GENETICS OF MIGRAINE AND RELATED SYNDROMES

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CHAPTER 1

GENERAL INTRODUCTION & SCOPE OF THE THESIS

1.1 CLINICAL CHARACTERISTICS OF MIGRAINE

Migraine with and without aura

Migraine is a common, disabling, episodic neurovascular headache disorder. The disease is characterized by recurrent attacks of headaches and associated autonomic and neurological symptoms. Migraine can be divided in two major subtypes: migraine without aura (MO) and migraine with aura (MA). MO attacks are typically characterized by recurrent, unilateral, pulsating headaches of moderate to severe intensity, lasting 4-72 hours that are aggravated by physical activity and often accompanied with nausea and/or vomiting, phonophobia and photophobia. Roughly one-third of migraine patients suffer from MA. MA is discriminated from MO by the presence of transient focal neurological symptoms that accompany the headache attacks. Aura symptoms usually last between 5 and 60 minutes and nearly always include visual symptoms (for an example see Figure 1). Less often sensory symptoms (i.e., paraesthesia) or dysphasic speech disturbances are experienced and, rarely motor symptoms.[1,2] Of all migraineurs, 33% have both types of migraine attacks.[3] Patients with MA suffer from less severe headache, some even may have attacks of migraine aura without headache. Prior to the aura and headache phase migraine patients may experience premonitory symptoms, such as mood disturbances, autonomic symptoms and concentration problems.[4] This premonitory phase can last up to 24 hours. Most frequently reported triggers for migraine attacks are too much or too little sleep, weather changes, missing a meal, certain food triggers (e.g., wine, chocolate, or cheese) and menstruation.[5] Some patients perceive higher mental stress levels before a migraine attack, however evidence suggests that this is more likely part of the premonitory phase of the migraine attack than a trigger.[6] Not the migraine attack itself, but the recurrence of attacks is clinically relevant and defines a migraine patient (according to ICHD-2 and 3 criteria: MO after 5 attacks without an aura and MA after 2 attacks with an aura).[7,8] As biomarkers for migraine are currently lacking, a diagnosis is based on a questionnaire and/or interview using diagnostic criteria that were provided by the International Headache Society in 1988; and in revised form in 2004 and 2013 (Table 1).[7,8]

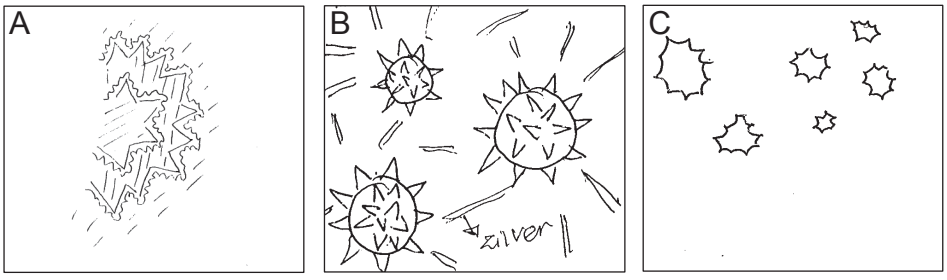

Figure 1. Drawings of visual aura symptoms by migraine patients from the Erasmus Rucphen Family (ERF) study. (**A**) Before the headache starts this patient experiences flashing star-shaped features that gradually increase and eventually cover one hemifield. These features last 10-30 minutes. (**B**) This patient describes the background to fade during minutes after which she sees dark-brown to black stars surrounded by silver colored rays lasting 10-15 minutes. After these visual disturbances the headache starts. (**C**) Before the headache this patient sees radar wheels which are present for 10-15 minutes.

Table 1. Diagnostic criteria for migraine with and without aura and familial hemiplegic migraine (ICHD-3 *beta*)[8]

Migraine without aura
A. At least 5 attacks fulfilling criteria B-D
B. Headache attacks lasting 4-72 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 diagnosis.
Migraine with aura
A. At least 2 attacks fulfilling criteria B and 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 four characteristics:
1. at least one aura symptom spreads gradually over ≥ 5 minutes, and/or two or more symptoms occur in succession
2. each individual aura symptom lasts 5-60 minutes
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 ischemic attack has been excluded.
Hemiplegic migraine
A. At least 2 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 symptom spreads gradually over ≥ 5 minutes, and/or two or more 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 or by another ICHD-3 diagnosis, and transient ischaemic attack and stroke have been excluded.

Familial and Sporadic Hemiplegic Migraine

Hemiplegic migraine (HM) is a rare autosomal dominantly inherited subtype of migraine with aura that is characterized by transient hemiparesis during the attacks (Table 1).[8] Two types of hemiplegic migraine are recognized. In Familial Hemiplegic Migraine (FHM) there

is at least one first- or second-degree family member with attacks of hemiplegic migraine.[7,8] In Sporadic Hemiplegic Migraine (SHM), the family history is negative for patients with hemiplegic attacks. FHM and SHM attacks are clinically indistinguishable,[9] suggesting a common pathophysiological basis. An extensive epidemiological Danish population-based study investigated the prevalence of hemiplegic migraine, as well as the headache and aura characteristics, in comparison to those in MA.[10-12] The prevalence of both hemiplegic migraine types is 0.01%.[10] The mean age at onset is around 17 years (95% CI: 15-18; range 1-45 years), which is lower than for familial MA, which on average starts at 21 years.[11,12] Besides motor aura symptoms, FHM patients can have sensory (98%), visual (89%) and aphasic (72%) symptoms. Furthermore, 69% of FHM patients has co-occurrence with Basilar Migraine (BM).[11,13] Basilar-type migraine symptoms include dysarthria, vertigo, visual symptoms simultaneously in temporal and nasal fields of both eyes, ataxia, decreased level of consciousness and bilateral paraesthesias.[7,8] In three-quarter of FHM patients, the sequence of aura symptoms in FHM is the following: visual - sensory - motor - aphasic - basilar-type.[11] Apart from the motor aura symptoms, which are only experienced by FHM patients, the composition of symptoms and the order of appearance during an attack are very similar for FHM and MA.[11,12] However, FHM patients are more likely to experience two or more aura symptoms, the duration of symptoms is longer for FHM, the presence of BM symptoms only co-occurs with FHM, and the presence of headache more often accompanies FHM than MA.[11,12] Severe atypical FHM attacks may be prolonged (up to six weeks) and accompanied by confusion, decreased consciousness, fever, seizures, and even coma.[14] A minor head trauma is reported in 9% of FHM patients as the initiating event.[11,14] In addition, cerebral or coronary angiography can trigger attacks.[14]

1.2 THE MIGRAINE SPECTRUM

Clinical similarities indicate that a migraine spectrum exists ranging from MO to MA and FHM. Arguments for this spectrum are: i) apart from the hemiparesis, the aura and headache characteristics of FHM and MO/MA are similar; ii) within FHM families, family members can have attacks of MA and/or MO; iii) about two-third of FHM patients also has non-hemiplegic migraine attacks.[15] Thus, it can be hypothesized that FHM genes and their pathways also provide insight in the common forms of migraine.

1.3 WHAT HAPPENS DURING A MIGRAINE ATTACK?

Traditionally, two hypotheses exist regarding the etiology of migraine: the *neuronal* hypothesis and the *vascular* hypothesis.[16] In the classical, purely, vascular hypothesis the migraine aura is caused by intracerebral vasoconstriction, whereas the headache is attributed to (rebound) vasodilatation of cerebral and meningeal blood vessels.[17] This hypothesis has nowadays been abandoned.[18] Instead, migraine is now considered a primary brain disorder with neuronal events affecting blood vessels, i.e., a neurovascular disorder.

The aura is thought to be caused by a phenomenon termed Cortical Spreading Depression (CSD),[19] a brief wave (lasting seconds) of intense neuronal and glial depolarization that

slowly (2-5 mm/min) propagates across the cerebral cortex.[19,20] This would account for the spreading character and propagation rate of the aura symptoms. The wave is followed by a relatively long-lasting (≥ 20 min) neuronal suppression, which would explain the occurrence of positive and negative phenomena in aura symptoms.[21] Functional neuroimaging studies revealed similarities between blood flow changes in patients during visual aura's and CSD in experimental animals suggesting that CSD indeed occurs in humans.[22]

The origin of the headache is considered to be related to a dysfunction of certain brainstem nuclei.[23] Whether the first neurologic event in migraine is CSD or brainstem dysfunction is still a point of controversy and the two theories may not be mutually exclusive. However, in both scenarios, vasodilatation of cerebral and meningeal vessels, if present, is considered a secondary phenomenon occurring after activation of the trigeminovascular system (TGVS).[24] How does this system works? The TGVS consists of the cranial blood vessels, innervated by sensory afferent fibres of the ophthalmic division of the trigeminal nerve. Activation of these fibres leads to activation of second-order neurons in the trigeminal nucleus caudalis (TNC) and the two uppermost levels of the spinal cord dorsal horn, together termed the trigeminocervical complex. Impulses are relayed further forward to several thalamic nuclei, the ventrolateral area of the caudal periaquaductal grey region and the cerebral cortex, where the pain sensation is registered.

On a molecular level, activation of trigeminovascular efferents leads to the release of vasoactive neuropeptides (e.g., Calcitonin Gene Related Peptide (CGRP), Substance P, and Nitric Oxide (NO)), which are believed to cause neurogenic inflammation, central pain transmission, and headache. The role of neurogenic inflammation has never been demonstrated in patients, but in experimental animal models of migraine neurogenic inflammation of the dura and around the meningeal vessels has clearly been shown.[25] These experiments also provided evidence that CSD can activate the TGVS and induces vasodilatation of the middle meningeal artery.[25] A role for vasodilatation in migraine in humans is further supported by the fact that specific antimigraine drugs, the triptans and ergotamine, next to deactivation of the TGVS and inhibition of the release of vasoactive neuropeptides from perivascular nerve terminals, have a vasoconstrictive effect.[24] Recent studies have casted considerable doubt on whether our idea about the role of vasodilatation in migraine is correct. For instance, CGRP antagonists do not have a vasoconstrictive effect, but are effective in the treatment of migraine.[26] A study using a sensitive 3 Tesla MRA-technique, failed to show *in vivo* cerebral and meningeal vasodilatation in humans during migraine headache.[27] Nevertheless, although this study did not provide evidence for vasodilatation of *large* meningeal and cerebral vessels during migraine headache, it does not rule out a role for *small* cerebral vessels.

1.4 EPIDEMIOLOGY OF MIGRAINE

Clinical epidemiology

The prevalence of migraine is more or less similar over Western countries, but varies for age and sex. The one-year prevalence of migraine varies between 17.1-25.0% in women and 5.6-7.5% in men.[28,29] Onset of migraine is in more than 90% of patients below the age of 50. Peak age of

onset is 10-12 years old for males and 14-16 years old for females, although attacks may start at any age.[28,30] The average female-to-male ratio is 3:1. The prevalence of migraine increases with age with a male preponderance in children under 12 and female preponderance at later ages. The one-year prevalence in women peaks at 35-40 years (33%) and then declines. In men, the one-year prevalence peaks between 50-55 years (15%).[28] Among active migraineurs the median attack frequency is 1-1.5 per month and the median attack duration is 24 hours.[28,30] Approximately 5% of the general population have at least 18 days of migraine per year and over 1% have at least 1 day of migraine per week.[24] Each year, approximately 2.5% of patients with episodic migraine develop new-onset chronic migraine (with more than half of the days per month headache).[31]

Migraine greatly affects the quality of life[32] and is rated by WHO among the most disabling disorders.[33] It is the most costly neurological disorder in the EU.[34] Current acute migraine therapies are far from optimal as not all patients respond to acute therapies and headache recurrence is a common problem. In general, the efficacy of migraine prophylactic drugs is limited. At most, 50% will have a 50% reduction in attack frequency.[35] In addition, currently available prophylactic drugs have a large risk of causing adverse effects. Therefore, better, especially preventative treatment options are clearly needed. In order to identify novel treatment targets it is important to unravel the molecular biological mechanisms involved in migraine. Identification of migraine genes may provide more insight into these mechanisms.

Genetic epidemiology

Studies in families, twins and the general population showed that genetic factors play an important role in the pathogenesis of migraine, probably by lowering the threshold for migraine attacks.[36,37] First-degree relatives of probands with MO have an increased risk for MO of 1.9 and for MA of 1.4 compared with the general population. First-degree relatives of probands with MA had a 3.8-fold increased risk for MA, but no increased risk for MO.[36] Early age at onset and migraine severity and disability appear to be predictors for familial aggregation.[38]

In twins, families, or large (isolated) populations the proportion of genetic involvement in a disease can be calculated as heritability. Heritability estimates the proportion of variability in a trait (i.e., the phenotypic variance) that can be attributed to additive genetic factors and shared early environmental factors. Additive genetic effects are the sum of the independent effect of alleles. Thus, non-additive genetic effects caused by interaction between alleles at the same locus (dominance) or at two different loci (epistasis) are not measured. Heritability estimates in twin[39-44] and family-based[45] studies range from 0.33-0.53 for migraine in general, and 0.61-0.77 and 0.65-0.79 for MO and MA separately.[39-45]

Studies based on the Danish Twin Registry provided information on specific concordance rates for MO and MA. Concordance rates for monozygotic twins for both MO (28% *vs* 18%, p=0.04) and MA (34% *vs* 12%, p=0.04) are significantly higher than for dizygotic twins, indicating the importance of genetic factors.[46,47] In a study that includes almost 30,000 twin pairs affected by migraine from six countries, monozygotic correlations were at least twice the size of dizygotic correlations, also indicating a contribution of genes to the liability of migraine.[42] As neither the heritability estimates nor the concordance rates in monozygotic twins reach 100%, part of the migraine phenotype must be explained by environmental factors.

These family and twin data show that genetic factors play a role in migraine, however from these data no conclusion can be drawn about the pattern of inheritance. A large Danish population-based segregation analysis showed that MO and MA most likely have a multifactorial inheritance pattern, with a combination of genetic and environmental factors.[45]

1.5. GENETIC FINDINGS IN COMMON FORMS OF MIGRAINE

The most widely used strategies to map genes for migraine are linkage analysis and candidate gene association analysis.[48] These methods are aimed at identifying genetic variants with different effects sizes and allelic frequencies. The linkage approach generally yields genes with a large effect size and a low population allele frequency, whereas candidate gene association studies test potential migraine gene variants with a low effect size and relatively high allele frequencies in the population.

Linkage studies

Linkage analysis requires DNA and clinical information of one or more (large) families containing multiple affected members, a genome wide scan (a narrow grid of polymorphic markers evenly spaced over the genome) and a statistical test (expressed as the Logarithm of the odds (LOD) score) to find out which markers are inherited along with the disease in the families. When a chromosomal region (locus) is transmitted with the disease phenotype within families, this region is likely to contain the gene of interest. Linkage analysis has been successful in FHM (where one gene with a large effect size is implicated in one family), where it lead to the identification of three genes.[49-51] For complex migraine (MO and MA) linkage analyses can also be performed, but the power is much more limited compared to monogenic disorders. The contribution of genes to the total disease risk is small or modest, making it hard to detect them. Linkage studies have identified migraine susceptibility loci on chromosomes Xq24-q28, 1q31, 4q21, 4q24, 6p12 -p21.1, 10q22-q23, 11q24, 14q21.2-q22.3, 15q11-q13 and 19p13.[52-64] With the exception of a few loci (4q21-q24 and 10q22-q23), none have been clearly replicated and for all migraine loci the causative gene still needs to be identified. Next to the small effect size of the variants involved, this is likely due to genetic heterogeneity but also clinical heterogeneity in the end-diagnosis of migraine.

Association studies

Association studies are aimed at detecting genetic variants that are more common in people with migraine than in unaffected persons, ideally from the same population, i.e., allele frequencies are compared. Association studies may be targeted to a biological candidate gene or performed with a genome wide scan. The genetic variants examined are usually single nucleotide polymorphisms (SNPs), DNA variants that represent variation in a single base.[48] Genetic association studies have greater power than linkage studies to detect genes with a small effect and require collection of large numbers of cases and controls, instead of large families. A significantly increased frequency of an allele of a polymorphism would suggest either that it directly affects the risk of migraine or that the polymorphism is located very close to the locus involved in the disorder and is transmitted with this disease locus (i.e., linkage disequilibrium

(LD), the non-random association of alleles at two or more loci on a chromosome that is gradually lost over generations by recombination). Alternatively, the association is false positive and due to some underlying stratification or admixture (substructure) of the population.

Although a large number of candidate gene association studies were performed in migraine, many are of limited value because of important limitations (e.g., small sample size and consequent low power to detect association, no correction for multiple testing, and/or no clear description of migraine subtypes). Association studies have investigated the possible association between migraine and polymorphisms in genes with a hypothesized function in migraine pathways. Amongst others, genes with a neurotransmitter function (mainly serotonin, dopamine) or involvement in hormonal, inflammatory or vascular pathways have been investigated (for review see de Vries et al., 2009).[65] The only variant to date that was found to be associated with migraine with aura in several, but not all[66-68] studies is the C677T polymorphism of the *MTHFR* gene (methylenetetrahydrofolate reductase).[69] At the start of this thesis, Genome Wide Association studies for migraine had not been published yet.

Genetic isolates

An alternative approach to study genetics of migraine is to make use of a genetically isolated population. Genetic isolates have several advantages compared to outbred populations. In an isolate genetic variability is reduced because a small number of founders, the occurrence of population "bottlenecks" (such as famine, wars, or infectious disease epidemics) and the absence of migration leading to more inbreeding and genetic drift.[70] This makes it more likely that patients in an isolate have a disease due to a similar underlying genetic defect that is inherited from a common ancestor. Isolates may vary in genetic diversity, depending on the extent of genetic drift and founder effects.[71] Another advantage of genetically isolated populations is that subjects generally have a more uniform lifestyle, culture and environment, which are therefore better controlled. This makes it more likely that a difference between healthy and diseased subjects reflects genetic effects instead of environmental effects. Extensive genealogical records allow reconstruction of extended pedigrees and the collection of unascertained phenotype data prevents selection bias for specific diseases. A disadvantage of isolates may be that some genetic variants are isolate-specific and findings cannot be extrapolated to the general population. However, a genome wide linkage study on depression in ERF showed that this does not account for all variants, as the results found in ERF could be replicated in an independent population.[72]

In this thesis, migraine is studied a Dutch genetic isolate (The Erasmus Rucphen Family (ERF) study). The advantage of the ERF isolate compared to the Finish and Icelandic isolates[73] is that it is a much younger isolate (i.e., 10-20 generations) with high levels of LD,[74] making it particularly useful in the mapping of complex diseases such as migraine.[75]

1.6 GENETIC FINDINGS IN MONOGENIC FORMS OF MIGRAINE

Two types of monogenic migraine forms exists. Monogenic FHM, a migraine subtype at the severe side of the migraine spectrum and monogenic migraine syndromes in which migraine is part of the clinical spectrum.

Genetic and clinical spectrum of FHM and the relation with migraine relevant diseases

FHM is genetically heterogeneous: three genes have been identified. Clinically, hemiplegic migraine attacks of the three subtypes (FHM1, FHM2, and FHM3) cannot be distinguished. FHM may present as 'pure' FHM or be associated with various comorbid diseases. As FHM families exist without mutations in any of the three FHM genes, at least a fourth and probably more FHM genes exist. All three FHM gene products are intimately involved in the modulation of ion transport across neuronal and glial cell membranes, suggesting that FHM, and possibly also common types of migraine, at least in part, are cerebral 'ionopathies'.

FHM1 is caused by mutations in the *CACNA1A* gene located on chromosome 19p13.[51] *CACNA1A* encodes the pore-forming α1 subunit of voltage-gated neuronal $Ca_V2.1$ (P/Q-type) calcium channels and is involved in the modulation of release of neurotransmitters at peripheral and central synapses. To date, over 50 *CACNA1A* mutations have been associated with a wide variety of symptoms. Almost twenty FHM1 gene mutations are known that represent a broad clinical spectrum. More than half of them are associated with cerebellar signs (i.e., slowly progressive ataxia, dysartria and gaze-evoked nystagmus).[14] About 20% of FHM1 families have cerebellar signs.[14] Epilepsy can also be part of the clinical spectrum of FHM1 mutation carriers.[76] Several *CACNA1A* mutations are associated with a decreased level of consciousness during FHM attacks[77-79] or even coma,[14,80-83] sometimes in combination with seizures.[80,83] *CACNA1A* mutations can also cause Episodic Ataxia type 2 (EA2) and Spinocerebellar Ataxia type 6 (SCA6).[51,84] Whereas FHM1 is caused by missense mutations, EA2 mutations mostly are nonsense, frameshift, or splice site,[51] and rarely missense mutations.[85] SCA6 is a so-called polyglutamine disorder caused by small expansions of a CAG repeat located in the distal end of the *CACNA1A* gene.[84] In rare cases, epilepsy (i.e., absence and generalized tonic-clonic seizures) has also been reported in EA2 patients.[86,87]

FHM2 is caused by mutations in the *ATP1A2* gene that is located on chromosome 1q23. *ATP1A2* encodes the α2-subunit of Na+/K+ pumps.[49] Sodium potassium ATPases are responsible for translocating sodium ions out of the cell, while they import potassium ions. The release of sodium ions provides a steep sodium gradient essential for the import of glutamate through glutamate transporters. Thus, ATPases, directly or indirectly, modulate re-uptake of potassium and glutamate from the synaptic cleft into glia cells. Over 35 *ATP1A2* mutations have been identified. With the exception of a few, FHM2 mutations are mostly missense mutations located in the large intracellular loop, which harbors important regulatory domains for ion transport. Although most FHM2 mutations are associated with pure FHM,[49,88-91] additional clinical features have also been reported in *ATP1A2* mutation carriers. In clear contrast to FHM1, cerebellar signs are very rare in FHM2.[92-94] Other FHM2 associated clinical features include, epilepsy,[49,95,96] permanent mental retardation,[95] prolonged hemiplegia,[97] coma,[90] and alternating hemiplegia of childhood (AHC). Alternating hemiplegia of childhood is a rare neurological disorder, with unknown etiology, that resembles FHM and initially was regarded as a migraine variant.[98] A link between FHM and alternating hemiplegia of childhood (AHC) was shown by the identification of an *ATP1A2* mutation in a Greek family with atypical AHC.[99,100] In contrast, the screening of the FHM genes *CACNA1A* and *ATP1A2* gene in a set

of *typical* AHC patients did not reveal any causal mutations.[101,102] *ATP1A2* mutations were also found in basilar-type migraine[103] and the common forms of migraine.[104]

FHM3 is caused by specific missense mutations in the *SCN1A* gene that is located on chromosome 2q24.[50] *SCN1A* encodes the α1-subunit of neuronal $Na_V1.1$ voltage-gated sodium channels that play an important role in the generation and propagation of action potentials. The FHM3 gene was originally identified in three related German families with the Q1489K mutation.[50] An association of *SCN1A* with FHM is rather surprising because over 150 mutations in this gene are known to cause childhood epilepsy (i.e., severe myoclonic epilepsy of infancy (SMEI) or generalized epilepsy with febrile seizures (GEFS+)).[105,106] With the identification of a second *SCN1A* L1649 mutation in an FHM family of North American decent, the link between *SCN1A* and FHM was firmly established.

FHM gene mutations can also be found in SHM patients, although the chance of identifying a causal mutation is much lower compared to FHM. Whereas in a Dutch clinical-based SHM sample of 39 SHM patients the prevalence of ATP1A2 mutations was 15 %, only in 1 % *ATP1A2* mutations were identified in 100 patients from a Danish population-based sample.[107,108] The prevalence of *CACNA1A* gene mutations in SHM patients lies between 1 and 5 %.[107-109] The chance of identifying an *ATP1A2* or *CACNA1A* mutation is further increased by early age at onset and presence of additional symptoms (such as ataxia or epilepsy).[110] No *SCN1A* mutations have been identified in SHM patients.[107]

Examples of monogenic syndromes associated with complex migraine

Migraine can also be part of the clinical spectrum of other monogenic syndromes. Hence, the identification of genes for these syndromes may further our understanding of the mechanisms involved in migraine. The monogenic syndromes CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy) and HVR (Hereditary Vascular Retinopathy) (later renamed RVCL (Retinal Vasculopathy with Cerebral Leukodystrophy) and CHARIOT (Cerebral Hereditary Angiopathy with Vascular Retinopathy and Impaired Organ Function caused by *TREX1* mutations)) are described below.

CADASIL

The clearest example of a monogenic syndrome in which migraine is prominent is Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL). CADASIL is an autosomal dominant late-onset arteriopathy that is clinically characterized by recurrent transient ischemic attacks (TIAs) and strokes and cognitive decline, psychiatric symptoms and dementia.[111] In about one-third of patients migraine with aura occurs, often as the presenting symptom several years before the onset of other symptoms.[112] CADASIL has distinct neuroradiological features that are usually manifest prior to the first stroke.[113,114] The most important features are white matter hyperintensities (WMH) and lacunar infarcts. WMHs are symmetrically distributed in the deep and periventricular white matter. Typical for CADASIL is the bilateral involvement of the anterior temporal lobes and external capsule. Histopathologically, CADASIL is characterized by the deposition of granular osmiophilic material (GOM) in the basement membrane and surrounding

extracellular matrix of vascular smooth muscle cells (VSMCs) as well as the degeneration and eventual disappearance of VSMCs. CADASIL is caused by mutations in the *NOTCH3* gene, located on chromosome 19q13.2-p13.1 encoding a cell surface receptor protein that, in human adult tissue, is solely expressed in vascular smooth muscle cells.[115,116] The pathophysiological mechanism that leads to increased aura prevalence in CADASIL is unknown, however increased CSD susceptibility, involvement of the migraine brainstem area or a direct shared genetic susceptibility have been suggested.[117]

Hereditary Vascular Retinopathy (later renamed RVCL and CHARIOT)

A second monogenic cerebrovascular syndrome where migraine is part of the clinical spectrum is hereditary vascular retinopathy (HVR).[118,119] HVR was reported in a large Dutch family and is primary characterized by a vascular retinopathy. Besides, migraine and Raynaud's phenomenon segregates in this family. HVR was mapped to chromosome 3p21.1-p21.3.[120] Genetic testing revealed that two additional families with overlapping clinical features were also linked to this locus: cerebroretinal vasculopathy (CRV)[121] and hereditary endotheliopathy with retinopathy, nephropathy and stroke (HERNS).[122] Although many genes in the linked region were sequenced, the causal gene had not been identified at the start of this thesis. Despite being linked to the same locus, there seemed a considerable variation in clinical symptoms between families. For example, nephropathy appeared specific for HERNS. The occurrence of cerebral mass lesions was reported in both the HERNS and CRV family, but not in the HVR family, where only a minor degree of cerebral white matter change was observed in some patients. Migraine was most prominent in the HVR family. The presence of different 3p21 haplotypes suggested that the clinical variation might be caused by different mutations in the same gene, although the presence of different retinopathy genes in the same region could not be excluded.[120] Two other families with a similar phenotype were also reported in the literature.[123,124] In a German case report in addition to retinopathy and an intracerebral mass lesion, the elevation of liver enzymes and colonic teleangiectasias was striking.[123] An Australian family presented with a clinical picture that resembled that of HERNS.[124] As migraine and Raynaud's phenomenon were also present in branches of the Dutch HVR family without retinopathy, one can speculate whether the genetic defect underlying the retinopathy may also (at least in part) be responsible for the increased prevalence of migraine and Raynaud's phenomenon. A genetic, family-based, association study demonstrated that the chromosome 3 locus indeed seem to enhance the susceptibility for Raynaud's phenomenon and migraine in the HVR family.[125]

1.7 COMPLEX MIGRAINE AND USE OF COMORBID DISEASES TO IDENTIFY GENETIC FACTORS

Research into comorbidity of the common forms of migraine may provide insight in shared epidemiological risk factors or pathophysiological mechanisms involved in both diseases. The term comorbidity refers to the greater than coincidental association of two conditions in the same individual.[126] Comorbidity itself is no evidence for a shared etiological background between

two diseases.[127] First of all, the association may be spurious, for example as a consequence of biased ascertainment or diagnostic uncertainty. Second, a simple unidirectional causal relationship may underlie the association. Third, shared environmental risk factors may play a major role. It can also be that comorbidity may be caused by a common genetic predisposition. Identification of such shared genetic risk factors may define previously unexpected biological pathways that link diseases together.[128] The study of comorbidity of migraine with and without aura may provide useful endophenotypes for genetic studies. Endophenotypes may assure more robust and consistent phenotyping of patients, decreasing clinical heterogeneity. Furthermore, endophenotypes may be helpful in the identification of genetic risk factors by reducing genetic heterogeneity. Migraine has been associated with a wide range of neurological, psychiatric and cardiovascular conditions.[126] A clear example is epilepsy, which is found to be comorbid with common migraine in population based studies,[129,130] and often co-occurs with FHM. A possible shared genetically determined pathophysiological pathway is suggested by the finding that many epilepsy genes as well as the known FHM genes encode ion transporters.[76,131] Besides epilepsy, in this thesis, depression and the association with cardiovascular diseases are under discussion.

Depression

Depression, like migraine, is a chronic episodic disorder. A depressive episode according to the DMS-IV criteria is characterized by a period of at least 14 days with depressed mood or loss of interest or pleasure combined with cognitive and vegetative symptoms leading to impairment in social, occupational or other important areas of functioning.[132] For clinical practice, various well validated screening instruments for depression have been developed that measure depressive symptoms, such as the Hospital Anxiety and Depression Scale (HADS) and the Centre for Epidemiological Studies Depression scale (CES-D).[133,134] These scales can be used for scientific purposes as well because they enable efficient assessment of depression in large populations.

Migraine and depression co-occur more frequently within subjects than to be expected by chance. Population-based studies range considerably with respect to the increased risk of depression in migraineurs, but in most studies odds ratios are at least doubled and consistently higher for patient with MA than for MO.

In migraineurs comorbid depression is often associated with chronification of migraine,[135,136] decreased quality of life[137] and the development of medication overuse headache.[135-137] The pathogenesis of both conditions, especially how episodes are being triggered and how symptoms may become chronified, is poorly understood. Interestingly, the comorbidity is bidirectional. Not only migraine patients have an increased risk to develop depression, but depressive patients also have an increased risk to develop migraine.[138] This bidirectional relationship suggests that migraine and depression share common etiological factors. This might be due to shared genetic factors, however this has never been investigated.

Cardiovascular disease

Several observational studies have shown that migraine, specifically MA, is a risk factor for ischemic stroke. A meta-analysis from 2005 of eleven retrospective case-control and three prospective cohort studies showed pooled increased relative risks of 1.83 (95% Confidence

Interval (CI) 1.06-3.15) for MO, and 2.27 (95% CI 1.61-3.19) for MA.[139] This risk was higher in young female migraineurs below 45 years old and highest when using oral contraceptives. Additional support for an association between migraine and ischemic stroke comes from imaging studies. In the population-based CAMERA study evidence was presented that MA patients have a fourteen-fold increased risk of silent infarct-like lesions in the posterior circulation territory of the brain (i.e., the cerebellar region), a risk which increases with increasing attack frequency.[140] The specific MRI characteristics of these lesions suggest an infarct origin.[141] Also, from the CAMERA study it became clear that in women with migraine the risk for deep white matter lesions is increased compared to controls, with no difference between MO and MA patients. This risk also increases with higher attack frequency. Whether vascular changes in migraine are restricted to the cerebral vasculature or also systemically present is not yet clear. Systemic vascular dysfunction is suggested by the association of migraine with an unfavorable cardiovascular risk factor profile,[142] prothrombotic and vasoactive factors,[143,144] and the relation with systemic endothelial dysfunction.[145] The reported association of migraine with ischemic heart disease points to systemic vascular dysfunction, although at the start of this thesis no large follow-up studies were performed and cross-sectional studies reported conflicting results.[146-150]

SCOPE OF THE THESIS

In this thesis clinical and genetic aspects of migraine and related syndromes are investigated. The studies described can be divided in three main parts.

The first part focuses on syndromes associated with FHM gene mutations. FHM may manifest in a 'pure' form or present as a more extensive syndrome with additional clinical features (**chapter 2**). The clinical spectrum of FHM1-3 and the relation with closely related migraine relevant diseases such as Alternating hemiplegia of Chilhood (AHC) (**chapter 3**), Early Seizures and Cerebral Edema after Trivial Head Trauma (ESCEATHT) (**chapter 4**), epilepsy (**chapter 5 and 6**) and episodic ataxia (**chapter 7**) is studied. The description of this clinical spectrum is important to learn more about the genotype-phenotype correlation and because of the implications for genetic testing. In addition, these studies may finally lead to more insight in the common forms of migraine, by providing pathofysiological clues.

In the second part a monogenic syndrome is studied where migraine is part of the clinical spectrum. **Chapter 8** describes the identification of *TREX1* as the causal gene for Retinal Vasculopathy with Cerebral Leukodystrophy (RVCL), formerly known as HVR, HERNS, CRV, and later (chapter 9) renamed CHARIOT. Next, in **chapter 9**, the clinical spectrum and the genotype-phenotype correlation is evaluated in eleven CHARIOT families with five different *TREX1* mutations**.** How the mutated *TREX1* gene causes disease is unknown. Therefore, in **Chapter 10** we explore the hypothesis whether endothelial dysfunction plays a role by using clinical tests assessing hemodynamic changes in *TREX1* mutation carriers compared to a control group and patients with CADASIL. The identification of the underlying gene and the study of the functional consequences and the clinical spectrum of CHARIOT may also improve our insight in (novel) pathways involved in migraine.

For the last part of this thesis migraine patients were identified in the Erasmus Rucphen Family (ERF) study, a genetically isolated population in the South-West of the Netherlands. The study of migraine and comorbid disorders can be used to define endophenotypes that are more robust and thought to have reduced genetic and clinical heterogeneity. Consequently, identification of genetic risk factors may be more successful. Comorbidity of migraine with depression (**chapter 11**) and atherosclerosis (**chapter 12**) was investigated. For depression it was also studied whether shared genetic factors underlie the comorbid relation with migraine.

A general discussion of the findings presented in this thesis and suggestions for future research are given in **Chapter 13**.

