diagnosis, and 5.33 for LCA migrainous headache). In contrast, considering only affected males, the linkage signal was below the level of nominal evidence of linkage (highest NPL_{pairs} LOD score 0.20 at the same location). For the Finnish females, all of the studied phenotypes except LCA severe migraine showed significant evidence of linkage. For the joint analysis, considering only females and nuclear families produced a significant LOD score of 4.11 (at 106 cM with the TCA photophobia phenotype, 2.52 for MA end diagnosis, and 3.19 for LCA migrainous headache). Female-specific results are detailed in Figure 4.

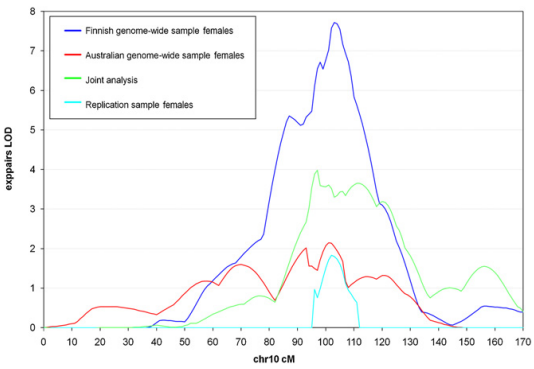

Figure 4. Female-Specific Multipoint Linkage Results on Chromosome 10

The graphs show the results obtained with the TCA pulsation phenotype, which gives the highest evidence of linkage in each sample.

Pedigree Identifier	D10S										
	1652	537	535	109	1730	605	569	1786	1686	1687	185
18	n/a	5	5	3	n/a	6	8	5	8	2	n/a
43	n	n	6	4	n/a	2	1	8	1	3	n/a
85	n	9	5	3	8	5	7	7	7	2	5
119	n	n	7	2	9	2	1	6	1	3	n/a
126	n	n	6	2	4	4	8	3	5	n	n
127	n	n	n	n	n	n	n	3	8	1	2
151	n	n	n	3	4	2	8	3	7	1	2
158	n	n	2	5	8	5	9	7	6	3	5
189	9	9	4	2	7	1	8	1	3	6	5
203	n	n	n	n	n	n	8	5	8	2	5
221	n	n	2	4	5	2	5	7	1	5	2
230	n	2	5	7	4	2	5	2	6	3	5
257	9	6	3	2	7	2	5	3	2	1	5
291	n	n	n	n	n	n	n	2	2	1	2
523	n	n	n	n	n	n	8	3	8	8	5
573	n	n	n	1	n/a	2	5	2	2	1	5
732	n/a	n	6	3	4	5	1	5	1	5	n/a
736	n/a	8	6	2	9	4	1	6	8	4	n/a
778	n/a	n	n	n	n	5	1	5	7	4	n/a
19703	n	4	5	2	7	2	8	7	8	5	10
23901	9	4	5	7	4	2	3	7	5	2	n
				KCNMA1				NRG3		GRID1	

Figure 5. Haplotype Distribution among the Finnish Families with Family-Specific LOD Scores over 1.00 at the Location of the Highest Linkage Signal in Finns, 103 cM

This figure shows the family-specific haplotype segregating with the affection status on chromosome 10q22-q23, for the roughly 30 Mb area spanning the markers D10S1652 and D10S185 around the linkage peak. The lightly shaded area represents the haplotype block shared by affected members of the family, and the darker shading indicates the region shared by all affected family members across families. "N/a" denotes an unavailable genotype, and "n" denotes multiple different alleles in affected family members. The bottom of the figure shows the largest transcribed genes in the region, in scale relative to the distances between the microsatellites.

Haplotype Analysis at 10q22-q23

Because the Finnish study sample is from a population with a limited number of founders and multiple bottlenecks in the population history, we performed a haplotype shared-segment analysis to further restrict the linked region and identify the most probable location of the disease predisposing variants. The analysis was conducted in those 21 Finnish families that had a family-specific nonparametric linkage (NPL) score greater than 1.00 (as measured by GENEHUNTER) at the location of the highest linkage signal (97.5 cM), both from the genome-wide sample and the replication sample. These families contain 178 individuals, of which 99 (or 56%) are considered affected according to our clinical MA end diagnosis. Ninety-five percent (94 out of 99 subjects) shared the family-specific haplotype between markers D10S1786 (103.3 cM) and D10S1686 (104.9 cM). Considering the locations of the flanking markers (D10S569 at 97.5 cM and D10S1687 at 107.3 cM), the detected haplotype is between 1.6 and 9.8 cM wide (1.6 – 9.6 Mb). Restricting the area to this region

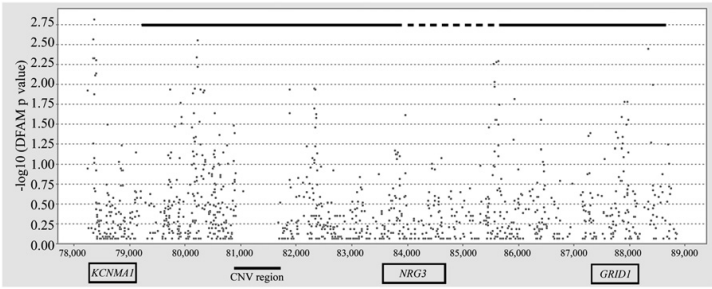

Figure 6. Results of the Follow-Up Association Study that Used 39 Trios, 256 Unrelated Cases, and 230 Controls in the DFAM Association Analysis

The dotted line at the top of the figure denotes the minimum known length of the family-specific segregating microsatellite haplotype (see Figure 6), and the solid line denotes its maximum possible length. The bottom of the figure shows the largest transcribed genes in the region, in scale according to the isoform with the largest genomic size, as well as the area of the known CNV.

was accomplished on the basis of three and four informative recombinations, respectively. Results of this analysis are detailed in Figure 5.

Follow-up Association Study

The trio and case-control samples were analyzed with the DFAM analysis option of PLINK,[34] which allows for the combination of trio and case-control data. None of the SNPs showed association exceeding the significance threshold of 6.73×10^{-5}. The highest association was detected with SNP rs1873695 (p value 9.22×10^{-4}; *KCNMA1* intronic), with several adjacent SNPs showing a similar level of association (rs2131218: 0.0035, rs16934025: 0.0019). Three other regions show association scores under the 0.005 level with several adjacent markers: rs10458664 (0.0035, outside any known gene), rs7906586 (p = value 0.0025, outside any known gene), and rs2691052 (0.002, outside any known gene) (see Figure 6 for results).

Discussion

In the present study, we detected highly significant evidence of linkage to 10q22-q23 and replicated the finding in two diverse populations. The locus was detected with all three phenotyping methods used, which alternatively concentrate on the presence of aura, IHS symptom clustering, or the individual migraine symptoms. The consistency of linkage findings across studies with different ascertainment schemes and phenotyping methods provides compelling evidence for the strength of this finding.

As is often the case in complex traits, the linkage peaks defining the detected 10q22-q23 region are relatively broad (between 8 cM and 21 cM wide, depending on the method of analysis), and the number of susceptibility loci within this region cannot be predicted. However, given that the analysis peaks all converge on a narrow (under 5 cM) area, which contains the area defined by the shared-segment analysis, there is therefore strong evidence for constraining the peak between 97.5 cM and 104.9 cM (see Figure 3). This interval contains two obvious functional candidate genes. *KCNMA1* is a Maxi-K, calcium-level detecting potassium channel, which is involved in ion transport in a similar manner to the three known genes involved in the molecular pathology of familial hemiplegic migraine (see Table S1A), a related Mendelian disorder (*CACNA1A*, *ATP1A2*, and *SCN1A*, MIM 141500, MIM 602481, and MIM 609634, respectively). *NRG3*, located directly in the middle of the narrowest peak, is a gene belonging to the neuregulin family of growth and differentiation factors that are related to epidermal growth factor, which plays a role in oligodendrocyte survival.

Overall, there is an encouraging consistency between the results of this study and the previous Finnish[11] and Australian[8] studies. An overview of current and previous results at 10q22-q23 can be seen in Figure 7. The three other chromosomal areas showing evidence of significant or suggestive linkage to migraine in more than one report and in more than one population prior to this study are on chromosomes 4q21-q31, 15q11-q13, and 18q12. The previously reported chromosome 4q locus seems to be exceptionally broad; whereas the linkage in the Icelandic population is reported at 4q21,[12] previous Finnish studies identified two peaks at 4q24[9] and at 4q28-q31,[11] making the total linked region up 50 cM wide. In this study, we

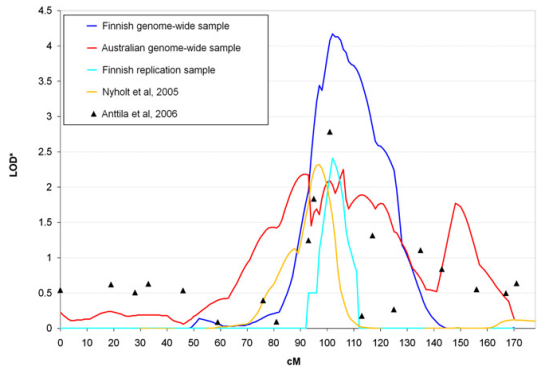

Figure 7. Previously Reported Migraine Linkage Results at the 10q22-q23 Locus Plotted Together with Results of This Study

were unable to replicate this locus, although closer examination of the family-specific results reveals the existence of a subset of families (approximately one-fourth of the total sample in both Australians and Finns) with family-specific Z scores up to 2.4, even though the overall evidence of linkage was nominal. This was also the case with the previously reported 17p13 locus.[11] Similarly, the previously reported 15q11-q13 locus was undetectable in our study. The chromosome 18q12 locus has been detected in both the Icelandic[12] and Finnish samples[9,11] (although the Icelandic linkage was observed after a broader definition of migraine was applied and only females considered as affecteds), as well as in the previous Australian study.[10] This locus is also replicated here, with the same TCA attack-length phenotype as in the previous Finnish study, bringing the total number of study samples showing linkage to this locus to four. Finally, three loci previously linked to migraine in only one study sample are also replicated, two in their respective populations: the locus on 8q21 detected in Australians (NPL_{qtl} LOD score of 2.63) is within 10 cM from a previously detected Australian locus,[8] a previously reported locus on 14q21 detected in a large Italian family[14] is replicated in the Australian sample (NPL_{qtl} LOD score of 2.21 at 26 cM), and the peak on Xp22 (NPL_{pairs} LOD score of 3.05 in Finns) is only 7 cM from a locus on Xp21, detected in the previous Finnish study.[9,11] In total, of the seven loci reported in Finns so far (4q, 10q*, 12q, 15q, 17p, 18q*, and Xp*), this study replicates three (denoted by asterisks), as well as three of the five reported in Australians (5q, 8q*, 10q*, 13q, and 18p-18q*). Finally, given that only one (2p12) of the four loci with suggestive or higher evidence of linkage in this study (2p, 8q, 10q, and Xp) has not been reported previously, overall the extended phenotyping methods seem to facilitate replication, especially considering that only one locus (4q21-q24) has been replicated with end-diagnosis-based approaches. An overview of results at each OMIM-listed migraine loci can be seen in Table S1B.

A closer look into previous migraine scans showed that the 10q22-q23 locus had been implicated in our two scans[8,11] with suggestive evidence of linkage. The present and the two previous studies suggest that complementing the classical clinical migraine diagnosis with alternative phenotyping strategies can facilitate the identification of susceptibility-locus identification. Both the trait-component analysis and the latent-class analysis approaches have proven useful in this respect, although they have different premises and represent conceptual approaches. It is of interest to note that if only the MA end diagnosis had been used as the study phenotype, the 10q22-q23 locus would have been detected with suggestive or significant evidence of linkage in only one of the five recent migraine study samples in the Australian and Finnish populations (including the three reported here; corresponding success rates 2/5 for LCA, and 5/5 for TCA).[8,11] There are two likely explanations for the greater sensitivity of TCA and LCA over the clinical diagnosis: (1) LCA and especially TCA may better reflect underlying processes in migraine pathophysiology, and/or (2) these two methods can utilize the questionnaire-based information in a more optimal way to find informative individuals within the MA and MO patient pools, thus including more cases and informative meioses for the linkage analysis. Although advantages and disadvantages exist for any analytical approach, these and previous results suggest that the trait-component analysis may offer substantial gains over analysis of clinical (migraine with aura) or empirical (e.g., LCA) end diagnoses especially when the diagnostic information is incomplete. Given that both the end diagnosis and the latent classes are based on combining information from phenotype profiles, it is perhaps not surprising that they both lose more power compared to TCA when the amount of available information is less than complete. This could explain the differences in results between the phenotyping methods in the current Australian study sample. Furthermore, using individual trait components directly allows additional efforts to be concentrated on increasing the size of the study samples, without the need to collect progressively more and more detailed clinical diagnostic information to optimize the formulation of the end diagnosis. On the other hand, it is possible that the TCA findings are the result of detecting genes involved in the symptom-specific processes and not involved in the primary pathophysiology of migraine.

The role of pulsating pain trait is of particular interest. Repeatedly, pulsation seems to be the most sensitive individual trait for linkage-based locus identification, providing all of the highest results in the Australian sample and many of those in the Finnish sample. This is evident in the previous two other genome-wide scans as well; the best signal in the previous Australian study (5q21)[8] is predominantly driven by pulsation, as is the best locus in the previous Finnish study (17p13),[11] which showed significant evidence of linkage only with pulsation. In addition, it plays a major role in the 10q22-q23 results in this study. The reason for this remains speculative. One possible explanation is that pulsating pain is a symptom that is more easily recognized by patients and thus is more consistently recorded in interviews and questionnaires. This does not, however, exclude the possibility that pulsation is indeed the most characteristic symptom of a particular type of migraine and reflects some yet-unknown neurovascular mechanism and is thus associated with specific pathophysiological pathways. It should also be noted that pulsation or any other TCA trait was not used in the sample ascertainment; that is, the sample selection process is naive with respect to the traits. Another interesting finding is the role of hemiparesis symptoms; because the families contributing most to the 10q22-q23 locus suffered from a more severe form of migraine, they have a higher proportion of hemiplegic symptoms. After the families for the replication study were selected for a higher prevalence of this severe form, a similar effect was observed in the replication study, further underlining the contribution of this

clinical phenotype to the 10q22-q23 locus. Thus, we were able to extract a part of the clinical spectrum of migraine, concentrate on it in case selection, and predict and subsequently demonstrate linkage to a particular genetic locus with a small number of patients targeted for that particular aspect, which is both a novel and an encouraging finding. In addition, on the basis of the known ion-channel-centered molecular pathology of familial hemiplegic migraine,[42–44] this supports *KCNMA1* as a compelling candidate gene. Lastly, the difference between overall and sex-specific linkage results at 10q22-q23 seem to reflect a predominantly female-dominated inheritance pattern in the Finnish families linked to this locus; such a finding is somewhat to be expected because of the higher prevalence of migraine in women. However, this is not enough to explain the considerable increase in linkage signal when considering only females as affected. The same effect, though to a smaller degree, can also be seen in the Australian study sample, as well as in a previous Finnish[11] and an Icelandic study.[12] These results suggest that using gender as a covariate in future migraine studies might provide increase in power for the detection of new variants. Sex might also be an indicator for male-specific environmental or behavioral characteristics that hide the signal in men, perhaps related to the better ability of women to detect and elaborate on symptoms and signs in headache and migraine; whether this is related also to higher prevalence of migraine in women also needs to be examined.

Although no SNPs showed significant association in the follow-up study, four potentially interesting regions support additional studies. The highest association, although not high enough to be considered significant, was observed with one obvious candidate gene, *KCNMA1*. The three SNPs with the next highest scores were located outside known coding genes. However, given the high linkage signal in the region and the variance among the family-specific affected haplotypes, it is possible that this locus contains multiple susceptibility variants affecting migraine but that the sample used is too small to sufficiently discern between them. Thus, larger studies are warranted to see whether these findings can be replicated. The potential, suggestive association to *KCNMA1* is intriguing because the established FHM mutations are all located in proteins involved in ion transport.

