Cover Page

Universiteit Leiden

Author: Weller, Claudia
Title: Unraveling genetic mechanisms in headache syndromes
Issue Date: 2015-09-02

Unraveling genetic mechanisms in headache syndromes

Layout, illustrations and cover design: T. Kind

Cover: DNA sculpture, El Museu de les Ciències Príncipe Felipe, Valencia, Spain

ISBN 978 94 6182 585 8

Unraveling genetic mechanisms in headache syndromes

Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof.mr. C.J.J.M. Stolker,
volgens besluit van het College voor Promoties
te verdedigen op woensdag 2 september 2015
klokke 15.00 uur

door

Claudia Mandina Weller
geboren te 's-Gravenhage
in 1983

Promotiecommissie

Promotores:
Prof. dr. A.M.J.M. van den Maagdenberg
Prof. dr. M.D. Ferrari

Co-promotor:
Dr. G.M. Terwindt

Leden promotiecommissie:
Prof. dr. R.R. Frants
Prof. dr. J.J. Houwing-Duistermaat
Prof. dr. S.M. van der Maarel
Dr. B.P.C. Koeleman (UMC Utrecht)

The research presented in this thesis was performed at the Department of Human and Clinical Genetics, Leiden University Medical Center, Leiden, The Netherlands.
This work is funded by grants of the Netherlands Organization for Scientific Research (NWO) (903-52-291, M.D.F.; Vici 918.56.602, M.D.F.; 907-00-217, G.M.T.; Vidi 917-11-319, G.M.T.), the European Community (EC) (EUROHEAD, LSHM-CT-2004-504837, M.D.F. and A.M.J.M.v.d.M; EUROHEADPAIN nr 602633, M.D.F. and A.M.J.M.v.d.M), and the Centre for Medical Systems Biology (CMSB) and Netherlands Consortium for Systems Biology (NCSB), both within the framework of the Netherlands Genomics Initiative (NGI) (A.M.J.M.v.d.M). The funding agencies had no role in the design or conduct of the studies.

Contents

1

General introduction and methods

Claudia M. Weller

Adapted from *Migraine Genes - Clinical and Preclinical Perspectives*. C.M. Weller, B. de Vries, G.M. Terwindt, M.D. Ferrari, J. Haan, A.M.J.M. van den Maagdenberg. Book chapter for *The Migraine Brain - Imaging, Structure and Function*, Oxford University Press. 2012

General introduction

HEADACHE DISORDERS are called 'primary' when no causal underlying structural lesion is present. The diagnostic criteria of the International Headache Society (IHS) define four groups of primary headaches, that is (1) migraine, (2) tension-type headache, (3) cluster headache and other trigeminal autonomic cephalalgias, and (4) other primary headaches, examples of which are hemicrania continua and hypnic headache.[1] These main categories of primary headaches are thought to consist of multifactorial diseases, meaning that they are likely caused by a combination of multiple environmental and genetic factors. Whereas not much is known about genetic factors in cluster headache and tension-type headache, there is growing body of knowledge on the genetics of migraine and its variants. In this section, first genetic knowledge on migraine and cluster headache will be summarized, followed by a description of our research strategies and populations.

Migraine

Clinical aspects of migraine

Migraine is a paroxysmal neurovascular disorder that is characterized by recurrent attacks of throbbing, unilateral headache of moderate to severe intensity. Attacks are aggravated by physical exercise and last 4-72 hours and are often accompanied by nausea, vomiting, photophobia, and/or phonophobia. One-year migraine prevalence in the general population is 11%, with a clear female preponderance (males 6-8% and females 15-18%).[2-5] Due to the lack of objective reliable biomarkers, such as measurements of specific compounds in blood, migraine diagnoses are currently based on questionnaires and/or interviews using consensus criteria of the International Classification of Headache Disorders (ICHD-3beta) from the International Headache Society (IHS) (table 1).[1] Approximately one-third of migraine patients experience transient focal neurological symptoms, known as migraine auras, which can precede attacks of headache. Auras develop gradually and have duration of 5 to 60 minutes and include (in decreasing order of prevalence) visual, sensory, speech and/or motor symptoms.[6] Based on the presence or absence of the aura phase two main migraine subtypes are distinguished, migraine with aura and migraine without aura, which can co-occur in the same patient.[5,7]

Migraine pathophysiology

The prevailing view is that migraine is a neurovascular disorder.[8] The migraine aura is caused by cortical spreading depression (CSD), a wave of neuronal and glial depolariza-

Table 1 - International headache criteria for migraine without and migraine with aura (ICHD-3beta).

Migraine without aura
A. At least five attacks fulfilling criteria B-D
B. Headache attacks lasting 4 to 72 hours (untreated or unsuccessfully treated)
C. Headache has at least two of the following four characteristics:
1. Unilateral location
2. Pulsating quality
3. Moderate or severe pain intensity
4. Aggravation by or causing avoidance of routine physical activity (e.g., walking or climbing stairs)
D. During headache at least one of the following;
1. Nausea and/or vomiting
2. Photophobia and phonophobia
E. Not better accounted for by another ICHD-3 diagnoses
Migraine with aura
A. At least two attacks fulfilling criteria B-C
B. One or more of the following fully reversible aura symptoms:
1. Visual
2. Sensory
3. Speech and/or language
4. Motor
5. Brainstem
6. Retinal
C. At least two of the following characteristics:
1. At least one aura symptom spreads gradually over ≥5 minutes, and/or two or more symptoms occur in succesion
2. Each individual aura symptom lasts 5-60 minutes
3. At least one aura symptom is unilateral
4. The auro is accompanied, or followed within 60 minutes, by headache
D. Not better accounted for by another ICHD-3 diagnoses, and transient ischemic attack has been excluded

tion that moves slowly over the cortex.[9] Using functional MRI to study the aura in patients with migraine with aura, Hadjikhani and colleagues[10] were able to detect local increases in blood oxygen level-dependent signal that spread through the visual cortex at a rate of approximately 3 mm/min, a speed similar to what is seen when a CSD is evoked in an experimental animal. The headache itself is caused by activation of the trigeminovascular system which consists of the meningeal and superficial cortical blood vessels that are innervated by the trigeminal nerve and that project to the trigeminal nucleus caudalis in the brainstem, which in turn projects to higher-order pain centers (thalamus and cortex) leading to pain.[11]

Migraine is a genetic disorder

Migraine shows strong familial aggregation and is a multifactorial complex genetic disorder.[12-14] Such complex disorders are likely caused by a combination of environmental factors and multiple genetic factors, each with a small effect size meaning that the individual genetic factors increase disease risk only slightly. Population-based family studies

revealed that the relative risk of migraine for a first-degree relative of the index patient is 1.5 to 4 times higher compared to the general population. The risk is highest for patients with migraine with aura, an early age of onset, and high attack severity and disease disability.[12] Twin studies also revealed a higher genetic load in migraine with than migraine without aura.[15,16] A large study of approximately 30,000 twins from six different countries showed that genetic and environmental factors play an almost equal role in migraine susceptibility.[17] Heritability, which is defined as the contribution of genetic factors to susceptibility for a disease, was estimated to range from 34 to 57% in that study. Shared environmental factors seemed to play a minor role, as migraine prevalence was similar in twins raised together and twins raised apart.[18,19]

There is debate whether migraine with and migraine without aura are two separate disease entities or merely different expressions of the same disease. The first view is supported by observations that there is no increased co-occurrence of both types of migraine in Danish twins[20] and the general Danish population.[6,12] In contrast, a study of 210 Finnish migraine families suggested the existence of a migraine continuum with pure migraine with aura and pure migraine without aura on both ends of the spectrum and a combination of both types of attacks in between.[21] The idea that migraine indeed is a continuum is supported by other studies[22,23] as well as by clinical observations that headache characteristics are identical in both migraine with and without aura and that a large number of patients experience both types of attacks.[24]

Approaches to Discover Migraine Genes

Hemiplegic migraine as a monogenic model for common migraine

Identifying genes and biological pathways of a monogenic subtype of a disease is likely to provide useful insight into the pathophysiology of the complex disease form. Gene mutations underlying monogenic disease have a large effect size and are expected to have clear consequences on either the level or the amino acid sequence of the affected protein, which can be investigated in cellular and animal disease model systems. With respect to genetic migraine research, Familial Hemiplegic Migraine (FHM) is a rare monogenic subtype of migraine with aura and insight into its pathophysiology may therefore serve to help unraveling part of the pathophysiology of common forms of migraine as well.

FHM is characterized by a transient hemiparesis during the aura phase, which may last several days. Diagnostic criteria for FHM were determined by the IHS[1] (table 2). Hemiplegic migraine occurring in isolated cases is called Sporadic Hemiplegic Migraine (SHM) and apart from the absence of an affected first-degree relative, diagnostic criteria are identical to those in FHM. Because of its clinical presentation, a significant number

Table 2 - International headache criteria for familial hemiplegic migraine (IHS 2013).

Hemiplegic migraine
A. At least two attacks fulfilling criteria B and C
B. Aura consisting of both of the following:
1. Fully reversible motor weakness
2. Fully reversible visual, sensory and/or speech/language symptoms
C. At least two of the following four characteristics:
1. At least one aura symptoms develops gradually over ≥ 5 minutes, and/or different aura symptoms occur in succession
2. Each individual non-motor aura symptom lasts 5–60 minutes, and motor symptoms last <72 hours
3. At least one aura symptom is unilateral
4. The aura is accompanied, or followed within 60 minutes, by headache
D. Not better accounted for by another ICHD-3 diagnosis, and transient ischaemic attack and stroke have been excluded.

of patients with hemiplegic migraine initially are diagnosed with stroke or epilepsy. Except for the hemiparesis, the visual and sensory aura symptoms are identical to those seen in migraine with aura, although duration of the aura often is significantly longer than in migraine with aura patients.[25] The headache characteristics in hemiplegic migraine patients and patients with the common forms of migraine are identical. Moreover, the majority of hemiplegic migraine patients experience attacks of common migraine in addition to their hemiplegic attacks.[26] Thus, from a clinical perspective hemiplegic migraine seems to be a valid model to study the common forms of migraine.[7]

Hemiplegic migraine genes

Genetic studies in FHM resulted in the discovery of three genes.[27] As several FHM families do not have a gene mutation in one of the known genes, likely more FHM genes exist. *CACNA1A*, the first FHM gene (FHM type 1; FHM1) is located on chromosome 19p13 and encodes the α1 pore-forming subunit of $Ca_V2.1$ calcium channels.[28] Some FHM1 mutations lead to pure hemiplegic migraine, whereas other mutations are associated with additional clinical symptoms such as cerebellar ataxia or epilepsy and have been shown to cause gain-of-function of $Ca_V2.1$ channel activity.[27,29] FHM1 is allelic with two other monogenic disorders: episodic ataxia type-2 (EA2)[28], and spinocerebellar ataxia type-6 (SCA6).[30] EA2 is a paroxysmal disorder that is characterized by vertigo, ataxia and nausea and approximately half of the patients suffer from migraine headaches and attacks can be accompanied by hemiplegia. EA2 mutations cause loss-of-function of $Ca_V2.1$ channel activity. SCA6 is a late-onset cerebellar ataxia disorder caused by moderate polyglutamine repeat expansion in the carboxyl-terminal part of the α1 pore-forming subunit.[30] It is still unclear how expansion of the glutamine stretch causes SCA6 and to what extent the pathoanatomical consequences of the different types of *CACNA1A* mutations overlap.

The second FHM gene (FHM2), *ATP1A2*, is located on chromosome 1q23 and encodes the α2 subunit of sodium-potassium pumps.[31] In contrast to FHM1 mutations, hardly

Table 3 - Monogenic Disorders Associated with Migraine.

Syndrome	Symptoms	Migraine subtype	Gene	References
CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy)	Recurrent stroke Cognitive deterioration Psychiatric disease	MA	*NOTCH3* Encodes a cell surface receptor on vascular smooth muscle cells	Joutel et al. 1996[52] Dichgans et al. 1998[51] Liem et al. 2010[53]
RVCL (Retinal Vasculopathy with Cerebral Leukodystrophy)	Retinopathy Cognitive disturbances Depression Raynaud phenomenon Liver and kidney dysfunction	MO	*TREX1* Encodes the major mammalian 3'-5' exonuclease, involved in DNA repair and apoptosis after DNA damage. Evidence is accumulating for additional functions	Grand et al. 1988[132] Jen et al. 1997[133] Terwind et al. 1998[58] Richards et al. 2007[56]
MELAS (Mitochondrial Encephalopathy, Lactic Acidosis and Stroke-like episodes)	Stroke-like episodes Encephalopathy with seizures and/or dementia Myopathy (lactic acidosis and/or ragged red fibers (RRF) on muscle biopsy)	MA	Specific mutations in mitochondrial genes	Montagna et al.1988[73]
HIHRATL (Hereditary Infantile Hemiparesis, Retinal Arteriolar Tortuosity and Leukoencephalopathy)	Porencephaly Cerebral and retinal microangiopathy	MA	*COL4A1* Encodes a collagen IV alpha chain in the basement membrane	Gould et al. 2006[61] Vahedi et al. 2007[134]
POLG-related disorders	Ataxia Ophthalmoplegia Epilepsy Liver failure	MA and MO	*POLG* Encodes the mitochondrial DNA polymerase gamma that is important for ATP homeostasis and normal cellular function	Winterthun et al.2005[76] Tzoulis et al. 2006[75]
TGFR2-related disorder	Aortic dissection Joint hypermobility Skin abnormalities Arthralgia	Unspecified	*TGFR2* Encodes the transforming growth factor beta receptor 2, which is involved in regulation of cellular processes and formation of extracellular matrix	Law et al. 2006[63]
Episodic ataxia type 6 (EA6)	Episodic ataxia Epilepsy	Hemiplegic migraine	*SLC1A3* Encodes the excitatory amino acid transporter 1 (EAAT1) which removes glutamate from the synaptic cleft	Jen et al.2005[71]
Proximal renal tubular acidosis (pRTA)	Renal dysfunction Ocular abnormalities	Hemiplegic migraine, MA, MO	*SLC4A4* Encodes a sodium bicarbonate cotransporter involved in regulating intracellular pH	Suzuki et al. 2010[72]
Advanced sleep phase syndrome	Early sleep time Early morning awakening	MA and MO	*CSNK1D* Encodes the casein kinase 1δ that phosphorylates the human circadian clock protein PER2	Brennan et al. 2013[135]

Note: MA = migraine with aura; MO = migraine without aura.

any recurrent mutations were reported for *ATP1A2.*[32,33] Another striking difference with FHM1 mutations is that almost all *ATP1A2* mutations are associated with pure hemiplegic migraine, that is without any additional clinical symptoms,[31,34-37] although some mutations are associated with epileptic seizures,[35,38] benign familial childhood convulsions,[39] febrile seizures[40] and mental retardation.[35] FHM2 mutations cause loss-of-function of the Na^+,K^+ ATPase.[27,29]

The third FHM gene (FHM3), *SCN1A*, is located on chromosome 2q24 and encodes the α1 subunit of neuronal $Na_V1.1$ sodium channels.[41] *SCN1A* mutations seem to account for only a small proportion of FHM families. Interestingly, the *SCN1A* gene is a well-known epilepsy gene with many mutations causing monogenic forms of childhood epilepsy, i.e. Dravet syndrome (also known as severe myoclonic epilepsy of infancy (SMEI)) or generalized epilepsy with febrile seizures (GEFS+).[42] At the start of the research described in this thesis, only five FHM3 mutations had been reported,[41,43-46] although from the clinical description in patients it is far from certain that one of them, mutation T1174S, is an FHM3 mutation as patients with the mutation may not have hemiplegic migraine.[45] Mutations L1649Q and Q1489K are associated with pure familial hemiplegic migraine,[43] whereas mutations Q1489H, and L263V have been associated with childhood epilepsy and generalized tonic-clonic seizures[41,44,46] in addition to FHM, at least in some of the mutation carriers. Some patients with FHM3 mutations Q1489H and F1499L were also reported to suffer from 'elicited repetitive daily blindness' (ERDB), which occurred apart from their hemiplegic migraine attacks.[46,47] FHM3 mutations seem to cause either gain or loss of function of $Na_V1.1$ channel activity.[27]

The first screens of FHM genes in hemiplegic migraine patients without a positive family history of other patients with hemiplegic migraine (so-called sporadic hemiplegic migraine = SHM) revealed mutations, predominantly in *ATP1A2*, in only a small proportion of patients.[48-50] Riant and coworkers, however, recently identified mutations in *CACNA1A* and *ATP1A2* in 23 out of 25 SHM patients with an age-of-onset before 16 years, of which most had additional symptoms such as epilepsy, learning difficulties, cerebellar ataxia and/or coma.[33] Three-quarter of the mutations had occurred *de novo* and mutation carriers thus represent the first patients of new FHM families. The question remains what is causing SHM in the patients that do not have an FHM gene mutation, which is a rather large proportion of patients in most studies.[48-50] Possibilities are that either other FHM genes may cause hemiplegic migraine in those patients or that SHM (especially when the phenotype is not severe) is due to a combination of multiple low-risk genetic variants, similar to what is predicted to occur in common forms of migraine. Support for the latter hypothesis comes from the observation that migraine with aura is frequent in families of SHM patients.[24] Also it would fit a view of migraine being a spectrum of disorders.

Investigating Monogenic Disorders in which Migraine is a Part of the Phenotype

Migraine can also be part of non-FHM monogenic disorders, which may be a useful source for identifying genes that may shed light on the pathophysiological mechanisms involved in migraine. In fact, the number of examples that are relevant to migraine is increasing (table 3).

Migraine as part of the phenotype of monogenic vasculopathies

Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL) is a monogenic syndrome characterized by recurrent stroke, cognitive deterioration, and psychiatric disease.[51] CADASIL is caused by mutations in the *NOTCH3* gene,[52] which encodes a cell surface receptor that is expressed in vascular smooth muscle cells. About one-third of CADASIL patients also suffer from migraine, predominantly migraine with aura, often with migraine as the presenting clinical symptom.[53] Whether there is a genetic link between migraine and Notch3 is still under debate, also because genetic association studies investigating *NOTCH3* polymorphisms gave conflicting results.[54,55]

Retinal Vasculopathy with Cerebral Leukodystrophy (soon to be renamed to Cerebral Hereditary Angiopathy with Vascular Retinopathy and Impaired Organ Function caused by TREX1 Mutations (abbreviated as CHARIOT)) is a monogenic vascular syndrome caused by mutations in the *TREX1* gene[56] that encodes the major 3'-5'-mammalian exonuclease, an enzyme thought to be involved in clearing cytosolic DNA.[57] RVCL patients suffer from a number of features that can include pronounced retinopathy, kidney and liver dysfunction, Raynaud phenomenon and various neurological features such as cognitive disturbances, depression and migraine.[58] In advanced stages of the disease, brain imaging shows characteristic contrast-enhancing white matter lesions.[58] A small genetic family-based study seemed to suggest a potential role for the RVCL gene as a susceptibility gene in migraine and Raynaud phenomenon.[59]

An increased prevalence of migraine with aura was reported in a rare angiopathy that can be described as Heriditary Infantile Hemiparesis, Retinal Arteriolar Tortuosity, and Leucoencephalopathy (or its acronym HIHRATL) with clinical symptoms of porencephaly and cerebral and retinal microangiopathy, hemiparesis, and stroke.[60] The causal gene is *COL4A1*, which encodes type IV collagen, an integral component of the vascular basement membrane.[61] The association adds to growing evidence for a link between migraine and early-onset cerebral angiopathies that is remarkable, but the mechanisms underlying this association are still poorly understood.[62] An additional piece of information that links affected blood vessels with migraine comes from a genetic study in a large pedigree in which patients suffer from familial aortic dissection and several other blood vessel

abnormalities.[63] Ten out of 14 carriers of the R460H mutation in the transforming growth factor factor-β receptor 2 (*TGFBR2*) gene also suffer from migraine.

In particular it needs to be further investigated whether endothelial dysfunction, which was also observed for migraine in several studies,[64-67] may underlie the extensive vasculopathy seen with CADASIL and RVCL. Given the high occurrence of migraine with aura in CADASIL patients, increased susceptibility for CSD may well explain the link with migraine. For RVCL this explanation seems unlikely, since the disease seems to be linked with both migraine without and with aura. Interestingly, prevalence of cardiovascular disease is increased in common forms of migraine, especially in migraine with aura patients. The basis for the comorbidity of migraine and cardiovascular disease is yet unknown, but these findings support the notion that shared pathophysiological processes could be involved.[68,69]

Other monogenic disorders in which migraine is part of the phenotype

There are several other monogenic disorders in which migraine can be prominent (table 3). First of all, in Familial Advanced Sleep Phase Syndrome (FASPS) there is a severe disruption of the sleep-wake cycle and other circadian rhythms. The disease is caused by missense mutations in the *CSNK1D* gene encoding Casein Kinase 1d (CK1δ), which is involved in the phosphorylation of the circadian clock protein Per2.[70] In two independent families a pathogenic *CSNK1D* mutation co-segregated with both FASPS and migraine with aura. Second, a complex monogenic phenotype of episodic ataxia, hemiplegic migraine, and seizures was reported for a 10-year-old boy with a P290R mutation in *SLC1A3* that encodes the excitatory amino acid transporter 1 (EAAT1), which removes glutamate from the synaptic cleft.[71] The missense mutation causes a dramatic loss in glutamate uptake in a cellular assay. Third, Suzuki and co-workers[72] reported various homozygous mutations in the Na^+-HCO_3^- co-transporter NBCe1 in patients with proximal renal tubular acidosis and ocular anomalies and, in addition, various clinical presentations of migraine, that is migraine without or with aura, hemiplegic migraine, and even episodic ataxia. Although the mutations themselves clearly are pathogenic in the sense that they are the cause of the renal and ocular problems, it is less obvious why they would cause the migraine and hemiplegia phenotypes that are very common and sometimes hard to diagnose.

A fourth monogenic syndrome that is associated with migraine is MELAS (Mitochondrial Encephalomyopathy, Lactic Acidosis, and Stroke-like syndrome) which is caused by a mitochondrial DNA (mtDNA) 3243 A>G tRNALeu point mutation.[73] Fifth, many carriers of mutations in the *POLG* gene, which encodes the nuclear polymerase-γ that is essential for the maintenance of mitochondrial DNA, suffer from migraine as well.[74-76] Lastly, Alternating Hemiplegia of Childhood (AHC) can be considered a monogenic model that has relevance to migraine. AHC is a rare syndrome characterised by recurrent

hemiplegic attacks, movement disorders, seizures, and developmental delay starting before the age of 18 months.[1] Due to the considerable clinical overlap in severely affected hemiplegic migraine patients and AHC patients, it is sometimes impossible to establish the correct diagnosis.[77] The clinical overlap prompted investigating the FHM genes *CACNA1A* and *ATP1A2* in AHC patients. A *CACNA1A* mutation was found in monozygous twins with overlapping clinical features of both disorders[78] and an *ATP1A2* mutation was identified in a Greek atypical AHC family.[79,80] No *CACNA1A* or *ATP1A2* mutations were found in any of the patients meeting all diagnostic criteria.[81,82] Due to the overlapping clinical and genetic features, advances in genetic research of AHC may also provide important information about the genetics of monogenic and complex forms of migraine.

Genetic Studies in Common Forms of Migraine

Identifying genes involved in complex disorders has proven difficult, especially before the era of genome-wide association studies (GWAS) (see below). Two main hypotheses are used to explain the genetic origin of complex diseases.[83] One hypothesis proposes that common disease is caused by common variants (CD-CV), which means that relatively frequent genetic variants cause disease, each with a small effect size cause disease but none of them is sufficient. In contrast, the common disease - rare variant (CD-RV) hypothesis assumes that multiple, relatively rare variants with a larger effect size may explain susceptibility to disease. To date, most findings in complex diseases test the first hypothesis only although a few rare variants with a moderate effect have been detected, such as Factor V Leiden in deep venous thrombosis.[84]

Linkage and candidate gene association studies in migraine

Until a few years ago, the main approach used in genetic studies of common migraine was family-based linkage analysis, which led to the identification of many chromosomal susceptibility regions, but did not result in the discovery of migraine genes. A second popular approach consisted of candidate gene association studies that search for significant differences in allele frequencies between migraine cases and controls in genes that had emerged from other knowledge of migraine pathophysiology. These association studies tested only one or at best a few DNA polymorphisms in such a gene. Candidate gene-based association studies in theory are a powerful tool, if carefully designed to overcome methodological issues regarding sample size, selection of cases and controls, selection of variants, correction for multiple testing, and replication of findings in independent populations. Unfortunately, the great majority of candidate gene association studies performed in migraine suffered from one or more methodological weakness and led to the conclusion that most results must be false positives.[85] Among the selected candidate genes that were most often tested are genes in the dopaminergic and serotonergic systems, hormone

receptors, and inflammatory pathways (for review see De Vries et al, 2009[27]). The best replicated finding is the association with the C677T polymorphism in the 5',10'-methylenetetrahydrofolate reductase (*MTHFR*) gene that increases the risk of migraine in carriers of the T-allele,[86-91] although other large and well-designed studies could not find such association.[92,93] Two meta-analyses showed an association of this polymorphism with migraine with aura, but not with migraine without aura.[94,95] *MTHFR* codes for an enzyme with an important role in homocysteine and folate metabolism.[96] Carriers of the T-allele have increased homocysteine concentrations, which is a well-known risk factor for cardiovascular disease. It is hypothesized that high homocysteine levels may induce vascular endothelial dysfunction and thereby increase migraine risk.

Genome-wide association studies in migraines

Over the last few years, genome-wide association studies (GWAS) have become the most used approach to identify genes that confer susceptibility to complex disorders. In a GWAS, hundreds of thousands of SNPs that are distributed over the genome are tested in a hypothesis-free manner for association with a disease trait. For each SNP, the allele frequencies are compared between cases and controls. Significant differences in allele frequency either pinpoint the SNP itself as a genetic susceptibility factor or provide statistical evidence that a causal gene variant is in close vicinity, i.e. is the causal variant is in linkage disequilibrium. Several major migraine GWA studies have been performed investigating so-called end-diagnoses migraine with aura[97] or migraine without aura[98] in two well-defined clinic-based studies, migraine in a population-based cohort[99], and, most recently, a systematic migraine meta-analysis.[97] Over a dozen migraine susceptibility gene variants have been identified that point to genes that cluster into five main different pathways related to (i) glutamatergic neurotransmission; (ii) synapse development and plasticity; (iii) pain sensing; (iv) metalloproteinases; and (v) vasculature & metabolism. The pathophysiological and clinical implications of these variants, however, still have to be determined.

Reduction of clinical heterogeneity for genetic studies of common migraine

Apart from genetic heterogeneity, the gene hunt in common migraine is also complicated by extensive clinical heterogeneity regarding for instance presenting symptoms or age-of-onset in addition to the lack of reliable biomarkers to establish a migraine diagnosis. Clearly, diagnostic criteria of the IHS that are useful to diagnose attacks in the clinic are less suited for genetic research, because multiple combinations of symptoms lead to the same end-diagnosis, not necessarily through the same pathophysiological mechanism. Two types of strategies can be applied to reduce clinical heterogeneity among participants of genetic studies in migraine. The first approach takes advantage of the well-known comorbidity of migraine with various disorders.[100] Although the observation can be

spurious due to selection bias or reflect a unidirectional causal relationship, that is migraine causes (or is caused by) the co-morbid disorder, it may also be that shared genetic and/or environmental factors underlie both migraine *and* the co-morbid disorder.[101] Migraine patients are at increased risk of epilepsy, ischemic stroke, myocardial infarction, major depressive disorder, anxiety disorders, bipolar disorder, asthma, and chronic pain disorders[102,103] and genetic heterogeneity might be reduced by selecting those migraine patients that also suffer from the co-morbid disorder. The second type of approach uses other classification than the ICHD-2 criteria[1] as a trait in genetic analysis. Two examples of this latter strategy are trait component analysis[104,105] (TCA; using individual symptoms instead of ICHD-2 diagnosis as traits in the analysis)) and latent class analysis[106-108] (LCA, using different classes of patients based on clustering of symptoms), which led to the discovery of various chromosomal regions potentially harboring migraine susceptibility genes. Until now, no susceptibility genes have been found using TCA or LCA.

An attractive alternative could be the use of a (set of) migraine biomarker(s) as a trait in genetic studies to search for novel migraine susceptibility genes. Past research on migraine biomarkers was hypothesis-driven and tested a small number of metabolites in small study populations[66,109-117] and failed to identify a clinically useful migraine biomarker. Current high-throughput proton nuclear magnetic resonance (^{1}H NMR) spectroscopy enables generating a profile of tens to hundreds low-molecular-weight metabolites from a blood sample in a single measurement.[118,119] Interestingly, GWAS using metabolite concentrations have led to the identification of genetic variants with much larger effect sizes than commonly encountered in GWAS using 'just' clinical diagnoses.[120] Combining the existing knowledge on genetics of metabolites with yet unidentified migraine biomarkers could be an attractive approach to identify novel migraine susceptibility genes.

Studying the genetics of cluster headache

Cluster headache is a rare, disabling, primary headache disorder.[1] Clinical characteristics and treatment options partly overlap with migraine and involvement of the trigeminovascular system may be a key feature of both disorders.[11,121,122] Cluster headache is much rarer than migraine and has a lifetime prevalence of only 0.12%.[123] In contrast to migraine, there is a striking male preponderance with a male-to-female ratio of 3:1.[123,124] Cluster headache is characterised by attacks of unilateral severe (supra)orbital and/or temporal pain accompanied by restlessness and/or ipsilateral autonomic symptoms, that is eyelid oedema, conjunctival injection, miosis, ptosis, lacrimation, nasal congestion, rhinorrhoea, forehead and facial sweating. Attacks have duration from 15 to 180 minutes and occur up to eight times a day.[1] Approximately 90% of patients have the episodic form of the disease. Patients with episodic cluster headache experience bouts of frequent attacks lasting weeks to months, followed by remission lasting several months to years. The

remaining 10% has chronic cluster headache, characterised by short (less than one month) or absent periods of remission.

Cluster headache is considered a complex genetic disorder, although an autosomal dominant pattern of transmission has been suggested in some cases.[124] The relative risk for family members of cluster headache patients is estimated to be 5-18 for first-degree relatives and 1-3 for second-degree relatives. Due to the low prevalence of the disorder, no large cohorts of patients are available for large-scale genetic studies such as GWAS. Therefore, genetic research in cluster headache has been limited mainly to candidate gene studies. Multiple small-scale studies have been performed, but many of them lack a replication sample, thereby precluding a final conclusion regarding the association of the reported genes with cluster headache. To date, only one genetic factor (i.e. a missense variant in the hypocretin type 2 receptor gene *HCRTR2*) was found to be associated with cluster headache in two of the three small conducted studies and in a meta-analysis of these studies.[125-128]

Study populations

Leiden University Migraine Neuro-Analysis (LUMINA) population

The Leiden University Migraine Neuro-Analysis LUMINA program was initiated in March 2008 and inclusion is still ongoing. The aim of the program is to enroll a large number of self-reported migraine patients for genetic and epidemiological studies through the project's website (www.lumc.nl/hoofdpijn). Self-reported migraine patients from the Dutch population were invited to complete a validated screening questionnaire. Screen-positives were subsequently asked to complete a newly developed extended web-based questionnaire aiming to diagnose migraine based on the ICHD-2 criteria. Many previously developed questionnaires are good at assessing headache characteristics, but fail to make reliable aura diagnoses. Discriminating between migraine with and without aura is of particular interest in genetic studies and such migraine type information can be reliably obtained using questionnaire-based aura diagnoses with our LUMINA questionnaire. Five years after the start of the project, approximately 5,000 migraine patients have been collected and have contributed to the identification of susceptibility genes for common forms of migraine in various GWAS.[97,98,129]

Leiden University Cluster headache Analysis (LUCA) population

The Leiden University Cluster headache Analysis (LUCA) program is the cluster headache counterpart of the LUMINA study and started in April 2010. Genetic studies in cluster

headache are greatly hampered by the combination of low prevalence and putative complex genetic background of the disorder. The LUCA study aimed to collect a large group of self-reported cluster headache patients for genetic and epidemiological studies. Self-reported cluster headache patients were invited to complete a screening questionnaire. Screen-positive participants were asked to complete the newly developed web-based extended questionnaire to enable diagnosing cluster headache for genetic and epidemiological studies. Three years after the start of the project, 917 patients cluster headache patients were enrolled in the program, of which 560 provided a DNA sample, resulting in the largest single sample set of cluster headache patients to date.

Erasmus Rucphen Family (ERF) Study

The ERF population originates from a genetically isolated region in the southwest of the Netherlands.[130] The study population consists of the 3,465 living descendants of 22 founder couples that had at least six children baptized in the community church between 1850 and 1900. The pedigree of this large family is characterized by multiple consanguinity and increased inbreeding. The occurrence of disease in isolated populations is influenced by a less heterogeneous genetic and environmental background than the occurrence of disease in the general outbred population. Identification of susceptibility genes in isolated populations is therefore considered easier because genetic drift (that is rare variants tend to disappear or become overrepresented), facilitates investigating rare variants underlying common disease, while isolated populations are equally well suited to detect common variants associated with disease, as these variants in general have similar allele frequencies in genetic isolates and the general outbred population.[131]

Scope and outline of the thesis

The studies presented in this thesis aim to advance genetic knowledge of primary headache disorders with a focus on migraine and cluster headache.

In **Chapter 2** the identification is reported of two novel mutations in the *SCN1A* gene in Spanish families with familial hemiplegic migraine. The identification of such mutations is important as the overwhelming majority of mutations in this gene cause epilepsy and it is still unclear why certain *SCN1A* mutations cause FHM instead. **Chapter 3** describes a mutation in the *SLC2A1* gene in a patient that links hemiplegic migraine with alternating hemiplegia of childhood, which may be of use to understand how one mutation causes features of both monogenic disorders and may be of relevance in dissecting mechanisms of migraine pathophysiology. Also attempts were made to investigate the possible role of the *SLC2A1* and *ATP1A3* genes in familial and sporadic hemiplegic migraine.

Chapters 4 and **5** are dedicated to the validation of extended web-based questionnaires that were developed to enrol self-reported headache patients for large-scale genetic studies. **Chapter 4** focuses on the LUMINA program that aims to recruit migraine patients and addresses the difficulties in obtaining reliable aura diagnoses using questionnaire data. In **Chapter 5** a similar approach was applied to recruit patients with cluster headache for the LUCA (Leiden University Cluster headache Analysis) program. One aim was to select which questions are best predictors for cluster headache diagnosis in the Dutch cluster headache population, generating a shorter questionnaire suitable for case-finding in large population-based studies.

Chapter 6 contains the results of a candidate gene study and subsequent meta-analysis investigating the role of a missense variant in the *HCRTR2* gene in cluster headache susceptibility. That variant had been associated with cluster headache in several small-scale association studies and *HCRTR2* is considered the only replicated cluster headache susceptibility gene. Using patients recruited in the LUCA program this association was re-investigated, now including the LUCA population as the largest single sample set of patients with cluster headache to date.

Chapter 7 contains the results of a genetic epidemiology investigation using the genetically isolated population of the Erasmus Rucphen Family study that aimed to investigate whether atherosclerosis is the cause of the previously observed increased rate of cardiovascular disease in migraine patients. The study also addresses, to certain extent, the question how useful it can be to select migraine patients that have a comorbid disease, in this case cardiovascular disease. In **Chapter 8** the same ERF cohort was used for metabolic profiling in serum using nuclear magnetic resonance (NMR) spectroscopy in a first attempt to identify molecular biomarkers for migraine. This study aims to identify a set of metabolites that may predict disease status.

Finally, **Chapter 9** provides a general discussion of the main findings presented in this thesis in relation to current literature, their implications and possibilities for future research.

References

1. Headache classification subcommittee of the International Headche Society. The International Classification of Headache Disorders: 3rd edition (beta version). *Cephalalgia* 2013; 33:629-808.
2. Rasmussen BK, Jensen R, Schroll M and Olesen J. Epidemiology of headache in a general population--a prevalence study. *J Clin Epidemiol* 1991; 44: 1147-1157.
3. Launer LJ, Terwindt GM and Ferrari MD. The prevalence and characteristics of migraine in a population-based cohort: the GEM study. *Neurology* 1999; 53: 537-542.
4. Lipton RB and Stewart WF. Migraine headaches: epidemiology and comorbidity. *Clin Neurosci* 1998; 5: 2-9.
5. Goadsby PJ, Lipton RB and Ferrari MD. Migraine--current understanding and treatment. *N Engl J Med* 2002; 346: 257-270.
6. Russell MB and Olesen J. A nosographic analysis of the migraine aura in a general population. *Brain* 1996; 119: 355-361.
7. Ferrari MD. Migraine. *Lancet* 1998; 351: 1043-1051.
8. Goadsby PJ. Recent advances in understanding migraine mechanisms, molecules and therapeutics. *Trends Mol Med* 2007; 13: 39-44.
9. Lauritzen M. Pathophysiology of the migraine aura. The spreading depression theory. *Brain* 1994; 117: 199-210.
10. Hadjikhani N, Sanchez Del Rio M, Wu O et al. Mechanisms of migraine aura revealed by functional MRI in human visual cortex. *Proc Natl Acad Sci U S A* 2001; 98: 4687-4692.
11. Ferrari MD and Goadsby PJ, 2007, Migraine as a cerebral ionopathy with abnormal central sensory processing. *Neurobiology of disease*, edited by (Elsevier Academic Press, pp. 333-348.
12. Russell MB and Olesen J. Increased familial risk and evidence of genetic factor in migraine. *BMJ* 1995; 311: 541-544.
13. Stewart WF, Bigal ME, Kolodner K et al. Familial risk of migraine: variation by proband age at onset and headache severity. *Neurology* 2006; 66: 344-348.
14. Stewart WF, Staffa J, Lipton RB and Ottman R. Familial risk of migraine: a population-based study. *Ann Neurol* 1997; 41: 166-172.
15. Ulrich V, Gervil M, Kyvik KO et al. Evidence of a genetic factor in migraine with aura: a population-based Danish twin study. *Ann Neurol* 1999; 45: 242-246.
16. Gervil M, Ulrich V, Kyvik KO et al. Migraine without aura: a population-based twin study. *Ann Neurol* 1999; 46: 606-611.
17. Mulder EJ, Van Baal C, Gaist D et al. Genetic and environmental influences on migraine: a twin study across six countries. *Twin Res* 2003; 6: 422-431.
18. Ziegler DK, Hur YM, Bouchard TJJ et al. Migraine in twins raised together and apart. *Headache* 1998; 38: 417-422.

19. Svensson D, Ekbom K, Pedersen NL et al. A note on cluster headache in a population-based twin register. *Cephalalgia* 2003; 23: 376-380.
20. Russell MB, Ulrich V, Gervil M and Olesen J. Migraine without aura and migraine with aura are distinct disorders. A population-based twin survey. *Headache* 2002; 42: 332-336.
21. Kallela M, Wessman M, Havanka H et al. Familial migraine with and without aura: clinical characteristics and co-occurrence. *Eur J Neurol* 2001; 8: 441-449.
22. Nyholt DR, Gillespie NG, Heath AC et al. Latent class and genetic analysis does not support migraine with aura and migraine without aura as separate entities. *Genet Epidemiol* 2004; 26: 231-244.
23. Ligthart L, Boomsma DI, Martin NG et al. Migraine with aura and migraine without aura are not distinct entities: further evidence from a large Dutch population study. *Twin Res Hum Genet* 2006; 9: 54-63.
24. Russell MB and Ducros A. Sporadic and familial hemiplegic migraine: pathophysiological mechanisms, clinical characteristics, diagnosis, and management. *Lancet Neurol* 2011; 10: 457-470.
25. Thomsen LL, Eriksen MK, Roemer SF et al. A population-based study of familial hemiplegic migraine suggests revised diagnostic criteria. *Brain* 2002; 125: 1379-1391.
26. Ducros A, Denier C, Joutel A et al. The clinical spectrum of familial hemiplegic migraine associated with mutations in a neuronal calcium channel. *N Engl J Med* 2001; 345: 17-24.
27. de Vries B, Frants RR, Ferrari MD and van den Maagdenberg AM. Molecular genetics of migraine. *Hum Genet* 2009; 126: 115-132.
28. Ophoff RA, Terwindt GM, Vergouwe MN et al. Familial hemiplegic migraine and episodic ataxia type-2 are caused by mutations in the Ca2+ channel gene CACNL1A4. *Cell* 1996; 87: 543-552.
29. van den Maagdenberg AM, Haan J, Terwindt GM and Ferrari MD. Migraine: gene mutations and functional consequences. *Curr Opin Neurol* 2007; 20: 299-305.
30. Zhuchenko O, Bailey J, Bonnen P et al. Autosomal dominant cerebellar ataxia (SCA6) associated with small polyglutamine expansions in the alpha 1A-voltage-dependent calcium channel. *Nat Genet* 1997; 15: 62-69.
31. De Fusco M, Marconi R, Silvestri L et al. Haploinsufficiency of ATP1A2 encoding the Na+/K+ pump alpha2 subunit associated with familial hemiplegic migraine type 2. *Nat Genet* 2003; 33: 192-196.
32. Castro MJ, Stam AH, Lemos C et al. Recurrent ATP1A2 mutations in Portuguese families with familial hemiplegic migraine. *J Hum Genet* 2007; 52: 990-998.
33. Riant F, Ducros A, Ploton C et al. De novo mutations in ATP1A2 and CACNA1A are frequent in early-onset sporadic hemiplegic migraine. *Neurology* 2010; 75: 967-972.
34. Riant F, De Fusco M, Aridon P et al. ATP1A2 mutations in 11 families with familial hemiplegic migraine. *Hum Mutat* 2005; 26: 281.

35. Jurkat-Rott K, Freilinger T, Dreier JP et al. Variability of familial hemiplegic migraine with novel A1A2 Na+/K+-ATPase variants. *Neurology* 2004; 62: 1857-1861.
36. Kaunisto MA, Harno H, Vanmolkot KR et al. A novel missense ATP1A2 mutation in a Finnish family with familial hemiplegic migraine type 2. *Neurogenetics* 2004; 5: 141-146.
37. Pierelli F, Grieco GS, Pauri F et al. A novel ATP1A2 mutation in a family with FHM type II. *Cephalalgia* 2006; 26: 324-328.
38. Deprez L, Weckhuysen S, Peeters K et al. Epilepsy as part of the phenotype associated with ATP1A2 mutations. *Epilepsia* 2008; 49: 500-508.
39. Vanmolkot KR, Kors EE, Hottenga JJ et al. Novel mutations in the Na+, K+-ATPase pump gene ATP1A2 associated with familial hemiplegic migraine and benign familial infantile convulsions. *Ann Neurol* 2003; 54: 360-366.
40. de Vries B, Stam AH, Kirkpatrick M et al. Familial hemiplegic migraine is associated with febrile seizures in an FHM2 family with a novel de novo ATP1A2 mutation. *Epilepsia* 2009; 50: 2503-2504.
41. Dichgans M, Freilinger T, Eckstein G et al. Mutation in the neuronal voltage-gated sodium channel SCN1A in familial hemiplegic migraine. *Lancet* 2005; 366: 371-377.
42. Escayg A and Goldin AL. Sodium channel SCN1A and epilepsy: mutations and mechanisms. *Epilepsia* 2010; 51: 1650-1658.
43. Vanmolkot KR, Babini E, de Vries B et al. The novel p.L1649Q mutation in the SCN1A epilepsy gene is associated with familial hemiplegic migraine: genetic and functional studies. Mutation in brief #957. Online. *Hum Mutat* 2007; 28: 522.
44. Castro MJ, Stam AH, Lemos C et al. First mutation in the voltage-gated Nav1.1 subunit gene SCN1A with co-occurring familial hemiplegic migraine and epilepsy. *Cephalalgia* 2009; 29: 308-313.
45. Gargus JJ and Tournay A. Novel mutation confirms seizure locus SCN1A is also familial hemiplegic migraine locus FHM3. *Pediatr Neurol* 2007; 37: 407-410.
46. Vahedi K, Depienne C, Le Fort D et al. Elicited repetitive daily blindness: a new phenotype associated with hemiplegic migraine and SCN1A mutations. *Neurology* 2009; 72: 1178-1183.
47. Le Fort D, Safran AB, Picard F et al. Elicited repetitive daily blindness: a new familial disorder related to migraine and epilepsy. *Neurology* 2004; 63: 348-350.
48. Terwindt G, Kors E, Haan J et al. Mutation analysis of the CACNA1A calcium channel subunit gene in 27 patients with sporadic hemiplegic migraine. *Arch Neurol* 2002; 59: 1016-1018.
49. de Vries B, Freilinger T, Vanmolkot KR et al. Systematic analysis of three FHM genes in 39 sporadic patients with hemiplegic migraine. *Neurology* 2007; 69: 2170-2176.
50. Thomsen LL, Kirchmann M, Bjornsson A et al. The genetic spectrum of a population-based sample of familial hemiplegic migraine. *Brain* 2007; 130: 346-356.
51. Dichgans M, Mayer M, Uttner I et al. The phenotypic spectrum of CADASIL: clinical findings in 102 cases. *Ann Neurol* 1998; 44: 731-739.