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PART 1

A MONOGENIC MIGRAINE SYNDROME: FHM

CHAPTER 2

CACNA1A R1347Q: A FREQUENT RECURRENT MUTATION IN HEMIPLEGIC MIGRAINE

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ABSTRACT

Of the 18 missense mutations in the *CACNA1A* gene which are associated with familial hemiplegic migraine type 1 (FHM1), only mutations S218L, R583Q and T666M were identified in more than two independent families. Including the four novel families presented here, of which two represent *de novo* cases, the R1347Q mutation has now been identified in six families. A genotype-phenotype comparison of R1347Q mutation carriers revealed a wide clinical spectrum ranging from (trauma-triggered) hemiplegic migraine with and without ataxia, loss of consciousness and epilepsy. R1347Q is the third most frequent mutation in hemiplegic migraine patients and should therefore be screened with priority for confirmation of clinical diagnosis. This study clearly demonstrates that the availability of multiple families better reflects the full clinical spectrum associated with FHM1 mutations.

INTRODUCTION

Familial hemiplegic migraine (FHM) is a rare autosomal dominant inherited subtype of migraine with aura, mainly characterized by transient hemiparesis during the attacks.[1] The disease is genetically heterogeneous as mutations in three genes, *CACNA1A* (FHM1), *ATP1A2* (FHM2) and *SCN1A* (FHM3) are involved,[2-5] and FHM families exist who are not linked to these genes. Mutations in all three genes cause a broad spectrum of clinical symptoms (for review see Kors et al., 2004[6]). FHM1 mutations are associated with clinical symptoms of hemiplegic migraine, ataxia, attacks of confusion, alterations of consciousness, epilepsy, fever and even fatal brain edema.[7-11]

To date 18 FHM1 mutations in the *CACNA1A* gene have been reported, of which six are recurrent. The T666M mutation has been found in 21 unrelated families, R583Q was identified in seven families and S218L in three; mutations R1347Q, R1668W, I1710T and I1811L were reported twice.

The identification of recurrent mutations and comparison of detailed clinical information of mutation carriers make genotype-phenotype correlations feasible. Moreover, the detection of mutation-specific clinical symptoms could benefit diagnostic testing. Here, we present four unrelated families carrying the R1347Q *CACNA1A* mutation, establish R1347Q as the third most frequent recurring *CACNA1A* mutation and describe an expansion of the clinical spectrum associated with this mutation.

MATERIALS AND METHODS

Clinical diagnosis

Diagnoses were made by neurologists according to the International Classification of Headache Disorders-second edition (ICHD-II) criteria.[1] All subjects gave informed consent.

Mutation analysis

Genomic DNA was isolated from peripheral blood cells according to standard methods.[12] All 47 exons of the *CACNA1A* gene were analysed using direct sequencing analysis. Exons were amplified by PCR using exon-specific primer sets and genomic DNA of each proband. Details of intron-exon structure, primer sequences and conditions are available from the authors upon request. Double-strand sequencing was done by Cycle sequencing (Prism Big Dye Terminators Cycle Sequencing kit, Applied Biosystems, Foster City, CA) using the dideoxy termination method and an ABI3700 sequencer (Applied Biosystems, Foster City, CA).

Carrier detection

For detection of the R1347Q mutation (nt 4040 G>A, *CACNA1A* reference sequence: X99897), exon 25 was amplified by PCR using primers that were extended with an M13-tail (underlined): "exon25F" (5'TGTAAAACGACGGCCAGTCTACCCAACCTGACCTCTGC-3') and "exon25R" (5'-CAGGAAACAGCTATGACCCCATACACGATGGCTAGGATG-3'), resulting in a 329-bp product. Subsequently, PCR products were digested with restriction enzyme *Hinf*I using

standard protocols, and electrophoresed on a 3% agarose gel. The R1347Q mutation causes a loss of a *Hinf*I site, which results in an undigested product of 329 bps for the mutant allele and wild-type products of 124 and 205 bps.

Haplotype analysis

For haplotyping, genetic markers D19S221, D19S1150 and D19S226 were tested. For primer sequences and PCR conditions, see http://gdbwww.gdb.org/. After PCR, amplified products were separated using an ABI 3700 DNA sequencer. All genotypes were analyzed and independently double scored using Genescan and Genotyper 2.1 software (Applied Biosystems, Foster City, CA).

RESULTS

Pedigrees of the four hemiplegic migraine families are shown in Figure 1. We identified a heterozygous nt 4040 G>A substitution in exon 25 (*CACNA1A* reference sequence: X99897), in the probands of all four families. The mutation causes an Arginine to a Glutamine substitution at codon 1347 that is located in the S4 segment of the protein domain III (see Figure 2). The R1347Q mutation co-segregated in all eight family members with hemiplegic migraine from whom DNA was available. In addition, the mutation was found in one person who reported hemisensory aura symptoms, but no weakness. Unique haplotypes showed no

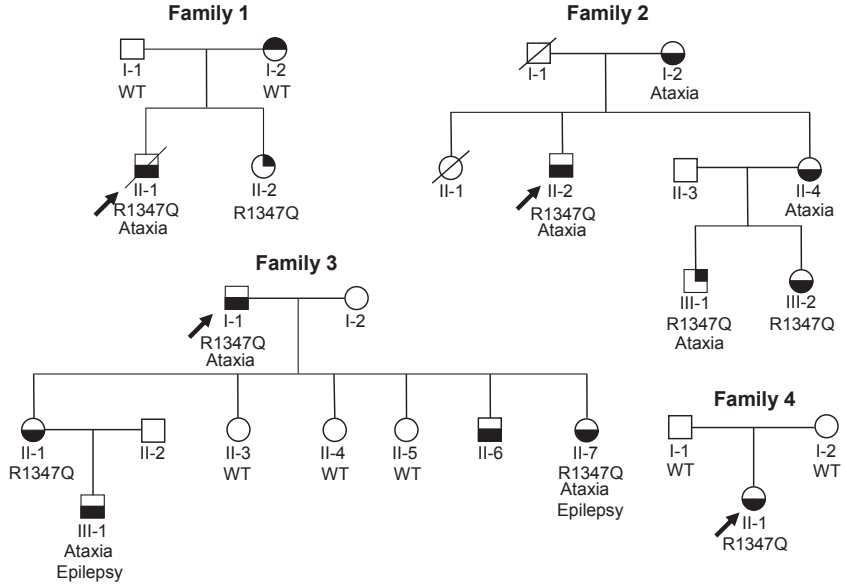

Figure 1. The pedigrees of the four R1347Q families.The arrow indicates the proband. Circles indicate females, squares indicate males. The following symbols indicate the clinical diagnosis: black lower half: hemiplegic migraine; black upper right quadrant: migraine with aura; black upper left quadrant: migraine without aura. The presence or absence of the R1347Q mutation is indicated by "R1347Q" and "WT" (wild-type), respectively.

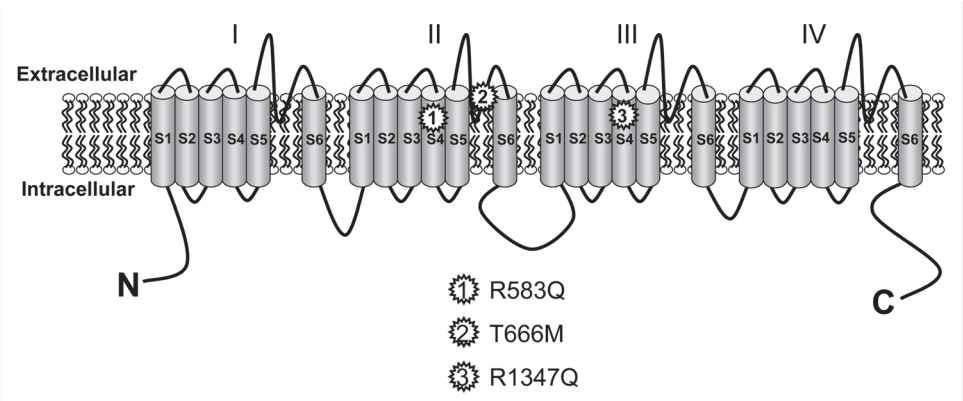

Figure 2. Schematic representation of the $Ca_v2.1\alpha1$ calcium channel subunit protein encoded by the *CACNA1A* gene. Each of the four protein domains (I to IV) has six transmembrane segments (S1-S6). The location of the three most frequent recurrent mutations is indicated.

relation between the families, indicating that all mutations occurred independently (data not shown). The mutation was not found in 200 control chromosomes by restriction enzyme analysis with *Hinf*I.

Family 1

The proband (II-1) was a 54-year-old male who since the age of three had hemiplegic migraine attacks, with a frequency of one every four to six weeks. Severe hemiplegic migraine attacks could last up to a week. During these attacks he could only be aroused briefly by vigorous stimulation and had an elevated temperature. From the age of three he gradually developed gait ataxia and dysarthria over the years. After the FHM attacks his gait ataxia and dysarthria worsened for days to weeks, but subsequently returned to the pre-ictal level. Interictal examination at the age of 51 showed saccadic pursuit eye movements, mild dysarthria, abnormal tandem gait, dysdiadochokinesis, intention tremor on finger-to-nose testing and overshoot on finger chase testing. Cerebral MRI showed evidence of cerebellar atrophy. The patient died at age 54 of a colon carcinoma. No autopsy was performed. His mother, now aged 82, has suffered from migraine without aura since puberty and recently started to have attacks of visual aura always followed by a non-migrainous headache (ICHD-II 1.2.2). The sister of the proband has migraine with visual aura since the age of 34. Mother and sister never experienced migraine attacks associated with hemiplegia.

Family 2

The proband (II-2) is a now 54-year-old male who has suffered from attacks of hemiplegic migraine since the age of three. Some attacks were associated with drowsiness and fever. Cerebral MRI scans were unremarkable. Interictal neurological examination at age 47 showed mild cognitive deterioration, saccadic ocular pursuit, gaze-evoked nystagmus, mild limb ataxia and more marked gait ataxia. Since the age of 47 he has been treated with acetazolamide which has dramatically reduced the number of attacks. His mother (I-2)

suffers from hemiplegic migraine and has a marked interictal gait ataxia. His eldest sister (II-1), who suffered from schizophrenia, died at the age of 19 in her sleep after a presumed epileptic attack. His youngest sister (II-4) suffers from typical attacks of hemiplegic migraine associated with mild confusion and disorientation, somnolence, general malaise and deafness. Interictal neurological examination at age 38 showed saccadic ocular pursuit, mild dysarthria, mild symmetrical limb ataxia and marked gait ataxia. Her son (III-1) now aged 18 has had a total of about 10 attacks of migraine with hemisensory symptoms since the age of five, but no weakness was reported. Some episodes were provoked by head trauma. Between episodes he has a gaze-evoked nystagmus to the left, mild dysarthria and mild gait ataxia. Her daughter (III-2), now aged 17 had episodes of blurred vision and paraesthesia in both upper and lower limbs, followed by weakness, and accompanied by headache, nausea and vomiting. Attacks are often brought on by flickering light. She had episodes of loss of consciousness, which were considered to be of epileptic origin and were successfully treated with valproate.

Family 3

Family 3 is a Jewish family that was originally reported in 1997.[13] The proband (I-1) is a 70-year-old male who has had attacks of hemiplegic migraine since the age of 26. He currently has an attack once every three to four months. None of the attacks has ever been accompanied by seizures. Interictal neurological examination reveals dysmetria on finger-to-nose testing, dysdiadochokinesis and an abnormal tandem gait. One of his children, II-6, now aged 29, has had three attacks of hemiplegia with dysarthria lasting between five and fifteen minutes not accompanied by headache. His interictal neurological examination is normal. Individual II-7 is a now 27-year-old woman who has suffered from attacks of hemiplegic migraine once every three months since the age of six. Attacks are sometimes associated with focal seizures always on the same side as the hemiplegia. She never experiences seizures independently from hemiplegic attacks. She has progressive ataxia**.** Individual II-1, aged 36 years, has had two events of acute hemiplegia at the ages of five and six years. She has a normal neurological examination. Her 17-year-old son (III-1) has had attacks of hemiplegic migraine since the age of three years now occurring once every three to six months. In addition, he has progressive ataxia. He also suffers from focal (tonic or clonic) seizures, independent of the hemiplegic migraine attacks, which can be secondary generalized. Three other daughters of I-1 do not suffer from any type of migraine.

Family 4

The proband (II-1) of this family is a 13-year-old girl. Her first attack of hemiplegic migraine occurred when she was nineteen months old. Later she had on average one to two attacks a year; the last one occurring two years ago. The attacks are always precipitated by trivial head trauma and include severe headache accompanied by nausea and vomiting, hemiplegia, hemianesthesia, psychotic alterations and stupor. Attacks always completely resolved within three days. She has a normal psychomotor and mental development. An MRI made 6 years ago showed no cerebellar atrophy. Neither of her parents nor any other family member has migraine or ataxia.

DISCUSSION

We identified the *CACNA1A* R1347Q mutation in four unrelated hemiplegic migraine families. This makes R1347Q the third frequently occurring FHM1 mutation. The mutation changes a positively charged Arginine to a neutral Glutamine and is located in the S4 segment of the third protein domain. The positively charged amino acids of the S4 segment act as the 'voltage sensor', which detect changes in the membrane electrical field. Mutations that neutralize highly conserved Arginine residues (i.e., R192Q, R195K, R583Q R1347Q, and R1677W) affect channel functioning by altering the voltage dependence[14] and are associated with disease.

The R1347Q mutation was previously identified in four hemiplegic migraine patients: two in a Portuguese family[15] and two in a German family.[16] All patients also had progressive cerebellar ataxia. With the present study, the total number of families with the R1347Q mutation has increased to six and the total number of mutation carriers to 13. Three additional hemiplegic migraine patients - two with ataxia - likely have the mutation, but genetic confirmation was not possible.

The newly identified R1347Q families reveal a broader clinical spectrum than previously reported. Attacks start at a young age in the majority of patients (range 19 months- 26 years; median four years) and the frequency of attacks is highly variable, from two attacks in a lifetime to one attack per month. Most mutation carriers had attacks of hemiplegic migraine: six with and three without interictal cerebellar signs. Patient III-1 in family 2 had no weakness during attacks, but hemisensory symptoms. In two patients attacks were triggered by head trauma and in two other patients attacks were associated with altered consciousness and fever. The young proband from family 4 had psychotic alterations and stupor. The proband of family 1 suffered from remarkably long hemiplegic attacks, which could last up to a week. After the attacks, there was often a temporary worsening of gait ataxia and dysarthria. The hemiplegic attacks of the proband of family 2 responded to acetazolamide, which has been reported before.[17]

Epilepsy now also appears to be part of the phenotypic spectrum of R1347Q, being present in two hemiplegic migraine patients of family 3. Patient II-7 had focal seizures ipsilateral to the hemiplegia during hemiplegic migraine attacks. Patient III-1 suffered from focal seizures that occurred independently from hemiplegic migraine attacks. *CACNA1A* mutations have been associated with epilepsy; in most cases occurring during hemiplegic attacks.[7-11;18,19] In only one FHM1 family did epileptic seizures occur independently from hemiplegic migraine attacks.[10]

In conclusion, although hemiplegic migraine and progressive ataxia are the predominant clinical features in these families, the R1347Q mutation, like other FHM1 *CACNA1A* mutations, shows considerable phenotypic variation. Identification of recurrent mutations has implications for genetic screening, as they should be screened with priority, that is before screening the entire *CACNA1A* gene.

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CHAPTER 3

CACNA1A MUTATION LINKING HEMIPLEGIC MIGRAINE AND ALTERNATING HEMIPLEGIA OF CHILDHHOOD

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ABSTRACT

Familial hemiplegic migraine (FHM) and alternating hemiplegia of childhood (AHC) are severe neurological disorders that share clinical features. Therefore, FHM genes are candidates for AHC. We performed mutation analysis in the *CACNA1A* gene in a monozygotic twin pair with clinical features overlapping with both AHC and FHM and identified a novel *de novo* *CACNA1A* mutation. We provide the first evidence that a *CACNA1A* mutation can cause atypical AHC, indicating an overlap of molecular mechanisms causing AHC and FHM. These results also suggest that *CACNA1A* mutation scanning is indicated in patients with a severe neurological phenotype that includes paroxysmal (alternating) hemiplegia.

INTRODUCTION

Alternating hemiplegia of childhood (AHC) and familial hemiplegic migraine (FHM) are clinically very similar disorders.[1,2] AHC is typically characterized by episodes of alternating hemiplegia or quadriplegia and progressive neurological features beginning before the age of 18 months.[1] FHM is a rare subtype of migraine with aura associated with hemiparesis and in some cases ataxia, mental retardation, movement disorders or other neurological abnormalities.[3] For FHM, three genes have been identified: *CACNA1A*, *ATP1A2* and *SCN1A*, which all play a role in ion transport.[4-6]

Except for one Greek family with atypical AHC and an *ATP1A2* mutation, no genes have been identified for AHC.[7,8] Here we describe a monozygotic twin pair suffering from a complex phenotype of early-onset ataxia, alternating hemiplegia, epilepsy, migraine-like attacks and mental retardation, which clinically overlaps with both AHC and FHM. We identified a novel *de novo CACNA1A* mutation in both patients, confirming a genetic overlap between AHC and FHM.

SUBJECTS AND METHODS

Subjects

Standardized criteria were used for the diagnosis of FHM[3] and AHC.[1] All subjects gave informed consent. This study was approved by the local ethics committee of the University of Greifswald.

Genetic analysis

Genomic DNA was isolated from peripheral leucocytes using a standard salting out extraction method.[9] The 47 coding exons and adjacent sequences of the *CACNA1A* gene were scanned for mutations by direct sequencing. In brief, all exons were amplified by polymerase chain reaction, using genomic DNA as a template. Direct sequencing was done by Cycle Sequencing (Prism Big Dye Terminators Cycle Sequencing kit; Applied Biosystems, Foster City, CA, USA) using the dideoxy termination method and an ABI3700 automated sequencer (Applied Biosystems). One hundred healthy controls were screened by direct sequencing. Detailed information is available from the authors upon request.

RESULTS

The monozygotic German twin brothers, now aged 17 years, were spontaneously born at term after an uneventful pregnancy. Their complex clinical features are summarized in the Table 1.

One twin brother (patient I in Table 1) was severely affected and had a delayed psychomotor development. He is still not able to walk without support and is not able to speak. Between the ages of 2 and 7 years he suffered from generalized atonic seizures up to three times daily, often followed by unconsciousness lasting from seconds to several hours. Subsequently, he

Table 1. Clinical features in monozygotic twins compared with AHC and FHM

	AHC	FHM1	Patient I	Patient II
Onset of (alternating) hemiplegic attacks	0-18 months*	>2 yr to adolescence**	10 yr	12 yr
Hemidystonic spells	+	–	–	–
Quadriplegia	+	–	+	–
Choreoathetosis	+	–	+	–
Ataxia	+	+	+	+
Nystagmus	+	+	–	–
Strabismus	+	–	+	+
Mental retardation	+	–	+	+
Autonomic symptoms	+	+	+	+
Positive effect of Sleep	+	–	–	–
Epilepsy	+	+	+	+
Aura symptoms	–	+	NA	+
Migrainous headache	–	+	NA	+

(+) or (–) indicates presence or absence of a symptom, respectively. NA indicates not applicable due to inability of verbal expression of the patient. * Typically alternating hemiplegia. ** Typically non-alternating hemiplegia.

developed mental and psychomotor regression with ataxic and athetotic limb movements. At age 11 years, when he was hospitalized because of severe constipation and abdominal pain, episodes of abrupt stops in movement, tachycardia and swallowing automatisms were observed. Ictal EEG recordings showed mainly occipitotemporal bilateral synchronic sharp–slow wave activity (not shown). At the age of 12 years he experienced a period of alternating hemiplegia, starting on the left side and accompanied by fever. Cerebral magnetic resonance imaging (MRI) showed ictal right cortical swelling (Fig. 1). After 12 days the left-sided symptoms resolved, but there after right-sided weakness occurred, which lasted for days. EEG revealed slow wave activity over the left hemisphere (data not shown). As he is still not able to express himself, the presence and severity of migraine symptoms (headache, phonophobia, photophobia, nausea) are difficult to assess. At present, he is tetraspastic with athetotic and ataxic movements, and has intermittent convergent strabismus.

The other twin brother (patient II in Table 1) was delivered shortly after his twin brother. Early infant psychomotor development was delayed. At the age of 1.5 years he was able to speak single words. Between the ages of 3 and 8 years he suffered twice a year from atonic episodes accompanied by loss of consciousness for 1 h, followed by ataxia. Ictal EEGs showed no signs of epilepsy (data not shown). Since the age of 10 years he has experienced several episodes of right- or left-sided hemiplegia, and one episode with alternating hemiplegia for several days. One of the hemiplegic episodes was accompanied by reduced consciousness and fever and lasted several weeks. Cerebral single photon emission computed tomography showed ictal left-sided hypoperfusion in the parieto-frontotemporal cortex (Fig. 2). At the age of 12 years, treatment with flunarizine was started, with doubtful effect. During hemiplegic

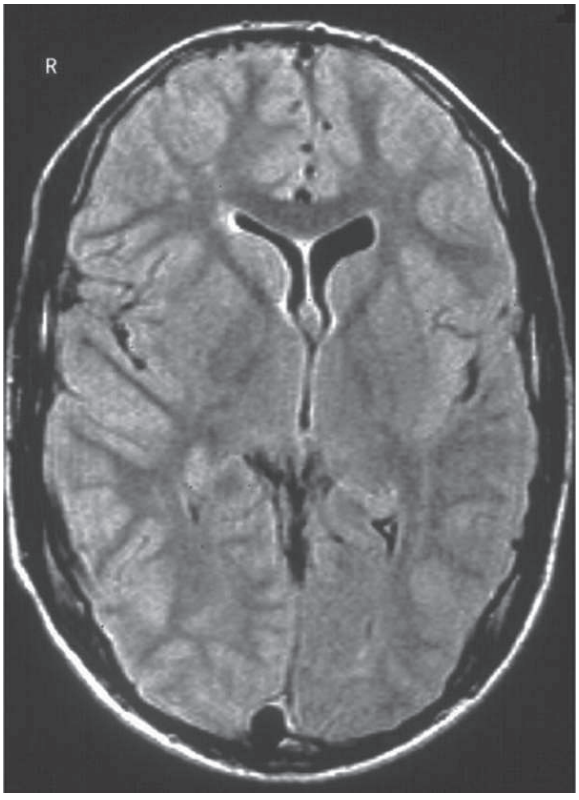

Figure 1. Fluid-attenuated inversion recovery (FLAIR) image shows ictal diffuse cortical swelling of the right hemisphere of Patient I.

attacks he complained of frontal throbbing headache with nausea, sometimes accompanied by phonophobia. Ophthalmic examination shows a strabismus. Except for the twin pair, no other family members suffer from attacks of hemiplegia, epilepsy or migraine.

Monozygosity of the twins was confirmed and false paternity was excluded by genetic, multimarker analysis (data not shown). Extensive metabolic screening in both twins revealed no abnormalities. Cerebral MRI, showing diffuse ictal swelling in patient I (see above), was without severe abnormalities in either of the twins, and did not reveal cerebellar atrophy. Direct sequencing of all 47 exons of the *CACNA1A* gene revealed a heterozygous 5361 G>T substitution (Genbank Ac. no. X99897) in exon 33 in both twins. This point mutation resulted in an amino acid change from a valine to a phenylalanine at position 1696. The mutation is located within the transmembrane segment S5 of the fourth domain, which together with segment S6 forms the inner part of the pore of the $Ca_v2.1\alpha1$ subunit. The parents did not carry the mutation, indicating that V1696F is a *de novo* mutation. Screening of 100 subjects from the general Dutch population with no history of migraine or epilepsy was performed by sequence analysis of exon 33 and was negative.

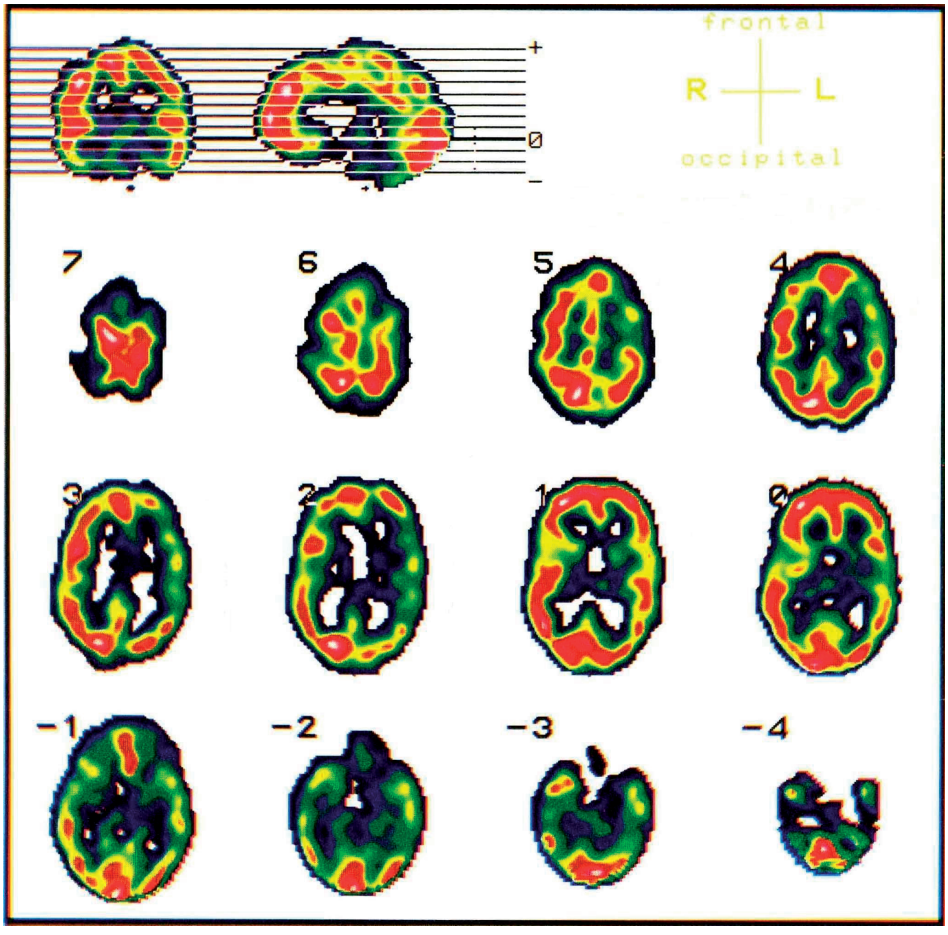

Figure 2. Single photon emission computed tomography (SPECT) scan shows ictal left- shows ictal left-sided hypoperfusion contralateral to the hemiparesis in the parietofrontal (severe) and temporal (mild) cortex of Patient II.

DISCUSSION

We have identified a novel *de novo CACNA1A* V1696F mutation in two monozygotic twin brothers with complex clinical features in part fulfilling the criteria of both AHC[1] and FHM[3] (Table 1). The presence of hemiplegia and other aura symptoms as well as migrainous headache are in favour of FHM. Episodes of alternating hemiplegia, developmental delay, mental retardation, choreoathetotic movements, strabismus and chronic ataxia are supportive of AHC. The severity of the phenotype, interictal symptoms and the relatively young age at onset of these patients are, in particular, very rare for FHM1 and more common in AHC. As no positive effect of sleep was observed and the age at onset was after 18 months, we name this overlap syndrome 'atypical AHC'. Although both monozygotic twin brothers are genetically

identical, their clinical symptoms and attack frequency vary in severity, with patient I being more severely affected.

Several lines of evidence indicate that V1696F is the disease-causing mutation in this family. Val1696 is highly conserved across multiple calcium channel homologues and across species (data not shown). The mutation was not identified in a large number of control chromosomes. The mutation occurred *de novo*, thus strengthening the evidence that it is the V1696F mutation that caused the disease. In a French FHM family another mutation affecting the same residue Val1696 (V1696I) caused hemiplegic migraine without cerebellar signs.[10] Finally, electrophysiological analysis of mutant $Ca_v2.1\alpha1$ containing Iso1696 has revealed that loss of the valine residue is associated with altered channel kinetics compatible with a phenotype of an increased Ca^{2+} influx.[11]

Notably, change of Val1696 to a phenylalanine (V1696F) or an isoleucine (V1696I) causes phenotypes of different severity. Two out of three V1696I mutation carriers had hemiplegic migraine without cerebellar signs or other severe neurological features.[10] Both V1696F mutation carriers, however, had a very severe phenotype of atypical AHC. Apparently, substitution of the valine residue for a bulky phenylalanine in the transmembrane domain has a more dramatic effect on channel functioning.

Previously, sporadic patients with typical AHC have been scanned for mutations in the *CACNA1A* (FHM1), *ATP1A2* (FHM2) and *SLC1A3* genes, but no mutations were found.[12-14] Now, we present the first *CACNA1A* mutation in patients with atypical AHC.

Previously an *ATP1A2* mutation was identified in a Greek family with atypical AHC, with a similar overlapping FHM/AHC phenotype.[7,8] Of note, both *CACNA1A* and *ATP1A2* are involved in ion transport, and mutations in these genes are predicted to increase concentrations of K^+ and glutamate in the synaptic cleft as a result of either increased neurotransmitter release (*CACNA1A* mutations) or impaired removal of K^+ and neurotransmitter (*ATP1A2* mutations).[15,16] Elucidating the molecular basis in this German family with complex clinical features strengthens the evidence for a common pathogenesis of FHM and AHC.

Our results suggest that in severely affected paroxysmal (alternating) hemiplegic patients with mental retardation and an age at onset of alternating episodes beyond 18 months, *CACNA1A* mutation scanning is indicated.

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CHAPTER 4

EARLY SEIZURES AND CEREBRAL EDEMA AFTER TRIVIAL HEAD TRAUMA ASSOCIATED WITH THE *CACNA1A* S218L MUTATION

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ABSTRACT

Objective: To study the clinical spectrum of *CACNA1A* S218L mutation carriers with special attention for "early seizures and cerebral edema after trivial head trauma" (ESCEATHT), a combination of symptoms which resembles the "juvenile head trauma syndrome".

Patients and Methods: We sequenced all exons of *CACNA1A* in two patients with ESCEATHT. Both patients also had hemiplegic migraine and ataxia. Subsequently, we screened the literature for S218L mutation carriers.

Results: In both patients we found a *de novo* S218L mutation in the *CACNA1A* gene. In addition, we identified 11 *CACNA1A* S218L carriers from literature. From these 13 S218L mutation carriers, twelve (92%) patients had ataxia or cerebellar symptoms. Nine (69%) had hemiplegic migraine that could be triggered by trivial head trauma. Three mutation carriers had the complete ESCEATHT phenotype. Seven (54%) had seizures (four had early post traumatic seizures) and five (38%) had edema as detected by MRI/CT.

Conclusions: The *CACNA1A* S218L mutation is associated with FHM, ataxia and/or ESCEATHT. A minority of S218L mutation carriers have the complete ESCEATHT phenotype, but a high percentage of patients had one or more ESCEATHT symptoms. As the S218L mutation enhances the propensity for cortical spreading depression (CSD), we postulate a role for CSD not only in hemiplegic migraine, but also in early seizures and cerebral edema after trivial head trauma. As this combination of symptoms is part of the unexplained "juvenile head trauma syndrome", a similar molecular mechanism may underlie this disorder.

INTRODUCTION

Post-traumatic seizures are classified as early, when they occur within a week after head injury.[1] Early seizures may increase the probability of epilepsy later in life.[2,3] The risk of early post-traumatic seizures increases with greater severity of the injury, the presence of intracranial haemorrhage, and a younger age.[1,4,5] Early seizures may occur, although rarely, after *trivial* head trauma, then usually in combination with sometimes very severe cerebral edema.[4,6-8] In children, this is often called "juvenile head trauma syndrome".[6-8] Apart from a young age, the risk factors and the pathogenetic mechanisms for "*early* seizures and cerebral edema after *trivial* head trauma" (ESCEATHT) are unknown.[3]

Epilepsy is a common comorbid disorder in patients with migraine with aura.[9,10] The increased risk is bidirectional and there are several clinical, therapeutical, genetic and electrophysiological similarities between both episodic brain disorders, suggesting common pathogenetic pathways.[9,10] Indeed, genes for Familial Hemiplegic Migraine (FHM) - a hereditary subtype of migraine with aura in which attacks are associated with hemiparesis - have also been implicated in epilepsy.[10] Seizures are not uncommon in patients with FHM1,- 2 and -3.[10,11] Notably, *trivial* head trauma is a known trigger for attacks of both FHM[12] and migraine with aura (MA).[13,14] Three genes have been identified for FHM, all encoding proteins involved in ion transportation: *CACNA1A* (FHM1),[15] *ATP1A2* (FHM2),[16] and *SCN1A* (FHM3).[17] The *CACNA1A* gene encodes the α1-subunit of $Ca_v2.1$ (P/Q-type) Ca^{2+} channels that modulate neurotransmitter release.[18] *CACNA1A* is expressed at the neuromuscular junction and throughout the central nervous system, in particular in cerebellar Purkinje cells. Functional studies of FHM1 mutations predict enhanced neuronal excitability and have shown increases of neuronal Ca^{2+} influx, neurotransmitter release, and propensity to cortical spreading depression (CSD).[19,20] CSD is a brief (seconds) wave of intense neuronal and glial depolarization that is slowly (2-5 mm/min) propagating over the cerebral cortex. A wave is associated by transient loss of membrane ionic gradients and by massive surges of extracellular potassium, neurotransmitters, and intracellular calcium. The depolarization wave is followed by a potent, relatively long-lasting (≥ 20 min) neuronal suppression.[21] These electrophysiological and secondary molecular events are accompanied by transient neuronal swelling and loss of dendritic spines due to temporary tissue hypoxia,[22] and cerebral edema as a result of increased permeability of blood vessels through upregulation of matrix metalloproteinases.[23] In humans, CSD is the likely underlying electrophysiological substrate of the migraine aura.[24]

One particular type of *CACNA1A* mutation, the S218L mutation, was found in patients who suffered from particularly severe attacks of FHM which were triggered by trivial head trauma and were associated with often fatal excessive cerebral edema.[12,25,26] In a transgenic animal model, the S218L mutation greatly enhances the propensity for CSD.[19]

Based on the above clinical observations and experimental findings, we hypothesized that FHM1 gene mutations (e.g., the S218L mutation) may confer an increased risk of (symptoms of) ESCEATHT, probably through increased susceptibility for CSD. We investigated this in two patients with FHM, ataxia and ESCEATHT and in a subsequent review of the literature.