A timely question is how linkage signals, as the chromosome 10q22-q23 locus reported here, correspond to association signals in WGA studies. Recent studies provide an opportunity to compare loci identified with these two different strategies that are based on different hypothesis. Although linkage studies are best suited to position relatively penetrant and possibly rare variants, WGA studies are designed to test the "common-diseases-common-variant" hypothesis. Although the number of WGA studies is still limited, some trends can be observed; there are examples of identification of both previously unidentified loci and confirmation of loci identified in linkage studies. Importantly, the WGA studies have identified new robustly replicated loci in regions not previously linked or associated to disease traits.[45–49] However, in cases such as prostate cancer (8q24)[50–52] Crohn's disease (16q12, *NOD2*),[53,54] type II diabetes (10q23-q26),[55–57] and MS (5p13),[58,59] some of the strongest associations are observed in regions where linkage has previously been detected and replicated in several studies. Thus, it is relevant to hypothesize that the linkage to chromosome 10q22-q23 region detected consistently in several migraine study samples could represent a region where a relatively highly penetrant and perhaps common variant(s) is/are associated to migraine. Another possibility is that a strong linkage observed in several study samples indicates that there are several susceptibility variants or even genes within the linked locus.

Regardless of the various constraints involved, we detected strong linkage at the 10q22-q23 locus in all three samples assessed in this study. The detection of linkage to the 10q22-q23 locus with different phenotyping methods and different ascertainment protocols provides strong support for the presence of a gene(s) in this region influencing migraine susceptibility. In addition, our study demonstrates the advantages of using of IHS clinical traits directly in migraine genetics and allowed the confirmation of a number of previously reported genomic regions being coinherited with migraine.

Supplemental Data

Two tables are available at http://www.ajhg.org/.

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Web Resources

The URLs for data presented herein are as follows:

matSpD, http://genepi.qimr.edu.au/general/daleN/matSpD/
Migraine Questionnaire used in the SSAGA-1 "Older Cohort," http://genepi.qimr.edu.au/general/daleN/Migraine/SSAGA1MigraineQuestionnaire.pdf
Migraine Questionnaire used in the Twin89 "Younger Cohort," http://genepi.qimr.edu.au/general/daleN/Migraine/Twin89MigraineQuestionnaire.pdf
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/
PLINK, http://pngu.mgh.harvard.edu/purcell/plink/
snpSpD, http://genepi.qimr.edu.au/general/daleN/SNPSpD/

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A high-density association screen of 155 ion transport genes for involvement with common migraine

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The clinical overlap between monogenic Familial Hemiplegic Migraine (FHM) and common migraine subtypes, and the fact that all three FHM genes are involved in the transport of ions, suggest that ion transport genes may underlie susceptibility to common forms of migraine. To test this leading hypothesis, we examined common variation in 155 ion transport genes using 5257 single nucleotide polymorphisms (SNPs) in a Finnish sample of 841 unrelated migraine with aura cases and 884 unrelated non-migraine controls. The top signals were then tested for replication in four independent migraine case–control samples from the Netherlands, Germany and Australia, totalling 2835 unrelated migraine cases and 2740 unrelated controls. SNPs within 12 genes (*KCNB2, KCNQ3, CLIC5, ATP2C2, CACNA1E, CACNB2, KCNE2, KCNK12, KCNK2, KCNS3, SCN5A* and *SCN9A*) with promising nominal association ($0.00041 < P < 0.005$) in the Finnish sample were selected for replication. Although no variant remained significant after adjusting for multiple testing nor produced consistent evidence for association across all cohorts, a significant epistatic interaction between *KCNB2* SNP rs1431656 (chromosome 8q13.3) and *CACNB2* SNP rs7076100 (chromosome 10p12.33) (pointwise $P = 0.00002$; global $P = 0.02$) was observed in the Finnish case–control sample. We conclude that common variants of moderate effect size in ion transport genes do not play a major role in susceptibility to common migraine within these European populations, although there is some evidence for epistatic interaction between potassium and calcium channel genes, *KCNB2* and *CACNB2*. Multiple rare variants or *trans*-regulatory elements of these genes are not ruled out.

INTRODUCTION

Migraine (OMIM no. 157300) is a common neurovascular disorder with a strong genetic component. Although genetic variants have been well established for Mendelian forms of migraine, no susceptibility variants associated with common forms of migraine have been consistently reported. So far, most association studies for headache traits have been performed in relatively small study samples and typically for only one or two genes per study. Thus, comprehensive candidate gene association studies saturating large gene families or whole genome association studies have not been reported for headache traits as yet.

The best insight to understand the genetic background underlying pathogenetic mechanisms in migraine is provided by studies in Familial Hemiplegic Migraine (FHM), a Mendelian subtype of migraine with aura (MA) associated with hemiparesis. Families with FHM type 1 (*FHM1*) (OMIM no. 141500) have missense mutations in *CACNA1A* (OMIM no. 601011) (1), which encodes the pore-forming $\alpha 1$ subunit of neuronal voltage-gated $Ca_v2.1$ (P/Q-type) Ca^{2+} channels, type 2 (*FHM2*) (OMIM no. 602481) patients show mutations in *ATP1A2* (OMIM no. 182340) (2), which encodes the $\alpha 2$ subunit of Na^+/K^+ ATPases that are expressed in astrocytes in the adult brain, whereas type 3 (*FHM3*) (OMIM no. 609634) patients have mutations in the $\alpha 1$ subunit of the neuronal voltage-gated Na^+ channel $Na_v1.1$ (*SCN1A*) (OMIM no. 182389) (3).

All three FHM genes are involved in the transport of ions and suggest that disturbances of ions and neurotransmitter levels in the synaptic cleft play a key role in migraine pathophysiology by influencing neuronal excitability (4,5). Experimental evidence points to cortical spreading depression (CSD)—a wave of neuronal and glial depolarization followed by long-lasting suppression of neuronal activity that propagates slowly over the cerebral cortex—as the key event for episodic activation of the trigeminovascular system, resulting in migraine headache (6,7). *CACNA1A FHM1* mutations are predicted to cause increased release of glutamate (the predominant excitatory amino acid transmitter in brain) in the cortex, thus increasing neuronal cortical network excitability rendering the cortex more susceptible to CSD (8). *ATP1A2 FHM2* mutations result in reduced activity or decreased affinity for K^+ of the Na^+/K^+ pump and thus impaired clearance of K^+ and glutamate from the synaptic cleft. *SCN1A FHM3* mutations are believed to have effects similar to *CACNA1A* mutations, in the sense that they predict increased neuronal excitability.

A relevant hypothesis is that the same pathways involved in rare Mendelian forms of migraine might also be involved in the pathogenesis of more common forms of migraine. This is especially appropriate as the main symptoms of headache and aura (as well as the accompanying symptoms of nausea, photophobia and phonophobia) of FHM attacks are very similar to those of MA, and both types of attack occur in FHM patients (9). In fact, migraine without aura (MO) and MA, frequently co-occur. A Dutch study found that 42% of active migraineurs with aura also reported having migraine attacks without aura (10). Moreover, MO and MA frequently coexist within a family; a Headache Centre in Italy reported that 45% of MA families also had MO cases (11) and the co-occurrence of FHM, MA and MO has been reported in families (12,13). Furthermore, changes in the presenting symptoms of migraine attacks from hemiplegic to severe headache with or without aura in later life (12), as well as the development of aura among subjects with MO and the converse (12,14), suggest overlapping aetiology.

The overlap in symptomatology among migraine subtypes and the fact that all three known FHM genes are involved in the transport of ions, strongly suggests that ion channels and ion transporters, may also underlie susceptibility to the more common forms of MO and MA. The relevance of ion channels and transporters is also apparent from the fact that effective migraine prophylactic agents such as valproate and topiramate act on ion channels or transporters (15).

There are several reports that rare mutations in FHM genes may also play a role in families with MA and/or MO (16,17).

A systematic investigation of rare predisposing variants in common migraine has become feasible because of recently improved technology to perform extensive sequencing in a large numbers of samples, but has not been performed. However, an equally relevant hypothesis is that common variants in either coding or non-coding regions of ion transport genes might predispose to migraine. Association of common variants to common traits can be studied with tools provided by HapMap (18) and modern genotyping techniques.

Our multicentre collaboration (the Migraine Genetics Consortium; MGC) assembled the largest cohort of patients with common migraine for a genetic mapping study to date. To investigate the potential role of genes involved in the transport of ions in common migraine, we performed the first comprehensive screen of common genetic variants in highly plausible candidate ion transport genes. A total of 5257 single nucleotide polymorphisms (SNPs) were examined in 155 genes in a large well-characterized Finnish case–control sample consisting of 841 unrelated MA cases and 884 unrelated non-migraine controls. Twelve genes producing nominally significant ($P < 0.005$) association signals in the Finnish sample were subsequently tested for replication in four independent migraine case–control samples from the Netherlands, Germany and Australia involving altogether 2835 migraine cases and 2740 controls.

Also, given the reported functional interaction between M-type *KCNQ2* and *KCNQ3* potassium channels (19) and their modulation by the *KCNE2* subunit (20), plus an account of compound heterozygosity for two novel allelic *ATP1A2* missense mutations in the proband of a FHM family (21), we also tested for interactions between SNPs with nominally significant ($P < 0.005$) association signals in the Finnish sample.

RESULTS

Of the 5269 SNPs passing Perlegen's quality control (QC) criteria analyzed in the Finnish case–control cohort, PLINK identified 12 SNPs with MAF < 0.01 and subsequently excluded them from further analysis. Importantly, the PLINK identity-by-state (IBS)-based cluster analysis determined that one cluster best fit the data, thus providing no evidence for population stratification within the Finnish cohort. Absence of stratification was further supported with PLINK reporting a genomic inflation factor (based on median chi-squared) of 1 (values >1 indicate population structure) and mean chi-squared statistic of 0.945 (a mean of 1 is expected under the null hypothesis of no stratification).

No evidence for association with MA was observed for SNPs within the footprints of the three FHM genes. Briefly, none of the 25 *ATP1A2* and 26 *SCN1A* SNPs produced point-wise *P*-values <0.05. Although five of the 87 *CACNA1A* SNPs (rs10416717, rs2419233, rs7249323, rs11882861, rs2074880) did produce nominal point-wise *P*-values ≤ 0.05 (0.0131, 0.01822, 0.04142, 0.04257, 0.04915, respectively), they were not significant after adjustment for testing multiple SNPs.

Allelic association results $-\log_{10}(P)$ for all 5257 SNPs are presented in Figure 1 (also see Supplementary Material, Table S1). The smallest observed point-wise *P*-value from allelic association analysis of 5257 SNPs in the Finnish 841 MA cases and 884 controls was obtained for SNP rs13276133 ($\chi^2_1 = 12.49$; point-wise $P = 0.00041$) residing in the potassium voltage-gated channel, Shab-related, member 2 (*KCNB2*) gene on chromosome 8q13.3. The next smallest *P*-value obtained for SNPs within *KCNQ3* on 8q24.22 (rs13267466; $P = 0.00064$) and *CLIC5* on 6p12.3 (rs2095771; $P = 0.00074$) also showed encouraging evidence for association. An additional nine genes (*ATP2C2, CACNA1E*, *CACNB2*, *KCNE2, KCNK12*, *KCNK2*, *KCNS3*, *SCN5A* and *SCN9A*) produced promising association signals ($P < 0.005$) (Table 1). However, after multiple test correction for the analysis of 5257 SNPs via permutation, our smallest point-wise *P*-value (rs13276133; $P = 0.00041$) was not globally significant with a global EMP2 *P*-value of 0.7713. Hence, none of the tested SNPs reached global statistical significance (EMP2 $P \leq 0.05$) (Table 1).

Exploratory analysis of haplotypes spanning two to six contiguous SNPs also did not produce significant evidence for association (data not shown), with the smallest observed point-wise *P*-value ($\chi^2_1 = 13.82$; point-wise $P = 0.00020$) obtained for the specific 3-allele G-T-T haplotype of SNPs rs1998822–rs7076100–rs11598027 within the *CACNB2* gene on chromosome 10p12.33. The omnibus 3-allele haplotype test for this three SNP window (correcting for haplotype tests for all observed 3-allele combinations) only produced a χ^2_1 of 18.12 with point-wise $P = 0.0012$, compared with the single SNP point-wise $P = 0.00198$ for rs11598027. Given that these results are not corrected for testing, all other two to six SNP haplotypes, analysis of haplotypes in these data did not produce stronger evidence for association compared with the single SNP analyses.

In addition to the primary MA phenotype, we also performed exploratory association analyses with strict International Headache Society (IHS) migraine criteria (i.e. excluding the 114 MA individuals with aura symptoms not strictly fulfilling the IHS aura criteria, but diagnosed as MA by our clinical neurologist; Table 2) and the key trait components of pulsation, photophobia and phonophobia (Table 1, Supplementary Material, Table S1). Although these exploratory analyses did not provide increased evidence for association between MA and SNPs within the three FHM genes, they did produce the smallest observed *P*-value ($\chi^2_1 = 16.00$; point-wise $P = 0.000063$), obtained for SNP rs12996816 residing in the *KCNS3* gene on chromosome 2p24.2 with the pulsation phenotype; however, this result does not surpass the primary analysis global significance threshold of point-wise $P \leq 0.000019$. Furthermore, given an additional global multiple test correction is required for examining four additional traits; these exploratory analyses do not alter the overall (negative) interpretation of our individual SNP association results.

Although no single SNP or haplotype reached statistical significance, multiple small effect association signals in multiple genes may nonetheless be present. To examine this possibility, we plotted the observed distribution of the 5257 rank-ordered chi-square (χ^2_1) values obtained from the single SNP allelic association analysis using MA as trait, against the distribution of χ^2_1 values expected under the null hypothesis of no

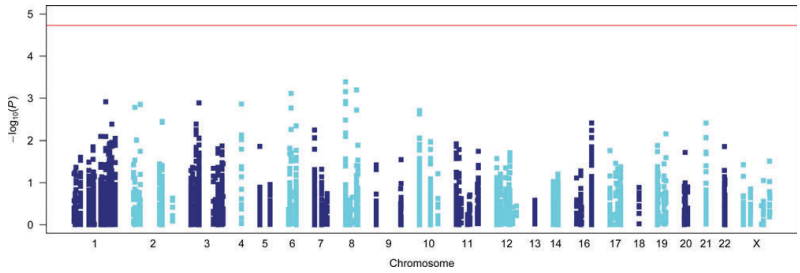

Figure 1. Allelic association results for Finnish MA cases versus controls. Horizontal red line indicates the required global significance threshold [$-\log_{10}(P) = 4.7286$] to ensure an overall type I error rate of 5%.

association. The resulting QQ-plot (Fig. 2) shows a definite, although non-significant preponderance of χ^2_1 values ranging from around 9.573 (point-wise $P = 0.00198$) to 10.19 (point-wise $P = 0.00141$) [i.e. the top 11–15 χ^2_1 values].

Applying the false discovery rate (FDR) approach to the 5257 rank-ordered allelic association *P*-values produced *q*-values (FDRs) of 0.673 and 0.692 for the 1st–11th and 12th–15th smallest *P*-values, respectively. Assigned *q*-values quickly and substantially increased in magnitude for the 16th (*q*-value = 0.773), 17th–23rd (*q*-value = 0.950) and remaining 24th–5257th ranked *P*-values (*q*-value = 0.9997). These results indicate that there are a maximum of four to five likely true-positives contained within the top 15 most significant allelic associations [i.e. true positive probability (1 − *q*-value) multiplied by the total number in the set with equivalent or smaller *q*-values: (1 – 0.6922) × 15 = 4.62]. Also, our stage-1 nominal significance threshold for allelic association with MA in the Finnish cohort demarcates *q*-values of 0.950 and 0.9997, thus supporting $P < 0.005$ as a reasonable compromise between following-up true risk loci and the total number of loci to follow-up (i.e. minimizing the multiple-test burden while maintaining high replication power).