52. Joutel A, Corpechot C, Ducros A et al. Notch3 mutations in CADASIL, a hereditary adult-onset condition causing stroke and dementia. *Nature* 1996; 383: 707-710.
53. Liem MK, Lesnik Oberstein SAJ, Van der Grond J et al. CADASIL and migraine: A narrative review. *Cephalalgia* 2010;
54. Borroni B, Brambilla C, Liberini P et al. Investigating the association between Notch3 polymorphism and migraine. *Headache* 2006; 46: 317-321.
55. Schwaag S, Evers S, Schirmacher A et al. Genetic variants of the NOTCH3 gene in migraine--a mutation analysis and association study. *Cephalalgia* 2006; 26: 158-161.
56. Richards A, van den Maagdenberg AM, Jen JC et al. C-terminal truncations in human 3'-5' DNA exonuclease TREX1 cause autosomal dominant retinal vasculopathy with cerebral leukodystrophy. *Nat Genet* 2007; 39: 1068-1070.
57. Mazur DJ and Perrino FW. Excision of 3' termini by the Trex1 and TREX2 3'-->5' exonucleases. Characterization of the recombinant proteins. *J Biol Chem* 2001; 276: 17022-17029.
58. Terwindt GM, Haan J, Ophoff RA et al. Clinical and genetic analysis of a large Dutch family with autosomal dominant vascular retinopathy, migraine and Raynaud's phenomenon. *Brain* 1998; 121: 303-316.
59. Hottenga JJ, Vanmolkot KR, Kors EE et al. The 3p21.1-p21.3 hereditary vascular retinopathy locus increases the risk for Raynaud's phenomenon and migraine. *Cephalalgia* 2005; 25: 1168-1172.
60. Vahedi K, Massin P, Guichard JP et al. Hereditary infantile hemiparesis, retinal arteriolar tortuosity, and leukoencephalopathy. *Neurology* 2003; 60: 57-63.
61. Gould DB, Phalan FC, van Mil SE et al. Role of COL4A1 in small-vessel disease and hemorrhagic stroke. *N Engl J Med* 2006; 354: 1489-1496.
62. Dichgans M and Hegele RA. Update on the genetics of stroke and cerebrovascular disease 2006. *Stroke* 2007; 38: 216-218.
63. Law C, Bunyan D, Castle B et al. Clinical features in a family with an R460H mutation in transforming growth factor beta receptor 2 gene. *J Med Genet* 2006; 43: 908-916.
64. Tietjen GE, Al-Qasmi MM, Athanas K et al. Altered hemostasis in migraineurs studied with a dynamic flow system. *Thromb Res* 2007; 119: 217-222.
65. Vanmolkot FH, Van Bortel LM and de Hoon JN. Altered arterial function in migraine of recent onset. *Neurology* 2007; 68: 1563-1570.
66. Hamed SA, Hamed EA, Ezz Eldin AM and Mahmoud NM. Vascular risk factors, endothelial function, and carotid thickness in patients with migraine: relationship to atherosclerosis. *J Stroke Cerebrovasc Dis* 2010; 19: 92-103.
67. Rossato A, Veronese F, Maggioni F et al. Autonomic dysfunction and endothelial changes in migraine sufferers. *Panminerva Med* 2011; 53: 13-18.
68. Bigal ME, Kurth T, Santanello N et al. Migraine and cardiovascular disease: A population-based study. *Neurology* 2010; 74: 628-635.

69. Kurth T, Chabriat H and Bousser MG. Migraine and stroke: a complex association with clinical implications. *Lancet Neurol* 2012; 11: 92-100.
70. Xu Y, Padiath QS, Shapiro RE et al. Functional consequences of a CKIdelta mutation causing familial advanced sleep phase syndrome. *Nature* 2005; 434: 640-644.
71. Jen JC, Wan J, Palos TP et al. Mutation in the glutamate transporter EAAT1 causes episodic ataxia, hemiplegia, and seizures. *Neurology* 2005; 65: 529-534.
72. Suzuki M, Van Paesschen W, Stalmans I et al. Defective membrane expression of the Na(+)-HCO(3)(-) cotransporter NBCe1 is associated with familial migraine. *Proc Natl Acad Sci U S A* 2010; 107: 15963-15968.
73. Montagna P, Gallassi R, Medori R et al. MELAS syndrome: characteristic migrainous and epileptic features and maternal transmission. *Neurology* 1988; 38: 751-754.
74. Hudson G and Chinnery PF. Mitochondrial DNA polymerase-gamma and human disease. *Hum Mol Genet* 2006; 15 Spec No 2: R244-52.
75. Tzoulis C, Engelsen BA, Telstad W et al. The spectrum of clinical disease caused by the A467T and W748S POLG mutations: a study of 26 cases. *Brain* 2006; 129: 1685-1692.
76. Winterthun S, Ferrari G, He L et al. Autosomal recessive mitochondrial ataxic syndrome due to mitochondrial polymerase gamma mutations. *Neurology* 2005; 64: 1204-1208.
77. Verret S and Steele JC. Alternating hemiplegia in childhood: a report of eight patients with complicated migraine beginning in infancy. *Pediatrics* 1971; 47: 675-680.
78. de Vries B, Stam AH, Beker F et al. CACNA1A mutation linking hemiplegic migraine and alternating hemiplegia of childhood. *Cephalalgia* 2008; 28: 887-891.
79. Bassi MT, Bresolin N, Tonelli A et al. A novel mutation in the ATP1A2 gene causes alternating hemiplegia of childhood. *J Med Genet* 2004; 41: 621-628.
80. Swoboda KJ, Kanavakis E, Xaidara A et al. Alternating hemiplegia of childhood or familial hemiplegic migraine? A novel ATP1A2 mutation. *Ann Neurol* 2004; 55: 884-887.
81. Haan J, Kors EE, Terwindt GM et al. Alternating hemiplegia of childhood: no mutations in the familial hemiplegic migraine CACNA1A gene. *Cephalalgia* 2000; 20: 696-700.
82. Kors EE, Vanmolkot KR, Haan J et al. Alternating hemiplegia of childhood: no mutations in the second familial hemiplegic migraine gene ATP1A2. *Neuropediatrics* 2004; 35: 293-296.
83. Manolio TA, Collins FS, Cox NJ et al. Finding the missing heritability of complex diseases. *Nature* 2009; 461: 747-753.
84. Bertina RM, Koeleman BP, Koster T et al. Mutation in blood coagulation factor V associated with resistance to activated protein C. *Nature* 1994; 369: 64-67.
85. Eising E, de Vries B, Ferrari MD et al. Pearls and pitfalls in genetic studies of migraine. *Cephalalgia* 2013; 33: 614-625.
86. Kowa H, Yasui K, Takeshima T et al. The homozygous C677T mutation in the methylenetetrahydrofolate reductase gene is a genetic risk factor for migraine. *Am J Med Genet* 2000; 96: 762-764.

87. Kara I, Sazci A, Ergul E et al. Association of the C677T and A1298C polymorphisms in the 5,10 methylenetetrahydrofolate reductase gene in patients with migraine risk. *Brain Res Mol Brain Res* 2003; 111: 84-90.
88. Lea RA, Ovcaric M, Sundholm J et al. The methylenetetrahydrofolate reductase gene variant C677T influences susceptibility to migraine with aura. *BMC Med* 2004; 2: 3.
89. Oterino A, Valle N, Bravo Y et al. MTHFR T677 homozygosis influences the presence of aura in migraineurs. *Cephalalgia* 2004; 24: 491-494.
90. Oterino A, Valle N, Pascual J et al. Thymidylate synthase promoter tandem repeat and MTHFD1 R653Q polymorphisms modulate the risk for migraine conferred by the MTHFR T677 allele. *Brain Res Mol Brain Res* 2005; 139: 163-168.
91. Scher AI, Terwindt GM, Verschuren WM et al. Migraine and MTHFR C677T genotype in a population-based sample. *Ann Neurol* 2006; 59: 372-375.
92. Todt U, Freudenberg J, Goebel I et al. MTHFR C677T polymorphism and migraine with aura. *Ann Neurol* 2006; 60: 621-2; author reply 622-3.
93. Kaunisto MA, Kallela M, Hamalainen E et al. Testing of variants of the MTHFR and ESR1 genes in 1798 Finnish individuals fails to confirm the association with migraine with aura. *Cephalalgia* 2006; 26: 1462-1472.
94. Rubino E, Ferrero M, Rainero I et al. Association of the C677T polymorphism in the MTHFR gene with migraine: a meta-analysis. *Cephalalgia* 2009; 29: 818-825.
95. Schurks M, Rist PM and Kurth T. MTHFR 677C>T and ACE D/I polymorphisms in migraine: a systematic review and meta-analysis. *Headache* 2010; 50: 588-599.
96. Goyette P, Sumner JS, Milos R et al. Human methylenetetrahydrofolate reductase: isolation of cDNA, mapping and mutation identification. *Nat Genet* 1994; 7: 195-200.
97. Anttila V, Stefansson H, Kallela M et al. Genome-wide association study of migraine implicates a common susceptibility variant on 8q22.1. *Nat Genet* 2010; 42: 869-873.
98. Freilinger T, Anttila V, de Vries B et al. Genome-wide association analysis identifies susceptibility loci for migraine without aura. *Nat Genet* 2012; 44: 777-782.
99. Chasman DI, Schurks M, Anttila V et al. Genome-wide association study reveals three susceptibility loci for common migraine in the general population. *Nat Genet* 2011; 43: 695-698.
100. Stam AH, van den Maagdenberg AM, Haan J et al. Genetics of migraine: an update with special attention to genetic comorbidity. *Curr Opin Neurol* 2008; 21: 288-293.
101. Bigal ME, Serrano D, Buse D et al. Acute migraine medications and evolution from episodic to chronic migraine: a longitudinal population-based study. *Headache* 2008; 48: 1157-1168.
102. Diener HC, Kuper M and Kurth T. Migraine-associated risks and comorbidity. *J Neurol* 2008; 255: 1290-1301.
103. Buse DC, Manack AN, Fanning KM et al. Chronic Migraine Prevalence, Disability, and Sociodemographic Factors: Results From the American Migraine Prevalence and Prevention Study. *Headache* 2012;

104. Anttila V, Kallela M, Oswell G et al. Trait components provide tools to dissect the genetic susceptibility of migraine. *Am J Hum Genet* 2006; 79: 85-99.
105. Anttila V, Nyholt DR, Kallela M et al. Consistently replicating locus linked to migraine on 10q22-q23. *Am J Hum Genet* 2008; 82: 1051-1063.
106. Nyholt DR, Morley KI, Ferreira MA et al. Genomewide significant linkage to migrainous headache on chromosome 5q21. *Am J Hum Genet* 2005; 77: 500-512.
107. Lea RA, Nyholt DR, Curtain RP et al. A genome-wide scan provides evidence for loci influencing a severe heritable form of common migraine. *Neurogenetics* 2005; 6: 67-72.
108. Chen CC, Keith JM, Nyholt DR et al. Bayesian latent trait modeling of migraine symptom data. *Hum Genet* 2009; 126: 277-288.
109. Ferrari MD, Odink J, Bos KD et al. Neuroexcitatory plasma amino acids are elevated in migraine. *Neurology* 1990; 40: 1582-1586.
110. Martinez F, Castillo J, Rodriguez JR et al. Neuroexcitatory amino acid levels in plasma and cerebrospinal fluid during migraine attacks. *Cephalalgia* 1993; 13: 89-93.
111. Bockowski L, Sobaniec W and Zelazowska-Rutkowska B. Proinflammatory plasma cytokines in children with migraine. *Pediatr Neurol* 2009; 41: 17-21.
112. Vanmolkot FH and de Hoon JN. Increased C-reactive protein in young adult patients with migraine. *Cephalalgia* 2007; 27: 843-846.
113. Uzar E, Evliyaoglu O, Yucel Y et al. Serum cytokine and pro-brain natriuretic peptide (BNP) levels in patients with migraine. *Eur Rev Med Pharmacol Sci* 2011; 15: 1111-1116.
114. Uzar E, Evliyaoglu O, Toprak G et al. Increased asymmetric dimethylarginine and nitric oxide levels in patients with migraine. *J Headache Pain* 2011;
115. Guldiken S, Guldiken B, Demir M et al. Soluble CD40 ligand and prolactin levels in migraine patients during interictal period. *J Headache Pain* 2011; 12: 355-360.
116. Fusayasu E, Kowa H, Takeshima T et al. Increased plasma substance P and CGRP levels, and high ACE activity in migraineurs during headache-free periods. *Pain* 2007; 128: 209-214.
117. Kurth T, Ridker PM and Buring JE. Migraine and biomarkers of cardiovascular disease in women. *Cephalalgia* 2008; 28: 49-56.
118. Dunn WB, Broadhurst D, Begley P et al. Procedures for large-scale metabolic profiling of serum and plasma using gas chromatography and liquid chromatography coupled to mass spectrometry. *Nat Protoc* 2011; 6: 1060-1083.
119. Beckonert O, Keun HC, Ebbels TM et al. Metabolic profiling, metabolomic and metabonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts. *Nat Protoc* 2007; 2: 2692-2703.
120. Suhre K and Gieger C. Genetic variation in metabolic phenotypes: study designs and applications. *Nat Rev Genet* 2012; 13: 759-769.
121. Holle D, Katsarava Z and Obermann M. The hypothalamus: specific or nonspecific role in the pathophysiology of trigeminal autonomic cephalalgias? *Curr Pain Headache Rep* 2011; 15:

101-107.
122. Obermann M, Yoon MS, Dommes P et al. Prevalence of trigeminal autonomic symptoms in migraine: a population-based study. *Cephalalgia* 2007; 27: 504-509.
123. Nesbitt AD and Goadsby PJ. Cluster headache. *BMJ* 2012; 344: e2407.
124. Russell MB. Epidemiology and genetics of cluster headache. *Lancet Neurol* 2004; 3: 279-283.
125. Rainero I, Gallone S, Valfre W et al. A polymorphism of the hypocretin receptor 2 gene is associated with cluster headache. *Neurology* 2004; 63: 1286-1288.
126. Baumber L, Sjostrand C, Leone M et al. A genome-wide scan and HCRTR2 candidate gene analysis in a European cluster headache cohort. *Neurology* 2006; 66: 1888-1893.
127. Schurks M, Kurth T, Geissler I et al. Cluster headache is associated with the G1246A polymorphism in the hypocretin receptor 2 gene. *Neurology* 2006; 66: 1917-1919.
128. Rainero I, Rubino E, Valfre W et al. Association between the G1246A polymorphism of the hypocretin receptor 2 gene and cluster headache: a meta-analysis. *J Headache Pain* 2007; 8: 152-156.
129. Anttila V, Winsvold BS, Gormley P et al. Genome-wide meta-analysis identifies new susceptibility loci for migraine. *Nat Genet* 2013;
130. Aulchenko YS, Heutink P, Mackay I et al. Linkage disequilibrium in young genetically isolated Dutch population. *Eur J Hum Genet* 2004; 12: 527-534.
131. Pardo LM, MacKay I, Oostra B et al. The effect of genetic drift in a young genetically isolated population. *Ann Hum Genet* 2005; 69: 288-295.
132. Grand MG, Kaine J, Fulling K et al. Cerebroretinal vasculopathy. A new hereditary syndrome. *Ophthalmology* 1988; 95: 649-659.
133. Jen J, Cohen AH, Yue Q et al. Hereditary endotheliopathy with retinopathy, nephropathy, and stroke (HERNS). *Neurology* 1997; 49: 1322-1330.
134. Vahedi K, Boukobza M, Massin P et al. Clinical and brain MRI follow-up study of a family with COL4A1 mutation. *Neurology* 2007; 69: 1564-1568.
135. Brennan KC, Bates EA, Shapiro RE et al. Casein kinase idelta mutations in familial migraine and advanced sleep phase. *Sci Transl Med* 2013; 5: 183ra56.

2

Two novel *SCN1A* mutations causing pure familial hemiplegic migraine

Claudia M. Weller[1*], Nadine Pelzer[2*]
Boukje de Vries[1], Mercè Artigas López[3]
Oriol De Fàbregues[4], Julio Pascual[5]
María A. Ramos Arroyo[3], Stephany C. Koelewijn[1]
Anine H. Stam[2], Joost Haan[2,6]
Michel D. Ferrari[2], Gisela M. Terwindt[2]
Arn M.J.M. van den Maagdenberg[1,2]

Departments of [1]Human Genetics, [2]Neurology Leiden University Medical Center, Leiden
Department of [3]Medical Genetics, Complejo Hospitalario de Navarra, Pamplona, Spain
Department of [4]Neurology, University Hospital Vall d'Hebron, Barcelona, Spain
Department of [5]Neurology, University Hospital Central de Asturias and Ineuropa, Oviedo, Spain
Department of [6]Neurology, Rijnland Hospital, Leiderdorp
*** equally contributed**

Cephalalgia 2014; 34(13):1062-9

Abstract

Background – Familial hemiplegic migraine (FHM) is a rare monogenic subtype of migraine with aura, characterized by motor auras. FHM is known to be caused by mutations in the *CACNA1A, ATP1A2* and *SCN1A* genes (FHM1, FHM2 and FHM3, respectively). Sofar, less than 5% of FHM cases are explained by mutations in the *SCN1A* gene. Two Spanish FHM families without mutations in *CACNA1A* and *ATP1A2* were screened for mutations in the *SCN1A* gene.
Methods – We assessed the clinical features of both FHM families and performed direct sequencing of all coding exons (and adjacent sequences) of the *SCN1A* gene.
Results – FHM patients in both families had pure hemiplegic migraine with highly variable severity and frequency of attacks. We identified a novel *SCN1A* missense mutation p.Ile1498Met in all three tested hemiplegic migraine patients of one family. In the other family, novel *SCN1A* missense mutation p.Phe1661Leu was identified in six out of eight tested hemiplegic migraine patients. The mutation p.Ile1498Met affects a part of the protein that is responsible for adequate occlusion of the ion pore of the $Na_v1.1$ channel.
Conclusions – We identified two mutations causing pure FHM, thereby increasing the number of FHM3 mutations to seven. *SCN1A* mutations are an infrequent cause of FHM.

Key Words: migraine ■ ion channel defects ■ familial hemiplegic migraine ■ *SCN1A* gene ■

Introduction

FAMILIAL HEMIPLEGIC MIGRAINE (FHM) is a rare monogenic subtype of migraine with aura (MA) that is characterized by transient hemiparesis during the aura phase. Headache and aura characteristics are identical in FHM and MA patients, apart from the motor auras and possible longer duration of auras in FHM.[1] Mutations in three neuronal or glial ion transporter genes (*CACNA1A*, *ATP1A2*, *SCN1A*) are known to cause FHM.[2] Thus far, over 70 mutations in the *CACNA1A* (FHM1)[3] and *ATP1A2* (FHM2)[4] genes have been identified and mutations in these genes seem to account for the majority of cases with FHM [5]. Only five mutations were reported for the third FHM (FHM3) gene *SCN1A*[5,6] encoding the α1 subunit of neuronal voltage-gated $Na_V1.1$ sodium channels that are primarily expressed on inhibitory neurons.[7,8] Of these, *SCN1A* mutations p.Leu1649Gln and p.Gln1489Lys cause pure FHM.[6,9] The other FHM3 mutations either cause FHM and seizures occurring separately from the hemiplegic migraine attacks (p.Gln1489His[10] and p.Leu263Val[11]) or FHM and 'elicited repetitive daily blindness' (p.Gln1489His and p.Phe1499Leu mutations)[10]). Functional analyses of pure FHM3 mutations predict a loss-of-function effect on inhibitory neurons, thereby leading to overall neuronal hyperexcitability[12], although this is debated by others[13]. Notably, *SCN1A* is a well-known epilepsy gene with several hundred mutations causing various forms of childhood epilepsy: Dravet syndrome (also known as severe myoclonic epilepsy of infancy (SMEI)) and the milder generalized epilepsy with febrile seizures (GEFS+).[14] Here we report the identification of two novel FHM3 mutations in two families with pure hemiplegic migraine.

Subjects and Methods

Patients

Family 1

This four-generation Spanish family includes four hemiplegic migraine patients (see Figure 1, Family 1 and Table 1). The family members were interviewed and neurologically examined (ODF). Diagnoses were established according to the International Classification of Headache Disorders, 2nd edition (ICHD-2).[1] The diagnosis of the proband's deceased father was based on information provided by the proband.

The proband (II-4) had her first migraine attack at age 11 and continued to have on average two attacks per month until the age of 73. These hemiplegic attacks were usually preceded by flashing lights or scotoma, speech problems and/or unilateral sensory symptoms. Auras developed gradually during 5 to 15 minutes, but on several occasions hemi-

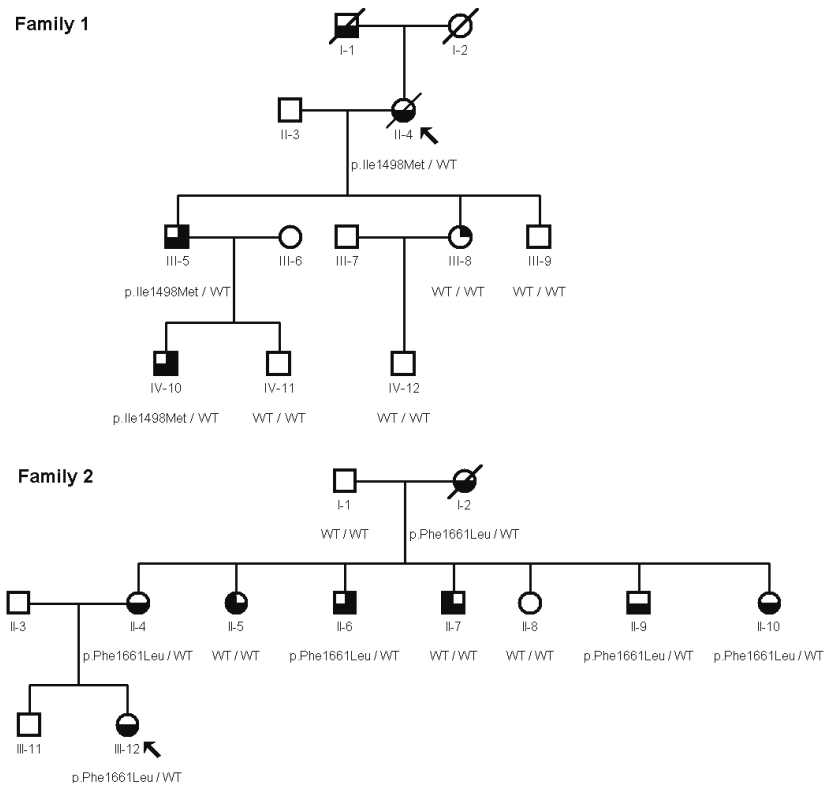

Figure 1 - Pedigrees of FHM families 1 and 2. The arrow indicates the proband. Symbols: black lower-half: hemiplegic migraine; right upper square: migraine with aura (MA); left upper square: migraine without aura (MO). WT: wild-type allele. p.Ile1498Met and p.Phe1661Leu: heterozygous carrier of the respective SCN1A mutations.

plegia occurred instantaneously, causing her to fall. In general, hemiplegia lasted 30 to 60 minutes and then diminished gradually. After these auras, she suffered from a typical migraine headache, i.e. unilateral pulsatile headache that increased with movement and was best relieved by sleep, accompanied by photophobia and phonophobia. At the age of 69 years, she experienced an episode of benign paroxysmal positional vertigo that lasted for fifteen days and then resolved gradually over the course of two weeks. At age 70, she presented with prolonged hemiplegia for ten days, which completely recovered during the subsequent three weeks. Cerebral MRI (see Figure 2) and SPECT (data not shown) during the hemiplegic phase showed vasogenic edema and cortical hypoperfusion in the right hemisphere. Further extensive diagnostic workup for cerebrovascular disease and epilepsy, including electroencephalography, cerebrospinal fluid analysis, echography of the heart and carotid arteries, laboratory analysis of coagulation factors and autoimmune titers, and skin biopsy for excluding CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy) showed no abnormalities.

Table 1 - Characteristics of Familial Hemiplegic Migraine patients in family 1 and family 2.

	Gender	Age (years)	Age at onset of FHM (years)	Average attack frequency (at different ages)	Aura symptoms during FHM attacks				Other migraine subtypes	Other medical history	Genotype
					Visual	Sensory	Motor	Speech			
Family 1											
I-1	male	deceased (at age 80)	?	?	?	?	yes	?	?	dementia	?
II-4 (proband)	female	deceased (at age 79)	11	2x/ month	yes	yes	yes	yes	none	benign paroxysmal, positional vertigo, dementia (Alzheimer's)	p.Ile1498Met / WT
III-5	male	58	?	2x/ year	?	?	yes	?	MA	rheumatoid arthritis	p.Ile1498Met / WT
IV-10	male	33	15	1x/ 4 years	no	yes	yes	no	MA	?	p.Ile1498Met / WT
Family 2											
I-2	female	deceased (at age 86)	13	youth: 1-2x/ month lower at older age	yes	yes	yes	yes	none	ischemic stroke, dementia, premature ovarian failure	p.Phe1661Leu / WT
II-4	female	53	14	14-30: 3-4x/ year >30: 1x/ several years	yes	yes	yes	yes	none	premature ovarian failure	p.Phe1661Leu / WT
II-5	female	52	20	1x/ month	yes	yes	yes	no	MO	?	WT / WT
II-6	male	50	14	1x/ week	yes	yes	yes	yes	MA	?	p.Phe1661Leu / WT
II-7	male	49	14	rarely	yes	yes	yes	yes	MO	?	WT / WT
II-9	male	46	14	14-40: 1x/ month >40: 1-2x/ year	yes	yes	yes	yes	none	?	p.Phe1661Leu / WT
II-10	female	43	11	2x/ year	yes	yes	yes	yes	none	?	p.Phe1661Leu / WT
III-12 (proband)	female	20	9	2x/ month	yes	yes	yes	yes	none	premature ovarian failure	p.Phe1661Leu / WT

MA= migraine with aura; MO= migraine without aura; WT: wild-type allele; p.Ile1498Met and p.Phe1661Leu: heterozygous carrier of the respective SCN1A mutations. The symbol '?' indicates this information was unavailable.

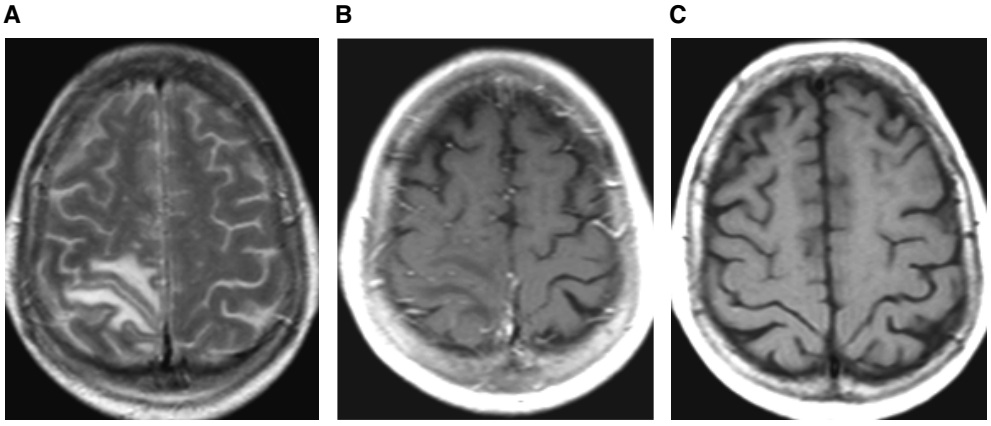

Figure 2 - Cerebral MRI of the proband of family 1 during prolonged aura (*A+B*) and after full recovery (*C*). *A+B*: MR FLAIR T2-weighted image in axial plane and gadolineum-enhanced T1-weighted image showing a subcortical area of white matter in the perirolandic region on the right is hyperintense on image A and relatively hypointense on image *B*, with blurring of the cortical sulci adjacent to the convexity. *C*: T1-weighted MR image showing the resolution of the radiological abnormalities, in agreement with the patient's clinical recovery.

From age 72, she showed progressive cognitive deterioration and was diagnosed with Alzheimer's disease. She died at the age of 79 years of urinary sepsis. Post-mortem examination of the brain showed multiple senile plaques and confirmed the diagnosis of Alzheimer's disease. No ischemic lesions were found.

The proband's father (I-1) had similar FHM attacks. He suffered from dementia from age 70 and died at age 80. Patient III-5, now 58 years old, suffered from MA and FHM with an attack frequency of two per year until he was 44. No hemiplegic attacks have occurred over the last 13 years. Patient IV-10, currently 33 years old, has experienced four hemiplegic migraine attacks from age 15 to 31. His attacks started with hypesthesia and hemiparesis during 20 minutes, followed by migraine headache. An MRI scan performed during one of these attacks showed no abnormalities. He also suffers from migraine with visual aura, with an attack frequency of one per three months.

Family 2

This three-generation family is also of Spanish origin. Eight family members were diagnosed with FHM (see Figure 1, Family 2 and Table 1). Their diagnoses were established according to the ICHD-2 criteria[1], and confirmed by a neurologist specialized in headache (JP). After the passing of the proband's grandmother, additional information was provided by her children.

The proband (III-12) had been suffering from approximately three migraine attacks per month since she was nine years old, with visual, sensory, and speech auras. At the age of 16 she presented at the emergency department with right-sided hemiparesis, accom-

panied by scotoma, photopsia, right-sided hypesthesia, contralateral headache and vomiting lasting for three hours. She was diagnosed with hemiplegic migraine.

The proband's mother (II-4) had three to four FHM attacks per year from age 14 to 30, later decreasing to one attack per several years. All attacks include visual, sensory, motor and speech symptoms lasting 30-60 minutes. The five affected siblings of the proband's mother experience multiple aura types, in varying combinations during separate attacks, followed by typical migraine headaches (for summary see Table 1).

The proband's grandmother (I-2) had suffered migraine attacks since the age of 13. She always experienced visual, sensory and dysphasic auras. Hemiparesis occurred on most occasions. At age 85 she was diagnosed with dementia. She died one year later after a stroke.

Genetic analysis

Genomic DNA was isolated from peripheral leukocytes according to standard procedures [15]. All 26 exons and flanking intronic sequences of the *SCN1A* gene were amplified by PCR and analyzed for mutations by direct sequencing. Detailed information is available from the authors upon request.

Results

In Family 1, a novel heterozygous point mutation in exon 24 (c.4460G>C; p.Ile1498Met) of the *SCN1A* gene was identified. The mutation co-segregated with FHM in this family (Figure 1, Family 1) and was absent in 161 healthy controls. The p.Ile1498Met mutation is located in the intracellular loop between transmembrane domains III and IV (Figure 3A) and affects the "IFMT motif". This motif encodes the hydrophobic latch that is responsible for adequate occlusion of the ion pore, which leads to inactivation of the $Na_v1.1$ channel, thereby regulating the generation and propagation of action potentials.[7,8] The IFMT motif is named after the four amino acids encoding it, i.e. isoleucine (I), phenylalanine (F), methionine (M), and threonine (T). Mutation p.Ile1498Met leads to the substitution of isoleucine for a methionine residue at the first position of the IFMT motif. Both are hydrophobic nonpolar amino acids with similar molecular weight. However, isoleucine is aliphatic while methionine is a sulfur-containing amino acid and is important for the function of the *SCN1A*-encoding protein. Analysis of this amino acid in homologous and orthologous sodium channel α1 subunits shows high conservation of isoleucine^{1498} (Figure 3B).[10]

Mutation scanning in Family 2 revealed a novel heterozygous point mutation in exon 26 (c.4981C>T; p.Phe1661Leu) of the *SCN1A* gene. This mutation partially co-segregated with FHM in this family as two of the eight tested clinically affected individuals (II-5

and II-7) did not have the mutation and are thus diagnosed as phenocopies (Figure 1, Family 2). The p.Phe1661Leu mutation was not found in the probands unaffected aunt nor in 161 healthy control subjects. Phenylalanine[1661] is located in domain IV, in the intracellular loop between the voltage-sensing S4 segment and part of the transmembrane pore (S5) (Figure 3A).[14] It is highly conserved within all orthologues and homologues except for the *SCN7A* gene, which, notably, has a leucine at the equivalent position (Figure 3B). Although both amino acids are hydrophobic, phenylalanine is a large aromatic amino acid, whereas leucine is smaller and lacks an aromatic ring.

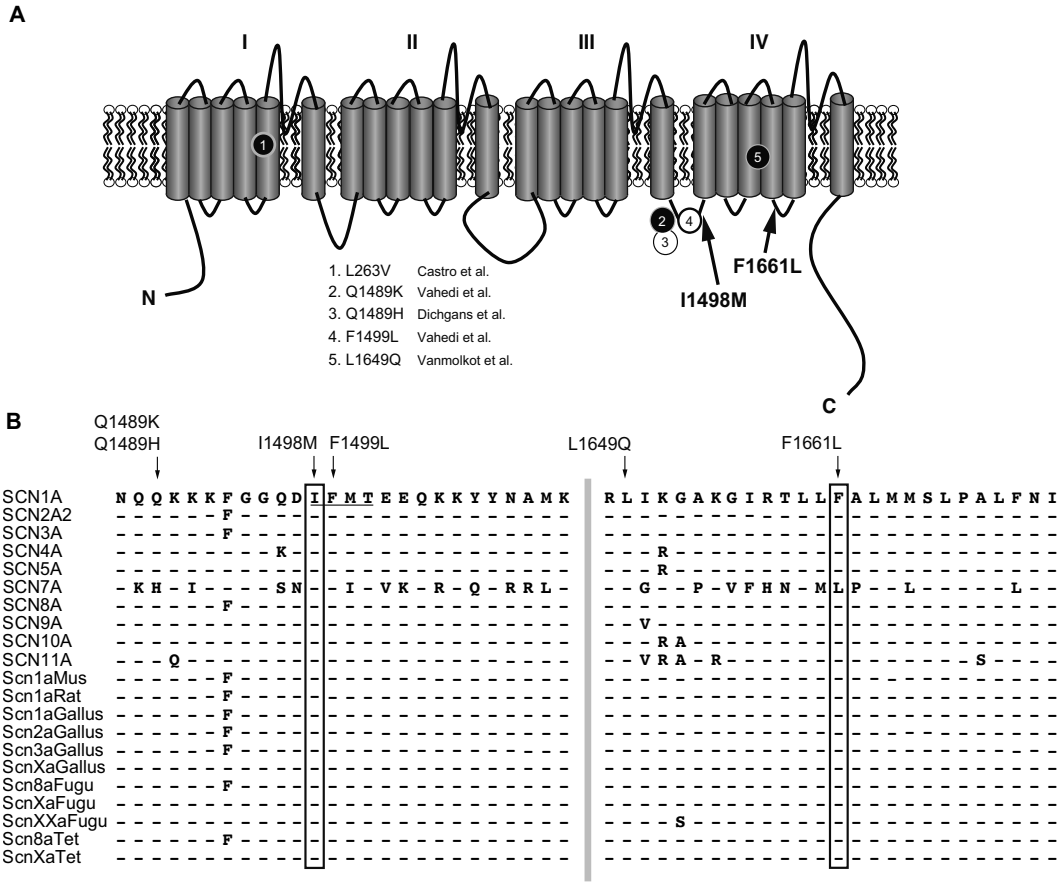

Figure 3 - *A*: Topology of the *SCN1A* gene product. Arrows indicate the location of the novel p.Ile1498Met (I1498M) and p.Phe1661Leu (F1661L) FHM3 *SCN1A* mutations. Previously identified FHM3 mutations are indicated by numbers 1- 5. Figure adapted from De Vries et al[4]. *B*: Conservation of the I[1498] and F[1661] amino acid residues. Alignment of the amino acid sequence of alpha1 subunits of sodium channels from various species, indicating the conservation of the amino acids that are affected by FHM3 mutations. Isoleucine[1498] (I[1498]) is completely conserved across homologues and orthologues of the human *SCN1A* gene. Phenylalanine[1661] (F1661) is highly conserved in all homologues and orthologues, except for *SCN7A*.

Discussion

We report two Spanish families with pure hemiplegic migraine (i.e. without other paroxysmal or permanent neurological symptoms such as epilepsy or ataxia) and two novel *SCN1A* mutations (p.Ile1498Met and p.Phe1661Leu). Attacks in both families started at a young age, ranging from age 9 to 20 years. Attack frequency was highly variable, ranging from one attack per week to one attack per several years. Prolonged duration of auras, varying from three hours to three weeks, and co-occurrence of FHM attacks with attacks of migraine with and without aura were observed in both families, as has been reported for other FHM families.[16]

Ictal MRI abnormalities (Figure 2), as observed in the proband of Family 1, have not been reported in FHM3 patients, but are similar to observations made in FHM1 and FHM2.[16] Post-mortem examination revealed that the dementia in patient II-4 of Family 1 was due to Alzheimer's disease. Dementia in patient I-2 of Family 2 is also unlikely related to the *SCN1A* mutation, given the age at onset after 80.

Although functional testing of the mutations has not been performed, we are confident that mutations p.Ile1498Met and p.Phe1661Leu are pathogenic. First of all, p.Ile1498Met is present only in the three family members with hemiplegic migraine and absent from a panel of 161 control subjects. Second, isoleucine[1498] is a highly conserved amino acid in α1 subunits of vertebrate sodium channels. Third, the mutation is located in the same intracellular loop as three of the five previously described FHM3 mutations (p.Gln1489Lys, p.Gln1489His, p.Phe1499Leu) (Figure 3A).[6,10] Of these, the p.Phe1499Leu mutation is also located in the IFMT motif, which encodes the hydrophobic latch, and is essential for the function of the neuronal voltage-gated $Na_V1.1$ sodium channel.[7,8] Interestingly, none of the more than 600 mutations causing Dravet syndrome or GEFS+ affect the IFMT motif.[14,17] It is hypothesized that a defective hydrophobic latch will lead to delayed sodium channel inactivation with concomitant decreased neuronal inhibition.

Several lines of evidence indicate that the p.Phe1661Leu mutation in Family 2 also is pathogenic. The mutated phenylalanine[1661] is a highly conserved amino acid across homologues and orthologues except for *SCN7A*, a gene that shares only 52% overall sequence similarity with the *SCN1A* gene. The p.Phe1661Leu mutation is absent from our panel of healthy control subjects and co-segregates in six of eight hemiplegic migraine patients. The remaining two hemiplegic migraine patients should be considered phenocopies. Phenocopies have been described before in FHM families.[18-20]

The p.Phe1661Leu FHM3 mutation is the first mutation to co-localize with a known Dravet mutation, i.e. p.Phe1661Ser. In the Dravet patient, phenylalanine[1661] is mutated into a much smaller, nucleophilic serine residue.[21] Substitution of the phenylalanine for a leucine apparently has a less detrimental effect on protein function, which is in agree-

ment with the much milder phenotype in our FHM family. The importance of phenylalanine[1661] for sodium channel function is further illustrated by the fact that mutating the corresponding residue in *SCN4A*, causing paramyotonia congenita, showed alterations of the kinetic properties of the channel. [22]

In conclusion, we identified two novel *SCN1A* mutations causing pure FHM with large intra-familial variation in severity and frequency of the attacks. Our findings increase the number of FHM-causing *SCN1A* mutations to seven. Although this still needs to be proven by functional studies, the p.Ile1498Met mutation most likely disrupts the fast inactivation of the $Na_V1.1$ channels and decreased neuronal inhibition. If true, this would support current insights that decreased sodium channel function is the consequence of FHM3 mutations.

Acknowledgements

Source of funding

This work was supported by grants of the Netherlands Organization for Scientific Research (NWO) (920-03-473, A.H.S., 903-52-291, M.D.F. and R.R.F.; VICI 918.56.602, M.D.F.; VIDI 91711319, G.M.T.; the European Community (EC); and the Centre for Medical Systems Biology (CMSB) in the framework of the Netherlands Genomics Initiative (NGI) (M.D.F. and A.M.J.M.v.d.M). They had no role in the design or conduct of the study.

References:

1. Headache classification subcommittee of the International Headache Society. The International Classification of Headache Disorders: 2nd edition. *Cephalalgia* 2004; 24 Suppl 1: 9-160.
2. van den Maagdenberg AM, Haan J, Terwindt GM and Ferrari MD. Migraine: gene mutations and functional consequences. *Curr Opin Neurol* 2007; 20: 299-305.
3. Ophoff RA, Terwindt GM, Vergouwe MN et al. Familial hemiplegic migraine and episodic ataxia type-2 are caused by mutations in the Ca2+ channel gene CACNL1A4. *Cell* 1996; 87: 543-552.
4. De Fusco M, Marconi R, Silvestri L et al. Haploinsufficiency of ATP1A2 encoding the Na+/K+ pump alpha2 subunit associated with familial hemiplegic migraine type 2. *Nat Genet* 2003; 33: 192-196.
5. de Vries B, Frants RR, Ferrari MD and van den Maagdenberg AM. Molecular genetics of migraine. *Hum Genet* 2009; 126: 115-132.
6. Dichgans M, Freilinger T, Eckstein G et al. Mutation in the neuronal voltage-gated sodium channel SCN1A in familial hemiplegic migraine. *Lancet* 2005; 366: 371-377.

7. Patton DE, West JW, Catterall WA and Goldin AL. Amino acid residues required for fast Na(+)-channel inactivation: charge neutralizations and deletions in the III-IV linker. *Proc Natl Acad Sci U S A* 1992; 89: 10905-10909.
8. Rohl CA, Boeckman FA, Baker C et al. Solution structure of the sodium channel inactivation gate. *Biochemistry* 1999; 38: 855-861.
9. Vanmolkot KR, Babini E, de Vries B et al. The novel p.L1649Q mutation in the SCN1A epilepsy gene is associated with familial hemiplegic migraine: genetic and functional studies. Mutation in brief #957. Online. *Hum Mutat* 2007; 28: 522.
10. Vahedi K, Depienne C, Le Fort D et al. Elicited repetitive daily blindness: a new phenotype associated with hemiplegic migraine and SCN1A mutations. *Neurology* 2009; 72: 1178-1183.
11. Castro MJ, Stam AH, Lemos C et al. First mutation in the voltage-gated Nav1.1 subunit gene SCN1A with co-occurring familial hemiplegic migraine and epilepsy. *Cephalalgia* 2009; 29: 308-313.
12. Kahlig KM, Rhodes TH, Pusch M et al. Divergent sodium channel defects in familial hemiplegic migraine. *Proc Natl Acad Sci U S A* 2008; 105: 9799-9804.
13. Cestele S, Scalmani P, Rusconi R et al. Self-limited hyperexcitability: functional effect of a familial hemiplegic migraine mutation of the Nav1.1 (SCN1A) Na+ channel. *J Neurosci* 2008; 28: 7273-7283.
14. Escayg A and Goldin AL. Sodium channel SCN1A and epilepsy: mutations and mechanisms. *Epilepsia* 2010; 51: 1650-1658.
15. Miller SA, Dykes DD and Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988; 16: 1215.
16. Russell MB and Ducros A. Sporadic and familial hemiplegic migraine: pathophysiological mechanisms, clinical characteristics, diagnosis, and management. *Lancet Neurol* 2011; 10: 457-470.
17. Claes LR, Deprez L, Suls A et al. The SCN1A variant database: a novel research and diagnostic tool. *Hum Mutat* 2009; 30: E904-20.
18. Terwindt GM, Ophoff RA, Haan J et al. Variable clinical expression of mutations in the P/Q-type calcium channel gene in familial hemiplegic migraine. Dutch Migraine Genetics Research Group. *Neurology* 1998; 50: 1105-1110.
19. Thomsen LL, Kirchmann M, Bjornsson A et al. The genetic spectrum of a population-based sample of familial hemiplegic migraine. *Brain* 2007; 130: 346-356.
20. Riant F, De Fusco M, Aridon P et al. ATP1A2 mutations in 11 families with familial hemiplegic migraine. *Hum Mutat* 2005; 26: 281.
21. Claes L, Ceulemans B, Audenaert D et al. De novo SCN1A mutations are a major cause of severe myoclonic epilepsy of infancy. *Hum Mutat* 2003; 21: 615-621.
22. Mitrovic N, Lerche H, Heine R et al. Role in fast inactivation of conserved amino acids in the IV/S4-S5 loop of the human muscle Na+ channel. *Neurosci Lett* 1996; 214: 9-12.

3

A novel *SLC2A1* mutation linking hemiplegic migraine with alternating hemiplegia of childhood

Claudia M. Weller[1]*, Wilhelmina G. Leen[2]*
Brian G.R. Neville[3], John S. Duncan[4]
Boukje de Vries[1], Marije A. Geilenkirchen[1]
Joost Haan[3,5], Erik-Jan Kamsteeg[6], Michel D. Ferrari[7]
Arn M.J.M. van den Maagdenberg[1,7]
Michel A.A.P. Willemsen[8], Hans Scheffer[6]
Gisela M. Terwindt[7]

Departments of [1]Human Genetics, [7]Neurology, Leiden University Medical Center, Leiden
Departments of [2]Neurology, [6]Human Genetics and [8]Paediatric Neurology, Radboud University Medical Centre, Nijmegen
[3]Neurosciences Unit, Institute of Child Health, UCL Medical School and Great Ormond Street Hospital for Children NHS Trust, UK
[4]UCL Institute of Neurology, UK
Department of [5]Neurology, Rijnland Hospital, Leiderdorp, the Netherlands
*** equally contributed**

Cephalalgia. 2015 Jan;35(1):10-5

Abstract

Background – Hemiplegic migraine (HM) and alternating hemiplegia of childhood (AHC) are rare episodic neurological brain disorders with partial clinical and genetic overlap. Recently, *ATP1A3* mutations were shown to account for the majority of AHC patients. In addition, a mutation in the *SLC2A1* gene was reported in a patient with atypical AHC. We therefore investigated whether mutations in these genes may also be involved in HM. Furthermore, we studied the role of *SLC2A1* mutations in a small set of AHC patients without *ATP1A3* mutations.
Methods – We screened 42 HM patients (21 familial and 21 sporadic patients) for *ATP1A3* and *SLC2A1* mutations. In addition, four typical AHC patients and one atypical patient with overlapping symptoms of both disorders were screened for *SLC2A1* mutations.
Results – A pathogenic de novo *SLC2A1* mutation (p.Gly18Arg) was found in the atypical patient with overlapping symp- toms of AHC and hemiplegic migraine. No mutations were found in the HM and the other AHC patients.
Conclusion – Screening for a mutation in the *SLC2A1* gene should be considered in patients with a complex phenotype with overlapping symptoms of hemiplegic migraine and AHC.

Key Words: Hemiplegic migraine (HM) ■ alternating hemiplegia of childhood (AHC) ■ *SLC2A1* gene, exercise-induced dystonia ■ GLUT1 deficiency syndrome

Introduction

HEMIPLEGIC MIGRAINE (HM) and alternating hemiplegia of childhood (AHC) are two rare episodic neurological brain disorders in which hemiplegia is a prominent symptom. Hemiplegic migraine is a subtype of migraine with aura that is characterized by transient hemiparesis during the aura phase.[1] HM can present as a familial (familial hemiplegic migraine; FHM) or a sporadic (sporadic hemiplegic migraine; SHM) disease. HM can occur in a pure form or with additional symptoms such as cerebellar ataxia, intellectual disability, and seizures.[2] Mutations in the *CACNA1A*, *ATP1A2* and *SCN1A* genes are well known to cause HM. The *CACNA1A* gene codes for a subunit of voltage-gated neuronal CaV2.1 calcium channels, the *ATP1A2* gene for a subunit of Na^+/K^+ ATPases, and the *SCN1A* gene codes for a subunit of neuronal $Na_V1.1$ sodium channels.[3–5] Functional studies of HM gene mutations suggest a net increase in neurotransmitter levels (that is glutamate in the cortex) in the synaptic cleft as a key mechanism in HM,[6] either due to an increased Ca_V 2.1 function or reduced Na_V 1.1 channel function in excitatory and inhibitory neurons, respectively, or due to reduced functionality of glial Na^+/K^+ ATPases. Recently, truncating mutations in the *PRRT2* gene have been reported in HM patients, of whom the majority also have phenotypes that are frequently associated with such *PRRT2* mutations (i.e. paroxysmal kinesigenic dyskinesia, benign familial infantile seizures, and infantile convulsion choreoathetosis syndrome).[7–13] Based on this association, *PRRT2* has been suggested as the fourth FHM gene.