MATERIAL AND METHODS

Patients

Subjects were interviewed and clinical headache diagnoses were established according to International Headache Society criteria (ICHD-2 criteria).[27] Seizures were classified according to the criteria of the International League Against Epilepsy (ILAE).[28] ESCEATHT in this study was defined as an episode with Early Seizures (within 7 days after head trauma) and associated Cerebral Edema (on MRI or CT-scan) After a Trivial Head Trauma. This study was approved by the ethical committee of the Leiden University Medical Centre. All individuals gave informed consent. Clinical details of affected family members are shown in the results section.

Search for other S218L mutation carriers

We screened the literature for publications describing patients with the S218L mutation and reviewed the clinical descriptions.[12,25,26,29]

Mutation analysis

Genomic DNA was isolated using a standard salting out extraction method.[30] Direct sequencing of all exons of the *CACNA1A* gene was performed with genomic DNA of both patients, using the dideoxy termination method and an ABI3700 sequencer (Prism Big Dye Terminators Cycle Sequencing kit; Applied Biosystems, Foster City, USA). Detection of the *CACNA1A* S218L mutation was performed as described previously.[12]

RESULTS

In Table 1 we present the clinical data of thirteen patients with the *CACNA1A* S218L mutation: two are new cases (*patients 1* and *2*) and the remaining patients were from the literature of which seven were published by us.

Genetic testing of new cases

The *CACNA1A* S218L mutation located in exon 5 was identified in both patients (*1* and *2*). Mutation screening was negative in their parents, indicating that the mutation had occurred *de novo*. For both patients, false paternity was excluded by haplotype analysis of genetic markers of the chromosome 19p13 *CACNA1A* locus (data not shown).

Clinical description Patient 1

This now 11 year-old girl of Dutch origin was born after 36 weeks of pregnancy. Shortly after birth spontaneous apneas were observed but after resuscitation and artificial ventilation for 24 hours she breathed spontaneously. When she grew up, psychomotor and mental development appeared to be delayed and she developed severe ataxia. At age three, she suffered a fall on the back of her head, without initial loss of consciousness. After 30 minutes she became comatose and developed left-sided hemi-convulsions for approximately 2 hours. Subsequently, a left-sided paresis with hemispatial neglect was present for one week. She recovered completely. A cerebral Magnetic

Resonance Imaging (MRI) performed two days after hospital admission showed edema in the right parieto-occipital cortex and to a lesser extent in the right temporal and (posterior) frontal cortex. Increased signal on diffusion-weighted images showed that the edema likely is of cytotoxic origin (Fig. 1). Also extensive cerebellar atrophy was present (Fig. 2). At the age of four, she developed a prolonged period of stupor after a trivial head injury, which was not further documented.

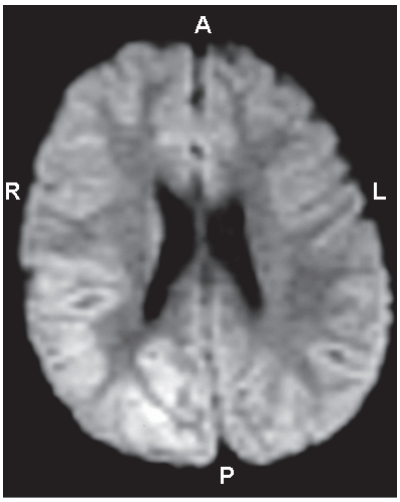

Figure 1. Coronal Diffusion Weighted Image (b=1,000s/mm^2) of patient 1 performed during the first coma episode at age three with left-sided hemi-convulsions and subsequent left sided paresis. Increased signal in the right parieto-occipital cortex indicates cytotoxic oedema. A: anterior; R: right; L: left; P: posterior.

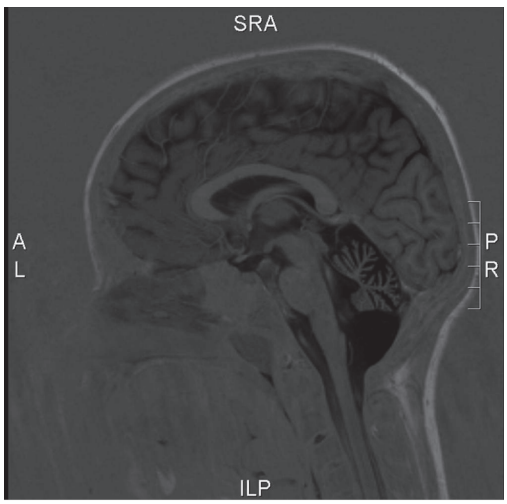

Figure 2. T2 sagittal brain MRI of patient 1 at age three showing marked cerebellar atrophy.

Table 1. Comparison of clinical symptoms of patients with an S218L *CACNA1A* mutation

Patient with S218L mutation	Family	Gender	Migraine attacks (*=attacks with coma)	Migraine attacks triggered by trivial head injury¥	Comatose episodes without clear seizures or migraine
1	Sporadic	Female	HM*	Yes	No
2	Sporadic	Male	HM*	Yes	No
3	Family I	Male	HM*	Yes	Yes
4	Family I	Male	HM*	Yes	No
5	Family I	Male	HM*	Not reported	No
6	Family I	Female	MA	Yes	No
7	Family II	Female	No	No	No
8	Family II	Female	No	No	No
9	Family II	Male	HM*	No	No
10	Sporadic	Female	HM*	Yes	No
11	Family III	Male	HM*	No	No
12	Family III	Female	HM*	No	No
13	Family III	Male	No	No	No

HM: hemiplegic migraine; MA: migraine with aura; AAO: age at onset; GTC: generalized tonic-clonic; §: seizures not well documented; #: seizures occurred more than 7 days after head trauma; ¥: trivial head trauma included fall on the back of head, heading the ball during soccer, hitting the head.
Patients 1, *2* and *7* suffered from episodes of early seizures and cerebral edema after trivial head trauma (ESCEATHT).
Patient 5 suffered from hemiplegic migraine attacks and in addition had one attack starting with a generalized tonic-clonic seizure followed by aphasia and right hemiparesis; it is unknown whether cerebral edema was present or whether trivial head trauma preceded this attack.

At the age of 6 years, a third episode occurred. Two minutes after a fall, of which it is unclear whether she had hit her head, she lost consciousness and was transported to the hospital. Some minutes after admission, and 30 minutes after the trauma, she developed a seizure with right-sided rhythmic clonic contractions and gaze deviation to the right. The seizure was successfully treated with phenytoin. Remarkably during the recovery phase it was noticed that there was a left-sided hemiparesis, which resolved in a few days. Her parents did not have epilepsy, migraine or ataxia.

MRI/CT-detected edema	Seizures	Early post-traumatic seizures (AAO in years)	Ataxia/ Cerebellar atrophy	Other clinical symptoms	Reference
Yes	Two complex partial seizures	Yes (3)	Yes/Yes	Mild mental retardation	*This article*
Yes	Single generalized seizure	Yes (15)	Yes /No	-	*This article*
Yes	No	No	Yes/Yes	Hallucinations	[12; 29]
Unknown	No	No	Yes/Yes	Mild mental retardation	[12; 29]
Unknown	Single generalized seizure	Unknown (59)	Yes/Yes	Psychotic episodes	[12; 29]
No	No	No	Yes/Yes	MA	[12; 29]
Yes	Single generalized seizure/seizures after birth[§]	Yes (16)	Clumsy/Yes	Hypotonic after birth, squint; fatal coma	[12]
No	No	No	Yes/Unknown	Hypotonic after birth, squint	[12]
No	No	No	Yes /Unknown	-	[12]
Yes	At least one partial seizure	No (5)[#]	Yes /No	Hallucinations	[25]
No	5 episodes of GTC seizures	Yes (5)	Yes/Yes	-	[26]
Unknown	No	No	Unknown/Yes	-	[26]
Unknown	Simple febrile seizures, once secondary GTC seizure	No (2, 4)	No/Unknown	-	[26]

Patient 10 had at least one partial seizure during a prolonged comatose episode.
Patient 11 had 5 episodes of GTC seizures triggered by mild head trauma at age of 5 years; no MRI/CT scan was made; seizures ceased spontaneously. At age 15 and 17 years, *patient 11* had an hemiplegic migraine attack, the latter associated with coma, no cerebral edema was seen on CT and MRI scan.
Patient 13 had a few episodes of simple febrile seizures between ages 2 and 4 years old and 1 secondarGTS during fever; no trivial head trauma was described.

Clinical description Patient 2

Since the age of eight years, this now 19 year-old boy of Dutch origin suffers from attacks of hemiplegic migraine 4 to 6 times a year. At the age of 1.5 years he fell off his bike and hit his head. He initially was conscious and alert but within three to four hours became somnolent and started vomiting. There were no focal neurological signs. He recovered spontaneously from

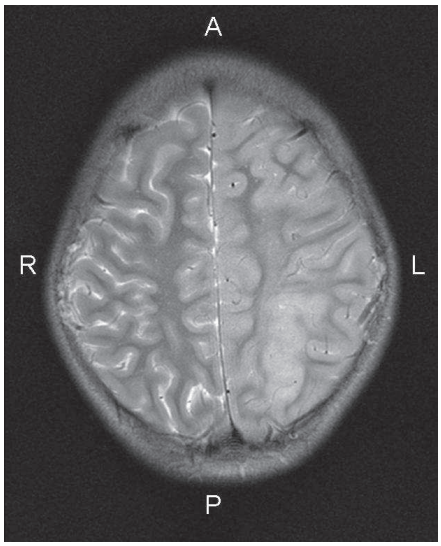

Figure 3. T2 axial brain MRI of patient 2 showing severe left-sided cortical edema. MRI was performed during the coma episode at age 15 with right-sided hemiparesis and generalized seizures. A: anterior; R: right; L: left; P: posterior.

this episode within 24 hours. At the age of 15 he developed a headache while playing soccer and heading the ball several times. Shortly thereafter, he became agitated, restless and developed aphasic speech**.** In the hospital his initial Glasgow coma score was 7 with a hemiparesis on the right side. A cerebral MRI scan showed a swollen left hemisphere with perfusion defects, but no parenchymous abnormalities or cerebellar atrophy (Fig. 3). Diffusion-weighted imaging showed increased signal indicating that the edema was of cytotoxic origin. Seven days after admission he suffered from a generalized seizure. A post-ictal EEG showed generalized slowing, but no epileptic activity. Phenytoin was started. Recovery of clinical symptoms was gradually within several weeks. At discharge, he still had cognitive disturbances and dysphasia. Only after several weeks he recovered fully. Several years later, he again got kicked against the head while playing soccer and became drowsy and confused. This time he developed neither hemiparesis nor seizures. This attack spontaneously resolved within five hours. His motor development is slightly delayed and he is ataxic. He is currently following secondary school without obvious learning difficulties.

Clinical spectrum in S218L patients from literature

A review of the literature revealed eleven additional case descriptions of S218L mutation carriers (Table 1, *patients 3-13*). In five out of eleven cases (45%), seizures were reported that occur in three patients after trivial head trauma (27%). In two patients (*patients 7* and *11*), post-traumatic seizures were generalized. In *patient 10*, seizures were of the partial type. *Patient 7* had a particularly severe phenotype. After a trivial head injury, she developed an early post-traumatic generalized seizure that was followed by extensive cerebral edema that resulted in a fatal coma.[12] Also *patient 10* had a severe phenotype after a trivial head injury

and did not entirely recover from a period of prolonged coma during which she had a partial seizure.[25] Finally, *patient 11* suffered from five separate episodes of generalized tonic-clonic seizures that were all triggered by trivial head injury.[26] Edema detected with MRI or CT-scan and associated with coma episodes was present in three patients of which two also had post-traumatic seizures. Ten patients showed cerebellar atrophy (91%) or ataxia and eight had migraine with (7) or without (1) hemiplegia. A family history of migraine was reported in 10 patients (originating from three families).

DISCUSSION

We screened two patients with ESCEATHT for mutations in the *CACNA1A* gene. Both proved to carry a *de novo CACNA1A* S218L mutation, which we previously showed to be associated with FHM1 and mild head trauma-triggered severe and sometimes fatal cerebral edema.[12] In the literature we identified eleven additional S218L mutation carriers, of which seven were published by us.[12] Twelve (92%) of mutation carriers displayed cerebellar symptoms and nine (69%) carriers had attacks of hemiplegic migraine. A minority of S218L mutation carriers had the full ESCEATHT phenotype (23%), but a high percentage of patients had one or more symptoms of ESCEATHT. Seven had seizures (54%), of which at least four (31%) were early seizures provoked by mild head trauma and of which three (23%) were associated with cytotoxic cerebral edema.[12,25,26] One patient had only edema without early seizures and one patient had trauma triggered early seizures without cerebral edema. In 6 out of 7 patients, seizures occurred in childhood (range 2 to 16 years) except for a patient who had seizures at the age of 59 years. Ten (77%) cases were familial and three (23%) sporadic. The three sporadic cases occurred *de novo*.

As earlier studies had not systematically looked for the presence of the complete spectrum of ESCEATHT symptoms, the true prevalence of early seizures and cerebral edema might be higher. Of note, in four patients (*patients* 4, 5, 12 and 13), no MRI or CT scan was made during attacks, therefore the presence of cerebral edema could not be investigated. Although, most S218L patients have a severe phenotype (with or without ESCEATHT), there is always the possibility that some cases with a mild phenotype may not have been included in this study, because of a possible publication bias.

The present findings suggest a possible pathogenetic role for $Ca_v2.1$ Ca^{2+} channels and CSD also in ESCEATHT. FHM1 mutations have been shown to increase the cellular influx of Ca^{2+} leading to enhanced release of neurotransmitters such as glutamate and a reduced trigger threshold for CSD.[20,31] The S218L mutation causes rather dramatic changes in $Ca_v2.1$ Ca^{2+} channel function, matching the severe clinical phenotype and the observation that seemingly harmless events may trigger attacks with sometimes fatal cerebral edema.[12,25,26,29] Detailed electrophysiological studies revealed a particularly low threshold for activation and a very slow inactivation of S218L-mutated $Ca_v2.1$ Ca^{2+} channels.[32] This would predict a vastly increased propensity for CSD as was seen in transgenic mice in which we introduced the S218L mutation.[19] As a result of the S218L mutation, even weak and otherwise harmless stimuli may readily depolarize mutated $Ca_v2.1$ Ca^{2+} channels and trigger multiple and prolonged waves

of CSD that are associated with severe and protracted cytotoxic cerebral edema and cell loss.[12] Enhanced release of glutamate will increase the activation of NMDA receptors, further affecting brain cells (e.g., through accumulation of intracellular Ca^{2+} and production of nitric oxide) and further worsening cell swelling. Trivial head trauma may also cause mechanical strain through transient mitochondrial dysfunction and delayed long lasting small neuronal depolarizations, thereby increasing neuronal vulnerability.[33] Notably, CSD has already been implicated in the pathophysiology of epilepsy.[34,35] Both CSD and epilepsy are characterized by spreading of neuronal depolarization.[21] The particular activation characteristics of S218L-mutated $Ca_v2.1$ Ca^{2+} channels may link CSD and epileptic seizures.

Although several carriers of other *CACNA1A* mutations have epilepsy as part of their hemiplegic migraine attacks, the combination with cerebral edema was only reported for the Y1384C mutation.[36,37] As attacks were not precipitated by trivial head trauma, a diagnosis of ESCEATHT is not applicable for this patient. For the FHM2 *ATP1A2* gene, several mutations (e.g., G301R and G615R) are associated with cerebral edema during severe attacks of hemiplegic migraine,[38,39] but a diagnosis of ESCEATHT (that includes early seizures and the trigger factor trivial head trauma) is only reported for the G615R mutation.[38] It remains an open question to what extent findings in S218L mutation carriers also have implications for common MA. As none of the severe associated clinical features is common in MA patients, it seems that the presence of mutated $Ca_V2.1$ channels results in particularly severe CSDs in S218L mutation carriers.

In conclusion, we postulate that CSD in S218L mutation carriers, in addition to hemiplegic migraine, is involved in causing early seizures and cerebral edema after trivial head trauma. Increased susceptibility to CSD might also play a role in the "juvenile head trauma syndrome", which is remarkably similar to ESCEATHT.[6-8] Although the *full* syndrome of ESCEATHT is present in only a minority of S218L mutation carriers, an important conclusion from this study is that they are at risk for developing the complete devastating phenotype. We propose that patients with this syndrome, especially when associated with permanent cerebellar symptoms and a history of migraine, are screened for the *CACNA1A* S218L mutation. Preventative therapeutic advice should be given to avoid activities that can cause even mild head trauma (i.e., contact sports) or wear a protective helmet.

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CHAPTER 5

FAMILIAL HEMIPLEGIC MIGRAINE IS ASSOCIATED WITH FEBRILE SEIZURES IN AN FHM2 FAMILY WITH A NOVEL DE NOVO *ATP1A2* MUTATION

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Epilepsia 2009;50:2503-4

INTRODUCTION

Febrile seizures are the most common form of convulsions between the age of 6 months and 5 years,[1] but genetic factors have not been identified. Here we investigated the molecular basis of febrile seizures in a small family with co-occurring hemiplegic migraine[2] and febrile seizures (Fig. 1). Intermittent ataxia and diffuse encephalopathic episodes are also present in this family.

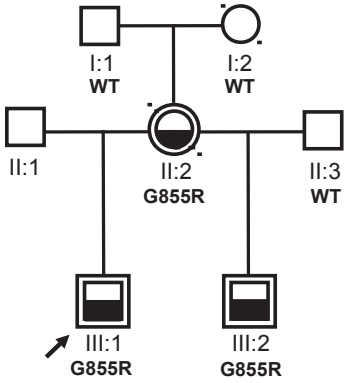

Figure 1. Pedigree of the FHM2 family. The arrow indicates the proband. Squares indicate male subjects and circles indicate female subjects. To indicate clinical diagnosis; with lower-half–filled symbols represent FHM and double-lined symbols represent febrile seizures. Individuals heterozygous for the ATP1A2 mutation are indicated by G855R. Wild-type (WT) indicates that the individual does not have the mutation.

RESULTS

Using direct sequencing (see Vanmolkot et al., 2003[3]), we identified a novel de novo heterozygous 2563 G>A substitution in exon 18 resulting in an amino acid change from a glycine to an arginine at position 855 in the *ATP1A2* FHM2 gene[4] that encodes the α2 subunit of Na+, K+-ATPase pumps. Only, the proband (III-1), the affected mother (II-2), and affected brother (III-2) carry this *ATP1A2* mutation. Gly855 is evolutionary conserved (Fig. 2A), and the mutation was not identified in 300 control chromosomes.

An ouabain challenge assay[5] for the mutant p.Gly855Arg construct, unlike wildtype, showed complete loss of cell survival, indicating that the mutation has functional consequences at the protein level (Fig. 2B).

CLINICAL DESCRIPTIONS

This now 13-year-old proband (III-1) (Fig. 1) experienced, from the age of 9 months to 3 years, five complex febrile seizures and one simple febrile seizure. The complex seizures either lasted more than 15 min or started focally. There were also episodes of several seizures occurring

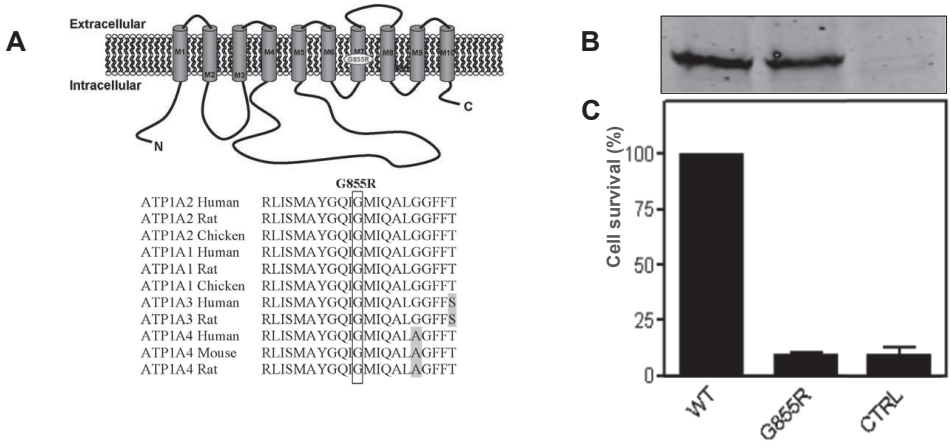

Figure 2. Genetic and functional data on mutant p.Gly855Arg. (**A**) Alignment of amino acid sequences of several vertebrate sodium-potassium ATPase a-subunits; Gly855 is represented as a black box. (**B**) Upper panel shows Western blot analysis of transfected HeLa cells. Lower panel shows graphic representation of ouabain cell survival assay. Bars represent cell survival after 5 days of ouabain treatment [error bars indicate standard error of the mean (SEM)].

sequentially. From the age of 7 months to 5 years, he also experienced several nonfebrile seizures, which typically had a focal onset and were secondary generalized. These seizures usually lasted up to 5 min, but sometimes were prolonged (up to 40 min), and at times occurred in clusters. Seizures stopped at age 5.

From age 2.5, he experienced headache attacks accompanied by transient hemiparesis as well as frequent unprovoked episodes of ataxia lasting a few minutes to days. In addition, he had episodes of rapidly progressive drowsiness down to Glasgow Coma scale (GCS) 6–7, without any focal neurologic deficits or epilepsy. The patient has ongoing behavioral problems and mild learning difficulties.

His now 3-year-old half-brother (III-2) had one complex (focal) febrile seizure lasting 15 min when he was 7 months old. Since the age of 21 months he had episodes of hemiplegia, and recurrent encephalopathic episodes often preceded by headache and hemiplegia, and one episode of unsteadiness after minor head injury. The mother (II-2), now age 31 years, had two simple febrile seizures at age 2 and also had attacks of hemiplegic migraine. The father of the proband (II-1) and the father (II-3) of his half-brother and their grandparents (I-1 and I-2) never had hemiplegic migraine, ataxia, or seizures.

CONCLUSION

We feel that the *ATP1A2* p.Gly855Arg mutation is the causal mutation in this family for a number of reasons: (1) FHM and febrile convulsions were present only in the three mutation carriers and not in non–mutation carriers; (2) the mutation was not identified in a panel of 150 healthy control individuals, and (3) functional studies revealed that the mutant has a

deleterious effect on cell survival. Febrile seizures are reported in only some mutation carriers of three FHM2 families.[3,6,7] Future identification of additional families with co-occurring hemiplegic migraine and febrile seizures may shed light on the association between *ATP1A2* gene mutations and febrile seizures. We, therefore, recommend genetic analysis of the *ATP1A2* gene in patients with febrile seizures.

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DISCLOSURE

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines. None of the authors has any conflict of interest to disclose.

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CHAPTER 6

FIRST MUTATION IN THE VOLTAGE-GATED $NA_V1.1$ SUBUNIT GENE *SCN1A* WITH CO-OCCURRING FAMILIAL HEMIPLEGIC MIGRAINE AND EPILEPSY

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ABSTRACT

Almost all mutations in the *SCN1A* gene, encoding the α1 subunit of neuronal voltage-gated $Na_V1.1$ sodium channels, are associated with severe childhood epilepsy. Recently, two mutations were identified in patients with pure familial hemiplegic migraine (FHM). Here, we identified a novel *SCN1A* L263V mutation in a Portuguese family with partly co-segregating hemiplegic migraine and epilepsy. The L263V mutation segregated in five FHM patients, three of whom also had epileptic attacks, occurring independently from their hemiplegic migraine attacks. L263V is the first *SCN1A* mutation associated with FHM and co-occurring epilepsy in multiple mutation carriers, and is the clearest molecular link between migraine and epilepsy thus far. The results extend the clinical spectrum associated with *SCN1A* mutations and further strengthen the molecular evidence that FHM and epilepsy share, at least in part, similar molecular pathways.

INTRODUCTION

Both migraine and epilepsy are complex episodic neurological diseases with genetic and environmental factors playing a role in their pathogenesis.[1,2] Epidemiological studies have indicated that there is a clear bidirectional increased risk of migraine and Epilepsy.[3] This suggests that migraine and epilepsy, at least in part, may share pathophysiological mechanisms.[4]

Most genes that are involved in rare monogenetic forms of epilepsy encode subunits of channels transporting sodium, potassium or chloride ions (for a recent review see Heron et al, 2007[2]). Gene identification in migraine has been successful only for familial hemiplegic migraine (FHM), a rare, monogenic, autosomal dominant subtype of migraine with aura with unilateral motor weakness during the aura phase.[5] All three FHM genes identified thus far are involved in ion transport.[6] The FHM genes encode subunits of $Ca_V2.1$ calcium channels (*CACNA1A*: FHM1),[7] sodium-potassium ATPases (*ATP1A2*: FHM2)[8] and $Na_v1.1$ sodium channels (*SCN1A*: FHM3).[9]

Despite their comorbidity, co-occurring migraine in epileptic mutation carriers has not been mentioned, probably because migraine in a patient or family with epilepsy will often be regarded coincidental because of the high prevalence of migraine in the general population. Epilepsy, however, has specifically been reported in some FHM mutation carriers.[10] In FHM1 mutation carriers, seizures may occur during severe hemiplegic migraine attacks.[11] Seizures have been described in two FHM1 I1710T mutation carriers in one family independently of hemiplegic attacks[12] and causing status epilepticus during hemiplegic migraine attacks in a sporadic patient.[13] Several carriers of FHM1 mutation S218L, which is associated with severe FHM attacks triggered by trivial head trauma, had epileptic seizures.[14,15] In FHM2 mutation carriers various types of childhood and adult epilepsy have been reported with different FHM2 mutations.[8,16-18] In rare cases, epilepsy is part of particularly severe phenotypes with alternating hemiplegia, coma with permanent cerebellar signs or mental retardation.[19-21]

A recent molecular link between migraine and epilepsy has come from the identification of the first two FHM mutations in *SCN1A*, a well-known epilepsy gene.[9,22] Over 150 mutations in the *SCN1A* gene have been reported with generalized epilepsy with febrile seizure plus additional symptoms (GEFS+) and severe myoclonic epilepsy of infancy (SMEI).[23,24] Both diseases are severe epileptic phenotypes occurring in childhood. The majority of SMEI mutations occur *de novo* and are either nonsense or frameshift mutations resulting in protein truncation and consequent loss-of-function or missense mutations with a wide range of functional consequences.[25] The milder GEFS+ phenotype is mostly caused by missense mutations, showing either loss- or gain-of-function effects.[26] Up to now, no associated migraine has been reported for any of the epilepsy mutations. It is not known why most *SCN1A* mutations cause childhood epilepsy, whereas only two cause hemiplegic migraine. A clinical continuum of migraine and epilepsy is probably associated with *SCN1A* mutations as a result of altered neuronal excitability.[27]

Here we describe the first *SCN1A* mutation with several mutation carriers with partly co-segregating FHM and epilepsy, which provides additional support for the clinical continuum hypothesis. Our results underscore the complex relation of migraine and epilepsy and provide further molecular evidence that the two diseases share common pathways.

METHODS

Clinical description

We investigated a Portuguese FHM family with partially co-segregating epilepsy (Fig.1A). All subjects were interviewed and the clinical headache diagnoses were established according to International Headache Society criteria (International

Classification of Headache Diseases criteria),[5] whereas seizures were classified according to the criteria of the International League Against Epilepsy.[28] Diagnoses were made prior to genetic analyses by a neurologist experienced in migraine and epilepsy. This study was approved by the ethics committee of the Hospital Geral de Santo António, Porto. All individuals gave written informed consent. Clinical details of affected family members are shown in Table 1. Six patients suffered from typical hemiplegic migraine attacks, with an age of onset varying from 10 to 18 years and an attack frequency of less than one to three per year. None of the patients reported (inter)ictal cerebellar abnormalities. Generalized tonic-clonic seizures (and in one case additional complex partial seizures) were co-segregating with hemiplegic migraine in three family members (i.e., II-2, II-5 and III-4), and probably in one deceased person (I-1) [seizures similar to those of the other family members were reported by his wife (I-2)]. Onset of seizures was between 4 and 8 years. Interictal EEG recording was unremarkable in patients II-2, II-5 and III-4. Structural lesions in these patients were excluded on the basis of computed tomography (i.e., II-2 and II-5) or magnetic resonance imaging (i.e., III-4) investigations (data not shown). Febrile convulsions were not reported in any of the family members. Epileptic seizures occurred independently from FHM attacks, and the age at onset was generally somewhat later than for the FHM attacks. Treatment with a low daily dose (400 mg/day) of carbamazepine in patients II-2 and II-5, who had a body weight

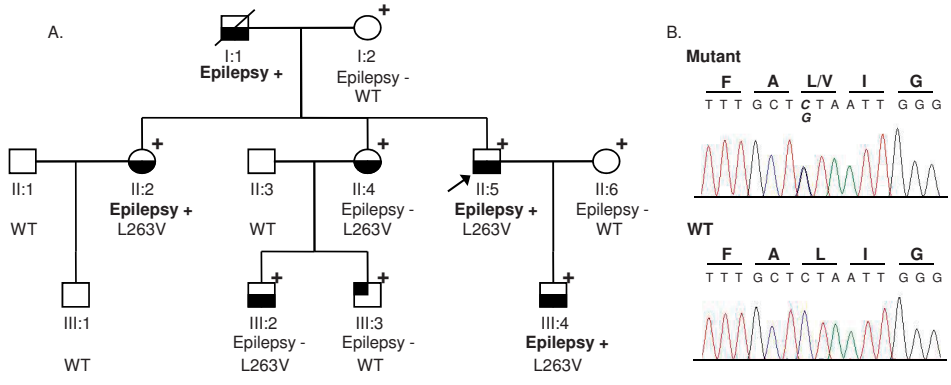

Figure 1. Pedigree of the Portuguese family with FHM and epilepsy. (**A**) Symbols with lower half filled represent individuals with FHM; the individual with the upper-left quarter filled has migraine without aura; double lined symbols represent individuals with epileptic seizures. The arrow indicates the proband. The "+" shows individuals that were clinically evaluated; "ha" indicates that clinical information was obtained heteroanamnestically. The presence of the heterozygous mutation is indicated by L263V / WT and the absence by WT / WT (wild type). (**B**) Electropherogram of the relevant part of exon 6 showing the heterozygous C>G nucleotide change (L263V) in the proband (Mutant) and healthy subject (WT) are shown.

Table 1. Summary of the clinical features of *SCN1A* mutation carriers

	II-2	II-4	II-5	III-2	III-4
Hemiplegia attacks					
Age/Age at onset *(years)*	59/14	57/16	52/18	35/12	12/10
Aura characteristics*	+/+/+/-	+/+/+/+	+/+/+/+	-/+/+/+	+/+/+/+
Duration	15 min-1 h	1 h	1 h	1 h	15 min-1 h
Frequency *(per year)*	< 1	2-3	1	1-2	> 1
Headache	Bilateral	Bilateral	Bilateral	Unilateral	Unilateral
Pain character	Throbbing	Throbbing	Non-throbbing	Throbbing	Non-throbbing
Severity	Severe	Severe	Mild	Moderate	Moderate
Nausea/vomiting	+/+	+/+	-/-	+/-	+/+
Photophobia/Phonophobia	+/+	+/+	+/+	-/-	+/+
Seizures/epilepsy					
Age at onset *(years)*	6	N.A.	4	N.A.	8
Type of epilepsy	GTC and CP	-	GTC	-	GTC
Frequency *(per year)*	1-3	N.A.	1-3	N.A.	1-4
Treatment (daily dose of carbamazepine in mg)	400	N.A.	400	N.A.	600

*Characteristics of aura: visual symptoms/sensory symptoms/hemiplegia/aphasia; "-": absent; "+":present; GTC: generalized tonic-clonic seizure; CP: complex partial seizure; N.A.: not applicable.

of < 60 kg, was successful with respect to both epileptic and hemiplegic attacks as patients remained attack free. Patient III-4 occasionally had epileptic attacks when treated with a daily dose of 600 mg of carbamazepine, but he did not tolerate a higher dose. Psychomotor development of all individuals was normal.

Haplotype analysis

Genomic DNA was isolated from peripheral leucocytes using a standard salting-out extraction method.[29] The involvement of the FHM1 (19p13) locus was excluded on the basis of genetic markers D19S221, D19S1150, D19S226 as described before.[7]

In addition, involvement of the FHM2 and FHM3 loci was investigated by analysing genetic markers D1S2624, D1S2707, D1S2844 (FHM2; 1q23)[16] and D2S156, D2S2330 and D2S2381 (FHM3;2q24),[9] respectively (data not shown). Oligonucleotide primer sequences were obtained from the Human Genome Database (http://www.gdb.org/). Polymerase chain reaction (PCR) amplification was performed using standard conditions. PCR products were detected on an automated fragment run analyser (ABI 3700 DNA sequencer; Applied Biosystems, Foster City, CA, USA). All genotypes were analysed and independently scored by M-J.C. and K.R.J.V. using Genescan and Genotyper 2.1 software (Applied Biosystems). Haplotype was constructed evaluating the segregation and assuming a minimal number of recombinations.

Mutation screening

All exons and flanking intronic regions of the *ATP1A2* and *SCN1A* genes were amplified by PCR, using genomic DNA of the proband as a template. Direct sequencing was performed on PCR products using Cycle Sequencing (Prism Big Dye Terminators Cycle Sequencing kit; Applied Biosystems) and an ABI3700 DNA sequencer (Applied Biosystems). Sequencing was performed in the forward and reverse direction. For detection of the *SCN1A* mutation L263V (C→G, nt position 787, Ac. no. NM_006920), exon 6 was amplified by PCR using primers 'E6F' (5′-TTGCTTCTCCACTAGCGTTG-3′) and 'E6R' (5′-ACTTGAGGGGCTGGATATCC-3′), resulting in a 489-bps product that was subjected to direct sequencing. One hundred and fifty healthy controls (i.e., Portuguese blood donors without migraine history) were screened for the *SCN1A* mutation by direct sequencing.