A total of 66 SNPs within 12 genes (Table 1) were selected for follow-up in the replication cohorts (see Supplementary Material, Tables S2a and S2b for estimates of power to replicate the Finnish association results). The 66 SNPs were primarily selected upon the basis of having the smallest allelic association results among all the Finnish MA cases and controls ($P < 0.005$). The SNPs within these 12 genes were also selected if they produced nominal evidence (point-wise $P < 0.05$) for MA and/or produced more significant *P*-values, compared with the primary MA phenotype, for association with the traits examined in the exploratory association analyses (Table 1, Supplementary Material, Table S1). In addition to the investigation of multiple effects within each gene, we selected multiple associated SNPs within the 12 most promising genes to help guard against potential assay failure in the follow-up replication studies. Counts of case–control individuals successfully genotyped are provided in Table 3.

All 66 SNPs, except those which failed, were tested first in the Leiden sample with MA and MO (one SNP failed). Following the Leiden results, 60 SNPs were successfully tested in the population-based Brisbane sample with MA and MO. Finally, 44 SNPs were successfully tested in the Cologne and Munich clinic-based German MA samples. We note that due to the Munich cohort consisting of only migraine cases, two German-specific analyses were performed; (i) Cologne cases versus Cologne controls and (ii) combined Cologne cases and Munich cases versus Cologne controls. *Post hoc* analyses (not presented) in the replication cohorts containing a mixture of migraine sub-groups did not identify significant allele frequency differences between MO, MA and the latent class analysis (LCA)-based migraine groups, thus supporting the combination of these sub-groups. After QC checks, some genotype assays were failed in each cohort.

The results from association analysis of the 66 replication SNPs are presented in Table 4. Although a small number of SNPs showed significant evidence for replication (EMP2 $P \leq 0.05$) in the Brisbane [*CLIC5* SNP rs3729618 (EMP2 $P = 0.0459$)] and Cologne cohort [*KCNE2* SNPs rs1467847 (EMP2 $P = 0.0139$) and rs1013063 (EMP2 $P = 0.0193$)], none of the SNPs produced consistent evidence for replication across the cohorts. Furthermore, none of the SNPs were significant in the combined replication cohort Cochran-Mantel-Haenszel (CMH) test after correction for testing 66 SNPs. Analysis of the Leiden, Brisbane and/or combined replication MA subset did not alter the overall results. Due to the associated *CLIC5* alleles in the Finnish and Brisbane cohorts being in opposite directions, the *KCNE2* associations remain as the sole result which could justify further investigation. See Supplementary Material, Table S3 for point-wise replication association analysis results for all 66 replication SNPs in the four individual replication cohorts and combined replication sample.

Interestingly, a SNP × SNP epistasis analysis of the 66 replication SNPs in the Finnish case–control sample detected an interaction between *KCNB2* SNP rs1431656 on chromosome 8q13.3 and *CACNB2* SNP rs7076100 on chromosome 10p12.33 (point-wise $P = 0.0000199$), which remained significant after conservatively correcting for all 2145 possible interaction tests [i.e. treating the 66 replication SNPs as independent] (global $P = 0.042$). Accounting for non-independence between SNPs due to intermarker linkage disequilibrium (LD) (22), the global *P*-value for the rs1431656 × rs7076100 interaction is $P = 0.022$. This interaction was driven by the rs1431656–rs7076100 G-A haplotype providing increased protection against migraine

Table 1. Single SNP association results for single nucleotide polymorphisms selected for replication from Finnish case–control cohort

Chr	NCBI build 36 position (bp)	dbSNP rs no.	Primary gene HGNC symbol	Case minor allele	Case MAF	Control MAF	Allelic *P*	Allelic OR	Allelic EMP2 *P*
1	179680697	rs12073221	CACNA1E	C	0.3165	0.275	0.008168	1.22	1
1	179691156	rs3856087	CACNA1E	A	0.3356	0.2837	0.001215	1.28	0.9839
1	179702677	rs644796	CACNA1E	C	0.467	0.4255	0.01537	1.18	1
1	179709099	rs505738	CACNA1E	C	0.4619	0.4268	0.0382	1.15	1
1	213353044	rs4300189	KCNK2	A	0.1391	0.1599	0.08803	0.85	1
1	213365431	rs11120499	KCNK2	T	0.2218	0.2576	0.01368	0.82	1
1	213395890	rs12567520	KCNK2	A	0.09869	0.1179	0.06979	0.82	1
1	213398386	rs2363556	KCNK2	G	0.1391	0.1612	0.0698	0.84	1
1	213428215	rs1556905	KCNK2	A	0.3254	0.3502	0.129	0.90	1
1	213428830	rs10494994	KCNK2	A	0.1663	0.2043	0.004157	0.78	1
1	213458463	rs12143625	KCNK2	C	0.1901	0.2244	0.01314	0.81	1
1	213467066	rs6670661	KCNK2	G	0.3061	0.333	0.09116	0.88	1
1	213476729	rs6686529	KCNK2	G	0.1902	0.2254	0.01113	0.81	1
2	17908169	rs12996816	KCNS3	T	0.1177	0.08541	0.001656	1.43	0.9952
2	17916155	rs10178489	KCNS3	G	0.2996	0.2661	0.02894	1.18	1
2	47660997	rs17568951	KCNK12	C	0.1946	0.1534	0.001409	1.33	0.9909
2	166879155	rs11890824	SCN9A	A	0.4512	0.4745	0.1714	0.91	1
2	166889773	rs11898284	SCN9A	G	0.1795	0.1433	0.003754	1.31	1
2	166902637	rs12622743	SCN9A	G	0.1798	0.1433	0.003573	1.31	1
3	38599347	rs3922843	SCN5A	A	0.2429	0.2036	0.005644	1.26	1
3	38602829	rs7645358	SCN5A	G	0.1611	0.1799	0.1435	0.88	1
3	38609146	rs7374540	SCN5A	C	0.4007	0.4274	0.1114	0.90	1
3	38653505	rs7427106	SCN5A	G	0.1397	0.1753	0.004134	0.76	1
3	38656222	rs9311195	SCN5A	G	0.1534	0.1878	0.007321	0.78	1
3	38672343	rs9861643	SCN5A	T	0.1974	0.22	0.1033	0.87	1
6	45993676	rs11758298	CLIC5	T	0.1308	0.1471	0.1677	0.87	1
6	45995399	rs1416167	CLIC5	T	0.2903	0.2571	0.02875	1.18	1
6	45996051	rs3729618	CLIC5	G	0.2905	0.2576	0.03071	1.18	1
6	46008544	rs3777567	CLIC5	A	0.2655	0.2197	0.001715	1.28	0.9966
6	46012610	rs2095771	CLIC5	A	0.25	0.2017	0.0007735	1.32	0.9358
6	46018442	rs3777580	CLIC5	C	0.321	0.2778	0.005546	1.23	1
6	46026947	rs3777585	CLIC5	T	0.1871	0.1586	0.0265	1.22	1
8	73598648	rs12550268	KCNB2	A	0.382	0.4189	0.02711	0.86	1
8	73601624	rs1431659	KCNB2	A	0.2271	0.2704	0.003311	0.79	0.9999
8	73609424	rs5007872	KCNB2	G	0.2187	0.2669	0.001311	0.77	0.9876
8	73614029	rs349358	KCNB2	C	0.1249	0.1489	0.04	0.82	1
8	73618324	rs349355	KCNB2	A	0.1244	0.1493	0.03353	0.81	1
8	73651525	rs349335	KCNB2	C	0.1013	0.131	0.006741	0.75	1
8	73653731	rs1431656	KCNB2	G	0.2525	0.3056	0.0006998	0.77	0.9172
8	73659142	rs1542709	KCNB2	A	0.2368	0.2773	0.00673	0.81	1
8	74006869	rs7006287	KCNB2	G	0.4006	0.4552	0.001205	0.80	0.983
8	74012757	rs11782118	KCNB2	A	0.365	0.4184	0.001352	0.80	0.9892
8	74016115	rs922772	KCNB2	C	0.4518	0.4072	0.008148	1.20	1
8	74017814	rs13276133	KCNB2	C	0.3556	0.4145	0.000409	0.78	0.7713
8	133408579	rs13267466	KCNQ3	C	0.1198	0.1602	0.0006373	0.71	0.897
10	18458186	rs7897594	CACNB2	G	0.3719	0.4117	0.01682	0.85	1
10	18460390	rs11596974	CACNB2	A	0.3641	0.3991	0.03481	0.86	1
10	18799543	rs7076100	CACNB2	A	0.419	0.4559	0.02933	0.86	1
10	18800641	rs11598027	CACNB2	T	0.147	0.1117	0.001975	1.37	0.9986
10	18803689	rs1409202	CACNB2	A	0.147	0.1122	0.002354	1.36	0.9998
10	18822886	rs8181477	CACNB2	C	0.4613	0.504	0.01226	0.84	1
10	18823994	rs11014504	CACNB2	C	0.4457	0.4819	0.03356	0.86	1
16	82964929	rs17740111	ATP2C2	C	0.1942	0.2347	0.003894	0.79	1
16	82971665	rs7350833	ATP2C2	C	0.2643	0.2333	0.03534	1.18	1
16	82974143	rs10459853	ATP2C2	G	0.1285	0.1619	0.005822	0.76	1
16	83002938	rs247820	ATP2C2	C	0.4388	0.4791	0.01756	0.85	1
16	83040732	rs247889	ATP2C2	A	0.4214	0.3776	0.008578	1.20	1
16	83055346	rs429313	ATP2C2	A	0.2811	0.2474	0.02536	1.19	1
16	83062088	rs394533	ATP2C2	T	0.3214	0.2874	0.03043	1.17	1
16	83063868	rs400922	ATP2C2	G	0.2935	0.256	0.01439	1.21	1
16	83065318	rs390208	ATP2C2	A	0.2843	0.2471	0.01406	1.21	1
21	34612656	rs2834442	KCNE2	T	0.2771	0.3222	0.003897	0.81	1
21	34620260	rs1013063	KCNE2	T	0.3526	0.3907	0.0205	0.85	1
21	34636414	rs1467847	KCNE2	G	0.35	0.3815	0.05496	0.87	1
21	34639061	rs2834462	KCNE2	T	0.2762	0.3171	0.008623	0.82	1
21	34643430	rs8128316	KCNE2	C	0.3909	0.4304	0.01872	0.85	1

Table 2. Demographic factors of the Finnish case and control groups

	Cases Twins (population-based sample)	Familial patients (clinic-based sample)	Controls Twins
n	279	619	900
Migraine status	MA 260 (93%) Other 19 (7%)	MA 524 (85%) Other 95 (15%)	No migraine No family history of migraine
Gender distribution % female	81.7	79.2	75.6
Mean age (years)	56	48	57
Age range (years)	47–80	13–91	48–66
Age range at the time of assessment (years)	36–69	10–88	39–57

Other; migraine aura symptoms not fulfilling the International Headache Society aura criteria, but classified as migraine with aura by a clinical neurologist.

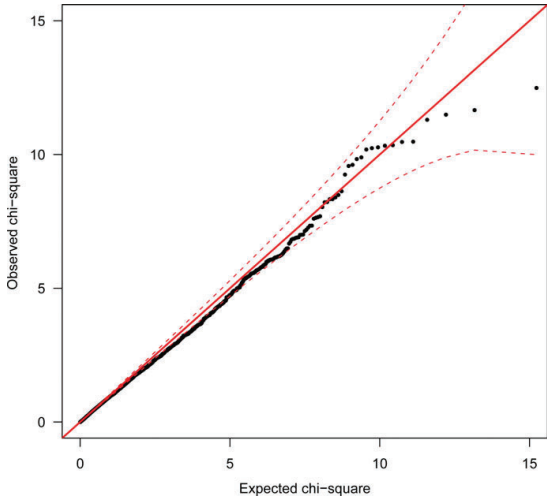

Figure 2. QQ-plot of Finnish case–control allelic association results for clinical migraine with aura end-diagnosis (closed black circles). The solid red line represents the expected (reference) distribution under the null. The broken red lines indicate the point-wise 95% CI envelope based on the standard errors of order statistics for an independent sample from the reference distribution.

occurrence (Table 5). An interaction between these two SNPs was not observed in any of the replication samples (Table 5 for comparison of population sample allele frequencies). However, other SNPs within these same genes, *KCNB2* and *CACNB2*, provided suggestive evidence for interaction in the Brisbane cohort and marginally significant evidence for interaction in the combined Cologne and Munich cohort (i.e. between *CACNB2* SNP rs8181477 and *KCNB2* SNPs rs7006287 and rs11782118, point-wise $P = 0.004017$ and $P = 0.004454$, respectively). A more detailed description of the interaction analysis is provided in the Supplementary Material, Appendix.

DISCUSSION

Triggered by the observation that all three genes for FHM, a monogenic subtype of MA, encode ion transporters, we investigated the role of ion transport genes in the common forms of migraine. To this end, we performed the first comprehensive screen of common genetic variants in highly plausible candidate ion transport genes. We tested a total of 5257 SNPs covering 155 of such genes initially in a large well-characterized Finnish case–control sample. The most significantly associated 66 SNPs in 12 genes were subsequently tested in four additional independent cohorts, but no consistent replication was detected across all cohorts.

Supplementary Material, Tables S2a and S2b contain estimates of the power to replicate association for the four most significantly associated SNPs [rs13276133, rs1431656 (*KCNB2*); rs13267466 (*KCNQ3*); rs2095771 (*CLIC5*)] in the Finnish cohort, plus the single *CLIC5* SNP (rs3729618) and two *KCNE2* SNPs (rs1013063, rs1467847) producing significant evidence for replication (EMP2 $P \leq 0.05$) in the individual Brisbane and Cologne cohorts, respectively. The combined replication sample had very high (>99%) power to replicate the four most significant Finnish association signals [rs13276133, rs1431656 (*KCNB2*); rs13267466 (*KCNQ3*); rs2095771 (*CLIC5*)], while the individual Leiden, Cologne, combined Cologne and Munich, and Brisbane cohorts each had 68–71%, 54–57%, 61–65% and 79–82% power to replicate at a nominal point-wise threshold of $P \leq 0.05$. At a globally significant replication threshold (adjusted for testing all 66 SNPs selected for replication) of $P \leq 0.001$ [estimated using SNPSpD (22)], the individual cohorts' power to replicate dropped considerably to 19–22%, 11–12%, 14–17% and 30–34%, respectively. However, the power to replicate ($P \leq 0.001$) remains high (85–89%) in the combined replication sample.

Despite our use of large and well-characterized migraine case–control cohorts (total of 2835 cases and 2740 controls), only the potassium voltage-gated channel, Isk-related, member 2 (*KCNE2*) gene on chromosome 21q22.11 produced marginally convincing evidence for replication in the Cologne cohort. However, the lack of association evidence in the remaining three replication cohorts, including a similar sample from Munich, cast doubt on the robustness of this finding. Furthermore, analysis of the *KCNE2* SNPs (rs1013063, rs1467847) in the combined replication sample produced non-significant CMH point-wise *P*-values of 0.187 and 0.161, and global EMP2 *P*-values of 1 and 0.9995, respectively (see Supplementary Material, Table S3).

While no single SNP produced significant evidence for association in the Finnish case–control sample, SNP × SNP epistasis analysis of the 66 SNPs selected for replication detected a significant interaction between *KCNB2* SNP rs1431656 on chromosome 8q13.3 and *CACNB2* SNP rs7076100 on chromosome 10p12.33 (point-wise

Table 3. Replication cohort sizes for individuals successfully genotyped

Replication cohort	Cases Total	Male	Female	Controls Total	Male	Female
Leiden	800	192	608	946	520	426
Cologne	601	136	465	651	159	492
Munich	288	40	248	0	0	0
Brisbane	1146	203	943	1143	691	452
Total	2835	571	2264	2740	1370	1370

$P = 0.0000199$; global $P = 0.022$). Although this specific interaction was not observed in our replication cohorts, the significance of the rs1542709 × rs7076100 interaction in the Finnish sample and supporting evidence for inter-genic SNP × SNP interaction in the combined Cologne and Munich replication cohort, indicate further study of *KCNB2* and *CACNB2* variants and their combined contribution to migraine susceptibility may be warranted.