AHC is characterized by intellectual disability and by recurrent attacks of hemiplegia, movement disorders, and seizures starting before the age of 18 months.[14] The *ATP1A3* gene was recently shown to be the major cause of AHC with mutations in more than 70% of patients.[15–17] *ATP1A3* codes for another subunit of Na^+/K^+ ATPases and is expressed in neurons of basal ganglia, cerebellum, and hippocampus. Na^+/K^+ ATPases are involved in the regulation of sodium and potassium gradients across glial (in the case of *ATP1A2*) or neuronal (in the case of *ATP1A3*) plasma membranes, thereby affecting sodium-coupled ion transport, neuronal excitability, and/or osmoregulation. In the respective cell types, FHM *ATP1A2* and AHC *ATP1A3* mutations result in a reduction of sodium-potassium pump activity.[15–17]

In addition to typical AHC and HM patients there are also atypical patients who either do not fulfill all criteria of AHC or present with clinical symptoms of both AHC and HM. The atypical presentation makes it difficult, if not impossible, to determine the correct clinical diagnosis.[18] Identification of gene mutations in atypical patients, therefore, may shed light on possible overlapping pathophysiological mechanisms between AHC and HM, and may confirm clinical diagnoses. Until now, an *ATP1A2* mutation was identified in a Greek family with atypical AHC in which patients did not fulfill all diag-

Table 1 - Diagnostic criteria for alternating hemiplegia of childhood and sporadic hemiplegic migraine.

Alternating Hemiplegia of Childhood (Sweney et al., Pediatrics 2009)[14]	**Our patient**	**Sporadic hemiplegic migraine (International Classification of Headache Disorders, 2nd ed.)**[1]	**Our patient**
A) Onset of symptoms before 18 months of age	No	A) At least two attacks fulfilling criteria B and C	Yes
B) Repeated attacks of hemiplegia involving either side of the body	Yes	B) Aura consisting of fully reversible motor weakness and at least one of the fol- lowing: 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	 Yes Yes Yes
C) Other paroxysmal disturbances including tonic or dystonic spells, oculomotor abnormalities, and autonomic phenomena during bouts in isolation	Yes	C) At least two of the following: 1. At least one aura symptom develops gradually over ≥ 5 minutes and/or different aura symptoms occur in succession over ≥5 minutes 2. Each aura symptom lasts ≥5 minutes and <24 hours 3. Headache fulfilling criteria B–D for migraine without aura begins during the aura or follows onset of aura within 60 minutes	Yes
D) Episodes of bilateral hemiplegia or quadriplegia as generalization of a hemiplegic episode, or bilateral from the beginning	No	D) No first- or second-degree relative has attacks fulfilling these criteria A-E	Yes
E) Immediate disappearance of symptoms upon sleeping, which later may resume after waking	No	E) Not attributed to another disorder	No
F) Evidence of developmental delay and neurologic abnormalities including choreoathetosis, dystonia, or ataxia	Yes	Other paroxysmal and permanent features described in SHM: -Attacks with impaired consciousness -Migraine with aura -Epilepsy -Cerebellar signs -Intellectual disability	 No Yes Yes Yes Yes

nostic criteria of AHC.[19,20] In addition, a *CACNA1A* mutation was identified in two German monozygous twins who displayed clinical features both of AHC and FHM,[18] and a mutation was identified in the glucose transporter 1 GLUT1 gene *SLC2A1* in an atypical AHC patient.[21] The GLUT1 protein is pivotal for glucose transport into the brain, and mutations in *SLC2A1* are a well-known cause of GLUT1 deficiency syndrome[22], which is characterized by developmental delay, medication-resistant epilepsy, and move-

ment disorders including ataxia and dystonia. Because of the partially overlapping clinical manifestations of AHC and HM, the *CACNA1A* and *ATP1A2* genes earlier were screened in typical AHC fulfilling all diagnostic criteria as well, but no mutations have been identified.[23,24]

Because of the clinical and genetic overlap of AHC and HM, we investigated (i) in our cohort of HM patients (who screened negative for mutations in known HM genes) whether mutations are found in the coding regions of *SLC2A1* and in those exons of *ATP1A3* in which previously AHC mutations had been identified; and (ii) whether *SLC2A1* mutations are present in patients with AHC who screened negative for *ATP1A3* mutations. Our results revealed no mutations in *ATP1A3* or *SLC2A1* in our cohort of HM patients. However, we did identify a p.Gly18Arg mutation in *SLC2A1* in a patient with a complex phenotype consisting of clinical features of AHC and HM (Table 1). In our study we did not provide evidence that AHC genes *ATP1A3* and *SLC2A1* play a role in FHM or SHM. We suggest considering screening for mutations in SLC2A1 in atypical patients with overlapping symptoms of HM and AHC.

Methods

Patients

For mutation screening of the *SLC2A1* and *ATP1A3* genes we selected 42 patients with HM (21 FHM and 21 SHM) for whom mutations in FHM genes (*CACNA1A*, *ATP1A2*, and *SCN1A*) had been excluded prior to the study by direct sequencing of all exons and flanking intronic regions. HM diagnoses were established according to the International Classification of Headache Disorders, 2nd ed. (ICHD-2; see Table 1 for SHM criteria).[1] We also included five *ATP1A3*-negative patients referred to us with a suspected AHC diagnosis in our screen of *SLC2A1* mutations. Four of these patients fulfilled all diagnostic criteria for AHC, as outlined by Sweney et al. (see Table 1).[14]. The fifth patient was atypical and met diagnostic criteria for SHM, but presented with a particularly severe phenotype of intellectual disability, seizures, and exercise-induced dystonia in addition to hemiplegic attacks, and thus symptoms fulfilled three of the six diagnostic criteria for AHC (see Table 1).

Three of the five AHC patients were sequenced for the *CACNA1A* and *ATP1A2* genes, including the atypical patient. This was performed as part of a large mutation screen prior to the discovery of the *ATP1A3* gene as a major gene for AHC. As these results were negative.[23,24] we did not continue to screen for these genes in AHC patients. *SCN1A* screening was also performed in a group of AHC patients (data not published) and was negative as well. As *SCN1A* mutations have not been found in three large series of sporadic hemiplegic migraine patients with or without associated neurological symptoms,[25–27] we

did not perform *SCN1A* screening in the five AHC patients. *PRRT2* was screened only in the atypical AHC patient, as screening in a large subset of our HM patient cohort showed no mutations (data not published). The local medical ethics committee approved the study and all patients or their parents provided written informed consent.

Mutation screening of *ATP1A3* and *SLC2A1*

Ethylenediaminetetraacetic acid (EDTA) blood samples were collected from all patients and DNA was isolated using a standard salting out method.[28] For the five AHC patients, genetic analysis of the *ATP1A3* gene was performed by direct sequencing of all exons (and flanking intronic sequences) (*ATP1A3* reference sequence: Genbank Accession Number NM_152296.4). For the 42 HM patients, *ATP1A3* exons (and flanking intronic sequences) containing AHC-causing mutations (that is exons 5, 7–9, 13–16, 17, 18, 20–22) were sequenced. Genetic analysis of the *SLC2A1* gene was performed by direct sequencing of all 10 exons and flanking intronic sequences (*SLC2A1* reference sequence: Genbank Accession Number NM_006516). Multiplex ligation-dependent probe amplification was performed for *SLC2A1* to search for intragenic deletions, using the SALSA multiplex ligation-dependent probe amplification (MLPA) kit P138 (MRC Holland, Amsterdam, the Netherlands) according to the manufacturer's instructions.

Results

We did not identify any causal mutations in the *ATP1A3* or *SLC2A1* gene in the 42 HM patients and four typical AHC patients. In contrast, in the atypical patient with overlapping features of AHC and SHM, we identified a novel heterozygous p.Gly18Arg mutation (c.52G>C) in exon 2 of the *SLC2A1* gene. This mutation was not found in 150 healthy controls nor in single-nucleotide polymorphism (SNP) databases (dbSNP, 1000G, Ensemble, ESP5400 and GoNL). It was also absent in the unaffected parents, and thus had occurred de novo. This patient showed a unique combination of symptoms, which were published 15 years ago without a genetic diagnosis.[29] At the age of 20 years, she presented with progressive cerebellar ataxia and exercise-induced dystonia responding to oral corticosteroid treatment, and moderate intellectual disability. No ocular abnormalities were observed. From the age of 11 years, she had experienced episodes of hemiplegia, which did not resolve upon falling asleep. Hemiplegic attacks were always accompanied by inability to speak and confused speech. Flashing lights and ipsilateral numbness were present in some of the attacks. Hemiplegia, visual, and sensory symptoms were followed by contralateral headache and vomiting. Furthermore, she suffered from simple and complex partial seizures. Interictal electroencephalograms (EEGs) showed epileptiform activity, predominantly located right parietal. Brain magnetic resonance imaging scans

(MRIs) were normal apart from a small non-specific white matter abnormality. Interictal cerebrospinal fluid (CSF) showed normal cell count, low glucose concentration of 2.4 mmol/l (reference: 2.5–3.7 mmol/l) and low lactate concentration of 1.2 mmol/l (reference: 1.3–1.9 mmol/l) (30). Blood glucose was not simultaneously measured.

Clinical follow-up at the age of 32 years revealed that no hemiplegic attacks had occurred during the last 10 years. Limb paresthesiae and hemianopsia lasting for several minutes without headache continued to occur and were classified as migraine with aura. Exercise-induced dystonia led to daily falls during walking. Ataxia had remained stable and seizures were never completely responsive to treatment with lamotrigine, sodium valproate, carbamazepine or clobazam. In retrospect, the CSF glucose and lactate concentrations were recognized as borderline low. The patient currently is considering starting a ketogenic diet.

Discussion

In this study we identified a novel pathogenic heterozygous *SLC2A1* p.Gly18Arg mutation in an atypical sporadic patient with overlapping symptoms of both AHC and HM. In 2009, Rotstein et al.[21] found a p.Arg93Trp *SLC2A1* mutation in a patient with atypical AHC features with intellectual disability, hemiplegic attacks (but otherwise not typical for HM either), episodes of ataxia, microcephaly and hypoglycorrhagia. Characteristically, mutations in *SLC2A1* cause autosomal dominant glucose transporter type 1 (GLUT1) deficiency syndrome.[31] The classical phenotype of GLUT1 deficiency syndrome consists of developmental delay, medication-resistant epilepsy, and movement disorders including ataxia and dystonia. A diagnosis of GLUT1 deficiency syndrome is suspected when hypoglycorrhachia (i.e. a low CSF glucose in a normoglycemic patient) is present. Symptoms improve with a ketogenic diet. Over the last few years it has become apparent that the clinical spectrum of the GLUT1 deficiency syndrome is broader.[31,32]

Our patient expands the clinical phenotype of *SLC2A1* mutations with episodes of HM and migraine with aura (without hemiplegia). The episodes of hemiplegia in our and Rotstein's[21] patient differ from classic AHC because in contrast to classical AHC patients (i) the age of onset in these two patients was after 18 months; (ii) sleep had no beneficial effect on the attacks; (iii) dysconjugated eye movements were absent; and (iv) quadriplegic attacks were absent. Therefore, these patients do not meet the diagnostic criteria for AHC (Table 1).[1] Moreover, the hemiplegia in our patient meets the ICHD-2 diagnostic criteria for SHM (Table 1),[1] but no mutations in *CACNA1A* and *ATP1A2* were found, so far the only two genes with strong evidence for implication in the sporadic form of HM. The phenotype of our patient combines symptoms from GLUT1 deficiency syndrome and some of the severest presentations of SHM, both of which can present

with intellectual disability, seizures and ataxia. The exercise-induced dystonia that is prominently present in our patient is not described in patients with SHM.[2]

Different lines of evidence indicate that the p.Gly18Arg *SLC2A1* mutation is the disease-causing mutation in our patient. The mutation had occurred de novo and was not found in healthy controls. Moreover, glycine at position 18 is a conserved amino acid in the first transmembrane segment of the GLUT1 protein. This segment is part of the central aqueous channel of the protein.[33] Since the nonpolar glycine residue is replaced by a positively charged arginine residue, the mutation most likely interferes with normal GLUT1 function by disrupting glucose transport across the aqueous central channel. Finally, CSF glucose was low in combination with a low normal CSF lactate, which is within the range observed in *SLC2A1* mutation carriers.[30] The exercise-induced dystonia improved with oral corticosteroid treatment, possibly due to corticosteroid-induced hyperglycemia leading to increased GLUT1-mediated glucose transport in the brain, as has been shown by experiments in rats.[34]

The absence of *SLC2A1* mutations in the four classical AHC patients is in line with a previous mutation screening in 23 classical AHC patients[35] that was conducted before the identification of *ATP1A3* as the causal gene in typical AHC patients[15,16] We did not identify causal mutations in *ATP1A3* exons implicated in AHC in our HM patients. In conclusion, we propose that mutation screening of *SLC2A1* should be considered in patients with a complex phenotype with overlapping symptoms of AHC and HM, especially if hypoglycorrhachia is detected in CSF. Diagnosing patients with a similar complex phenotype and *SLC2A1* mutations may be of importance also because symptoms may ameliorate upon treatment with a ketogenic diet.

Acknowledgements

Source of funding

This work was supported by grants from the Netherlands Organization for Scientific Research (NWO) (903-52-291, M.D.F.; Vici 918.56.602, M.D.F.; Vidi 917-11-319, G.M.T), NWO 'AGIKO-stipendium'; grant number 92003529, W.G.L.); the European Community (EC) (EUROHEAD, LSHM-CT-2004-504837, M.D.F.; EUROHEAD-PAIN (M.D.F. & A.M.J.M.v.d.M.) and the Centre for Medical Systems Biology (CMSB) in the framework of the Netherlands Genomics Initiative (NGI) (A.M.J.M.v.d.M).

References

1. Headache Classification Subcommittee of the International Headache Society. The International Classification of Headache Disorders: 2nd ed. *Cephalalgia* 2004; 24 Suppl 1: 9–160.
2. Russell MB and Ducros A. Sporadic and familial hemiplegic migraine: Pathophysiological mechanisms, clinical characteristics, diagnosis, and management. *Lancet Neurol* 2011; 10: 457–470.
3. Ophoff RA, Terwindt GM, Vergouwe MN, et al. Familial hemiplegic migraine and episodic ataxia type-2 are caused by mutations in the $Ca2^{+}$ channel gene CACNL1A4. *Cell* 1996; 87: 543–552.
4. De Fusco M, Marconi R, Silvestri L, et al. Haploinsufficiency of ATP1A2 encoding the Na^{+}/K^{+} pump alpha2 subunit associated with familial hemiplegic migraine type 2. *Nat Genet 2003*; 33: 92–196.
5. Dichgans M, Freilinger T, Eckstein G, et al. Mutation in the neuronal voltage-gated sodium channel SCN1A in familial hemiplegic migraine. *Lancet* 2005; 366: 371–377.
6. van den Maagdenberg AM, Haan J, Terwindt GM, et al. Migraine: Gene mutations and functional consequences. *Curr Opin Neurol* 2007; 20: 299–305.
7. Marini C, ContiV,Mei D, et al. PRRT2mutations in familial infantile seizures, paroxysmal dyskinesia, and hemiplegic migraine. *Neurology* 2012; 79: 2109–2114.
8. Riant F, Roze E, Barbance C, et al. PRRT2 mutations cause hemiplegic migraine. *Neurology* 2012; 79: 2122–2124.
9. Cloarec R, Bruneau N, Rudolf G, et al. PRRT2 links infantile convulsions and paroxysmal dyskinesia with migraine. *Neurology* 2012; 79: 2097–2103.
10. Gardiner AR, Bhatia KP, Stamelou M, et al. PRRT2 gene mutations: From paroxysmal dyskinesia to episodic ataxia and hemiplegic migraine. *Neurology* 2012; 79: 2115–2121.
11. Dale RC, Gardiner A, Antony J, et al. Familial PRRT2 mutation with heterogeneous paroxysmal disorders including paroxysmal torticollis and hemiplegic migraine. *Dev Med Child Neurol* 2012; 54: 958–960.
12. Castiglioni C, Lopez I, Riant F, et al. PRRT2 mutation causes paroxysmal kinesigenic dyskinesia and hemiplegic migraine in monozygotic twins. *Eur J Paediatr Neurol* 2013; 17: 254–258.
13. van Vliet R, Breedveld G, de Rijk-van Andel J, et al. PRRT2 phenotypes and penetrance of paroxysmal kinesigenic dyskinesia and infantile convulsions. *Neurology* 2012; 79: 777–784.
14. Sweney MT, Silver K, Gerard-Blanluet M, et al. Alternating hemiplegia of childhood: Early characteristics and evolution of a neurodevelopmental syndrome. *Pediatrics* 2009; 123: e534–e541.
15. Heinzen EL, Swoboda KJ, Hitomi Y, et al. De novo mutations in ATP1A3 cause alternating hemiplegia of childhood. *Nat Genet* 2012; 44: 1030–1034.
16. Rosewich H, Thiele H, Ohlenbusch A, et al. Heterozygous de-novo mutations in ATP1A3 in

patients with alternating hemiplegia of childhood: A whole-exome sequencing gene-identification study. *Lancet Neurol* 2012; 11: 764–773.
17. Ishii A, Saito Y, Mitsui J, et al. Identification of ATP1A3 mutations by exome sequencing as the cause of alternating hemiplegia of childhood in Japanese patients. *PLoS One* 2013; 8: e56120.
18. de Vries B, Stam AH, Beker F, et al. CACNA1A mutation linking hemiplegic migraine and alternating hemiplegia of childhood. *Cephalalgia* 2008; 28: 887–891.
19. Bassi MT, Bresolin N, Tonelli A, et al. A novel mutation in the ATP1A2 gene causes alternating hemiplegia of childhood. *J Med Genet* 2004; 41: 621–628.
20. de Vries B, Haan J, Stam AH, et al. Alternating hemiplegia of childhood: No mutations in the glutamate transporter EAAT1. *Neuropediatrics* 2006; 37: 302–304.
21. Rotstein M, Doran J, Yang H, et al. Glut1 deficiency and alternating hemiplegia of childhood. *Neurology* 2009; 73: 2042–2044.
22. De Vivo DC, Trifiletti RR, Jacobson RI, et al. Defective glucose transport across the blood-brain barrier as a cause of persistent hypoglycorrhachia, seizures, and developmental delay. *N Engl J Med* 1991; 325: 703–709.
23. Haan J, Kors EE, Terwindt GM, et al. Alternating hemiplegia of childhood: No mutations in the familial hemiplegic migraine CACNA1A gene. *Cephalalgia* 2000; 20: 696–700.
24. Kors EE, Vanmolkot KR, Haan J, et al. Alternating hemiplegia of childhood: No mutations in the second familial hemiplegic migraine gene ATP1A2. *Neuropediatrics* 2004; 35: 293–296.
25. Terwindt G, Kors E, Haan J, et al. Mutation analysis of the CACNA1A calcium channel subunit gene in 27 patients with sporadic hemiplegic migraine. *Arch Neurol* 2002; 59: 1016–1018.
26. de Vries B, Freilinger T, Vanmolkot KR, et al. Systematic analysis of three FHM genes in 39 sporadic patients with hemiplegic migraine. Neurology 2007; 69: 2170–2176.
27. Riant F, Ducros A, Ploton C, et al. De novo mutations in ATP1A2 and CACNA1A are frequent in early-onset sporadic hemiplegic migraine. *Neurology* 2010; 75: 967–972.
28. Miller SA, Dykes DD and Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988; 16: 1215.
29. Neville BG, Besag FM and Marsden CD. Exercise induced steroid dependent dystonia, ataxia, and alternating hemiplegia associated with epilepsy. *J Neurol Neurosurg Psychiatry* 1998; 65: 241–244.
30. Leen WG, Willemsen MA, Wevers RA, et al. Cerebrospinal fluid glucose and lactate: Age-specific reference values and implications for clinical practice. *PLoS One* 2012; 7: e42745.
31. Leen WG, Klepper J, Verbeek MM, et al. Glucose transporter-1 deficiency syndrome: The expanding clinical and genetic spectrum of a treatable disorder. *Brain* 2010; 133: 655–670.
32. Brockmann K. The expanding phenotype of GLUT1-deficiency syndrome. *Brain Dev* 2009; 31: 545–552.
33. Mueckler M and Makepeace C. Model of the exofacial substrate-binding site and helical folding

of the human Glut1 glucose transporter based on scanning mutagenesis. *Biochemistry* 2009; 48: 5934–5942.

34. Chipkin SR, van Bueren A, Bercel E, et al. Effects of dexamethasone in vivo and in vitro on hexose transport in brain microvasculature. *Neurochem Res* 1998; 23: 645–652.

35. Vuillaumier-Barrot S, Panagiotakaki E, Le Bizec C, et al. Absence of mutation in the SLC2A1 gene in a cohort of patients with alternating hemiplegia of childhood (AHC). *Neuropediatrics* 2010; 41: 267–269.

Validation of the web-based LUMINA questionnaire for recruiting large cohorts of migraineurs

4

W.P.J. (Ron) van Oosterhout[1], Claudia M. Weller[2]
Anine H. Stam[1], Floor Bakels[1], Theo Stijnen[3]
Michel D. Ferrari[1], Gisela M. Terwindt[1]

Departments of [1]Neurology,
[2]Human Genetics, [3]Medical Statistics
Leiden University Medical Center, Leiden

Cephalalgia 2011;31(13):1359–1367

Abstract

Objective – To assess validity and reliability of a self-administered web-based migraine-questionnaire in diagnosing migraine aura for the use of epidemiological and genetic studies.

Methods – Self-reported migraineurs enrolled via the LUMINA-website and completed a web-based questionnaire on headache and aura symptoms, after fulfilling screening criteria. Diagnoses were calculated using an algorithm based on the International Classification of Headache Disorders (ICHD-2), and semi-structured telephone-interviews were performed for final diagnoses. Logistic regression generated a prediction rule for aura. Algorithm-diagnoses and predicted diagnoses were subsequently compared to the interview-derived diagnoses.

Results – In one year, we recruited 2,397 migraineurs, of which 1,067 were included in the validation. A seven-question subset provided higher sensitivity (86%vs.45%), slightly lower specificity (75%vs.95%) and similar positive predictive value (86%vs.88%) in assessing aura when comparing with the ICHD-2 based algorithm.

Conclusions – This questionnaire is accurate and reliable in diagnosing migraine aura among self-reported migraineurs, and enables detection of more aura cases with low false-positive rate.

Key Words: migraine ■ headache ■ questionnaire validation ■ cohort study ■ screening in epidemiology

Introduction

MIGRAINE is a common brain disorder characterized by recurrent, disabling attacks of headache, autonomic features (migraine without aura; MO), and, in one third of patients, transient neurological aura symptoms (migraine with aura; MA). In western countries, the overall migraine prevalence in the general population is at least 12 percent, two-thirds of which concerns females[1-4]. Since no biomarker for migraine exists, diagnosis according to the headache classification of the International Headache Society (IHS)[5] relies exclusively on the headache history. A careful history taken by a headache specialist is the gold standard for making a valid migraine and aura diagnosis.

Large-scale studies with several thousands of participants are important to obtain information for epidemiological and genetic migraine research and may yield important insights in migraine pathophysiology. Migraine is a complex genetic disorder, i.e. multiple genetic and environmental factors contribute to migraine susceptibility.

Twin and population-based family studies showed that genetic factors play an important role in migraine susceptibility, especially in the MA subtype[6-12]. However, genetic linkage studies using migraine subtypes as an end diagnosis did not yield gene variants thus far. Clinical heterogeneity in migraine and aura diagnosis may have hampered the identification of such variants. Recently, in a large genome wide association analysis (GWA) with a large set of clinic-based migraineurs, a first-ever genetic risk factor was identified associated with common types of migraine, in patients that were largely recruited from specialist headache clinics with a clinic-based migraine diagnosis[13]. However, population-based large-scale studies exclude the possibility of a face-to-face examination, and, therefore, a less time-consuming and less costly diagnostic strategy has to be chosen. A web-based questionnaire represents an attractive and inexpensive alternative for a clinic interview. Several groups have reported on the use of internet to recruit headache and other patients for clinical research[14-18]. However, reliably diagnosing aura remains an issue.

The availability of a validated, aura-specific questionnaire is important when large numbers of cases are needed, especially in studies with self-reported migraineurs from the general population[19,20]. We developed the LUMINA (Leiden University MIgraine Neuro-Analysis) website and designed and validated a self-reporting, web-based questionnaire to reliably diagnose migraine headache and aura symptoms, using only a limited number of questions. In this paper, we will present the validation of this web-based migraine and aura questionnaire.

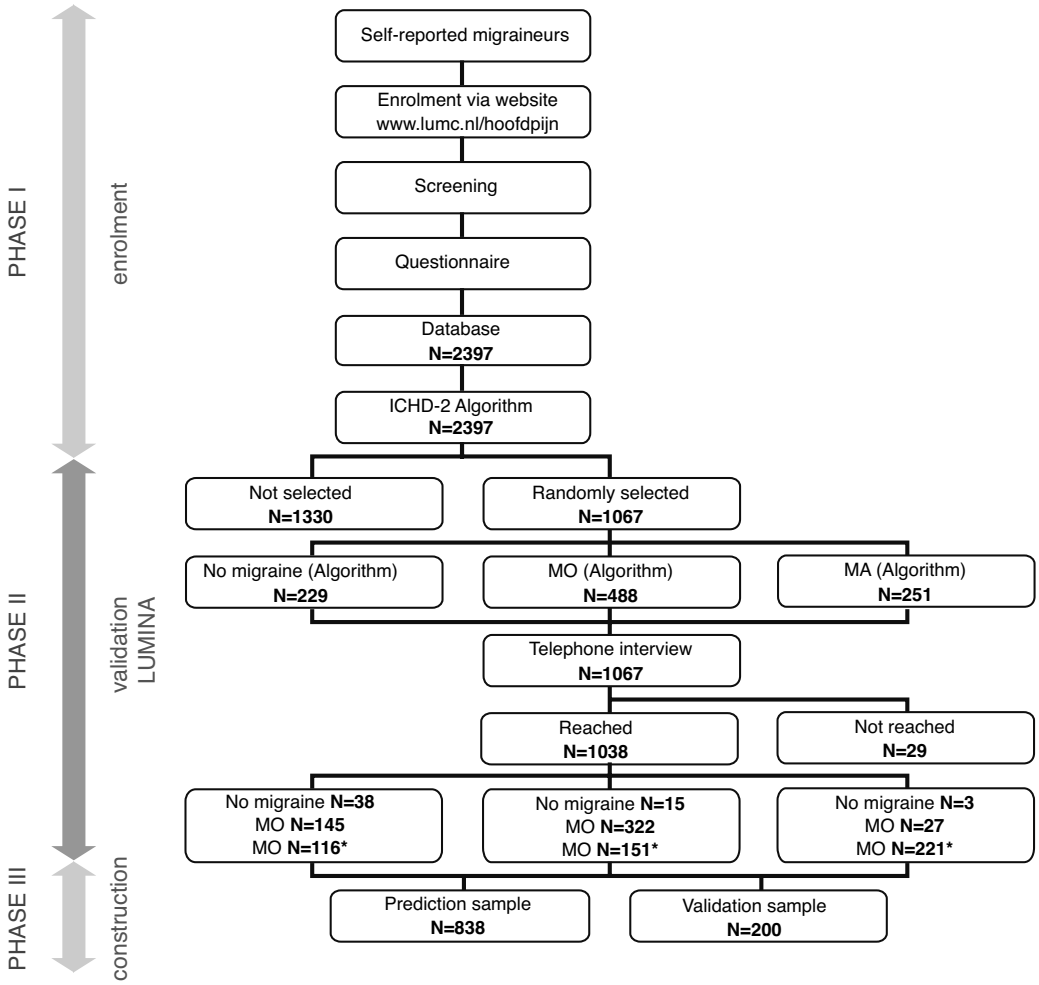

Figure 1 - Flowchart of (semi-)automated study flow. Screening = screening questionnaire; questionnaire = extended questionnaire; Alg.= ICHD-2 based algorithm diagnosis; Int.= interview diagnosis; MA = migraine with aura; MO = migraine without aura; *In the total MA group, 91.6% (447/488) reported visual aura symptoms.

Methods

Subjects

Participants were Dutch adults aged 18 to 74 years with migraine (MA and MO), who were informed via the lay press nationwide to enrol via the especially designed LUMINA website. Additionally, patients from our outpatient headache clinic were invited by a letter. In this clinic-based study, all participants were self-reporting migraineurs, of which approxiamtely 90% had previously been diagnosed with migraine by a physician.

Study flow

Study flow is depicted in Figure 1. Patients who visited the website were informed about the study and could enrol directly. The first step was to fulfil the screening criteria, using a simple screening questionnaire that was validated previously in the population-based GEM-study[3]. This screening questionnaire included five questions asking whether the patient i) had severe headaches in the past 12 months; ii) what the headache severity was; iii) had suffered from headaches which were preceded by visual disturbances; iv) had been diagnosed with migraine by a physician; and v) had ever used anti-migraine medication. After fulfilling these criteria, cases received a unique user ID-code via e-mail to log on to the study website, where they could participate in an extended, web-based questionnaire study. Having completed the extended questionnaire, a number of randomly selected participants were contacted by telephone by WPJvO, CMW, and AHS, who are experienced in diagnosing migraine. This semi-structured telephone interview detailed questions on headache and aura characteristics including ICHD-2 migraine and aura criteria[5] with special attention for visual, sensory, motor and speech aurasymptoms, was used as the gold standard. Median interview duration was 10-15 minutes, ranging up to 30 minutes if necessary. Afterwards, a final diagnosis was made: in case of ambiguity, a headache specialist (GMT) was consulted. Patients were excluded from the analysis if they could not be reached by telephone after five failed telephone contact attempts. The study was approved by the local medical ethics committee. All participants provided written informed consent.

Construction of questionnaire

The extended questionnaire (accessible via www.lumc.nl/hoofdpijn) was based on the ICHD-2 criteria[5] and incorporated 127 items on migraine headache and aura characteristics, premonitory symptoms, trigger factors, allodynia, and medication use and was presented to participants as a digital web-form. The questions were to be answered by choosing from categorical alternatives. On the web-form multicolour exemplary illustrations were shown with the most characteristic visual aura features (hemianopsia, scotoma, fortification spectra, visual blurring) and sensory aura features (anatomical distribution).

ICHD-2 based algorithm

After completion of the extended questionnaire, an algorithm based on ICHD-2 criteria[5] migraine criteria was run and individual diagnosis was determined. The algorithm had the following possible outcomes: 'no migraine'; 'migraine without aura'; and 'migraine with aura'. In the analysis, the algorithm outcomes were dichotomised into 'aura' and 'no aura' (Figure 2).

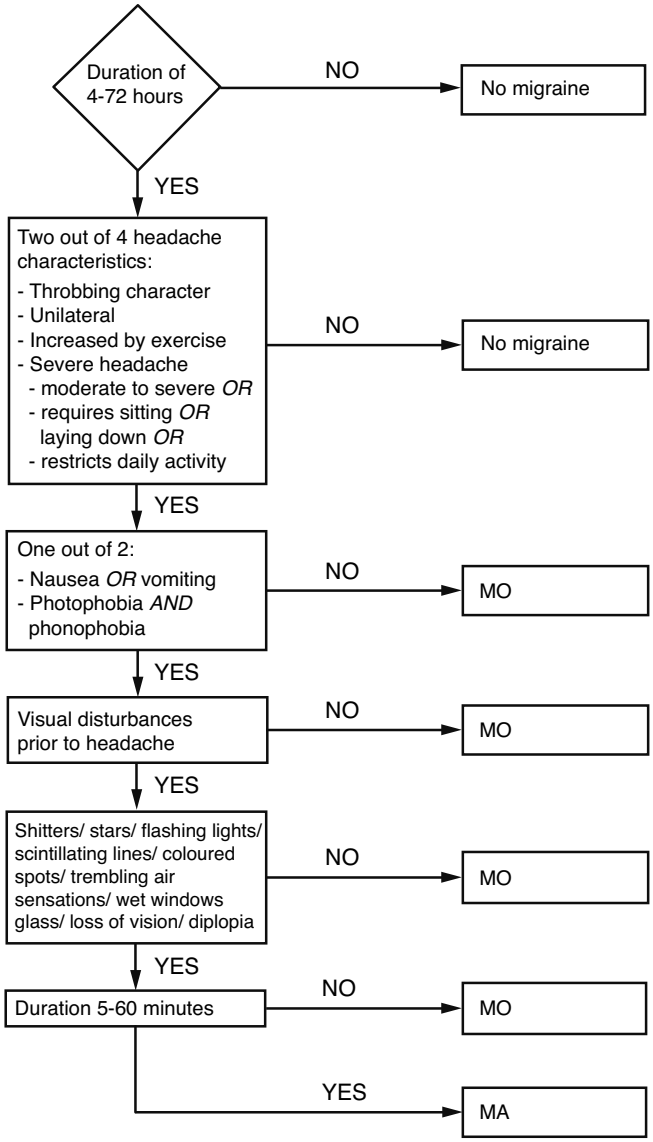

Figure 2 - Structure of ICHD-II based algorithm used in LUMINA study. MO = migraine without aura; MA = migraine with aura;

Statistical analysis

Descriptive statistics

Descriptive statistics were performed on demographic and clinical variables, on the algorithm based diagnoses and on the interview-derived diagnoses. Results are reported as mean ± SD or as percentage. Differences in between-groups means were analyzed with independent sample t-tests and ANOVAs. Proportions were compared using Chi-square tests. All items from the extended questionnaire that concerned ICHD-2 migraine criteria were evaluated separately. Likelihood ratios were calculated using standard formulas for positive likelihood ratio (LR+, sensitivity/[1 – specificity]) and negative likelihood ratio (LR-, [1 – sensitivity]/ specificity).

Questionnaire validation process

The questionnaire validation process was divided into two phases and was aimed at identifying a combination of items that were better predictors for diagnosing migraine aura than the ICHD-2 based algorithm, with the interview-derived diagnosis as the gold standard. In phase I, a sample of 838 self-reported migraineurs (approximately 80% of total group) was randomly selected and used as a training sample (see Figure 1) to derive a predictive model. These patients fulfilled set screening criteria from the five-item LUMINA screener before they could enter the extended questionnaire. Logistic regression (see below) was used to develop the predictive model that included questionnaire items most contributing to predict subcategories 'aura' and 'no aura'. Subsequently, we compared both the ICHD-2 based algorithm diagnoses and the diagnoses predicted by the logistic model, to the gold standard. In phase II, we validated this derived predictive model in an independent validation sample, consisting of 200 patients, approximately 20% of our sample (see Figure 1).

Phase I: Development of prediction rule

In phase I, a prediction rule for the aura subcategories 'aura' vs. 'no aura' was developed using a multivariate logistic regression analysis. Relevant, individual, dichotomized items (n=33) were selected from the extended questionnaire and were used as predictor variables for aura in the model. Selection of items was made by the authors (WPJvO; CMW; GMT) and was based on clinical relevance to migraine aura, and sensitivity, specificity, PPV, NPV en likelihood ratios of individual items. Inter-item correlation was assessed for relevant items using Spearman's rank coefficients and when items correlated with coefficients >0.9, one of these items was excluded from the analysis. A forward selection strategy using the likelihood ratio test was performed to identify items that were significant ($p<0.05$) predictors for the outcome of aura. For each subject in this sample (n=838), a prediction score was calculated using these items. Subsequently, a receiver operator

characteristics (ROC) curve was generated to assess the optimum cut off point for this prediction score. Using the method proposed by Halpern et al.[21], an optimum cut-off (highest sensitivity and specificity) was determined from the ROC curve. Therefore, the logistic model resulted in a selection of the 33 items with significant ($p<0.05$) contribution in the aura prediction.

Phase II: Validation of prediction rule

The derived predictive rule was subsequently validated in the second sample (validation sample; n=200; see Figure 1). Validity of this predictive model was assessed by checking whether the selected items contributed significantly ($p<0.05$) for the prediction in the second sample too. Subsequently, the sensitivity and specificity from the ROC optimum in the training sample were compared with these parameters in the validation sample, using the same cut-off value.

Overall outcome measures

Sensitivity, specificity, positive and negative predictive values were calculated to compare the fit of the three different models with the interview-derived aura diagnosis as the gold standard. These models were: 1) ICHD-2 based algorithm; 2) predictive model from phase I; and 3) validation of predictive rule in phase II.

All data analyses were performed using SPSS 16.0.2 (SPSS inc., IBM, USA). P values less than 0.05 were considered significant. When appropriate, categorical items were dichotomized into binary variables for the analysis in an attempt to simplify the instrument.

Table 1 - Baseline characteristics of total study population and separate study samples.

		Selection for study		Telephone interview		Sample	
Characteristic	**Total (n = 2397)**	**Not selected (n=1330)**	**Selected (n=1067)**	**Not reached (n=29)**	**Reached (n=1038)**	**Training (n=838)**	**Validation (n=200)**
Age, years	42.8±11.9	41.6±12.0	44.3±11.6	43.9±11.1	44.4±11.6	44.6±1.7	43.3±11.5
Female	84.8	83.9	85.8	89.7	85.6	85.0	88.5
Ever M diagnosis	88.9	87.8	90.2	100	89.9	90.2	89.0
Anti-M drug use	82.8	80.3	85.8	93.1	85.6	85.2	87.5
Algorithm diagnosis M	87.1	97.3*	71.4*	79.3	72.4	72.1	73.5

Values are mean ± SD or %. * $p<0.001$ (χ^2-test). M = migraine.

Table 2 - Sensitivity, specificity, and positive and negative predictive values as well as the corresponding likelihood ratios for diagnosis of migraine aura based on the IHCD-II-based algorithm (in both the total group and training sample) and the derived seven-item prediction model (in both the training sample and in the validation sample)

Characteristic	ICHD-2 based algorithm		Model	
	Total sample (n=1038)	Predictive sample (n=838)	Training sample (n=838)	Validation sample (n=200)
Sensitivity, %	45	44	83	86
Specificity, %	95	95	74	75
PPV MA, %	88	89	74	74
PPV MO (=NPV MA), %	70	64	83	86
Positive likelihood ratio	8.2	8.7	3.1	3.5
Negative likelihood ratio	0.6	0.6	0.2	0.2

MA = migraine with aura; MO = migraine without aura; NPV = negative predictive value; PPV = positive predictive value.

Receiver Operator Characteristics (ROC) curve

From the data in the training sample, we generated an ROC curve by plotting the sensitivity of the questionnaire against one minus the specificity. As a graphical representation of the trade-off between false negative and false positive rates for every possible cut-off point, the ROC curve reflects the trade-offs between sensitivity and specificity, and plots the false positive rate on the X axis and the true positive rate on the Y-axis. The area under the curve is a measure of correlation between the prediction of the questionnaire and the gold standard diagnosis. The closer the area under the curve (AUC) is to 1, the better the test is. To validate the derived logistic model, we compared the ROC from the prediction sample (n=838) to the ROC of the validation sample (n=200).

Results

General results

Over a 1-year period, from April 2008 until April 2009, 2,397 subjects fulfilled the set screening criteria and completed the extended questionnaire (Figure 1). During this time period, a total of 1,067 subjects (44.5%) were randomly selected for the semi-structured telephone interview, of which 1,038 (97.3%) were reached and could be used in the analysis. A total of 29 subjects (2.7%) were not included in the analysis because they could not be reached by telephone, after having tried at least five times. From these 1,038 subjects, 838 (79.4%) were randomly selected and used for the prediction model and the remaining sample of 200 subjects (18.9%) was used for validation (Figure 1).

Baseline characteristics of the total study population and separate prediction and validation samples are depicted in Table 1. Almost 90% of self-reported migraineurs had previously been diagnosed with migraine by a physician. Age, gender, prevalence of previous migraine diagnosis and use of anti-migraine medication did not differ significantly between selected subjects and non-selected subjects, nor between subjects that were reached compared to those that could not be reached for telephone interview (see Table 1). In the selected subjects (n=1,067; with special attention to patients which fulfilled ICHD-2 migraine criteria except for attack duration), the algorithm diagnosis of 'no-migraine' was more prevalent (28.6% [305/1,067] vs. 2.7% [36/1,330]; p<0.001) compared to non-selected subjects (n=1,330).

Screening questionnaire

In total, 94.6 percent of subjects (982/1,038) fulfilling the screening criteria, fulfilled ICHD-2 migraine criteria in the telephone interview. We considered everyone fulfilling the screening criteria to be migraineur. We used a logistic model to predict individual aura vs. no aura status.

Algorithm diagnosis

From the total sample of 1.038 subjects, the ICHD-2 based algorithm classified 488 subjects as MO patients, 251 as having MA, and 299 subjects as non-migraineurs (Figure 1). Of these, 243 were misclassified as non-migraineurs due to reporting of longer than actual attack duration. Table 2 summarizes the sensitivity, specificity, positive and negative predictive values as well as the corresponding likelihood ratios for the ICHD-2 based

Table 3 - Sensitivity, specificity, predictive values and likelihood ratios of individual questionnaire headache items vs. the interview diagnosis of migraine headache.

	Question									
	Yes		No							
Variable	M.	No M.	M.	No M.	Sens.	Spec.	PPV	NPV	LR+	LR-
Duration 4-72 hrs	721	19	249	49	0.74	0.72	0.97	0.16	2.64	0.36
Throbbing	670	232	40	96	0.94	0.29	0.74	0.71	1.32	0.21
Unilateral	863	57	46	72	0.95	0.56	0.94	0.61	2.16	0.89
Increase by activity	878	57	63	40	0.93	0.41	0.94	0.39	1.58	0.17
Severe	516	11	455	56	0.53	0.84	0.98	0.11	3.31	0.56
Nausea	867	63	36	72	0.96	0.53	0.93	0.67	2.04	0.08
Vomiting	627	87	64	260	0.91	0.75	0.88	0.80	3.64	0.12
Photophobia	859	91	25	63	0.97	0.41	0.90	0.72	1.64	0.07
Phonophobia	809	128	30	71	0.96	0.36	0.86	0.70	1.50	0.11

M = migraine; Sens. = sensitivity; Spec. = specificity; PPV = positive predictive value; NPV = negative predictive value; LR+ = positive likelihood ratio; LR- = negative likelihood ratio.

Table 4 - Sensitivity, specificity, predictive values and likelihood ratios of individual questionnaire aura items vs. the interview diagnosis of migraine aura.

	Question									
	Yes		No							
Variable	M.	No M.	M.	No M.	Sens.	Spec.	PPV	NPV	LR+	LR-
Visual aura symptoms										
Suffer from visual disturbances?	436	235	42	278	0.91	0.54	0.65	0.87	1.98	0.17
Shitters	335	117	143	396	0.70	0.77	0.74	0.74	3.04	0.39
Stars	201	71	277	442	0.42	0.86	0.74	0.62	3.00	0.67
Flashes	178	42	300	471	0.37	0.92	0.81	0.61	4.63	0.68
Scintillating lines	223	25	255	488	0.47	0.95	0.90	0.66	9.40	0.56
Figures	111	29	367	484	0.23	0.94	0.79	0.57	3.83	0.82
Coloured spots	153	70	325	443	0.32	0.86	0.69	0.58	2.29	0.79
Trembling air sensations	488	412	25	66	0.14	0.95	0.73	0.54	2.80	0.91
Wet window glass	118	71	360	442	0.25	0.86	0.62	0.55	1.79	0.87
Loss of vision	283	62	195	451	0.59	0.88	0.82	0.70	4.92	0.47
Diplopia	146	72	332	441	0.31	0.86	0.67	0.57	2.21	0.80
Other specific visual disturbances	87	67	391	446	0.18	0.87	0.57	0.53	1.38	0.94
Sensory aura symtpoms										
Sensory numbness/ tingling	114	268	13	623	0.90	0.70	0.30	0.98	3.00	0.14
Unilateral	111	236	16	655	0.87	0.73	0.32	0.98	3.22	0.18
5-60 min	49	50	78	841	0.39	0.94	0.50	0.92	6.50	0.65
Start before headache	94	154	33	737	0.74	0.83	0.38	0.96	4.35	0.31
Motor aura symptoms										
Muscle weakness	20	203	6	802	0.77	0.80	0.09	0.99	3.85	0.29
Unilaterality	14	59	12	946	0.54	0.94	0.19	0.99	9.00	0.49
Duration 5-60 minutes	6	47	20	958	0.23	0.95	0.11	0.98	4.60	0.81
Starts prior to headache	14	128	12	877	0.54	0.87	0.10	0.99	4.15	0.53
Pinching	13	117	13	888	0.50	0.88	0.10	0.99	4.17	0.57
Arm lifting problem	10	62	16	943	0.39	0.94	0.14	0.98	6.50	0.65
Crippled walking	9	51	17	954	0.35	0.95	0.15	0.98	7.00	0.68
Facial asymmetry	8	26	18	979	0.31	0.97	0.24	0.98	10.33	0.71
Speech disturbances										
Speech problems	132	366	8	489	0.94	0.57	0.27	0.98	2.19	0.11
Stiff mouth/ tongue	66	103	74	752	0.47	0.88	0.39	0.91	3.92	0.60
Wrong words	80	96	60	759	0.57	0.89	0.46	0.93	5.18	0.48
Expressive aphasia	119	311	21	544	0.85	0.64	0.28	0.96	2.36	0.23
Dysarthria	73	98	67	757	0.52	0.89	0.43	0.92	4.73	0.54
Prior to headache	102	154	38	701	0.73	0.82	0.40	0.95	4.06	0.33

Abbreviations as in Table 3.

Table 5 - Significantly correlated questions (n = 7) with regression coefficients, odds ratios and significance levels derived from the logistic regression model (training sample; n = 838).

	B	OR (95% CI)	p-value
Did you have visual disturbances before headache in the past 12 months?	0.729	2.07 (132-3.26)	0.002
Did the visual disturbances last 5-60 minutes?	1.658	5.25 (3.08-8.96)	<0.001
Have you had scintillating lines before or during your headache in the past 12 months?	1.210	3.35(2.06-5.45)	<0.001
Have you had loss of vision before or during your headache in the past 12 months?	0.913	2.49(1.63-3.80)	<0.001
Did you suffer from numbness or a tingling feeling in your face/ unilateral arm/ leg that started prior to headache in the past 12 months?	0.631	1.88(1.07-3.29)	0.027
Did you use nonsense words prior or during your headache in the past 12 months?	0.680	1.97(1.22-3.19)	0.005
Did you use a triptan in the past 12 months?	-0.561	0.57 (0.39-0.83)	0.003

B = regression coefficient; CI = confidence interval; OR = odds ratio..

algorithm diagnosis of migraine aura in the total sample (n=1,038). Similar values for this classification in the training sample (n=838) suggest this sample is a good representation of the whole group. In both the total group and the training sample, sensitivity for aura was approximately 0.45, specificity 0.95, positive predictive value (PPV) 0.88 and negative predictive value (NPV) 0.70 (Table 2). Additionally, we calculated characteristics of all individual questionnaire items that reflect migraine headache and migraine aura criteria and summarized those in Table 3 and 4. The results show individual sensitivity ranging up to 0.97 (photophobia; nausea) and PPV up to 0.98 (headache severity; headache duration).