RESULTS

Haplotype analysis in the Portuguese family was compatible with both the FHM2 (i.e., 1q24) and FHM3 (i.e., 2q24) locus as single haplotypes co-segregated with disease in all five FHM patients for which DNA was available (data not shown). Sequence analysis of the *ATP1A2* gene did not reveal a causative mutation. In contrast, in the *SCN1A* gene a novel heterozygous missense mutation was identified, changing a single nucleotide in exon 6 (nt 787 C→G) (Fig. 1B). This C to G nucleotide transversion replaces a leucine for a valine at position 263 of the $Na_v1.1$ a1 subunit (Fig. 2A). The L263V mutation co-segregated with hemiplegic migraine in all five FHM individuals and was absent in 300 control chromosomes. Notably, three of the five FHM patients with the L263V mutation also had epileptic seizures. Patient I-1 most probably is the fourth patient from this family suffering from hemiplegic migraine and epilepsy, but no DNA was available to confirm the presence of the L263V mutation. No other associated neurological symptoms such as ataxia were reported for any of the family members. Taxonomy analysis showed a strong evolutionary conservation of Leu263 amino acid among different voltage-gated sodium channels a subunits (Fig. 2B).

DISCUSSION

Here we report a novel L263V missense mutation in the *SCN1A* gene encoding the α1 subunit of voltage-gated sodium $Na_V1.1$ channels in a Portuguese family with FHM and partially co-segregating epilepsy. Several factors indicate that L263V is the causative mutation in this family with FHM and epilepsy: (i) L263V co-segregated in all five patients with hemiplegic migraine, three of them also had epilepsy (a fourth, deceased person probably had the mutation and epilepsy); no phenocopies or cases of incomplete penetrance were observed; (ii) L263V was absent in 300 control chromosomes; and (iii) Leu263 shows strong evolutionary conservation among several voltage-gated sodium channel subunit homologues across species; and (iv) its location in transmembrane domain DIS5 of the protein suggests an important functional role. α1 subunits of $Na_V1.1$ sodium channels consist of four homologous domains (DI–DIV), each containing six highly conserved transmembrane segments (S1–S6), which form the

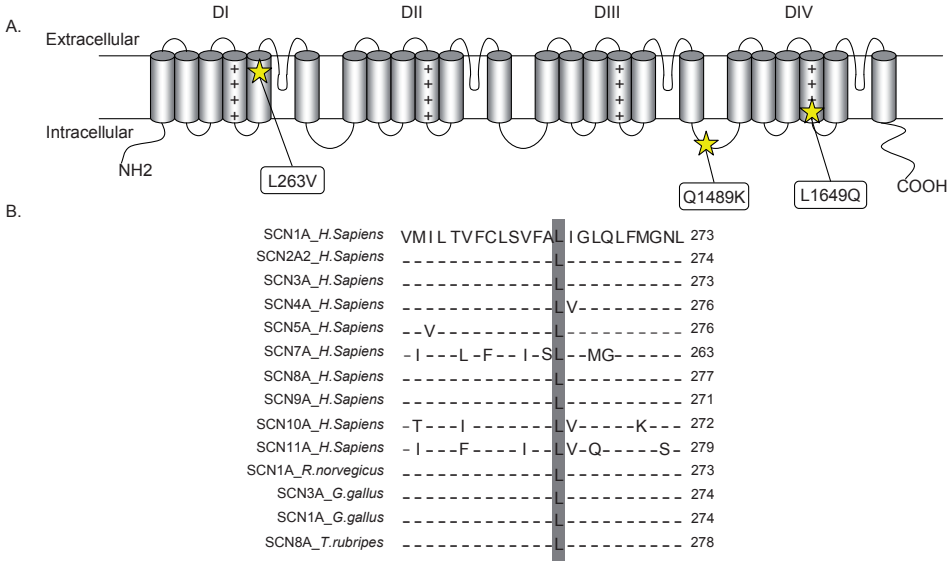

Figure 2. Location of FHM3 *SCN1A* mutations and conservation of Leu263. (**A**) Schematic representation in the $Na_v1.1$ α1 subunit with the location of the FHM3 mutations. (**B**) Evolutionary conservation of the relevant part of the D1S5 transmembrane segment. Amino acid numbering for each sequence is presented. The gray box (with L) represents conservation of residue Leu[263]. Protein accession numbers are as follows: *Homo sapiens*: SCN1A (P35498), SCN2A2 (Q99250), SCN3A (Q9NY46), SCN4A (P35499), SCN5A (Q14524), SCN7A (Q01118), SCN8A (Q9UQD0), SCN9A (Q15858), SCN10A (Q9Y5Y9), SCN11A (Q9UI33); *Rattus norvegicus* SCN1A (P04774); *Gallus gallus*: SCN1A (ENSGALP00000017793) and SCN3A (ENSGALP00000017960); and *Takifugu rubripes* SCN8A (NEWSINFRUP00000180009). All sequences were retrieved from NCBI or Ensembl databases.

central pore of the sodium channel.[30] Mutation L263V is located in the highly conserved S5 segment of the first domain (DIS5). S5 and S6 segments and S5-S6 linkers are important for ion selectivity and gating kinetics of the channel.[31-32] No particular functional role has been established for DIS5, but closely located mutation I252N has been found associated with SMEI.[33] Various other missense *SCN1A* mutations have been identified in S5 segments and are associated with different epileptic phenotypes of GEFS+[34,35], SMEI[36,37] or SMEB (borderline SMEI).[37–39]

The L263V mutation is the third FHM3 mutation. The first two FHM3 mutations, Q1489K and L1649Q, were identified in large German and US families, respectively, and are associated with FHM without additional neurological features such as epilepsy or ataxia.[9,22] Only in three of the 18 Q1489K mutation carriers with hemiplegic migraine were seizures reported that occurred only during infancy [two patients (in different families) with one episode; one patient with more attacks].[9] The Portuguese family with the L263V mutation displays co-occurring hemiplegic migraine and generalized tonic-clonic epilepsy with a high penetrance. It is not clear how this mutation can cause epilepsy in certain attacks and hemiplegic migraine in other attacks. A detailed analysis of the functional consequences of the L263V mutation and the two other FHM3 mutations is underway to answer that question.

In conclusion, this study has strengthened the molecular link between migraine and epilepsy, supporting the existence of a continuum of chronic episodic disorders. In addition, it has expanded the clinical spectrum associated with FHM3 *SCN1A* mutations.

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CHAPTER 7

EPISODIC ATAXIA ASSOCIATED WITH EAAT1 MUTATION C186S AFFECTING GLUTAMATE REUPTAKE

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ABSTRACT

Background: Episodic ataxia (EA) is variably associated with additional neurologic symptoms. At least 4 genes have been implicated. Recently, a mutation in the *SLC1A3* gene encoding the glutamate transporter EAAT1 was identified in a patient with severe episodic and progressive ataxia, seizures, alternating hemiplegia, and migraine headache. The mutant EAAT1 showed severely reduced uptake of glutamate. The syndrome was designated EA6 and shares overlapping clinical features with EA2, which is caused by mutations in *CACNA1A*.

Objective: To test the role of the *SLC1A3* gene in EA.

Design: Genetic and functional studies. We analyzed the coding region of the *SLC1A3* gene by direct sequencing.

Patients: DNA samples from 20 patients with EA (with or without interictal nystagmus) negative for *CACNA1A* mutations were analyzed.

Main Outcome Measures: We identified 1 novel EAAT1 mutation in a family with EA and studied the functional consequences of this mutation using glutamate uptake assay.

Results: We identified a missense C186S mutation that segregated with EA in 3 family members. The mutant EAAT1 showed a modest but significant reduction of glutamate uptake.

Conclusions: We broadened the clinical spectrum associated with *SLC1A3* mutations to include milder manifestations of EA without seizures or alternating hemiplegia. The severity of EA6 symptoms appears to be correlated with the extent of glutamate transporter dysfunction.

INTRODUCTION

Episodic ataxias (EAS) are rare genetic disorders characterized by recurrent episodes of cerebellar ataxia variably associated with additional neurologic features. Different subtypes of EA are defined on the basis of genetic loci and clinical manifestations.[1]

The most common and best characterized subtypes of EA are EA1 and EA2. EA1 is caused by missense mutations in the *KCNA1* gene encoding a subunit of neuronal $K_v1.1$ K^+ channels.[2] EA1 usually presents with short-lasting attacks that often are triggered by exertion, stress, or startle. Patients show persistent interictal motor unit activity (myokymia). EA2 is caused by mutations in the *CACNA1A* gene encoding the pore-forming subunit of neuronal $Ca_v2.1$ Ca^{2+} channels.[3] Mostly, nonsense, frameshift, splice site, and missense mutations have been described, resulting in either a complete loss[4] or partial impairment[5,6] of $Ca_v2.1$ channel function. The episodes in EA2 last longer than in EA1, up to several hours,[7] and are often associated with vertigo and migrainous headache and can be triggered by exercise, fatigue, and stress.[8] Acetazolamide may prevent attacks.[9] Between attacks, nystagmus usually occurs. Many patients have interictal ataxia in addition to the attacks. The EA3, EA4, and EA5 subtypes are rarer and less well-defined disorders compared with EA1 and EA2.[1]

The EA6 subtype was identified in a 10-year-old patient with a severe phenotype of episodic and progressive ataxia, seizures, alternating hemiplegia, and migraine headache.[10] A heterozygous de novo P290R missense mutation was identified in the *SLC1A3* gene by use of a candidate gene approach. *SLC1A3* encodes the glial excitatory amino acid transporter EAAT1, which is involved in glutamate removal from the synaptic cleft.[11,12] Functional analysis of the mutant EAAT1 protein showed marked reduction of glutamate uptake in vitro.[10]

In the present study, we performed a mutation analysis of the *SLC1A3* gene (OM/M 600111) in 20 patients with EA2-like symptoms but without *CACNA1A* mutations. In 1 family, we found an EAAT1 mutation that segregated with the disease in 3 patients. Functional studies revealed a moderate impairment of glutamate reuptake.

METHODS

Patients

We investigated 20 patients who were referred for molecular confirmation of EA2 in whom no mutations were found in the *CACNA1A* gene. These patients showed typical EA2-like symptoms, including interictal nystagmus but no myokymia, attacks of mild ataxia with a duration of several hours, and a positive response to acetazolamide. Except for 2 patients from the United States, all patients came from Europe, mostly the Netherlands. Family members of the proband with the *SLC1A3* mutation (Fig. 1) underwent neurologic examination by experienced neurologists (S.L.M.B. and A.H.S). All patients gave informed consent, and the study was approved by the local review board.

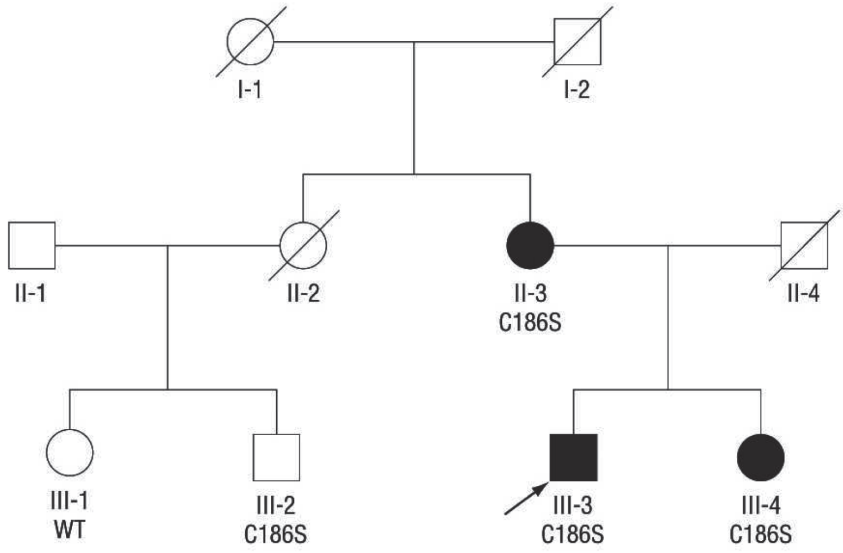

Figure 1. Pedigree of the episodic ataxia family with the EAAT1 186S mutation. Episodic ataxia is indicated by a filled black square or circle for males or females, respectively. C186S indicates heterozygosity for the mutation. WT indicates homozygosity for the wild-type allele.

Genetic studies

Genomic DNA was isolated from peripheral leukocytes using a standard salting out extraction method.[13] All exons and flanking intronic regions of the *SLC1A3* gene were amplified by polymerase chain reaction (PCR), using genomic DNA as a template. Direct sequencing was performed by cycle sequencing (Prism Big Dye Terminators Cycle Sequencing kit; Applied Biosystems, Foster City, California) using the dideoxy termination method and an ABI3700 automated sequencer (Applied Biosystems). Two hundred healthy controls were screened for the mutation by PCR analysis of exon 5 and subsequent restriction digestion of PCR products with restriction enzyme *Alu*I.

Functional studies

Functional studies[10] on glutamate uptake of wild-type and mutant EAAT1 were performed as described previously. In brief, full-length wild-type complementary DNA (EAAT1-WT) was cloned into a mammalian expression vector pcDNA3.1 (Invitrogen; Carlsbad, California). The mutant construct (EAAT1 186S) was generated by performing sitedirected mutagenesis (QuikChange; Stratagene; La Jolla, California). For functional analyses of the *SLC1A3* C186S mutation, 2 µg of wild-type (EAAT1-WT) or mutant (EAAT1-186S) EAAT1 complementary DNA constructs were transfected into COS7 cells. One day after transfection, the cells were dissociated and plated onto 60-mm-diameter tissue culture dishes. The cells were incubated with 1.5 mL of 1µM L-glutamic acid containing 1 µCi/mL of L-[3,4-3H]-glutamic acid for 2 minutes at room temperature. A total of 4 independent and masked experiments were performed, each in triplicate.

RESULTS

Genetic studies and clinical features associated with EAAT1 mutation

Mutation analysis of the *SLC1A3* gene in 20 patients revealed in 1 patient a heterozygous c.556 T_A substitution (*SLC1A3* reference sequence; GenBankNM004172) that changed a cysteine to a serine at position 186 (C186S) of the EAAT1 protein (Fig. 2A and 2B). The mutation was absent in 200 Dutch control individuals. C186S was identified in the proband (III-3), clinically affected family members II-3 and III-4, and 1 asymptomatic family member (III-2) (Fig. 1).

Clinical information of the affected family members is summarized in the Table. The proband (III-3) is a 35-year-old man who has had episodes of ataxia since early childhood. Attacks gradually changed over time. Initially, vertigo, nausea, and vomiting were the most bothersome symptoms. Later in life, truncal and gait ataxia during the attacks became more prominent. Attacks are often associated with nausea, vomiting, photophobia, phonophobia, vertigo, diplopia, slurred speech, and blurred vision. No headache was reported. Typically,

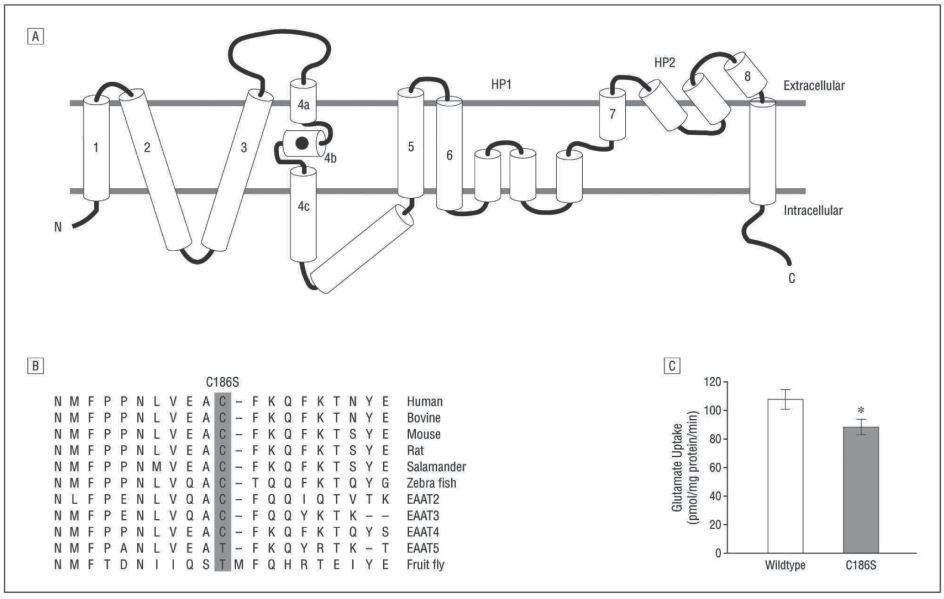

Figure 2. EAAT1 C186S mutation. (**A**) Schematic representation of the EAAT1 protein and the location of the mutated Cys186 amino acid in transmembrane segment 4b (indicated by a black dot) (the structure is adapted from Yernool et al[14]). (**B**) Conservation of the mutated residue Cys186 highlighted in gray. The protein sequences were obtained from GenBank (*Homo sapiens*, NP_004163; *Bos taurus*, NP_46411; *Mus musculus*, NP_683740; *Rattus norvegicus*, NP_062098; salamander, O57321; *Danio rerio*, NP_997805; *Drosophila melanogaster*, NP_477428; human EAAT2, AY066021; human EAAT3, NP_004161; human EAAT4, NM_005062; human EAAT5, NP_006662). (**C**) Glutamate uptake assay in COS7 cells expressing mutant EAAT1-186S (mean [SEM], 88.2[5.5]) or wild-type EAAT1-186C (mean [SEM], 107.8 [6.9]). The results are the mean (SEM) of the 4 experiments, each in triplicate. The values are picomoles of glutamate transported per milligram of protein per minute of incubation. Asterisk indicates significant reduction of glutamate uptake compared with wild type (p=0.029).
Error bars indicate SEM. HP indicates helical hairpin.

Table. Summary of clinical features of patients with episodic ataxia carrying the EAAT1 C186S Mutation

Clinical Feature	Mother (II-3)	Proband (III-3)	Sister (III-4)
Age at examination, y	56	35	28
Age of onset, y	<10	3	14
Ataxia	+	+	+
Vertigo	+	+	+
Diplopia/Visual blurring	-/-	+/+	-/-
Nausea/Vomiting	+/+	+/+	+/+
Photophobia/Phonophobia	+/+	+/+	+/-
Attack duration	Hours	Hours	Hours
Attack frequency	~ 10 / year	1-2 / month	~ 6 / year
Triggers	Emotional stress	Emotional stress, fatique, alcohol, caffeine	Emotional stress, fatique, exercise
Response to acetazolamide	+	+	+
Interictal gaze evoked nystagmus	-	+	-
Headache	-	-	+

attacks were provoked by emotional stress, fatigue, or consuming alcohol or caffeine. Attack duration was usually between 2 and 3 hours. Currently, his average attack frequency is once a month. Interictal neurologic examination revealed a horizontal gaze-evoked nystagmus without gait or truncal ataxia. Interictal electroencephalographic recording revealed no epileptic activity, and magnetic resonance imaging revealed no abnormalities (data not shown). His mother (II-3) and sister (III-4) were also diagnosed as having EAs. The 56-year-old mother (II-3) has had episodes of ataxia similar to those of the proband since elementary school. Her attacks are also associated with vertigo, nausea, vomiting, photophobia, phonophobia, and slurred speech. The attacks were not associated with headache. She now has approximately 10 attacks per year, which may last for several hours and can be triggered by stress. The 28-year-old sister (III-4) has had episodes of ataxia since the age of 14 years. Associated symptoms include vertigo, nausea, vomiting, and mild photophobia. Sometimes, the day after an attack, she experiences bilateral headache not associated with nausea, vomiting, phonophobia, or photophobia. Reported triggers are exercise, fatigue, and stress. Currently, she has on average 6 attacks a year. Typically, attacks last several hours. Acetazolamide significantly reduced the frequency of attacks in all 3 affected family members. His 40-year-old cousin (III-2) is an asymptomatic carrier of the C186S EAAT1 mutation. He experienced 4 attacks of migraine without aura and has tensiontype headache, but does not exhibit signs or symptoms related to ataxia. Individuals I-1, I-2, and II-2 were considered healthy based on limited heteroanamnestic information. His grandfather had died at the age of 98 years. His grandmother had complained about dizziness, but no neurologic examination was performed during her lifetime. No relevant clinical information is available for individual II-2, who died of an unrelated cause. Non-mutation carrier III-1 is asymptomatic.

Functional study of EAAT1 mutation C186S

To investigate the functional consequences of the EAAT1 C186S mutation, radioactive glutamate uptake assays were performed in COS7 cells. The low level of endogenous glutamate uptake activity has long established the COS7 cells as being well suited for functional studies of glutamate transporters.[15] We measured glutamate uptake in COS7 cells transfected with the wild-type (EAAT1-186C) or the mutant construct (EAAT1-186S). An 18% reduction in glutamate uptake was observed in cells expressing the mutant (mean [SEM], 88.2[5.5]) compared with the wild-type (mean [SEM], 107.8[6.9]) EAAT1, measured in picomoles per milligram of total protein per minute of incubation (p=0.029; Fig. 2C).

COMMENTS

We scanned the *SLC1A3* gene for mutations in 20 patients with EA2-like symptoms without *CACNA1A* mutations because of overlapping clinical features between EA2 and EA6. We found a novel nucleotide change c.556T_A in the *SLC1A3* gene, resulting in EAAT1 mutation C186S, in a family with EA and interictal nystagmus but without migraine, seizures, cerebellar atrophy, or alternating hemiplegia. Our genetic and functional data suggest that mutation C186S is pathogenic. First, the mutation C186S segregated with all 3 symptomatic family members but was not identified in a large panel of controls. The asymptomatic mutation carrier (III-2) had migraine without aura, but given the relatively high prevalence of migraine it is unlikely that these attacks are caused by the EAAT1 mutation. Therefore, he likely represents a nonpenetrant case of EA. Second, Cys186 is highly conserved among species (Fig. 2B). Our functional studies revealed a reduced glutamate reuptake for the mutant EAAT1 (Fig. 2C). Cys186 resides in transmembrane segment 4B (Fig. 2A) on the outer perimeter of the human EAAT1 transporter protein that is implicated in intersubunit contact.[15] The 4B-4C loop was recently shown to undergo substrate-dependent conformational changes and has been hypothesized to be important in stabilizing the trimeric structure of the transporter and coordinating the cooperativity for sodium binding.[16] Clinical severity of EA6 appears to be well correlated with glutamate reuptake capability of mutant EAAT1. The P290R mutation leads to a complete loss of glutamate reuptake and is associated with a severe EA phenotype with months-long attacks, seizures, and alternating hemiplegia.[10] In contrast, the C186S mutation has a mild effect on glutamate reuptake and is correlated with a milder EA phenotype. Although it is hard to predict from cellular studies how a mild increase in extracellular glutamate will affect cerebellar functioning in patients, it is well known that ion and neurotransmitter pathways are complex and tightly regulated. Subtle changes in these pathways have been associated with clinical manifestations.[17,18] Since we found a mutation in only 1 of 20 patients with *CACNA1A*-negative EA2-like symptoms, other genes must be involved. Likely candidate genes are components of ion and neurotransmitter pathways involved in the regulation of cerebellar neuronal excitability.

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FINANCIAL DISCLOSURE

Non reported.

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PART 2

A MONOGENIC MIGRAINE ASSOCIATED SYNDROME: RVCL/CHARIOT

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CHAPTER 8

C-TERMINAL TRUNCATIONS IN HUMAN 3'-5' DNA EXONUCLEASE TREX1 CAUSE AUTOSOMAL DOMINANT RETINAL VASCULOPATHY WITH CEREBRAL LEUKODYSTROPHY

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Autosomal dominant retinal vasculopathy with cerebral leukodystrophy is a microvascular endotheliopathy with middle-age onset. In nine families, we identified heterozygous C-terminal frameshift mutations in *TREX1*, which encodes a 3'-5' exonuclease. These truncated proteins retain exonuclease activity but lose normal perinuclear localization. These data have implications for the maintenance of vascular integrity in the degenerative cerebral microangiopathies leading to stroke and dementias.

We have previously described three families sharing common features of retinal and cerebral dysfunction. Visual loss, stroke and dementia begin in middle age, and death occurs in most families 5 to 10 years later. These diseases map to 3p21.1-p21.3 (ref. 1) and are called cerebroretinal vasculopathy (CRV),[2] hereditary vascular retinopathy (HVR)[3,4] and hereditary endotheliopathy, retinopathy, nephropathy and stroke (HERNS).[5] We now designate these illnesses as autosomal dominant retinal vasculopathy with cerebral leukodystrophy (RVCL) (OMIM 192315). The neurovascular syndrome features a progressive loss of visual acuity secondary to retinal vasculopathy, in combination with a more variable neurological picture.[1-7] In a subset of affected individuals, systemic vascular involvement is evidenced by Raynaud's phenomenon and mild liver (micronodular cirrhosis)[2,5] and kidney (glomerular) dysfunction.[5]

This retinal vasculopathy is characterized by telangiectasias, microaneurysms and retinal capillary obliteration starting in the macula. Diseased cerebral white matter has prominent small infarcts that often coalesce to pseudotumors. Neuroimaging studies demonstrate contrast-enhancing lesions in the white matter of the cerebrum and cerebellum. Histopathology shows ischemic necrosis with minimal inflammation and small blood vessels occluded with fibrin.[5] The white matter lesions resemble post-radiation vascular damage.[2] Ultrastructural studies of capillaries show a distinctive, multilamellar subendothelial basement membrane.[5] By combining haplotypes in the three RVCL families, we narrowed the disease gene to a 3-cM region between markers D3S1578 and D3S3564 that encompassed ~10 Mb, containing over 120 candidate genes.[1] We then sequenced the full coding region and intron-exon boundaries of 33 candidate genes within this region (Supplementary Table 1).

Here we report the identification of mutations in *TREX1* (NM_033627), encoding DNA-specific 3' to 5' exonuclease DNase III. In the CRV[2] and HVR[3,4] pedigrees, a heterozygous 1-bp insertion (3688_3689insG) leads to V235fs and a consequent premature stop. In HERNS,[5] a heterozygous 4-bp insertion (3727_3730dupGTCA) results in a frameshift at T249 (Fig. 1a, b).

Next, we examined six families with putative RVCL (Supplementary Table 2).[2,6,7] In each, we identified frameshift mutations affecting the C terminus of TREX1. In three, the alteration was V235fs, the same as that in the CRV and HVR pedigrees. Haplotype analysis suggests that they are not related (data not shown). We did not detect any of the mutations in panels of chromosomes matched by ancestry or location (Supplementary Methods). In the CRV and HERNS families, all affected individuals over the age of 60 (but none of the unaffected individuals over the age of 60) carried a *TREX1* mutation (100% penetrance). In the HVR[3,4] family, 10 of the 11 mutation carriers over 60 years of age have retinopathy.

TREX1 (DNase III) is a DNA-specific 3' to 5' exonuclease ubiquitously expressed in mammalian cells.[8-10] It is thought to function as a homodimer, with a preference for single-stranded DNA and mispaired 3' termini.[8] TREX1 is a part of the SET complex[11] that normally resides in the cytoplasm but translocates to the nucleus in response to oxidative DNA damage.[12]

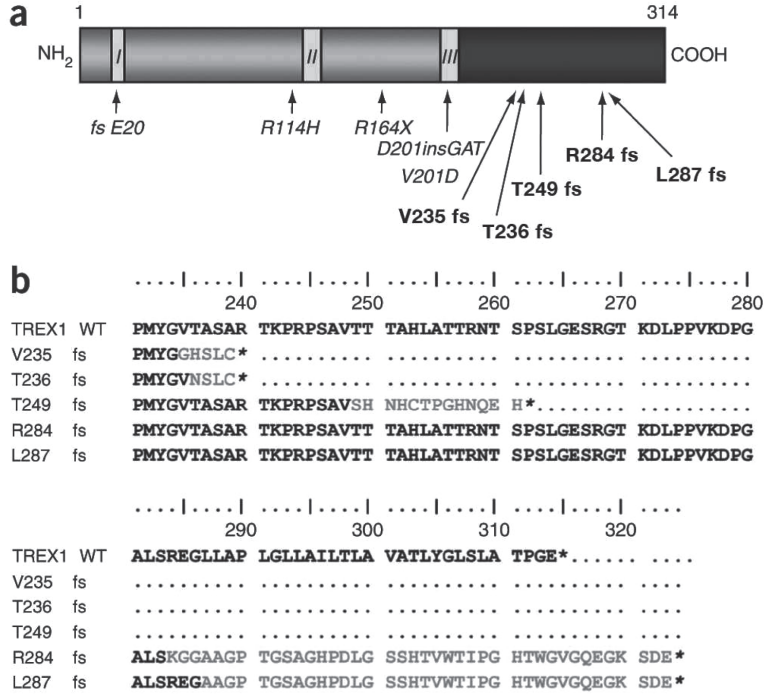

Figure 1. Diagram of TREX1 protein. (**a**) TREX1 has three exonuclease domains. Mutations in italics are associated with AGS[13], and those in boldface at the C terminus are associated with RVCL. (**b**) Comparison of the amino acid sequence of the C terminus of wild-type (WT) TREX1 with RVCL associated mutations. The abnormal sequence introduced by the frameshifts is depicted in gray.

Recently, homozygous mutations in *TREX1* have been reported to cause Aicardi-Goutière syndrome (AGS).[13] AGS is a rare, familial, early-onset progressive encephalopathy featuring basal ganglia calcifications and cerebrospinal fluid lymphocytosis, mimicking congenital viral encephalitis.[14] Notably, mutations associated with AGS disrupt the enzymatic sites in TREX1. This loss of exonuclease function[13] (Fig. 1) is hypothesized to cause the accumulation of altered DNA that triggers a destructive autoimmune response.[13] No phenotype was reported for the heterozygous carriers of these mutations; however, a heterozygous mutation in *TREX1* causing familial chilblain lupus has been reported recently.[15]

The distinctive clinical course and pathology of RVCL compared with AGS suggests separate disease mechanisms. The frameshift mutations observed in RVCL are downstream of the regions encoding the catalytic domains, whereas in AGS, homozygous mutations occur that alter exonuclease function. The heterozygous mutations observed in RVCL did not impair the enzymatic activity of TREX1 (Fig. 2a), in comparison with the R114H substitution in AGS.[13]

To investigate how the RVCL TREX1 proteins differ from the wild type, we performed expression studies using confocal microscopy on cells transfected with TREX1 tagged with a fluorescent protein (Fig. 2b and Supplementary Fig. 1). The wild-type TREX1 labeled with

fluorescent protein (FP-TREX1) localized to the perinuclear region. In contrast, the TREX1 proteins FP-V235fs and FP-T249fs were diffusely distributed in the cytoplasm and the nucleus, as was the case for the fluorescent protein alone (Fig. 2b and Supplementary Videos 1-4 online). Protein blotting confirmed that the expressed proteins were of the correct size (Fig. 2c). These results suggest a perinuclear targeting signal within the C terminus of TREX1. Consequently, we generated a construct containing the C-terminal 106 amino acid residues of TREX1 (FP-C-106). This protein showed a perinuclear localization pattern identical to that of the wild-type TREX1 protein (Fig. 2b). The TREX1 protein containing amino acid change R114H, found in AGS, also had the same pattern as the wild-type protein. In contrast, the protein with the alteration closest to the C terminus of TREX1, FP-287fs, was diffusely distributed, like the other two truncated proteins (data not shown).

The TREX1 proteins found in individuals with RVCL lack part of the C terminus. In haploinsufficient individuals, this may prevent an interaction with the SET proteins and therefore may prevent formation of the SET complex. The SET complex is hypothesized to target DNA repair factors, including TREX1, to damaged DNA under conditions of oxidative

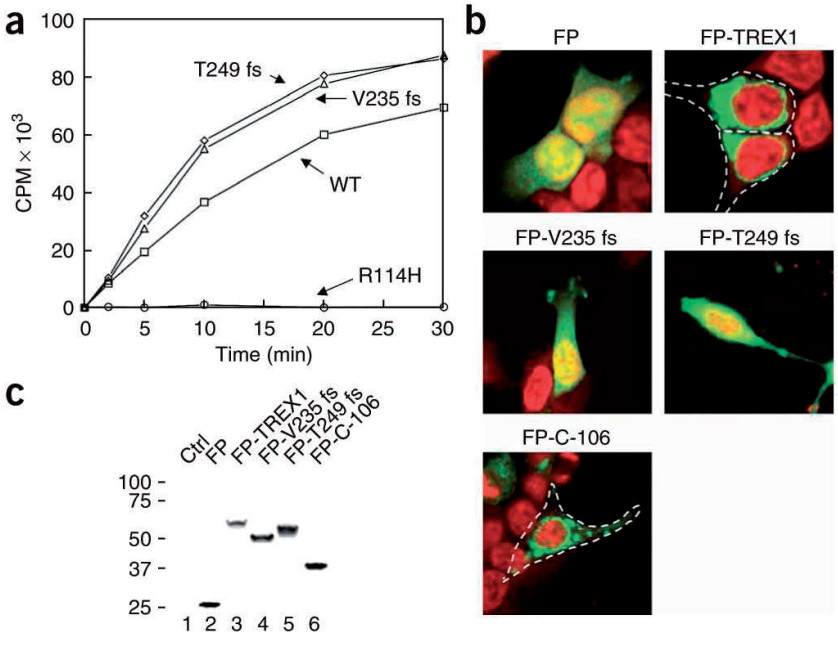

Figure 2. Functional consequences of RVCL associated *TREX1* mutations. (**a**) Assessment of 3'-5' exonuclease activity using equivalent amounts of purified recombinant proteins expressed in E. coli. (**b**) Confocal microscopy of HEK293T cells showing transiently expressed fluorescent protein (FP)-tagged TREX1 proteins (green), TOPRO3 staining of nuclei (red) and overlay (yellow). Similar expression patterns were obtained for wild-type protein and for proteins derived from constructs containing mutations associated with AGS and RVCL in CHO, HL-60 and HeLa cells (data not shown). (**c**) Protein blot analysis of untransfected cells (1) and cells transfected with enhanced yellow fluorescent protein (eYFP) (2), wild-type TREX1 (3), TREX1 mutants (4,5) and the C-terminal 106 amino acids (6), all linked to eYFP.

stress.[11,12] Lack of sufficient TREX1 associated with the SET complex may result in failure of granzyme A-mediated cell death.[12] Alternatively, the dissemination of untethered TREX1 in the nucleus and cytoplasm may have detrimental effects, specially on endothelial cells. The clinical syndromes in these families and the study of their mutations should deepen our understanding of exonuclease function, homeostasis of the endothelium and events leading to premature vascular aging. RVCL and cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) represent two examples of monogenic disease featuring a cerebral microangiopathy for which the genetic defects are now known and from which we can gain new insights into the origin of strokes and dementia. We obtained consent from all participants in this study, and the study was approved by the Office for Protection of Research Subjects at UCLA and the Human Research Protection Office at Washington University School of Medicine.