To investigate how the reported results would compare to those potentially obtained in a high-density genome-wide association (GWA) study, we examined the SNP coverage across the gene footprints provided by the Illumina HumanCNV370-Duo and HumanHap550-Duo BeadChips (array marker lists were downloaded from http://illumina.com.au on 19 December 2007). These two arrays were selected owing to both their popularity and design. More specifically, in contrast to the Affymetrix arrays 5.0 and 6.0 (containing 440 794 and 906 600 SNPs, respectively), which are not designed with reference to LD structure, the Illumina arrays contain SNPs selected to tag LD bins—analogous to the approach employed in the design of our ion transport Perlegen array. However, although the Affymetrix 6.0 array was not designed with reference to LD structure, it is worth noting that it has been shown to provide similar LD coverage to the Illumina HumanHap550 [http://www.ashg.org/genetics/ashg07s/index.shtml; (23)].

Of the 370 405 SNPs present on the HumanCNV370 array, 3272 are located within the 155 gene 'footprints' (includes extension of 20 kb upstream of transcription start and 10 kb downstream of transcription end). Of the 561 467 HumanHap550 SNPs, 5270 are located within the 155 gene 'footprints'. Given the usable number of Illumina array SNPs would likely be reduced after QC, these counts indicate that our study provides at least as much coverage of common variation as that provided by the high resolution HumanHap550 array [which captures 90% of all HapMap Phase I + II loci (MAF > 0.05) with $r^2 > 0.8$] and far greater coverage than the HumanCNV370 array. Hence, the results of our detailed association screen of 155 ion transport genes are highly relevant to the burgeoning field of whole GWA studies. Indeed, the results of our study may be readily compared with GWA studies as they come to pass.

Furthermore, although our study was well-powered and had excellent SNP coverage, the absence of significant association within these 155 highly plausible migraine candidate genes at a moderate significance threshold (approximately $P < 10^{-5}$) compared to that required for GWA studies (recent guidelines recommend $P < 10^{-7}$) (24,25), indicates that common variation of moderate effect in these candidate genes does not play a major role in common migraine susceptibility within the samples examined. These results therefore suggest that either common variation of smaller effect in these candidate genes, or other 'less obvious' genes and pathways likely underlie the genetic susceptibility of the common forms of migraine. Alternatively, the investigated ion transport genes contribute to migraine through multiple, relatively rare and/or possibly interacting variants, or variation in distant *cis*- or *trans*-regulatory elements not tested in this study design. Although sample sizes in excess of 10 000 cases and 10 000 controls will be required to detect rare variants (MAF < 0.1) (26), a GWA study in large, well-characterized samples would be an unbiased way to test the hypothesis whether common variants in any part of the genome are associated with common forms of migraine. Indeed, efforts are now underway within the MGC to perform a GWA study utilizing at least 2000 unrelated MA cases. The results of the MGC GWA study should ultimately determine whether common genetic variants underlie susceptibility to common migraine and this approach would not restrict our thinking to previously known or predicted pathways. It enables testing of less predictable regions of the genome and allows examination of SNP × SNP interaction on a true genome-wide scale.

In addition, pathway-based approaches can be readily applied to GWA studies to yield biological insights that are otherwise undetectable by focusing only on individual genes and/or regions that have the strongest evidence for association (27). For example, a typical pathway-based approach might rank all genes by their significance of association and then investigate whether a particular group of genes is enriched at the significant end of the ranked list more than that expected by chance. Application of such pathway-based approaches, where multiple genes in the same pathway contribute to disease aetiology, but common variations in each of the causal genes make modest contributions to disease risk, has enormous potential to both detect novel and confirm hypothesized causal pathways and disease mechanisms other than ion transportation (e.g. dopaminergic and serotonergic pathways).

MATERIALS AND METHODS

Finnish discovery cohort

For this cohort, patients with a diagnosis of MA were drawn from the ongoing Finnish Migraine Study. MA was chosen as the principal diagnosis, as the Finnish patient pool has been primarily ascertained for this disorder and because the inclusion of aura as a diagnostic criterion reduces the possibility that other headache disorders would be included in the case sample (28). Patients were recruited from four headache clinics across Finland. Index cases were selected based on clinical examination by a neurologist from patients with at least three affected first-degree relatives; all available family members were also recruited into the study. Index cases and family members were diagnosed based on the IHS criteria (29,30) using the validated Finnish Migraine-Specific Questionnaire for Family studies (31). For the Finnish study, all participants gave informed consent and approval to conduct the research was obtained from the Helsinki University Central Hospital Ethics Committee. Over 6000 individuals in more than 800 families have taken part in this study and

Table 4. Replication association analysis results

Chr	NCBI build 36 position (bp)	dbSNP rs no.	Primary gene HGNC symbol	Finnish allelic *P* (MA)	Finnish allelic EMP2 *P* (MA)	Leiden replication allelic EMP2 *P*	Cologne replication allelic EMP2 *P*	Cologne + Munich replication allelic EMP2 *P*	Brisbane replication allelic EMP2 *P*	Replication CMH pointwise *P*	Replication CMH EMP2 *P*
1	179680697	rs12073221	CACNA1E	0.00817	1	0.9985				0.1543	0.9993
1	179691156	rs3856087	CACNA1E	0.00122	0.9839		1	1	0.9999	0.4142	1
1	179702677	rs644796	CACNA1E	0.01537	1	1	1	1	1	0.7915	1
1	179709099	rs505738	CACNA1E	0.0382	1	1	1	1	1	0.872	1
1	213353044	rs4300189	KCNK2	0.08803	1	1			1	0.8259	1
1	213365431	rs11120499	KCNK2	0.01368	1	1	1	1	1	0.8649	1
1	213395890	rs12567520	KCNK2	0.06979	1	1				0.3703	1
1	213398386	rs2363556	KCNK2	0.0698	1	1			1	0.7654	1
1	213428215	rs1556905	KCNK2	0.129	1	1			1	0.664	1
1	213428830	rs10494994	KCNK2	0.00416	1	1	1	1	1	0.7921	1
1	213458463	rs12143625	KCNK2	0.01314	1	1			1	0.9547	1
1	213467066	rs6670661	KCNK2	0.09116	1	1			1	0.9523	1
1	213476729	rs6686529	KCNK2	0.01113	1	0.9967			1	0.696	1
2	17908169	rs12996816	KCNS3	0.00166	0.9952	1	1	1	1	0.3029	1
2	17916155	rs10178489	KCNS3	0.02894	1	1	1	1	1	0.9674	1
2	47660997	rs17568951	KCNK12	0.00141	0.9909	1	1	1	1	0.6712	1
2	166879155	rs11890824	SCN9A	0.1714	1	1				0.2164	1
2	166889773	rs11898284	SCN9A	0.00375	1	1			1	0.8456	1
2	166902637	rs12622743	SCN9A	0.00357	1	1				0.3085	1
3	38599347	rs3922843	SCN5A	0.00564	1	1	0.9948	0.9995	1	0.08824	0.9832
3	38602829	rs7645358	SCN5A	0.1435	1	1			1	0.3676	1
3	38609146	rs7374540	SCN5A	0.1114	1	1			1	0.6739	1
3	38653505	rs7427106	SCN5A	0.00413	1	0.998	1	1	1	0.4155	1
3	38656222	rs9311195	SCN5A	0.00732	1	1	1	1	1	0.8305	1
3	38672343	rs9861643	SCN5A	0.1033	1	1			1	0.4628	1
6	45993676	rs11758298	CLIC5	0.1677	1	1			1	0.2692	1
6	45995399	rs1416167	CLIC5	0.02875	1	1			0.06909	0.03557	0.8129
6	45996051	rs3729618	CLIC5	0.03071	1	1	0.8306	0.9745	**0.0459**	0.3258	1
6	46008544	rs3777567	CLIC5	0.00172	0.9966	1	0.6888	0.938	0.2892	0.9135	1
6	46012610	rs2095771	CLIC5	0.00077	0.9358	1	0.7314	0.941	0.3037	0.6519	1
6	46018442	rs3777580	CLIC5	0.00555	1	1	1	1	0.5138	0.09749	0.9884
6	46026947	rs3777585	CLIC5	0.0265	1	1			0.1563	0.03622	0.8174
8	73598648	rs12550268	KCNB2	0.02711	1	1	0.9129	0.9347	0.9683	0.7111	1
8	73601624	rs1431659	KCNB2	0.00331	0.9999	1	0.6798	0.9853	0.9953	0.6447	1
8	73609424	rs5007872	KCNB2	0.00131	0.9876	1	0.7153	0.9728	1	0.7553	1
8	73614029	rs349358	KCNB2	0.04	1	1	0.9995	1	0.9996	0.5346	1
8	73618324	rs349355	KCNB2	0.03353	1	1	1	1	1	0.5737	1
8	73651525	rs349335	KCNB2	0.00674	1	1	1	1	1	0.6819	1
8	73653731	rs1431656	KCNB2	0.0007	0.9172	1	0.8643	0.9822	1	0.978	1
8	73659142	rs1542709	KCNB2	0.00673	1	1	0.9223	0.9938	1	0.9925	1
8	74006869	rs7006287	KCNB2	0.00121	0.983	0.9863	1	1	1	0.1335	0.998
8	74012757	rs11782118	KCNB2	0.00135	0.9892	0.9891	1	1	1	0.04141	0.8557
8	74016115	rs922772	KCNB2	0.00815	1	1	0.9997	0.9999	1	0.06849	0.9585
8	74017814	rs13276133	KCNB2	0.00041	0.7713	0.9975	1	1	0.9998	0.04397	0.8723
8	133408579	rs13267466	KCNQ3	0.00064	0.897	1	1	1	0.9996	0.5143	1
10	18458186	rs7897594	CACNB2	0.01682	1	0.9998	1	1	0.3177	0.003368	0.1659
10	18460390	rs11596974	CACNB2	0.03481	1	1			0.7107	0.05995	0.939
10	18799543	rs7076100	CACNB2	0.02933	1	0.9984			0.8954	0.6093	1
10	18800641	rs11598027	CACNB2	0.00198	0.9986	0.9998	1	1	1	0.9374	1

Continued

Table 4. *Continued*

Chr	NCBI build 36 position (bp)	dbSNP rs no.	Primary gene HGNC symbol	Finnish allelic *P* (MA)	Finnish allelic EMP2 *P* (MA)	Leiden replication allelic EMP2 *P*	Cologne replication allelic EMP2 *P*	Cologne + Munich replication allelic EMP2 *P*	Brisbane replication allelic EMP2 *P*	Replication CMH pointwise *P*	Replication CMH EMP2 *P*
10	18803689	rs1409202	CACNB2	0.00235	0.9998	0.9328	1	0.9999	1	0.9421	1
10	18822886	rs8181477	CACNB2	0.01226	1	1	0.9947	1	1	0.6363	1
10	18823994	rs11014504	CACNB2	0.03356	1	1			1	0.2729	1
16	82964929	rs17740111	ATP2C2	0.00389	1	1	1	1		0.6789	1
16	82971665	rs7350833	ATP2C2	0.03534	1	1	1	1	1	0.5818	1
16	82974143	rs10459853	ATP2C2	0.00582	1	1			0.9994	0.4499	1
16	83002938	rs247820	ATP2C2	0.01756	1	1	1	1	1	0.5331	1
16	83040732	rs247889	ATP2C2	0.00858	1	0.7355	0.9927	0.4468	1	0.5936	1
16	83055346	rs429313	ATP2C2	0.02536	1	1	0.4746	0.1494	0.9999	0.323	1
16	83062088	rs394533	ATP2C2	0.03043	1	1	0.2917	0.1177	0.859	0.54	1
16	83063868	rs400922	ATP2C2	0.01439	1	1	0.9851	0.8241		0.06808	0.9575
16	83065318	rs390208	ATP2C2	0.01406	1	1	0.9896	0.7866	1	0.4388	1
21	34612656	rs2834442	KCNE2	0.0039	1	1	0.2738	0.4455	1	0.3635	1
21	34620260	rs1013063	KCNE2	0.0205	1	1	**0.0193**	0.1565	1	0.1872	1
21	34636414	rs1467847	KCNE2	0.05496	1	1	**0.0139**	0.146	1	0.1605	0.9995
21	34639061	rs2834462	KCNE2	0.00862	1	1	0.708	0.9251	1	0.2167	1
21	34643430	rs8128316	KCNE2	0.01872	1	1		0.7774	1	0.7774	1

recruitment is under progress. From the total cohort of migraineurs in the Finnish family study, 898 unrelated individuals with MA were selected for this case–control study. Control subjects for the study were 900 unrelated individuals drawn from the Finnish Twin Cohort (32). One twin was selected from each pair, and these subjects were matched with respect to gender and age to cases. Control subjects have all responded negatively to the question of lifetime occurrence of migraine and also report no family history of migraine. Table 2 details key characteristics of patient findings for the Finnish study (33).

Replication cohorts

Leiden cohort. The Leiden sample is a well-phenotyped population-based migraine cohort from the Genetic Epidemiology of Migraine (GEM) study from the Netherlands. DNA was available from 823 unrelated migraine cases (555 MO; 268 MA) and 946 unrelated non-migraine controls. The GEM cohort is embedded within the 'MORGEN' project—a population-based study designed to monitor risk factors for and the prevalence of chronic diseases of public health importance in Dutch adults 20–65 years of age. The sample for the MORGEN project was selected randomly within equal-size strata of a five-year age group and gender from two Dutch county population registries. During 1995–1996, a total of 6491 individuals (52.7%) participated in the study. Respondents signed a general informed consent for the MORGEN project, and a specific informed consent for the GEM Study. For case-finding, MORGEN participants were mailed an extensive self-administered questionnaire that included questions about sociodemographic characteristics, medical history, psychosocial functioning and five migraine screening questions [adapted from Stewart *et al*. (34)]. Screen-positive participants completed a more detailed questionnaire that focused on signs and symptoms of migraine headache and aura as outlined in the IHS criteria. Very special care was given to diagnose aura and those reporting visual aura symptoms were also asked to draw what they saw. A semi-structured telephone interview was obtained in a random sample of (83%) screen-positive and (5%) screen-negative participants to clarify the signs and symptoms of migraine headache and aura reported in the stage 2 questionnaire, and to validate the earlier questionnaire. After a screening procedure, final (lifetime) diagnosis of migraine was made in 863 participants, 620 of whom had active migraine (10). The lifetime prevalence of migraine in women was 33% and the 1-year prevalence was 25%. In men, the lifetime prevalence was 13.3% and the 1-year prevalence was 7.5%. Among patients with migraine in the past year, 63.9% had MO, 17.9% had MA and 13.1% had both MA and MO. The prevalence of migraine was significantly higher in women and not associated with socioeconomic status. Migraine patients suffered a median of 12 migraine attacks per year; 25% had at least two attacks per month. The Leiden control sample was drawn from participants who were screened negative on the five migraine screening questions and matched the cases with respect to age and gender. The GEM sample has been used for genetic and clinical research, including assessing

Table 5. Allele frequency data for rs1431656 and rs7076100 interaction

Single nucleotide polymorphism	Allele	Finland Cases	Controls	Leiden Cases	Controls	Brisbane Cases	Controls
rs1431656	G	0.25	0.31	0.27	0.26	0.28	0.27
	A	0.75	0.69	0.73	0.74	0.72	0.73
rs7076100	A	0.42	0.46	0.40	0.38	0.41	0.44
	T	0.58	0.54	0.60	0.62	0.59	0.56
Haplotype	G–A	0.08	0.17	0.10	0.10	0.12	0.12
	G–T	0.17	0.14	0.17	0.17	0.16	0.15
	A–A	0.34	0.29	0.30	0.28	0.29	0.32
	A–T	0.41	0.40	0.43	0.45	0.43	0.41

the cardiovascular risk profile and impact of migraine on quality of life (35–37).