Phase I: Derivation of predictive model

Using logistic regression, 7 questions (from the 33 included; none showed Spearman rank correlation >0.9) showed a significant impact on the likelihood of having a migraine aura in accordance to the gold standard derived from the telephone interview. These questions are summarized in Table 5, which also shows significance levels and regression coefficients derived from the logistic model. The questions show partial overlap with the questions used in the ICHD-2 based algorithm. This model explained between 35.4% (Cox and Snell R Square) and 47.3% (Nagelkerke adjusted R Squared) of variance, and correctly classified 651/838 (77.8%) of subjects.

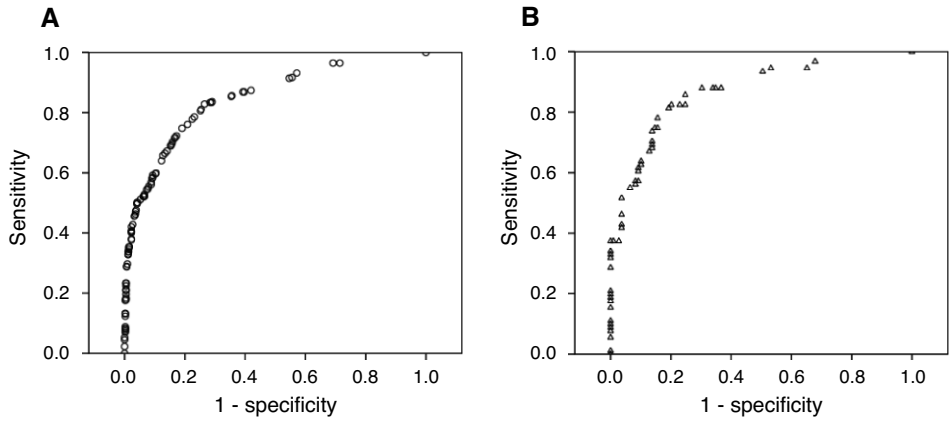

Figure 3 - Receiver operator characteristics (ROC) curves for (*A*) the derived prediction rule in the initial training sample (n=838) and (*B*) in the validation sample (n=200). The area under the ROC-curve (C-statistic) for the prediction rule was 0.85 (95% CI 0.83-0.88) in the training sample and 0.87 (95% CI 0.82-0.92) in the validation sample.

ROC curve

From the data in the predictive cohort, we generated an ROC curve by plotting the sensitivity of the questionnaire against one minus the specificity (Figure 3A). This analysis resulted in an optimal cut off point for the used logistic model at 0.35 with AUC of 0.85 (95% C.I. 0.83-0.88), yielding a 7 item questionnaire with a sensitivity of 0.83 and a specificity of 0.74. Compared to the ICHD-2 based algorithm outcome, this approach therefore resulted in a vast increment in sensitivity, with only small decrement of specificity (Table 2).

Phase II: Validation of derived prediction rule

Using the predictive model and cut-off point (0.35) derived from the training sample (n=838), we validated this model in a second, independent sample (n=200) of subjects who also fulfilled the set screening criteria. This analysis showed the model to have approximately similar sensitivity and specificity in this validation sample (Table 2). In the validation cohort, the ROC curve yielded an AUC of 0.87 (95% C.I. 0.82-0.92), which is comparable to the output from the training cohort (Figure 3B). When using this cut off from the training cohort, migraine aura diagnosis was predicted correctly in 160/200 (80.0%) of subjects.

Test-retest reliability

For a random selection of 44 patients who completed the extended questionnaire a second time, with a mean test-retest interval of 155 days (median 89 days, range 1-422 days), test-retest reliability was found to be good with a test-retest kappa for algorithm diagnostic group of 0.59 (95% CI 0.38-0.80). Test-retest interval did not influence agreement (linear regression, p=0.852).

Discussion

Our study has been the first one to validate a web-based questionnaire for purposes of diagnosing aura cases using a large sample of self-reported migraineurs. Few previous studies on migraine screeners and questionnaires have focussed on migraine aura, and the numbers of MA cases used to validate the questionnaire instruments in these studies were limited to n=8-186[17,19,22-24] respectively, in comparison to the large number of 488 aura cases in our study. Physicians frequently rely on aura as a cardinal symptom of migraine, as suggested by the 1.9 fold higher rate of medical diagnosis in interview settings when comparing MA cases to cases of MO[25]. Our study shows that, in self-reported migraineurs, a distinction between MA and MO can be made via a self-administered web-based questionnaire, with a focus on visual aura symptoms. The difficulty in diagnosing other aura types might be explained by the lack of perceptions and recognition of verbal and other non-visual auras by patients[26]. For diagnosing patients with these specific aura symptoms a clinical interview is needed. However, since the vast majority of the self-reported aura cases suffer from visual auras and only a small minority suffers from non-visual auras[27], we believe this number is neglectable when recruiting aura cases from a population of self-reported migraineurs. Perhaps the most helpful item identifying aura cases is the duration of the aura phenomena, since this question enables to distinguish visual aura symptoms from non-specific visual disturbances. Additionally, our data show aura patients are less likely to use triptans for rescue medication, which might be an indicator of lower headache severity.

We show that the question addressing the duration of the headache may hamper correct identification of migraine cases in a web-based questionnaire setting because some migraineurs overestimate the duration of an attack. Conversely, a question addressing headache severity should be included because this is helpful in distinguishing aura cases with migraineous headache from patients with non-specific headache.

The strength of our study includes the large samples of both the training (n=838) and validation sample (n=200), which are representative for the population studied. Both outclinic patients and other patients (most of whom are treated by their own GP or neurologist elsewhere) were included via the same web-based flow. We found no clinical

or demographic differences between these populations that could have affected the predictive model. Secondly, the use of a telephone interview as a gold standard by well-trained physicians with consultation of a headache specialist assured precise categorisation of migraineurs. Although we did not had a face-to-face interview as gold standard, we feel that our thorough semi-structured telephone interview safeguarded a very reliable migraine and aura diagnosis. Thirdly, the use of a validated screening instrument prior to our new questionnaire resulted in a group of self-reported migraineurs in which 95% could in fact be diagnosed with migraine. Fourth, we used a web-based questionnaire that was easy to fill out and send in for participants. With this approach, we successfully recruited large samples of migraineurs and contributed to the identification of the first genetic risk factor for the common forms of migraine[13]. We included a selected population of self-reported migraineurs, that had already been diagnosed with migraine by a physician, or otherwise thought they suffered from migraine, in which our questionnaire shows a high reliability in diagnosing aura. Our study did not aim to validate the questionnaire as a screening instrument for migraine in a naïve, general population.

The World Wide Web as a tool for recruiting patients and conducting research has several advantages. First, a large and diverse subject population can be reached at low cost[16]. Secondly, internet research imposes fewer burdens on participants, compared to non-internet research[15]. Thirdly, available software permits data entry and analysis in a secure Web database. Fourth, investigators may be able to increase patient awareness and participation on clinical research. However, there might be certain challenges too[28]. Internet users tend to be younger and better educated than the patient population as a whole; visually impaired and minority groups may be underrepresented; and the symptoms expressed by participants may be more severe then is typical. We feel, however, these potential biases haven't pivotally influenced our data. Additionally, the so-called 'virtual Munchhausen syndrome', i.e. individuals referring themselves for studies for which they are not truly eligible, may compromise the validity of results[29]. In our study, we have no evidence that data have been influenced by subjects masquerading electronically as patients. This is in accordance with previous migraine research[15]. Even with such biases, altogether, the internet represents an appropriate aid to conduct research aimed at collecting clinical headache data from large numbers of patients.

We conclude that our web-based recruitment system in combination with an automated study flow is a very successful instrument to truly distinguish MA and MO in self-reported migraine patients. We propose to use our identified seven questions that have a higher accuracy in identifying aura cases from a population of self-reported migraineurs than an ICHD-2 based algorithm.

Acknowledgements

Source of funding

This work was supported by grants of the Netherlands Organization for Scientific Research (NWO) (903-52-291, M.D.F.; Vici 918.56.602, M.D.F; 907-00-217 GMT; Vidi 917-11-319 GMT), and by a grant from the Centre for Medical Systems Biology (CMSB) established by the Netherlands Genomic Initiative/ Netherlands Organisation for Scientific Research (NGI/NWO).

Reference list

1. Stewart WF, Shechter A, Rasmussen BK. Migraine prevalence. A review of population-based studies. *Neurology*. 1994 Jun;44:S17-S23.
2. Scher AI, Stewart WF, Liberman J, Lipton RB. Prevalence of frequent headache in a population sample. *Headache*. 1998 Jul;38:497-506.
3. Launer LJ, Terwindt GM, Ferrari MD. The prevalence and characteristics of migraine in a population-based cohort - The GEM Study. *Neurology*. 1999 Aug 11;53:537-542.
4. Stovner LJ, Zwart JA, Hagen K, Terwindt GM, Pascual J. Epidemiology of headache in Europe. *Eur J Neurol*. 2006 Apr;13:333-345.
5. Headache Classification Subcommittee of the International Headache Society. The International Classification of Headache Disorders: 2nd edition. *Cephalalgia*. 2004;24 Suppl 1:9-160.
6. Russell MB, Rasmussen BK, Thorvaldsen P, Olesen J. Prevalence and Sex-Ratio of the Subtypes of Migraine. *International Journal of Epidemiology*. 1995 Jun;24:612-618.
7. Ulrich V, Gervil M, Kyvik KO, Olesen J, Russell MB. Evidence of a genetic factor in migraine with aura: a population-based Danish twin study. *Ann Neurol*. 1999 Feb;45:242-246.
8. Ulrich V, Gervil M, Kyvik KO, Olesen J, Russell MB. The inheritance of migraine with aura estimated by means of structural equation modelling. *J Med Genet*. 1999 Mar;36:225-227.
9. Gervil M, Ulrich V, Kyvik KO, Olesen J, Russell MB. Migraine without aura: a population-based twin study. *Ann Neurol*. 1999 Oct;46:606-611.
10. Ulrich V, Gervil M, Fenger K, Olesen J, Russell MB. The prevalence and characteristics of migraine in twins from the general population. *Headache*. 1999 Mar;39:173-180.
11. Gervil M, Ulrich V, Kaprio J, Olesen J, Russell MB. The relative role of genetic and environmental factors in migraine without aura. *Neurology*. 1999 Sep 22;53:995-999.
12. Stewart WF, Staffa J, Lipton RB, Ottman R. Familial risk of migraine: a population-based study. *Ann Neurol*. 1997 Feb;41:166-172.
13. Anttila V, Stefansson H, Kallela M, et al. Genome-wide association study of migraine implicates a common susceptibility variant on 8q22.1. *Nat Genet*. 2010 Aug 29.
14. de Groen PC, Barry JA, Schaller WJ. Applying World Wide Web technology to the study of patients with rare diseases. *Ann Intern Med*.1998 Jul 15;129:107-113.

15. Lenert LA, Looman T, Agoncillo T, Nguyen M, Sturley A, Jackson CM. Potential validity of conducting research on headache in internet populations. *Headache*. 2002 Mar;42:200-203.
16. Strom L, Pettersson R, Andersson G. A controlled trial of self-help treatment of recurrent headache conducted via the Internet. *J Consult Clin Psychol*. 2000 Aug;68:722-727.
17. Hagen K, Zwart JA, Vatten L, Stovner LJ, Bovim G. Head-HUNT: validity and reliability of a headache questionnaire in a large population-based study in Norway. *Cephalalgia*. 2000 May;20:244-251.
18. Cady RK, Borchert LD, Spalding W, Hart CC, Sheftell FD. Simple and efficient recognition of migraine with 3-question headache screen. Headache. 2004 Apr;44:323-327.
19. Kirchmann M, Seven E, Bjornsson A, et al. Validation of the deCODE Migraine Questionnaire (DMQ3) for use in genetic studies. *Eur J Neurol*. 2006 Nov;13:1239-1244.
20. Hagen K, Stovner LJ, Zwart JA. Potentials and pitfalls in analytical headache epidemiological studies--lessons to be learned from the Head-HUNT study. *Cephalalgia*. 2007 May;27:403-413.
21. Halpern EJ, Albert M, Krieger AM, Metz CE, Maidment AD. Comparison of receiver operating characteristic curves on the basis of optimal operating points. *Acad Radiol*. 1996 Mar;3:245-253.
22. Valentinis L, Valent F, Mucchiut M, Barbone F, Bergonzi P, Zanchin G. Migraine in adolescents: validation of a screening questionnaire. *Headache*. 2009 Feb;49:202-211.
23. Kallela M, Wessman M, Farkkila M. Validation of a migraine-specific questionnaire for use in family studies. *Eur J Neurol*. 2001 Jan;8:61-66.
24. Gervil M, Ulrich V, Olesen J, Russell MB. Screening for migraine in the general population: validation of a simple questionnaire. *Cephalalgia*. 1998 Jul;18:342-348.
25. Leone M, Filippini G, D'Amico D, Farinotti M, Bussone G. Assessment of International Headache Society diagnostic criteria: a reliability study. *Cephalalgia*. 1994 Aug;14:280-284.
26. Facheris MF, Vogl FD, Hollmann S, et al. Adapted Finnish Migraine-Specific Questionnaire for family studies (FMSQ(FS)): a validation study in two languages. *Eur J Neurol*. 2008 Oct;15:1071-1074.
27. Rasmussen BK, Olesen J. Migraine with Aura and Migraine Without Aura - An Epidemiologic-Study. *Cephalalgia*. 1992 Aug;12:221-228.
28. Rothman KJ, Cann CI, Walker AM. Epidemiology and the internet. *Epidemiology*. 1997 Mar;8:123-125.
29. Soetikno RM, Mrad R, Pao V, Lenert LA. Quality-of-life research on the Internet: feasibility and potential biases in patients with ulcerative colitis. *J Am Med Inform Assoc*. 1997 Nov;4:426-435.

5

Stepwise web-based questionnaires for diagnosing cluster headache: LUCA and QATCH

Leopoldine A. Wilbrink[1,2*] , Claudia M. Weller[3*]
Carlo Cheung[1], Theo Stijnen[4], Joost Haan[1,5]
Michel D. Ferrari[1], Gisela M. Terwindt[1]

Departments of [1]Neurology, [3]Human Genetics
[4]Medical statistics & BioInformatics
Leiden University Medical Center, Leiden
Department of [2]Neurosurgery, Maastricht University
Medical Centre, the Netherlands
Department of [5]Neurology, Rijnland Hospital,
Leiderdorp, the Netherlands
*** equally contributed**

Cephalalgia 2013;33(11):924–931

Abstract

Background – Cluster headache (CH) is a primary headache disorder that is diagnosed based on the patient's history. For large-scale epidemiologic and genetic studies, a web-based, preferably short, questionnaire can be a feasible alternative to replace time-consuming clinical interviews.
Methods – Self-reported CH patients were enrolled via our research website. Participants meeting screening criteria were directed to the Leiden University Cluster headache Analysis program (LUCA) questionnaire. Individual diagnoses were calculated using an algorithm based on International Headache Society criteria. Subsequently, semi-structured telephone interviews were carried out to validate the LUCA questionnaire. The shorter Quick Ascertainment of Cluster Headache (QATCH) questionnaire for diagnosing CH was constructed by using logistic regression to select the most predictive questions.
Results – Via our website 437 self-reported CH patients were recruited. Of these, 291 patients were included in this cross-sectional study. The LUCA questionnaire was valid and accurate. Using logistic regression, three questions (QATCH) provided similar sensitivity (53.8% vs. 57.2%), specificity (88.9% vs. 87.5%), positive predictive value (95.5% vs. 95.9%) and negative predictive value (30.8% vs. 28.8%) compared with the LUCA questionnaire.
Conclusion – The web-based LUCA questionnaire was accurate and reliable in diagnosing CH among self-reported patients. Males with headache attacks of short duration and long headache-free intervals (months to years) are very likely to have CH.

Key Words: cluster headache ■ questionnaires ■ genetics ■ validation ■ LUCA

Introduction

CLUSTER HEADACHE (CH) is one of the so-called 'trigeminal autonomic cephalgias' (TACs) that are characterized by severe, short-lasting headache attacks accompanied by ipsilateral facial autonomic symptoms[1]. CH consists of attacks of severe, strictly unilateral, orbital, supraorbital and/or temporal pain, lasting 15 to 180 minutes and occurring from once every other day to a maximum of eight times a day[2]. The attacks are associated with one or more of the following symptoms: restlessness or ipsilateral autonomic symptoms, i.e. conjunctival injection, lacrimation, nasal congestion, rhinorhea, forehead and facial sweating, miosis, ptosis, and eyelid edema. CH can be either episodic or chronic, with the vast majority of patients having episodic CH. Episodic CH is characterized by periods of weeks to months with frequent attacks that alternate with symptom-free periods of several months to years. About 10% of CH patients have chronic CH without attack-free periods or attack-free periods of less than one month. Recent epidemiologic studies have documented that the life-time prevalence of CH ranges from 0.05 to 0.4%[3]. CH is more prevalent in men (ratio male to female of 4.4:1) with a peak age of onset between the ages of 20 and 29 years[3,4].

CH is a complex genetic disorder; i.e. multiple genetic and environmental factors contribute to CH susceptibility[3]. Based on a prevalence of 0.2%, the relative risk for first-degree family members of CH patients varies between five and 18 and for second-degree relatives between one and three[3]. Thus far one genetic factor, i.e. a variant in the hypocretin type 2 receptor gene *HCRTR2*, was found to be associated with CH, albeit inconsistently[5].

More research is necessary to elucidate the genetics of CH. Genome-wide association studies (GWAS) aim to identify genetic factors by testing hundreds to thousands of single-nucleotide polymorphisms of affected individuals for associations with complex genetic diseases or particular traits[6]. Recent GWAS have been successful for migraine[7–9]. However, at least several hundred but preferably thousands of patients are needed for this type of study. Direct diagnostic interviews of such a large number of subjects are time consuming and expensive; therefore, a short reliable questionnaire would be preferable. An important pitfall of using such self-administered questionnaires is the inclusion of false-positive cases. This is a major concern for genetic research for which it is of the utmost importance to obtain reliable diagnoses.

Studies validating self-administered screening questionnaires for CH so far were small and included highly selected patients[10–13]. We aimed to develop web-based questionnaires for recruiting large numbers of patients with self-reported primary headache syndromes such as CH and migraine. Recently, we published the first results of our Leiden University Migraine Neuro Analysis (LUMINA) study in which we described a reliable web-

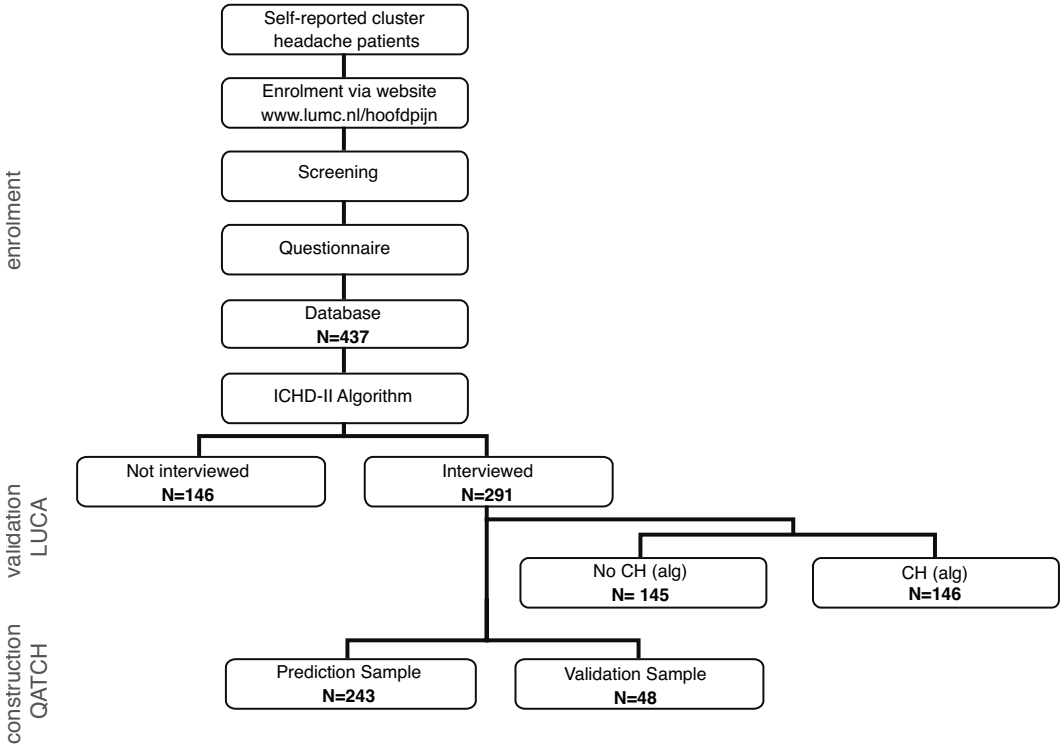

Figure 1 - Flowchart study flow. Screening: screening questionnaire; Questionnaire: extended questionnaire (LUCA); CH: cluster headache; alg.: International Classification of Headache Disorders: 2nd edition (ICHD-2)-based algorithm diagnosis; hoofdpijn: Dutch for headache.

based screening questionnaire for migraine[14] that has been used in our GWAS for migraine[7,9].

In the present cross-sectional study, we developed and validated our Dutch, web-based CH questionnaire 'Leiden University Cluster headache Analysis program' (LUCA questionnaire) to diagnose CH patients. In addition, we assessed which questions from the LUCA questionnaire contribute most in assessing CH diagnosis to develop an ultra-short 'Quick Ascertainment of Cluster Headache' (QATCH) questionnaire to select patients in a large cohort.

Materials and methods

Subjects

Male and female patients aged 18 years and older were recruited via our headache research website (www.lumc.nl/hoofdpijn), which was developed in 2008 for research of primary headache disorders in the Netherlands. We aimed to recruit self-reported CH patients as

our previous efforts to collect a population-based cohort in the Netherlands in the large population-based GEM-study[15] led to identification of only a few CH patients (personal communication, GMT and MDF, 1996). CH patients who participated in previous studies by our group, in which patients were recruited via general practitioners and tertiary headache centers throughout the Netherlands, were invited by letter to complete the web-based questionnaire[16]. Announcements were made in local newspapers and on national medical TV programs. Self-reported CH patients were able to participate on their own initiative. Approval for this study was obtained by the local medical ethical committee. All participants provided written informed consent.

Study flow

The study flow is depicted in the Figure 1. Our study consisted of a three-step procedure. Phase 1 consisted of the actual enrollment of participants with a screening questionnaire, the LUCA questionnaire and a calculated diagnosis by algorithm based on the International Classification of Headache Disorders: 2nd edition (ICHD-2) (the LUCA questionnaire is available by request at www.lumc.nl/clusterheadache). In phase 2 we validated the accuracy of the extended questionnaire (LUCA) by performing a direct interview. In the last phase of the study we aimed to develop a shorter questionnaire (QATCH) for diagnosing CH with at least the same accuracy as the LUCA questionnaire.

Phase I: Enrollment of patients

First the subjects were informed about the study and were asked to complete a short screening questionnaire (Supplementary Figure 1) on the headache website. The purpose of this screening questionnaire, which was minimally adapted from previous questionnaires in migraine (questions on restlessness, autonomic symptoms and attack duration were added and questions on possible aura symptoms were removed)[15], was to exclude participants who were very unlikely to suffer from CH. The screening questionnaire was validated previously in 31 CH patients, 29 migraine patients and four tension-type headache patients at our outpatient clinic and was found to have a sensitivity of 100%, a specificity of 58%, a positive predictive value (PPV) of 69% and a negative predictive value (NPV) of 100% (unpublished data). Because we have an open-access website, we decided that if the screening conditions were not met, the data of the subjects were not to be stored in our database. This prevents storage of junk information of subjects from whom we could not obtain informed consent, and who could not be used for validation of the questionnaire. If the screening conditions were met, patients were registered in our system and received an email in which they were asked to complete the LUCA questionnaire.

The LUCA questionnaire was based on the ICHD-2 criteria[2] and consisted of 142 items on CH and more specific and detailed questions concerning CH symptoms, demo-

graphical information, co-existing headaches and treatments of CH. The answers consisted of categorical alternatives.

Upon completion of the LUCA questionnaire, a second algorithm based on the ICHD-2 criteria was run automatically to determine the individual diagnoses (not shown). The following criteria, all to be fulfilled for receiving CH diagnoses, were used for the algorithm: severe pain, unilateral pain, temporal or orbital pain, presence of one or more autonomic symptoms or restlessness, occurrence of at least five CH attacks in the past, untreated attack duration between 15 minutes and three hours and a headache attack frequency of at least one on every other day to a maximum of eight times a day. The outcome categories of the algorithm were "cluster headache" (all items fulfilled) and "no cluster headache" (not all items fulfilled).

We also used a more lenient algorithm; criterion "untreated attack duration between 15 minutes and three hours" was changed to "treated or untreated attack duration between 15 minutes and three hours."

Phase II: Validation of the LUCA questionnaire

Semi-structured telephone interview. Within two months after completion of the LUCA questionnaire, enrolled participants were approached for a semi-structured telephone interview to diagnose CH according to the ICHD-2 criteria[2]. The interview diagnosis was used as the gold standard. Interviews were performed by a medical student (CC) trained in diagnosing CH under supervision of the study physicians (LAW and CMW). The interviewer and supervisors were blinded for the automatically calculated algorithm diagnosis. If participants were not reached after two attempts, they were excluded from the validation procedure. Final diagnoses were made directly after the interview by CC and LAW/CMW. In case of ambiguous symptoms or when the diagnoses determined by CC and one of the study physicians did not correspond, a headache specialist (JH), also blinded for the calculated diagnoses, was consulted, and a final diagnosis was made.

Statistical analysis of the LUCA questionnaire. All data analyses were performed using SPSS 17 (SPSS, IBM, US). Baseline characteristics of participants who were successfully interviewed and participants who were not reached were compared using two-sided χ^2 tests for categorical data and Student's t tests for continuous variables. Alpha was set to 0.05. Algorithm and interview diagnoses were compared to assess the sensitivity, specificity, the (prevalence dependent) PPV and NPV, respectively, and the positive and negative likelihood ratios for the entire LUCA questionnaire.

Phase III: Toward the short QATCH questionnaire

Development of a prediction rule. In this phase of the study, we aimed to identify a subset of questions from the extended LUCA questionnaire to construct a shorter questionnaire (named Quick Ascertainment of Cluster Headache, abbreviated as QATCH) that predicts the CH diagnoses in our participants equally well or even better. To select these questions, we assessed the contribution of the individual 142 LUCA items to the CH diagnoses by calculating sensitivity, specificity, PPV and NPV, and positive and negative likelihood ratios, and selected questions based on PPVs of more than 0.90 and/or a positive likelihood ratio of more than 1.5.

To develop a prediction rule, the successfully interviewed study population was randomly divided into a training set (80% of participants) and a validation set (20% of participants) to construct and validate the generated regression model. The selected LUCA items were incorporated in a forward logistic regression model on the 80% sample to assess their contribution to discriminating CH patients from non-CH patients.

Validation of the prediction rule. The questions that contributed significantly to the CH diagnoses in the forward regression model were incorporated in the prediction rule. The regression coefficients of these items were used to assign weights to the questions and to calculate the predicted probabilities for a CH diagnosis based on this model. Subsequently, a receiver operating characteristics (ROC) curve was computed for determining the optimal cut-off value for a CH diagnosis based on our prediction rule in the 80% group according to the method of Halpern et al[17].

The area under the curve (AUC) was assessed as a measure of correlation between the prediction of the short questionnaire and the gold standard (interview) diagnosis. Finally, interview diagnoses were compared with diagnoses obtained by our new model to detemine sensitivity and specificity of our newly generated short questionnaire. In addition, we assessed the performance of this test in our population by means of the PPV and NPV in the 20% validation sample.

Table 1 - Baseline characteristics of total study population and separate study samples.

Characteristic	Total	Telephone interview	Not reached
Number of participants	437	291	146
Age in years: mean (SD)	46.4 (11.9)	47.3 (11.7)[a]	44.5 (12.0)[a]
Gender (% male)	64.8%	65.9%	62.3%
Physician CH diagnosis	93.6%	93.8%	93.1%
Use of anti-CH drugs (prophylactic and/or acute)	89.9%	90.7%	88.3%
Algorithm diagnosis CH	49.2%	49.8%	47.9%
Duration of CH in years (SD)	17.1 (11.3)	18.0 (11.3)[b]	15.3 (11.2)[b]
Years of education (SD)			

CH: cluster headache; [a]$p = 0.018$ (t test); [b]$p = 0.046$ (t test); [c]$p = 0.0049$ (t test).

Table 2 - Sensitivity, specificity, positive and negative predictive values as well as the corresponding likelihood ratios for diagnosis of cluster headache based on: 1) the ICHD-2-based algorithm; 2) the ICHD-2-based lenient algorithm, including patients with headache duration of 15–180 minutes upon treatment.

Characteristic	ICHD-II based algorithm total sample (*n* = 291)	ICHD-II lenient algorithm total sample (*n* = 291)
Sensitivity (%)	57.2	70.4
Specificity	87.5	70.8
PPV	95.9	92.5
NPV	28.8	67.6
Positive likelihood ratio	4.58	2.42
Negative likelihood ratio	0.49	0.41

ICHD-2: International Classification of Headache Disorders: 2nd edition; PPV; positive predictive value; NPV: negative predictive value; Gold standard: direct interview.

Results

Phase I: Enrollment of patients

The recruitment of patients started in April 2010, and two months later a total of 437 participants met the screening criteria and completed the LUCA questionnaire (Figure 1). In this study, all participants were self-reported CH patients, of whom 94% also reported a physician diagnosis of CH (Table 1).

Phase II: Validation of LUCA questionnaire

Telephone interview. From these 437 participants, a total of 146 (33%) were excluded in the analysis because they could not be reached by telephone after at least two attempts. Thus 291 participants (67%) were interviewed. In total 83.5% of subjects (243/291) fulfilled ICHD-2 criteria in the telephone interview.

Baseline characteristics of 291 included patients were compared to 146 excluded patients. Interviewed subjects were significantly older (p = 0.018), had longer duration of CH (p = 0.046) and fewer years of education (p = 0.0049) but absolute differences were small. There were no significant differences with respect to gender, proportions of patients using anti-CH medication (prophylactic and acute), the proportion of patients with a physician diagnosis of CH, or algorithm diagnosis (Table 1).

Statistical analysis of the LUCA questionnaire. We interviewed 291 participants of the LUCA study and established a CH diagnosis in 243 (83%) of them. Of these 243 subjects, 139 were also diagnosed as having CH by our LUCA questionnaire. Using the interview as the gold standard, the algorithm of the LUCA questionnaire had a sensitivity of 57.2%

Table 3 - Short diagnostic cluster headache questionnaire: QATCH.

	Score
Untreated attack duration 15–180 minutes	2.5
Attack-free period (four months–three years)	1.5
Male gender	1
Female gender	0

QATCH: Quick Ascertainment of Cluster Headache; Cluster headache is diagnosed with a score ≥1.5 (after fulfilling the screening questionnaire). Scores are regression coefficients of the model. *P* values can be found in the text in the Results section.

and a specificity of 87.5%. The PPV was 95.9% and the NPV was 28.8% in this population (Table 2). A total of 152 subjects (52.2%) stated that they had an attack duration of 15 minutes to three hours, despite using symptomatic medication. A total of 252 subjects (86.6%) stated that their attacks lasted 15 minutes to three hours without or with attack medication (lenient algorithm) and 183 subjects (75.3%) stated that their attacks lasted 15 minutes to three hours without attack medication (strict algorithm).

With the use of the more lenient algorithm, which also includes patients with headache duration between 15 to 180 minutes upon treatment, the sensitivity increased to 70.4%, and the NPV increased to 67.6% with only a slightly decrease of the PPV to 92.5% (Table 2).

Phase III: Toward a short CH questionnaire

Development of a prediction rule. Nine variables from our LUCA questionnaire met our selection criteria for PPV of more than 0.90 and/or a positive likelihood ratio of more than 1.5. These nine variables were: untreated attack duration between 15 minutes to three hours, average attack-free period of four months to three years, pain on top of the eyeball, unilateral miosis, moderate to good response to oxygen, good response to sumatriptan, male sex, and smoking. Of the nine variables incorporated in our regression analysis, three contributed significantly to the model (QATCH questionnaire): i) untreated attack duration between 15 minutes and three hours ($p < 0.001$); ii) pain-free period between four months and three years ($p < 0.007$); and iii) male gender ($p = 0.053$). The regression coefficients were rounded off and used to assign points to the three items. Predicted probabilities for CH were calculated for the validation set using the regression coefficients of the model.

Validation of the prediction rule. From the data of the validation sample, we generated an ROC curve by plotting the sensitivity against one-specificity of the new three-item questionnaire. This analysis resulted in an optimal cut-off value of 1.5 (Table 3). The area under the curve (AUC) value was 0.817. Using this optimal cut-off value, all cases with

a score equal to or higher than 1.5 are classified as positive CH (Table 3). The three items were weighted according to the calculated regression coefficients of the model; having an untreated attack duration between 15 and 180 minutes (2.5 points), having an attack-free period between four months and three years (1.5 points) and male gender (1 point). In our 20% validation population (n = 48), 22 subjects were diagnosed as CH according to this three-item model. Our three-item questionnaire had a sensitivity of 53.8%, a specificity of 88.9%, a PPV of 95.5% and an NPV of 30.8% when compared to interview diagnoses (gold standard). This new three-item questionnaire was named the Quick AscerTainment of Cluster Headache (QATCH) questionnaire.

Discussion

The main objective of this study was to validate a web-based questionnaire to diagnose CH patients for future large-scale epidemiologic and genetic studies. The LUCA questionnaire proved to be a valid and reliable method for diagnosing CH in a population of self-reported CH patients. Our second objective was to construct a shorter questionnaire to diagnose CH in large samples. The QATCH questionnaire represents a practical alternative for diagnosing CH patients as the combination of the screening and QATCH questionnaire works as well as combining the screening questionnaire with the much longer LUCA questionnaire. The QATCH questionnaire indicates that males with untreated headache attacks lasting 15–180 minutes and attack-free periods of four months to three years are very likely to have CH under the condition that the including criteria are fulfilled. This does not imply that females or chronic CH patients are excluded by using this questionnaire, as the questions are weighted and not all three items are obligatory to receive the diagnosis of CH. Male gender alone is not enough to receive the diagnosis of CH. In fact, untreated attack duration of 15 minutes to three hours or attack-free periods of four months to three years are enough to receive the diagnosis of CH in a pre-screened population for research purposes.

Regression analysis indicated that these three questions about gender, untreated attack duration and duration of attack-free periods are good predictors of CH diagnosis in our training set (80% of study population) and that the resulting QATCH questionnaire performed equally well in the validation set (20% of study population). A similar approach was successfully applied to select items for migraine questionnaires by our group as well as by others[14,18]. However, it is important to keep in mind that the QATCH questionnaire was developed in a population of self-reported CH patients with enriched CH prevalence by application of a screening questionnaire, resulting in a study population of only 48 non-CH subjects. Therefore, the screening questionnaire should be included as a primary step, and the results cannot be generalized to application in the general

population. The screening questionnaire was based on the validated migraine screening questionnaire and adapted for CH[15]. Its sole purpose is exclusion of self-reported CH patients that were highly unlikely to have CH, and it was validated in our outpatient headache clinic. A drawback is that the QATCH questionnaire is not validated without the use of a screening questionnaire as a first step. It is uncertain if our ultra-short three-item QATCH questionnaire performs equally well without application of the screening questionnaire, which has 10 items and a more complicated algorithm. Another limitation could be the length of our LUCA questionnaire, which could have led to a low response. Despite the 20 minutes that it takes to fill in the questionnaire, we have a response rate of approximately 90%. In our opinion the length of our LUCA questionnaire would not be a major drawback.

The LUCA questionnaire was designed to diagnose CH fulfilling all ICHD-2 criteria[2]. As a consequence, the questionnaire had high specificity but low sensitivity. This is mostly explained by the observation that many patients use medication to treat their attacks, resulting in unknown untreated attack duration, which makes it hard to establish a diagnosis that is in full agreement with ICHD-2 criteria as these require knowledge of untreated attack duration. Fifteen percent of participants responded to the question about attack duration without medication "do not know," of whom 80% answered they did not know because they always use medication. This leads to an underestimation of the number of CH patients diagnosed by our LUCA questionnaire, resulting in a relatively low sensitivity. Because of this shortcoming we also calculated sensitivity, specificity, PPV and NPV for the questionnaire using a more lenient algorithm. When altering the necessary item "duration of attack without medication use between 15 minutes and three hours" to "duration of attack without or with medication use between 15 minutes and three hours," the sensitivity increased to ~70% with the PPV remaining as high as ~92%. For genetic studies in CH, both algorithms have advantages and disadvantages. The strict algorithm reduces the number of false-positives in this study, which is important for identifying genetic variants with a small effect on the disease. However, using the strict algorithm also means that many CH patients will be excluded because of the low sensitivity. The lenient algorithm could be an attractive alternative because it has a much higher sensitivity and for GWAS recruitment of sufficient numbers of patients is a concern in rare disorders such as CH.

The strength of our study consists of the large sample size in comparison with other questionnaire studies performed in CH that included only up to 30 CH patients and that did not validate their findings on a randomly selected validation sample as we did[10–12]. Secondly, the web-based step-wise procedure with a screening questionnaire as the first step prevents the collection of junk information and non-CH headache patients in a very easy and semi-automated way. Thirdly, the application of a semi-structured

telephone interview as a gold standard ensured precise categorization of CH patients. Our LUCA questionnaire focused on the detection of self-reported CH patients from the general adult population, but did not aim to diagnose CH in the naïve general population. Further validation studies are needed to assess the generalizability of this model (screening questionnaire plus the QATCH) or using the QATCH on its own in other study designs.

The LUCA questionnaire proved to be a valid and reliable method for diagnosing CH in a population of self-reported CH patients who filled out a screening questionnaire. Being a male and suffering from headache attacks of 15 minutes to three hours with pain-free periods (of four months to three years) are good predictors for a valid diagnosis of CH according to our QATCH questionnaire. Our web-based, step-wise procedure is an easy and semi-automated way to easily collect large numbers of CH patients for genetic-epidemiologic studies.

Acknowledgements

Source of funding

This work was supported by grants of the Netherlands Organization for Scientific Research (NWO) (903-52-291, M.D.F.; Vici 918.56.602, M.D.F; 907-00-217 GMT; Vidi 917-11-319 GMT), and by a grant from the Centre for Medical Systems Biology (CMSB) established by the Netherlands Genomic Initiative/ Netherlands Organisation for Scientific Research (NGI/NWO).

Acknowledgments

We thank Drs A.H. Stam and W.P.J. van Oosterhout for critically reviewing the manuscript.

Reference list

1. Halker R, Vargas B and Dodick DW. Cluster headache: Diagnosis and treatment. Semin Neurol 2010; 30: 175–185.
2. Headache Classification Committee of the International Headache Society. The International Classification of Headache Disorders: 2nd edition. Cephalalgia 2004; 24(Suppl 1): 9–160.
3. Russell MB. Epidemiology and genetics of cluster headache. Lancet Neurol 2004; 3: 279–283.
4. Ekbom K, Svensson DA, Traff H, et al. Age at onset and sex ratio in cluster headache: Observations over three decades. Cephalalgia 2002; 22: 94–100.
5. Rainero I, Rubino E, Valfre W, et al. Association between the G1246A polymorphism of the

hypocretin receptor 2 gene and cluster headache: A meta-analysis. J Headache Pain 2007; 8: 152–156.

6. Manolio TA. Genomewide association studies and assessment of the risk of disease. N Engl J Med 2010; 363: 166–176.
7. Freilinger T, Anttila V, De Vries B, et al. Genomewide association analysis identifies susceptibility loci for migraine without aura. Nat Genet 2012; 44: 777–782.
8. Chasman DI, Schurks M, Anttila V, et al. Genome-wide association study reveals three susceptibility loci for common migraine in the general population. Nat Genet 2011; 43: 695–698.
9. Anttila V, Stefansson H, Kallela M, et al. Genome-wide association study of migraine implicates a common susceptibility variant on 8q22.1. Nat Genet 2010; 42: 869–873.
10. Dousset V, Laporte A, Legoff M, et al. Validation of a brief self-administered questionnaire for cluster headache screening in a tertiary center. Headache 2009; 49: 64–70.
11. Fritsche G, Hueppe M, Kukava M, et al. Validation of a German language questionnaire for screening for migraine, tension-type headache, and trigeminal autonomic cephalgias. Headache 2007; 47: 546–551.
12. Torelli P, Beghi E and Manzoni GC. Validation of a questionnaire for the detection of cluster headache. Headache 2005; 45: 644–652.
13. Yoon MS, Obermann M, Fritsche G, et al. Populationbased validation of a German-language self-administered headache questionnaire. Cephalalgia 2008; 28: 605–608.
14. van Oosterhout WP, Weller CM, Stam AH, et al. Validation of the web-based LUMINA questionnaire for recruiting large cohorts of migraineurs. Cephalalgia 2011; 31: 1359–1367.
15. Launer LJ, Terwindt GM and Ferrari MD. The prevalence and characteristics of migraine in a population-based cohort: The GEM study. Neurology 1999; 53: 537–542.
16. van Vliet JA, Eekers PJ, Haan J, et al. Evaluating the IHS criteria for cluster headache—a comparison between patients meeting all criteria and patients failing one criterion. Cephalalgia 2006; 26: 241–245.
17. Halpern EJ, Albert M, Krieger AM, et al. Comparison of receiver operating characteristic curves on the basis of optimal operating points. Acad Radiol 1996; 3: 245–253.
18. Lainez MJ, Dominguez M, Rejas J, et al. Development and validation of the Migraine Screen Questionnaire (MS-Q). Headache 2005; 45: 1328–1338.

Cluster headache and the hypocretin receptor 2 reconsidered: a genetic association study and meta-analysis

6

Claudia M. Weller[1*], Leopoldine A. Wilbrink[2,3*]
Jeanine J. Houwing-Duistermaat[4]
Stephany C. Koelewijn[1], Lisanne S. Vijfhuizen[2]
Joost Haan[2,5], Michel D. Ferrari[2], Gisela M. Terwindt[2]
Arn M.J.M. van den Maagdenberg[1,2*]
Boukje de Vries[1*]

Departments of [1]Human Genetics, [2]Neurology, [4]Medical statistics, Leiden University Medical Center, Leiden
Department of [3]Neurosurgery, Maastricht University Medical Center, the Netherlands
Department of [5]Neurology, Rijnland Hospital, Leiderdorp, the Netherlands
*** equally contributed**

Cephalalgia: in press

Abstract

Background – Cluster headache is a severe neurological disorder with a complex genetic background. A missense single nucleotide polymorphism (rs2653349; p.Ile308Val) in the *HCRTR2* gene that encodes the hypocretin receptor 2 is the only genetic factor that is reported to be associated with cluster headache in different studies. However, as there are conflicting results between studies we re-evaluated its role in cluster headache.

Methods – We performed a genetic association analysis for rs2653349 in our large LUCA (Leiden University Cluster headache Analysis programme) study population. Systematic selection of the literature yielded three additional studies comprising five study populations, which were included in our meta-analysis. Data were extracted according to predefined criteria.

Results – A total of 575 cluster headache patients from our LUCA study and 874 controls were genotyped for *HCRTR2* SNP rs2653349 but no significant association with cluster headache was found (odds ratio 0.91 (95% confidence interval 0.75-1.10), p=0.319). In contrast, the meta-analysis that included in total 1,167 cluster headache cases and 1,618 controls from the six study populations, which were part of four different studies, showed association of the SNP with cluster headache (random effect odds ratio 0.69 (95% confidence interval 0.53-0.90), p=0.006). The association became weaker, as the odds ratio increased to 0.80, when the meta-analysis was repeated without the initial single South European study with the largest effect size.

Conclusions – Although we did not find evidence for association of rs2653349 in our LUCA study, which is the largest investigated study population thus far, our meta-analysis provides genetic evidence for a role of *HCRTR2* in cluster headache. Regardless, we feel that the association should be interpreted with caution as meta-analyses with individual populations that have limited power have diminished validity.

Key Words: Meta-analysis ■ Genetic association ■ *HCRTR2* gene ■ G1246A polymorphism ■ rs2653349

Introduction

CLUSTER HEADACHE is a primary headache disorder of largely unknown aetiology that is characterized by severe, short-lasting headache attacks accompanied by ipsilateral facial autonomic symptoms and/or restlessness occurring up to eight times a day.[1,2] A role for genetic factors in the aetiology of cluster headache was overlooked for a long time, but has been considered in several studies since the early 1990s.[3-8] Four family studies showed that relatives of cluster headache patients have a higher disease risk,[9-12] but estimates of the relative risk varied considerably between studies, ranging from 14 to 45.[9-11,13] As cluster headache is not as rare as previously thought, it is likely that the disease risk in these studies may have been overestimated.[11] In addition, one of the studies with the highest estimated relative risk[9] may have overestimated the occurance of cluster headache by partly using heteroanamnestic information instead of direct interview or questiannaire data from all affected relatives. Recalculation of the relative risk using a cluster headache prevalence of 0.2% showed a relative risk of 5-18 for first-degree relatives, and 1-3 for second-degree relatives,[13] suggesting a considerably smaller but still relevant contribution of genetic factors in cluster headache.[11] Case reports[3-7,14] on monozygotic twins, both affected by cluster headache, provide further support for a role of genetic factors in the disease. Complex segregation analysis suggested that low-penatrant autosomal dominant genetic risk factor may play a role in a small subset of patients.[34] Most likely, cluster headache is a complex disorder caused by both genetic and environmental factors.[13]

Several genetic studies aimed to identify genes involved in cluster headache but were unsuccessful.[15-18] The role of the *CACNA1A* gene was investigated both in a kindred with three affected family members[16] as well as in an association study,[15,16] but results were negative. Single nucleotide polymorphism (SNP) rs1801133 in *MTHFR* showed no association with cluster headache either.[17] A mitochondrial mutation reported to cause MELAS was identified in a single cluster headache patient without a family history of this disorder,[18] but later studies[19,20] failed to replicate the involvement of mitochondrial mutations in cluster headache. Thus far, genetic research in cluster headache has only led to the identification of one replicated possible genetic susceptibility factor: SNP rs2653349 (G1246A) in *HCRTR2* that encodes the hypocretin type 2 receptor[22-24]. This receptor is expressed in the posterior hypothalamus, which is thought to play an important role in cluster headache.[21] The role of the *HCRTR2* SNP was investigated in five small cohorts in three studies[22-24], comprising in total 593 cases and 599 healthy controls. The minor A allele of SNP rs2653349 was associated with a reduced risk for cluster headache (i.e. the A allele being more frequent in controls than in cases). Rainero *et al.* performed a meta-analysis of all five cohorts and reported association of the *HCRTR2* SNP with cluster

headache[25]. However, the accuracy of the estimated effect size differed largely between studies and the meta-analysis is, therefore, difficult to interpret because (i) there were differences in the statistical models used (i.e. other models than the additive model that is thought to underlie genetic susceptibility of most complex disorders[26]) and ii) there was considerable statistical heterogeneity between the studies.