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COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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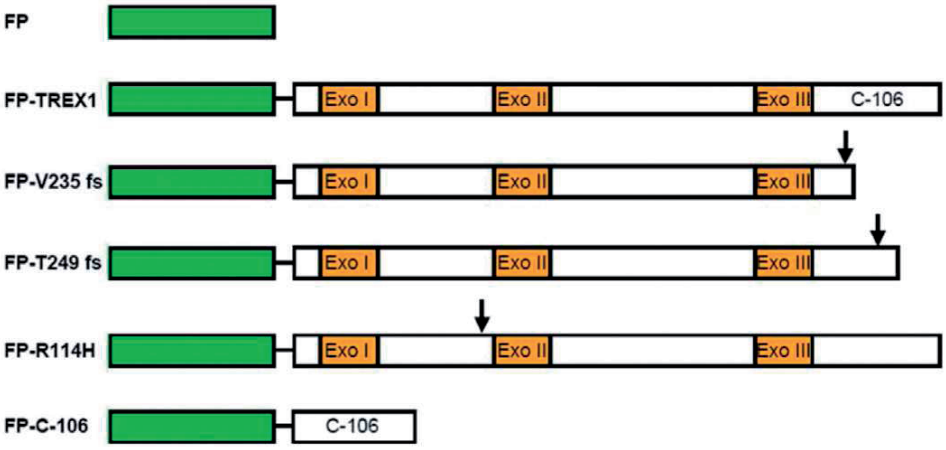
SUPPLEMENTARY MATERIAL



Supplementary Fig. 1. Schematic representation of FP constructs expressed in mammalian cells. The FP was cloned at the amino-terminus of TREX1 and mutants. A carboxyl-terminal segment of the last 106 amino acids of wild-type TREX1 was also prepared (C-106). The arrows indicate approximate sites of the mutations.

Supplementary Table 1. The 32 candidate genes sequenced prior to the discovery of *TREX1* as the causative gene for RVCL

Gene	Name	Gene ID	OMIM
AMIGO3	Adhesion molecule with Ig-like domain 3	386724	N/A
ATRIP	ATR interacting protein	11277	606605
CACNA1D	Voltage-dependent L-type calcium channel subunit alpha-1D	776	114206
CACNA2D2	Calcium channel voltage dependent, alpha- 2/ Delta Subunit 2	9254	607082
CCR1	Chemokine (C-C motif) receptor 1	1230	601159
CCR2	Chemokine (C-C motif) receptor 2	1231	601267
CCR3	Chemokine (C-C motif) receptor 3	1232	601268
CCR9	Chemokine (C-C motif) receptor 9	10803	604738
CELSR3	Cadherin, EGF LAG seven-pass G-type receptor 3	1951	604264
CSPG5	Chondroitin sulfate proteoglycan 5	10675	606775
CTNNB1	Catenin (cadherin-associated protein), beta 1,	1499	116806
CX3CR1	Chemokine (C-X3-C motif) receptor 1	1524	601470
CXCR6	Chemokine (C-X-C motif) receptor 6	10663	605163
DAG1	Dystroglycan 1 (dystrophin-associated glycoprotein 1)	1605	128239
ENTPD	Ectonucleoside triphosphate diphosphohydrolase 3	956	603161
GNAT1	Guanine nucleotide binding protein, alpha transducing activity polypeptide 1	2779	139330
GPX1	Glutathione peroxidase 1	2876	138320
LAMB2	Laminin, beta 2 (laminin S)	3913	150325
MAP4	Microtubule-associated protein 4	4134	157132
PH4	PH-4 hypoxia-inducible factor prolyl 4-hydroxylase	54681	N/A
PLXNB1	Plexin B1	5364	601053
RASSF1	Ras association (RalGDS/AF-6) domain family 1	11186	605082
RHOA	Ras homolog gene family, member A	387	165390
RIS1	TMEM158 transmembrane protein 158 (RIS-1 Ras-induced senescence 1)	25907	N/A
RPL29	Ribosomal protein L29	6159	601832
RPSA	Ribosomal protein SA	3921	150370
SEMA3F	Semaphorin 3F	6405	601124
Scotin	Scotin	51246	607290
SEMA3B	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphoring) 3B	7869	601281
STAB1	Stabilin 1	23166	608560
TRAIP	TRAF interacting protein	10293	605958
VIPR1	Vasoactive intestinal peptide receptor 1	7433	192321

N/A, not applicable. GeneID, and OMIM identities are indicated.

Supplementary Table 2. Mutations identified in TREX1 in RVCL

#	Mutation	Frameshift	Reference	Geographical (Background)
1	3688_3689insG	V235	Grand et al	North America (European)
2	3688_3689insG	V235	Storimans et al Terwindt et al	Netherlands
3	3727_3730dupGTCA	T249	Jen et al	North America (Chinese)
4	3688_3689insG	V235	Grand et al	North America (Ashkenazi-Jewish)
5	3691_3692insA	T236	Weil et al	Germany
6	3835_3836insA	R284	Cohn et al	Australia
7	3688_3689insG	V235	Unpublished	North America
8	3688_3689insG	V235	Unpublished	Australia
9	3843_3844insG	L287	Unpublished	Netherlands

Supplementary Table 3. Primers and PCR conditions for *TREX1* exon

Primer	Primer Sequence Forward	Primer Sequence Reverse
1	tgtaaaacgacggccagtatggtggtgagagggacagacc	caggaaacagctatgaccaaagatgagggtctgcatggg
2	tgtaaaacgacggccagtgaatgtgctggtcccactaagg	caggaaacagctatgaccaaggctaggagcaggttggc
3	tgtaaaacgacggccagtctctccctgtgtgtggctcc	caggaaacagctatgaccttgtgacagcagatggtcttgg
4	tgtaaaacgacggccagtctaggcagcatctacactcgcc	caggaaacagctatgaccatcctgctagggaaagtgaggg

The appropriate universal sequencing primer was used for either reading the forward or reverse strand of all amplicons. Forward sequencing primer (5'-GTAAAACGACGGCCAGT-3'); reverse sequencing primer (5'-CAGGAAACAGCTATGACC-3'). Incubation conditions: temperature, 60°C; Mg2+concentration, 1.5 mM.

SUPPLEMENTARY VIDEOS 1-4 LEGENDS

Supplementary Video 1.

Confocal microscopy video showing functional consequences of RVCL-associated *TREX1* mutations as modeled in transiently expressed HEK293T cells. Fluorescent protein (green) and TOPRO3 stained nuclei (blue). Fluorescence expression pattern of fluorescent protein (FP) alone. The protein is diffusely distributed in the cytoplasm and in the nucleus.

Supplementary Video 2.

Confocal microscopy video showing functional consequences of RVCL-associated *TREX1* mutations as modeled in transiently expressed HEK293T cells. Fluorescent protein (green) and TOPRO3 stained nuclei (blue). Fluorescence expression pattern of wild type TREX1 tagged with the fluorescent protein (FP-TREX1). This fusion protein is found in a perinuclear compartment and is excluded from the nucleus.

Supplementary Video 3.

Confocal microscopy video showing functional consequences of RVCL-associated *TREX1* mutations as modeled in transiently expressed HEK293T cells. Fluorescent protein (green) and TOPRO3 stained nuclei (blue). Fluorescence expression pattern of mutant TREX1, tagged with the fluorescent protein (FP-V235 fs). The mutant form of TREX1 exhibits an expression pattern identical to the fluorescent protein (FP) alone.

Supplementary Video 4.

Confocal microscopy video showing functional consequences of RVCL-associated *TREX1* mutations as modeled in transiently expressed HEK293T cells. Fluorescent protein (green) and TOPRO3 stained nuclei (blue). Fluorescence expression pattern of carboxyl-terminal 106 amino acids of TREX1 tagged with the fluorescent protein (FP-C-106). This fusion protein is found in a perinuclear compartment and is excluded from the nucleus. This pattern is identical to the native exonuclease, implicating this short stretch of amino acids in mediating the perinuclear localization of the protein.

SUPPLEMENTAL METHODS

Samples

We analyzed DNA samples from nine families with clinical symptoms of RVCL (Supplementary Table 2). Informed consent was obtained from all patients, in accordance with procedures and regulations of the Institutional Review Boards.

Mutation detection

Genomic DNA was isolated from peripheral blood leukocytes or immortalized cell lines from consenting subjects as approved by IRB. In specific cases (Washington University Genome Sequencing Center), Phi29-based whole genome amplification was performed

on genomic DNA samples. Gene sequences of candidate genes were extracted from GenBank (www.ncbi.nlm.nih.gov) and Ensembl (www.ensembl.org) databases. Primers to amplify the coding exons and exon-intron boundaries of candidate genes were designed with the PrimerDesign script that is based on the use of the Primer 3 program. Universal (forward and reverse) tails were added to the 5' ends of amplification primers to serve as the sequencing primer sites (primer sequences and PCR conditions are available on request). Direct sequencing of purified PCR products was done by using dye-terminator chemistry and electrophoresed on a MegaBase500 (Amersham Biosciences, Princeton, NJ) capillary sequencer or either the ABI3700 or ABI3730 automated sequencer (Applied Biosystems, Foster City, CA). The sequence traces were assembled and scanned for variations from the reference sequence using the PolyScan informatics suite or Vector NTI suite 9.0.0 program (Invitrogen, Carlsbad, CA). The tagged variations were then manually reviewed. All detailed protocols are available on request.

For further mutational analysis of *TREX1*, primers were designed to amplify the coding exons of *TREX1* (Supplementary Table 3). Purified PCR amplification products were sequenced using dye-terminator chemistry and electrophoresed on a MegaBase500 (Amersham Biosciences, Princeton, NJ) capillary sequencer or an ABI3700 sequencer (Applied Biosystems, Foster City, CA). Sequencing was analyzed using PolyPhred. Anonymized control samples were screened by sequencing. Controls were matched with the ethnic origins of the mutations. The RVCL *TREX1* mutations were not detected in 192 Caucasian (HD100CAU, Coriell), 192 Chinese (HD100CHI, Coriell) and 300 Dutch control alleles.

Haplotype analysis

For 20 microsatellite markers in the chromosome 3p21.1-p21.3 region, standard PCRs were performed using a PTC200 thermal cycler (Bio-Rad Laboratories, Foster City, CA). PCR products were analyzed on an ABI3700 sequencer (Applied Biosystems, Foster City, CA) and genotypes were assigned using GENESCAN and GENOTYPER software (Applied Biosystems, Foster City, CA). Two investigators scored genotypes independently. In addition, 13 SNPs in the region closely flanking *TREX1* were typed by direct sequencing. Disease haplotypes were constructed by inspection of segregation.

Molecular cloning, mutagenesis, expression, and purification of E. coli proteins

Mutations (V235fs, T249fs and R114H) were constructed using the cDNA clone encoding TREX1 variant 1 (Origene TC304415) as a template for site-directed mutagenesis. Oligos used for mutagenesis were as follows:

V235fs (5' ATGTATGGGGGTCACAGCCTCTG 3' and 5' CAGAGCGTGTGACCCCCATACA TG 3')

T249fs (5' TCTGCTGTCAGTCACAACCACTGC 3' and 5' CAGTGGTTGTGACTGACAGCA GATG 3')

R114H (5' GCCTTCCTGCGGCACCAGCCACAGCCCTGG and 3' ACCAGGGCTGTGGC TGGTGCCGCAGGAAGGC).

In the case of TREX1, V235fs, T249fs and R114H, the inserts were subcloned by PCR using the TREX1 cDNA clone as a template into the *E. coli* expression vector pET28a+-1 {a derivative of pET28a+ (Novagen) created in house} containing an N- terminal 6x His epitope tag. Correct clones were transformed into the *E. coli* strain BL21CodonPlus (DE3)-RIL (Stratagene, La Jolla, CA). Cells containing the TREX1 plasmids were grown at 37°C to an $Absorbance_{600}$ of 0.6. Isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM and the cultures incubated at 37°C for an additional 3h. Cells were then harvested and the pellets were stored at -80°C. For purification of recombinant proteins, cells were resuspended in cold sonication buffer (50 mM Tris pH 8.0, 500 mM NaCl, 10% glycerol, 5 mM beta mercaptoethanol, 1 mM imidazole, and 1 mM PMSF). The cell suspension was sonicated and centrifuged at 15,000 g at 4°C for 20 min to obtain a cleared lysate. His-tagged proteins were batch adsorbed to Ni-NTA Agarose (Qiagen, Valencia, CA) for 1 h at 4°C. The beads were washed extensively in wash buffer (sonication buffer containing 25 mM imidazole) and packed into a 5 ml polypropylene (Qiagen) column. After additional washes, fractions were collected during elution with five column volumes of elution buffer (sonication buffer containing 250 mM imidazole). Fractions containing His-TREX1 proteins were identified by SDS-PAG electrophoresis and Western blotting with an anti-HIS-HRP conjugated antibody (Clontech, Mountain View, CA) or, alternatively, with Coomassie Brilliant Blue staining. The samples were pooled, concentrated and aliquots of the purified proteins frozen at -80°C.

Exonuclease assays

1 µg of Poly(dA) (GE Healthcare, Princeton, NJ) was labeled at the 3' end with ^{32}P dATP (GE Healthcare) using Terminal Transferase (Roche Diagnostic Corp., Indianapolis, IN). Reactions containing 50 mM Tris pH 8.5, 4 mM MgCl2, 1 mM DTT, 10 µg BSA, 0.01 g radiolabeled poly(dA) substrate, and recombinant exonuclease TREX1 protein were incubated in a total volume of 100 l at 37°C. Aliquots were removed at the indicated times and ethanol precipitated in the presence of 50 µg denatured calf thymus DNA (Sigma-Aldrich, St. Louis, MO). Ethanol-soluble radioactivity released into the supernatant was measured by scintillation counting.

Generation of N-terminally-tagged TREX1 constructs

To directly visualize TREX1 within the living cell, all TREX1 forms were N-terminally tagged with the enhanced yellow fluorescent protein (eYFP). For clarity, the epitope tag is designated hereafter as fluorescent protein (FP) tag. At the wavelength employed it gives green fluorescence. The FP coding sequence was excised via *Eco*RI/*Bsr*GI from sT-DAF-eY.[1] This fragment was utilized in a three-fragment ligation with *Eco*RI/*Xba*I-digested CD59dGPI[2] and the respective *Bsr*GI/*Xba*I-digested PCR- derived TREX1 forms (see below). This resulted in amino-terminal tagging of TREX1 FP-TREX1, FP-V235fs and FP-T249fs). Wild-type FP was expressed from the second cistron of sT-DAF. Wild-type TREX1 (FP-TREX1) was used as a template DNA to generate the Aicardi-Goutières R114H mutant (FP-R114H) by site-directed mutagenesis as described above. To study the effect on cellular localization of the carboxyl-terminus of TREX1, a FP-tagged fusion protein containing the last 106 amino acids of native

TREX1 was generated (FP-C106). A 646 bp *BsrGI*/*Bsa*I fragment was excised from FP-TREX1, to remove the entire amino-terminus including all exonuclease sites. The cohesive ends were then blunted and the linearized 4532 bp fragment relegated, resulting in FP-C106. All PCR-derived DNA fragments and ligation products were verified by DNA sequencing.

Connfocal Microscopy

HEK293T cells (7 x 10^5 on cover slides in 6-well plates) were transiently transfected overnight with 1.5 µg of each construct using TransIT-293 (Mirus, Madison, WI), according to manufacturer's directions. Following two washes in PBS, the cells were fixed for 30 min at room temperature (RT) in PBS containing 2% paraformaldehyde. Following two washes in PBS, the cover slides were incubated for 30 min at RT in PBS containing a 1/2000 dilution of Topro3 (Molecular Probes, Carlsbad, CA) to visualize the nuclei, washed again with PBS and mounted on slides over night with ProLong Gold (Molecular Probes). Samples were examined using a Zeiss LSM 510 laser scanning confocal microscope and images were processed using Image Examiner Software (Zeiss, Jena, Germany).

Expression, SDS-PAGE, and Western blotting

Following overnight transient transfection of HEK293T cells (described above), cells were washed with PBS, lysed with 1% Nonidet P-40, 0.05% SDS in PBS with 2 mM PMSF for 15 min at 4°C, and centrifuged at 12,000 g for 10 min. Supernatants were immediately evaluated or frozen at -80 °C. The Western blot was loaded with 5 x 105 cell equivalents per lane on non-reduced and electrophoresed (10% SDS-PAG). Following transfer to nitrocellulose, the blots were probed with 1:4000 dilution of monoclonal anti-GFP antibody JL-8 that recognizes both GFP and YFP (Clontech Laboratories) and then HRP donkey anti-mouse IgG (Amersham Biosciences).

Accession codes

GenBank: cDNA and amino acid numbering was determined using the TREX1 protein AAK07616 and nucleotide sequence NM_033627 (with the A at 2986 as the first base of the initiating ATG codon).

URLs

The UCSC Genome Browser is available at http://genome.ucsc.edu/. The Marshfield chromosome 3 genetic map is found at http://research.marshfieldclinic.org/genetics.

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CHAPTER 9

THE CLINICOPATHOLOGIC SPECTRUM OF CEREBRAL HEREDITARY ANGIOPATHY WITH VASCULAR RETINOPATHY AND IMPAIRED ORGAN FUNCTION CAUSED BY *TREX1* MUTATIONS (CHARIOT). A REVIEW OF 78 MUTATION CARRIERS FROM 11 UNRELATED FAMILIES

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Submitted

ABSTRACT

Background: We have shown that Cerebroretinal Vasculopathy (CRV), Hereditary Vascular Retinopathy (HVR), and Hereditary Endotheliopathy, Retinopathy, Nephropathy and Stroke (HERNS) are one disease caused by C-terminal frame-shift mutations in the DNA exonuclease *TREX1*. Here we define the clinicopathologic spectrum of this newly recognized and commonly misdiagnosed syndrome which we have renamed CHARIOT.

Methods: Standardized review of clinical, radiological, pathological and genetic findings in 11 unrelated families with CHARIOT.

Findings: We identified five distinct *TREX1* mutations in 78 subjects. Sixty-five mutation carriers had the characteristic clinical syndrome, similar across all mutations, of a vascular retinopathy followed by progressive focal neurological symptoms in association with white matter hyperintensities and contrast-enhancing frontoparietal mass lesions, often featuring massive edema. Mean (± SD) age at diagnosis was 42·9 ± 8·3 and at death 53·1 ± 9·6 years. Other cerebral manifestations were migraine (59%), cognitive decline (56%), psychiatric disturbances (42%) and seizures (17%). Non-cerebral involvement was typically mild and included anemia (74%), impaired liver (70%) and renal (50%) function, hypertension (60%) and Raynaud's phenomenon (40%). Pathological examination demonstrated a systemic vasculopathy with luminal narrowing and multi-laminated basement membranes. Presymptomatic mutation carriers (N=13; mean age: 35·1 ± 10·6 years) had Raynaud's phenomenon (54%), migraine (42%) and psychiatric symptoms (23%).

Interpretation: CHARIOT is an autosomal dominant, progressive, systemic small-vessel disease mainly characterized by progressive blindness due to vascular retinopathy, relentless neurological decline caused by cerebral mass and white matter lesions, and premature death. We propose diagnostic criteria to aid clinical recognition of this new disease.

INTRODUCTION

Cerebroretinal Vasculopathy (CRV),[1] Hereditary Vascular Retinopathy (HVR)[2,3] and Hereditary Endotheliopathy, Retinopathy, Nephropathy and Stroke (HERNS)[4] are autosomal dominant diseases initially described as independent entities. By concentrating on shared features of vascular retinopathy and brain lesions our international consortium mapped a common locus to chromosome 3p21·1-p21·3[5] and subsequently identified pathogenic heterozygous C-terminal frame-shift mutations in the *TREX1* gene. *TREX1* encodes a 3′-5′ DNA exonuclease involved in clearing cytosolic nucleic acids.[6] Thus, the three diseases were united into a single disorder and termed Retinal Vasculopathy with Cerebral Leukodystrophy.[6] Here we report a retrospective analysis of 78 *TREX1* mutation carriers from 11 unrelated families and provide the first comprehensive characterization of its genetic, clinical, neuro-radiological and pathological spectrum. The disease is commonly misdiagnosed as brain tumor, multiple sclerosis, or a central nervous system vasculitis. Prompted by the emerging clinical picture, pathogenesis, and absence of "leukodystrophy", we renamed the disease CHARIOT: Cerebral Hereditary Angiopathy with vascular Retinopathy and Impaired Organ function caused by *TREX1* mutations. To facilitate clinical recognition of CHARIOT, we formulated diagnostic criteria.

METHODS

We retrospectively evaluated medical records of 78 *TREX1* mutation carriers (35 females; 43 males) from 11 unrelated families from The Netherlands (3 families; n=37), USA (5 families; n=32), Australia (2 families; n=5), and Germany (1 family; n=4). Some genetic and clinical data from families 1-5 and 7-11 have been previously published in brief;[1-9] family 6 was recently identified and not reported before (Supplementary Table S1 and S2).

The institutional ethics committee at each participating institution approved the study and all living subjects provided written informed consent. Demographic and relevant clinical information was obtained from the medical records prior to September 1, 2009. All patients were personally interviewed and examined by one or more of the authors.

Vascular retinopathy was diagnosed from ophthalmologic reports of fundoscopic examination and fluorescein angiography. Cerebral lesions were identified by review of images on brain computed tomography (CT) or magnetic resonance imaging (MRI). Migraine,[10] liver and renal dysfunction, anemia, Raynaud's phenomenon[11] and hypertension were diagnosed according to established international criteria. Pathological findings were obtained from biopsy and autopsy reports in families 1, 2, 5 and 7-11. Neuropathologic data of families 1 and 7-9 have in part been previously published.[1, 4, 8, 9]

Descriptive statistics are based on the number of subjects for whom relevant data were available.

RESULTS

Study population

Five distinct C-terminal frame-shift *TREX1* mutations were identified (Chapter 8, Figure 1a). A clinical diagnosis of CHARIOT was made in 65/78 mutation carriers based on the presence of vascular retinopathy (n=64) or cerebral mass lesion with unknown retinopathy status (n=1) (Mutation carriers with symptomatic CHARIOT; MC+). Thirteen mutation carriers did not have vascular retinopathy or cerebral mass lesions at the time of inclusion in the study (MC-). Clinical, demographic, and neuro-radiological characteristics of living and deceased mutation carriers are summarized in Table 1. The clinical phenotype was similar across all mutations (Supplementary Table S1).

Mutation carriers with symptomatic CHARIOT (MC+)

Initial presentation and diagnosis (Table 1)

All 65 MC+ had developed a vascular retinopathy and/or cerebral mass lesion by middle age. Mean (± SD) age at clinical diagnosis was 42·9 ± 8·3 years (range 25-61). At the time of diagnosis, visual disturbances were present in 50/65 (77%) MC+; ten of them also had neurological symptoms. Asymptomatic retinopathy was discovered on screening because of neurological symptoms (n=4) or a family history of CHARIOT (n=5). For the remaining six MC+, documentation of initial symptoms (n=3) or visual function and retinal examinations were unavailable at the time of diagnosis (n=3). Thus, retinopathy was present at initial diagnosis in all MC+ with available data. Although the majority sought medical attention because of visual symptoms, a quarter did so because of neurological symptoms. A typical case of CHARIOT is described in the legend of Figure 1.

Vascular Retinopathy

Visual symptoms attributable to retinopathy included decreased acuity and field defects. Retinopathy could be visualized by fundoscopy, but was better appreciated with fluorescein angiography. Early stages were characterized by telangiectasias, micro-aneurysms and cotton wool spots (Fig. 2A, B) and, in the later stages, by perifoveal capillary obliteration and neovascularization (Fig. 2C, D). Histopathologic examination of the retina at autopsy (n=8) was consistent with scattered micro-infarcts. The retinal arteries had thickened hyalinized walls (Fig. 3A) and there were focal areas of disruption to the ganglion cells and inner nuclear layer of the retina, usually accompanied by vascular changes. In some, the pathologic process had progressed to retinal hemorrhage and neovascularization.

Cerebral Manifestations

All MC+ showed progressive focal neurological symptoms and/or cognitive impairment in association with an ever-increasing number and size of brain lesions (Table 1). Of the MC+ who were still alive at the time of the study, 12/30 (40%) had focal neurological symptoms and 11/29 (38%) had cognitive impairment, manifesting as bradyphrenia, apathy, irritability, and impaired memory and judgment. Of the MC+ who had already died, 28/29 (97%) had focal

Table 1. Manifestations of CHARIOT

	MC+		
	Living	**Deceased**	**MC-^**
Demographics			
Number of mutation carriers	30	35	13
Age at last follow-up or death			
Mean ± SD (yr)	47·3± 8·1	53·1± 9·6	35·1± 10·6
Range (yr)	34-62	32-72	18-58
Major signs/symptoms*			
Retinopathy	100 (30/30)	100 (34/34)	0 (0/12)
Age at diagnosis retinopathy			
Mean ± SD (yr)	41.2± 8.4	43.7± 7.7	N/A
Range (yr)	25-56	30-61	N/A
Cerebral			
Focal neurological	40 (12/30)	97 (28/29)	0 (0/13)
Cognitive decline	38 (11/29)	75 (21/28)	8 (1/13)
Migraine	48 (12/25)	75 (12/16)	42 (5/12)
Seizures	7 (2/27)	26 (7/27)	0 (0/12)
Psychiatric	31 (9/29)	52 (17/33)	23 (3/13)
Neuroradiology*			
White matter disease	95 (19/20[#])	100 (28/28)	33 (1/3)
Mass occupying lesions	75 (15/20)	91 (21/23^^)	0 (0/3)
White matter hyperintensities**	95 (18/19)	100 (16/16)	33 (1/3)
Calcifications***	71 (5/7)	45 (9/20)	Not Tested
Other organs involved*			
Liver[v]	65 (11/17)	74 (17/23)	Not Tested
Kidney[v]	50 (9/18)	50 (13/26)	0 (0/1)
Possible associations*			
Anemia	67 (8/12)	77 (17/22)	Not Tested
Hypertension	47 (9/19)	68 (21/31)	0 (0/2)
Raynaud's phenomenon	52 (14/27)	30 (10/33)	54 (7/13)
Gastrointestinal bleeding/telangiectasias	3 (1/30)	24 (8/34)	0 (0/13)

MC+: Mutation carriers with retinopathy or cerebral mass lesions; MC-: Mutation carriers without retinopathy or cerebral mass lesions. * Unless indicated otherwise, the disease manifestations presented in the table are shown as percentage of subjects followed by the number of subjects. The denominator varies according to the number of individuals with available data. [#] One subject with no evidence of white matter hyperintensities had an MRI done within 1 year of diagnosis with retinopathy. ^ Ten mutation carriers are from family 1 (mutation V235fs), 2 mutation carriers from family 11 (mutation L287fs), 1 from family 8 (mutationT249fs, this patient committed suicide at age 30). ^^ Five patients were excluded since the last neuroimaging available was more than 5 years before their death. ** Based on MRI scans only. *** Based on CT scans only. [v] Based on laboratory values. N/A:Not Applicable.

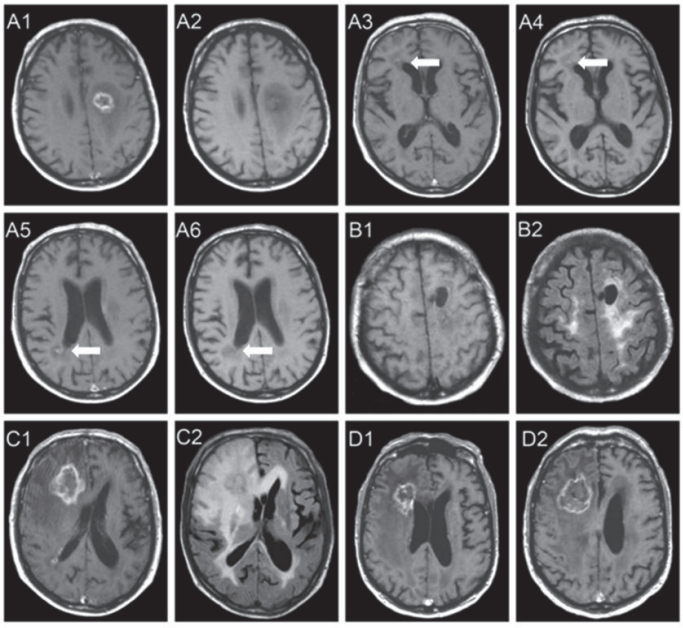

Figure 1. Presentation and progression of a typical case of CHARIOT.
At age 52, this man (family 2, V235fs mutation) reported progressive bilateral loss of vision. Ophthalmologic evaluation revealed a vascular retinopathy. At age 58 he developed a slowly progressive right-sided hemiparesis. He became intermittently irritable and passive and complained of headaches. His medical history revealed Raynaud's phenomenon and paroxysmal atrial fibrillation. In the left frontal white matter, there was a rim-enhancing lesion with mass-effect and surrounding edema on MRI (gadolinium enhanced T1-weighted in A1, non-enhanced T1-weighted in A2). This lesion demonstrated focal calcifications on CT (not shown). Two smaller rim-enhancing lesions were noted periventricularly in the right frontal (T1-weighted in A3, non-enhanced T1-weighted in A4) and parietal lobes (gadolinium enhanced T1-weighted in A5, non-enhanced T1-weighted in A6). A biopsy of the left fronto-parietal lesion revealed tissue necrosis. Dexamethasone (60 mg for 10 days) was started with slight improvement of the hemiparesis. Four months later, his headaches became worse and he developed word-finding difficulties and a wide-based gait in addition to his right-sided hemiparesis. Routine laboratory investigation showed a mild anemia and mildly impaired renal and liver function. There was a mild increase in cerebrospinal fluid protein with normal cell count and no oligoclonal bands. Antinuclear antibodies, extractable nuclear antigens, anticardiolipin IgG and IgM and anti-neutrophilic cytoplasmic antibodies were negative. On MRI, the left frontal lesion had diminished in size, showed now only minimal enhancement, although the surrounding edema and/or gliosis remained as a large zone of confluent T2 hyperintensities, with in this small nodular foci of faint enhancement (gadolinium enhanced T1-weighted in B1, FLAIR in B2).
Half a year later his condition worsened and he became easily agitated with emotional lability, disorientation, apathy and urinary incontinence. Additionally, he developed a left-sided hemiparesis with facial weakness and could walk only with assistance. MRI showed at the location of the pre-existing punctate enhancing white matter lesion now a large irregularly rim-enhancing lesion with central necrosis, and a large zone of surrounding edema extending in the corpus callosum, basal ganglia and parietal and temporal lobe, with some mass-effect of the right lateral ventricle; the pre-existing lesion adjacent to the parietal horn of the right lateral ventricle did not change significantly (gadolinium enhanced T1-weighted in C1, FLAIR in C2). A second biopsy showed mainly necrotic tissue. Corticosteroids provided temporary improvement of his gait. A repeat MRI two months later showed persistence of the right frontal lesion (gadolinium enhanced T1-weighted in D1 and D2). Open biopsy and partial debulking of the right frontal lesion was performed. Pathology showed largely necrotic tissue with scattered inflammatory cells, mainly around the vessel walls, which were thickened with adventitial fibrosis. In the following year, his condition deteriorated and he died of aspiration pneumonia at age 60. An autopsy was performed (data included in Figure 3).

neurological symptoms on examination prior to death and at least 21/28 (75%) had cognitive impairment. The single deceased MC+ without focal neurological symptoms or cognitive impairment died of heart disease two years after diagnosis of retinopathy.

Formal neuropsychological testing was performed in six MC+ with cognitive decline: five had dementia and one had mild cognitive impairment. In the five MC+ tested because of mild subjective complaints or scientific interest, no abnormalities were found.

Other brain symptoms included: migraine (24/41; 59%), seizures (9/54; 17%), and personality changes and psychiatric complaints, in particular depression and anxiety (26/62; 42%) (Table 1). In the 22 migraineurs in whom the subtype could be determined, six (27%) had migraine with aura and 16 (73%) had migraine without aura. In 14/17 (82%) migraineurs with a recorded age of migraine onset, the attacks had begun well before the visual or cerebral symptoms.

Premature death

Thirty-five MC+ were deceased at a mean age of 53·1 $\pm$ 9·6 years (range 32-72), primarily from complications of the neurological decline. One MC+ had died of heart disease. Mean survival time from symptom onset was 9·0 $\pm$ 6·7 years (range <1-26 years).

Neuroimaging

Neuroimaging was available for 48 MC+: both MRI and CT for 14, only MRI for 21, and only CT for 13. All lesions were restricted to the white matter with sparing of gray matter. Two types of lesions were regularly observed, often together: (i) focal, non-enhancing T2-hyperintense lesions scattered throughout the periventricular and deep white matter; and (ii) mass lesions that were T2-hyperintense and T1-hypointense, enhanced with gadolinium contrast, and usually were surrounded by extensive edema displacing adjacent structures leading to sulcal effacement (Fig. 1 and 4).

White matter hyperintensities typically were present early in the clinical course and were detected on MRI in all MC+ except for one who had been diagnosed with retinopathy for less than a year (34/35; 97%). Although non-specific, the lesions were excessive for the relatively young patient age and indicative of CHARIOT when found in combination with retinopathy and a family history of CHARIOT symptoms.

Mass lesions were observed in 36/43 (84%) MC+, sometimes at initial diagnosis but more frequently with advanced disease (Table 1). They were commonly associated with focal neurological symptoms (31/36; 86%). In all individuals with mass lesions, there was a lesion in the fronto-parietal lobe (26/26; 100%); additional lesions were sometimes seen in the cerebellum (1/26) and occipital lobe (1/26). The space occupying lesions usually grew with time, but also could remain stable or diminish in size (Fig. 4a).[12] Often they developed superimposed on pre-existing white matter hyperintensities. In five cases, restricted diffusion was observed, which was most pronounced at the center of the lesions and could persist for months. In 14/27 (52%) cases calcifications were seen on CT. Hemorrhage was not a typical feature (1/36).