Cologne/Kiel cohort. All participants were diagnosed by the revised criteria of the IHS (30) (601 MA patients and 651 matched, healthy control individuals) and have provided their written informed consent; the study was approved by the Local University Ethics Committees. All patients were interviewed personally or by telephone by an experienced neurologist and a detailed questionnaire was completed for each of them. The complete patient ascertainment was performed by a single highly specialized headache centre. The questionnaire included a comprehensive assessment of (i) the type, frequency, localization and duration of aura symptoms, (ii) the possible existence of motor symptoms such as weakness or hemiplegia, (iii) the properties, frequency, localization and duration of the headache attack, (iv) a history of medication, (v) the existence of other possible diseases and (vi) the family history. All control individuals were unrelated, of German origin and were also gender-matched. Control individuals were interviewed to exclude those who suffer from frequent headaches or migraine. From each family, the index case, provided that he/she had a positive family history, has been included in the case–control sample [for more detailed cohort information see, for example, Netzer *et al*. (38)].

Munich cohort. All subjects were unrelated and were diagnosed with MA based on the revised criteria of the IHS and have given their written informed consent. All patients were personally seen and examined by a headache specialist. The majority of subjects were sampled through a headache clinic (approximately 60%) or a headache specialist in private practice (30%). The remaining patients were recruited at the Department of Neurology (Klinikum Großhadern, Munich). All participants received an extensive, validated questionnaire (31) used in studies on the genetics of migraine. The questionnaire includes a comprehensive assessment of (i) premonitory symptoms, (ii) the type, frequency, localization and duration of aura symptoms, (iii) the presence/absence of motor symptoms, (iv) the properties, frequency, localization and duration of the headache attack, (v) a history of medication, (vi) comorbidities, (vii) family history and (viii) ethnic origin. The study was approved by the Local Ethics Committee in Munich.

Brisbane cohort. The Australia-wide sample is a large population-based migraine cohort which has been used in a number of studies addressing various aspects of migraine genetics (39). The Brisbane sample is made up of two cohorts drawn from the Australian Twin Registry. In 1993–1995, a telephone interview comprising a diagnostic assessment of psychiatric disorders, including alcohol use and abuse, anxiety, depression and phobias, was conducted to an older cohort of twins, born 1902–1964. A total of 5996 individual twins completed the 1993 interview (40). Between 1996 and 2000, a younger cohort of twins (born 1964–1975) undertook a similar extensive semi-structured telephone interview, designed to assess physical, psychological and social manifestations of alcoholism and related disorders (41); 6265 individual twins completed the interview. For the Australian study, all participants gave informed consent and approval to conduct the research was obtained from the Queensland Institute of Medical Research (QIMR) Human Research Ethics Committee. Both cohort samples were not selected with regard to personal or family history of alcoholism or other psychiatric or medical disorders including migraine. Migraine symptom data were obtained during the course of the telephone interviews. The wording of questions was identical between cohorts. However, the younger cohort were asked questions relating to all IHS symptoms, whereas the older cohort had a somewhat more restricted number of questions (no questions regarding attack frequency, duration and pain intensity). The two cohorts were combined, thus allowing LCA to impute class membership in the older cohort, based on the pattern of all 10 symptoms observed in the younger cohort. To examine the accuracy of imputed class memberships, we compared the classification results for the younger dataset utilizing all the 10 available IHS symptoms, to the classification results for the younger dataset utilizing only the seven IHS symptoms that were available for the older cohort. Compared to using all 10 symptoms, when only the seven symptoms were used, these analyses found 98.5% and 96.0% of individuals were correctly classified as unaffected and affected, respectively; thus indicating that the three missing symptoms in the older cohort have negligible effect on the accuracy of individual LCA classifications (42). Given that we have previously shown LCA classified migraine to be a valid and accurate measure for genetic research (39), we combined Brisbane cases satisfying IHS criteria (64%) with cases classified as affected via our LCA approach (36%).

The Brisbane case–control replication sample consists of 1152 unrelated migrainous headache (39,42) cases of which 971 report visual aura and 1152 unrelated Australian twin

Table 6. Breakdown of candidate gene categories

Genes (*n*)	Gene category
26	Voltage-gated calcium channels
74	Voltage-gated potassium channels
14	Voltage-gated sodium channels
21	Chloride channels
20	ATPase ion transporters

controls screened negative for migraine. All controls have participated in the same survey and indicated that neither the study subject nor any first-degree relative had migraine.

Candidate gene selection

We targeted a comprehensive listing of ion channels and ion-channel modulators expressed in human brain for the association screen. Microarrays for genotyping were designed with the goal that they will have utility in a number of disorders beyond migraine in which ion-transporters are also predicted to play an important role, including epilepsy and cardiac arrhythmias. Therefore, we targeted all the relevant ion channels with sufficient SNP marker density to have high power to detect association. During the month of October 2005, we selected an inclusive list of ion-transporters from publicly available databases, including the ion-channel categorization on the Kyoto Encyclopedia of Genes and Genomes (KEGG) website http://www.genome.jp/kegg/ a text-based search of ion channels, ATPases and related proteins at the University of California Santa Cruz ('Golden Path') database http://genome.ucsc.edu/ and a screen of the Ensembl database http://www.ensembl.org/index.html for other ion-transport genes (Biological Process Gene Ontology term ID: 0030001). These genes were then further screened for expression in brain or heart using the eGenetics/SANBI databases via the Ensembl website. Additional screening for molecular function was performed using the data available in the National Center for Biotechnology Information (NCBI) Online Mendelian Inheritance in Man (OMIM) database http://www.ncbi.nlm.nih.gov/omim. This iterative process yielded a comprehensive listing of 155 ion channel and ion-channel modulators (see Supplementary Material, Table S1). Table 6 shows a breakdown of the major categories.

Polymorphism selection

Polymorphism selection for the ion-transport screen was primarily based on Perlegen's 'LD bin-map' of SNPs that are validated in their assay, have a minor allele frequency (MAF) >0.1, and have been tested in their screen of almost 1.6 million SNPs in 71 Americans of European, African and Asian ancestry to define whole genome patterns of variability (43). Perlegen has identified tag SNPs within each bin that are in LD with every other SNP in the bin with $r^2 > 0.8$ using the algorithm of Carlson *et al*. (44). Such representative tag SNPs were selected from LD bins spanning each of the 155 gene 'footprints' (transcriptional footprint extended 20 kb upstream of transcription start and 10 kb downstream of transcription end). The mean and median gene footprint was 136 280 bp and 67 100 bp, respectively.

In designing the microarrays, one-tag SNP was selected from bins containing one to two SNPs. For bins containing three or more SNPs, two-tag SNPs were selected to guard against possible assay failure of a single-tag SNP, thus minimizing the potential for loss of the substantial allelic variation contained within larger LD bins. A total of 5975 SNPs were selected for genotyping. These 5975 tag SNPs provide an overall mean and median per-gene resolution of one SNP every 5034 bp and 3552 bp, respectively; and effectively test a total of 11 148 SNPs with $r^2 > 0.8$, thus providing a very high resolution to test all common alleles within these genes.

Genotyping

Genotyping of the ion-transport SNPs utilized microarrays designed by Perlegen Sciences, Inc. (Mountain View, CA, USA) and manufactured by Affymetrix, Inc. (Santa Clara, CA, USA). These assays have been documented to have a $>99\%$ concordance with other genotyping methods (43,45). Genotyping assays and QC was performed by Perlegen. A total of 59 individuals were excluded from further analysis due to an overall call rate of $<80\%$ and the data quality remaining poor after repeat genotyping of the sample. An additional 14 individuals were excluded from further analysis due to evidence of familial relatedness after examination of allele sharing among pairs of individuals using the genotypic relative risk program (46).

A total of 706 SNPs were excluded from further analysis according to at least one of the following QC criteria: (i) call rate of $<95\%$, (ii) $P < 10^{-4}$ for deviation from Hardy–Weinberg equilibrium and (iii) X and Y chromosome SNP performance (i.e. for non-pseudoautosomal X chromosome SNPs, females can have up to two alleles, whereas males can have only one allele; for Y chromosome SNPs, females should have zero alleles while males should have one allele). The resulting 5269 tag-SNPs still provide a very high resolution to test all common alleles within the 155 ion-transport genes with an overall mean and median per-gene resolution of one SNP every 5968 bp and 4323 bp, respectively.

For the replication study, SNP genotypes were determined in a 384-well format using the Sequenom iPLEX system (Sequenom, San Diego, CA, USA) in which the distinction between genotypes is based on the mass differences between the primer extension products of the two alleles. Assays were designed using the AssayDesign software (Sequenom); primer information is available from the corresponding author upon request. The multiplex polymerase chain reaction and primer extension reactions were performed under standard conditions according to the manufacturer's instructions using 20 ng of genomic DNA as a template.

Power of Finnish case–control sample to detect association

To account for non-independence between SNPs due to intermarker LD, we determined the approximate effective number of independent SNPs (M_{eff}) following the SNPSpD approach of Nyholt (22) and applying equation (5) of Li and Ji (47). Utilizing observed genotype data for the final 5269 SNPs passing

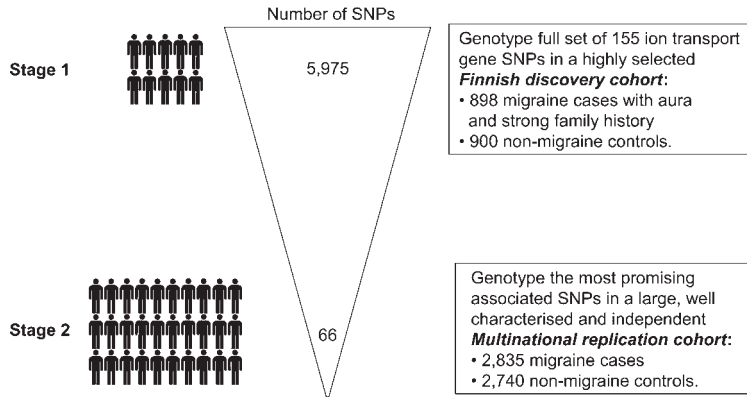

Figure 3. Overview of study design.

Perlegen's QC protocols, we calculated the intermarker LD (pair-wise r^2) for SNPs located on the same chromosome. Examination of the eigenvalues after spectral decomposition of the resulting chromosome-specific correlation matrices ($\sqrt{r^2}$) determined an equivalent of 2677 total independent SNPs to be tested for association. Consequently, to ensure an overall type I error rate of 5%, we require a Bonferroni-adjusted significance level (α) of $P = 0.05/2677 = 0.000019$ $[-\log_{10}(\alpha) = 4.7386]$.

Power calculations were performed for a case–control study assuming a disease prevalence for MA of 5% using the Genetic Power Calculator (48). In short, power calculations for our Finnish discovery cohort assumed loci with dominant and multiplicative modes of inheritance and were based on our post-QC sample of 841 cases and 884 controls. The Finnish cohort has over 80% power to detect dominant disease-predisposing alleles of frequency 0.10, 0.25 and 0.5 contributing a GRR of 1.78, 1.70 and 2.32, respectively. For the general multiplicative model, there is 80% power to detect risk alleles of frequency 0.10, 0.25 and 0.5 contributing a GRR of 1.65, 1.45 and 1.40, respectively. These calculations demonstrate our Finnish discovery cohort to have high power to detect ion-transport gene associations of moderate effect.

Statistical analysis

The following statistical analyses were performed using the PLINK analysis program (version 1.00) (http://pngu.mgh.harvard.edu/purcell/plink/) (49). PLINK QC options were utilized with default thresholds: maximum missing genotypes per person (*--mind* option) ≤ 0.10, maximum missing genotypes per SNP (*--geno* option) ≤ 0.10, MAF (*--maf* option) ≥ 0.01 and Hardy–Weinberg disequilibrium (exact) P-value ≥ 0.001 in controls (*--hwe* option). Cluster analysis based on pair-wise IBS distance (*--cluster* option) of the SNPs passing both Perlegen and PLINK QC criteria was utilized to identify possible population structure within the Finnish case–control cohort. Single marker basic allelic association (χ^2_1) tests (*--assoc* option) were performed for each of the post-QC SNPs. PLINK's max(T) permutation procedure (*--mperm* option) was used with 10 000 iterations to obtain accurate global empirical P-values (i.e. point-wise P-values are for the single test only, whereas global P-values are adjusted for testing all analyzed SNPs) ('EMP2' column of *--mperm* output). Genome-wide significant association is therefore obtained at a global EMP2 P-value of 0.05 or smaller. Although the primary analysis in the Finnish cohort tested for association to MA, we also performed exploratory association analysis to strict IHS criteria, and the individual migraine trait components of 'pulsation', 'photophobia' and 'phonophobia' to help select SNPs for replication.

The FDR approach (50–52), which allows a controlled proportion of positive results to be false, while detecting more true-positives was also used to help select and interpret the most promising SNP associations for replication. This was done using the default settings of the QVALUE computer program (version 1.1) (53). A preponderance of χ^2_1 values suggestive of association was also examined via a QQ-plot, by plotting the distribution of observed chi-square (χ^2_1) values obtained from the single SNP allelic association analysis of MA in Finns against the distribution of χ^2_1 values expected under the null hypothesis of no association.

Given that recent results from two-stage association studies indicate the most significant SNP in the total sample is not necessarily the most significant SNP in the first stage, to balance guarding against not following-up a true risk locus with the number of loci to follow-up (i.e. minimizing the multiple-test burden to maintain high replication power), SNPs producing nominally significant ($P < 0.005$) allelic association with MA in the Finnish cohort, together with supporting evidence for association to strict IHS migraine and individual trait components were selected for genotyping in the replication cohorts. The nominal significance threshold of $P < 0.005$ was selected after examination of the QQ-plot and FDR results.

Given PLINK's clustering procedure (*--cluster* option) requires whole-genome level data to give accurate results (probably $>10\,000$ SNPs), to allow for potential population differences between the replication cohorts, a CMH analysis (*--mh* option) was used to test for overall association in the

replication samples, controlling for cohort. The CMH test for $2 \times 2 \times K$-stratified tables is based on an 'average' odds ratio (OR) that controls for the potential confounding due to the cluster (cohort) variable. To account for differences in ascertainment and diagnoses in addition to potential genetic differences at the population level, the Leiden (Dutch), Cologne and Munich (German), and Brisbane (Australian) cohorts were each assigned to the separate clusters, resulting in a CMH test that controls for four separate clusters. A schematic overview of our study design is provided in Figure 3.

To further examine peak association results and guard against missing a strong association signal by only analyzing observed/single SNP data, exploratory association analysis of haplotypes spanning two to six contiguous SNPs were also performed using a sliding window approach via PLINK's *--hap-window* and *--hap-assoc* options.

Given the strong prior hypothesis for involvement of these genes in migraine, we also tested for SNP × SNP epistasis using PLINK's *--epistasis* option. For our total 5257 SNPs there are 13 815 396 possible SNP × SNP interactions. Therefore, although it is possible for loci with no individual effects to still have a component of genetic variance attributable to epistatic effects, following the recommendation of Blangero *et al*. (54), to minimize epistasis multiple-testing burden, analyses were restricted to SNPs producing nominally significant ($P < 0.005$) allelic association with MA in the Finnish cohort.

Finally, the PS program (55) was used to perform power calculations in the replication cohorts. Briefly, the power to replicate promising association results was estimated in the individual and combined replication cohorts utilizing the control allele frequency and OR observed in the Finnish case–control cohort.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* Online.