Using our web-based Leiden University Cluster headache Analysis (LUCA) study population,[27] we re-evaluated the possible association of *HCRTR2* SNP rs2653349 with cluster headache in the largest single study thus far and we subsequently performed new meta-analyses combining our results with those of previous studies.

Materials and Methods

Patient recruitment for the LUCA study

For the LUCA study, self-reported cluster headache patients aged 18 years or older were recruited via our Dutch headache research website (www.lumc.nl/hoofdpijn), which was developed for genetic and epidemiological research on primary headache disorders. Individual diagnoses were established using an extended web-based questionnaire according to the International Classification of Headache Disorders, 2nd edition (ICHD-2)[1] and were validated in a subset of patients by a telephone interview.[27] The medical ethics committee of the Leiden University Medical Centre approved our study and all participants provided written informed consent.

Genetic association study in the LUCA population

All participants that met our algorithm criteria[27] for cluster headache and had provided a DNA sample were included. Our control population consisted of anonymous blood donors of whom no specific health information is available. Based on the low prevalence (0.2%) of cluster headache in the general population, we expected a negligible effect of possibly including one or two patients with cluster headache in our control group. Power calculations were performed using the Genetic Power Calculator (http://pngu.mgh.harvard.edu/~purcell/gpc/),[28] under the assumption of an additive model.

Genotyping of SNP rs2653349 (i.e. DNA variant G1246A) was performed using a TaqMan assay for which probes and primers were designed by Applied Biosystems. A standard PCR reaction was carried out using the TaqMan Universal PCR Master Mix reagent and the genotyping was performed on a Lightcycler LC-480 machine combined with LightCycler®480 1.5.0 software, version 1.5.0.39 (Roche Applied Science) to analyse the genotype clusters. Data analysis was performed using PLINK software version 1.07

(http://pngu.mgh.harvard.edu/~purcell/plink/) by calculating odds ratios (ORs) and 95% confidence intervals (95% C.I.) for the association between the *HCRTR2* SNP rs2653349 and cluster headache. We assumed an additive genetic model, and performed uncorrected and corrected analyses with age and sex as covariates. Association analysis was performed with the major allele (G) as reference. A significance level of 0.05 was used.

Meta-analysis: study selection

According to the guidelines for systematic reviews of genetic association studies[29] two researchers (C.M.W. and L.A.W.) individually searched the literature for genetic studies on the role of *HCRTR2* in cluster headache. We searched the MEDLINE, EMBASE, Pubmed, Web of science, CINAHL, PsychINFO, Academic search Premier, ScienceDirect, LWW, Pubmed Central, Goand Science Citation Index databases (to July 6, 2012) using the following search terms: ("Cluster Headache"[Mesh] OR cluster headache OR Cluster Headaches OR Ciliary Neuralgia OR Ciliary Neuralgias OR Neuralgic Migraine OR Neuralgic Migraines OR Histamine Cephalgia OR Histamine Cephalgias OR Horton Syndrome OR Horton's Syndrome OR Hortons Syndrome OR trigeminal autonomic cephalalgias OR trigeminal autonomic cephalalgia) AND (HCRTR2 OR hypocretin receptor-2 OR HCRTR-2 OR HCRTR 2 OR hypocretin receptor 2 OR HCRTR OR hypocretin receptor OR hypocretin receptors OR OX2R OR orexin OR orexin B OR hypocretin 2 OR rs2653349 OR 2653349 OR G1246A OR G 1246 A OR G1246 A OR G 1246A OR G922A OR G 922 A OR G922 A OR G 922A). We considered all articles published in English. References of all primary articles and reviews on cluster headache genetics were manually searched. Studies were eligible for inclusion if they met the following pre-specified criteria:

1. Study design: only cross-sectional, case–control or cohort studies are eligible.
2. Study must include cluster headache patients and healthy controls.
3. Genotype frequencies of the single nucleotide polymorphism (SNP) *HCRTR2* rs2653349 must be listed in the manuscript. Alternatively, sufficient data to calculate the frequencies must be available from the paper or from the authors upon request.
4. The largest study with extractable data will be selected if multiple studies use the same study population.

Meta-analysis: statistical analysis

Meta-analysis of all study populations was performed in R version 2.15 using the metagen function from the meta package (www.r-project.org). We assumed an additive genetic

model, and modelled the OR both as fixed effect, which assumes homogeneity across studies, and as random effect, which incorporates the between-study variability. The random effect model was defined as our primary model because of the high inter-study variability with respect to sample sizes and estimated effect sizes. The between-study heterogeneity was tested using the I^2 statistic, a Cochrane's Q statistic-based measure that describes the amount of variation due to heterogeneity rather than chance on a continuous scale from 0 to 1. I^2 values of approximately 25%, 50% and 75% are indicative for low, moderate and high between-study heterogeneity, respectively.[30] A funnel plot was generated using SPSS Statistics 20.0 (IBM, Armonk, NY, USA) to check for publication bias. All analyses were performed with the major allele (G) as reference, and a significance level of 0.05 was used.

Table 1 - Baseline characteristics of LUCA cluster headache patient population

	LUCA population N=575
Sex (male, %)	404 (70.3)
Age (year [SD])	49.6 (11.8)
Cluster headache type (episodic)	421 (73.2)
First-degree family member cluster headache (%)	133 (23.2)
Second-degree family member cluster headache (%)	27 (4.7)

Table 2 - Study populations included in the meta-analysis.

			Genotype frequencies			**Allele frequencies**		**Hardy-Weinberg equilibrium**	
Study	**Group**	**N**	**GG**	**GA**	**AA**	**G**	**A**	**χ^2-test**	**p-value**
This study - The Netherlands	Cases	575	351 (61.0%)	206 (35.9%)	18 (3.1%)	908 (79.0%)	242 (21.0%)	3.55	0.059
	Controls	874	522 (59.7%)	307 (35.1%)	45 (5.2%)	1351 (77.3%)	397 (22.7%)	$2.5 \cdot 10^{-4}$	0.987
Rainero et al. - Italy	Cases	109	103 (94.5%)	4 (3.7%)	2 (1.8%)	210 (96.3%)	8 (3.7%)	25.21	$1 \cdot 10^{-6}$
	Controls	211	163 (77.3%)	43 (20.4%)	5 (2.4%)	369 (87.4%)	53 (12.6%)	1.10	0.295
Schurks et al. - Germany	Cases	226	173 (76.5%)	46 (20.4%)	7 (3.1%)	392 (86.7%)	60 (13.3%)	3.04	0.081
	Controls	266	166 (62.4%)	93 (35.0%)	7 (2.6%)	425 (80.0%)	107 (20.0%)	2.06	0.151
Baumber et al. - UK	Cases	63	41 (65.1%)	20 (31.7%)	2 (3.2%)	102 (81.0%)	24 (19.0%)	0.05	0.815
	Controls	89	57 (64.0%)	27 (30.3%)	5 (5.6%)	141 (79.2%)	37 (20.8%)	0.55	0.457
Baumber et al. - Denmark	Cases	96	56 (58.3%)	38 (39.6%)	2 (2.1%)	150 (78.1%)	42 (21.9%)	2.40	0.121
	Controls	72	37 (51.4%)	31 (43.1%)	4 (5.6%)	105 (72.9%)	39 (27.1%)	0.58	0.444
Baumber et al. - Sweden	Cases	98	68 (69.4%)	26 (26.5%)	4 (4.1%)	162 (82.7%)	34 (17.3%)	0.55	0.459
	Controls	106	67 (63.2%)	32 (30.2%)	7 (6.6%)	166 (78.3%)	46 (21.7%)	1.32	0.251
Meta-analysis	Cases	1167	792 (67.9%)	340 (29.1%)	35 (3.0%)	1924 (82.4%)	410 (17.6%)	0.04	0.838
	Controls	1618	1012 (62.6%)	533 (32.9%)	73 (4.5%)	2557 (79.0%)	679 (21.0%)	0.07	0.791

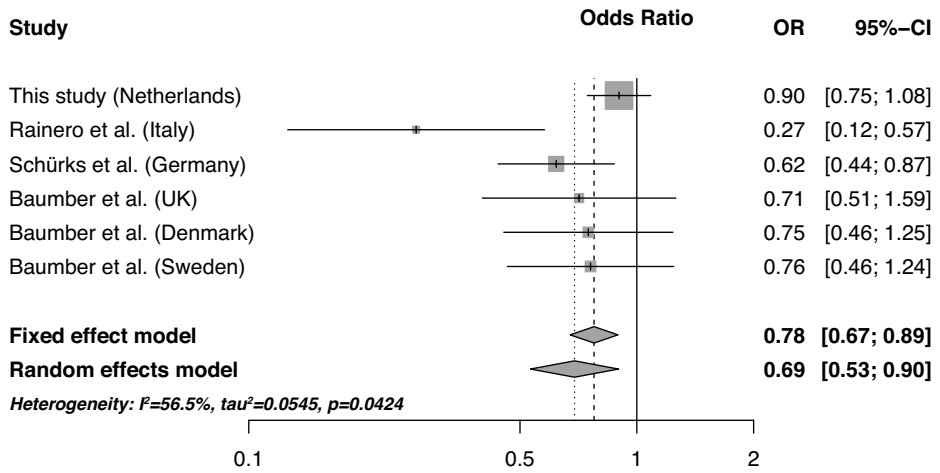

Figure 1 - Forest plot representing the results of the meta-analysis of the rs2653349 polymorphism of the *HCRTR2* gene assuming an additive model.

Results

A total of 587 participants from the LUCA study met our algorithm criteria[27] for cluster headache, provided blood for DNA isolation, and were included in the study. The majority of patients (73%) had the episodic form of the disorder and approximately 1 in 4 patients reported familial occurrence of cluster headache. Genotypes were obtained from 575 patients (clinical characteristics are summarized in Table 1) and 874 control subjects, and were in Hardy-Weinberg equilibrium (Table 2). No significant association between SNP rs2653349 and cluster headache was observed in our LUCA study (uncorrected OR: 0.90 (95% CI 0.75-1.08), p=0.265; corrected OR 0.91 (95% CI 0.75-1.10) p=0.319).

We identified 123 references meeting our aforementioned search criteria. Of these, a total of five studies reported on the association of SNP rs2653349 with cluster headache.[22-25,31] Three of these papers[22,25,31] described the same Italian study population and only the original research paper was included.[22] The Danish, Swedish and British populations from the study by Baumber et al.[23] were included as separate populations, leading to a total number of six study populations for our meta-analysis.

Subsequently, a meta-analysis was performed of three previously published studies (that included five study populations) and our own study.[22-24] These studies comprise in total 1,167 cluster headache patients and 1,618 control subjects. Genotype and allele frequencies of the various study populations are shown in Table 2. Genotype frequencies in the controls were in Hardy-Weinberg equilibrium in all studies. Our meta-analysis, assuming an additive model, replicated the previously reported association between the

Table 3 - Association with cluster headache in the study populations and the meta-analysis (additive model).

			Risk of cluster headache for carriers of A allele of rs2653349		Power for a given odds ratio				
Study	**Group**	**N**	**OR**	**p-value**	**OR 0.90**	**OR 0.80**	**OR 0.67**	**OR 0.50**	**OR 0.25**
This study - The Netherlands	Cases Controls	575 874	0.90 (0.75-1.08)	0.2646	0.16	0.43	0.97	1.00	1.00
Rainero et al. - Italy	Cases Controls	109 211	0.27 (0.12-0.57)	0.0003	0.07	0.15	0.37	0.77	1.00
Schurks et al. - Germany	Cases Controls	226 266	0.62 (0.44-0.87)	0.0055	0.06	0.18	0.53	0.98	1.00
Baumber et al. - UK	Cases Controls	63 89	0.71 (0.51-1.59)	0.7092	0.06	0.23	0.25	0.66	1.00
Baumber et al. - Denmark	Cases Controls	96 72	0.75 (0.46-1.25)	0.2694	0.06	0.12	0.30	0.73	1.00
Baumber et al. - Sweden	Cases Controls	98 106	0.76 (0.46-1.24)	0.2687	0.07	0.12	0.33	0.79	1.00
Meta-analysis	Cases Controls	1167 1618	0.69 (0.53-0.90)	0.0056	0.36	0.89	1.00	1.00	1.00

Power calculations were performed using a cluster headache prevalence of 0.02% with D' set to 1 (assuming perfect linkage disequilibrium of the marker allele and the disease allele) and equal prevalence of marker and high risk allele. Odds ratios are displayed below 1 because of the observed effect size of the A allele of rs2653349. The range of odds ratios used for the power calculations correspond to ORs of 1.11 – 1.25 – 2 – 4 for heterozygous carriers of the G allele.

A allele of the *HCRTR2* SNP and a decreased risk of cluster headache (random effect OR 0.69 (95% CI 0.53-0.90), p=0.006) (Figure 1)). The results of our meta-analysis indicated the presence of low to moderate between-study heterogeneity (I^2=0.57, p=0.042). Notably, the funnel plot graphically demonstrates asymmetry, indicating a publication bias favouring studies with an OR significantly deviating from 1, mainly caused by the initial study from Rainero *et al.*[22] (Figure 2). That study population is the only study from Southern Europe with allele frequencies that differ greatly from the other investigated populations that were all from Northern Europe. Remarkably, the absolute number of A alleles (8/218 alleles) among the cluster headache cases in the study population from Rainero *et al.* is very low compared to the other populations which makes it difficult to get reliable estimates of the allele frequency. We, therefore, performed an additional meta-analysis that contained only the populations from Northern Europe, so combining only data of Danish, Swedish, British, German and Dutch populations. The heterogeneity across these studies was negligible (I^2=0%, p=0.437), hence the random effect estimates correspond to the fixed effect estimates (OR 0.80 (95% CI 0.70-0.93), p=0.0031). Although the effect size decreased, the association remained.

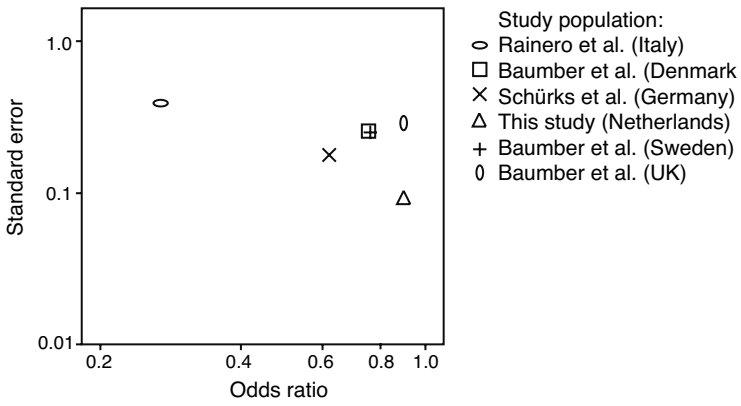

Figure 2 - Funnel plot of the six studies included in the meta-analysis of the rs2653349 (G1246A) polymorphism of the *HCRTR2* gene. The vertical axis shows the standard error of the log odds ratio for each study assuming an additive model, while the horizontal axis, the odds ratio, is again plotted in a log10 scale.

Discussion

Genetic factors have been implicated in cluster headache and several candidate gene association studies have been performed[16-21,22-24] aimed at identifying such factors but most studies did not produce convincing results, except for the genetic association with SNP rs2653349, which is located in the *HCRTR2* gene, that showed association for the (protective) A allele when using additive models. We decided to re-evaluate the association with this SNP by performing (i) a genetic association study in our LUCA population and (ii) meta-analyses of data from the LUCA study with data from earlier studies.

Our LUCA programme using validated questionnaire-based diagnoses[28] gave us the opportunity to include the largest number of cluster headache patients to date in a genetic study. Despite having adequate power (Table 3) to detect associations with similar effect sizes as reported by Rainero et al.[22] and Schürks et al. [24] no association (p=0.26) was found between the *HCRTR2* SNP rs2653349 and cluster headache. A subsequent meta-analysis of all selected previous studies together with the LUCA study revealed, however, a significant association of rs2653349 with cluster headache, but validity of the meta-analysis is questionable because all previously published studies had insufficient power due to small numbers of cases and controls (Table 3). The first study, by Rainero *et al.*[22], reported an association with a much larger effect than the other studies and seemed to drive the observed association effect. However, a second meta-analysis in which we removed that study from the analysis resulted in a large reduction of between-study heterogeneity with a slightly improved p-value for association (p=0.0031), albeit with a marked increase in

odds ratio (from 0.69 to 0.80). This indicates that the effect of the association is smaller when the first study is removed. Although our LUCA study *a priori* had by itself sufficient power to detect moderate effects on disease risk and has an acceptable power of 80% for detecting variants with an OR ≥ 1.31 (which is equivalent to OR ≤ 0.76 in case of a protective effect) (Table 3), it is underpowered to study genetic variants with small effect sizes that have been shown to underlie many genetic complex diseases.[26] Notably, a Cochrane review demonstrated that underpowered studies tend to have a large influence on the outcomes of meta-analyses if not at least two well-powered studies were included.[32] Moreover, a recent review, provocatively entitled "power failure: why small sample size undermines the reliability of neuroscience", addressed the important notion that low power not only decreases the chance to detect a true effect, but also decreases the chance that a significant finding from a small study reflects a true effect.[33] Such random fluctuations in allele frequencies may be driving the results of our meta-analyses. Thus, despite the apparent positive outcome of our meta-analyses, in our opinion, there is still no robust evidence for a true association between *HCRTR2* SNP rs2653349 and cluster headache.

In conclusion, our LUCA study and subsequent meta-analysis illustrate that even doubling of the sample size does not lead to a definite conclusion on the role of the *HCRTR2* gene in cluster headache. We demonstrated that such a role (if any) is likely smaller than previously reported. Future genetic studies in cluster headache patients need to include much larger numbers of patients and controls and may benefit also from focusing on hypothesis-free gene variant discovery, as is the case with GWAs.

Acknowledgements

This study is funded by grants of the Netherlands Organization for Scientific Research (NWO) (903-52-291, M.D.F.; Vici 918.56.602, M.D.F.; 907-00-217, G.M.T.; Vidi 917-11-319, G.M.T.), the European Community (EC) (EUROHEAD, LSHM-CT-2004-504837, M.D.F. and A.M.J.M.v.d.M; EUROHEADPAIN nr 602633, M.D.F. and A.M.J.M.v.d.M), and the Centre for Medical Systems Biology (CMSB) and Netherlands Consortium for Systems Biology (NCSB), both within the framework of the Netherlands Genomics Initiative (NGI) (A.M.J.M.v.d.M). The funding agencies had no role in the design or conduct of the study. We are grateful to all study participants and their relatives, general practitioners and neurologists for their contributions.

References

1. Headache classification subcommittee of the International Headace Society. The International Classification of Headache Disorders: 2nd edition. *Cephalalgia* 2004; 24 Suppl 1: 9-160.
2. Nesbitt AD and Goadsby PJ. Cluster headache. *BMJ* 2012; 344: e2407.
3. Sjaastad O and Salvesen R. Cluster headache: are we only seeing the tip of the iceberg? *Cephalalgia* 1986; 6: 127-129.
4. Schuh-Hofer S, Meisel A, Reuter U and Arnold G. Monozygotic twin sisters suffering from cluster headache and migraine without aura. *Neurology* 2003; 60: 1864-1865.
5. Couturier EG, Hering R and Steiner TJ. The first report of cluster headache in identical twins. *Neurology* 1991; 41: 761.
6. Roberge C, Bouchard JP, Simard D and Gagne R. Cluster headache in twins. *Neurology* 1992; 42: 1255-1256.
7. Sjaastad O, Shen JM, Stovner LJ and Elsas T. Cluster headache in identical twins. *Headache* 1993; 33: 214-217.
8. Svensson D, Ekbom K, Pedersen NL et al. A note on cluster headache in a population-based twin register. *Cephalalgia* 2003; 23: 376-380.
9. Kudrow L and Kudrow DB. Inheritance of cluster headache and its possible link to migraine. *Headache* 1994; 34: 400-407.
10. Leone M, Russell MB, Rigamonti A et al. Increased familial risk of cluster headache. *Neurology* 2001; 56: 1233-1236.
11. Russell MB, Andersson PG and Thomsen LL. Familial occurrence of cluster headache. *J Neurol Neurosurg Psychiatry* 1995; 58: 341-343.
12. Sjostrand C. Genetic aspects of cluster headache. *Expert Rev Neurother* 2009; 9: 359-368.
13. Russell MB. Epidemiology and genetics of cluster headache. *Lancet Neurol* 2004; 3: 279-283.
14. Eadie MJ and Sutherland JM. Migrainous neuralgia. *Med J Aust* 1966; 1: 1053-1057.
15. Haan J, van Vliet JA, Kors EE et al. No involvement of the calcium channel gene (CACNA1A) in a family with cluster headache. *Cephalalgia* 2001; 21: 959-962.
16. Sjostrand C, Giedratis V, Ekbom K et al. CACNA1A gene polymorphisms in cluster headache. *Cephalalgia* 2001; 21: 953-958.
17. Schurks M, Neumann FA, Kessler C et al. MTHFR 677C>T polymorphism and cluster headache. *Headache* 2011; 51: 201-207.
18. Shimomura T, Kitano A, Marukawa H et al. Point mutation in platelet mitochondrial tRNA(Leu(UUR)) in patient with cluster headache. *Lancet* 1994; 344: 625.
19. Cortelli P, Zacchini A, Barboni P et al. Lack of association between mitochondrial tRNA(Leu(UUR)) point mutation and cluster headache. *Lancet* 1995; 345: 1120-1121.
20. Seibel P, Grunewald T, Gundolla A et al. Investigation on the mitochondrial transfer RNA(Leu)(UUR) in blood cells from patients with cluster headache. *J Neurol* 1996; 243: 305-307.

21. May A, Bahra A, Buchel C et al. Hypothalamic activation in cluster headache attacks. *Lancet* 1998; 352: 275-278.
22. Rainero I, Gallone S, Valfre W et al. A polymorphism of the hypocretin receptor 2 gene is associated with cluster headache. *Neurology* 2004; 63: 1286-1288.
23. Baumber L, Sjostrand C, Leone M et al. A genome-wide scan and HCRTR2 candidate gene analysis in a European cluster headache cohort. *Neurology* 2006; 66: 1888-1893.
24. Schurks M, Kurth T, Geissler I et al. Cluster headache is associated with the G1246A polymorphism in the hypocretin receptor 2 gene. *Neurology* 2006; 66: 1917-1919.
25. Rainero I, Rubino E, Valfre W et al. Association between the G1246A polymorphism of the hypocretin receptor 2 gene and cluster headache: a meta-analysis. *J Headache Pain* 2007; 8: 152-156.
26. Manolio TA, Collins FS, Cox NJ et al. Finding the missing heritability of complex diseases. *Nature* 2009; 461: 747-753.
27. Wilbrink LA, Weller CM, Cheung C et al. Stepwise web-based questionnaires for diagnosing cluster headache: LUCA and QATCH. *Cephalalgia* 2013;
28. Purcell S, Cherny SS and Sham PC. Genetic Power Calculator: design of linkage and association genetic mapping studies of complex traits. *Bioinformatics* 2003; 19: 149-150.
29. Sagoo GS, Little J and Higgins JP. Systematic reviews of genetic association studies. Human Genome Epidemiology Network. *PLoS Med* 2009; 6: e28.
30. Higgins JP, Thompson SG, Deeks JJ and Altman DG. Measuring inconsistency in meta-analyses. *BMJ* 2003; 327: 557-560.
31. Rainero I, Gallone S, Rubino E et al. Haplotype analysis confirms the association between the HCRTR2 gene and cluster headache. *Headache* 2008; 48: 1108-1114.
32. Turner RM, Bird SM and Higgins JP. The impact of study size on meta-analyses: examination of underpowered studies in Cochrane reviews. *PLoS One* 2013; 8: e59202.
33. Button KS, Ioannidis JP, Mokrysz C et al. Power failure: why small sample size undermines the reliability of neuroscience. *Nat Rev Neurosci* 2013; 14: 365-376.
34. Russell MB, Andersson PG, Thomsen LL, et al. Cluster headache is an autosomal dominantly inherited disorder in some families: a complex segregation analysis. *J Med Genet* 1995; 32: 954-956

7

Migraine is not associated with enhanced atherosclerosis

Anine H. Stam[1*], Claudia M. Weller[2*]
A. Cecile J.W. Janssens[3], Yurii S. Aulchenko[4]
Ben A. Oostra[4], Rune R. Frants[2]
Arn M.J.M. van den Maagdenberg[1,2]
Michel D. Ferrari[1], Cornelia M. van Duijn[4]
Gisela M. Terwindt[1]

Departments of [1]Neurology, [2]Human Genetics
Leiden University Medical Center, Leiden
Departments of [3]Epidemiology, [4]Clinical Genetics
Erasmus University Medical Center, Rotterdam
*** equally contributed**

Cephalalgia 2013;33(4):228-235

Abstract

Aim – Migraine, in particular with aura, has been associated with an increased risk for ischemic stroke and coronary heart disease. The underlying mechanism is unknown. In a cross-sectional case control study we investigated whether an enhanced risk of atherosclerosis in migraineurs explains this increased cardiovascular risk.
Methods – Subjects were participants from the population-based Erasmus Rucphen Family study. Atherosclerosis was assessed in 360 migraineurs (209 without aura and 151 with aura) and 617 subjects without migraine or severe headache. Atherosclerosis was quantified by Intima Media Thickness, Pulse Wave Velocity and Ankle-Brachial Index.
Results – Migraineurs, especially with aura, were found more likely to smoke, have diabetes or a modestly decreased HDL-cholesterol. No differences were found for the atherosclerosis parameters.
Conclusions – In this large population-based study, migraineurs have no increased risk of atherosclerosis. Therefore, enhanced atherosclerosis is an unlikely explanation for the increased cardiovascular risk seen in migraineurs.

Key Words: all cerebrovascular disease ■ stroke ■ migraine

Introduction

MIGRAINE is a prevalent neurovascular brain disorder that is characterized by recurrent disabling attacks of headache associated with nausea, vomiting, and photoand phonophobia (migraine without aura) (MO); in one-third of migraineurs, attacks may be preceded by transient focal neurological aura symptoms (migraine with aura) (MA).[1] Both neuronal as well as vascular mechanisms have been implicated. There is observational, prospective and imaging evidence that migraine increases the risk of ischemic stroke almost two fold.[2-4] Patients may also be at increased risk for major cardiovascular disease, including coronary heart disease and peripheral artery disease.[2,5,6] This suggests that vascular dysfunction in migraineurs is not restricted to the cerebral vasculature, but is also systemically present.

The underlying mechanism for the association between migraine and ischemic cardiovascular disease is unknown. An adverse cardiovascular risk profile and associated atherosclerosis has been suggested. Some studies found an unfavorable cardiovascular risk profile, in particular for MA patients[5,7,8], while other studies did not find any[9] or only a modest association[10]. Framingham Risk Scores for coronary heart disease (FRS-CHD) were elevated in migraineurs in most[5,7,8,11], but not all studies[9,12]. None of the studies reported on the Framingham Risk Score for ischemic stroke (FRS-Stroke) in migraine patients, probably because the electrocardiogram (ECG) data that are necessary to calculate this risk score were not available.

The atherosclerotic process, one of the strongest risk factors for stroke and coronary heart disease, can be assessed by means of non-invasive preclinical functional and structural markers of changes in the vessel wall.[13] In the present study, we investigated atherosclerosis in a large group of migraine patients from the Erasmus Rucphen Family (ERF) study.

Methods

Study population

We performed a case-control study nested within the population-based ERF study. The ERF study is a family-based cross-sectional study in a genetically isolated community in the Southwest of the Netherlands.[14] The study population includes 3465 individuals who were ascertained for genetic-epidemiological studies based on their genealogical background (i.e. not selected on phenotypes of interest). All individuals 18 years or older were invited to participate. For this epidemiological study we did not use genetic or genealogical data, but the genetic and environmental homogeneity of the population can be

an advantage. The study was approved by the Medical Ethical Committee of the Erasmus Medical Centre Rotterdam and all participants gave written informed consent.

Migraine patients and controls

Migraineurs were identified using a three-stage screening procedure assessing lifetime occurrence of migraine, which was previously validated in a population-based study[15] and which was based on the second edition of the International Classification of Headache Disorders (ICHD-II).[1] The sensitivity of this casefinding procedure was 0.93 and the specificity was 0.36. The positive predictive value was 0.65 and the negative predictive value was 0.91. Thus the screening procedure picked up many false-positives and missed some true-positives.[15] Only patients with "definite" MA and MO are included. Patients with probable migraine are excluded from the study.

Details on the migraine case-finding procedure have been published before.[16] In brief, in the first stage, participants were asked to fill out five screening questions on headache and aura symptoms. Then, screenpositives received a detailed questionnaire on headache and aura symptoms. Finally, screen-positives were interviewed by telephone for further clarification of their answers. Subjects who were screen-positive but did not return the extensive headache questionnaire and subjects who could not be screened because they did not (completely) fill out the screening questions were directly contacted by telephone. Telephone interviews were performed by the principal study physician (A.H.S.), who is experienced in diagnosing migraine patients, and by well-trained medical students supervised by A.H.S. A final diagnosis was made only after the telephone interview and in consultation with a neurologist specializing in headache (G.M.T.).

The control group consisted of subjects who 1) did not report severe headache in their lifetime (only headache up to pain severity score ≤4 on a scale from 0 to 10); 2) did not report visual aura symptoms; 3) had never been diagnosed with migraine by a physician; and 4) never used specific antimigraine medication. Thus, subjects with mild headache in combination with visual aura are excluded from the control group. To exclude false-negative control cases, we used an even more conservative definition for non-migraineurs than in our previous population-based study, as from this study we know that about 10% of migraineurs are missed using this screening procedure.[15]

Assessment of cardiovascular parameters

Participants (n=3465) were asked to attend the research center located within the community. Extensive clinical examinations were performed, including the collection of fasting blood samples, anthropometric measurements, cardiovascular assessments, and personal interviews by physicians. Participants were also asked to fill out questionnaires assessing

various variables, including symptoms of headache and their level of education. The interview included questions on cigarette smoking status, alcohol consumption, and medical history. Alcohol consumption was measured in units per week and was categorized as <28 consumptions per week and ≥28 consumptions per week. Current smoking was categorized as yes or no. Participants took all prescription medication to the research center, and antihypertensive treatment and the use of oral contraceptives (OAC) was verified by a physician. Height and weight were measured with the participant in light underclothing and body mass index (kg/m2) was computed. An ECG was performed, and each was scored by an experienced cardiologist. Blood pressure was measured twice on the right arm in a sitting position after at least 5 minutes of rest using an automated device (OMRON 711, Automatic IS; Vernon Hills, IL, USA). The average of the two measures was used for the brachial blood pressure in the analyses. Plasma concentrations of triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and total cholesterol (TC) were determined according to standard procedures, as described previously.[14] Diabetes was defined as the use of blood glucose-lowering medication or sober glucose levels of ≥7mmol/l or both. Total plasma adiponectin was analyzed with the Human Adiponectin radioimmunoassay (RIA) kit (catalog number: HADP-61HK) of Linco Research (St. Charles, MO, USA). Total plasma C-reactive protein (CRP) was analyzed with the US C-reactive protein enzyme-linked immunosorbent assay (ELISA) (catalog number: DSL-10-42100) of Diagnostic Systems Laboratories Inc. (Webster, TX, USA). All measurements were performed according to the manufacturers' protocol. Plasma CRP levels showed a high level of kurtosis. Therefore, upper plasma CRP levels exceeding three times the standard deviation of the mean were removed from further analyses.

We used the FRS-CHD[17] and FRS-Stroke[18] to summarize the data. The FRS-CHD estimates the 10-year probability of CHD, defined as myocardial infarction or CHD death. The score assigns points for age, HDL-C, TC, smoking status, and systolic blood pressure stratified by sex and treatment for hypertension. The FRS-Stroke estimates the 10-year probability of stroke. The score assigns points for age, systolic blood pressure, treatment for hypertension, history of diabetes mellitus, smoking status, cardiovascular disease (i.e. history of myocardial infarction, angina pectoris, coronary insufficiency, intermittent claudication or congestive heart failure), and atrial fibrillation and left ventricular hypertrophy on ECG, all stratified by sex. The individual points for both the FRS-CHD and FRS-Stroke scores are summed and converted into 10-year risk percentages.

Functional and structural vascular assessment

The atherosclerotic process can be assessed by means of non-invasive preclinical functional and structural markers of changes in the vessel wall, including intima media thickness (IMT), pulse wave velocity (PWV, measure of arterial stiffness), and ankle-brachial index (ABI).[13] These measures correlate with central (IMT, PWV) or peripheral (ABI) atherosclerosis.[13] IMT was measured by ultrasonography with a 7.5 MHz linear array transducer (ATL Ultra-Mark IV; Advanced Technological Laboratories, Bothell, WA, USA) of the left and right common carotid artery. The maximum carotid IMT was determined as the mean of the maximum IMT of near and far wall of both common carotid arteries.[14] Carotid-femoral PWV was measured by means of an automatic Complior SP device with subjects in the supine position. The time delay between the rapid upstroke from the base point of simultaneously recorded pulse wave curves in the carotid and the femoral arteries was assessed, and the distance between the carotid and the femoral arteries was measured over the surface of the body with a tape measure. PWV was calculated as the ratio between the distance traveled by the pulse wave and the time delay and expressed in meters per second.[14] Ankle blood pressure was measured in both the posterior tibial arteries by using an 8-MHz continuous Doppler probe. Blood pressure was measured twice in the right arm in the sitting position. The ABI was calculated for each leg by dividing the ankle systolic pressure by the mean brachial systolic pressure. The lowest of the two ABI values was used in the analysis. Patients with ABI >1.4 were excluded from the analysis as this indicates non-compressible vessels.[19]

Statistical methods

Analyses were performed separately for all migraine, MO and MA. A two-sided p value of alpha <0.05 was considered significant. General characteristics were compared between groups using a Student's t test for age and a Chi-square test for sex and education. Means for continuous variables and proportions for dichotomous variables were calculated for cardiovascular risk parameters. Means of cardiovascular parameters were compared according to migraine status using analyses of covariance, adjusted for age, sex and education. Mean PWV, IMT and ABI were corrected for age, sex, education, HDL-C and smoking. Proportions were compared by binary logistic regression with the risk factor as the outcome (dependent) variable and migraine status as the independent variable, adjusted for age, sex and education. Odds ratios for having an FRS-CHD or FRS-Stroke >10% were calculated using logistic regression adjusted for age, sex and education. We had 80% power to detect a difference of 0.23 m/s for PWV, 0.03 mm for IMT and 0.016 for ABI. These power calculations are based on the number of participants with data available for that parameter.

Analyses were performed using SPSS version 16.0 (SPSS, Chicago, IL, USA). Significant results were corrected for inbreeding using the SOLAR 2.1.2 software package

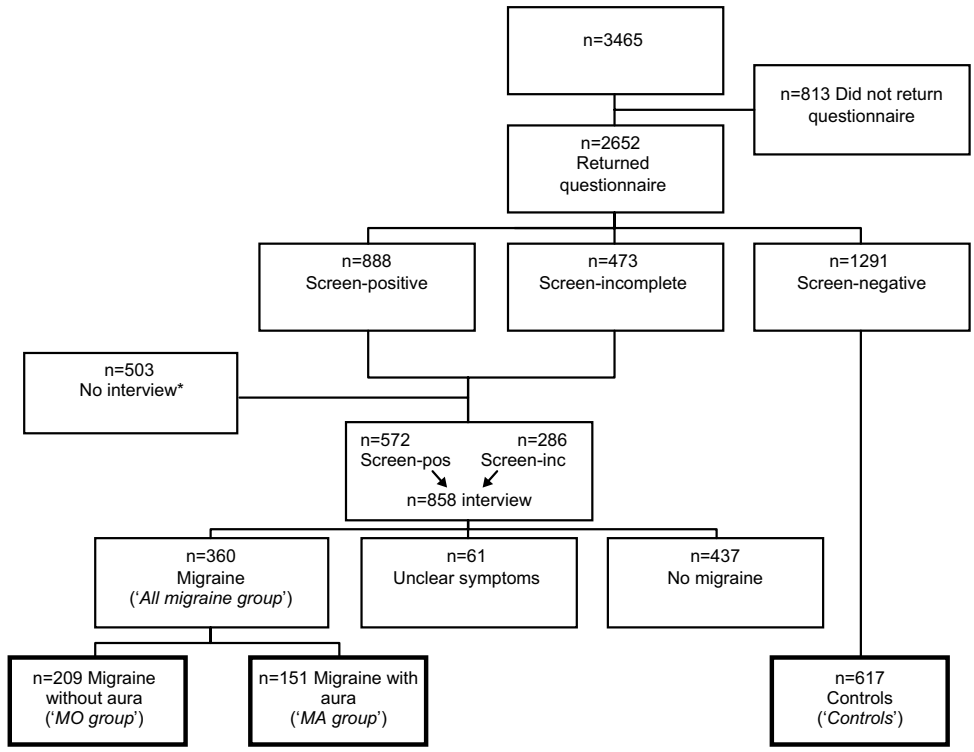

Figure 1 - Flowchart of ascertainment of migraine cases and controls in the Erasmus Rucphen Family study. *Subjects were deceased or declined participation (n=122), had an incorrect (n=140) or unavailable (n=10) telephone number, gave no permission for a telephone interview (n=130), were not answering the phone (at least 3 attempts were made) (n=72) or indicated on the phone that they did not want to cooperate with the interview (n=29).

(Southwest Foundation for Biomedical Research, San Antonio, TX, USA), which is necessary because subjects are part of a genetic isolate. The absolute numbers of cases and controls with diabetes was too small to correct for inbreeding. The coefficient of inbreeding per individual was calculated based on available genealogical information using PEDIG software. The inbreeding coefficient indicates, for each person in ERF, the probability that two alleles at a given locus in an individual are identical by descent and thus is a measure for relatedness. Subjects of the ERF population are the living descendants of 22 couples with at least six children baptized in the community church between 1850 and 1900. Thus, they are part of a large extended pedigree and therefore are more related than individuals from the general population. In isolated populations such as ERF, genetic drift may lead to an increased risk of allele frequencies, which may (falsely) increase associations between traits. Correction for relatedness prevents this and better reflects the association in the general population.

Table 1 - Baseline characteristics of the study population by migraine subtype

	All migraine		MO	MA		Controls
	n=360	p-value	n=209	n=151	p-value	n=617
Age , yr	46.2 (12.3)	0.149	47.0 (12.7)	45.1 (11.6)	0.105	47.8 (15.3)
% Female	75%	<0.001	77%	73%	MA: <0.001 MO: <0.001	47%
Education, % Higher	5	0.017	5	4	MA: 0.04 MO: 0.10	9
Medium	64		63	66		65
Lower	31		32	30		26
Body Mass Index, kg/m^2	27.1 (5.1)	0.10	27.2 (5.3)	26.9 (4.9)	0.24	26.8 (4.5)
SBP, mm Hg	135.3 (18.2)	0.22	135.4 (18.6)	135.2 (17.6)	0.42	139.4 (19.5)
DBP, mm Hg	79.2 (9.2)	0.13	79.3 (9.5)	79.0 (8.8)	0.33	79.7 (9.8)
LDL-C, mmol/L	3.75 (0.97)	0.10	3.73 (1.01)	3.78 (0.93)	0.19	3.66 (0.94)
HDL-C, mmol/L	1.30 (0.35)	0.02	1.35 (0.36)	1.23 (0.34)	MA: <0.001 MO: 0.53	1.28 (0.34)
Triglycerides, mmol/L	1.27 (0.67)	0.36	1.25 (0.66)	1.31 (0.69)	0.46	1.31 (0.77)
Total cholesterol, mmol/L	5.6 (1.1)	0.32	5.6 (1.1)	5.6 (1.0)	0.60	5.5 (1.0)
Diabetes, %	5.5	0.01	5.5	5.4	MA: 0.02 MO: 0.04	3.2
Current smoking, %	45	0.005	41	49	MA: <0.001 MO: 0.11	31
Alcohol consumption, u/w	5.4 (7.8)	0.06	5.0 (7.8)	5.8 (7.8)	0.15	8.2 (10.4)
CRP, mg/L	5.3 (4.3)	0.24	5.0 (4.1)	5.6 (4.4)	0.23	5.4 (4.5)
Adiponectin, mmol/L	11.1 (6.0)	0.58	11.3 (6.0)	10.8 (6.1)	0.69	10.2 (5.2)
FRS-Stroke, %	5.7 (0.3)	0.55	5.8 (0.4)	5.6 (0.3)	0.80	5.9 (0.2)
FRS-CHD, %	5.0 (0.2)	0.82	5.0 (0.4)	5.0 (0.3)	0.97	5.1 (0.2)
FRS-CHD > 10% (% of subjects)	12		12	11		22

Continuous values are mean (SD), categorical values are proportions. P-values are adjusted for age, sex and education. Significant p-values were adjusted for inbreeding as well, except for diabetes. SD is standard deviation, u/w = units (glasses) per week, MA is migraine with aura, MO is migraine without aura, SBP is systolic blood pressure, DBP is diastolic blood pressure, LDL-C is low density lipoprotein cholesterol, HDL-C is high density lipoprotein cholesterol, CRP is C-reactive protein. Education level was divided in lower (primary or elementary school or unfinished secondary school), medium (secondary school or vocational technical training) and higher (college or university). FRS-Stroke is Framingham risk score for stroke, FRS-CHD is Framingham Risk Score for coronary heart disease; percentages for these scores represent the 10 year risk of stroke or coronary heart disease.

Results

Recruitment of migraine cases and controls

Results on ascertainment of migraine cases and controls have been described elsewhere and are depicted in the flowchart (Figure 1).[18] In total, 3465 subjects participated in the study, from wtahom we recruited 360 migraine patients (151 MA, 209 MO) and 617 control subjects. Comparing subjects included (n=977) versus those not included (n=2488) in the present study showed that included subjects were significantly younger (p<0.001) and higher educated (p<0.03) compared with those not included, but there was no difference with regard to gender (p=0.20).

Cardiovascular parameters

Baseline characteristics are shown in Table 1. In line with the higher prevalence of migraine in women, more women were present in the migraine group. Migraineurs, in particular MA, had lower levels of education. Smoking was more prevalent in migraineurs, in particular in MA (49% versus 31% of controls, p<0.001). MA patients had a modestly decreased HDL-C (uncorrected difference 0.05 mmol/l, p<0.001). After correction for menopause status, alcohol consumption and smoking status, which are known to influence HDL-cholesterol levels, the difference between MA patients and controls remained significant. Migraine patients were more likely to have diabetes (5.4% MA, 5.5% MO versus 3.2% controls, p<0.05). The mean 10-year probability of CHD (FRS-CHD) or stroke (FRS-Stroke) was similar in migraine patients and controls (Table 1). None of the odds ratios for having a 10-year risk for FRS-Stroke or FRS-CHD larger than 10% reached statistical significance (data not shown).

Functional and structural measures for atherosclerosis

ABI data were available for 408 control subjects, 179 MO patients and 140 MA patients, IMT data for 470 control subjects, 167 MO patients and 123 MA patients, PWV data for 542 control subjects, 185 MO patients and 133 MA patients. Baseline characteristics including a predicted 10-year FRS-Stroke and FRS-CHD were not significantly different between the subjects with and without missing data. No difference was observed in mean IMT, carotid-femoral PWV, or ABI between migraineurs and controls (Table 2). All values observed for these three measurements were within the normal range.[13]

Discussion

The aim of this study was to investigate whether migraine patients are at increased risk for atherosclerosis. Our main finding is that atherosclerosis, assessed by three complementary noninvasive measures, is no more prevalent in migraineurs than in controls.

Several studies assessed atherosclerosis in migraine patients using comparable functional and structural markers, but with contradictory results. One clinic-based study found a small decrease in ABI in migraine patients compared to controls.[20] In line with our findings, another larger clinic-based study found no difference.[21] Three studies on PWV found increased values in migraine patients but were clinic based and excluded patients with known cardiovascular risk factors[21–23], which hampers comparison of results with our population-based study, where no selection for migraine or cardiovascular disease risk was made. Seven studies measured IMT in migraine patients with conflicting results.[9,23–27] Comparison between these studies is hampered by different case selection methods, such as clinic based[23,25–28] versus population based[9,24], and exclusion of participants with known cardiovascular risk factors[23,25–28] versus correction for these risk factors[9,24]. The two largest population-based studies[9,24], with more than 100 migraine patients (unselected with regard to cardiovascular risk factors), found slightly decreased values of IMT, which is in line with our findings.

Compared to previous studies, our study has important strengths. We assessed the largest group of migraine patients thus far, including a large group of MA patients (n=120). Three complementary measurements that quantified both central and peripheral atherosclerosis were used. Migraine diagnoses were made after a telephone interview in consultation with a neurologist and according to criteria of the International Headache Society. Our sample was unselected for a particular disease phenotype, and included a broad age range of adults (18–87 years), thus preventing ascertainment bias. Participants were from a population-based study with a relatively homogenous genetic and environmental background. Finally, data on a large number of cardiovascular risk factors were available and allowed us to calculate the FRS-CHD as well as the FRS-Stroke.

Our study has some limitations. First, we were unable to assess directly the mediating effect of atherosclerosis in migraineurs with a cardiovascular event because of the size of the study population (i.e. ≤10 migraineurs had a myocardial infarction or stroke). Second, data on functional and structural measures for atherosclerosis were missing for a substantial proportion of cases and controls. We do not think this affected our outcome since power calculations were based on the available number of participants and showed a reasonable power to detect differences in mean values. Moreover, baseline characteristics were not significantly different between subjects with and without missing data, making selection bias unlikely. Third, we were unable to assess the effect of migraine attack

frequency. This would have been interesting as the association of migraine with cardiovascular disease varies by attack frequency.[29] Fourth, IMT and ABI are indicative of clinical atherosclerotic disease and perhaps limit conclusions on variability in the normal range. However, we have also included a measure that measures subclinical arterial stiffness (PWV). Last, as our study population has a homogeneous genetic and environmental background, future studies are needed to assess to what extent our findings can be replicated in other populations of migraine patients. Although our study has a larger percentage of MA, we feel this is not a disadvantage of our study as the risk for cardiovascular events is highest among MA cases.