Neuropathology

Neuropathologic examination was performed on 13 autopsy and 7 biopsy specimens from 20 MC+. The gross pathology at autopsy demonstrated minimal to marked involvement of the periventricular white matter, particularly the fronto-parietal lobes and occasionally the

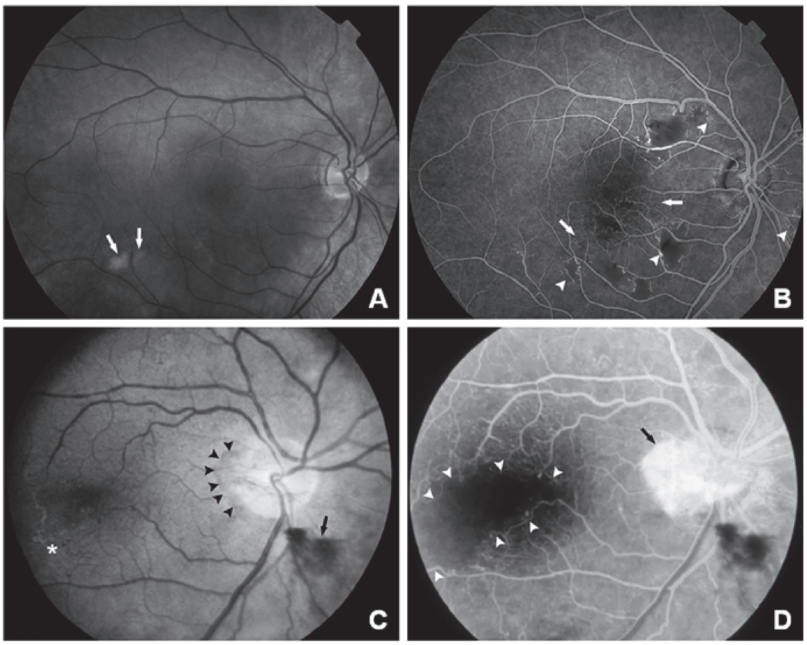

Figure 2. Fundoscopic (A and C) and fluorescein angiogram (B and D) images of the vascular retinopathy. Right eye of a 33-year-old man with cotton-wool spots (arrows, A), extensive areas of capillary obliteration with non-perfusion (arrows, B), and intraretinal microvascular abnormalities (arrowheads, B). Right eye of a 48-year-old woman with a neovascular membrane (arrowheads, C) and preretinal hemorrhage (arrow, C). Temporal to the macula, vascular sheathing and occlusion is present (asterix, C). The same eye shows profuse leakage from the membrane on the disc (arrow, D) and a large avascular region involving the fovea (arrowheads, D).

brainstem and cerebellum. Microscopic examination showed similar findings for both mass lesions and the smaller, scattered lesions seen on neuroimaging. Multiple, often confluent, foci of coagulation necrosis were identified in the white matter with sparing of the grey matter. The larger affected areas had extensive necrosis with focal calcification (Fig. 3B).

On microscopic evaluation a striking vasculopathy affecting the medium and small caliber arteries characterized these necrotic foci and adjacent white matter (Fig. 3B). Fibrinoid necrosis, adventitial fibrosis, luminal narrowing and mural hyalinization with collagenous material were hallmarks of the vasculopathy (Fig. 3B, C, D). Occasionally, vascular telangiectasias were observed. In some cases, a modest chronic inflammatory cell infiltrate, consisting predominantly of perivascular and parenchymal lymphocytes and plasma cells, was found near ischemic lesions. The cellular infiltrate was most consistent with a reaction to ischemic brain tissue without evidence of destruction or invasion of the vascular wall.

Focal calcifications and reactive astrocytosis were frequent findings. Myelin loss was substantial at autopsy. Neurofilament immunolocalization showed concomitant axon loss and frequently large numbers of swollen axonal spheroids, consistent with an ischemic process. Electron microscopy showed irregular thickening and splitting of the basement membranes in vessel walls (Fig. 3E), especially in the media with signs of smooth muscle cell and pericyte degeneration.

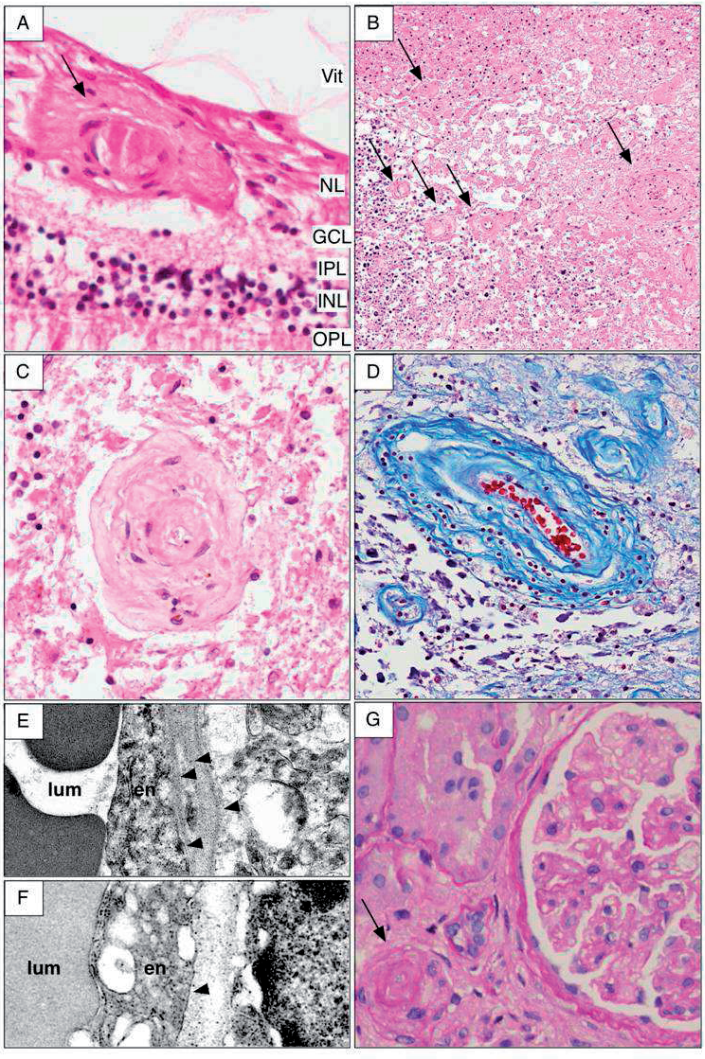

Figure 3. Representative histopathologic findings in the retina, brain and kidney. Microscopic examination of various organs shows a characteristic vasculopathy. The vessels of the inner layers of the retina often demonstrate damage to the walls with occasional deposition of amorphous material [arrow; panel A, hematoxylin and eosin (H&E) stain; vitreous (Vit); nerve fiber layer (NL); ganglion cell layer (GCL); inner plexiform layer (IPL); inner nuclear layer (INL); outer plexiform layer (OPL)] or thickened collagenous walls. The brain also shows a prominent vasculopathy in the white matter, most often adjacent to and in sites of coagulation necrosis. Small to medium sized vessels demonstrate vascular wall thickening with varying degrees of luminal narrowing (arrows; panel B; H&E; brain). In some cases, this progresses to a frank fibrinoid necrosis. In areas with extensive white matter ischemic damage, granular calcifications are particularly evident (dark blue staining in lower left of panel B). The vasculopathy may result in luminal obliteration leading to parenchymal necrosis (panel C; H&E; brain). There is concentric collagenous thickening of the vessel walls, mostly involving the medial layer of the vessels (panel D;Trichrome stain; brain). Ultrastructural examination of affected vessel walls in the brain demonstrates multilaminated basement membranes with duplication of the lamina densa [arrowheads; panel E; electron microscopy; lumen (lum); endothelial cell (en)] in contrast to that found in unaffected regions (arrowhead; panel F; electron microscopy). In the kidney, the vasculopathy is manifested by arteriosclerosis (arrow; panel G; H&E) and glomerulosclerosis.

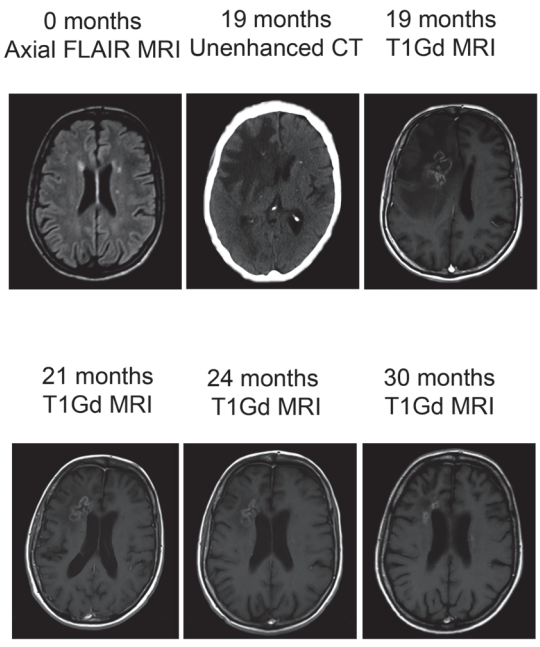

Figure 4a. Characteristic dynamic changes over time of contrast-enhancing cerebral mass lesions and white matter hyperintensities in a patient with CHARIOT. The first MRI shows punctate T2-hyperintense periventricular lesions (0 months, Axial FLAIR). Nineteen months later, a right frontal rim-enhancing lesion with associated calcification (unenhanced CT) and perifocal edema (gadolinium enhanced T1-weighted MRI) has developed on the location of a pre-existing punctate white matter hyperintensity. MRIs at two, five and eleven months after corticosteroid treatment for several weeks with clinical improvement show progressive reduction of the cerebral edema and contrast enhancement (gadolinium enhanced T1-weighted MRIs). Note the slight contrast-enhancement (at 19-30 months) and associated calcification also in the punctate T2 hyperintensities periventricularly on the left site.

Other affected organs

Liver

Liver-chemistry was abnormal in 28/40 (70%) MC+, typically showing modest elevations of alkaline phosphatase and gamma glutamyltransferase. Histologic examination of biopsy and autopsy specimens showed pathologic changes in 13 MC+ of whom three had normal laboratory parameters. The predominant finding was nodular regenerative hyperplasia (Supplementary Fig. S1). Other findings included micro- or macro-vesicular steatosis, periportal inflammation and portal or bridging fibrosis.

Kidney

In 22/44 (50%) MC+ renal disease was manifested clinically by a mild to moderate increase in serum creatinine (< 2·0 mg/dL) and/or proteinuria (< 2 g/24hr). Pathological findings on biopsy or autopsy specimens were noted in 18 MC+ including five who had normal laboratory values. The predominant lesions were arteriolosclerosis or arteriolonephrosclerosis and focal to diffuse global glomerulosclerosis (Fig. 3G). In one patient, renal disease was detected prior to retinopathy.

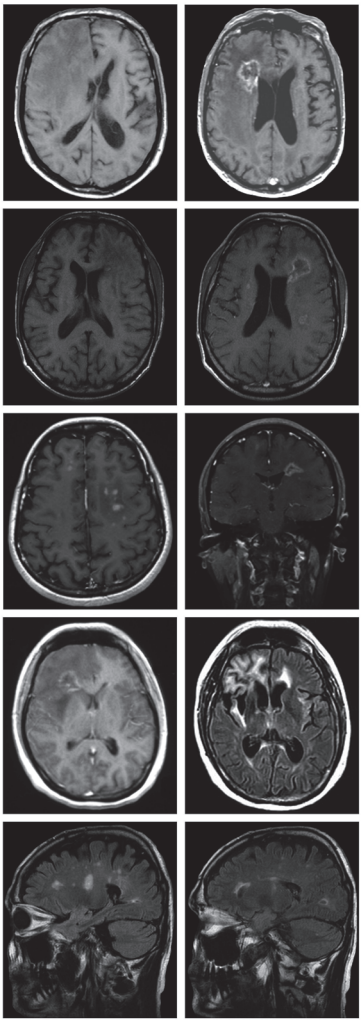

Figure 4b. Cerebral MRI scans of patients with different frame-shift mutations.
V235fs: Axial T1-weighted (left) and axial gadolinium enhanced T1-weighted (right) MRI images of a 59-year-old man (family 2) with a right frontal rim-enhancing lesion with mass-effect and surrounding edema.
T236fs: Axial T1-weighted (left) and axial gadolinium enhanced T1-weighted (right) images of a 40-year-old man (family 7) showing a left frontal rim-enhancing lesion, a smaller rim-enhancing lesion left parietal, and an enhancing punctateright frontal periventricular white matter hyperintensity.
T249fs: Axial (left) and coronal (right) gadolinium enhanced T1-weighted images (family 8) show a left frontal rim-enhancing lesion with mass-effect and some enhancing punctate T2 hyperintensities in the right frontal lobes.
R284fs: Axial T1-weighted (left) and FLAIR (right) images of a 32-year-old woman (family 10) showing a large right frontal rim-enhancing lesion and non-enhancing periventricular white matter hyperintensities frontal left.
L287fs: Saggital T1-weighted images of a 58-year-old man (family 11) reveal small and medium sized non-enhancing periventricular and subcortical white matter hyperintensities.

Other possible clinical associations

Other findings observed in MC+ more frequently than in the general population included anemia, hypertension, and Raynaud's phenomenon (Table 1). Normocytic and normochromic anemia (25/34; 74%) was typically mild to moderate (hematocrit 27-36%). Of those with anemia, six had documented microscopic gastrointestinal bleeding or telangiectasias. Hypertension was present in 30/50 (60%) MC+, often with concomitant renal disease (18/26; 69%). Raynaud's phenomenon found in 24/60 (40%) MC+ was mild, without ischemic injury or pulp infarcts, and did not require treatment. Autoimmune markers were positive in 3/18 (17%) MC+. Anti-nuclear antibodies were detected by immunofluorescence on Hep2 substrate in two subjects (speckled pattern with a titer of 1:80 and 1:640 respectively) and anti-cardiolipin IgG by ELISA in a third.

Mutation carriers without retinopathy or cerebral lesions (MC-)

Thirteen *TREX1* mutation carriers had no evidence of retinopathy or cerebral lesions at the time of their last examination. Their mean age at last follow-up was 35·1 $\pm$ 10·6 years (range 18-58). The most common clinical symptoms noted were Raynaud's phenomenon (7/13; 54%), migraine (5/12; 42%), and psychiatric complaints (3/13; 23%). One MC- committed suicide in his twenties. Three MC- underwent MRI scans, which showed only mild white matter hyperintensities in one at 45 years of age.

DISCUSSION

We present here the genetic and clinicopathologic spectrum of CHARIOT, a newly recognized autosomal dominant, systemic small vessel disease caused by C-terminal frame-shift mutations in *TREX1*. We identified 5 different mutations in 78 subjects from 11 unrelated families and found a strikingly similar disease profile. CHARIOT should be considered in individuals in middle age with a vascular retinopathy and neuropsychiatric symptoms, particularly if similar features are present in family members. Brain imaging will often reveal contrast-enhancing mass lesions, white matter hyperintensities and focal calcifications. Genetic testing of *TREX1* can confirm the diagnosis or identify pre-symptomatic patients. Proposed diagnostic criteria and other supporting features for CHARIOT are summarized in Table 2.

Based on our extensive clinical experience of over 20 years with CHARIOT, once patients develop retinopathy, all will suffer progressive neurological decline and most will die within ten years, usually from pneumonia or sepsis in the setting of severe general debilitation. To date, no mutation carrier has lived a normal lifespan without developing CHARIOT, thus suggesting 100% penetrance and premature mortality. The 13 MC- were on average ten years younger than the MC+ and thus may still develop the full CHARIOT syndrome.

Many patients with CHARIOT also have migraine and Raynaud's phenomenon, typically preceding the onset of retinopathy and cerebral lesions. Compared to the general population, migraine prevalence among CHARIOT patients was five times higher in males and nearly three times greater in females, using the same diagnostic criteria.[13] The prevalence of Raynaud's phenomenon was approximately two times higher in both sexes.[14] These statistics suggest that

Table 2. Proposed diagnostic criteria for CHARIOT

Major Diagnostic Criteria
Vascular retinopathy (with in the early phase hemorrhages, intraretinal microvascular abnormalities and/or cotton wool spots)
Signs and symptoms of progressive focal and/or global brain dysfunction with neuroimaging showing contrast-enhancing cerebral mass lesions and/or cerebral white matter hyperintensities
Family history of autosomal dominant inheritance with middle-age onset of disease manifestations#
C-terminal frame-shift mutation in *TREX1*
Supportive features
Microvascular liver disease (nodular regenerative hyperplasia)
Microvascular kidney disease (arterio- or arteriolonephrosclerosis, glomerulosclerosis)
Possibly associated features
Migraine
Raynaud's phenomenon
Anemia consistent with blood loss and/or chronic disease
Hypertension
Microscopic gastrointestinal bleeding

De novo mutations may be possible although none have been reported to date.

TREX1 mutations have a causal relationship to migraine and Raynaud's phenomenon as has previously been reported in a genetic study of family 2.[15] As both migraine and Raynaud's phenomenon are common in the general population, their presence alone cannot be used for a definitive clinical diagnosis of CHARIOT. The same applies to other CHARIOT-associated symptoms such as nephropathy and hepatic dysfunction. Although typically occurring later, they may represent the initial manifestation of CHARIOT, but are too non-specific for establishing the diagnosis.

Not surprisingly, many patients were initially misdiagnosed. The vascular retinopathy was commonly confused with diabetic retinopathy but the differential diagnosis also includes branch retinal vein occlusion, hypertension, sickle cell disease, collagen vascular disease and radiation, or idiopathic retinopathy.[1,16] The early white matter lesions in CHARIOT were often confused with multiple sclerosis, multi-infarct dementia, vasculitis, or other hereditary white matter diseases.[17,18] Ring-enhancing mass lesions with a necrotic central core and surrounding edema were commonly mistaken for neoplasms or tumefactive multiple sclerosis in several patients leading to multiple brain biopsies and surgical resection. Some patients with rapid progression of neurological symptoms were diagnosed with acute ischemic stroke, but diffusion restriction corresponding to a vascular territory was not seen. Interestingly, in some of our patients, diffusion restriction indicative of cytotoxic edema was observed in the center of a mass lesion.

The clinical and histopathologic findings are consistent with the hypothesis that the systemic small vessel vasculopathy of CHARIOT is caused by an endotheliopathy which

disrupts the vascular basal membrane and leads to progressive loss of microvascular blood flow. The histopathologic findings in the brain are reminiscent of delayed radiation necrosis, a condition which is believed to be secondary to endothelial cell-dysfunction.[19] Electron microscopy showed multi-laminated capillary basement membranes in the brain, kidney, stomach, appendix, omentum, and skin.[4] Capillary occlusion is suggested by retinal and cerebral vessel wall thickening and lumen obliteration, as well as a number of other pathologies. These include nodular regenerative liver hyperplasia, which is likely due to diminished hepatic blood flow,[20] and renal arteriosclerosis, arteriolosclerosis, and glomerulosclerosis, which, like microscopic gastrointestinal bleeding, are probable manifestations of small vessel disease.

Several studies support a role for *TREX1* in immunity. Absence of functional *TREX1* results in accumulation of single-stranded DNA in cells and multi-organ inflammation in knock-out mice.[21-23] In humans, mutations that abolish *TREX1* exonuclease activity are associated with three different autoimmune diseases: autosomal recessive Aicardi-Goutières syndrome (AGS), autosomal dominant familial chilblain lupus (FCL), and systemic lupus erythematosus (SLE). AGS is associated with complete absence of exonuclease activity and mimics an *in utero* viral encephalopathy, possibly secondary to activation of the immune system by host DNA.[24] Heterozygous *TREX1* mutations are found in FCL, an autoimmune disease that primarily affects the skin.[25,26] Rare variants in *TREX1* have also been associated with SLE in some patients.[27] Further support for a role in innate immunity comes from studies demonstrating that *TREX1* degrades HIV-1 DNA generated during infection, thereby preventing an interferon-mediated immune response.[28]

In contrast to the mutations seen in other *TREX1* diseases, CHARIOT *TREX1* frame-shift mutations result in a mislocalized but functional exonuclease and are likely associated with a gain-of-function or toxic effect. Unlike the role for immunity in AGS, FCL and SLE, the cerebral pathology of CHARIOT does not resemble an autoimmune disease as features of vasculitis are missing. However, auto-immunity may play a role, especially in the derailment of mass lesions at a certain age.

Since the initial description of the disease in 1988,[1] we have identified ten additional families, expanded and better delineated the clinical phenotype and discovered the causal gene. However, treatment to prevent, reverse or halt the disease is still lacking. Immunosuppressive agents, including cyclophosphamide, were given to a few individuals without benefit (Atkinson, unpublished data). Intra-vitreal bevacizumab was effective for the proliferative retinopathy in a single patient.[29] Corticosteroids can reduce cerebral vasogenic edema, but do not improve the underlying lesions. Further research is needed to determine the pathogenesis of CHARIOT and develop effective treatments for this devastating disorder.

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AUTHORS' CONTRIBUTIONS

The study was designed and coordinated, the data were analysed, and the first drafts and various revisions of the manuscript were written by AHS, PHK, GMT, JPA and MDF under supervision by JPA, GMT, JH and MDF who also take overall responsibility. Figures were designed by AHS, JPA, PTVMJ, GRK. All authors were involved in the data collection, literature search, data interpretation, vouch for the completeness and accuracy of their data, contributed to drafting and the final report, and participated in the decision to submit the findings for publication.

ROLE OF THE FUNDING SOURCE

The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author (GMT), AHS, PHK, JPA and MDF had full access to all the data in the study and had final responsibility for the decision to submit for publication.

CONFLICT OF INTEREST

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SUPPLEMENTARY MATERIAL

Table S1. Manifestation of CHARIOT for clinically affected and unaffected individuals subdivided by family and mutation status

	MC-^	**MC+**		
Mutation	**V235fs; T249fs; L287fs**	**V235fs**		
Origin	**Dutch; US**	**Dutch; US; Australia**		
Original disease name	***HVR; HERNS***		***CRV; US***	***HVR; Dutch***
Family number	**2, 8, 11**	**1, 2, 3, 4, 5, 6**	***1***	***2***
Demographics				
Number of mutation carriers	13	43	18	20
Age at last follow-up or death				
Mean ± SD (yr)	35·1 ± 10·6	52·6 ± 8·3	51·0 ± 5·9	55·4 ± 9·7
Range (yr)	18-58	35-72	41-62	35-72
Major signs/symptoms*				
Retinopathy	0 (0/12)	100 (42/42)	100 (17/17)	100 (20/20)
Age at diagnosis retinopathy				
Mean ± SD (yr)	N/A	45·1 ± 6·9	45·0 ± 4·7	45·5 ± 8·0
Range (yr)	N/A	35-61	40-55	35-61
Cerebral				
Focal neurological	0 (0/13)	66 (25/38)	94 (15/16)	41 (7/17)
Cognitive decline	8 (1/13)	62 (23/37)	88 (15/17)	38 (6/16)
Migraine	42 (5/12)	54 (13/24)	50 (1/2)	60 (12/20)
Psychiatric	23 (3/13)	45 (19/42)	56 (10/18)	40 (8/20)
Seizure	0 (0/12)	14 (6/43)	28 (5/18)	5 (1/20)
Neuroradiology*				
White matter disease	33 (1/3)	97 (30/31[#])	100 (14/14)	92 (11/12[#])
Mass occupying lesions	0 (0/3)	65 (20/31)	79 (11/14)	33 (4/12)
White matter hyperintensities**	33 (1/3)	96 (23/24)	100 (8/8)	91 (10/11)
Calcifications***	Not Tested	56 (9/16)	56 (5/9)	57 (4/7)
Other organs involved*				
Liver[V]	Not Tested	65 (20/31)	56 (9/16)	90 (9/10)
Kidney[V]	0 (0/1)	34 (10/29)	36 (5/14)	27 (3/11)
Possible associations*				
Anemia	Not tested	70 (21/30)	80 (12/15)	50 (6/12)
Hypertension	0 (0/2)	53 (19/36)	56 (10/18)	40 (6/15)
Raynaud's phenomenon	54 (7/13)	49 (20/41)	6 (1/18)	85 (17/20)
Gastrointestinal bleeding/ telangiectasias	0 (0/13)	16 (7/43)	33 (6/18)	5 (1/20)

MC+: Mutation carriers with retinopathy or cerebral mass lesions; MC-: Mutation carriers without retinopathy or cerebral mass lesions. * Unless indicated otherwise, the disease manifestations presented in the table are shown as percentage of subjects followed by the number of subjects. The denominator varies according to the number of individuals with available data. ^ Ten mutation carriers from family 1 (mutation V235fs), 2 mutation carriers from family 11 (mutation L287fs), 1 from family 8 (mutationT249fs, this patient committed suicide at age 30).

T236fs	**T249fs**		**R284fs**	**L287fs**
German	**US**		**Australia**	**Dutch**
		HERNS; US		
7	**8, 9**	**8**	**10**	**11**
4	11	10	3	4
41^^	46·4 ± 9·1	44·4 ± 7·8	40·7 ± 5·0	49·0 ± 17·1
N/A	32-60	32-53	36-46	34-68
100 (4/4)	100 (11/11)	100 (10/10)	100 (3/3)	100 (4/4)
39·8 ± 6·9	33·4 ± 2·6	33·4 ± 2·6	34·0 ± 8·5	44·3 ± 11·9
30-46	30-37	30-37	25-42	33-56
75 (3/4)	82 (9/11)	80 (8/10)	67 (2/3)	33 (1/3)
67 (2/3)	36 (4/11)	30 (3/10)	67 (2/3)	33 (1/3)
0 (0/2)	88 (7/8)	88 (7/8)	100 (3/3)	25 (1/4)
33 (1/3)	27 (3/11)	30 (3/10)	100 (3/3)	0 (0/3)
0 (0/4)	100 (1/1)	Unknown	67 (2/3)	0 (0/3)
100 (2/2)	100 (11/11)	100 (10/10)	100 (3/3)	100 (1/1)
100 (2/2)	100 (11/11)	100 (10/10)	67 (2/3)	100 (1/1)
100 (2/2)	100 (6/6)	100 (5/5)	100 (2/2)	100 (1/1)
Not Tested	38 (3/8)	29 (2/7)	67 (2/3)	Not Tested
100 (4/4)	100 (1/1)	Not Tested	67 (2/3)	100 (1/1)
50 (1/2)	78 (7/9)	75 (6/8)	100 (3/3)	100 (1/1)
Not Tested	100 (1/1)	Not Tested	100 (3/3)	Not Tested
Not Tested	78 (7/9)	75 (6/8)	67 (2/3)	100 (2/2)
0 (0/2)	0 (0/11)	0 (0/10)	0 (0/2)	100 (4/4)
50 (2/4)	0 (0/11)	0 (0/10)	0 (0/3)	0 (0/3)

^^ Data on one patient only. N/A: Not Applicable. [#] One subject with no evidence of white matter hyperintensities had an MRI done within 1 year of diagnosis with retinopathy. ** Based on MRI scans only. *** Based on CT scans. [V] Based on laboratory values. The five patients that were excluded from the neuro-imaging section in the deceased category of Table 1 (because the last scan was made more than 5 years before death) are included in this table.

Table S2. The 11 CHARIOT families with five different mutations in the *TREX1* gene

Family	Origin	Mutation (DNA)	Mutation (protein)	Number of mutation carriers	Reference
1	North America (European)	3688_3689insG	V235fs	18	1
2	Netherlands	3688_3689insG	V235fs	30	2, 3
3	North America (Ashkenazi-Jewish)	3688_3689insG	V235fs	1	1
4	North America	3688_3689insG	V235fs	1	6
5	Australia	3688_3689insG	V235fs	2	6
6	Netherlands	3688_3689insG	V235fs	1	Unpublished
7	Germany	3691_3692insA	T236fs	4	8
8	North America (Chinese)	3727_3730dupGTCA	T249fs	11	4
9	North America	3727_3730dupGTCA	T249fs	1	9
10	Australia	3835_3836insA	R284fs	3	7
11	Netherlands	3843_3844insG	L287fs	6	6

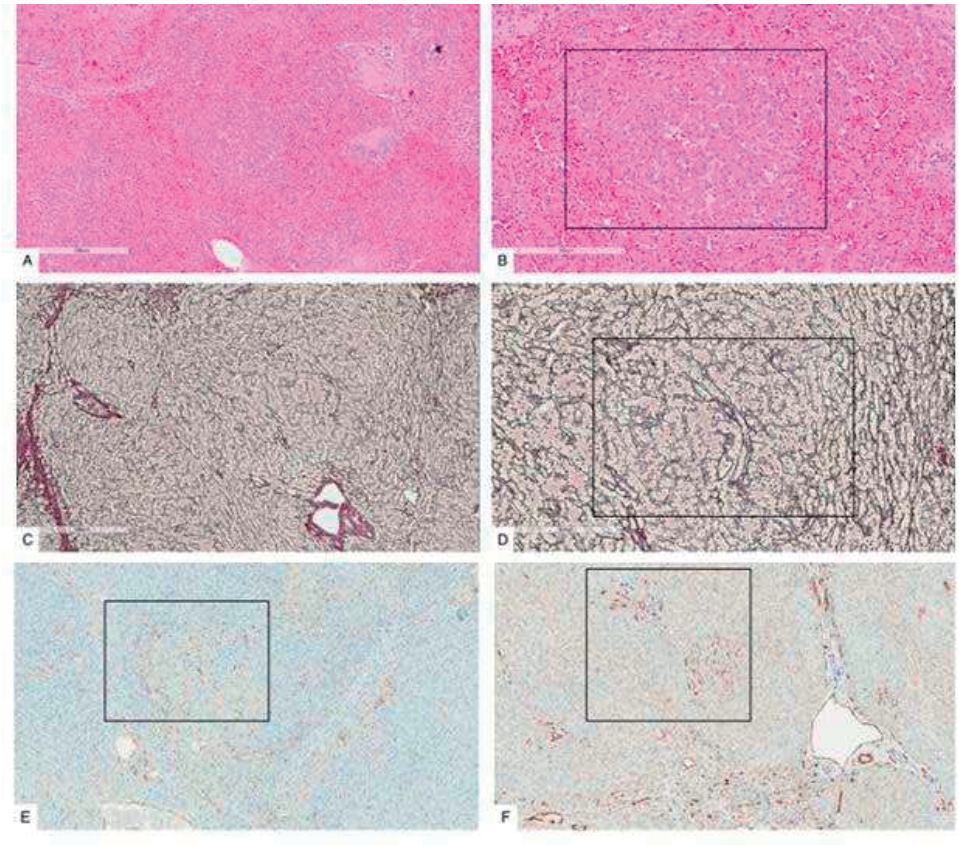

Figure S1. Regenerative hyperplasia and capillarization of the liver. The liver shows an abnormal parenchymal architecture (Panel A and B; hematoxylin and eosin (H&E) stains and panel C and D, reticulin stains) In A, the portal tracts (seen on the right) and terminal hepatic venules are unevenly spaced; in addition, a vague nodule is present centrally. Within the boxed area in B, the nodule is comprised of hepatic cords delineated from surrounding parenchyma by blood-filled sinusoids. The cords in the latter regions are atrophic (see panel C) compared to the more hypertrophic cords in the center (boxed area in panel D). None of the cords, however, are greater than 2 nuclei broad. Panels E and F are immunohistochemical stains highlighting aberrant activation of hepatic stellate cells (boxed area in E, anti-smooth muscle actin) and altered sinusoidal endothelial cells with expression of anti-CD34 (boxed area in F). The latter signifies a process of closure of sinusoidal endothelial fenestrae and presence of basement membrane. These are features of "capillarization" of the sinusoids.

CHAPTER 10

CEREBRAL HEREDITARY ANGIOPATHIES RVCL AND CADASIL DISPLAY DISTINCT IMPAIRED VASCULAR FUNCTIONALITY

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Work in progress

SUMMARY

Retinal Vasculopathy with Cerebral Leukodystrophy (RVCL) and Cerebral Autosomal Dominant Arteriopathy with Subcortical infarcts and Leukoencephalopathy (CADASIL) are cerebral small vessel diseases, which serve as monogenic models for common complex neurovascular disorders, such as stroke, dementia and migraine. Impaired vascular reactivity is likely to play a role in the pathophysiology of both monogenic disorders, but it remains unclear whether this is due to impaired endothelium-dependent or -independent (i.e., mediated by direct relaxation of smooth muscle cells) mechanisms. In this study, vascular function tests were performed at different levels of the vascular bed to investigate functional changes in (arterial) stiffness, vasodilatation of resistance vessels, and endothelial function of conduit arteries. Eighteen RVCL and 23 CADASIL patients with *TREX1* and *NOTCH3* mutations, respectively, were compared with 26 matched control subjects. Data shown are uncorrected means ± SD and corrected *p*-values.

Vascular stiffness was assessed by pulse wave analysis and pulse wave velocity. CADASIL patients displayed an elevated Aortic Augmentation Index compared to controls (22,8 ± 13,3 vs. 16,0 ± 12,7 %, $p = 0.007$); in RVCL patients a similar trend was shown (21,0 ± 13,3 %, $p = 0.06$). Pulse wave velocity was increased in RVCL patients compared to controls (7,8 ± 1,6 vs. 7,0 ± 1,4 m/s, $p = 0.01$) and showed a trend in CADASIL patients (7,5 ± 1,5 m/s, $p = 0.07$ versus controls). Vasodilatation of dermal microcirculation was reduced in CADASIL but not in RVCL, compared to control subjects as characterized by both an attenuated capsaicin-induced dermal blood flow increase 40 minutes after capsaicin application (1,38 ± 0,88 vs. 2,22 ± 1,20 Arbitrary Units (AU), $p = 0.02$) and a lower area under the curve over 40 minutes (0,52 ± 0,43 vs. 0,93 ± 0,60 AU, $p = 0.02$). Endothelium-dependent vasodilatation as assessed by flow-mediated dilatation was decreased in RVCL versus controls (2,32 ± 3,83 vs. 5,76 ± 3,07 %, $p = 0.02$ versus controls), but not in CADASIL. After correction for shear rate and blood viscosity, flow-mediated dilatation remained reduced in RVCL (3,31 ± 7,24 vs. 10,07 ± 5,73 %, $p = 0.03$ versus controls).