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IV

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Genome-wide association study of migraine implicates a common susceptibility variant on 8q22.1

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Migraine is a common episodic neurological disorder, typically presenting with recurrent attacks of severe headache and autonomic dysfunction. Apart from rare monogenic subtypes, no genetic or molecular markers for migraine have been convincingly established. We identified the minor allele of rs1835740 on chromosome 8q22.1 to be associated with migraine ($P = 5.38 \times 10^{-9}$, odds ratio = 1.23, 95% CI 1.150–1.324) in a genome-wide association study of 2,731 migraine cases ascertained from three European headache clinics and 10,747 population-matched controls. The association was replicated in 3,202 cases and 40,062 controls for an overall meta-analysis P value of 1.69×10^{-11} (odds ratio = 1.18, 95% CI 1.127–1.244). rs1835740 is located between *MTDH* (astrocyte elevated gene 1, also known as *AEG-1*) and *PGCP* (encoding plasma glutamate carboxypeptidase). In an expression quantitative trait study in lymphoblastoid cell lines, transcript levels of the *MTDH* were found to have a significant correlation to rs1835740 ($P = 3.96 \times 10^{-5}$, permuted threshold for genome-wide significance 7.7×10^{-5}). To our knowledge, our data establish rs1835740 as the first genetic risk factor for migraine.

The recent boom of genome-wide association studies (GWAS) has had a major impact on our current view of genetic susceptibility to common traits and complex disorders. However, central nervous system disorders are under-represented among the conditions for which such associations have been found[1]. To our knowledge, no GWAS or common, robustly established linked genetic variants have been reported for major episodic neurological disorders (ICD-10 codes G40–G44, migraine, epilepsy and ataxias). However, there is substantial genetic information for rare Mendelian forms of migraine, epilepsy and ataxia, which classifies them as channelopathies associated with compromised neurotransmitter homeostasis[2]. So far, there is no evidence for the contribution of ion channel variants in common forms of these diseases[3,4].

Migraine is an episodic neurological disorder with complex pathophysiology, affecting 8% of males and 17% of females[5] in the European population. Migraine ranks among the 20 most disabling diseases and has been estimated as the most costly neurological disorder, with a considerable impact on public health[6]. Clinically, the International Classification of Headache Disorders (ICHD-II[7]) recognizes two main common forms of migraine: migraine with aura and migraine without aura. The two forms are distinguished from each other based on the presence of aura, a period of variable and diverse neurological symptoms that precede the headache phase. Individuals may have attacks of only migraine without aura, or only migraine with aura, or they may have a combination of both types in variable proportions. There is debate among the scientific community whether migraine with aura and migraine without aura attacks represent two distinct disorders or if they are merely variations of a single disease having a common complex genetic background. Migraine headache is believed to be caused by activation of the trigeminovascular system and the aura by cortical spreading depression, a slowly propagating wave of neuronal and glial

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Table 1 Study populations used in the two stages of the study

		Total	Men (%)	Women (%)	Individuals with both MA and MO (%)	Individuals with MA only (%)	Individuals with MO only (%)
Discovery stage							
Finland	Cases	1,064	19.8	80.2	94.4	5.6	0.0
	Controls	3,513	47.4	52.6	–	–	–
Germany	Cases	1,029	18.9	81.1	70.2	29.8	0.0
	Controls	2,317	45.1	54.9	–	–	–
The Netherlands	Cases	655	17.2	82.8	65.9	34.1	0.0
	Controls	4,917	41.7	58.3	–	–	–
Total GWAS	Cases	2,731	18.8	81.2	78.5	21.5	0.0
	Controls	10,747	44.3	55.7	–	–	–
Replication stage							
Iceland	Cases	900	22.5	77.5	63.0	21.8	15.2
	Controls	35,221	57.4	42.6	–	–	–
Denmark	Cases	1,116	22.4	77.6	26.3	43.3	30.5
	Controls	1,353	44.5	55.5	–	–	–
The Netherlands	Cases	349	18.3	81.7	59.8	40.2	0.0
	Controls	2,082	43.9	56.1	–	–	–
Germany	Cases	837	11.6	88.4	0.0	0.0	100.0
	Controls	1,406	37.3	62.7	–	–	–
Total replication	Cases	3,202	19.1	80.9	33.8	25.6	41.0
	Controls	40,062	55.6	44.4	–	–	–
Overall meta-analysis	Cases	5,933	19.0	81.0	54.4	23.7	22.1
	Controls	50,809	53.2	46.8	–	–	–

MA, migraine with aura; MO, migraine without aura.

depolarization[8–10]. However, these are considered to be downstream events, and it is unknown how migraine attacks are initiated.

To identify variants associated with the common forms of migraine, we carried out a two-stage GWAS in seven European migraine case collections (six clinic-based and one population-based) (**Supplementary Fig. 1**). In the discovery stage, we studied 3,279 migraineurs (1,124 Finnish, 1,276 German and 879 Dutch individuals) recruited from headache clinics and genotyped using Illumina arrays against population-matched controls (10,747 individuals) recruited from preexisting population-based GWAS (**Supplementary Note**). In the replication stage, a further 3,202 cases and 40,062 population-matched controls from Iceland, Denmark, The Netherlands and Germany were studied.

Diagnoses were made by headache experts using a combination of questionnaires and individual interviews that were based on the ICHD-II guidelines[7]. Due to the overlap between individuals having migraine with aura and those having migraine without aura, we analyzed the following diagnostic subgroups: (i) 'all migraine', defined as all individuals with migraine irrespective of subtype; (ii) 'migraine with aura only', defined as individuals who only have attacks where aura is present; (iii) 'both migraine with aura and migraine without aura', defined as individuals with attacks both with and without aura; and (iv) 'migraine without aura only', defined as individuals with only attacks of migraine without aura.

We used a multipopulation Cochran-Mantel-Haenszel (CMH) association analysis and a significance threshold of $P \leq 5 \times 10^{-8}$ in our analyses. In the discovery sample, 2,731 cases and 10,747 controls (**Table 1**) passed quality control steps, and 429,912 markers were successfully genotyped (Online Methods). A quantile-quantile plot of the CMH analysis (**Supplementary Fig. 2**) and an overall inflation factor (λ) of 1.08 were used as final quality control measures.

Only one marker, rs1835740 on chromosome 8q22.1, showed significant association with migraine in the multipopulation CMH analysis (**Fig. 1** and **Supplementary Fig. 3**). Eleven further loci were found with $P \leq 5 \times 10^{-5}$ (**Supplementary Table 1**). The minor allele (A) of marker rs1835740 was associated with migraine with $P = 5.38 \times 10^{-9}$ and odds ratios ranging between 1.21 and 1.33 (**Table 2**). Two nearby markers with the highest linkage disequilibrium (LD) to rs1835740 (rs982502, $r^2 = 0.59$, $P = 1.34 \times 10^{-4}$ and rs2436046, $r^2 = 0.69$, $P = 1.78 \times 10^{-5}$) also showed association with migraine (**Supplementary Table 2**). Haplotype analysis detected a 27-kb haplotype ($P = 5.35 \times 10^{-8}$) (**Supplementary Fig. 4** and **Supplementary Table 3**). In the HapMap Phase II data[11], the variant is located between two close recombination hotspots, and analysis using the ssSNPer program[12] demonstrated that no long-range LD to rs1835740 exists within a 5-Mb window, strongly suggesting that the causative variant in this region is tagged by the minor allele of rs1835740 (**Fig. 1**). The 2-Mb window around rs1835740 was also imputed against the 1000 Genomes data (August 2009 release), but no other marker showed evidence of association exceeding that for rs1835740 (**Fig. 1**). Conditional analysis of the SNPs around rs1835740 showed no additional independent signals (**Supplementary Table 2**). The proportion of genetic variance explained by the rs1835740 variant was estimated to be between

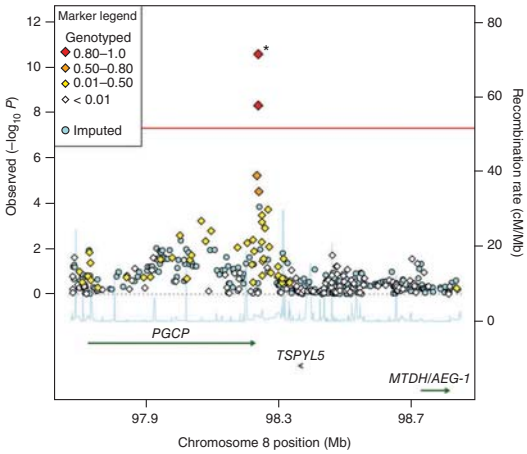

Figure 1 Cochran-Mantel-Haenszel association results for combined analysis of the three study populations between 97.5 Mb and 99.0 Mb on chromosome 8q22.1. Diamonds show the position and *P* value for each marker in the region, with colors representing the extent of linkage disequilibrium (measured in r^2) with the marker rs1835740, and blue circles indicate the locations and *P* values of the imputed markers. For rs1835740, *P* values are shown for both the original GWAS and the meta-analysis of all migraine samples in the study (denoted by asterisk). The blue graph shows the local recombination rate based on HapMap Phase II data[11]. The red line denotes the threshold for genome-wide significance ($P \leq 5 \times 10^{-8}$). This figure was generated using a modified version of the script available at http://www.broadinstitute.org/node/555.

Table 2 Association results for marker rs1835740 using the CMH test

	Diagnosis	*n* (cases)	*n* (controls)	Case alleles (MAF)	Control alleles (MAF)	*P*	OR	95% CI
GWAS								
Finland	All migraine	1,064	3,513	548/1,576 (0.258)	1,553/5,461 (0.221)	0.000447	1.22	1.093–1.368
Germany	All migraine	1,029	2,317	515/1,537 (0.251)	998/3,632 (0.216)	0.00142	1.22	1.079–1.378
The Netherlands	All migraine	655	4,917	329/963 (0.255)	2,086/7,742 (0.212)	0.000876	1.26	1.098–1.437
Discovery stage								
	MA only	589	10,747	313/859 (0.267)	4,637/16,385 (0.216)	3.07×10^{-5}	1.33	1.164–1.528
	Both MA and MO	2,142	10,747	1,071/3,193 (0.251)	4,637/16,385 (0.216)	2.69×10^{-6}	1.21	1.115–1.304
	All migraine	2,731	10,747	1,384/4,052 (0.255)	4,637/16,385 (0.216)	$\mathbf{5.38 \times 10^{-9}}$	1.23	1.150–1.324
Replication stage								
Denmark	MA only	483	1,353	244/722 (0.253)	562/2,144 (0.208)	**0.015**	1.29	1.050–1.583
	Both MA and MO	293	1,353	121/465 (0.206)	562/2,144 (0.208)	0.951	0.99	0.785–1.255
	MO only	340	1,353	153/527 (0.225)	562/2,144 (0.208)	0.333	1.11	0.900–1.362
	All migraine	1,116	1,353	518/1,714 (0.232)	562/2,144 (0.208)	0.069	1.15	0.989–1.344
Iceland	MA only	137	35,221	70/204 (0.255)	14,212/56,230 (0.202)	**0.0380**	1.36	1.017–1.812
	Both MA and MO	196	35,221	82/310 (0.209)	14,212/56,230 (0.202)	0.7256	1.05	0.812–1.350
	MO only	567	35,221	261/873 (0.230)	14,212/56,230 (0.202)	**0.0292**	1.18	1.017–1.376
	All migraine	900	35,221	413/1,387 (0.229)	14,212/56,230 (0.202)	**0.010**	1.18	1.041–1.334
The Netherlands	MA only	212	2,082	100/324 (0.236)	909/3,255 (0.218)	0.406	1.11	0.873–1.399
	Both MA and MO	137	2,082	66/208 (0.241)	909/3,255 (0.218)	0.382	1.14	0.853–1.513
	All migraine	349	2,082	166/532 (0.238)	909/3,255 (0.218)	0.250	1.12	0.925–1.350
Germany	MO only	837	1,406	396/1,278 (0.240)	629/2,183 (0.224)	0.3206	1.08	0.932–1.241
	MO only[a]	837	541	396/1,278 (0.240)	218/864 (0.201)	**0.0307**	1.23	1.019–1.480
Meta-analysis								
	All "MA only"	1,421	49,403	727/2,109 (0.256)	20,320/78,464 (0.206)	6.98×10^{-8}	1.29	1.173–1.408
	All "Both MA and MO"	2,768	49,403	1,340/4,176 (0.243)	20,320/78,464 (0.206)	1.09×10^{-5}	1.17	1.089–1.248
	All "MO only"	1,744	37,980	810/2,678 (0.232)	15,403/60,557 (0.203)	0.0105	1.12	1.028–1.230
	All "All migraine"	5,933	50,809	2,877/8,963 (0.243)	20,949/80,647 (0.206)	$\mathbf{1.69 \times 10^{-11}}$	1.18	1.127–1.244

MA, migraine with aura; MO, migraine without aura. Genome-wide significant values and successful replications are shown in boldface.
[a]Values in this row were calculated after excluding an outlier control sample. The German replication control set consisted of several small samples. The largest of these had a considerably deviating minor allele frequency (MAF) (MAF = 0.238, *n* = 865) compared to other German (average MAF = 0.216, *n* = 3,260) and Central European control sets (average MAF = 0.212, *n* = 9,560). Thus, values with both including and excluding the outlier control sample are presented in the case allele and control allele columns. The meta-analysis value includes all control samples (without the outlier control group, "all migraine without aura samples," $P = 0.00107$, OR = 1.18, 95% CI 1.068–1.298 and "all migraine samples," $\mathbf{P = 8.43 \times 10^{-13}}$, OR = 1.20, 95% CI 1.143–1.264.

1.5% and 2.5%, depending on the heritability estimate used, and the population attributable risk was estimated to be 10.7% using previous methodology[13].

To confirm and extend our results, we performed a replication study on the only marker with genome-wide significance in the discovery stage: rs1835740. The diagnostic subgroups used in the discovery stage were also applied to the replication stage. Replication was successful in two 'migraine with aura only' subsets (Danish, $P = 0.015$, OR = 1.29 and Icelandic, $P = 0.038$, OR = 1.36), in the Icelandic 'migraine without aura' set ($P = 0.0292$, OR = 1.18) and in the Icelandic 'all migraine' group ($P = 0.010$, OR = 1.18) (**Table 2**). Overall, the A allele of marker rs1835740 was overrepresented (OR = 1.05–1.36; **Table 2**) in each subset of all replication samples except in the Danish 'both migraine with aura and migraine without aura' group (OR = 0.99). The effect was consistently stronger in the 'migraine with aura only' groups than other migraine subgroups (**Fig. 2**). It should be noted that the majority of the groups that did not reach formal replication were small and had limited power. Meta-analysis was conducted using the CMH test for each diagnosis subgroup alone as well as for all migraine samples together, with the latter group showing a final $P = 1.69 \times 10^{-11}$ (**Table 2**).

Marker rs1835740 is located between two potentially interesting candidate genes, *MTDH* and *PGCP*. We analyzed the effect of this marker's genotype on the expression of genes within a 2-Mb window in fibroblasts, primary T cells and lymphoblastoid cell lines (LCL) obtained from umbilical cords[14]. In the expression quantitative trait locus (eQTL) analysis, the rs1835740 genotype was found to have significant correlation

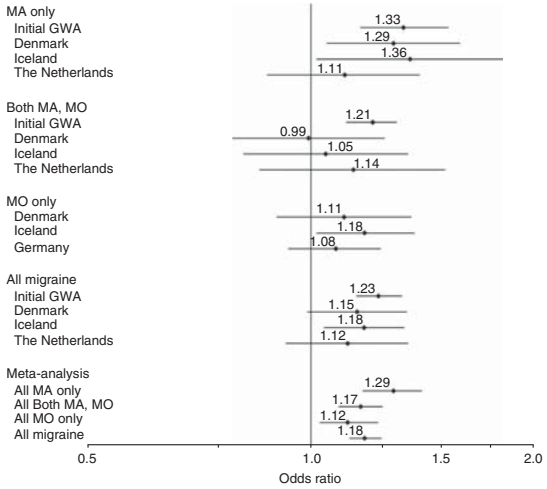

Figure 2 For each dataset, the horizontal line indicates the 95% CI, and the number above the line indicates the point estimate of the odds ratio. MA only, individuals whose attacks are always accompanied with aura; both MA, MO, individuals with attacks with and without aura; MO only, individuals whose attacks never include aura.