We assessed the FRS-Stroke in migraineurs and showed no increased odds for a higher score, which suggests that traditional cardiovascular stroke predictors are not relevant in the migraine-stroke association. This is in line with our data on atherosclerosis parameters. In addition, several previous observations support this conclusion. First, in most prospective cohort studies the increased risk of ischemic stroke appeared independent from traditional cardiovascular risk factors[30] with the exception of use of OAC and smoking in women. Second, population-based magnetic resonance imaging (MRI) studies showed no difference in cardiovascular risk factors between migraineurs with and without posterior circulation territory infarct-like lesions and white matter lesions.[31,32] Third, the Women's Health Study showed an increased risk for myocardial infarction in migraine patients with a high FRS-CHD, while increased stroke risk was observed among migraine patients with a low FRS-CHD.[11] This supports the idea that the mechanisms underlying the migraine-stroke association are different from atherosclerosis.

Based on these data, it seems unlikely that the higher risk of cerebroand cardiovascular disease in migraineurs is mediated by atherosclerosis, although it might be possible that the process of atherosclerosis plays a role on a subclinical level with endothelial dysfunction as a presumed early marker.[33] Larger, preferably prospective studies are necessary to further clarify the role of atherosclerosis in incident vascular events in migraineurs. Potential other explanations for the migraine-stroke relationship include the association of migraine with specific etiologies of stroke (i.e. micro emboli caused by patent foramen ovale), the association with a pro-inflammatory, pro-coagulatory state, the use of vasoconstrictive drugs in migraineurs or the (genetic) lowered threshold for spreading depression leading to either MA or ischemia.[34] The latter hypothesis is supported by recent data in migraine (familial hemiplegic migraine (FHM)1 CaV2.1) transgenic mice that, after transient ischemia, developed earlier onset of anoxic depolarization and more frequent peri-infarct depolarization resulting in larger infarcts and worse neurological outcomes compared to wild-type mice.[35] These data suggest that enhanced susceptibility to ischemic depolarization akin to spreading depression predisposes migraineurs to infarction during mild ischemic events, thereby increasing the stroke risk. In humans, MA is associated

Table 2 - Significantly correlated questions (*n*=7) with regression coefficients, odds ratios and significance levels derived from the logistic regression model (training sample; *n*=838).

	All migraine	MO	MA	Controls	p value
Common carotid IMT, mm	0.77 (0.16)	0.77 (0.17)	0.77 (0.14)	0.79 (0.20)	0.55
PWV, m/s	9.0 (1.5)	9.0 (1.5)	9.1 (1.5)	9.3 (1.9)	0.55
ABI	1.07 (0.11)	1.06 (0.11)	1.07 (0.10)	1.07 (0.12)	0.62

Values are mean (SE). Means and p values (all migraine versus controls) are corrected for age, sex, education, high-density lipoprotein cholesterol (HDL-C) and smoking. MO: migraine without aura; MA: migraine with aura. Ankle-brachial index (ABI) data were available for 408 control subjects, 179 MO patients and 140 MA patients, IMT (intima media thickness) data for 470 control subjects, 167 MO patients and 123 MA patients, pulse wave velocity (PWV) data for 542 control subjects, 185 MO patients and 133 MA patients. Baseline characteristics including predicted Framingham 10-year risk for stroke and coronary heart disease were not significantly different between the subjects with and without missing data. Power calculations were performed for each measure based on actual number of cases and controls with available data.

with strokes with good functional outcome[36], which seems contradictory to these mouse data. However, disability might be related to the size and type of the infarcts rather than of their specific underlying mechanism and thus does not rule out susceptibility to spreading depression as a causal factor in humans as well. More translational studies are needed to provide more insight in the migrainestroke relationship and to develop prophylactic treatment strategies to prevent cerebroand cardiovascular events in migraine patients.

Acknowledgements

Source of funding

This work was supported by grants from the Netherlands Organization for Scientific Research (NWO) (903-52-291, M.D.F. and R.R.F.; VICI 918.56.602, M.D.F.; 907-00-217, G.M.T.; 920-03-473, A.H.S.; VIDI 91711319, G.M.T.); the European Community (EC) (EUROHEAD, LSHM-CT-2004-504837, M.D.F.); and the Centre for Medical Systems Biology (CMSB) in the framework of the Netherlands Genomics Initiative (NGI) (M.D.F., R.R.F., and A.M.J.M.v.d.M). They had no role in the design or conduct of the study.

Reference list

1. Headache Classification subcommittee of the International Headache Society. The International Classification of Headache Disorders: 2nd edition. Cephalalgia 2004; 24(Suppl 1): 9–160.
2. Schurks M, Rist PM, Bigal ME, et al. Migraine and cardiovascular disease: Systematic review and meta-analysis. BMJ 2009; 339: b3914.

3. Spector JT, Kahn SR, Jones MR, et al. Migraine headache and ischemic stroke risk: An updated meta-analysis. Am J Med 2010; 123: 612–624.
4. Kruit MC, van Buchem MA, Hofman PA, et al. Migraine as a risk factor for subclinical brain lesions. JAMA 2004; 291: 427–434.
5. Bigal ME, Kurth T, Santanello N, et al. Migraine and cardiovascular disease: A population-based study. Neurology 2010; 74: 628–635.
6. Kurth T, Gaziano JM, Cook NR, et al. Migraine and risk of cardiovascular disease in women. JAMA 2006; 296: 283–291.
7. Scher AI, Terwindt GM, Picavet HS, et al. Cardiovascular risk factors and migraine: The GEM population-based study. Neurology 2005; 64: 614–620.
8. Winsvold BS, Hagen K, Aamodt AH, et al. Headache, migraine and cardiovascular risk factors: The HUNT study. Eur J Neurol 2011; 18: 504–511.
9. Schwaiger J, Kiechl S, Stockner H, et al. Burden of atherosclerosis and risk of venous thromboembolism in patients with migraine. Neurology 2008; 71: 937–943.
10. Kurth T, Ridker PM and Buring JE. Migraine and biomarkers of cardiovascular disease in women. Cephalalgia 2008; 28: 49–56.
11. Kurth T, Schurks M, Logroscino G, et al. Migraine, vascular risk, and cardiovascular events in women: Prospective cohort study. BMJ 2008; 337: a636.
12. Bensenor IM, Goulart AC, Lotufo PA, et al. Cardiovascular risk factors associated with migraine among the elderly with a low income: The Sao Paulo Ageing & Health Study (SPAH). Cephalalgia 2011; 31: 331–337.
13. Holewijn S, den Heijer M, Stalenhoef AF, et al. Non-invasive measurements of atherosclerosis (NIMA): Current evidence and future perspectives. Neth J Med 2010; 68: 388–399.
14. Sayed-Tabatabaei FA, van Rijn MJ, Schut AF, et al. Heritability of the function and structure of the arterial wall: Findings of the Erasmus Rucphen Family (ERF) study. Stroke 2005; 36: 2351–2356.
15. Launer LJ, Terwindt GM and Ferrari MD. The prevalence and characteristics of migraine in a population-based cohort: The GEM study. Neurology 1999; 53: 537–542.
16. Stam AH, de Vries B, Janssens AC, et al. Shared genetic factors in migraine and depression: Evidence from a genetic isolate. Neurology 2010; 74: 288–294.
17. Executive summary of the Third Report of The National Cholesterol Education Program (NCEP). Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). JAMA 2001; 285: 2486–2497.
18. D'Agostino RB, Wolf PA, Belanger AJ, et al. Stroke risk profile: Adjustment for antihypertensive medication. The Framingham Study. Stroke 1994; 25: 40–43.
19. Begelman SM and Jaff MR. Noninvasive diagnostic strategies for peripheral arterial disease. Cleve Clin J Med 2006; 73(Suppl 4): S22–S29.
20. Jurno ME, Chevtchouk L, Nunes AA, et al. Ankle-brachial index, a screening for peripheral

obstructive arterial disease, and migraine – a controlled study. Headache 2010; 50: 626–630.
21. Ikeda K, Hirayama T, Iwamoto K, et al. Pulse wave velocity study in middle-aged migraineurs at low cardiovascular disease risk. Headache 2011; 51: 1239–1244.
22. Schillaci G, Sarchielli P, Corbelli I, et al. Aortic stiffness and pulse wave reflection in young subjects with migraine: A case-control study. Neurology 2010; 75: 960–966.
23. Vanmolkot FH, Van Bortel LM and de Hoon JN. Altered arterial function in migraine of recent onset. Neurology 2007; 68: 1563–1570.
24. TzourioC,Gagnie`reB,ElAmraniM,etal.Relationship between migraine, blood pressure and carotid thickness. A population-based study in the elderly. Cephalalgia 2003; 23: 914–920.
25. Hamed SA, Hamed EA, Ezz Eldin AM, et al. Vascular risk factors, endothelial function, and carotid thickness in patients with migraine: Relationship to atherosclerosis. J Stroke Cerebrovasc Dis 2010; 19: 92–103.
26. Rossato A, Veronese F, Maggioni F, et al. Autonomic dysfunction and endothelial changes in migraine sufferers. Panminerva Med 2011; 53: 13–18.
27. Perko D, Pretnar-Oblak J, Sabovic M, et al. Endothelium-dependent vasodilatation in migraine patients. Cephalalgia 2011; 31: 654–660.
28. Liman TG, Neeb L, Rosinski J, et al. Peripheral endothelial function and arterial stiffness in women with migraine with aura: A case-control study. Cephalalgia 2012; 32: 459–466.
29. Kurth T, Schurks M, Logroscino G, et al. Migraine frequency and risk of cardiovascular disease in women. Neurology 2009; 73: 581–588.
30. Kurth T, Slomke MA, Kase CS, et al. Migraine, headache, and the risk of stroke in women: A prospective study. Neurology 2005; 64: 1020–1026.
31. Kruit MC, Launer LJ, Ferrari MD, et al. Infarcts in the posterior circulation territory in migraine. The population-based MRI CAMERA study. Brain 2005; 128: 2068–2077.
32. Scher AI, Gudmundsson LS, Sigurdsson S, et al. Migraine headache in middle age and late-life brain infarcts. JAMA 2009; 301: 2563–2570.
33. Tietjen GE. The role of the endothelium in migraine. Cephalalgia 2011; 31: 645–647.
34. Kurth T, Chabriat H and Bousser MG. Migraine and stroke: A complex association with clinical implications. Lancet Neurol 2012; 11: 92–100.
35. Eikermann-Haerter K, Lee JH, Yuzawa I, et al. Migraine mutations increase stroke vulnerability by facilitating ischemic depolarizations. Circulation 2012; 125: 335–345.
36. Rist PM, Buring JE, Kase CS, et al. Migraine and functional outcome from ischemic cerebral events in women. Circulation 2010; 122: 2551–2557.

^{1}H-NMR spectroscopy in serum suggests altered concentrations of several metabolites in active migraineurs

8

Claudia M. Weller[1*], Boukje de Vries[1*]
Aswin Verhoeven[2], Sibel Göraler[2]
Lisanne S. Vijfhuizen[1], Axel Meissner[2], Najaf Amin[3]
Lennart Karssen[3], Stefan Böhringer[4], Linda Broer[3]
Aaron Isaacs[3], André Deelder[2], Peter Henneman[1]
Ko Willems van Dijk[1,5], Ben A. Oostra[3]
Anine H. Stam[6], Cornelia M. van Duijn[3]
Michel D. Ferrari[6], Gisela M. Terwindt[6]
Arn M.J.M. van den Maagdenberg[1,6]

Departments of [1]Human Genetics, [2]Center for Proteomics and Metabolomics, [4]Medical Statistics, [5]Endocrinolgy and [6]Neurology, Leiden University Medical Center, Leiden
Departments of [3]Clinical Genetics Erasmus University Medical Center, Rotterdam
*equally contributed

Abstract

Objective – Migraine is a prevalent neurovascular disorder that is diagnosed based on consensus criteria due to the lack of reliable biomarkers. We here used a single-point measurement of serum samples to identify low-molecular-weight metabolites associated with migraine, which may help unraveling pathophysiological mechanisms involved in the disease.

Methods – From 289 migraine patients and 1,360 controls without severe headache from the Erasmus Rucphen Family (ERF) study fasting serum samples were available for proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy. A total of 100 signals representing at least 49 metabolites were detected. Using elastic net regression analysis we identified a subset of metabolites associated with lifetime or active migraine and cross-validated these analyses two-fold. Odds ratio, p-values and explained variance of the scores were calculated using logistic regression analysis.

Results – The subset of peaks that was identified for lifetime migraine status was not significantly associated with the outcome after age and sex correction. However, for active migraine we identified a set of 20 peaks, containing amino acids, lipids, pyruvate, dimethylglycine, 1,5-anhydrosorbitol and glucose, which significantly ($p = 6.2*10^{-3}$) associated with active migraine status after correction for age and sex.

Interpretation – Our results show that serum concentrations of several low-molecular weight metabolites measured by ^{1}H-NMR spectroscopy differ between migraine patients and controls, especially in active migraineurs. Future studies with similar NMR data containing all associated peaks could validate our findings. The associated metabolites provide important information that might help elucidate the pathophysiological processes underlying this disorder.

Key Words: ^{1}H-NMR spectroscopy ■ Migraine ■ Biomarker

Introduction

MIGRAINE IS A HIGHLY PREVALENT episodic neurovascular disorder that is characterized by recurrent throbbing, unilateral headache of moderate to severe intensity, which is aggravated by physical exercise and lasts 4-72 hours.[1] Attacks are accompanied by nausea, vomiting, photophobia, and/or phonophobia. Due to the lack of reliable biomarkers, migraine diagnoses is still based on history taking and interviews according to the consensus criteria of the International Classification of Headache Disorders (ICHD-2) from the International Headache Society.[2]

Various studies have attempted to find reliable clinical, genetic, radiological and CSF biomarkers to diagnose migraine patients.[3] Studies that focused on identifying biochemical biomarkers in blood until now focused on only a limited amount of molecules and these studies have been hypothesis-driven, that is they focused on e.g. neuroexcitatory amino acids,[4,5] inflammatory markers,[6-10] vasoactive neuropeptides,[11] and cardiovascular risk factors.[12,13]

In order to search for migraine biomarkers in serum in a hypothesis-free manner, we performed proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy, which is widely used to acquire metabolic profiles from large groups of patients because of the robustness and relatively low costs of this method.[14] With ^{1}H-NMR spectroscopy it is possible to quantify up to several hundreds of low-molecular weight metabolites in a single measurement in serum or plasma. We analyzed metabolite profiles in serum samples of migraine patients and controls from the Erasmus Rucphen Family population, a large Dutch population-based family study from the Southwest of the Netherlands in which we previously had identified migraine cases.[15] By comparing metabolic profiles of migraine patients and control individuals we set out to identify potential (sets of) biomarkers that may also provide valuable insights into pathophysiological mechanisms underlying migraine.

Material and Methods

Study population

The study flow is depicted in Figure 1. Subjects were participants of the Erasmus Rucphen Family (ERF) study. This is a population-based family study from a genetically isolated community in the Southwest of the Netherlands. The ERF study population includes 3,465 living descendants of 22 couples that had at least six children baptized in the community church between 1850 and 1900. Participants were not selected based on disease of interest. The extensive genealogy and pedigree of the population has been published previously.[16] The Medical Ethical Committee of the Erasmus Medical Center approved of the study and all participants provided written informed consent.

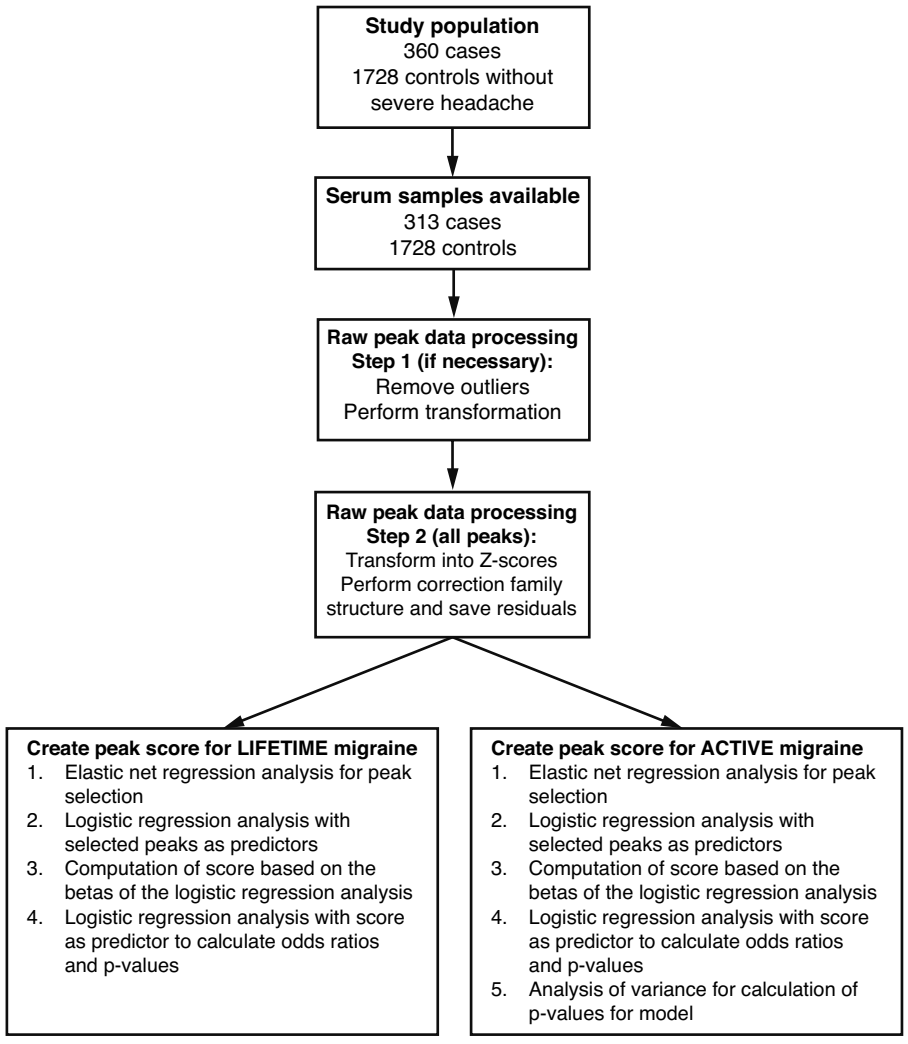

Figure 1 - Study flow.

Migraine diagnoses

Participants were selected between 2005 and 2007 using a validated three-stage screening procedure[17] assessing lifetime occurrence of migraine based on the second edition of the International Classification of Headache Disorders (ICHD-2).[2] Details on the migraine case-finding procedure have been published.[15] In short, stage one consisted of a five-item screening questionnaire on headache and aura symptoms. If the screening criteria were met, participants entered the second stage and completed a detailed questionnaire on headache and aura symptoms. The final stage consisted of telephone interviews with

participants meeting the screening criteria specified by step one. In addition, two groups of subjects were contacted by telephone directly: (i) screen-positives subjects who did not return the extended questionnaire and (ii) subjects providing no or incomplete data on the screening questionnaire. A study physician, who consulted a headache specialist in case of ambiguous symptoms, made final diagnoses. A total of 360 lifetime migraineurs and 1,728 controls were identified. Fasting serum samples were available from 313 of these migraine patients and 1,512 controls without severe headache, which were used for ^{1}H-NMR spectroscopy profiling.

^{1}H-NMR spectroscopy metabolite profiling: data processing and quality control

Details on the acquisition of NMR spectra can be found in the Supplementary Materials and Methods section. Good-quality NMR spectra were obtained from 289 migraine patients and 1,360 controls. Post-acquisition data processing and quality control (QC) on the set of ^{1}H-NMR spectra were carried out in Matlab® (R2009a, The Mathworks Inc., Natick, MA, USA). For QC, a set of spectroscopic parameters (i.e. shim values and intensity of the water signal) was examined and spectra were visually inspected. Spectra that failed the QC were not included for further analysis. Subsequently, the spectra that passed the QC were scaled with respect to the sensitivity of the experiment, which is inversely proportional to the pulse length. After subtracting a constant value as a simple baseline correction, the spectra were calibrated to the anomeric resonance of α-D-glucose (δ = 5.23 ppm).[18] Since there are small deviations of the peak position in the different ^{1}H-NMR spectra, alignment was performed using the correlation optimized warping algorithm by Tomasi et al.[19] This was performed actively for the Carr–Purcell–Meiboom–Gill (CPMG) spectra, after which the same warping was applied to the J-resolved spectra (JRES) projection. The peaks in the JRES projection were automatically deconvoluted by fitting the spectra with mixed Gauss-Lorentz lineshapes using the Simplex method. As the fitting algorithm incidentally converges to a local minimum, values further from the median than three times the interquartile range were discarded. Using partial least square regression, the remaining peak intensities were used to build a linear model that predicts the intensities directly from the unwarped spectrum, yielding also reasonable values for the cases where the deconvolution or warping algorithms failed.

Using the abovementioned procedure, 100 metabolite peaks were detected in the JRES projection and quantified in the ^{1}H-NMR spectra. In total, we had available spectra of 289 migraine patients and 1,360 controls for further analysis. For 82 peaks, metabolites could be assigned using information from the Human Metabolome Database (HMDB)[20] and the Pearson correlation coefficients between the peaks intensities. These 82 peaks represented 49 different metabolites (i.e. after subtracting the peaks representing EDTA,

Table 1 - Overview of peaks identified in the 2D J-resolved spectrum and performed transformations.

Peak number	Chemical shift (ppm)	Assignment (Jan 2012)	Trans-formation	Peak number	Chemical shift (ppm)	Assignment (Jan 2012)	Trans-formation
1	0,85113	unknown	none	51	2,93035	asparagine	none
2	0,87072	lipids CH3	LN	52	3,02340	lysine	none
3	0,89006	cholesterol	LN	53	3,03376	creatine/creatinine	none
4	0,92847	isoleucine	none	54	3,04941	ornithine	none
5	0,94597	leucine	none	55	3,14304	unknown	SD
6	0,95118	unknown	none	56	3,20231	choline	none
7	0,95702	leucine	none	57	3,21429	phosphorylcholine	none
8	0,97200	unknown	none	58	3,24004	glucose	none
9	0,98082	valine	none	59	3,26710	1,5-anhydrosorbitol	none
10	0,98612	unknown	none	60	3,33236	proline	LN
11	0,99919	isoleucine	none	61	3,34504	1,5-anhydrosorbitol	none
12	1,03145	valine	none	62	3,35396	unknown	none
13	1,06258	unknown	none	63	3,38052	unknown	none
14	1,11139	ketoisovalerate	none	64	3,40064	glucose	none
15	1,13461	unknown	SD	65	3,48648	glucose	none
16	1,16301	isopropyl alcohol	SD	66	3,53111	glucose	none
17	1,17437	ethanol	SD	67	3,55110	glycine	none
18	1,19057	3-hydroxybutyrate	SD + LN	68	3,55914	glycerol	none
19	1,21117	unknown	none	69	3,58832	1,5-anhydrosorbitol	none
20	1,26482	lipids CH2	LN	70	3,59782	valine	none
21	1,31981	lactate	none	71	3,62232	myoinositol	SD
22	1,38997	unknown	none	72	3,65021	ethanol	SD + LN
23	1,40660	unknown	none	73	3,65855	isoleucine	none
24	1,42426	unknown	none	74	3,71204	glucose	none
25	1,47045	alanine	none	75	3,72103	glucose	none
26	1,70571	unknown	none	76	3,74475	unknown	none
27	1,90859	acetate	SD	77	3,75932	glucose	none
28	1,99964	lipids	none	78	3,77643	alanine	none
29	2,03401	N-acetyl glycoproteins	none	79	3,80094	glucose	none
30	2,06653	O-acetyl glycoproteins	none	80	3,81746	unknown	none
31	2,10168	glutamine/glutamate	none	81	3,82382	glucose	SD
32	2,11814	glutamine/glutamate	none	82	3,83140	unknown	none
33	2,13291	acetylcarnitine	none	83	3,83856	glucose	SD
34	2,22215	lipids (CH2CO)	LN	84	3,87709	1,5-anhydrosorbitol	none
35	2,26037	valine	none	85	3,89397	glucose	none
36	2,27276	acetoacetate	SD	86	3,92001	creatine	none
37	2,30052	3-hydroxybutyrate	SD	87	3,93298	unknown	none
38	2,34857	glutamate	SD	88	3,95567	serine	none
39	2,36196	pyruvate	none	89	3,97538	phenylalanine/histidine	none
40	2,39235	3-hydroxybutyrate	SD	90	4,04386	creatinine	none
41	2,42815	glutamine	none	91	4,10334	lactate	LN
42	2,44561	glutamine	none	92	4,12106	proline	none
43	2,46282	glutamine	none	93	4,23715	threonine	none
44	2,52733	citrate	SD	94	4,50117	unknown	SD
45	2,59803	unknown	none	95	5,17855	mannose	none
46	2,63742	methionine	none	96	5,22921	glucose	SD
47	2,66908	citrate	SD	97	5,29802	lipids (CH=CH)	LN
48	2,70842	dimethylamine	none	98	6,89014	tyrosine	none
49	2,89562	unknown	none	99	7,18628	tyrosine	none
50	2,91618	dimethylglycine	SD	100	8,44976	formate	none

LN: log transformation performed; SD: outliers > 4 standard deviations from the mean removed.

see Table 1 for peak assignment). The other 18 peaks could not be annotated yet and were discarded from further analysis.

^{1}H-NMR metabolite profiling: acquisition of NMR spectra

Stored fasting serum EDTA samples were thawed at 4°C and were mixed by inverting the containers ten times. Samples (300 µL) were mixed with 300 µL 75 mM disodium phosphate buffer in H_2O/D_2O (80/20) with a pH of 7.4 containing 6.15 mM NaN_3 and 4.64 mM sodium 3-[trimethylsilyl] d4-propionate (TSP) using a Gilson 215 liquid handler in combination with a Bruker SampleTrack system (Bruker, Karlsruhe, Germany). Samples were transferred into 5mm SampleJet NMR tubes (Bruker, Karlsruhe, Germany) in 96 tube racks using a modified Gilson 215 tube filling station (Gilson, Middleton, WI, US) and kept at 6°C on a SampleJet sample changer (Bruker, Karlsruhe, Germany) while queued for acquisition.

All proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy experiments were acquired on a 600 MHz Bruker Avance II spectrometer (Bruker, Karlsruhe, Germany) equipped with a 5 mm TCI cryogenic probe head with Z-gradient system and automatic tuning and matching. All experiments were recorded at 310K. Temperature calibration was done prior to each batch of measurements using the method of Findeisen et al.[21] The duration of the $\pi/2$ pulses were automatically calibrated for each individual sample using a homonuclear-gated nutation experiment on the locked and shimmed samples after automatic tuning and matching of the probe head.[22]

For water suppression pre-saturation of the water resonance with an effective field of $\gamma B_1 =$ 25 Hz was applied during the relaxation delay.[18] J-resolved spectra (JRES)[23] were recorded with a relaxation delay of 2 s and a total of one scan for each increment in the indirect dimension. A data matrix of 40 x 12,288 data points was collected covering a sweep width of 78 x 10,000 Hz. A sine-shaped window function was applied and the data was zero-filled to 256 x 16,384 complex data points prior to Fourier transformation. The resulting data matrix was tilted along the rows by shifting each row (k) by 0.4992*(128-k) points and symmetrised about the central horizontal lines in order to compensate for the skewness of the multiplets in the F1 dimension. For T2-filtered ^{1}H-NMR spectra a standard 1D CPMG pulse sequence,[24,25] was used with a relaxation delay of 4 s. A pulse train of 130 refocusing pulses with individual spin echo delays of 0.6 ms were applied resulting in a total T2 filtering delay of 78 ms. 73,728 data points covering a spectral width of 12,019 Hz were collected using 16 scans. The FID was zero-filled to 131,072 complex data points and an exponential window function was applied with a line broadening factor of 1.0 Hz prior to Fourier transformation. The spectra were automatically phase and baseline corrected.

Quality control, scaling and calibration of the NMR spectra

Further data processing was performed in Matlab® (R2009a, The Mathworks Inc., Natick, MA, USA). The spectra and associated data were converted into Matlab files using in-house code These files can be quickly loaded into Matlab for further processing. First, the spectra were combined into one file while removing superfluous information. For CPMG this included dropping the imaginary part of the spectrum, while for the JRES spectra the sum projection along the indirect dimension was taken. Quality control (QC) on the set of ^{1}H-NMR spectra was carried out by examining a set of spectroscopic parameters such as shim values and intensity of the water signal, and subsequently visually inspecting the spectra. The spectra that failed the QC were not included for further analysis. The spectra were then scaled with respect to the sensitivity of the receiver coil. This sensitivity is inversely proportional to the pulse length, which is dependent on the tuning of the RF coil. After subtracting a constant value as a simple baseline correction, the spectra were calibrated with respect to the anomeric resonance of α-D-glucose (δ = 5.23 ppm).[20] Since there are small deviations of the peak position in the different ^{1}H-NMR spectra, alignment was performed using the correlation optimized warping algorithm by Tomasi et al.[19] This was performed actively for the CPMG spectra, after which the same warping was applied to the JRES projection. The peaks in the JRES projection were automatically deconvoluted by fitting the spectra with mixed Gauss-Lorentz lineshapes using the Simplex method. As the fitting algorithm incidentally gets stuck in local minima, values further from the median than 3 times the interquartile range are discarded. Using PLS regression, the remaining peak intensities were used to build a linear model that predicts the intensities directly from the unwarped spectrum, yielding also values for the cases where the deconvolution values were discarded and eliminating the problem of faulty warping.

The peaks were assigned using information from the Human Metabolome Database[26] and the Pearson correlation coefficients between the peaks intensities.

^{1}H-NMR spectroscopy metabolite profiling: statistical analyses

Demographic characteristics of cases and controls were compared using a Student t test for continuous variables and χ^2 statistics for dichotomous variables. Raw ^{1}H-NMR peak data were processed in four steps. First, we removed outliers from the data by filtering out all data points more than four standard deviations (SD) away from the mean. This was necessary for 19 of the 100 peaks. Second, the distribution was checked for normality, and data were transformed if necessary. We performed log-transformations on nine peaks (of which two were also processed in the first step). For the remaining 74 peaks additional processing was not necessary. Analyses were performed using SPSS software version 20.0 (SPSS-IBM, NY). Third, we adjusted the peak data for family structure by using the

Table 2 - Demographic characteristics of the study population.

Variable	Cases (N=289)	Controls (N=1,360)	p-value
Age (years)	46.5 ±12.1	48.7 ±14.5	0.013*
Female sex (%)	76.1	49.5	<0.000*
Education (%)			0.185
high	3.8	6.6	
medium	63.1	62.8	
low	33.1	30.6	

*Difference significant between migraine cases and controls ($p < 0.05$).

information from the kinship matrix in a linear regression analysis in GenABEL.[27] Fourth, the residuals from this linear regression model were transformed into Z-scores and used for further analyses.

After processing of the raw data we aimed to identify subsets of predictive peaks for (1) life-time diagnosis and (2) a diagnosis of active migraine (defined as having migraines in the last 12 months). We used a special form of regression analysis, called elastic net regression analysis, to overcome the problems encountered in conventional logistic regression analyses in studies like ours where the number of predictors (i.e. the ^{1}H-NMR peak data, age and sex) is much larger than the number of observations on the outcome measure (i.e. migraine status). We used the glmnet package for elastic net regression analysis[28] with alpha set to 0.5 and 50-fold cross-validation using R software version 2.14.2 (www.r-project.org). In this first cross-validation step we validated the selection of the peaks by performing our regression analysis on 50 randomly chosen samples of our study population. We performed an additional cross-validation step to assess the fit of the selected model on different subpopulations of our sample.

After identification of the set of associated peaks we entered these variables in a logistic regression model to determine the weights for each peak for this population. Subsequently, we used the betas from this regression analysis to transform the set of associated peaks into a weighted score per individual participant. This score was used in a second logistic regression analysis to calculate odds ratios, p-values and the proportion of explained variance. To correct for confounding by differences in age and gender distribution we also calculated adjusted odds ratios and p-values by performing a logistic regression analysis with these confounders as covariates.

To validate the findings from the previous analysis we performed analysis of variance (ANOVA) in which we compared the performance of the identified scores for migraine with the performance of a model containing only information on age and sex. The resulting p-value can be interpreted as the likelihood that all peaks identified by the elastic net analysis are only associated with migraine status by chance.

Table 3 - ^{1}H-NMR peaks associated with lifetime prevalence of migraine

Chemical shift (ppm)	Metabolite	Beta
0,928474601	Isoleucine	-,045
0,999191706	Isoleucine	-,206
1,406597219	Unknown	,177
2,637416682	Methionine	-,100
3,588320905	1,5-anhydrosorbitol	-,163

Table 4 - ^{1}H-NMR peaks associated with active migraine

Chemical shift (ppm)	Metabolite	Beta
0,890058516	Cholesterol	-,0101
0,928474601	Isoleucine	-,084
0,957021758	Leucine	-,0036
0,999191706	Isoleucine	,001
1,264820045	Lipds CH2	-,029
1,406597219	Unknown	,161
2,222145112	Lipids (CH*2CH=CH)	-,018
2,361963346	Pyruvate	,224
2,637416682	Methionine	-,084
2,916183974	Dimethylglycine	-,196
3,353959417	Unknown	-,159
3,588320905	1,5-anhydrosorbitol	-,114
3,59781855	Valine	-.023
3,721027815	Glucose	-,0318
3,95566792	Serine	,152
4,043855495	Creatinine	-,0098
4,121059468	Proline	-,122
4,50117004	Unknown	-,111
5,229206003	Glucose	-,239

Results

Demographic characteristics

We compared several demographic characteristics in our migraine patients compared to the control group (see Table 2). Migraine patients tended to be younger and, as can be expected, they were more often female than the controls. There was no significant difference in educational level between cases and controls. Of the 289 migraineurs in our study, 150 patients (58%) reported at least one migraine attack in the 12 months preceding the interview and were assigned to the group of "active migraineurs".

^{1}H-NMR score for life-time diagnosis of migraine

Using the complete group of 289 migraineurs we identified a subset of five peaks that were associated with migraine status using the elastic net regression analysis (see Table 3). One of these peaks could not be annotated yet, so it is unclear which metabolite it represents. The other four peaks represent isoleucine (2 peaks), methionine and 1,5-anhydrosorbitol. This score containing these five metabolites explained 3.6% of the variance in migraine status and was significantly associated with migraine status in the uncorrected analysis (OR = 2.72 (1.94 – 3.81), $p = 6.8*10^{-9}$). As it is known that NMR spectra are highly dependent on age and sex we corrected the score for these confounders. Unfortunately, after correction the association with migraine was no longer significant (OR = 1.40 (0.95 – 2.06), p = 0.088).

^{1}H-NMR score for diagnosis of active migraine

When including only the 150 active migraineurs, we identified a subset of 20 associated peaks using elastic net regression analysis (see Table 4). Notably, all five peaks that were part of the subset associated with lifetime migraine were also part of the 20 peaks that associated with active migraine status. Three of these 20 peaks have not been annotated yet. The remaining 17 peaks represent 14 metabolites: leucine, isoleucine (2 peaks), methionine, proline, serine, valine, dimethylglycine, glucose (two peaks), 1,5-anhydrosorbitol, cholesterol, lipids CH2, lipids CH2CO, creatinine and pyruvate. This score explained 8.7% of the variance and was significantly associated with active migraine status in the uncorrected analysis (OR = 2.72 (2.09 – 3.53), $p = 9.5*10^{-14}$). After correcting the score for age and sex it remained strongly associated (OR = 1.74 (1.27 – 2.40), $p = 6.2*10^{-3}$). The outcome of our ANOVA analysis was also significant ($p = 1.5*10^{-12}$), adding to the evidence for involvement of these metabolites in active migraine.

Table 5 - Associated serum metabolites previously studied in migraine patients and controls.

Metabolite	Concentration compared to controls	Number of patients	Number of controls	Reference
Creatinine	No difference	5,087	22,539	Kurth, Ridker & Buring 2008
Glucose (fasting)	No difference	111	463	Schwaiger et al. 2008
HDL-cholesterol	No difference	620	5135	Scher et al. 2005
	No difference	111	463	Schwaiger et al. 2008
	No difference	165	1285	Benseñor et al. 2011
	Decreased	5,087	22,539	Kurth et al. 2008
	Decreased	3,412	28,115	Winsvold et al. 2011
Total cholesterol	Increased	620	5,135	Scher et al. 2005
	Increased	5,087	22,539	Kurth et al. 2008
	No difference	111	463	Schwaiger et al. 2008
	No difference	165	1,285	Benseñor et al. 2011
	Decreased	3,412	28,115	Winsvold et al. 2011
Pyruvate	Increased	14	12	Okada et al. 1998
Leucine	No difference	31	9	Ferrari et al. 1990
Isoleucine	No difference	31	9	Ferrari et al. 1990
Methionine	No difference	31	9	Ferrari et al. 1990
Proline	No difference	31	9	Ferrari et al. 1990
Serine	No difference	31	9	Ferrari et al. 1990
Valine	No difference	31	9	Ferrari et al. 1990

Discussion

The aim of this study was to search for (a set of) serum metabolites associated with migraine status. Using ^{1}H-NMR spectroscopy metabolic profiling we were able to identify a subset of metabolites significantly associated with active migraine, but not with lifetime migraine in the large population-based Erasmus Ruchen Family (ERF) sample. Our study has several strengths compared with previous studies on plasma concentrations of metabolites in migraine patients. Our sample consists of a large number of well-characterized migraine patients and controls that are not affected by severe headaches. Participants were selected based on their ancestry and came from a relatively homogeneous population. Using ^{1}H-NMR spectroscopy, which is considered a highly robust method [14], we were able to test a large number of metabolites for their association with migraine in a hypothesis-free manner.

In our 150 migraine patients with active migraine in the year preceding the interview and the 1,360 controls we found an association with 22 peaks representing 16 known metabolites. In contrast, in the combined analysis of the same controls with our 150

patients with active and 139 patients with inactive migraine (i.e. lifetime migraine status) the subset of peaks associated with lifetime migraine was no longer significant after correcting for age and sex. The five peaks that showed some association with lifetime migraine status were also present among the 22 peaks that were associated with active migraine. The fact that we only saw significant association with active migraine may be due to the fact that that group is more homogeneous compared to the group with lifetime migraine, which allows smaller concentration differences to be detected. Alternatively, one could envisage that some metabolites undergo persistent concentration changes and are therefore picked up in our lifetime migraine analysis, while concentrations of other metabolites alter only temporarily, explaining why these metabolites are only significantly associated with active migraine status.

The 22 peaks associated with active migraine represent 19 annotated peaks and three unknown metabolites, which is a common problem in this type of studies, as the human metabolome has not been annotated completely.[14] The 19 annotated peaks represent leucine, isoleucine, methionine, proline, serine, valine, dimethylglycine, glucose, 1,5-anhydrosorbitol, cholesterol, lipids CH2, lipids CH2CO, creatinine, myoinositol, acetate and pyruvate. Of these metabolites, 1,5-anhydrosorbitol (inert metabolite from the diet that competes with glucose for reabsorption in the kidneys), myoinositol (involved in cerebral calcium homeostasis) and dimethylglycine (produced upon metabolizing choline into glycine) have not been studied in the serum of migraine patients before (www.hmdb.com). The other metabolites have been studied to some extent in migraine (for overview, see Table 5). Comparing the previous studies with our study is difficult, because they all used conventional platforms to assess metabolite concentrations. For the cardiovascular disease markers (total cholesterol, HDL-cholesterol, creatinine), results of these studies are not unequivocal. In our study, all lipid levels were decreased in migraine patients, which is in line with the findings from another European population-based study,[29] but is different from findings in other studies.[12,30-32] It should be noted, though, that comparison of these results is hampered because [1]H-NMR spectroscopy subdivides the lipids in different subclasses than conventional analyses.

Pyruvate levels in our migraine patients were higher than in controls. A small study with only 14 migraine patients also found increased plasma concentrations of pyruvate and suggested a role for abnormal mitochondrial metabolism in the pathophysiology of migraine.[33] Interestingly, lactate and pyruvate concentrations are also raised in patients with mitochondrial myopathy, encephalopathy, lactic acidosis with stroke-like episodes (MELAS) in which migraine frequently occurs as one of the presenting symptoms.[34]
Lastly, we found an association of migraine status with several amino acids. We found decreased concentrations of leucine, isoleucine, methionine and proline and increased concentrations of serine and valine in our migraineurs compared to controls. Some studies

from the early nineties addressed the role of neuroexcitatory amino acids in migraine patients and measured interictal serum levels using conventional assays.[4,35-37] Most of these studies focused mainly on plasma levels of glutamate and glutamine, but all amino acids associated with migraine in the ERF population were also measured in the study by Ferrari et al.[4] No differences were found between cases and controls in that study. This may be due to the small sample size of this study (including only 31 migraine patients) and the technical advances in ^{1}H-NMR spectroscopy that made it possible to detect much smaller differences in concentration than with the older met, but future studies using state of the art detection methods are necessary to seek confirmation of the association of migraine with these various amino acids.

Over the years, the important role of glutamate in monogenic and complex forms of migraine has been established firmly in several genetic and functional studies.[38] Interictal plasma glutamate levels also have been studied in several studies. Glutamate levels were shown to be increased in three of the four studies, either in the migraine without aura patient group[35] or in all migraine patients.[4,37] In contrast, a small study in 34 children with migraine found decreased plasma glutamate concentrations compared to controls.[36] Since all studies had relatively small sample sizes, it remains unclear to what extent plasma glutamate concentrations are altered in migraineurs. In our study, glutamine/glutamate and glutamate peaks were not associated with migraine status. It could be that there is indeed no difference between migraine patients and controls. An alternative explanation would be that our group with active migraine is too heterogeneous to pick up differences that could occur shortly after a migraine attack and do not persist for a long time.

Several limitations of our study have to be acknowledged as well. First, the set of metabolites that we studied covered only a small part of the human metabolome using our single metabolic profiling method.[14] Future complementary studies using different platforms may identify additional metabolites associated with migraine status. The second limitation is that our group of active migraine patients is defined by migraine attacks occurring in the last twelve months prior to the interview. Transient changes occurring shortly after a migraine attack in plasma concentrations may therefore be detectable in this study. The third limitation of our study is that we used the same population for discovery of the associated peaks as well as for assessing the magnitude of the association. Ideally, we would have been able to perform a replication study to validate our findings, but no ^{1}H-NMR spectroscopy study is currently available that measured at least the vast majority of the associated metabolites. We took specific precautions in our statistical analysis to minimize the lack of replication problem by using two cross-validations and performing the ANOVA analyses. In addition, the ANOVA analysis indicated that it is highly unlikely that none of these peaks has any association with active migraine. This shows that our set of associated peaks is an interesting starting point for further studies.

In conclusion, using [1]H-NMR spectroscopy, we were able to measure a large set of metabolites and perform a hypothesis-free metabolic-profiling study in migraine. We identified a subset of 22 metabolites that is associated with active migraine in our ERF population. For most of these metabolites, the relationship with migraine has not been studied before. Further studies are needed to validate our findings and to elucidate the possible pathophysiological basis of the association between migraine and each of these metabolites.

Acknowledgements

Source of funding

This work was supported by grants from the Netherlands Organization for Scientific Research (NWO) (903-52-291, M.D.F. and R.R.F.; VICI 918.56.602, M.D.F.; 907-00-217, G.M.T.; 920-03-473, A.H.S.; VIDI 91711319, G.M.T.); the European Community (EC) (EUROHEAD, LSHM-CT-2004-504837, M.D.F.); and the Centre for Medical Systems Biology (CMSB) in the framework of the Netherlands Genomics Initiative (NGI) (M.D.F., R.R.F., and A.M.J.M.v.d.M). They had no role in the design or conduct of the study.

References

1. Ferrari MD and Goadsby PJ, 2007, Migraine as a cerebral ionopathy with abnormal central sensory processing. Neurobiology of disease, edited by (Elsevier Academic Press, pp. 333-348.
2. Headache classification subcomittee of the International Headache Society. The International Classification of Headache Disorders: 2nd edition. Cephalalgia 2004; 24 Suppl 1: 9-160.
3. De Vries B, Haan J, Frants RR et al. Genetic biomarkers for migraine. Headache 2006; 46: 1059-1068.
4. Ferrari MD, Odink J, Bos KD et al. Neuroexcitatory plasma amino acids are elevated in migraine. Neurology 1990; 40: 1582-1586.
5. Martinez F, Castillo J, Rodriguez JR et al. Neuroexcitatory amino acid levels in plasma and cerebrospinal fluid during migraine attacks. Cephalalgia 1993; 13: 89-93.
6. Bockowski L, Sobaniec W and Zelazowska-Rutkowska B. Proinflammatory plasma cytokines in children with migraine. Pediatr Neurol 2009; 41: 17-21.
7. Vanmolkot FH and de Hoon JN. Increased C-reactive protein in young adult patients with migraine. Cephalalgia 2007; 27: 843-846.
8. Uzar E, Evliyaoglu O, Yucel Y et al. Serum cytokine and pro-brain natriuretic peptide (BNP) levels in patients with migraine. Eur Rev Med Pharmacol Sci 2011; 15: 1111-1116.
9. Uzar E, Evliyaoglu O, Toprak G et al. Increased asymmetric dimethylarginine and nitric oxide levels in patients with migraine. J Headache Pain 2011;

10. Guldiken S, Guldiken B, Demir M et al. Soluble CD40 ligand and prolactin levels in migraine patients during interictal period. J Headache Pain 2011; 12: 355-360.
11. Fusayasu E, Kowa H, Takeshima T et al. Increased plasma substance P and CGRP levels, and high ACE activity in migraineurs during headache-free periods. Pain 2007; 128: 209-214.
12. Kurth T, Ridker PM and Buring JE. Migraine and biomarkers of cardiovascular disease in women. Cephalalgia 2008; 28: 49-56.
13. Hamed SA, Hamed EA, Ezz Eldin AM and Mahmoud NM. Vascular risk factors, endothelial function, and carotid thickness in patients with migraine: relationship to atherosclerosis. J Stroke Cerebrovasc Dis 2010; 19: 92-103.
14. Dunn WB, Broadhurst D, Begley P et al. Procedures for large-scale metabolic profiling of serum and plasma using gas chromatography and liquid chromatography coupled to mass spectrometry. Nat Protoc 2011; 6: 1060-1083.
15. Stam AH, de Vries B, Janssens AC et al. Shared genetic factors in migraine and depression: evidence from a genetic isolate. Neurology 2010; 74: 288-294.
16. Aulchenko YS, Heutink P, Mackay I et al. Linkage disequilibrium in young genetically isolated Dutch population. Eur J Hum Genet 2004; 12: 527-534.
17. Launer LJ, Terwindt GM and Ferrari MD. The prevalence and characteristics of migraine in a population-based cohort: the GEM study. Neurology 1999; 53: 537-542.
18. Price WS. Water Signal Suppression in NMR Spectroscopy. Annual Reports on NMR Spectroscopy 1999; 38: 289-354.
19. Tomasi G, van den Berg F and Andersson C. Correlation optimized warping and dynamic time warping as preprocessing methods for chromatographic data. Journal of Chemometrics 2004; 18: 231-241.
20. MacIntyre DA, Jimenez B, Lewintre EJ et al. Serum metabolome analysis by 1H-NMR reveals differences between chronic lymphocytic leukaemia molecular subgroups. Leukemia 2010; 24: 788-797.
21. Findeisen M, Brand T and Berger S. A 1H-NMR thermometer suitable for cryoprobes. Magn Reson Chem 2007; 45: 175-178.
22. Wu PS and Otting G. Rapid pulse length determination in high-resolution NMR. J Magn Reson 2005; 176: 115-119.
23. Aue W, Karhan J and R E. Homonuclear broad band decoupling and two-dimensional J-resolved NMR spectroscopy. The Journal of Chemical Physics 1976; 64: 4226-4227.
24. Meiboom S and Gill D. Modified Spin-Echo Method for Measuring Nuclear Relaxation Times. Review of Scientific Instruments 1958; 29: 688-691.
25. Nicholson JK, Foxall PJ, Spraul M et al. 750 MHz 1H and 1H-13C NMR spectroscopy of human blood plasma. Anal Chem 1995; 67: 793-811.
26. Wishart DS, Jewison T, Guo AC et al. HMDB 3.0--The Human Metabolome Database in 2013. Nucleic Acids Res 2013; 41: D801-7.