We identified endothelial dysfunction in *TREX1*-mutated RVCL patients and confirmed impaired smooth muscle cell relaxation in resistance vessels of *NOTCH3*-mutated CADASIL patients. Increased vascular stiffness illustrates reduced vascular functionality in both syndromes. Our findings not only improve insight in RVCL and CADASIL pathophysiology, but may also be important to understand common disorders that are part of the disease spectrum of these neurovascular disorders, such as stroke, dementia, and migraine.

INTRODUCTION

Neurovascular diseases such as migraine, (vascular) dementia and ischemic stroke are clinically and genetically heterogeneous disorders with a high population prevalence and disease burden.[1] Comorbidity of these diseases suggests, at least to some extent, shared underlying pathophysiological mechanisms. For example, stroke and dementia commonly co-occur in cerebral small vessel disease.[2] Moreover, migraineurs with aura have a doubled risk of ischemic stroke[3] and an increased risk of posterior circulation territory infarcts[4,5] and deep white matter hyperintensities.[4,6] However, as these common disorders are multifactorial and genetically complex it is difficult to identify the causal genes and disease pathways. We envisage that RVCL (Retinal Vasculopathy with Cerebral Leukodystrophy) and CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy), which are monogenic forms of cerebral small vessel disease with a high prevalence of stroke, dementia, and migraine, may serve as useful disease models for the common disorders.[7,8,9,10]

RVCL is caused by heterozygous C-terminal frameshift mutations in the *TREX1* gene, which encodes the major 3'-5' DNA exonuclease that is thought to be involved in clearing cytosolic nucleic acids.[9,11,12] In RVCL, truncated proteins seem to retain their exonuclease activity but lose normal perinuclear localization, which likely interferes with normal functioning of TREX1.[9] RVCL is a systemic vascular syndrome that primarily involves the smaller vessels in the retina and the brain.[8,13,14,15] Neurological manifestations may include cognitive disturbances, focal neurological symptoms, depression, and in 59% of *TREX1* mutation carriers migraine. In later disease stages, cerebral MRI scans frequently show characteristic contrast-enhancing intracerebral mass lesions. Several systemic symptoms can be present as well, including renal and liver dysfunction, anemia, and Raynaud's phenomenon.

CADASIL is caused by heterozygous mutations in the *NOTCH3* gene,[7] which encodes a cell surface receptor that is solely expressed on adult vascular smooth muscle cells. NOTCH3 is involved in a signal transduction pathway critical for development, homeostasis and differentiation of vascular smooth muscle cells (VSMCs).[16] Clinically, the disease is characterized by recurrent transient ischaemic attacks (TIAs) and strokes leading to cognitive decline,[17] psychiatric symptoms and dementia. Migraine with aura occurs in about one-third of patients, often as the first presenting symptom, years before the other symptoms.[18]

Both RVCL and CADASIL are small vessel vasculopathies but with different radiological features.[10] RVCL is characterized by contrast-enhancing cerebral mass lesions that develop in the end stage of the disease, typically in combination with (or preceded by the presence of) calcifications and non-specific white matter hyperintensities. The most prominent radiological features in CADASIL are white matter hyperintensities and lacunar infarcts. White matter hyperintensities are symmetrically distributed and located in the deep and periventricular white matter. Typical for CADASIL is the bilateral involvement of the anterior temporal lobes and external capsule. Other neuroradiological features in CADASIL are cortical morphologic changes,[19] subcortical lacunar lesions, lacunar infarcts and microbleeds.[18,20]

Thickening of the vessel wall leading to lumen stenosis, is a communal feature of the cerebral pathology of these arteriopathies. In addition, both in the cerebral and the systemic circulation of CADASIL patients, there is a remarkable degeneration of vascular smooth muscle cells and deposition of granular osmiophilic material (GOM) in the media/adventitia,[21] with a morphologically largely normal endothelium.[18] In contrast, in RVCL, muscle cell degeneration is minimal and electron microscopy shows irregular thickening and splitting of basement membranes in the vessel wall, which is primarily produced by endothelial cells.[22,23] These changes on cerebral vessels may affect their capability to dilate, which, in addition to luminal narrowing, may lead to hypoxia.

In CADASIL, previous studies showed reduced baseline cerebral blood flow and impaired hemodynamic reserve (i.e., vasodilation response to acetazolamide) related to the severity of white matter hyperintensities.[24-26] However, it is not yet clear whether this impaired vasoreactivity is caused by an endothelium-independent or -dependent mechanism.[27-30]

In RVCL, functional vascular properties have not been studied before. Besides radiological and other pathological alterations, the presence of Raynaud's phenomenon and migraine seems to point to altered vascular reactivity. In Raynaud's phenomenon impaired endothelium-dependent vasodilatation and a mismatch between endothelium derived vasoconstrictors and vasodilators has been observed.[31] Migraine has been linked to endothelial dysfunction,[32-36] vascular smooth muscle cell dysfunction,[37] increased peripheral arterial stiffness[34] and increased intima-media thickness.[36] However, controversy over the vascular theory in migraine pathogenesis [38-42] and migraine treatment[43,44] remains. Nevertheless, migraine (with aura) has repeatedly been linked with cardiovascular diseases[45] including ischemic stroke[3,46-48] and coronary events.[49,50]

By using different techniques at distinct levels of the vascular bed, we investigated vascular functional consequences of both RVCL and CADASIL *in vivo*, and compared results with those of a matched group healthy volunteers.

METHODS

Subjects

The study was approved by the Ethics Committee of Leiden University Medical Centre and conducted in accordance with the Declaration of Helsinki. All participants were at least eighteen years of age and gave written informed consent. RVCL and CADASIL patients with a confirmed mutation in the *TREX1* (i.e., V235fs) and the *NOTCH3* gene, respectively, were recruited from databases provided by the treating neurologist (GMT) and clinical geneticist. Subjects were invited to participate by a letter explaining the tests and study objectives. The control group consisted of spouses or friends of mutation carriers and volunteers recruited via public advertisements. Medical history, genetic and clinical information was obtained prior to the measurements and by a different investigator than the one performing the vascular measurements. During the measurements, participants were instructed not to reveal their mutation status or medical history to the investigator who performed the measurements to prevent unblinding.

Clinical assessment

Participants were asked in advance to fill out a questionnaire that included various variables on medical history such as neurological symptoms including (transient) ischemic attacks, other stepwise neurological deterioration, cognitive complaints including apathy, migraine (with or without aura), depression, visual symptoms, Raynaud's phenomenon, diabetes, thyroid function, cardiovascular disease, airway disease, cancer, multiple sclerosis, epilepsy, intestinal and stomach problems, liver disease, renal and urinary tract disease and dermatological diseases. The questionnaire also had questions on current medical condition, current medication, smoking, daily intake of alcohol, caffeine and drugs, and socio-demographic parameters such as educational level and ethnic origin. During the interview preceding the experiments, diagnosis of migraine and Raynaud's phenomenon was verified. Migraine diagnosis was made according to the International Classification of Headache Disorders-second edition (ICDH-2) and Raynaud's phenomenon was assessed according to the criteria of Miller.[51] Medication was also verified and subdivided in the following categories: anti-hypertensive, cholesterol lowering, acetylsalicylic acid (anti-platelet), anti-coagulant, acute and prophylactic migraine medication, analgesics (e.g., acetaminophen, paracetamol, NSAIDs), oral contraceptive. If possible, and in agreement with their treating physician, included subjects were asked to abstain from medication with vascular (side-)effects for one week prior to the measurements. Patients were instructed to abstain from alcohol and caffeine for 12 hours, from smoking and food for 6 hours and, if their health status allowed this, from medication for 7 days prior to the measurements. This was verified during the interview. Subsequently, weight and height were measured and BMI was calculated.

Biochemical measurements

Plasma concentrations of triglyceride (TG), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), total cholesterol (TC), blood fasting glucose levels, creatinine, ureum, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP), gamma-glutamyltransferase (GGT), lactate dehydrogenase (LDH), hemoglobin (Hb) and hematocrit (Ht) were determined according to standard procedures.

Experimental setup

All measurements were performed by the same investigator (SV) who was blinded for the diagnosis and mutation status of the subjects. Before starting the measurements, subjects had to lay down on a comfortable bed in supine position for acclimatization of at least 15 min in a room with stable ambient temperature (22 ± 1 °C).

Resting blood pressure and pulse rate were recorded at the right upper arm using a validated semi-automated oscillometric device (OMRON 705IT, OMRON Healthcare, Hoofddorp, The Netherlands). Median blood pressure and heart rate of 3 measurements was used for analyses. Subsequently, the following assessments were consecutively performed in a standardized order: i) pulse wave analysis; ii) pulse wave velocity; iii) capsaicin-induced changes in dermal blood flow; and iv) flow-mediated dilatation of the brachial artery. All these assessments have previously been described in detail, including reproducibility.[52-57]

Pulse Wave Analysis (peripheral arterial stiffness)

Augmentation index (AIx), which is a measure for arterial wave reflections and peripheral arterial stiffness, was determined via Pulse Wave Analysis (PWA) using SphygmoCor software (version 8.2; Atcor Medical, Australia). PWA was performed by applanation tonometry over the radial artery to obtain resting measurements of the peripheral AIx (Augmented Pressure / Pulse Pressure), expressing the reflected pressure wave as percentage of the forward wave (Supplementary Fig. 1). This non-invasive method has previously been shown a reproducible technique to evaluate peripheral arterial stiffness.[55] Based on 10-second PWA recordings performed at the radial artery, aortic (central) pressure waveforms can be reliably estimated by the software using a validated transfer function.[54] Subsequently, central pressure wave forms can be used for calculating the central AIx. Since AIx is influenced by heart rate,[53] an index normalized for heart rate at 75 beats/minutes (min) (AIx@HR75) was used. Mean values of at least three AIx@HR75 measurements per subject were used for statistical analysis.

Pulse wave velocity (aortic stiffness)

Aortic Pulse Wave Velocity (PWV) was measured with the same SphygmoCor device as PWA, by sequentially recording ECG-gated carotid and femoral artery pressure waves. The intersecting tangent algorithm[52] was used by the software to determine the characteristic points of the pressure wave and the R wave of the ECG for calculating the time delay. Based on 10-second recordings, the carotid-femoral transit time (T) was calculated. The carotid-femoral distance (L) used for calculating the PWV was obtained by subtracting the carotid-sternal notch distance from the sternal notch-femoral distance. PWV was calculated as L (cm) divided by T (m/s). If available, mean values of at least 3 PWV measurements per subject were used for statistical analysis.

Capsaicin-induced dermal blood flow (microcirculation in the skin)

Local application of capsaicin onto the skin results in binding to the Transient Receptor Potential Vanilloid type I receptor (TRPV1) at primary sensory neurons (Aδ- and C-fiber nociceptors). TRPV1 receptor binding of capsaicin induces a neurogenic inflammatory response due to predominant release of CalcitoninGene-Related Peptide (CGRP).[57] CGRP is a very potent vasodilator causing a local increase in dermal blood flow, which can be quantified by Laser Doppler Perfusion Imaging (LDPI)(PeriScan PIM II®; Perimed, Järfälla, Sweden). Reproducibility of this non-invasive test to evaluate dermal vascular reactivity to capsaicin was confirmed earlier.[56] After at least 20 min of supine rest the baseline dermal blood flow (DBF) was measured using LDPI. Subsequently, subjects received single topical doses of 1000 µg per 20 µL capsaicin solution (in ethanol/polysorbate 20/water) in two 10-mm rubber 'O'-rings on the volar surface of one forearm. In two rings on the opposite arm, placebo (i.e., vehicle) was applied. DBF was measured at 10, 20, 30 and 40 min after capsaicin/placebo administration. Results are presented as absolute and percentage change in DBF after capsaicin application compared to placebo.

Flow-mediated dilatation (endothelial function of conduit artery)

Flow-Mediated Dilatation (FMD) of the brachial artery was assessed following existing guidelines[58] using an echo-tracking system (Wall Track System, Pie Medical, Maastricht, The

Netherlands). The system consists of an ultrasound device (Esaote AU5, Esaote Biomedical, Genoa, Italy) equipped with a 7.5-10 MHz linear-array transducer connected to a data acquisition and processing unit. Measurements were performed as described earlier.[34] In brief, right brachial artery diameter was measured 5-10 cm proximal to the antecubital crease in a longitudinal plane, before and after an increase in shear stress induced by reactive hyperaemia. At baseline, diameter and velocity profiles were recorded 3 times and the mean was used for data analysis. After baseline measurements, a cuff (TMC7, D.E. Hokanson, Bellevue, USA), placed around the forearm, was rapidly inflated to 220 mm Hg. The cuff was released after 5 min, followed by recording of the peak velocity profile within the first 15 seconds (s). Diameter was measured at 0.5, 0.75, 1, 1.25, 1.5, 2, 3, 4 and 5 min after cuff release. Each diameter recording lasted 4 s.

After 15 min of recovery, following measuring baseline brachial artery diameter again, 400 μg sublingual nitroglycerin (NTG) was administered. Subsequently, the diameter was measured every minute for 6 min to assess NTG-induced endothelium-independent brachial artery dilatation. The endothelium-dependent FMD after 5 min of reactive hyperemia was expressed as the maximal absolute and percentage increase in diameter from baseline. FMD was corrected for the hyperemic stimulus using shear rate (= velocity/diameter) and additionally corrected for blood viscosity using shear stress (= shear rate*hematocrit) to calculate normalized FMD. Endothelium-independent vasodilatation capacity after administration of the exogenous NO donor (NTG) was expressed as the maximal absolute and percentage increase in diameter from baseline.

Statistics

P-values were calculated by independent sample t-test for normally distributed variables, Mann-Whitney U test for non-normally distributed variables and χ^2-test for categorical variables. Analysis of variance (ANOVA) was used to detect differences between controls and patient groups, followed by post-hoc Fisher LSD-test. Univariate associations were established using Spearman rank order correlations. Multiple linear regression analysis was used to adjust for confounders found with the univariate Spearman test. For all analyses p-values <0.05 were considered statistical significant. All statistical analyses were performed using SPSS 17.0 software (SPSS inc, IBM, Chicago, IL, USA).

RESULTS

Subjects

Eighteen RVCL and 23 CADASIL patients were included in the study and compared with 26 age-, BMI- and gender-matched healthy control subjects. Demographic details, clinical symptoms and laboratory values of all subjects are summarized in Table 1. All tests were well-tolerated. No subjects reported a migraine attack due to NTG when asked in a survey by phone in the first week after participation in the study. Success rates for abstaining from alcohol, caffeine and food for the predefined period prior to the measurements was 100%, except for smoking in the CADASIL group (72%; five patients smoked 1-4 cigarettes in the 6 hours prior to the measurements), abstention from alcohol in the CADASIL group (94%; one patient used 2 units 10 hours prior to the measurements), caffeine in the RVCL group (96%; one patient

Table 1. Subject demographics

Variable	Controls (n=26)	CADASIL (n=23)	RVCL (n=18)	*p-value*
Age (years)	49.0 ± 9.1	49.5 ± 12.7	50.1 ± 9.6	*0.939*
BMI (kg/m²)	25.2 ± 3.6	26.7 ± 4.3	25.2 ± 3.7	*0.362*
Female, n (%)	14 (54)	11 (48)	10 (56)	*0.867*
Smoking (Pack years)	3.0 ± 5.0	13.4 ± 16.0	3.7 ± 5.8	*0.002*
Retinopathy, n (%)	0 (0)	0 (0)	9 (56)†	*N.A.*
TIA, n (%)	0 (0)	8/21 (38)††	0 (0)	*N.A.*
Brain infarction, n (%)	0 (0)	2(9)	0 (0)	*N.A.*
Cognitive complains, n (%)	0 (0)	12 (52)	8 (44)	*N.A.*
Concentration problems, n (%)	0 (0)	8 (35)	5 (18)	*N.A.*
Depressive symptoms, n (%)	0	6 (26)	4 (22)	*N.A.*
Character change, n (%)	0 (0)	5 (28)	2 (9)	*N.A.*
Migraine, n (%)	0 (0)	6/22 (27)§	6 (33)	*N.A.*
Chronic medication use, n (%)	4 (15)	16 (70)	12 (67)	*N.A.*
Oral contraceptive use (% of women)	2 (14)	1 (9)	0 (0)	
Anti-hypertensives	0 (0)	7 (30) **(1)**	6 (33) **(2)**	
Cholesterol lowering medication	0 (0)	9 (39) **(2)**	6 (33) **(2)**	
Acetylsalicylic acid	0 (0)	13 (57) **(3)**	8 (44) **(4)**	
Prophylactic migraine medication	0 (0)	1 (4)	0 (0)	
Analgesics (acetominophen + NSAIDs)°	0 (0)	2 (9)	5 (28)	
Other°° (%)	2 (7.7)	9 (39)	8 (44)	
Laboratory parameters				
Glucose (mmol/L)	4.95 ± 0.37	5.05 ± 0.37	5.13 ± 0.38	*0.267*
TC (mmol/L)	5.25 ± 1.18	4.94 ± 1.06	5.27 ± 1.14	*0.548*
HDL-C (mmol/L)	1.51 ± 0.42	1.50 ± 0.47	1.70 ± 0.64	*0.373*
TG (mmol/L)	1.13 ± 0.76	1.00 ± 0.46	0.95 ± 0.59	*0.628*
Hb (g/dL)	8.98 ± 0.71	9.15 ± 0.73	8.10 ± 0.65	***0.000***
Ht (%)	0.43 ± 0.03	0.44 ± 0.04	0.40 ± 0.04	***0.001***
AST	23,2±6.45	24.3±6.15	28.2±8.50	*0.064*
ALT	23.3±10.6	23.8±12.1	26.8±12.9	*0.603*
GGT	21.7±12.1	24.9±6.7	99.6±117.1	***0.000***
ALP	68.4±13.4	69.7±22.7	95.73±65.0	***0.038***
LDH	163.5±31.6	158.8±19.6	147.2±22	*0.120*
Creatinine	70.0±12.3	71.4±12.9	87.4±44.8	*0.064*
Ureum	4.7±1.03	4.90±4.09	6.61±3.90	*0.014*

Values are means ± SD or number (percentage). *P*-values were calculated using a one-way ANOVA. Number of subjects that abstained from medication 7 days prior to the measurements are depicted in bold. N.A.: not applicable. † Retinopathy status for two patients unknown. †† Two patients could no be categorized as TIA or definitely no TIA. § One patient could not be classified as migraine or no migraine. ° All subjects used these painkiller for acute treatment of migraine, none of the subjects used acetaminophen or NSAIDs 24 hours prior to the measurements. °° Other medication included: glucose lowering drugs, proton pump inhibitors, gabapentin, carbamazepine, pyridoxine, folic acid, valproic acid, alfacalcidol, baclofen, cinnarezine, ursodeoxycholic acid, atovaquon.

used 1 unit 6 hours prior to the measurements). Success rates of abstaining from medication are depicted in Table 1.

Pulse Wave Analysis (PWA) and Pulse Wave Velocity (PWV)

Peripheral (brachial) and central (aortic) systolic, diastolic and pulse pressures are presented in Table 2. No differences in blood pressure values were observed between the control group and both patient groups. PWA software was unable to calculate the augmentation index and aortic pressure parameters in one subject (CADASIL patient) as the result of an atypical waveform. PWV measurements could not be performed reliably in two CADASIL patients due to obesity (BMI of 29.7 and 35.4).

Univariate analysis showed a trend towards increased AIx@HR75 in CADASIL patients versus control subjects (22,8 ± 13,3 vs. 16,0 ± 12,7%, $p = 0.08$) and a trend towards increased pulse wave velocity in RVCL patients versus control subjects (7,8 ± 1,6 vs. 7,0 ± 1,4 m/s, $p = 0.10$; Table 2). Univariate associations between clinical characteristics and PWA/PWV were assessed using Spearman correlation coefficients. (Supplementary Table 1)

Multivariate analysis resulted in a regression model for PWA (Multiple R = 0.77; Adjusted $R^2 = 0.57$) with age ($p < 0.001$; observed power > 0.99), gender ($p < 0.001$; observed power > 0.99), peripheral diastolic pressure ($p = 0.001$, observed power = 0.91) and subject group ($p = 0.02$; observed power = 0.71) as covariates. This regression model for PWA with covariates age, gender and diastolic blood pressure, revealed a significant difference in AIx@HR75 between control subjects and CADASIL patients ($p = 0.007$ and a trend in RVCL patients ($p = 0.06$) (Fig. 1).

Table 2. Blood pressure and univariate pulse wave analysis and pulse wave velocity data

Variable	Controls (n=26)	CADASIL (n=22)	*p-value*†	RVCL (n=18)	*p-value*‡
Peripheral systolic pressure (mm Hg)	129.2 ± 20.5	126.2 ± 11.1	*0.94*	132.8 ± 21.7	*0.71*
Peripheral diastolic pressure (mm Hg)	77.8 ± 10.1	75.4 ± 6.7	*0.40*	75.3 ± 8.8	*0.26*
Peripheral pulse pressure (mm Hg)	51.3 ± 12.3	50.8 ± 9.6	*0.98*	57.5 ± 15.8	*0.21*
Aortic systolic pressure (mm Hg)	119.6 ± 21.2	116.8 ± 12.3	*0.93*	122.8 ± 22.4	*0.61*
Aortic diastolic pressure (mm Hg)	79.5 ± 10.6	79.8 ± 8.2	*0.90*	76.6 ± 9.0	*0.22*
Aortic pulse pressure (mm Hg)	39.5 ± 11.7	39.2 ± 8.4	*0.77*	46.2 ± 16.9	*0.16*
Aortic Augmentation index @ HR75 (%)°	16.0 ± 12.7	22.8 ± 13.3	*0.08*	21.0 ± 13.3	*0.21*
PWV (m/s)°°	7.0 ± 1.4	7.5 ± 1.5	*0.29*	7.8 ± 1.6	*0.10*

Data presented as mean ± SD. † Difference between control subjects and CADASIL patients. ‡ Difference between control subjects and RVCL patients. ° PWA: pulse wave analysis; PWA software was unable to calculate the augmentation index and aortic pressure parameters in 1 subject (CADASIL patient) as the result of a atypical waveform. °° PWV: pulse wave velocity; PWV measurements could not be performed reliable in 2 subjects due to obesity.

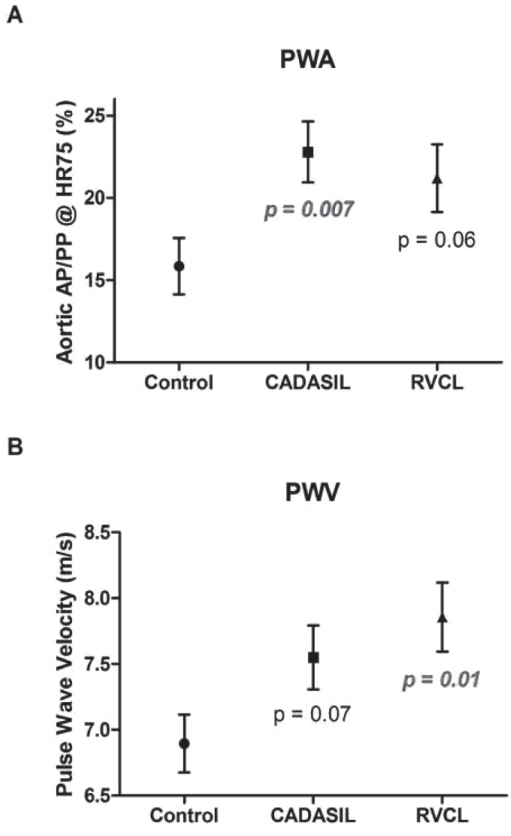

Figure 1. Increased vascular stiffness observed in CADASIL and RVCL patients measured with Pulse Wave Analysis (PWA) and Pulse Wave Velocity (PWV) respectively. (**A**) Aortic augmentation pressure corrected for heart rate and measured at the radial artery was increased in CADASIL patients using multivariate regression with age, gender and diastolic blood pressure as covariates (p=0.007). (**B**) Carotid-femoral aortic pulse wave velocity was increased in RVCL patients using multivariate regression with age and diastolic blood pressure as covariates (p=0.01).

Multivariate analysis resulted in a regression model for PWV (Multiple R = 0.70; Adjusted R^2 = 0.46; p < 0.001) with age (p = 0.003; observed power = 0.87), diastolic blood pressure (< 0.001; observed power >0.99) and subject group (p = 0.02; observed power = 0.72) as covariates. This regression model for PWV with covariates age and diastolic blood pressure, revealed a significant difference between control subjects and RVCL patients (p = 0.01) and a trend in CADASIL patients (p = 0.07) (Fig. 1).

Capsaicin-induced Dermal Blood Flow (DBF)

Results on capsaicin-induced DBF are summarized in Fig. 2. One subject did not undergo capsaicin testing due to a skin allergy to alcohol containing solutions. Another subject was excluded from analysis because of prophylactic anti-migraine medication (pizotifen) use. One subject was excluded because of coloured skin. Because measurements for RVCL and CADASIL

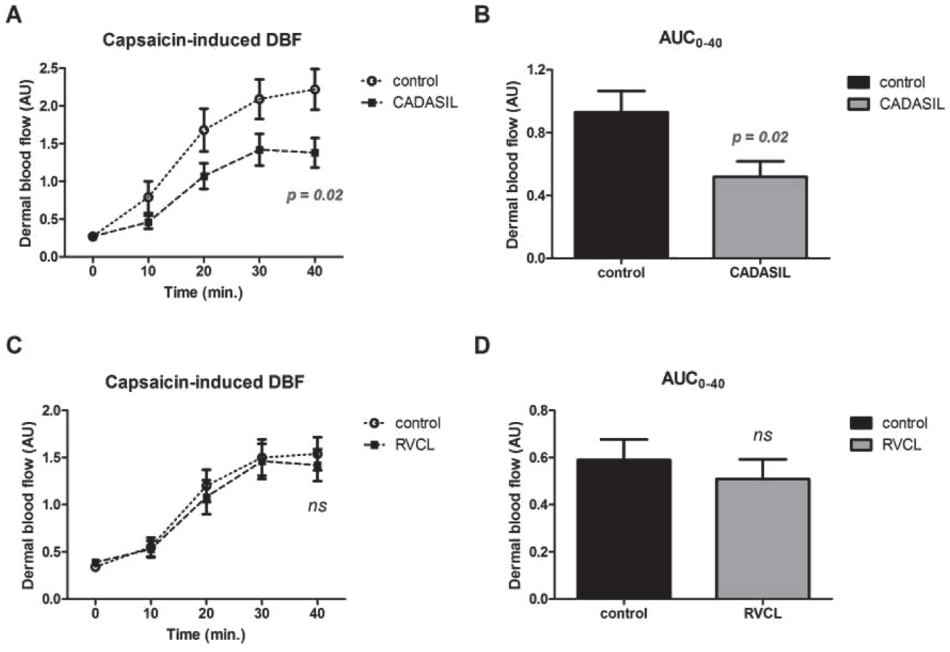

Figure 2. Microvascular reactivity of the skin after topical capsaicin application is reduced in CADASIL subjects. Dermal blood flow was assessed using laser Doppler perfusion imaging. (**A**) Maximal dermal blood flow increase 40 minutes after capsaicin application was lower in CADASIL patients than a matched control group (p=0.02). (**B**) Total dermal blood flow response over a period of 40 minutes after capsaicin application, displayed as area-under-the-curve, was reduced in CADASIL patients (p=0.02). (**C**) and (**D**) No changes in capsaicin-induced dermal blood flow were observed in RVCL patients, when compared to a matched control group.

were performed at different time points with different LDI scanners, separate matched control groups were used. No differences were found between RVCL patients and control subjects. CADASIL patients compared to control subjects displayed a lower increase in DBF 40 min after capsaicin application (1,38 ± 0,88 vs. 2,22 ± 1,20 Arbitrary Units (AU), p = 0.02) and a lower area under the curve over the total observation period of 40 min (0,52 ± 0,43 vs. 0,93 ± 0,60 AU, p = 0.02).

Flow-Mediated Dilatation (FMD)

FMD assessments were performed in 18 RVCL, 14 CADASIL patients and in 18 control subjects. Because of technical difficulties and movement artefacts, a valid set of measurements could only be obtained in 14 control subjects and 10 subjects of the two patient groups. Results are summarized in Fig. 3. In RVCL patients a decreased FMD was observed compared to control subjects (2,32 ± 3,83 vs. 5,75 ± 3,07 %, p = 0.02). In contrast, no significant differences were found between CADASIL patients and control subjects (3,77 ± 3,15 vs. 5,75 ± 3,07, p = 0.14) for any of the parameters. The decreased FMD in RVCL patients (Fig. 3) persisted after correction for shear rate (p = 0.02) and shear stress (2,32 ± 3,83 vs. 5,75 ± 3,07 %, p = 0.03) (Supplementary Table 3).

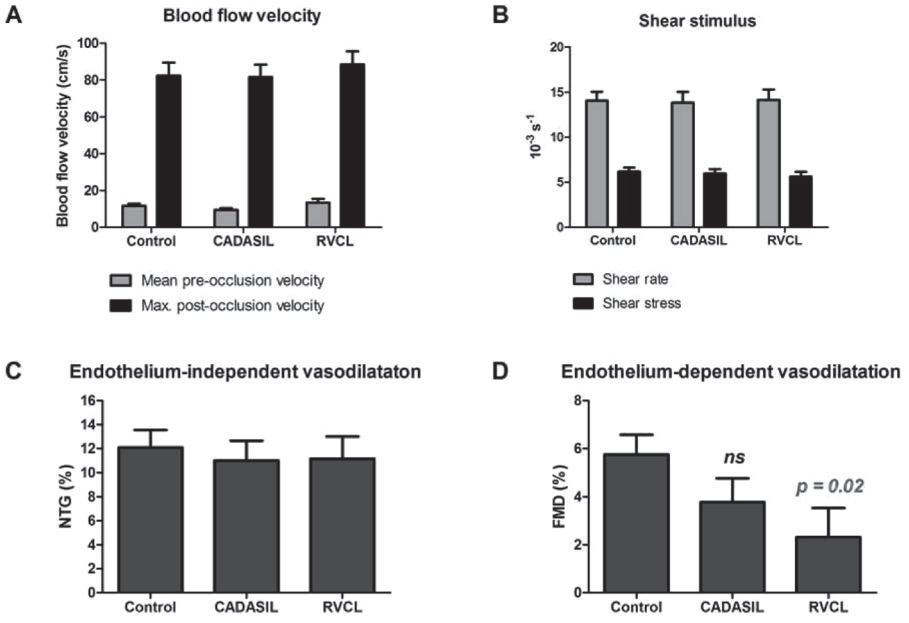

Figure 3. Endothelial dysfunction was found in RVCL patients using Flow Mediated Dilatation (FMD) at the brachial artery. (**A**) Blood flow velocity, measured pre- and post-occlusion hyperaemia, was comparable between all groups. (**B**) Shear rate, calculated as the blood flow velocity divided by artery diameter, and shear stress, calculated as shear rate multiplied by hematocrit, did not differ between all groups. (**C**) The endothelium-independent percentage increase in brachial artery diameter measured 6 minutes after sublingual nitroglycerin administration did not differ between groups. (**D**) The endothelium-dependent maximal increase in brachial artery diameter after 5 minutes of hyperaemia was decreased in RVCL patients (p=0.02).

DISCUSSION

This study provides a detailed assessment of distinct vascular properties of two monogenic cerebral small vessel diseases: RVCL and CADASIL. We are the first to find evidence for endothelial dysfunction in RVCL. In addition, we confirm impaired endothelial-independent microvascular reactivity in CADASIL. The observed increased arterial stiffness in both patient groups further illustrates RVCL and CADASIL as vasculopathies.

Endothelial dysfunction in RVCL

In RVCL patients, a decreased endothelium-dependent flow-mediated dilatation was observed in the brachial artery, supporting the hypothesis that *TREX1* mutations in RVCL cause endothelial dysfunction, as was previously hypothesized.[10,59]

In RVCL patients, capillary occlusion is suggested by histological examination of the retina and cerebral white matter, revealing thickening of the vessel wall with lumen obliteration. Additionally, nodular regenerative hyperplasia of the liver, arteriosclerosis, and glomerulosclerosis in the kidney, together with anemia and gastrointestinal bleeding, are

probably all manifestations of small vessel disease.[10,60] Thus, RVCL is a systemic vasculopathy with luminal narrowing, but in contrast to CADASIL, only minimal degeneration of vascular smooth muscle cells. Instead, fibrinoid necrosis of small and medium vessel wall, hyalinized vessels and reactive gliosis is found, which is may be a consequence of endothelial cell dysfunction. Remarkably, electron microscopy also shows irregular thickening and splitting of the basement membranes in the vessel wall[14], which may be produced by endothelial cells.[10,14]

Impaired microvascular reactivity and increased vascular resistance in CADASIL

In CADASIL patients, a decreased dermal blood flow response to capsaicin was observed. Capsaicin activates pre-synaptic TRPV1 receptors on Aδ- and C-fiber nociceptors and leads to relaxation of the VSMCs of the skin microvasculature upon CGRP release. A decreased dermal blood flow could result from defects at different levels in this pathway: i) less sensitivity of TRPV1 receptors to capsaicin; ii) impairment of CGRP release from nociceptors; iii) a decreased expression of functional TRPV1 and CGRP-receptors, or iv) a decreased relaxation of VSMCs in response to an endothelium-independent stimulus. Given the morphological alterations seen in the microvasculature of CADASIL patients, i.e., degeneration of vascular smooth muscle cells with a largely normal endothelium,[18] the last explanation seems most plausible. Thus, our findings suggest impaired endothelium independent VSMC dilatation in CADASIL.