Table 3 Association of rs1835740 genotype with gene expression levels

SNP	Gene	Strand	SNP coordinate	Gene start	Distance	SRC *P*
rs1835740	*UQCRB*	–	98,236,089	97,311,911	924,178	0.0013226
rs1835740	*MTDH*	+	98,236,089	98,725,583	489,494	0.0000396[a]
rs1835740	*HRSP12*	–	98,236,089	99,183,743	947,654	0.0028748

Genes with nominal or higher *P* values of expression association to rs1835740 genotype in the Spearman rank correlation test are shown.
[a]This value surpassed the significance threshold 7.7×10^{-5} (corresponding to a 0.001 permutation threshold after 10,000 permutations). Gene start refers to the location of 5′ end of the gene if on the positive strand and the 3′ end if on the negative strand. Locations and distances are given in base pairs and are according to NCBI build 36. SRC, Spearman rank correlation.

to the transcript levels of the nearby *MTDH* gene in LCLs (**Table 3** and **Supplementary Table 4**), with the risk allele A being associated with higher expression levels (**Fig. 3**). This is in line with previous studies, which have proven that expression analyses in LCL cells are informative in neurological and neuropsychiatric traits[15–17]. No significant association was detected in fibroblasts or primary T cells. The eQTL analysis suggested that rs1835740 is a *cis* regulator of *MTDH* in LCLs.

The location of the associating sequence variant, rs1835740, between two genes involved in glutamate homeostasis, *PGCP* and *MTDH,* suggests that this region contains elements that could regulate either or both of these flanking genes; the eQTL analysis pointed to the latter. Although *MTDH* has mainly been studied in relation to carcinogenesis[18], previous studies in cultured astrocytes have shown that *MTDH* downregulates *SLC1A2* (also known as *EAAT2* and *GLT-1*)[18–22], the gene encoding the major glutamate transporter in the brain. Furthermore, knock-out mice lacking the EAAT2 protein from their brains have been shown to suffer from lethal spontaneous epileptic seizures[23]. Despite the limitations in extrapolating eQTL findings from LCL cells directly to brain tissue, these data suggest a plausible link between the identified variant and glutamate regulation. This is a tempting hypothesis, as this neurotransmitter has long been suspected to play a key role in migraine pathophysiology[24].

Although the evidence provided here is indirect, accumulation of excess glutamate in the synaptic cleft through downregulation of EAAT2 or an increase in PGCP activity (or both) would provide a putative mechanism for the occurrence of migraine attacks. It is reasonable to speculate that this accumulation can increase susceptibility to migraine through increased sensitivity to cortical spreading depression, the likely mechanism for the migraine aura[9,10], as well as through glutamate involvement in central sensitization, which has been postulated to be the underlying mechanism of allodynia during a migraine attack[25].

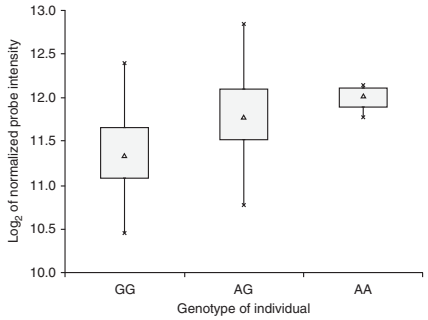

Figure 3 A box-plot of the quantified expression values for *MTDH*, ordered based on the sample genotype of rs1835740. Normalized expression levels in lymphoblastoid cell lines using Illumina's WG-6 v3 Expression BeadChip array are shown. In each group, the small pyramid indicates the median value, the shaded area represents the lower and upper quartiles, and the crosses show the minimum and maximum values in the expression data.

Neither this study nor our previous study[3] yielded evidence for association of ion channel genes to common forms of migraine. Thus, even if the contribution of ion channel genes is well established in Mendelian forms of paroxysmal neurological disorders, such as familial hemiplegic migraine (FHM)[26–29], their direct role in more common forms of paroxysmal neurological disorders remains open. Interestingly, previous studies suggested that the imbalance of glutamate release and clearance is a key component of the pathogenesis of FHM; the underlying mutation in FHM lies in *CACNA1A*, *ATP1A2* or *SCN1A*[30,31]. The results of the present study support the hypothesis that complementary pathways such as the glutamate system may tie the Mendelian channelopathies with the pathogenetic mechanisms of more common forms of episodic neurological disorders, such as migraine. Alterations in the functionally related EAAT1 transporter have been identified in other episodic phenotypes (such as episodic ataxia 6 (ref. 32) and a phenotype with episodic ataxia, hemiplegia and seizures[33]), providing an example of the link between EAAT transporters and episodic disorders. Future studies should be conducted to specifically test this hypothesis.

In summary, to our knowledge, we have identified the first robust genetic association to migraine. As our cases were mainly selected from specialized headache clinics, subsequent studies are needed to establish the contribution of rs1835740 in population-based migraine cohorts. These population-based cohorts may represent a different severity spectrum and possibly also a somewhat different underlying combination of genetic susceptibility variants. The effect of rs1835740 is stronger in individuals with migraine with aura than in those with migraine without aura, but further studies are needed to confirm the role of the variant in different migraine subgroups. This variant explains only a small fraction of the overall genetic variance in migraine, and future GWAS, perhaps with different ascertainment schemes, will likely identify additional loci explaining more of the genetic variance.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

Accession codes. Migraine, OMIM 157300.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

All authors contributed to the current version of the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Study design. We jointly analyzed samples from three migraine with aura collections from Finland, Germany and The Netherlands with population-matched controls obtained from preexisting studies. This discovery phase was followed by a replication study of the top SNP, rs1835740, in samples from individuals with migraine from Denmark, Iceland, The Netherlands and Germany. Characteristics of each study sample are described in **Table 1**, and the recruitment and ascertainment of cases and controls are described in the **Supplementary Note**.

Discovery stage genotyping. DNA was extracted from the subjects' blood samples using standard methods. Genotyping of the GWAS samples was done at the Wellcome Trust Sanger Institute on the Illumina 610K (for the Finnish and German samples) and the Illumina 550K (for the Dutch samples) SNP microarrays following the Infinium II protocol from the manufacturer (Illumina Inc.). Genotype calling was performed using the Illuminus software[34].

Replication stage genotyping. For the replication study, all Danish cases and 459 migraine-free controls were genotyped using the Centaurus platform (Nanogen Inc.), and 904 additional controls were genotyped at deCODE genetics using the Illumina HumanHap650 BeadArray. The Icelandic cases and controls were genotyped using the Illumina HumanHap 317K, 370K, 610K or 1M bead arrays at deCODE genetics. The Dutch replication cohort was genotyped using the TaqMan technology (Applied Biosystems, Life Technologies) at Leiden University Medical Center. The German replication cases were genotyped using Illumina HumanHap 610K array at the Institute of Human Genetics at the Helmholtz Zentrum, Munich.

Expression study. The GenCord resource, a collection of cell lines derived from umbilical cords of 75 newborns of Western European origin born at the maternity ward of the University of Geneva Hospital, was used for the expression study. Sample collection was performed on full-term or near-full-term pregnancies to ensure homogeneity for sample source age. Three cell types were derived: (i) primary fibroblasts, (ii) LCLs and (iii) primary T cells[14]. Total RNA was extracted from these cells and two one-quarter-scale MessageAmp II reactions (Ambion) were performed for each extraction with 200 ng of total RNA. 1.5 µg of cRNA was hybridized to Illumina's WG-6 v3 Expression BeadChip array to quantify transcript abundance[35]. Intensity values were log2 transformed and normalized independently for each cell type using quantile normalization for sample replicates and median normalization across all individuals. Each cell type was renormalized using the mean of the medians of each cell type's expression values. DNA samples were extracted from umbilical cord tissue LCLs with the Puregene cell kit (Gentra-Qiagen), and genotyping was performed using the Illumina 550K SNP array (Illumina Inc.) to obtain the SNP genotypes for the samples.

Statistical analysis of the genome-wide scan data. Stringent per-SNP and per-sample limits were implemented in order to obtain high-quality data. Quality control measures were as follows: exclusion of samples with call rates <97%, non-comparable ancestry as measured using multidimensional scaling plots from PLINK[36], possible contamination as identified by being an extreme heterozygosity outlier and cryptic relatedness (low-level relatedness to a large number of samples) and non-cryptic relatedness of $\hat{\pi}$ >12.5%. From the initial 3,279 cases and 12,369 controls, 2,731 cases and 10,747 controls passed all quality control criteria, and 531 cases and 1,622 controls were excluded. The majority of case exclusions were due to quality issues on the 550K chips, and the majority of control exclusions were due to low-level relatedness in the Dutch control set. SNPs were excluded for having a minor allele frequency of <1% or for departing from Hardy-Weinberg equilibrium with $P < 10^{-6}$ in cases or controls. Only completely overlapping SNPs from the three populations were used, leaving a total of 429,912 SNPs for analysis. To ascertain whether the control samples were properly matched to the cases, a population-specific inflation factor and an overall genomic inflation factor (λ) were estimated using the median χ^2 value from a 1 degree-of-freedom allelic χ^2 test. For the Finnish samples, $\lambda = 1.05$; for the German samples, $\lambda = 1.07$; for the Dutch samples, $\lambda = 1.09$; and the overall $\lambda = 1.08$, suggesting reasonably well matched controls in each case. Differences between cases and controls were assessed between each SNP and disease status using a two-tailed CMH test for $2 \times 2 \times K$ stratified data (where $K = 3$), as implemented in PLINK v1.06. To exclude long-range LD for the identified variant, we used the program ssSNPer[12] to demonstrate that no SNP within a 5-Mb window had high LD to rs1835740 in HapMap Phase II data.

Conditional analysis for secondary effects. In addition to rs1835740, two other SNPs on 8q22.1, rs2436046 and rs982502, showed a CMH $P < 10^{-3}$ (**Table 2** and **Fig. 2**). Based on our data, rs2436046 ($r^2 = 0.68$) and rs982502 ($r^2 = 0.59$) are in moderate LD with rs1835740. To evaluate whether these signals were independent from the top SNP association signal, the association between migraine and SNP alleles was tested using logistic regression, conditioning on rs1835740 as implemented in PLINK v1.06. Conditioning on rs1835740, no evidence of additional independent signals was found either for rs2436046 or rs982502 ($P = 0.89$ and $P = 0.47$) (**Supplementary Table 3**), suggesting that the moderate association of rs2436046 and rs982502 observed in the CMH test is the result of these SNPs being in LD with rs1835740.

Meta-analysis of discovery and replication samples. The CMH test was used for the meta-analysis, with a nominal covariate used to distinguish each sample collection from the others. For the replication in Icelandic and Danish samples, association analysis was carried out using a likelihood procedure[37], and results were adjusted for relatedness by dividing the χ^2 statistics by an inflation factor estimated through simulation[38].

Imputation. For each cohort, imputation of the untyped markers in the 2-Mb region around rs1835740 was carried out using IMPUTE v2 with the recommended options[39]. Haplotypes from the 1000 Genomes Project (August 2009 release) and haplotypes from HapMap Phase 3 were used as reference panels.

eQTL analysis. Association between genotypes and expression was analyzed using Spearman rank correlation for all SNPs with a 2-Mb window centered on the transcription start site of the gene. Significance was assessed by comparing the observed P values at a 0.001 threshold with the minimum P values from each of 10,000 permutations of the expression values relative to genotypes[35].

URLs. Control populations: Finland—Health2000 study, http://www.nationalbiobanks.fi; Finland—Helsinki Birth Cohort study, http://www.nationalbiobanks.fi; Germany—KORA S4/F4 study, http://www.helmholtz-muenchen.de/kora; Germany—PopGen study, http://www.popgen.de; Germany—HNR study, http://www.recall-studie.uni-essen.de/recall_info.html; Illumina iControlDB, http://www.illumina.com; The Netherlands—Rotterdam I and III studies, http://www.epib.nl/research/ergo.htm; the Netherlands—Lumina study, http://www.lumc.nl/hoofdpijn. Other URLs: International Headache Genetics Consortium, http://www.headachegenetics.org; ssSNPer, http://gump.qimr.edu.au/general/daleN/ssSNPer/; GWAS plotter, http://www.broadinstitute.org/node/555; HapMap Phase 2 and 3 data, http://www.hapmap.org.

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Supplementary Material

Genome-wide association study of migraine implicates a common susceptibility variant on 8q22.1

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Contents

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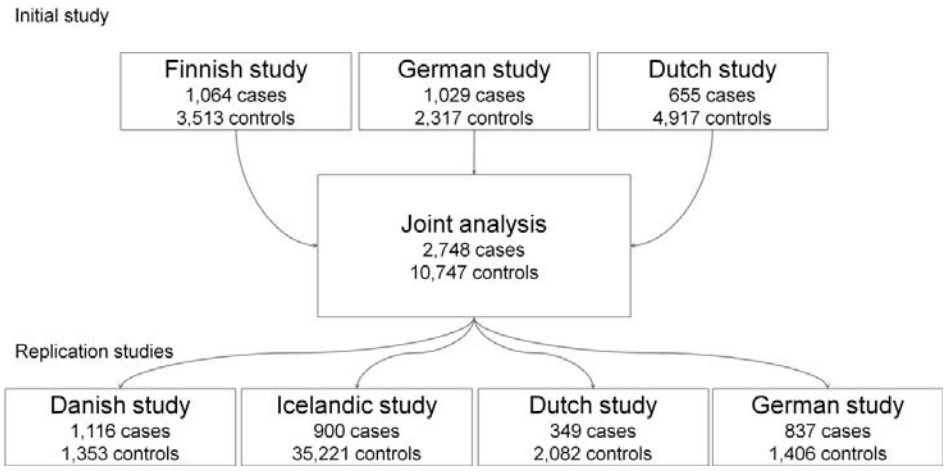
Supplementary Figures and Figure Legends

Supplementary Figure 1. Study design

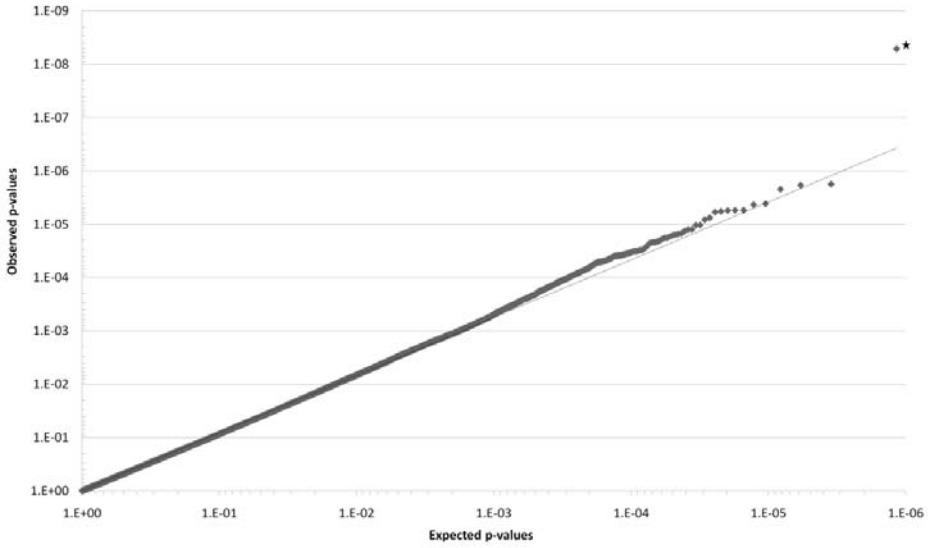
In the initial study, migraine with aura (MA) patients from three clinic-based collections were analyzed in a joint genome-wide association analysis. The most significant association signal was replicated in an independent Danish clinic-based sample and an Icelandic population-based sample, containing MA and migraine without aura (MO) samples, as well as in a German clinic-based MO-specific sample.