27. Aulchenko YS, Ripke S, Isaacs A and van Duijn CM. GenABEL: an R library for genome-wide association analysis. Bioinformatics 2007; 23: 1294-1296.
28. Friedman J, Hastie T and Tibshirani R. Regularization paths for generalized linear models via coordinate descent. Journal of statistical software 2010; 33: 1.
29. Winsvold BS, Hagen K, Aamodt AH et al. Headache, migraine and cardiovascular risk factors: The HUNT study. European Journal of Neurology 2011; 18: 504-511.
30. Scher AI, Terwindt GM, Picavet HS et al. Cardiovascular risk factors and migraine: the GEM population-based study. Neurology 2005; 64: 614-620.
31. Schwaiger J, Kiechl S, Stockner H et al. Burden of atherosclerosis and risk of venous thromboembolism in patients with migraine. Neurology 2008; 71: 937-943.
32. Benseñor IM, Goulart AC, Lotufo PA et al. Cardiovascular risk factors associated with migraine among the elderly with a low income: The São Paulo Ageing & Health Study (SPAH). Cephalalgia 2011; 31: 331-337.
33. Okada H, Araga S, Takeshima T and Nakashima K. Plasma Lactic Acid and Pyruvic Acid Levels in Migraine and Tension-Type Headache. Headache: The Journal of Head and Face Pain 1998; 38: 39-42.
34. Montagna P, Gallassi R, Medori R et al. MELAS syndrome: characteristic migrainous and epileptic features and maternal transmission. Neurology 1988; 38: 751-754.
35. Cananzi AR, D'andrea G, Perini F et al. Platelet and plasma levels of glutamate and glutamine in migraine with and without aura. Cephalalgia 1995; 15: 132-135.
36. D'Eufemia P, Finocchiaro R, Lendvai D et al. Erythrocyte and plasma levels of glutamate and aspartate in children affected by migraine. Cephalalgia 1997; 17: 652-657.
37. Alam Z, Coombes N, H Waring R et al. Plasma levels of neuroexcitatory amino acids in patients with migraine or tension headache. Journal of the neurological sciences 1998; 156: 102-106.
38. de Vries B, Frants RR, Ferrari MD and van den Maagdenberg AM. Molecular genetics of migraine. Hum Genet 2009; 126: 115-132.

9

General discussion

Claudia M. Weller

MIGRAINE IS A HIGHLY PREVALENT neurovascular disorder characterized by recurring attacks of severe headache with autonomic and other neurological symptoms.[1,2] Cluster headache is another, much rarer primary headache disorder with a lifetime prevalence of only 0.12%.[3] Current prophylactic and abortive treatment for both primary headache disorders is not optimal and novel drug targets should be identified to reduce the burden of the disease in many patients. Understanding the genetic factors involved in these disorders will advance our knowledge of the underlying molecular pathways that can point to useful targets for future drug discovery.

The research presented in this thesis had a number of aims to fill some of the gaps. The first aim was to search for mutations in the *SCN1A* gene that had already been linked to monogenic Familial Hemiplegic Migraine (FHM) and in the *SLC2A1*, *ATP1A3*, *PRRT2* genes that have been identified in diseases with overlapping symptoms with migraine types and/or are comorbid with migraine: autosomal dominant GLUT1 Deficiency Syndrome (GLUT1DS), Alternating Hemiplegia of Childhood (AHC), and Benign Familial Infantile Convulsions (BFIC), respectively. Mutation analyses in these genes were performed in various patient groups relevant to migraine. The second aim was to develop and validate web-based questionnaires for reliable ascertainment of ICHD-2 compatible diagnoses, that is migraine and cluster headache, which is of great use for recruitment of large numbers of patients for (large-scale) genetic studies. The third aim was to test a single nucleotide polymorphism in the hypocretin receptor type 2 gene *HCRTR2* that had been reported as the only replicated genetic susceptibility factor in cluster headache.[4-7] The fourth aim was to investigate whether atherosclerosis can explain the increased risk of ischemic stroke, myocardial infarction and peripheral arterial disease that is frequently reported in epidemiological and clinical studies on migraine, using the Erasmus Rucphen Family (ERF) study. The fifth, and final, aim is to use serum of participants of the ERF study for metabolomic profiling using proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy in order to identify a set of compounds that was able to predict a migraine diagnosis.

Mutation analysis in monogenic syndromes with relevance to migraine

The identification of causal gene mutations, and their subsequent functional characterization, in monogenic subtypes of a disease can be a useful approach to unravel biological pathways that may also have relevance to the complex disease form. Familial hemiplegic migraine (FHM) is a monogenic subtype of migraine with aura and serves as an autosomal dominant model for common migraine. Apart from the hemiparesis, aura symp-

toms in patients with hemiplegic migraine are identical to those in patients with migraine with aura and the headache characteristics are identical to those in patients with migraine with aura or without aura.[1,2,8] Moreover, many patients with hemiplegic migraine also have attacks of the common forms of migraine.[9] Thus far, mutations in the *CACNA1A* (FHM1), *ATP1A2* (FHM2) and *SCN1A* (FHM3) genes are known to cause FHM. *CACNA1A* encodes the pore-forming α1 subunit of $Ca_V2.1$ channels that are expressed at the plasma membrane of neurons in the central and peripheral nervous system. These channels control the flow of calcium across membranes and thereby release of neurotransmitters into the synaptic cleft.[10,11] *ATP1A2* encodes the α2 subunit of sodium-potassium pumps (Na^+,K^+-ATPase). These pumps catalyze the exchange of Na^+ and K^+ across the cell membrane and provide the steep sodium gradient that is essential for the transport of calcium and glutamate from the synaptic cleft.[12] And finally, *SCN1A* codes the pore-forming subunit of voltage-gated $Na_V1.1$ sodium channels that are mainly—but not exclusively—expressed on inhibitory neurons, where they are essential for generation and propagation of action potentials.[11,13,14]

Notably, hemiplegic migraine can also occur in isolated cases and is then called Sporadic Hemiplegic Migraine (SHM).[1,2] Mutations in the *CACNA1A* and *ATP1A2*, but not the *SCN1A*, gene have been identified in a significant proportion of SHM patients, particularly in severely affected patients with additional symptoms and early onset of disease.[4-7] Many mutations in *CACNA1A* (29 until now) and *ATP1A2* (over 30 until now) have been found associated with FHM or SHM. *CACNA1A* mutations found in patients with hemiplegic migraine are thought to cause increased activity of $Ca_V2.1$ channels with mutated channels opening more readily than normal channels.[19-21] Studies in transgenic knock-in mice confirmed that FHM1 is caused by hyperactive $Ca_V2.1$ channels.[15,16] Other mutations in this gene are associated with episodic ataxia type 2 (caused by decreased $Ca_V2.1$ channel activity[17]) or spinocerebellar ataxia type 6[18] (functional consequences of the causal CAG repeat expansion have not been elucidated yet[19]). Most mutations in *ATP1A2* lead to hemiplegic migraine and are associated with a reduced Na^+,K^+-ATPase function,[20-25] but certain mutations in *ATP1A2* have been reported to cause other neurological phenotypes, such as basilar migraine,[26] Alternating Hemiplegia of Childhood (AHC),[27] Benign Familial Infantile Convulsions (BFIC),[28] or even common migraine without aura.[29] Even mutation carriers with complex phenotypes presented as a combination of hemiplegic migraine and AHC have been reported.[30] This suggests that specific functional consequences of mutations in these genes affect the protein function of the gene product in a specific, yet largely unknown manner, resulting in the diverse spectrum of clinical phenotypes.

Chapter 2 focuses on the identification of novel *SCN1A* mutations in two families with FHM. Only five *SCN1A* mutations had been identified in FHM thus far.[31-35] An additional mutation was reported, but from the clinical description it is far from certain that this leads to hemiplegic migraine in the mutation carriers.[33,36,37] Of the five FHM3 mutations, p.L1649Q and p.Q1489K are associated with pure familial hemiplegic migraine,[35] whereas mutations p.Q1489H and p.L263V have been associated with childhood epilepsy and generalized tonic-clonic seizures,[31,32,34] in addition to hemiplegic migraine in the patient. Some patients with FHM3 mutations p.Q1489H and p.F1499L were also reported to suffer from "elicited repetitive daily blindness" (ERDB), which occurred apart from their hemiplegic migraine attacks.[34,38] The overwhelming majority of mutations in *SCN1A*, however, are association with various non-FHM phenotypes of severe childhood epilepsy, i.e. Dravet syndrome (also known as severe myoclonic epilepsy of infancy (SMEI)) or generalized epilepsy with febrile seizures plus (GEFS+).[39] Like with *CACNA1A* and *ATP1A2*, the genotype-phenotype associations seen with *SCN1A* are complex.

The p.1498M and p.F1661L mutations that were identified in **Chapter 2** are only the sixth and seventh causative FHM3 mutations. Both mutations cause pure FHM with highly variable severity and frequency of attacks in two Spanish families. With respect to the functional consequences of FHM3 mutations it seems that p.Q1489K and p. L1649Q show reduced activity of $Na_V1.1$ channels.[32,35,40] The third functionally tested FHM3 mutation, p.L263V, that in patients causes FHM and in the majority of mutation carriers also generalized tonic-clonic epilepsy, essentially revealed hyperactivity effects on $Na_V1.1$. channel functioning.[40] It was hypothesized that loss of $Na_V1.1$ activity primarily disturbs the functioning of inhibitory neurons, where $Na_V1.1$ channels are normally expressed[13,14], whereas increase of $Na_V1.1$ activity predominantly would affect excitatory neurons. The exact functional consequences of the p.F1661L mutation are difficult to predict, but the p.I498M mutation likely affects fast inactivation of $Na_V1.1$ channels, supporting decreased channel function as the underlying cause of FHM in *SCN1A* mutation carriers. From a recent *in vitro* study in which FHM3 mutation p.Q1489K was expressed in cultured neurons, however, it became clear that the functional consequences of FHM3 mutations can be very complex. Depending on the test paradigm, the mutation had functional consequences compatible with either a hyperexcitability or hypoexcitability (self-limiting hyperexcitability capacity) phenotype,[41] but this has not been tested in e.g. knock-in mice with FHM3 mutations, as they are not available.

The research performed in **Chapter 3** and an additional genetic study from our group[42] shows that knowledge on migraine genetic mechanisms can indirectly come from investigating genes that had been identified in diseases with overlapping symptoms with

migraine types and/or had been found to be comorbid with migraine. **Chapter 3** describes a study that combines and builds on knowledge that comes from various studies. In one Dutch study an FHM1 gene mutation in German twins lead to overlapping clinical symptoms of hemiplegic migraine and rather atypical AHC and it was concluded that hemiplegic migraine and AHC to some extent may share pathophysiological mechanisms.[43] The link with hemiplegic migraine is further strengthened by the fact that also a mutation in the FHM2 *ATP1A2* gene was found in atypical AHC patients.[27,30] Due to the overlapping clinical features, advances in genetic research of AHC may therefore provide important information about the genetics of migraine. Typical AHC is a rare syndrome with such considerable clinical overlap with hemiplegic migraine that it is sometimes impossible to determine the correct diagnosis in very severely affected patients.[44] In addition to recurrent hemiplegic attacks, patients with AHC suffer from movement disorders, seizures, and developmental delay starting before the age of 18 months.[1] Recently, the *ATP1A3* gene was identified as the major cause of AHC as over 70% of patients carry a mutation in that gene.[45-47] This gene is a close homologue of the FHM2 *ATP1A2* gene and encodes a Na^+/K^+ ATPase expressed in neurons of the basal ganglia, cerebellum and hippocampus. AHC-causing mutations in this gene are thought to reduce the activity of the pump, possibly via alteration of the three-dimensional structure of the protein[45,46,] thereby affecting osmoregulation, sodium-coupled ion transport and neuronal excitability.

In 2009, Rotstein et al.[48] identified an *SLC2A1* mutation in an atypical AHC patient who had presented with intellectual disability, hemiplegic attacks, episodes of ataxia, microcephaly and hypoglycorrhachia. Until then mutations in *SLC2A1* had been identified in patients with autosomal dominant GLUT1 Deficiency Syndrome (GLUT1DS).[49] A diagnosis of GLUT1DS is suspected by hypoglycorrhachia (a low cerebrospinal fluid glucose in a normoglycaemic patient) but needs to be confirmed by a positive *SLC2A1* mutation analysis. The *SLC2A1* gene encodes the GLUT1 protein that is pivotal for glucose transport into the brain. Recent research has indicated that the phenotype of GLUT1DS is much broader than the classical presentation with developmental delay, medication-resistant epilepsy, ataxia and dystonia.[50,51] Rotstein et al. illustrated this in their description of a patient with an *SLC2A1* mutation, presenting with GLUT1DS and AHC[48] Because of the overlapping clinical symptoms in FHM, AHC and GLUT1DS and because some of the genes (*ATP1A2* and *ATP1A3*) of some of these disorders suggest also some molecular overlap, we searched for causal mutations in *ATP1A3* and *SLC2A1* in i) patients that with overlapping clinical features of AHC and hemiplegic migraine, and ii) patients with familial or sporadic hemiplegic migraine. In addition, we searched for *SLC2A1* mutations in AHC patients tested negative for *ATP1A3* mutations.

In the study presented in **Chapter 3** it was shown that the *SLC2A1* and *ATP1A3* genes do not play a major role in hemiplegic migraine patients in which we previously had not identified any mutations in *CACNA1A*, *ATP1A2* or *SCN1A*. Notably, a novel p.Gly18Arg mutation was identified in a severely affected patient that had an atypical clinical presentation of hemiplegic migraine and was initially diagnosed with atypical AHC. By genetic analysis the phenotypic spectrum associated with *SLC2A1* mutations could be extended with (sporadic) hemiplegic migraine and exercise-induced dystonia improving on corticosteroid treatment. Our results also indicate that *ATP1A3* and *SLC2A1* mutation screening is not indicated in typical hemiplegic migraine, nor is *SLC2A1* mutation screening indicated in patients with classical AHC. However, in patients with a complex, atypical phenotype with overlapping symptoms of AHC and HM a lumbar puncture should be considered in order to detect a low cerebrospinal fluid glucose concentration, followed by *SLC2A1* mutation analysis.

The additional genetic study from our group[42] may have relevance to migraine, since Benign Familial Infantile Convulsions (BFIC) was shown to co-occur with FHM. In fact, an *ATP1A2* mutation that was identified in a Dutch-Canadian family was presented as the cause of both hemiplegic migraine and BFIC.[28] Since the identification of genes involved in such comorbid seizure disorders can also improve our understanding of migraine genetics, we aimed to determine the cause of the symptoms in four families with BFIC without any additional symptoms. The additional genetic study[42] describes the identification of the p.R217PfsX8 mutation in the *PRRT2* gene as the underlying cause in three of these families. The mutation affects a proline-rich transmembrane protein with unknown function that is thought to interact with the presynaptic synaptosomal-associated protein 25 kDA (SNAP25) that plays a role in calcium-triggered exocytosis by regulating fusion of synaptic vesicles to the plasma membrane.[52] Notably, recently (recurrent) mutations in *PRRT2* were shown to cause a variety of paroxysmal neurological phenotypes occurring in isolation or in various combinations.[53] *PRRT2* mutations were identified in a large proportion of patients with infantile seizures and/or paroxysmal dyskinesia, but thus far mutations were only identified in less than 1% of patients with hemiplegic migraine and episodic ataxia.[54-59] Although mutations in *PRRT2* account for only a few hemiplegic migraine cases in these studies and the relation between hemiplegic migraine and BFIC was shown recently to be more complex than previously thought,[60] these findings highlight that despite different phenotypes, these paroxysmal neurological disorders may in part have overlapping genetic characteristics. As the phenotype associated with *PRRT2* mutations is broad, unravelling the role of this protein can help improving our understanding of many episodic neurological disorders.

Validation of questionnaires for genetic studies of primary headache disorders

At the start of this thesis, rapid advances in genomic technologies had rendered it feasible to perform genome-wide association (GWA) studies testing hundreds of thousands of genetic variants in several thousand cases and controls in a single experiment. Statistical power is a major concern in such studies, because of stringent multiple testing correction combined with the limited effect size of common variants underlying common disease. Valid ascertainment of cases and controls is essential to prevent additional loss of power due to clinical heterogeneity. Obtaining valid ICHD-2 based diagnoses in large-scale population-based studies is challenging, as diagnoses in primary headache disorders are based on consensus criteria for which the gold standard is a careful history taken by a headache specialist. Because face-to-face examinations are too time-consuming and too costly in this setting, a different diagnostic strategy was needed. Several groups had reported on the use of internet to recruit (headache) patients for clinical research[61-65] and in **Chapters 4 and 5** we describe our recruitment strategy for collecting large numbers of self-reported primary headache patients from the general Dutch population using web-based questionnaires.

In **Chapter 4** the recruitment strategy is reported for the Leiden University Migraine Neuro-Analysis (LUMINA) program for inclusion of a large number of self-reported migraine patients for genetic and epidemiological studies through the project's website (www.lumc.nl/hoofdpijn). Self-reported migraine patients from the Dutch population were invited to complete a previously validated screening questionnaire. Screen-positives were subsequently asked to complete a newly developed extended web-based questionnaire aiming to diagnose migraine based on the ICHD-2 criteria. After the first year, we had recruited 2,397 self-reported migraine patients, of which we selected 1,067 participants for validation of the extended questionnaire using semi-structured telephone interviews. Nearly all patients meeting the screening criteria (95%) were diagnosed with migraine upon interview, so we decided to regard the entire group as being a migraine patient. The good performance of our screener with respect to headache characteristics is in agreement with many previously developed questionnaires, but making reliable aura diagnoses based on questionnaire data is notoriously difficult. Discriminating between migraine with and without aura is of particular interest in genetic studies and we hoped to improve questionnaire-based aura diagnoses with our LUMINA questionnaire. Upon validation we found it to be a valid instrument for making aura diagnoses as well, with a positive predictive value of 86%. We attempted to make an equally well or even better prediction of aura status using a subset of items from the extended questionnaire, which

were selected based on clinical relevance, sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and likelihood ratios. Using multivariate logistic regression analysis with a forward selection strategy we identified a seven-question subset with similar PPV in assessing aura diagnosis as compared to the ICHD-2 based algorithm. This subset of aura questions represents an attractive way to reliably diagnose aura status in large epidemiological studies. Currently, clinical data have been collected from approximately 5,000 migraine patients. In the large LUMINA study more than 3,500 DNA samples are present and the study population contributed significantly to the identification of susceptibility genes for both migraine with aura,[66], migraine without aura,[67] and the subsequent migraine meta-analysis (for specific details see section 10.3).[68]

In contrast to the rapid advances in the migraine field, the genetic basis of cluster headache (CH) remained poorly studied in candidate-gene based studies with a high risk of false-positive associations due to small sample sizes and lack of a replication cohort. Genetic studies in cluster headache are greatly hampered by the combination of low prevalence and putative complex genetic background of the disorder. To facilitate large-scale genetic studies in CH, we launched the LUCA program as the CH counterpart of the LUMINA study. Via the project's website self-reported cluster headache patients completed a screening questionnaire and screen-positives were asked to complete the newly developed web-based extended questionnaire. In Chapter 5 we report on the recruitment of the LUCA population and demonstrate that our extended questionnaire has positive predictive value of 96% and thus is a valid tool for diagnosing CH in a research setting. In addition, we constructed the shorter Quick Ascertainment of Cluster Headache (QATCH) questionnaire to diagnose CH in large samples as a practical alternative for diagnosing CH patients, because the combination of the screening and QATCH-questionnaire works as well as combining the screening questionnaire with the much longer LUCA-questionnaire. Validation in our own population showed promising results, but the QATCH-questionnaire should be validated in other populations as well.

Genetic studies in complex migraine and cluster headache

Recent findings from genetic studies in migraine

Identifying genes involved in complex disorders has proven difficult. Two main hypotheses are used to explain the genetic origin of complex diseases, either "the common disease is caused by common variants (i.e., CD-CV)" or "the common disease is caused by rare variants" (CD-RV).[69] The hypotheses propose that disease susceptibility is conferred by multiple genetic variants that either occur with a high frequency in the population but each only slightly increase disease risk or these genetic variants are much rarer but each

variant has a larger effect size. Genome-wide association studies (GWAS) typically test the common variant hypothesis as all tested variants have a frequency >5% in the general population. Next generation sequencing strategies can pick-up rarer disease variants. To date, most findings in complex diseases come from GWAS and therefore capture only common variants. Only few examples have been reported of rare variants with a moderate effect, for example, factor V Leiden in deep venous thrombosis [70] or rare protective variants in *IFIH1* for type 1 diabetes.[71]

Gene identification studies in common migraine are particularly challenging because, apart from genetic heterogeneity, there is also extensive clinical heterogeneity caused by large variations in presenting symptoms and age of onset. Due to the lack of reliable biomarkers, migraine diagnoses are based on questionnaire and/or interview data using the diagnostic criteria of the International Headache Society.[1] These criteria are useful to diagnose the type of attacks patients in a clinical setting, but are less suited for genetic research as attacks may vary within patients with multiple combinations of symptoms leading to the same diagnosis, but not necessarily through the same pathophysiological mechanism.

Linkage and candidate gene association studies in common migraine

Initial genetic successes in common migraine came with family-based linkage analysis that led to the identification of many chromosomal regions that are shared by affected family members more often than can be expected by chance. However, no migraine genes were identified.[72] Also candidate gene association studies were initially very popular. These studies tested for significant differences in allele frequencies between migraine cases and controls at locations in the genome with known variation in genes that were selected because they had been implicated in migraine pathophysiology. Among the most frequently tested candidate genes were genes in the dopaminergic and serotonergic systems, hormone receptors, and inflammatory pathways (for review see de Vries et al.[72]). These studies can produce valid results if carefully designed to overcome methodological issues regarding sample size, selection of cases and controls, selection of variants, correction for multiple testing, and replication of findings in independent populations, but this was rarely the case.

Genome-Wide Association Studies in Common Migraine

Over the last few years, genome-wide association (GWA) studies have become the standard approach to identify disease susceptibility variants. GWAs enable simultaneous testing of hundreds of thousands of common (that is with a frequency >5% in the general

population) DNA markers in several thousand cases and controls in a hypothesis-free manner Such large sample sizes are needed to correct for the large number of statistical tests performed. Since 2010, several migraine GWAS have been published that each investigated several thousand DNA samples from patients with a clinical diagnosis of migraine with aura[66] and migraine without aura[67] that came from established migraine clinics in the Netherlands (Leiden, LUMINA) but also other countries such as Germany and Finland. These studies, combined with a recent meta-analysis that studies in total 23,285 cases and 95,425 controls from almost 30 cohorts worldwide, yielded 13 DNA variants that confer susceptibility to migraine. The individual migraine GWAs[66,67,73] and subsequent meta-analysis[68] revealed susceptibility genes involved in several pathways (for review see Eising et al.[74]). The polymorphisms in the *MTDH*,[66] *LRP1*[67,73] and *MEF2D*[67] are involved in (glutamatergic) neurotransmission, a pathway that plays a central role in the pathophysiology of FHM. The *MEF2D* gene is also involved in development of neurons and synapses, just like four other susceptibility genes that were identified in these studies (*ASTN2*, *PRDM16*, *PHACTR1* and *TGFBR2)*. The latter two of these are also involved in brain vasculature function. Several variants in genes coding for metalloproteinases (*AJAT1*, [68], *MMP16*,[68] and *TSPAN2*[68], involved in degradation of extracellular matrix proteins, were also found to increase disease risk. Lastly, two variants in the *TRPM8* gene[67,73] involved in pain sensation and a variant in *c7orf10*[68] involved in glutaric acid metabolism were found to be associated with migraine.

Each of these variants only slightly increased disease risk with an odds ratio not exceeding ~1.2 and therefore explain only a few percent of the total genetic variance. This is in line with GWAS findings in other complex disorders. Due to their small effect sizes, but these variants have limited value from a clinical perspective. However, their identification may shed light on novel molecular disease pathways as the variants point to genes in pathways that have been implicated in migraine pathophysiology.

Future genetic studies in migraine

Despite current efforts that are expected to yield higher numbers of migraine gene variants by further increasing sample size and/or by applying more advanced statistical methodologies such as imputation strategies, it is expected that *common variants* in migraine at best will explain only a small portion of the genetic variance.[69] There may be common variants with very small effect sizes that remain undetected as they would require extremely high numbers of cases and controls. Alternatively, there may be rarer variants that are not captured by commercial array chips. More recent technical developments known as *next-generation sequencing* allow the identification of such rarer variants with a medium effect size either by *exome sequencing* (i.e. sequencing all coding exons of an individual)

or—in the more distant future—*whole genome sequencing*.[75] Other possibilities why current gene hunts capture only little of the variance in complex diseases may be that one has to take into account gene–gene interactions (epistasis) or even different genetic mechanisms such as epigenetic mechanisms, in which gene expression is modified by heritable changes in packaging of DNA.

Future genetic studies may also benefit from alternatives to current methods of diagnosing patients. In **Chapter 8** we explored whether endophenotyping of patients can be performed by analyzing serum using proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy. This technique is used frequently to acquire metabolic profiles from large groups of patients because of the robustness and relatively low costs of this method.[76] With ^{1}H NMR it may be possible to identify (sets of) low-molecular weight metabolites in serum that can distinguish migraine patients from controls. Using ^{1}H-NMR profiles of sera from migraine patients and controls of the Erasmus Rucphen Family (ERF) population in combination with elastic net regression analysis, we identified a subset of 14 annotated metabolites that was associated with active migraine status, which should be regarded as hypothesis-generating and provides a subset of metabolites that may be involved in migraine pathophysiology.

Recent findings from genetic studies in cluster headache

Cluster headache is considered a complex genetic disorder. Previous family studies using old data regarding cluster headache prevalence found up to 46-fold increased risks among first-degree relatives and up to eight-fold for second-degree relatives.[77-79,79,80] Recalculated with a prevalence of 0.2%, first-degree family members have 5-18 fold increased risk and second-degree relatives have 1-3 fold increased risk of having cluster headache.[81] Additional support for a genetic component in cluster headache comes from the observation of six monozygous twins both affected.[82-87] However, the only systematic twin study showed both monozygous and dizygous twins to be discordant for cluster headache.[88]

Genetic research in cluster headache is scarce and its genetic background is almost entirely unknown. Several studies were inspired by the knowledge on migraine genetics. A mitochondrial mutation reported to cause MELAS was identified in a single cluster headache patient without a family history of this disorder,[89] but this seems unrelated to the occurrence of cluster headache as additional studies failed to replicate involvement of mitochondrial mutations in cluster headache.[90,91] Multiple small-scale studies were performed, including studies that tested the role of the FHM1 gene *CACNA1A* or rs1801133 polymorphism in the *MTHFR* gene, but these studies were negative or lack a replication sample rendering it plausible that they are all chance findings.[92-94] Only one genetic factor

(i.e. a variant in the hypocretin type 2 receptor gene *HCRTR2*) was found to be associated with cluster headache in two of the three small studies and in a meta-analysis of these studies.[4-7] In **Chapter 6** the association of the previously found associated polymorphism (rs2653349) in *HCRTR2* was tested in our LUCA population but no association with cluster headache was found. In contrast, when we performed a novel meta-analysis including the LUCA population we found a significant association of this variant with cluster headache susceptibility. There is growing concern, though, that many associations found in small-scale candidate gene studies are false-positive findings, even if they were replicated in another (equally small) population. Although our meta-analysis results remained positive after removal of the first study with an unusually large effect size, we concluded that such hypothesis-driven studies are likely to produce invalid results and therefore, are not going to help us to unravel the genetic basis of cluster headache. Other strategies using large discovery and replication cohorts are needed. With recruiting and validating our LUCA population we made one of the first steps in that direction.

Mining the Erasmus Rucphen Family study for migraine research: studying comorbid vascular disease and serum profiling towards biomarker identification

Some studies took advantage of the fact that certain disorders are comorbid with migraine. Although the observation can be spurious due to selection bias or reflect a unidirectional causal relationship (that is migraine causes [or is caused by] the comorbid disorder), it may also be that shared genetic and/or environmental factors underlie both migraine *and* the comorbid disorder.[95] Taking advantage of comorbid disorders can be an attractive strategy for genetic migraine studies, as it might reduce genetic heterogeneity by selecting those migraine patients who also suffer from the comorbid disorder. Several examples that used this approach are discussed below.

Investigating Comorbid Depression in Migraine Genetics

Depression is one of the psychiatric disorders comorbid with migraine. A recent meta-analysis estimated the OR for depression in a migraine patient at 2.2. The risk appears highest for MA patients.[96] Conversely, depression also increases the risk of migraine (OR = 2.8–3.4), reflecting the well-established bidirectional relationship between both disorders that suggests involvement of shared pathophysiological mechanisms. Three recent studies investigated whether shared genetic factors for both disorders exist, although a direct comparison of the studies is complicated by methodological differences.[97-99] Two of the studies investigated twins[97, 98], of which one study included only male twin pairs [98] —

whereas the third study was performed in a genetically isolated population.[99] A decrease in heritability of migraine upon correction for the co-occurrence of depression was interpreted as evidence that part of the variance in migraine must be explained by genetic factors that play a role in both migraine and depression.[97,99] Of these two studies, the one by Stam and co-workers found the largest prevalence of depression and the biggest drop in heritability in MA. The third study by Schur and co-workers[98] concluded that the genetic architecture of migraine and depression is best described by a model that incorporated both genetic and environmental factors and estimated that shared genetic factors account for approximately 20% of the variance in migraine and depression. Preliminary data from a genome-wide linkage scan taking advantage of the comorbidity revealed some candidate chromosome regions with nominal evidence, but no susceptibility gene.[100]

Investigating Comorbid Cardiovascular Disease in Migraine Genetics

The prevalence of cardiovascular disorders seems to be increased in migraine patients as well. A two-fold increased risk of ischemic stroke has been documented in observational, prospective and imaging studies.[101-103] Several reports also indicate increased risk of peripheral arterial disease and coronary heart disease.[101,104,105] This suggests that generalized vascular dysfunction is an important pathophysiological characteristic in migraine, but the underlying mechanism is unclear. Proposed mechanisms include a cortical spreading depression-mediated mechanism[106,107], migraine-specific medication,[108] patent foramen ovale (PFO),[109,110] coagulation abnormalities,[111] endothelial dysfunction [112,113] and/or shared underlying genetic [114] or environmental risk factors.[115,116] Alternatively, an adverse cardiovascular risk profile and associated atherosclerosis could underlie the increased risk of cardiovascular disease in migraine patients. Some studies found an unfavorable cardiovascular risk profile, in particular for migraine with aura patients,[104,117,118] while other studies did not find any[119] or only a modest association.[120] Framingham Risk Scores for coronary heart disease (FRS-CHD) were elevated in migraineurs in most,[104,117,118,121] but not all studies.[119,122] None of the studies reported on the Framingham Risk Score for ischemic stroke (FRS-Stroke) in migraine patients, probably because the electrocardiogram (ECG) data that are necessary to calculate this risk score were not available.

In **Chapter 7** we investigated the grade of prevalent atherosclerosis in migraine patients and controls from the ERF population. We used three complementary non-invasive preclinical functional and structural markers of vessel wall properties, including carotid intima-media thickness (IMT), pulse-wave velocity (PWV), and ankle brachial index (ABI). Although we found modest associations of three established cardiovascular risk factors with migraine status, this did not lead to higher Framingham risk scores for coronary heart disease or stroke in migraineurs when compared to controls. This is in agree-

ment with our main finding that atherosclerosis is no more prevalent in migraineurs than in controls. Overall, it seems unlikely that the higher risk of cerebro- and cardiovascular disease in migraineurs is mediated by atherosclerosis, although it might be possible that the process of atherosclerosis plays a role on a subclinical level with endothelial dysfunction as a presumed early marker.[113] Larger, preferably prospective studies are necessary to further clarify the role of atherosclerosis in incident vascular events in migraineurs. It could be that enhanced susceptibility to ischemic depolarization akin to spreading depression predisposes migraineurs to infarction during mild ischemic events, thereby increasing the stroke risk. Recent findings from transgenic FHM1 mouse mutants provide further support that there seems a pathophysiological link between migraine and ischemic events. It was shown that the transgenic FHM1 mice have increased infarct size upon experimental induction of stroke by transient middle meningeal artery occlusion and that they have more pronounced peri-infarct depolarizations mimicking spreading depression events.[156] More translational studies are needed to provide insight in the comorbidity with cardiovascular disease and to develop prophylactic treatment strategies to prevent cerebro- and cardiovascular events in migraine patients.

Future perspectives

The research described in this thesis was directed at understanding the molecular mechanisms that underlie rare and common primary headache disorders. Although mutation analyses as performed in **Chapters 2 and 3** and our genetic study on BFIC[42] has provided some further insight in molecular mechanisms that have relevance to migraine, the search for migraine genes is far from over. The search for additional FHM genes is ongoing, as mutations in the three currently known FHM genes do not explain the occurrence of disease in all families. Over the last few years, exome sequencing has led to the identification of many genes underlying monogenic disorders and one may expect that exome sequencing efforts will lead to the identification of additional genes for FHM as well.

At the start of this thesis there were no well-established genetic risk factors for migraine despite some indications from candidate gene association studies and chromosomal loci, but no gene variants, from family-based linkage studies. In recent years, through technical developments in genotyping methodology combined with rapidly decreasing costs for genotyping costs, large-scale GWAS have shown their capability to identify disease gene variants at an unprecedented scale. The real challenge with GWAS findings in the coming years is to elucidate the role of these novel susceptibility genes in the pathophysiology of the disease.

One has to conclude that GWAS did not live up to the expectation with respect to being able to predict individual genetic risk to disease. In theory, one would expect that it is possible to identify individuals with multiple risk alleles that are at higher risk, but these calculations are not meaningful when only a fraction of the disease genes is known. At present a staggering ~80% of the disease heritability is still missing, as it is not within reach of current GWAS approaches. Future efforts should target the missing heritability in common disease and the identification of rarer gene variants with hopefully clinically relevant effect sizes that at least theoretically can be captured by next generation sequencing approaches seem nearest. In addition, some of the heritability in migraine may be explained by rare copy number variations as seems the case for neuropsychiatric disorders, such as autism spectrum disorder.[123] Defining intermediate and/or functional phenotypes, such as an altered response to a migraine trigger like nitric oxide or defining endophenotypes using biomarker identification strategies like ^{1}H-NMR spectroscopy should be tried as well. Finally, one has to take into account mechanisms such as epistasis, gene-environment interactions, and epigenetics. The potential of epistasis has not yet been investigated in migraine also because of substantially increased multiple-testing burden. Gene-environment interactions also have been understudied in GWAS, primarily due to lack of data on environmental exposures, which would require large prospective cohorts. Finally, epigenetic modifications, i.e. modifications to the genome other than changes in the DNA sequence, such as DNA methylation and histone modifications, may affect disease susceptibility by influencing gene expression, e.g. after exposure to environmental triggers.

Clearly the genetics in cluster headache is less advanced than in migraine. Genetic research in cluster headache is hampered by the combination of low disease prevalence and the putative complex genetic nature of the disorder. Although our LUCA population is the current largest DNA collection of cluster headache patients worldwide, collaboration with other groups investigating this disease is needed to obtain valid results. It is unclear whether GWAS in CH are feasible given the difficulties to collect sufficiently large sample sets for the discovery and replication studies. Perhaps exome sequencing should be tried when one assumes that a limited number of rare variants with high individual effect size underlie susceptibility to cluster headache, which is unclear at this moment.

One of the most challenging problems in genetic research of primary headaches, perhaps more so in migraine than in cluster headache, is the large clinical heterogeneity resulting from the ICHD-2 consensus criteria. More research efforts should go towards the identification of reliable disease biomarkers, which are currently lacking for all primary headaches. A first attempt to use ^{1}H-NMR spectroscopy is this thesis revealed a metabolic

signature in serum, which suggests that a neurological disorder can lead to metabolic changes detectable in blood in our study that in its current state is, at best, hypothesis-generating. A similar study performed using the ERF population as a validation study led to the identification of a metabolite score associated with coronary heart disease independent of traditional cardiovascular risk factors[124] thereby illustrating that metabolic profiling can be a promising tool for identifying novel pathways involved in the pathophysiology of diseases. Still, it would be interesting to perform biomarker discovery studies for brain diseases such as primary headaches in cerebrospinal fluid as this body fluid is more directly affected. As long as no biomarker for migraine exists, other strategies to reduce clinical heterogeneity remain an attractive strategy. In conclusion, genetic research has led to the discovery of the first genetic risks factors for migraine and more genes for primary headache disorders will be discovered in the coming years. Collaborative efforts of clinicians, geneticists, bio-informaticians and molecular biologists are needed to unravel novel pathophysiological mechanisms that may find new treatment options for these highly disabling disorders.

References

1. Headache classification subcommittee of the International Headache Society. The International Classification of Headache Disorders: 2nd edition. *Cephalalgia* 2004; 24 Suppl 1: 9-160.
2. The International Classification of Headache Disorders, 3rd edition (beta version). *Cephalalgia* 2013; 33: 629-808.
3. Nesbitt AD and Goadsby PJ. Cluster headache. *BMJ* 2012; 344: e2407.
4. Rainero I, Gallone S, Valfre W et al. A polymorphism of the hypocretin receptor 2 gene is associated with cluster headache. *Neurology* 2004; 63: 1286-1288.
5. Schurks M, Kurth T, Geissler I et al. Cluster headache is associated with the G1246A polymorphism in the hypocretin receptor 2 gene. *Neurology* 2006; 66: 1917-1919.
6. Baumber L, Sjostrand C, Leone M et al. A genome-wide scan and HCRTR2 candidate gene analysis in a European cluster headache cohort. *Neurology* 2006; 66: 1888-1893.
7. Rainero I, Rubino E, Valfre W et al. Association between the G1246A polymorphism of the hypocretin receptor 2 gene and cluster headache: a meta-analysis. *J Headache Pain* 2007; 8: 152-156.
8. Thomsen LL, Eriksen MK, Roemer SF et al. A population-based study of familial hemiplegic migraine suggests revised diagnostic criteria. *Brain* 2002; 125: 1379-1391.
9. Ducros A, Denier C, Joutel A et al. The clinical spectrum of familial hemiplegic migraine associated with mutations in a neuronal calcium channel. *N Engl J Med* 2001; 345: 17-24.
10. Catterall WA. Structure and function of neuronal Ca2+ channels and their role in neurotransmitter release. *Cell Calcium* 1998; 24: 307-323.
11. Westenbroek RE, Sakurai T, Elliott EM et al. Immunochemical identification and subcellular distribution of the alpha 1A subunits of brain calcium channels. *J Neurosci* 1995; 15: 6403-6418.
12. De Fusco M, Marconi R, Silvestri L et al. Haploinsufficiency of ATP1A2 encoding the Na+/K+ pump alpha2 subunit associated with familial hemiplegic migraine type 2. *Nat Genet* 2003; 33: 192-196.
13. Yu FH, Mantegazza M, Westenbroek RE et al. Reduced sodium current in GABAergic interneurons in a mouse model of severe myoclonic epilepsy in infancy. *Nat Neurosci* 2006; 9: 1142-1149.
14. Ogiwara I, Miyamoto H, Morita N et al. Na(v)1.1 localizes to axons of parvalbumin-positive inhibitory interneurons: a circuit basis for epileptic seizures in mice carrying an Scn1a gene mutation. *J Neurosci* 2007; 27: 5903-5914.
15. van den Maagdenberg AM, Pietrobon D, Pizzorusso T et al. A Cacna1a knockin migraine mouse model with increased susceptibility to cortical spreading depression. *Neuron* 2004; 41: 701-710.
16. van den Maagdenberg AM, Pizzorusso T, Kaja S et al. High cortical spreading depression sus-

ceptibility and migraine-associated symptoms in Ca(v)2.1 S218L mice. *Ann Neurol* 2010; 67: 85-98.

17. Ophoff RA, Terwindt GM, Vergouwe MN et al. Familial hemiplegic migraine and episodic ataxia type-2 are caused by mutations in the Ca2+ channel gene CACNL1A4. *Cell* 1996; 87: 543-552.
18. Zhuchenko O, Bailey J, Bonnen P et al. Autosomal dominant cerebellar ataxia (SCA6) associated with small polyglutamine expansions in the alpha 1A-voltage-dependent calcium channel. *Nat Genet* 1997; 15: 62-69.
19. Takahashi M, Obayashi M, Ishiguro T et al. Cytoplasmic Location of alpha1A Voltage-Gated Calcium Channel C-Terminal Fragment (Cav2.1-CTF) Aggregate Is Sufficient to Cause Cell Death. *PLoS One* 2013; 8: e50121.
20. Capendeguy O and Horisberger JD. Functional effects of Na+,K+-ATPase gene mutations linked to familial hemiplegic migraine. *Neuromolecular Med* 2004; 6: 105-116.
21. Koenderink JB, Zifarelli G, Qiu LY et al. Na,K-ATPase mutations in familial hemiplegic migraine lead to functional inactivation. *Biochim Biophys Acta* 2005; 1669: 61-68.
22. Segall L, Scanzano R, Kaunisto MA et al. Kinetic alterations due to a missense mutation in the Na,K-ATPase alpha2 subunit cause familial hemiplegic migraine type 2. *J Biol Chem* 2004; 279: 43692-43696.
23. Segall L, Mezzetti A, Scanzano R et al. Alterations in the alpha2 isoform of Na,K-ATPase associated with familial hemiplegic migraine type 2. *Proc Natl Acad Sci U S A* 2005; 102: 11106-11111.
24. Tavraz NN, Friedrich T, Durr KL et al. Diverse functional consequences of mutations in the Na+/K+-ATPase alpha2-subunit causing familial hemiplegic migraine type 2. *J Biol Chem* 2008; 283: 31097-31106.
25. Tavraz NN, Durr KL, Koenderink JB et al. Impaired plasma membrane targeting or protein stability by certain ATP1A2 mutations identified in sporadic or familial hemiplegic migraine. *Channels (Austin)* 2009; 3: 82-87.
26. Schoenen JE, Ambrosini A and de Noordhout AM. Single-fiber EMG in familial hemiplegic migraine. *Neurology* 2005; 64: 1666-7; author reply 1666-7.
27. Bassi MT, Bresolin N, Tonelli A et al. A novel mutation in the ATP1A2 gene causes alternating hemiplegia of childhood. *J Med Genet* 2004; 41: 621-628.
28. Vanmolkot KR, Kors EE, Hottenga JJ et al. Novel mutations in the Na+, K+-ATPase pump gene ATP1A2 associated with familial hemiplegic migraine and benign familial infantile convulsions. *Ann Neurol* 2003; 54: 360-366.
29. Todt U, Dichgans M, Jurkat-Rott K et al. Rare missense variants in ATP1A2 in families with clustering of common forms of migraine. *Hum Mutat* 2005; 26: 315-321.
30. Swoboda KJ, Kanavakis E, Xaidara A et al. Alternating hemiplegia of childhood or familial hemiplegic migraine? A novel ATP1A2 mutation. *Ann Neurol* 2004; 55: 884-887.

31. Castro MJ, Stam AH, Lemos C et al. First mutation in the voltage-gated Nav1.1 subunit gene SCN1A with co-occurring familial hemiplegic migraine and epilepsy. *Cephalalgia* 2009; 29: 308-313.
32. Dichgans M, Freilinger T, Eckstein G et al. Mutation in the neuronal voltage-gated sodium channel SCN1A in familial hemiplegic migraine. *Lancet* 2005; 366: 371-377.
33. Gargus JJ and Tournay A. Novel mutation confirms seizure locus SCN1A is also familial hemiplegic migraine locus FHM3. *Pediatr Neurol* 2007; 37: 407-410.
34. Vahedi K, Depienne C, Le Fort D et al. Elicited repetitive daily blindness: a new phenotype associated with hemiplegic migraine and SCN1A mutations. *Neurology* 2009; 72: 1178-1183.
35. Vanmolkot KR, Babini E, de Vries B et al. The novel p.L1649Q mutation in the SCN1A epilepsy gene is associated with familial hemiplegic migraine: genetic and functional studies. Mutation in brief #957. Online. *Hum Mutat* 2007; 28: 522.
36. Cestele S, Labate A, Rusconi R et al. Divergent effects of the T1174S SCN1A mutation associated with seizures and hemiplegic migraine. *Epilepsia* 2013;
37. Frosk P, Mhanni AA and Rafay MF. SCN1A mutation associated with intractable myoclonic epilepsy and migraine headache. *J Child Neurol* 2013; 28: 389-391.
38. Le Fort D, Safran AB, Picard F et al. Elicited repetitive daily blindness: a new familial disorder related to migraine and epilepsy. *Neurology* 2004; 63: 348-350.
39. Escayg A and Goldin AL. Sodium channel SCN1A and epilepsy: mutations and mechanisms. *Epilepsia* 2010; 51: 1650-1658.
40. Kahlig KM, Rhodes TH, Pusch M et al. Divergent sodium channel defects in familial hemiplegic migraine. *Proc Natl Acad Sci U S A* 2008; 105: 9799-9804.
41. Cestele S, Scalmani P, Rusconi R et al. Self-limited hyperexcitability: functional effect of a familial hemiplegic migraine mutation of the Nav1.1 (SCN1A) Na+ channel. *J Neurosci* 2008; 28: 7273-7283.
42. de Vries B, Callenbach PM, Kamphorst JT et al. PRRT2 mutation causes benign familial infantile convulsions. *Neurology* 2012; 79: 2154-2155.
43. de Vries B, Stam AH, Beker F et al. CACNA1A mutation linking hemiplegic migraine and alternating hemiplegia of childhood. *Cephalalgia* 2008; 28: 887-891.
44. Verret S and Steele JC. Alternating hemiplegia in childhood: a report of eight patients with complicated migraine beginning in infancy. *Pediatrics* 1971; 47: 675-680.
45. Heinzen EL, Swoboda KJ, Hitomi Y et al. De novo mutations in ATP1A3 cause alternating hemiplegia of childhood. *Nat Genet* 2012; 44: 1030-1034.
46. Rosewich H, Thiele H, Ohlenbusch A et al. Heterozygous de-novo mutations in ATP1A3 in patients with alternating hemiplegia of childhood: a whole-exome sequencing gene-identification study. *Lancet Neurol* 2012; 11: 764-773.
47. Ishii A, Saito Y, Mitsui J et al. Identification of ATP1A3 mutations by exome sequencing as the cause of alternating hemiplegia of childhood in Japanese patients. *PLoS One* 2013; 8:

e56120.
48. Rotstein M, Doran J, Yang H et al. Glut1 deficiency and alternating hemiplegia of childhood. *Neurology* 2009; 73: 2042-2044.
49. De Vivo DC, Trifiletti RR, Jacobson RI et al. Defective glucose transport across the blood-brain barrier as a cause of persistent hypoglycorrhachia, seizures, and developmental delay. *N Engl J Med* 1991; 325: 703-709.
50. Brockmann K. The expanding phenotype of GLUT1-deficiency syndrome. *Brain Dev* 2009; 31: 545-552.
51. Leen WG, Klepper J, Verbeek MM et al. Glucose transporter-1 deficiency syndrome: the expanding clinical and genetic spectrum of a treatable disorder. *Brain* 2010; 133: 655-670.
52. Lee HY, Huang Y, Bruneau N et al. Mutations in the novel protein PRRT2 cause paroxysmal kinesigenic dyskinesia with infantile convulsions. *Cell Rep* 2012; 1: 2-12.
53. Guerrini R and Mink JW. Paroxysmal disorders associated with PRRT2 mutations shake up expectations on ion channel genes. *Neurology* 2012; 79: 2086-2088.
54. Cloarec R, Bruneau N, Rudolf G et al. PRRT2 links infantile convulsions and paroxysmal dyskinesia with migraine. *Neurology* 2012; 79: 2097-2103.
55. Scheffer IE, Grinton BE, Heron SE et al. PRRT2 phenotypic spectrum includes sporadic and fever-related infantile seizures. *Neurology* 2012; 79: 2104-2108.
56. Marini C, Conti V, Mei D et al. PRRT2 mutations in familial infantile seizures, paroxysmal dyskinesia, and hemiplegic migraine. *Neurology* 2012; 79: 2109-2114.
57. Gardiner AR, Bhatia KP, Stamelou M et al. PRRT2 gene mutations: from paroxysmal dyskinesia to episodic ataxia and hemiplegic migraine. *Neurology* 2012; 79: 2115-2121.
58. Riant F, Roze E, Barbance C et al. PRRT2 mutations cause hemiplegic migraine. *Neurology* 2012; 79: 2122-2124.
59. Castiglioni C, Lopez I, Riant F et al. PRRT2 mutation causes paroxysmal kinesigenic dyskinesia and hemiplegic migraine in monozygotic twins. *Eur J Paediatr Neurol* 2012;
60. Pelzer N, de Vries B, Kamphorst JT et al. PRRT2 and hemiplegic migraine: A complex association. *Neurology* 2014;
61. de Groen PC, Barry JA and Schaller WJ. Applying World Wide Web technology to the study of patients with rare diseases. *Ann Intern Med* 1998; 129: 107-113.
62. Lenert LA, Looman T, Agoncillo T et al. Potential validity of conducting research on headache in internet populations. *Headache* 2002; 42: 200-203.
63. Strom L, Pettersson R and Andersson G. A controlled trial of self-help treatment of recurrent headache conducted via the Internet. *J Consult Clin Psychol* 2000; 68: 722-727.
64. Hagen K, Zwart JA, Vatten L et al. Head-HUNT: validity and reliability of a headache questionnaire in a large population-based study in Norway. *Cephalalgia* 2000; 20: 244-251.
65. Cady RK, Borchert LD, Spalding W et al. Simple and efficient recognition of migraine with 3-question headache screen. *Headache* 2004; 44: 323-327.

66. Anttila V, Stefansson H, Kallela M et al. Genome-wide association study of migraine implicates a common susceptibility variant on 8q22.1. *Nat Genet* 2010; 42: 869-873.
67. Freilinger T, Anttila V, de Vries B et al. Genome-wide association analysis identifies susceptibility loci for migraine without aura. *Nat Genet* 2012; 44: 777-782.
68. Anttila V, Winsvold BS, Gormley P et al. Genome-wide meta-analysis identifies new susceptibility loci for migraine. *Nat Genet* 2013;
69. Manolio TA, Collins FS, Cox NJ et al. Finding the missing heritability of complex diseases. *Nature* 2009; 461: 747-753.
70. Bertina RM, Koeleman BP, Koster T et al. Mutation in blood coagulation factor V associated with resistance to activated protein C. *Nature* 1994; 369: 64-67.
71. Ionita-Laza I, Buxbaum JD, Laird NM and Lange C. A new testing strategy to identify rare variants with either risk or protective effect on disease. *PLoS Genet* 2011; 7: e1001289.
72. de Vries B, Frants RR, Ferrari MD and van den Maagdenberg AM. Molecular genetics of migraine. *Hum Genet* 2009; 126: 115-132.
73. Chasman DI, Schurks M, Anttila V et al. Genome-wide association study reveals three susceptibility loci for common migraine in the general population. *Nat Genet* 2011; 43: 695-698.
74. Eising E, de Vries B, Ferrari MD et al. Pearls and pitfalls in genetic studies of migraine. *Cephalalgia* 2013; 33: 614-625.
75. Teer JK and Mullikin JC. Exome sequencing: the sweet spot before whole genomes. *Hum Mol Genet* 2010; 19: R145-51.
76. Dunn WB, Broadhurst D, Begley P et al. Procedures for large-scale metabolic profiling of serum and plasma using gas chromatography and liquid chromatography coupled to mass spectrometry. *Nat Protoc* 2011; 6: 1060-1083.
77. Kudrow L and Kudrow DB. Inheritance of cluster headache and its possible link to migraine. *Headache* 1994; 34: 400-407.
78. Leone M, Russell MB, Rigamonti A et al. Increased familial risk of cluster headache. *Neurology* 2001; 56: 1233-1236.
79. Russell MB, Andersson PG and Thomsen LL. Familial occurrence of cluster headache. *J Neurol Neurosurg Psychiatry* 1995; 58: 341-343.
80. El Amrani M, Ducros A, Boulan P et al. Familial cluster headache: a series of 186 index patients. *Headache* 2002; 42: 974-977.
81. Russell MB. Epidemiology and genetics of cluster headache. *Lancet Neurol* 2004; 3: 279-283.
82. Sjaastad O and Salvesen R. Cluster headache: are we only seeing the tip of the iceberg? *Cephalalgia* 1986; 6: 127-129.
83. Schuh-Hofer S, Meisel A, Reuter U and Arnold G. Monozygotic twin sisters suffering from cluster headache and migraine without aura. *Neurology* 2003; 60: 1864-1865.
84. Couturier EG, Hering R and Steiner TJ. The first report of cluster headache in identical twins. *Neurology* 1991; 41: 761.

85. Eadie MJ and Sutherland JM. Migrainous neuralgia. *Med J Aust* 1966; 1: 1053-1057.
86. Roberge C, Bouchard JP, Simard D and Gagne R. Cluster headache in twins. *Neurology* 1992; 42: 1255-1256.
87. Sjaastad O, Shen JM, Stovner LJ and Elsas T. Cluster headache in identical twins. *Headache* 1993; 33: 214-217.
88. Svensson D, Ekbom K, Pedersen NL et al. A note on cluster headache in a population-based twin register. *Cephalalgia* 2003; 23: 376-380.
89. Shimomura T, Kitano A, Marukawa H et al. Point mutation in platelet mitochondrial tRNA(Leu(UUR)) in patient with cluster headache. *Lancet* 1994; 344: 625.
90. Cortelli P, Zacchini A, Barboni P et al. Lack of association between mitochondrial tRNA(Leu(UUR)) point mutation and cluster headache. *Lancet* 1995; 345: 1120-1121.
91. Seibel P, Grunewald T, Gundolla A et al. Investigation on the mitochondrial transfer RNA(Leu)(UUR) in blood cells from patients with cluster headache. *J Neurol* 1996; 243: 305-307.
92. Haan J, van Vliet JA, Kors EE et al. No involvement of the calcium channel gene (CACNA1A) in a family with cluster headache. *Cephalalgia* 2001; 21: 959-962.
93. Sjostrand C, Giedratis V, Ekbom K et al. CACNA1A gene polymorphisms in cluster headache. *Cephalalgia* 2001; 21: 953-958.
94. Schurks M, Neumann FA, Kessler C et al. MTHFR 677C>T polymorphism and cluster headache. *Headache* 2011; 51: 201-207.
95. Bigal ME, Serrano D, Buse D et al. Acute migraine medications and evolution from episodic to chronic migraine: a longitudinal population-based study. *Headache* 2008; 48: 1157-1168.
96. Antonaci F, Nappi G, Galli F et al. Migraine and psychiatric comorbidity: a review of clinical findings. *J Headache Pain* 2011;
97. Ligthart L, Nyholt DR, Penninx BW and Boomsma DI. The shared genetics of migraine and anxious depression. *Headache* 2010; 50: 1549-1560.
98. Schur EA, Noonan C, Buchwald D et al. A twin study of depression and migraine: evidence for a shared genetic vulnerability. *Headache* 2009; 49: 1493-1502.
99. Stam AH, de Vries B, Janssens AC et al. Shared genetic factors in migraine and depression: evidence from a genetic isolate. *Neurology* 2010; 74: 288-294.
100. Ligthart L, Nyholt DR, Hottenga JJ et al. A genome-wide linkage scan provides evidence for both new and previously reported loci influencing common migraine. *Am J Med Genet B Neuropsychiatr Genet* 2008; 147B: 1186-1195.
101. Schurks M, Rist PM, Bigal ME et al. Migraine and cardiovascular disease: systematic review and meta-analysis. *BMJ* 2009; 339: b3914.
102. Spector JT, Kahn SR, Jones MR et al. Migraine headache and ischemic stroke risk: an updated meta-analysis. *Am J Med* 2010; 123: 612-624.
103. Kruit MC, van Buchem MA, Hofman PA et al. Migraine as a risk factor for subclinical brain lesions. *JAMA* 2004; 291: 427-434.

104. Bigal ME, Kurth T, Santanello N et al. Migraine and cardiovascular disease: A population-based study. *Neurology* 2010; 74: 628-635.
105. Kurth T, Gaziano JM, Cook NR et al. Migraine and risk of cardiovascular disease in women. *JAMA* 2006; 296: 283-291.
106. Lauritzen M. Pathophysiology of the migraine aura. The spreading depression theory. *Brain* 1994; 117: 199-210.
107. Hadjikhani N, Sanchez Del Rio M, Wu O et al. Mechanisms of migraine aura revealed by functional MRI in human visual cortex. *Proc Natl Acad Sci U S A* 2001; 98: 4687-4692.
108. Tietjen EG. Migraine and ischaemic heart disease and stroke: potential mechanisms and treatment implications. *Cephalalgia* 2007; 27: 981-987.
109. Diener HC, Kurth T and Dodick D. Patent foramen ovale and migraine. *Curr Pain Headache Rep* 2007; 11: 236-240.
110. Tepper SJ, Sheftell FD and Bigal ME. The patent foramen ovale-migraine question. *Neurol Sci* 2007; 28 Suppl 2: S118-23.
111. Tietjen GE, Al-Qasmi MM, Athanas K et al. Altered hemostasis in migraineurs studied with a dynamic flow system. *Thromb Res* 2007; 119: 217-222.
112. Vanmolkot FH, Van Bortel LM and de Hoon JN. Altered arterial function in migraine of recent onset. *Neurology* 2007; 68: 1563-1570.
113. Tietjen GE. The role of the endothelium in migraine. *Cephalalgia* 2011; 31: 645-647.
114. Scher AI, Terwindt GM, Verschuren WM et al. Migraine and MTHFR C677T genotype in a population-based sample. *Ann Neurol* 2006; 59: 372-375.
115. Bigal ME, Kurth T, Hu H et al. Migraine and cardiovascular disease: possible mechanisms of interaction. *Neurology* 2009; 72: 1864-1871.
116. Kurth T. Associations between migraine and cardiovascular disease. *Expert Rev Neurother* 2007; 7: 1097-1104.
117. Scher AI, Terwindt GM, Picavet HS et al. Cardiovascular risk factors and migraine: the GEM population-based study. *Neurology* 2005; 64: 614-620.
118. Winsvold BS, Hagen K, Aamodt AH et al. Headache, migraine and cardiovascular risk factors: The HUNT study. *European Journal of Neurology* 2011; 18: 504-511.
119. Schwaiger J, Kiechl S, Stockner H et al. Burden of atherosclerosis and risk of venous thromboembolism in patients with migraine. *Neurology* 2008; 71: 937-943.
120. Kurth T, Ridker PM and Buring JE. Migraine and biomarkers of cardiovascular disease in women. *Cephalalgia* 2008; 28: 49-56.
121. Kurth T, Schurks M, Logroscino G et al. Migraine, vascular risk, and cardiovascular events in women: prospective cohort study. *BMJ* 2008; 337: a636.
122. Benseñor IM, Goulart AC, Lotufo PA et al. Cardiovascular risk factors associated with migraine among the elderly with a low income: The São Paulo Ageing & Health Study (SPAH). *Cephalalgia* 2011; 31: 331-337.

123. Sebat J, Lakshmi B, Malhotra D et al. Strong association of de novo copy number mutations with autism. *Science* 2007; 316: 445-449.
124. Vaarhorst AA, Verhoeven A, Weller CM et al. A metabolomic profile is associated with the risk of incident coronary heart disease. *Am Heart J* 2014; 168: 45-52.e7.

Summary

Claudia M. Weller

MIGRAINE IS A HIGHLY PREVALENT, disabling brain disorder affecting approximately 15% of the population. Cluster headache is another primary headache disorder with a lifetime prevalence of 0.12%. Despite the availability of prophylactic and acute therapies, treatment is not effective in many patients with these headache disorders. There is a clear need to develop better treatments but this is hampered by a lack of knowledge of the underlying molecular disease mechanisms. The research described in this thesis was aimed at elucidating some of the molecular genetic mechanisms of these two headache disorders by means of clinical and genetic studies in complex and/or monogenic forms of the disease and in related disorders. Hopefully, knowledge that will come from studies like these can be used to the benefit of patients by improving clinical diagnoses and/or by providing useful drug targets for future drug development strategies.

Chapters 2 and 3 and an additional genetic study from our group describe four novel gene mutations in patients with pure familial hemiplegic migraine (FHM) (**Chapter 2**), a mixed phenotype of hemiplegic migraine and alternating hemiplegia of childhood (AHC) (**Chapter 3**), and benign familial infantile convulsions (additional study). In **Chapter 2** the identification is described of p.Ile1498Met p.Phe1661Leu missense mutations in the *SCN1A* gene in two Spanish families with FHM. These mutations, only the sixth and seventh FHM-causing mutations in this gene thus far, underline the importance of *SCN1A* testing in FHM families. Mutation carriers in these two families showed pure hemiplegic migraine with large variability in attack severity and frequency, which is in agreement with earlier findings in patients with other FHM mutations, be it in this gene or the other two hemiplegic migraine genes. Although no functional characterization of the mutation has been performed, the fact that the p.Ile1498Met mutation affects the important and evolutionary well-conversed so-called IFMT motif of the $Na_V1.1$ channel, and thus likely affects the inactivation properties of the channel, seems to add to existing evidence from functional studies on other FHM-causing *SCN1A* mutations that decreased channel activity is the most likely cause of FHM3 patients. The majority of the mutations in *SCN1A* are however not associated with FHM, but instead lead to severe childhood epilepsy. How particular mutations in this gene lead to one of these two different allelic disorders remains to large extent a mystery. In **Chapter 3** the involvement of two genes previously implicated in AHC in patients with hemiplegic migraine is investigated. No mutations were identified in the *ATP1A3* gene, the gene previously was found to be the major genetic factor causing AHC, but an *SLC2A1* mutation was found in a severely affected patient with hemiplegic migraine, intellectual disability, seizures and exercise-induced dystonia. Mutations of the glucose transporter 1 that is encoded by *SLC2A1* had been shown to lead to low glucose concentrations in the brain and leading to a wide spectrum of paroxysmal and permanent neurological symptoms. Our patient expands

the phenotypic spectrum with hemiplegic migraine attacks. In the additional genetic study we report a common heterozygous truncating mutation in the *PRRT2* gene in three families with BFIC, an autosomal dominantly inherited epilepsy syndrome with onset between 3 and 12 months of age. Similar truncating mutations in the *PRRT2* gene, which codes for proline-rich transmembrane protein 2, were first identified in patients with paroxysmal kinesigenic dyskinesia with or without infantile convulsions. With this study the phenotypic spectrum of *PRRT2* mutations is extended to benign familial infantile convulsions without associated paroxysmal dyskinesia. Although the exact function of the protein is still unknown, it is speculated that PRRT2, through binding to synaptic protein SNAP25, may be involved in vesicle docking and calcium-triggered neuronal exocytosis. Mutant PRRT2 protein may result in abnormal neurotransmitter release and neuronal hyperexcitability. This functional phenotype resembles the hyperexcitability phenotype seen with FHM mutations, in particular in FHM1. One can speculate whether *PRRT2* mutations are also found in FHM, which would provide an explanation why BFIC and FHM can co-occur in patients. **Chapters 2 and 3** and the additional genetic study together show that there seems to be a spectrum of clinical phenotypes, related to (common) migraine, that are associated with mutations in the *SLC2A1*, *ATP1A3*, *SCN1A* and *PRRT2* genes.

Chapters 4 and 5 describe the validation of our recruitment strategy for patients with migraine and cluster headache that can be used, and has been used in the case of migraine, for large-scale genetic and clinical studies. In **Chapter 4** we reported on the successful recruitment of some 2,400 self-reported migraine patients for our Leiden University Medical Centre Neuro-Analysis program (LUMINA) study. Good correlation was shown of our questionnaire-based migraine diagnoses with interview diagnoses. Virtually all patients meeting the screening criteria had a migraine diagnosis, but prediction of aura status by the ICHD-2 criteria-based algorithm was less accurate. To construct a prediction rule for aura status, 33 items were selected from the LUMINA questionnaire and a stepwise forward logistic regression analysis was run. A seven-question subset provided higher sensitivity, slightly lower specificity, and similar positive predictive value in assessing aura when comparing with the ICHD-2-based algorithm. The migraine questionnaire has been successfully used already in recent genome-wide association studies (GWAS) in which LUMINA samples played an important role in the identification of migraine susceptibility gene variants.

In **Chapter 5** a similar project named the Leiden University Cluster headache Analysis (LUCA) program is presented, launched for recruitment of self-reported cluster headache patients for genetic and clinical studies. For this study, 437 self-reported cluster headache

patients meeting our screening criteria were recruited. Semi-structured telephone interviews revealed a high positive predictive value of an algorithm-based cluster headache diagnosis. Despite its high accuracy, the LUCA questionnaire is too long to be applied in large-scale population-based studies focusing on multiple disorders at the same time. Therefore, the shorter Quick Ascertainment of Cluster Headache (QATCH) questionnaire was developed by incorporating the items with highest positive predictive value and positive likelihood ratio in a stepwise forward logistic regression model. The QATCH questionnaire contains three questions and indicates that men with headache attacks of short duration and long headache-free intervals are very likely to have CH.

In **Chapter 6** the LUCA study from **Chapter 5** was used for the largest candidate-gene association study in cluster headache thus far. The association was re-evaluated of single nucleotide polymorphism (SNP) rs2653349 in the *HCRTR2* gene that changes amino acid 1246 of the hypocretin receptor type 2 from a glycine residue into an alanine residue. Two small earlier studies had indicated that *HCRTR2* may be a genetic risk factor for cluster headache, although a third study was negative. No support for *HCRTR2* as a susceptibility gene in cluster headache was found by testing the SNP in our LUCA population. Adding the LUCA study to existing data sets in a subsequent meta-analysis, which doubled in sample size, showed, however, suggestive association. A subsequent meta-analysis including our LUCA study and without the initial study, that showed a very large protective effect (odds ratio of 0.27, which is equivalent to an odds ratio of 3.7 in case of a susceptibility-increasing variant), still showed a significance association, although the odds ratio was more modest (0.80 (equivalent to an odds ratio of 1.25 for a susceptibility-increasing variant)). Although our study could not define whether *HCRTR2* should be disregarded as a promising susceptibility gene for cluster headache, one could argue that the outcome of combining small candidate gene association studies in a meta-analysis, even if a significant result is obtained, should be treated with great caution.

Chapter 7 describes a study in the genetically isolated population from the Erasmus Rucphen Family (ERF) study that consists of 3,465 living descendants of 22 couples who had at least six children baptized in the community church between 1850 and 1900 and from whom extensive clinical and genetic data is available. In this population 360 migraine patients and 1,291 subjects without severe headache were identified. The study aimed to investigate whether atherosclerosis explains the increased risk of ischemic stroke, myocardial infarction and peripheral arterial disease that is frequently reported in epidemiological and clinical studies on migraine. The degree of atherosclerosis was evaluated by

using three non-invasive measurements of atherosclerosis but no difference was found between patients and controls. It was therefore concluded that increased atherosclerosis is an unlikely explanation for the higher rates of cardiovascular disease in migraine patients.

In **Chapter 8** the study in ERF was extended by focussing on metabolic profiling in serum using proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy. It was investigated whether a set of compounds could be identified that was associated with a migraine diagnosis. The study showed significant association for 14 metabolites: leucine, isoleucine, methionine, proline, serine, valine, dimethylglycine, glucose, 1,5-anhydrosorbitol, lipids (CH_3) / cholesterol, lipids (CH_2), lipids (CH_2CO), creatinine and pyruvate. Notably, these metabolites were associated with an active migraine status, i.e. patients having migraine in the last year, not with migraine *per se*, and provide a good starting point for further research into metabolic changes in migraine patients. Although currently not suitable for application in clinical practice, it is a proof of concept showing that a metabolite fingerprint of migraine status may be worth looking into in the search for a migraine biomarker.

Nederlandse samenvatting

Claudia M. Weller

MIGRAINE IS EEN ERNSTIGE HERSENAANDOENING die vaak voorkomt in de algemene bevolking met een prevalentie van circa 15%. Clusterhoofdpijn is ook een primair hoofdpijnsyndroom maar is veel zeldzamer dan migraine en treft slechts 0.12% van de bevolking op enig moment. Behandeling van deze hoofdpijnvormen met de momenteel beschikbare acute medicatie en profylaxe is ineffectief gebleken bij veel patiënten. Er is derhalve een duidelijke behoefte aan betere behandelmethoden, maar de ontwikkeling hiervan wordt gehinderd door het beperkte inzicht in de moleculaire mechanismen die aan deze ziekten ten grondslag liggen. Het onderzoek in dit proefschrift was gericht op het vergroten van het inzicht in de moleculair-genetische mechanismen die betrokken zijn bij het ontstaan van migraine en clusterhoofdpijn door middel van klinische en genetische studies in complexe en/of monogene vormen van beide ziekten en daaraan gerelateerde aandoeningen. Het uiteindelijke doel is dat kennis opgedaan uit dergelijke studies gebruikt kan worden voor het bevorderen van de zorg voor hoofdpijnpatiënten door verbeterde klinische diagnostiek en/of door het identificeren van nieuwe aangrijpingspunten voor toekomstige ontwikkeling van medicatie.

Hoofdstukken 2 en 3 en een aanvullende genetische studie beschrijven vier nieuwe genetische mutaties in patiënten met pure familiaire hemiplegische migraine (FHM) (**hoofdstuk 2)**, een gecombineerd fenotype van hemiplegische migraine en alternerende hemiplegie op de kinderleeftijd [alternating hemiplegia of childhood (AHC)] (**hoofdstuk 3**) en benigne familiaire infantiele convulsies [(BFIC); aanvullende studie]. In hoofdstuk 2 wordt de identificatie van de nieuwe *SCN1A* mutaties p.Ile1498Met en p.Phe1661Leu in twee Spaanse FHM-families beschreven. Deze mutaties zijn pas de zesde en zevende FHM-mutatie in dit gen en onderstrepen het belang van het onderzoek naar *SCN1A*-mutaties in de DNA-diagnostiek voor FHM. Mutatiedragers in deze twee families hadden pure familiare hemiplegische migraine, maar de frequentie en ernst van de aanvallen wisselde sterk. Deze grote mate van variabiliteit komt overeen met bevindingen bij eerdere FHM-mutaties in zowel het *SCN1A*-gen als in de twee andere FHM-genen. Ondanks dat er geen functionele studies werden verricht lijkt mutatie p.Ile1498Met additioneel bewijs te leveren voor de gangbare hypothese dat verminderde activiteit van het $Na_V1.1$-kanaal het mechanisme is dat tot ziekte leidt in patiënten met *SCN1A*-mutaties, omdat deze mutatie het belangrijke, evolutionair sterk geconserveerde IFMT-motief van dit kanaal verandert en daarmee waarschijnlijk de inactivatie van het kanaal verstoort. Verreweg de meeste *SCN1A*-mutaties veroorzaken echter geen FHM, maar ernstige vormen van epilepsie vanaf de kinderleeftijd. Het is nog grotendeels onduidelijk waarom bepaalde mutaties in dit gen leiden tot FHM en andere tot ernstige epilepsiesyndromen. In **hoofdstuk 3** wordt onderzocht of twee genen die eerder in verband zijn gebracht met AHC ook een rol spelen bij patiënten met hemiplegische migraine. Er werden geen

mutaties aangetroffen in het *ATP1A3*-gen, het gemuteerde gen bij een ruime meerderheid van de AHC-patiënten, maar er werd wel een *SLC2A1*-mutatie gevonden in een ernstig aangedane patiënt met hemiplegische migraine, verstandelijke beperking, epilepsie en door inspanning geïnduceerde dystonie. Mutaties in de door *SLC2A1* gecodeerde glucose transporter 1 leiden tot lage glucoseconcentraties in het brein en werden al eerder in verband gebracht met een breed spectrum van paroxysmale en permanente neurologische symptomen. Onze patiënt breidt dit spectrum verder uit met hemiplegische migraine en door inspanning geïnduceerde dystonie. De aanvullende genetische studie beschrijft een veel voorkomende truncerende mutatie in het *PRRT2*-gen in drie families met BFIC, een autosomaal dominant overervend epilepsiesyndroom waarvan de eerste symptomen optreden bij kinderen tussen de drie en twaalf maanden oud. Vergelijkbare truncerende mutaties in het *PRRT2*-gen, coderend voor het prolinerijk transmembraaneitwit 2, waren al eerder geïdentificeerd in patiënten met paroxysmale kinesigene dyskinesie met of zonder infantiele convulsies. Met deze studie werd het fenotypische spectrum van *PRRT2*-mutaties uitgebreid met benigne familiaire infantiele convulsies zonder geassocieerde paroxysmale dyskinesie. De exacte functie van PRRT2 is onbekend, maar de hypothese is dat het eiwit door binding aan het synaptische eiwit SNAP25 is betrokken bij aankoppeling van vesikels en calcium-geïnduceerde neuronale exocytose. Het mutante PRRT2-eiwit zou kunnen leiden tot aberrant gereguleerde afgifte van neurotransmitters en neuronale hyperexcitatie. Er kan worden gespeculeerd over het voorkomen van *PRRT2*-mutaties bij FHM-patiënten, wat zou kunnen verklaren waarom BFIC en FHM gezamenlijk kunnen voorkomen in dezelfde patiënt. De **hoofdstukken 2 en 3** en de aanvullende genetische studie illustreren gezamenlijk een spectrum van klinische fenotypes die zijn gerelateerd aan migraine en worden veroorzaakt door mutaties in de *SLC2A1*, *ATP1A3*, *SCN1A* en *PRRT2*-genen.

De **hoofdstukken 4** en **5** beschrijven de validatie van onze wervingsstrategie voor een grote groep van patiënten met migraine en clusterhoofdpijn die gebruikt kunnen worden (en voor migraine al gebruikt zijn) voor grootschalige genetische en klinische studies. In **hoofdstuk 4** rapporteren we de werving van circa 2400 zelfverklaarde migrainepatiënten voor onze Leiden University Medical Centre Neuro-analysis (LUMINA) studie. Er bleek een goede correlatie te bestaan tussen de op de vragenlijst gebaseerde migrainediagnoses en de diagnoses gesteld tijdens telefonische interviews. Vrijwel alle patiënten die voldeden aan de screeningscriteria bleken ook inderdaad migraine te hebben, maar de voorspelling van aurastatus op basis van de ICHD2-criteria was minder accuraat. Om een predictieregel voor aurastatus te ontwikkelen selecteerden we 33 vragen uit de LUMINA-vragenlijst voor een stapsgewijze logistische regressieanalyse. Dit resulteerde in een selectie van zeven vragen met hogere sensitiviteit, iets lagere specificiteit en vergelijkbare positief voorspellende

waarde voor aurastatus in vergelijking met het op de ICHD-2-criteria gebaseerde algoritme. De migrainevragenlijst is al met succes toegepast in recente genoomwijde associatiestudies waarin de DNA-monsters uit de LUMINA-studie een belangrijke rol hebben gespeeld bij de identificatie van meerdere genetische risicofactoren voor migraine.

In **hoofdstuk 5** presenteren we een vergelijkbaar project getiteld de Leiden University Cluster headache Analysis (LUCA) studie. Dit project werd gestart voor het recruteren van zelfverklaarde patiënten met clusterhoofdpijn voor genetische en klinische studies. Voor de validatiestudie werden 437 patiënten geworven die voldeden aan de screeningscriteria. De op de vragenlijst gebaseerde diagnose toonde een hoge positief voorspellende waarde voor de diagnose in een semigestructureerd telefonisch interview. Ondanks de accuratesse is de lengte van de LUCA-vragenlijst een probleem bij toepassing in grootschalige studies die zich richten op het gelijktijdig bestuderen van meerdere ziekten in dezelfde populatie. Daarom ontwikkelden we een korte vragenlijst door analyse van de vragen met de hoogste positief voorspellende waarden en hoogste positieve likelihood ratios in een stapsgewijze logistische regressieanalyse. Deze zogenaamde QATCH-vragenlijst bevat drie onderdelen en suggereert dat mannen met kortdurende hoofdpijn en langdurige pijnvrije intervallen een hoge kans hebben om clusterhoofdpijn te hebben.

In **hoofdstuk 6** gebruiken we de populatie uit de LUCA-studie uit **hoofdstuk 5** voor de grootste kandidaatgenstudie tot dusverre uitgevoerd in het onderzoek naar clusterhoofdpijn. We evalueerden de eerder gerapporteerde associatie van clusterhoofdpijn met het polymorfisme rs2653349 in het *HCRTR2*-gen. Dit polymorfisme verandert aminozuur 1246 van de hypocretinereceptor type 2 van een glycine naar een alanine. Twee eerdere kleine studies suggereerden dat dit polymorfisme het risico op het krijgen van clusterhoofdpijn beïnvloedt, maar een derde studie was negatief. Toen we de associatie onderzochten in onze LUCA-populatie werd er geen associatie gevonden. Vervolgens verrichtten we een nieuwe meta-analyse waarin de LUCA-populatie werd toegevoegd aan de eerder gepubliceerde populaties, waardoor de studie in omvang verdubbelde. De meta-analyse suggereerde wel dat er associatie bestond tussen dit polymorfisme en clusterhoofdpijn. Vervolgens werd een meta-analyse verricht zonder de initiële studie, waarin een zeer groot effect (odds ratio (OR) 0.27, corresponderend met een OR van 3.7 in geval van een risicoverhogend effect) werd gevonden. De OR nam daarop af tot 0.80 (equivalent aan OR 1.25 in geval van een risicoverhogend effect) maar de associatie bleef significant. Hoewel door de discrepantie in resultaten niet kon worden uitgesloten dat dit polymorfisme in *HCRTR2* het risico op clusterhoofdpijn beïnvloedt kan wel gesteld worden dat de resultaten van een meta-analyse op basis van kleine studies ook bij een significante associatie met grote voorzichtigheid moeten worden geïnterpreteerd.

Hoofdstuk 8 beschrijft een studie in de genetisch geïsoleerde Erasmus Rucphen Familie (ERF) studie, welke bestaat uit 3465 levende nakomelingen van 22 koppels die tussen 1850 en 1900 tenminste zes kinderen lieten dopen in de dorpskerk en van wie er uitgebreide klinische en genetische data beschikbaar is. In deze populatie werden 360 migrainepatiënten en 1291 controlepersonen zonder ernstige hoofdpijn geïdentificeerd. De in dit hoofdstuk beschreven studie was erop gericht om te onderzoeken of atherosclerose de verklaring vormt voor het frequent in de literatuur gerapporteerde verhoogde risico van migrainepatiënten op herseninfarcten, hartinfarcten en perifeer arterieel vaatlijden. De mate van atherosclerose werd beoordeeld door middel van drie niet-invasieve metingen, waarbij geen verschillen werden gevonden tussen de patiënten en controles. We concludeerden daarom dat atherosclerose een onwaarschijnlijke verklaring vormt voor het verhoogde risico op cardiovasculaire risico bij migrainepatiënten.

In **hoofdstuk 8** werd dezelfde ERF-populatie gebruikt voor een studie naar de metabolieten in het serum van migrainepatiënten en controles door gebruik te maken van waterstof kernspinresonantie spectroscopie (in het Engels proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy). Gebruikmakend van deze techniek werd gezocht naar een set van metabolieten geassocieerd met migraine status. De studie toonde significante associatie voor 14 metabolieten: leucine, isoleucine, methionine, proline, serine, valine, dimethylglycine, glucose, 1,5-anhydrosorbitol, CH_3-lipiden / cholesterol, CH_2-lipiden, CH_2CO-lipiden, creatinine en pyruvaat. Deze metabolieten waren geassocieerd met *actieve* migraine status, gedefinieerd als migraine optredend in het afgelopen jaar, en niet met een migraine diagnose op zich. Deze set metabolieten vormt een goed vertrekpunt voor verder onderzoek naar metabole veranderingen in migrainepatiënten. Hoewel deze metabolietenset niet geschikt is voor klinische toepassing toont de studie wel aan dat de methode geschikt kan zijn voor het zoeken naar een mogelijke metabole signatuur als biomarker voor migraine.

List of abbreviations

^{1}H-NMR	proton nuclear magnetic resonance (spectroscopy)
ABI	ankle-brachial index
AHC	alternating hemiplegia of childhood
BFIC	benign familial infantile convulsions
CADASIL	cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
CD-CV	common disease – common variant (hypothesis)
CD-RV	common disease – rare variant (hypothesis)
CHD	coronary heart disease
CPMG	Carr-Purcell-Meiboom-Gill (spectrum)
CSD	cortical spreading depression
CSF	cerebrospinal fluid
DNA	deoxyribonucleic acid
EA2	episodic ataxia type 2
ERF	Erasmusm Rucphen Family (study)
FHM	familial hemiplegic migraine
FRS-CHD	Framingham risk score for coronary heart disease
FRS-Stroke	Framingham risk score for ischemic stroke
GEFS+	generalised epilepsy with febrile seizures+
GLUT1DS	glucose transporter 1 deficiency (syndrome)
GWAs	genome-wide association studies
HMDB	Human Metabolome Database
ICHD-2	International Classification of Headache Disorders, 2nd edition
ICHD-3beta	International Classification of Headache Disorders, 3nd edition, beta
IHS	International Headache Society
IMT	intima-media thickness
JRES	J-resolved spectrum
LUCA	Leiden University Cluster headache Analysis (program)
LUMINA	Leiden University Migraine Neuro-Analysis (program)
MA	migraine with aura
MELAS	mitochondrial encephalopathy with lactic acidosis and stroke-like episodes
MO	migraine without aura
MRI	magnetic resonance imaging
NMR	nuclear magnetic resonance (spectroscopy)
OR	odds ratio
PCR	polymerase chain reaction
PWV	pulse-wave velocity

QATCH	quick ascertainment of cluster headache (questionnaire)
RVCL	retinal vasculopathy with cerebral leukodystrophy
SCA6	spinocerebellar ataxia type 6
SD	standard deviation
SHM	sporadic hemiplegic migraine
SMEI	severe myoclonic epilepsy of infancy
SNP	single nucleotide polymorphism
WT	wild-type

List of publications

List of publications

Weller CM*, Wilbrink LA*, Houwing-Duistermaat JJ, Koelewijn SC, Vijfhuizen LS, Haan J, Ferrari MD, Terwindt GM, Van den Maagdenberg, AMJM*, De Vries, B*. *Cluster headache and the hypocretin receptor 2 reconsidered: a genetic association study and meta-analysis*. Cephalalgia. 2014 Nov. (*Equal author contribution).

Weller CM*, Leen WG*, Neville BG, Duncan JS, Vries B de, Geilenkirchen MA, Haan J, Kamsteeg EJ, Ferrari MD, van den Maagdenberg AM, Willemsen MA, Scheffer H, Terwindt GM. *A novel SLC2A1 mutation linking hemiplegic migraine with alternating hemiplegia of childhood.* Cephalalgia. 2015 Jan;35(1):10-5. (*Equal author contribution).

Vaarhorst AAM*, Verhoeven A*, **Weller CM***, Bohringer S, Goraler S, Meissner A, Deelder AM, Henneman P, Gorgels APM, Van den Brandt PA, Schouten LJ, Van Greevenbroek MM, Merry AHH, Verschuren WMM, Van den Maagdenberg AMJM, Willems van Dijk K, Isaacs A, Boomsma D, Oostra BA, Van Duijn CM, Jukema JW, Boer JMA, Fesken E, Heijmans BT, Slagboom PE. *A metabolomics profile is associated with the risk of incident coronoray heart disease.* Am Heart J. 2014 Jul;168(1):45-52. (*Equal author contribution).

Weller CM*, Pelzer N*, de Vries B, López MA, De Fàbregues O, Pascual J, Arroyo MA, Koelewijn SC, Stam AH, Haan J, Ferrari MD, Terwindt GM, van den Maagdenberg AM. *Two novel SCN1A mutations identified in families with familial hemiplegic migraine.* Cephalalgia. 2014; 34(13):1062-9. (*Equal author contribution).

Wilbrink LA, **Weller CM**, Cheung C, Haan J, Ferrari MD. *Cluster-tic syndrome: a cross-sectional study of cluster headache patients.* Headache. 2013 Sep;53(8):1334-40.

Anttila V, Winsvold BS, Gormley P, Kurth T, Bettella F, McMahon G, Kallela M, Malik R, de Vries B, Terwindt G, Medland SE, Todt U, McArdle WL, Quaye L, Koiranen M, Ikram MA, Lehtimäki T, Stam AH, Ligthart L, Wedenoja J, Dunham I, Neale BM, Palta P, Hamalainen E, Schürks M, Rose LM, Buring JE, Ridker PM, Steinberg S, Stefansson H, Jakobsson F, Lawlor DA, Evans DM, Ring SM, Färkkilä M, Artto V, Kaunisto MA, Freilinger T, Schoenen J, Frants RR, Pelzer N, **Weller CM**, Zielman R, Heath AC, Madden PA, Montgomery GW, Martin NG, Borck G, Göbel H, Heinze A, Heinze-Kuhn K, Williams FM, Hartikainen AL, Pouta A, van den Ende J, Uitterlinden AG, Hofman A, Amin N, Hottenga JJ, Vink JM, Heikkilä K, Alexander M, Muller-Myhsok B, Schreiber

S, Meitinger T, Wichmann HE, Aromaa A, Eriksson JG, Traynor BJ, Trabzuni D, Rossin E, Lage K, Jacobs SB, Gibbs JR, Birney E, Kaprio J, Penninx BW, Boomsma DI, van Duijn C, Raitakari O, Jarvelin MR, Zwart JA, Cherkas L, Strachan DP, Kubisch C, Ferrari MD, van den Maagdenberg AM, Dichgans M, Wessman M, Smith GD, Stefansson K, Daly MJ, Nyholt DR, Chasman DI, Palotie A; North American Brain Expression Consortium; UK Brain Expression Consortium; International Headache Genetics Consortium. *Genome-wide meta-analysis identifies new susceptibility loci for migraine.* Nat Genet. 2013 Aug;45(8):912-7.

Wilbrink LA*, **Weller CM*,** Cheung C, Stijnen T, Haan J, Ferrari MD, Terwindt GM. *Stepwise web-based questionnaires for diagnosing cluster headache: LUCA and QATCH.* Cephalalgia. 2013 Aug;33(11):924-31. (*Equal author contribution).

Dibbens LM, de Vries B, Donatello S, Heron SE, Hodgson BL, Chintawar S, Crompton DE, Hughes JN, Bellows ST, Klein KM, Callenbach PM, Corbett MA, Gardner AE, Kivity S, Iona X, Regan BM, **Weller CM**, Crimmins D, O'Brien TJ, Guerrero-López R, Mulley JC, Dubeau F, Licchetta L, Bisulli F, Cossette P, Thomas PQ, Gecz J, Serratosa J, Brouwer OF, Andermann F, Andermann E, van den Maagdenberg AM, Pandolfo M, Berkovic SF, Scheffer IE. *Mutations in DEPDC5 cause familial focal epilepsy with variable foci.* Nat Genet. 2013 May;45(5):546-51.

Stam AH*, **Weller CM***, Janssens AC, Aulchenko YS, Oostra BA, Frants RR, van den Maagdenberg AM, Ferrari MD, van Duijn CM, Gisela MT. *Migraine is not associated with enhanced atherosclerosis.* Cephalalgia. 2013 Mar;33(4):228-35. (*Equal author contribution).

Wilbrink LA, Cheung C, **Weller CM**, Ferrari MD, Haan J. *Aurasymptomen bij clusterhoofdpijn: Resultaten van een deelonderzoek in de LUCA substudie.* Ned Tijdschr Geneeskd. 2013;157(1):A5306.

Koutsopoulos OS, Kretz C, **Weller CM**, Roux A, Mojzisova H, Böhm J, Koch C, Toussaint A, Heckel E, Stemkens D, Ter Horst SA, Thibault C, Koch M, Mehdi SQ, Bijlsma EK, Mandel JL, Vermot J, Laporte J. *Dynamin 2 homozygous mutation in humans with a lethal congenital syndrome.* Eur J Hum Genet. 2013 Jun;21(6):637-42.

Weller CM, De Vries B, Terwindt GM, Ferrari MD, Haan J, Van den Maagdenberg AMJM. *Migraine Genes – Clinical and Preclinical Persepectives.* Book chapter for The Migraine Brain – Imaging Structure and Function. Oxford University Press 2012.

De Vries B, Callenbach PM, Kamphorst JT, **Weller CM**, Koelewijn SC, ten Houten R, de Coo IF, Brouwer OF, van den Maagdenberg AM. *PRRT2 mutation causes benign familial infantile convulsions.* Neurology. 2012 Nov 20;79(21):2154-5.

Van Oosterhout WP, **Weller CM**, Stam AH, Bakels F, Stijnen T, Ferrari MD, Terwindt GM. *Validation of the web-based LUMINA questionnaire for recruiting large cohorts of migraineurs.* Cephalalgia. 2011 Oct;31(13):1359-67.

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, Palotie A; International Headache Genetics Consortium. *Genome-wide association study of migraine implicates a common susceptibility variant on 8q22.1.* Nat Genet. 2010 Oct;42(10):869-73.

Curriculum Vitae

Claudia Mandina Weller werd geboren op 19 april 1983 te 's-Gravenhage. In 2001 behaalde zij cum laude haar VWO-diploma aan het Alfrink College te Zoetermeer. In datzelfde jaar begon zij met de studie Geneeskunde aan de Universiteit Leiden. Het propedeutisch examen behaalde zij in 2002. Gedurende haar studie was zij werkzaam als ECG-laborant en gaf zij als student-assistent werkgroepen aan jongerejaars studenten. Daarnaast was zij als secretaris van de jaarvertegenwoordiging betrokken bij de evaluatie van het aangeboden onderwijs.

In 2005 startte zij met de coassistentschappen en ontving een beurs van de Stichting Werkgelegenheid Geneeskundigen (SWG) om gedurende vier maanden coschappen te lopen in het Horacio E. Oduber Hospitaal in Oranjestad, Aruba. In 2007 volgde zij gedurende vier maanden de semi-artsstage op de afdeling Klinische Genetica van het Leids Universitair Medisch Centrum.

Haar wetenschappelijke stage deed zij gedurende zes maanden bij de afdeling Klinische Genetica van het Leids Universitair Medisch Centrum in samenwerking met de afdeling Moleculaire Celbiologie van het Instituut Biologie Leiden onder leiding van Dr. S.A.J. Lesnik Oberstein en professor H.P. Spaink. In 2007 kreeg zij een beurs van de Danish Research Foundation voor deelname aan de International Summer School in Functional Genomics aan het Wilhelm Johannsen Centre for Functional Genome Research van de University of Copenhagen. In februari 2008 behaalde zij het doctoraalexamen (cum laude) en het artsexamen.

Van januari tot september 2008 werkte zij als arts-assistent op de afdeling Klinische Genetica. Per 1 juli van datzelfde jaar startte zij met haar promotieonderzoek naar de genetica van migraine en clusterhoofdpijn op de afdelingen Humane Genetica en Neurologie van het Leids Universitair Medisch Centrum onder supervisie van professor A.M.J.M. van den Maagdenberg, professor M.D. Ferrari en Dr. G.M. Terwindt. Vanaf 2013 combineerde zij de afronding van het onderzoek beschreven in dit proefschrift met een baan als jeugdarts bij de GGD Hollands Midden en als arts-assistent bij het Rijnlands Revalidatie Centrum in Leiden. Vanaf januari 2015 is zij werkzaam als transfusiearts in opleiding bij Sanquin Bloedvoorziening in Leiden.