Nature Genetics: doi: 10.1038/ng.652

Supplementary Figure 2. Quantile-quantile plot of the results in the Cochran-Mantel-Haenszel analysis

Asterisk denotes marker rs1835740. Black line represents the distribution of p-values under the null given study inflation factor lambda of 1.08.



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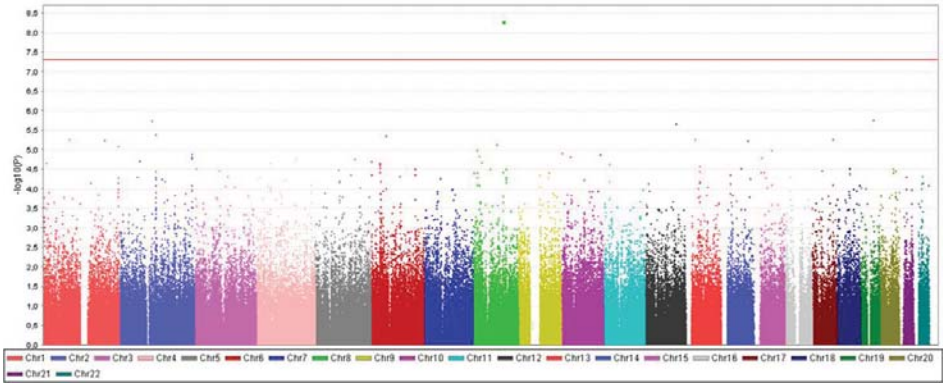
Supplementary Figure 3. Genome-wide Cochran-Mantel-Haenszel results for association between each marker and migraine with aura in the combined analysis of the three initial study populations

Red line denotes the threshold of genome-wide significance ($p \leq 5 \times 10^{-8}$). Only marker rs1835740 on 8q22.1 exceeded this threshold.



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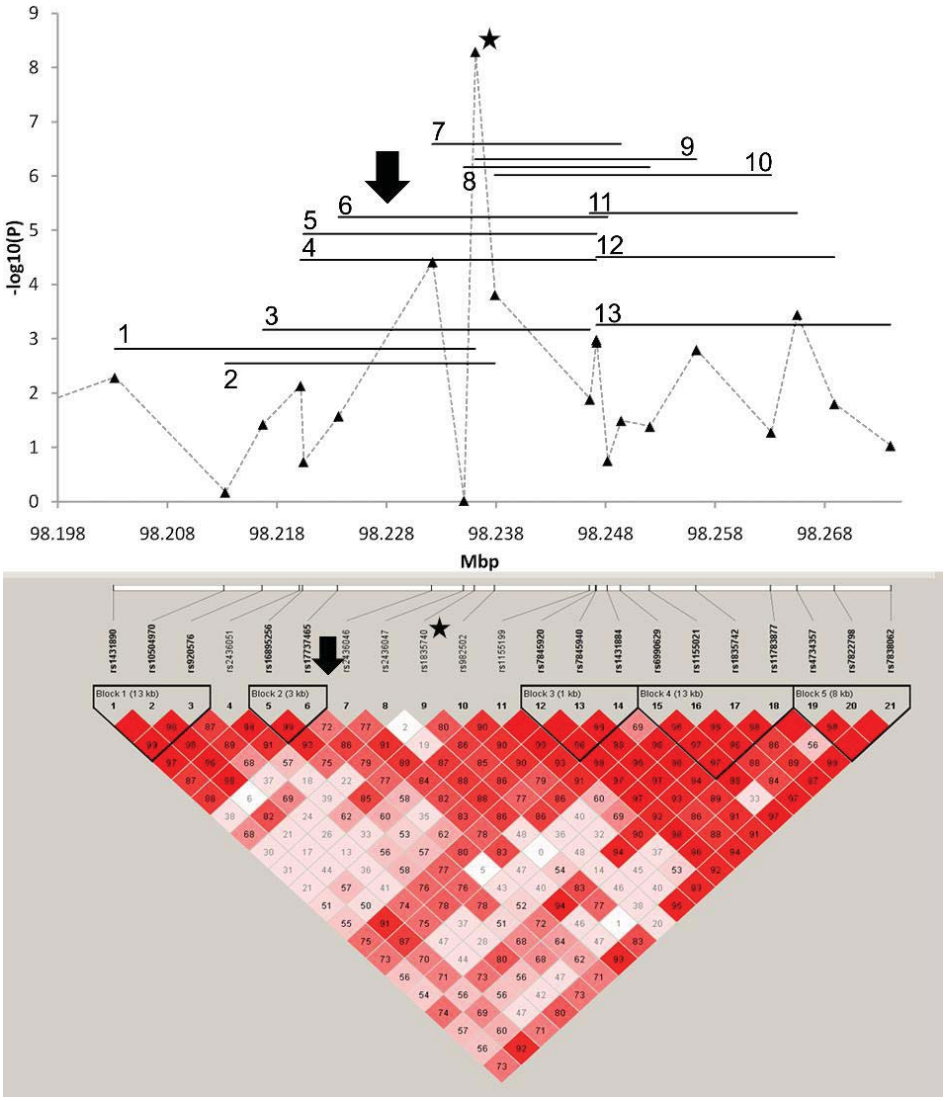
Supplementary Figure 4. Nine SNP sliding window haplotype analysis and local haplotype structure around marker rs1835740 on chromosome 8q22.1

In the upper part of the figure, the black pyramids show single-marker association results for each marker. The horizontal lines show the length and overall p-values for the nine marker sliding windows in the haplotype analysis. The lower part of the figure shows the Haploview D' matrix in the GWA study analysis data, with estimated LD blocks using the Gabriel *et al.* method[1]. Black stars denote the location of rs1835740 and the black arrows denote the 3' end of *PGCP* in either part of the figure.



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Supplementary Tables

Supplementary Table 1. Association signals with $p \leq 5 \times 10^{-5}$ and with multiple nearby associating SNPs

SNP	Chr	Location	p-value	OR	95% CI	Location	Gene
rs12084862	1	244269837	8.20E-06	1.17	1.09-1.25	intragenic	*SMYD3*
rs17528324	2	118572626	4.13E-06	1.27	1.15-1.41	intragenic	*INSIG2*
rs17862920	2	234492734	1.26E-05	0.776	0.693-0.870	intragenic	*TRPM8*
rs2038761	6	2625766	2.02E-05	0.865	0.809-0.925	intragenic	*MYLK4*
rs6456880	6	29071227	2.18E-05	0.873	0.819-0.929	intragenic	*ZNF311*
rs7753655	6	49644523	4.29E-06	0.852	0.796-0.912	intergenic	-
rs10888075	8	13804790	1.04E-05	1.21	1.11-1.31	intergenic	near *SGCZ*
rs10111769	8	21003036	1.49E-05	1.15	1.08-1.23	intergenic	-
rs2042600	11	19709275	2.28E-05	0.876	0.824-0.932	intragenic	*NAV2*
rs3794331	13	44951545	2.70E-05	1.28	1.14-1.43	intragenic	*COG3*
rs473422	15	56453633	1.03E-05	0.864	0.820-0.922	intergenic	near *AQP9*

Footnote: Locations and distances in basepairs, according to NCBI build 36. Only the SNP with the lowest p-value is reported for each locus.

Supplementary Table 2. Conditional analyses for the two SNPs with moderate linkage disequilibrium to rs1835740 in chromosome 8q22.1

Chr	SNP A	SNP B	r^2	SNP A p-value	SNP B p-value	SNP B given A
8	rs1835740	rs2436046	0.69	5.12×10^{-9}	1.78×10^{-5}	0.892
8	rs1835740	rs982502	0.59	5.12×10^{-9}	1.34×10^{-4}	0.466

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Supplementary Table 3. Nine SNP sliding window haplotype analysis on the chromosome 8q22.1 associated region from Supplementary Figure 2

Haplotype	First SNP	Last SNP	Chi-sq.	D.f.	Overall p-value
1	rs1431890	rs1835740	43.07	16	2.730E-04
2	rs10504970	rs982502	43.10	17	4.643E-04
3	rs920576	rs1155199	41.37	13	8.291E-05
4	rs2436051	rs7845920	48.48	14	1.093E-05
5	rs16895256	rs7845940	47.68	12	3.553E-06
6	rs17737465	rs1431884	46.52	10	1.156E-06
7	**rs2436046**	**rs6990629**	**51.62**	**9**	**5.327E-08**
8	rs2436047	rs1155021	48.93	10	4.196E-07
9	rs1835740	rs1835742	53.46	10	6.107E-08
10	rs982502	rs11783877	45.23	8	3.327E-07
11	rs1155199	rs4734357	41.91	8	1.410E-06
12	rs7845920	rs7822798	39.34	9	9.995E-06
13	rs7845940	rs7838062	32.98	8	6.208E-05

The nine SNP window in bold is the one referred to in the text. N.B. haplotype value shown in text is for the single haplotype, above values for the association of the whole haplotype distribution.

Supplementary Table 4. SNPs with nominal or higher p-values for association with expression levels of *MTDH/AEG-1*

SNP	Gene	SNP coordinate	Gene start	Distance	SRC p-value
rs11783750	*MTDH/AEG-1*	98 865 219	98 725 583	139 636	0.0018741
rs10105830	*MTDH/AEG-1*	98 307 895	98 725 583	417 688	0.0004235
rs1835740	*MTDH/AEG-1*	98 236 089	98 725 583	489 494	**0.0000396***
rs7845920	*MTDH/AEG-1*	98 247 132	98 725 583	478 451	0.0014652

Footnote: * indicates surpassing the significance threshold 7.7×10^{-5} (corresponding to a 0.001 permutation threshold after 10,000 permutations). SRC = Spearman rank correlation. Locations and distances in basepairs, according to NCBI build 36. Numbers in bold are statistically significant.

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Supplementary Note: Clinical subject ascertainment and control samples

Ethical aspects
Written informed consent was obtained from all participants, and the study was approved by the respective local research ethics committees of the Helsinki University Central Hospital, Pain Clinic Kiel in Kiel, the Department of Neurology at Klinikum Großhadern, Ludwig-Maximilians-University in Munich, and the University of Leiden Medical Centre. Informed consent was obtained from all patients.

Initial study
The initial genome-wide association study consisted of three patient samples, collected from headache clinics in Finland, Germany and the Netherlands.

In Finland, 1,124 Finnish migraine with aura (MA, and MA/MO) patients were recruited. Each patient belongs to a multigenerational family with at least three family members with migraine. Patients were examined by a neurologist, and fulfilled the validated Finnish Migraine Specific Questionnaire for Family Studies ($FMSQ_{FS}$[2]). In cases of insufficient or conflicting information, a follow-up interview was conducted by telephone. All patients were diagnosed by the same headache specialist (M. Kallela) according to the current International Headache Society diagnostic criteria (ICHD-II)[3].

In Germany, patient recruitment was done at two sites, in Kiel and in Munich. At the Pain Clinic in Kiel, a total of 994 German MA and MA/MO patients were recruited to a patient collection maintained at the Universities of Bonn and Cologne. All patients were diagnosed according to the ICHD-II[3] by headache specialists[4]. The detailed migraine anamnesis was obtained either by face-to-face interviews or by telephone interviews standardized by using a comprehensive migraine questionnaire. The second German set of 282 MA and MA/MO cases were recruited and examined by a headache specialist at the Klinikum Großhadern of the Ludwig-Maximilians-University, Munich. Phenotyping was based on a German translation of the $FMSQ_{FS}$[2]. Whenever the information was insufficient or conflicting, an additional telephone interview was performed. Information was obtained on all aspects of the ICHD-II[3] criteria as well as on other aspects (such as age at onset, prodromal symptoms, triggers, acute and prophylactic medication, family history, general past medical history, co-morbidity and place of birth).

In the Netherlands, 879 MA and MA/MO patients were available from the clinic-based Leiden University Migraine Neuro Analysis (LUMINA) study. Self-reported migraineurs were recruited via the project's website. A set of screening questions validated previously in a population-based study[5] was used first. Participants fulfilling the screening criteria completed then the extended questionnaire focusing on signs and symptoms of migraine headache and aura as outlined in ICHD-II[3]. Individual diagnoses were made using an algorithm based on these criteria. The algorithm diagnosis was validated by a semi-

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structured telephone interview performed by experienced study physicians or by well-trained medical students. Specific attention was paid to migraine aura. A subset of the patients was asked to participate upon visiting the outpatient clinic.

Replication studies

The replication phase of the study consisted of four separately recruited migraine patient samples from Denmark, Iceland, the Netherlands and Germany.

The Danish replication sample comprised 825 MA subjects of which 776 were successfully genotyped. Of these, 483 patients suffered from only MA attacks and 293 from both MA and MO attacks. Patients were selected from the Danish National Patient Register and from case files from neurological clinics, 1,365 took part in a screening telephone interview. If the proband was diagnosed with MA, the proband and selected relatives were diagnosed according to the ICHD-I[6] in a validated telephone interview (M. Kirchmann or A.H.). 305 Danish MO patients were selected from case files at the Danish Headache Center and diagnosed as mentioned above (ICHD-II[3]) in an extensive semi-structured telephone interview performed by trained physicians. In addition 81 MO subjects were identified during recruitment of the MA families. Thus, 386 MO patients were recruited and 340 successfully genotyped.

The Icelandic replication samples were recruited from three sources: first, a list of patients provided by two neurologists (401 potential participants), second, responses to an advertisement in the newsletter of the Icelandic Migraine Society (137 participants), and third, responses to a brief screening questionnaire mailed to a random sample of 20,000 Icelanders, aged 18–50 years and living in the Reykjavik area. All Icelandic recruits were asked to answer the comprehensive validated deCODE Migraine Questionnaire 2 or 3 (DMQ2 or DMQ3[7]). The questionnaire was designed based on ICHD-II[3]. The reliability of the MA and MO diagnoses based on the DMQ3 was assessed using a physician-conducted interview as an empirical index of validity. In total 1,612 subjects reporting five or more headache attacks were genotyped. Of them, 712 subjects reported atypical symptoms, preventing reliable IHS classification through questionnaire data only, and were excluded from the analysis. In total, the Icelandic sample consists of 567 MO patients, and 333 MA patients either with or without the MO attacks.

The German replication cohort includes 837 MO cases from the Department of Neurology of the Ludwig-Maximilians-University, Munich, Germany. Phenotyping followed the same protocol as described for the Munich patient sample.

The Dutch replication sample includes 356 Dutch MA or MA/MO patients that were recently recruited through the clinic-based Leiden University Migraine Neuro Analysis (LUMINA) study. The diagnosis and classification followed the same procedure as in the initial Dutch sample.

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Control samples

Population-matched control samples were obtained from previously genotyped studies (for links to studies, see URL section of Online Methods). 1,881 Finnish controls originated from the Helsinki Birth Cohort study[8] and 2,173 controls from the Health2000 study, genotyped on the Illumina 660K or 610K platforms. 840 German controls were obtained from the KORA S4/F4 study[9], 380 controls from the HNR study[10] and 677 from PopGen study[11], all genotyped on the Illumina 550K platform. In addition, 444 controls were obtained from Illumina iControlDB by querying all Caucasian samples genotyped on the Illumina 550K platform on June 30th, 2008 and filtering these samples based on stratification as observed from multidimensional scaling plots of all existing German samples, and keeping only those identified as being of German descent. 974 Dutch controls were obtained from the Rotterdam study I[12], genotyped on the Illumina 550K platform and imputed to cover all markers on the 610K platform. For each replication study, the group providing a replication dataset supplied a matched control cohort; the controls for the Danish and Icelandic replications were provided by deCODE, and German controls were obtained from the MARS study[13] and from GlaxoSmithKline[14] and Rotterdam study III.

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