Findings from other studies that measured microvascular vasoreactivity using different techniques (without involvement of CGRP) are in line with our results. For example, endothelium-independent skin vasoreactivity, as measured by cutaneous blood flow post-occlusive reactive hyperemia was altered in CADASIL patients.[27] In addition, lower dermal blood flow was observed after iontophoretic application of sodium nitroprusside, an exogenous Nitric Oxide (NO)-donor relaxing VSMCs. Impaired endothelium-independent vasoreactivity to glyceryltrinitrate, another exogenous NO-donor, was also found using plethysmographicendo-peripheral arterial tone (Endo-PAT) measurement at the fingertips of CADASIL patients.[30]

After measuring FMD at the brachial artery, we did not find evidence for endothelial dysfunction in CADASIL, nor did we find differences in endothelium-independent relaxation of the brachial artery after NTG administration. Thus, the vascular function of *conduit* vessels, in this case the brachial artery, remains unaltered in CADASIL. These results confirm findings of others[27,28], who also reported that FMD of the brachial artery in CADASIL patients was unaffected. One study[29] suggested endothelial dysfunction based on L-arginine induced vasoreactivity of the middle cerebral artery. However, these results might be due to baseline differences between CADASIL and control subjects. Stenborg et al.[28] reported impaired endothelium-dependent vasodilatation in forearm *resistance* arteries (i.e., the microcirculation) using venous occlusion plethysmography with intra-arterial acetylcholine infusion. Thus, although we find no evidence for endothelial dysfunction in conduit arteries of CADASIL patients, there is evidence for endothelial dysfunction of the microcirculation, which is in line with the labeling of CADASIL as a small vessel disease.

Increased vascular stiffness in both RVCL and CADASIL

We found an increased PWV and AIx in RVCL and CADASIL, which supports increased vascular stiffness in both diseases. Pulse wave velocity is a marker of aortic stiffness while the augmentation index is related to arterial stiffness in general as well as to the intensity and location of reflected waves, which is largely determined by the diameter and compliance of small arteries. In CADASIL, significance was only reached for the AIx. These results may suggest that the microcirculation is more severely affected in CADASIL than in RVCL. In RVCL significance was only reached for PWV, which may be related to endothelial dysfunction found in conduit vessels. Both PWA and PWV are validated methods which have been used for patient outcome predictions in stroke[61] and renal transplant patients,[62,63] cardiovascular disease risk in type 1 diabetes[64] as a marker for risk in cardiovascular disease[65] and brain abnormalities.[66,67] The augmentation index also predicts adverse cardiovascular events in coronary artery disease patients.[68] Consequently, a clinical implication of our results may be that for both RVCL and CADASIL, and possibly other cerebral hereditary angiopathies, intensive monitoring and treatment of cardiovascular risk factors may be important to prevent neurovascular and systemic vascular complications.[69]

Methodological considerations

Our study has important strengths. We are the first to assess four complementary vascular properties in two rare monogenic small vessel diseases (RVCL and CADASIL). All techniques were performed by one investigator (SV) who was blinded for the health status of the study subjects. A specific set of well-validated techniques was used to investigate vascular function at distinct levels of the vascular bed.[52,56,70,71] More specifically, FMD, the absolute standard for testing endothelial function, was performed according to the guidelines from the International Brachial Artery Reactivity Task Force.[58]

There are also some limitations of our study that need to be considered. Because CADASIL and RVCL are rare diseases, sample sizes are relatively small. This limits the power of our observations. Unfortunately, movement artifacts, possibly related to the fact that FMD was the last test performed during the examination (this was done because of the systemic effect of NTG), led to loss of some FMD data. Notably also is the slightly larger (non-significant) baseline brachial artery diameter in the RVCL group. Control subjects with a smaller baseline diameter who display the same absolute increase in diameter, would then automatically display higher % FMD. However, the absolute change in diameter of the RVCL group tended to be smaller than in the control group. Therefore, the validity of our results remains. Whereas the effect of hematocrit was corrected via the shear stress, the effect of slightly lower hemoglobin levels in RVCL patients may have affected our measurements as hemoglobin absorbs NO, and a lower hemoglobin results in an increased FMD.[72] However, correction for this would only strengthen our results and lead to an even lower FMD in RVCL. The slightly (not significant) reduced FMD observed in CADASIL might be the result of the mismatch in cigarette pack years, although it has to be said that mainly the two heavy smokers dramatically influenced the number of pack years. Furthermore, we did not measure endothelial function at the level of resistance arteries. Therefore, we are unable to state that the endothelial dysfunction we found

in RVCL can be extrapolated to small vessels. Therefore, iontophoresis or venous occlusion plethysmography with acetylcholine would be of interest as a follow-up study. Finally, the impact of medication use or stop of medication on our results is unknown. Although we attempted to minimize the effect by asking patients to stop their medication a week prior to the measurements, unfortunately only a few patients got permission to do so from their treating physician.

Possible implications for migraine

The increased prevalence of migraine in both CADASIL and RVCL might be related to the altered vascular properties found in the present and previous studies. In CADASIL, where patients mainly display migraine *with aura*, the increased prevalence of migraine may be due to an increased susceptibility for cortical spreading depression (CSD), which is the underlying mechanism of the migraine aura.[73,74] This hypothesis is strengthened by the fact that transgenic *NOTCH3* over-expressor mice were shown to have a decreased threshold for CSD.[75] Increased susceptibility to CSD may be due to alterations in smooth muscle cells and subsequent altered vasoreactivity of the microcirculation. These alterations may induce (subclinical) ischemia, which is a known trigger for CSD. In line with our results in the skin, indeed altered cerebral vasoreactivity in CADASIL patients has been found.[75]

A direct link with CSD is missing in the case of RVCL because migraine without aura is the more prevalent migraine subtype. A shared increased genetic susceptibility may underlie this co-morbidity, as was shown for RVCL in a family-based association study.[76] In light of the present data, an increased susceptibility for endothelial dysfunction may contribute to the development of migraine in RVCL. This seems supported by the fact that migraine itself has been linked to endothelial dysfunction.[32-36]

Conclusions

In RVCL patients endothelial dysfunction was demonstrated and, based on pulse wave velocity, increased vascular stiffness was suggested. In CADASIL patients impaired endothelium-independent vasoreactivity of the dermal microvasculature was shown. In combination with an increased augmentation index, suggestive for increased vascular resistance, these findings clearly label CADASIL as a small vessel disease. Future studies may link these vascular functional markers to disease progression and perhaps to individual symptoms of these syndromes, including, stroke, dementia, and migraine.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1. Univariate associations between clinical characteristics and pulse wave analysis (PWA) and pulse wave velocity (PWV)

Spearman rank order correlations(p<0.05)	
PWA	
Gender	-0.37
Age	0.55
Systolic blood pressure	0.38
Diastolic blood pressure	0.38
Mean arterial pressure	0.24
Packyears	0.31
HDL cholesterol	0.24
Hematocrite	-0.20
Hemoglobin	-0.20
Ureum	0.22
PWV	
Age	0.56
Systolic blood pressure	0.55
Diastolic blood pressure	0.55
Mean arterial pressure	0.37
Total cholesterol	0.26
gamma-GT	0.27
Ureum	0.32

Supplementary Table 2. Capsaicin induced dermal blood flow

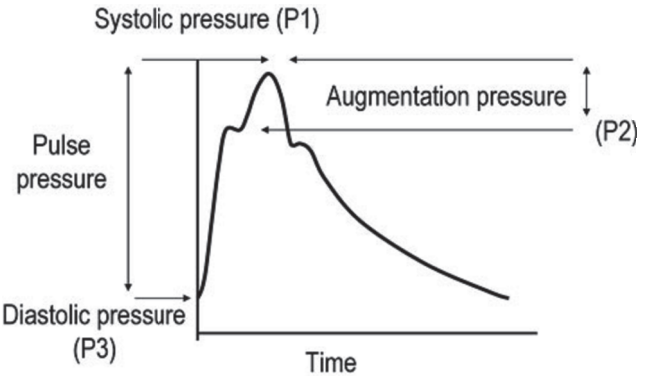
Dermal blood flow	**Control (n=20)**	**CADASIL (n=20)°**	***p-value*** †	**Control (n=18)**	**RVCL (n=18)**	***p-value*** ‡
Baseline (AU)	0.27 ± 0.15	0.27 ± 0.17	*0.98*	0.34 ± 0.08	0.39 ± 0.11	*0.12*
10 min (AU)	0.79 ± 0.94	0.46 ±0.39	*0.16*	0.55 ± 0.42	0.53 ± 0.37	*0.93*
20 min (AU)	1.68 ± 1.27	1.07 ± 0.76	*0.08*	1.20 ± 0.73	1.08 ± 0.77	*0.63*
30 min (AU)	2.09 ± 1.17	1.42 ± 0.94	*0.06*	1.50 ± 0.82	1.46 ± 0.79	*0.87*
40 min (AU)	2.22 ± 1.20	1.38 ± 0.88	*0.02*	1.54 ± 0.74	1.42 ± 0.72	*0.64*
AUC_{0-40} (AU)	0.93 ± 0.60	0.52 ± 0.43	*0.02*	0.59 ± 0.37	0.51 ± 0.35	*0.52*
Increase in DBF (%) 40min. post-capsaicin	607 ± 474	346 ± 343	*0.05*	332 ± 2.21	272 ± 2.01	*0.40*

Data presented as mean ± SD. AUC_{0-40}: area under the curve over the total observation period of 40 minutes. AU: arbitrary units. † Difference between control subjects and CADASIL patients. ‡ Difference between control subjects and RVCL patients. ° One subject did not undergo capsaicin test due to a skin allergy to alcohol containing solutions. One subject was excluded from analysis because of prophylactic anti-migraine medication (Pizotifen) use. One subject was excluded because of Indonesian coloured skin.

Supplementary Table 3. Flow mediated vasodilation (FMD) of the brachial artery

Variable	Controls (n=14)	CADASIL (n=10)	*p-value*†	RVCL (n=10)	*p-value*‡
Pre-occlusion diameter (µm)	5655 ± 1014	5934 ± 839	*0.48*	6438 ± 1103	*0.09*
Maximal post-occlusion diameter (µm)	5987 ±1109	6150 ± 828	*0.70*	6602 ± 1252	*0.22*
Absolute increase in diameter (µm)	332 ± 187	216 ± 169	*0.13*	165 ± 256	*0.08*
FMD (%)	5.76 ± 3.07	3.77 ± 3.15	*0.14*	2.32 ± 3.83	*0.02*
Pre-occlusion velocity (cm/s)	11.7 ± 4.1	9.5 ± 3.4	*0.18*	13.4 ± 6.3	*0.78*
Maximal post-occlusion velocity (cm/s)	82.4 ± 24.9	81.7 ± 20.5	*0.94*	88.5 ± 21.4	*0.56*
Shear rate (s^{-1})	0.014 ± 0.0034	0.014 ± 0.0036	*0.88*	0.014 ± 0.0035	*0.97*
Shear rate normalised FMD (s)	4.35 ± 2.31	2.99 ± 2.33	*0.20*	1.32 ± 2.91	*0.02*
Shear stress (s^{-1})	0.0062 ± 0.0016	0.0060 ± 0.0015	*0.78*	0.0056 ± 0.0015	*0.46*
Shear stress normalized FMD (s)	10.07 ± 5.73	6.96 ± 5.58	*0.23*	3.31 ± 7.24	*0.03*
Pre-NTG diameter (µm)	5710 ± 844	6198 ± 1019	*0.15*	6190 ± 1034	*0.22*
Maximal post-NTG diameter (µm)	6496 ± 899	6947 ± 891	*0.24*	6980 ± 1193	*0.27*
NTG (%)	12.08 ± 5.47	11.01 ± 5.23	*0.64*	11.16 ± 5.84	*0.70*

Data presented as mean ± SD. NTG: nitroglycerine. Peak shear rate was missing in 4 subjects, therefore no corrected FMD could be calculated in 2 healthy volunteers and in 1 of each patient groups. † Difference between control subjects and CADASIL patients. ‡ Difference between control subjects and RVCL patients



Supplementary Figure 1. Augmentation Index
Artery pressure waveform is recorded by applanation tonometry.The height of the late systolic peak (P1) above the inflection (P2) defines the augmentation pressure, and the ratio of augmentation pressure to PP defines the AIx (in percent). "In the human body, wave reflections originate in various locations, including peripheral bifurcations of conducting arteries and smaller muscular arteries. The geometry, number of arterioles, and the architecture of the microvascular network play an important role in wave reflection. Indeed, arterial and arteriolar constriction results in reflection points closer to the heart, leading to earlier aortic wave reflections. In addition, with increased arterial stiffness, as observed, for example, in older subjects or hypertensive patients, the reflected wave travels more rapidly along the arterial tree. Thus, both small and large arteries contribute to early reflected waves which arrive in early systole, superimpose on the forward wave, and boost the systolic pressure further, whereas blood pressure falls sharply in diastole with reduced diastolic fluctuations." Adapted from: Laurent S, Cockcroft J, Van Bortel L, Boutouyrie P, Giannattasio C, Hayoz D, et al. Expert consensus document on arterial stiffness: methodological issues and clinical applications. Eur Heart J 2006;27(21):2588-605.

PART 3

MIGRAINE AND COMORBIDITY IN A GENETIC ISOLATE

CHAPTER 11

SHARED GENETIC FACTORS IN MIGRAINE AND DEPRESSION: EVIDENCE FROM A GENETIC ISOLATE

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ABSTRACT

Objective: To investigate the co-occurrence of migraine and depression and assess whether shared genetic factors may underlie both diseases.

Methods: Subjects were 2,652 participants of the Erasmus Rucphen Family (ERF) genetic isolate study. Migraine was diagnosed using a validated three-stage screening method that included a telephone interview. Symptoms of depression were assessed using the Centre for Epidemiologic Studies Depression (CES-D) scale and the depression subscale of the Hospital Anxiety and Depression Scale (HADS-D). The contribution of shared genetic factors in migraine and depression was investigated by comparing heritability estimates for migraine with and without adjustment for symptoms of depression, and by comparison of the heritability scores of depression between migraineurs and controls.

Results: We identified 360 migraine cases, 209 had migraine without aura (MO) and 151 had migraine with aura (MA). Odds ratios for depression in migraine patients were 1.29 (95% confidence interval (CI) 0.98-1.70) for MO and 1.70 (95% CI 1.28-2.24) for MA. Heritability estimates were significant for all migraine (0.56), MO (0.77), and MA (0.96), and decreased after adjustment for symptoms of depression or use of antidepressant medication, in particular for MA. Comparison of the heritability scores for depression between migraine patients and controls showed a genetic correlation between the HADS-D score and MA.

Conclusions: There is a bidirectional association between depression and migraine, in particular MA, which can be explained, at least partly, by shared genetic factors.

INTRODUCTION

Migraine is a highly prevalent brain disorder that is characterized by recurrent headache attacks associated with nausea, vomiting, photo- and phonophobia (migraine without aura; MO). In one third of migraineurs, attacks are preceded by transient focal neurological symptoms (migraine with aura; MA).[1] Migraineurs often have symptoms of depression. Population-based odds ratios (OR) range from 2.0 to 5.8, with strongest associations for MA.[2-13] The quality of life of migraineurs with depression is decreased.[10,14] Co-morbid depression is a risk factor for chronification of migraine[15-17] and development of medication–overuse-headache.[18] Patients with depression also have an increased risk for migraine, with risk estimates of 2.8-3.4 for migraine, 2.2 for MO and 4.0 for MA.[2-4] This bidirectional relationship suggests that migraine and depression may share common etiological factors.

Twin- and family-based studies have shown that both migraine and depression have a strong genetic basis.[19-24] Heritability estimates range from 0.33-0.53 for migraine (33-53% of the trait is explained by additive genetic effects) to 0.61-0.77 for MO and 0.65-0.79 for MA.[19-23] For depression, heritability estimates range from 0.17-0.78.[24] Recent evidence suggests that both diseases share molecular pathways controlling serotonergic and glutaminergic neurotransmitter systems.[25,26] Shared genetic factors may therefore underlie the bidirectional comorbidity of migraine and depression.

Here we determined the co-occurrence of migraine and depression in a Dutch genetic isolate, investigated to what extent genetic factors are involved in migraine, and whether shared genetic factors may underlie the comorbidity of both disorders.

METHODS

Study Population

Subjects were participants of the Erasmus Rucphen Family (ERF) study, a family-based study in a genetically isolated community in the Southwest of the Netherlands.[27] In brief, the ERF study population includes 3,465 individuals that were ascertained based on genealogical background (not selected on phenotypes of interest), and are living descendants of 22 couples with at least six children baptized in the community church between 1850 and 1900. Founding couples are related through previous generations as demonstrated by extensive genealogical information from detailed church and municipal records. Hence, study participants were all members of a large extended pedigree. All individuals of 18 years and older were invited to participate. Spouses were invited only for family members that had children of 18 years and older.

Standard Protocol Approvals, Registrations, and Patients

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

Clinical evaluation

Extensive clinical information from ERF participants (n = 3,465) was available.[27] For this study relevant clinical data were obtained by questionnaires (symptoms of headache and depression and education level) or from a visit to the research centre (use of antidepressant medication).

Migraine

Migraineurs were identified between 2005 and 2007 using a three-stage screening procedure assessing lifetime occurrence of migraine, which was previously validated in a population-based study[28] and which was based on the Classification Criteria of the International Headache Society (IHS).[1] In brief, in the first stage, participants were asked to fill in five screening questions on headache and aura symptoms. Then, screen-positives received a detailed questionnaire on headache and aura symptoms. Finally, screen-positives were interviewed by telephone for further clarification of their answers. Subjects that were screen-positive but did not return the extensive headache questionnaire and subjects that could not be screened because they did not, or incompletely, filled in the screening questions, were directly contacted by telephone. Telephone interviews were performed by the principal study physician (AHS), who is experienced in diagnosing migraine patients, and by well-trained medical students supervised by AHS. A final diagnosis was only made after the telephone interview and in consultation with a neurologist specialized in headache (GMT).

Depressive symptoms

Symptoms of depression were assessed using the Centre for Epidemiological Studies Depression Scale (CES-D)[29] and the depression subscale of the Hospital Anxiety and Depression Scale (HADS-D).[30] Both scales are validated, reliable self-report measures of symptoms of depression.[31, 32] The CES-D consists of 20 items with total scores ranging from 0 to 60 and the HADS-D of seven items with scores ranging from 0 to 21. Higher scores indicate more symptoms of depression. Depression scores were analyzed both as continuous variables and as dichotomous variables. For the dichotomous variable, depression was defined as a CES-D score ≥ 16[31] and HADS-D score ≥ 8[32] or the use of antidepressant medication. CES-D scores were missing for 327 and HADS-D for 104 subjects. Missing CES-D and HADS-D data were imputed based on data of the other depression scale, age, sex and use of antidepressants using SPSS version 12.0, iterative expectation-maximization method. Depression scores were not imputed when both scores were missing.

Control group

The control group consisted of subjects that: (i) did not report severe headache (pain severity score ≤ 4 on a scale from 0 to 10); ii) did not report visual aura symptoms; (iii) had never been diagnosed with migraine by a physician; and (iv) never used specific anti-migraine medication. Thus, also 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 a non-migraineurs than in our previous population-based study.[28]

Statistical methods

For statistical analysis we used data from three migraine groups (i.e., all migraine cases, MO, and MA) and the control group. General characteristics of the subjects of each group were compared using chi-square statistics for dichotomous variables and a Student's t-test for continuous variables.

All analyses were corrected for inbreeding. The coefficient of inbreeding per individual was calculated based on available genealogical information using PEDIG software.[33] This coefficient reflects the probability that two alleles at a given locus in an individual are identical by descent.

To examine the co-occurrence of migraine and depression, we calculated the odds ratio (OR) for the risk of depression for each migraine group using multivariate mixed model regression analyses corrected for inbreeding, age, sex and education and taking into account pedigree relations between individuals (polygenic liability threshold model).[34]

Mean CES-D and HADS-D scores were calculated for each group. *P*-values comparing migraine subgroups versus controls were corrected for age, sex, level of education, use of antidepressants, inbreeding and pedigree relations by multivariate linear mixed model regression, considering each migraine group as an independent variable and HADS-D or CES-D data as the continuous dependent variable. Because both CES-D data and HADS-D data deviated from the normal distribution, data were transformed by taking the natural logarithm (CES-D) and by taking the square root (HADS-D).

We calculated the heritability estimates (h^2) as the ratio of the variance of a trait that is explained by additive polygenic effects to total phenotypic variance of the trait. The polygenic model was applied that assumes an infinite number of genetic factors with a small additive effect contributing to the trait variance. Analyses were adjusted for age, sex and inbreeding. As sibship effects, a combination of effects induced by sharing early childhood environment and dominant genetic variation, did not significantly influence heritability estimation, this covariate was excluded from the analyses.

Involvement of shared genetic factors underlying migraine and depression was assessed by two complementary methods. First, heritability estimates for migraine were computed with and without depressive symptoms as covariate (i.e., HADS-D, CES-D scores and use of antidepressants included) (see also[27]). A difference between adjusted and unadjusted heritability estimates represents that part of the genetic component in migraine that is shared with depression.

Second, we compared the heritability of depression in migraine patients to that in controls (i.e., assess whether a diagnosis of migraine influences the heritability of depression) by performing a bivariate polygenic analysis with both HADS-D and CES-D scores (i.e., depression score + migraine status, covariates: age, sex, inbreeding, use of antidepressant medication) (see for examples[35,36] except here, we compared migraine versus no migraine status, instead of gender, to estimate the genetic correlation). The significance of a genetic correlation was determined using a likelihood ratio test.

Descriptive analyses were performed using SPSS version 12.0 for windows (SPSS, Chicago, IL). SOLAR 2.1.2 software package (Southwest Foundation for Biomedical Research, San Antonio, Texas, USA) was used for calculation of heritability estimates, regression analyses and genetic correlations.

RESULTS

Recruitment of migraine patients

In total, 3,465 subjects participated in the ERF study. Figure 1 shows the ascertainment flow chart of migraine cases. The questionnaire with headache screening questions was returned by 2,652 (76.5%) subjects. Of these, 888 were screen-positive and 1,291 screen-negative; 473 subjects had not or only incompletely, filled out the screening questionnaire. Of the screen-positives, 572 (64.4%) subjects could be interviewed of which 305 proved to have migraine. Of the group that was incompletely screened, 286 (60.5%) subjects were interviewed of which 55 proved to have migraine. A diagnosis could not be made in 503 subjects because of a variety of mainly logistic reasons. Of the in total 2,149 ERF participants who were interviewed (n=858) or who were screen-negative (n=1291), 360 (16.8 %) had migraine. Of these, 209 (58%) had MO and 151 (42%) had MA. A total of 617 subjects gave negative answers to all screening questions, thereby fulfilling the criteria for the control group.

Descriptive data

The demographic characteristics of all groups are shown in Table 1. No significant differences were found for the mean age. In line with the higher prevalence of migraine in women, more

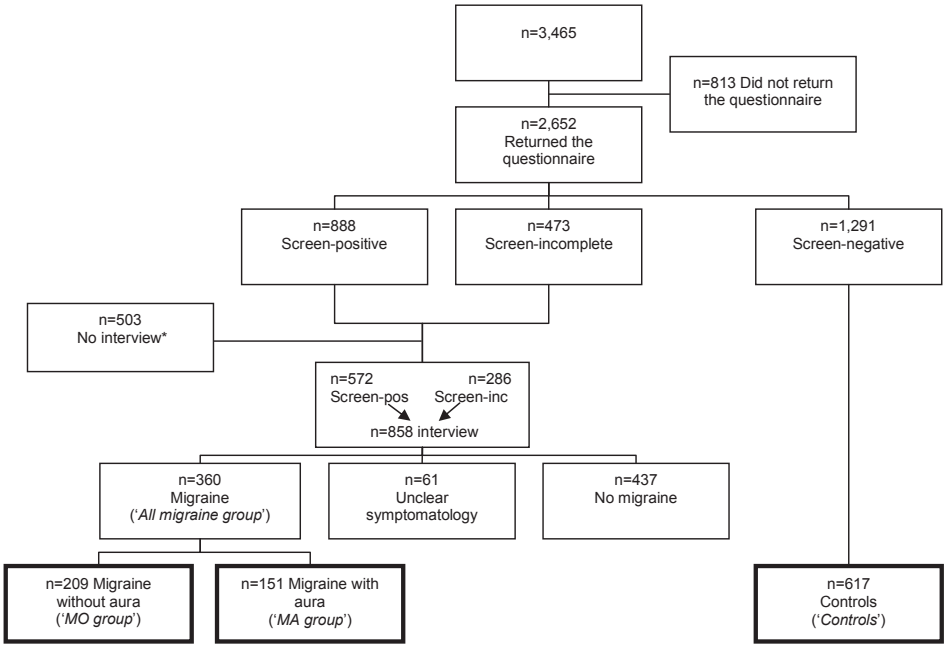

Figure 1. Flowchart of ascertainment of migraine cases and the controls in the Erasmus Rucphen Family study
* Subjects were deceased or declined participation (n=122), had an incorrect (n=140) or unavailable (n=10) telephone number, gave no permission for a telephone interview (n=130), were not answering 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).

Table 1. Distribution of sex, age, and education in the various groups

Characteristics	All migraine (n=360)	MO (n=209)	MA (n=151)	Controls (n=617)
Women, %	75*	77*	73*	47
Mean age in years (SD)	46.2 (12.3)	47.0 (12.7)	45.1 (11.6)	47.8 (15.3)
Education, %				
Higher	5§	5	4§	9
Medium	64	63	66	65
Lower	31	32	30	26

MO: migraine without aura; MA: migraine with aura. Education: Higher: college or university, Medium: secondary school or vocational technical training, Lower : primary or elementary school or unfinished secondary school. *$p < 0.001$ compared to controls; §$p < 0.05$ compared to controls. SD: standard deviation.

women were present in the migraine groups. Overall, patients with migraine, in particular those with MA, had a lower education than controls.

Depression in migraineurs

Of the 2,652 participants that completed the questionnaire, HADS-D scores were obtained for 2,548 subjects and CES-D scores for 2,325. After imputation, HADS-D and CES-D scores were available for 2,584 subjects. Of these, 583 met our definition for depression. Of these, 91 were included in the migraine group and 82 in the non-migraine control group. The remaining subjects were not included in the current assessment because they did not meet the inclusion criteria for either the all migraine or control group.

Twenty-five percent (91/360) of migraineurs and 13% (82/617) of controls were found to be depressed (Table 2). Depression was more often seen in MA (32%; 48/151) than in MO (21%; 43/209). The ORs were 1.43 (95% CI 1.15 -1.78) for all migraine, 1.29 (95% CI 0.98-1.70) for MO, and 1.70 (95% CI 1.28-2.24) for MA. Mean CES-D scores were higher ($p < 0.001$) in migraine patients compared to controls (all migraine: 12.0 ± 10.2, MO: 11.1 ± 9.7, MA: 13.2 ± 10.7, and controls: 7.5 ± 7.4). The mean HADS-D score was higher ($p < 0.01$) in the all migraine and MA groups compared to controls (all migraine: 6.1 ± 4.4 and MA: 6.7 ± 4.6), but was not significantly different between the MO and control group (MO: 5.7 ± 4.2 and controls: 4.9 ± 3.7).

Table 2. Depression in migraine patients and controls

Group	Depression (n, (%))	OR (95% CI)	*p* value
Controls (n=617)	82 (13%)	1.00	-
MO (n=209)	43 (21%)	1.29 (0.98-1.70)	0.07
MA (n=151)	48 (32%)	1.70 (1.28-2.24)	< 0.001

MO: migraine without aura; MA: migraine with aura. Prevalence of depression per group is given. Depression was defined as scoring both CES-D ≥ 16 and HADS-D ≥ 8 or use of antidepressants. Odds ratios for the risk of depression in migraine patients are adjusted for sex, age, education and inbreeding.

Heritability estimates of migraine

In total 325 migraine patients (188 MO and 137 MA) and 562 control subjects were present in the ERF extended pedigree.[27] Heritability estimates were 0.56 (95% CI 0.26-0.86) for all migraine, 0.77 (95% CI 0.38-1.00) for MO, and 0.96 (95% CI 0.51-1.00) for MA (Table 3). These estimates decreased after adjustment for depressive symptoms to 0.51 (95% CI 0.19-0.83) for all migraine, 0.75 (95% CI 0.32-1.00) for MO, and 0.81 (95% CI 0.31-1.00) for MA. The decrease was highest in patients with MA (15%), compared to a decrease of 4% in the all migraine group and 2% in MO patients. This would suggest that shared genetic factors particularly underlie the comorbidity of depression and MA.

Table 3. Heritability estimates of migraine with and without adjustment for depression

	h^2 (95% CI)[a]	h^2 (95% CI) (with adjustment for depression)[b]	*p* value[c]
All migraine (n=325)	0.56 (0.26-0.86) **	0.51 (0.19-0.83) **	0.81
MO (n=188)	0.77 (0.38-1.00) **	0.75 (0.32-1.00) **	0.95
MA (n=137)	0.96 (0.51-1.00) **	0.81 (0.31-1.00) *	0.65

MO: migraine without aura; MA: migraine with aura. [a]Heritability estimates are based on a polygenic model, covariates: age, sex and inbreeding coefficient. [b]Heritability estimates are based on a polygenic model, covariates: age, sex and inbreeding coefficient, HADS-D, CES-D, use of antidepressant medication. [c]P value for the difference in heritability estimates before and after adjustment for depression. *P* values for heritability estimates: **p <0.001 and *p <0.01.

Next we compared the heritability for depression in migraineurs and controls. A correlation of 1 indicates that there is no difference in the genetics of depression between patients and controls, while a deviation from 1 suggests shared genetic factors for depression and migraine. Only for HADS-D and MA a reduced genetic correlation was found (correlation of 0.36) indicating that genetic factors causing symptoms of depression in MA patients differ to those involved in depression in controls. In MO patients the correlation coefficient was 1 indicating no difference in genetic factors for depression in MO and controls. For CESD there was no difference seen between migraine patients and controls.

DISCUSSION

We investigated the co-occurrence of migraine and depression in a large Dutch genetic isolate and to what extent shared genetic factors are involved. Our study is particularly suited to address these questions because of the following reasons: (i) our three-step diagnostic procedure - including a telephone interview for clarification and confirmation of the clinical symptoms and a final diagnosis according to the Classification Criteria of the IHS[1] guaranteed a highly reliable diagnosis of migraine; (ii) the presence of depression was assessed using two different depression scales; (iii) our study population includes a very large number of well-characterized subjects covering a broad age range from 18 to 91 years.

We found an increased risk of depression and depressive symptoms in migraine patients, in particular in those with aura. These findings are in agreement with previous studies from outbred populations.[2-13] ORs for depression in migraine patients in those studies were larger (2.0 – 5.8 for MA) than ORs observed here (1.7 for MA), which may be due to differences in the diagnostic methods, and definitions. This seems particularly true for depression. While several studies addressed *life-time* prevalence of depression,[2,4,5,9] which is notoriously difficult to assess reliably, we studied *current* depression, which is reliable to diagnose and will result in lower prevalence data. One might thus argue that presence of lifetime depression was underestimated in our study.

Some 23.5% of the 3,465 subjects were non-responders, which may have introduced a selection bias leading to an overrepresentation of very depressed patients or severe migraine patients in the non-responder population. However, such a bias in this particular study is less likely, as we used a general questionnaire designed to collect information on a large number of traits, not only related to headache and depression.

Heritability estimates in our study were significant for all three migraine groups and were substantially higher for MA than for MO. This is in accordance with studies in outbred populations,[19-23] confirming the hypothesis that the genetic contribution in MA is stronger than in MO. Also in line with these studies, a lower heritability estimate was observed for 'all migraine' than for MO or MA, which indicates that MA and MO share some, but not all, genetic factors. We observed a remarkably high heritability for MA of over 90% in ERF. This suggests that most of the variance in MA in ERF is explained by genetic factors. This finding may be particularly relevant for gene discovery and suggests that MA is the most promising migraine subtype to search for migraine genes. Moreover, our observation that heritability estimates for MA decreased when adjusting for depression, indicates that *shared* genetic factors may underlie depression and MA. For MO, a smaller decrease in heritability estimates was observed, suggesting that a small fraction of the comorbidity with depression is explained by shared genetic factors for this migraine subtype.

In our bivariate analysis, we found remarkably different results when using the two depression scales. When depression was determined by using the HADS-D scale, we found evidence for shared genetic factors for migraine with aura and depression. However, no such association was observed when depression was determined using the CES-D scale. This discrepancy may seem remarkable as both scales have been validated for the assessment of symptoms of depression[31,32] and there is a high correlation for both depression scales in our study (Pearson correlation coefficient rho = 0.75; $p < 0.001$). However, both scales assess slightly different symptoms, which may well explain our findings. As the HADS-D was specifically designed to prevent noise signal from somatic disorders,[32] it excludes 'physical symptoms' of depression, such as insomnia, fatigue or loss of appetite. In the CES-D scale, however, these symptoms are included. Although admittedly difficult to prove, we would like to argue that these physical symptoms are more likely a consequence of the migraine attacks than due to a depression. Consequently, we propose that the CES-D score, in a way, is confounded by migraine-related symptoms and therefore less suited to assess shared genetic factors in migraine and depression.

Previous studies found a bidirectional association between migraine, in particular those with aura, and depression.[2-4] This suggests that common pathogenetic pathways, at least partly, underlie both disorders, rather than that one is the consequence of the other. Thus, migraine patients may develop depression as a result of the demoralizing experience of recurrent and disabling headaches. However, then a correlation with disease disability would be expected, which was not found in previous studies.[2] Also, an even stronger association with MO should be seen, which in general is the more disabling form of migraine[1] and usually associated with higher attack frequencies. Our study provides evidence that this bidirectional relationship can be explained, at least partly, by shared underlying genetically determined disease mechanisms. Support for our findings comes from a recent twin study that also suggested that shared genetic risk factors may underlie migraine and depression.[37] Unfortunately that study has important limitations. First of all, it is unclear whether a correct migraine diagnosis was made as a diagnosis was based on 'self-reported physician's diagnosis', and not on well-defined IHS criteria. A similar criticism can be made with respect to the diagnosis of depression. This may well have led to misclassification and underreporting of migraine and depression. Second, unlike in our study, the sample consisted of female subjects only, and results therefore cannot be extrapolated to male patients. Third, no distinction was made between MA and MO. This last limitation is very relevant in light of our observation that shared genetic effects between migraine and depression are particular evident for migraine with aura.

Identification of common genetic factors may significantly improve the insight into the molecular basis of these common and highly disabling episodic brain disorders.

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CHAPTER 12

MIGRAINE IS NOT ASSOCIATED WITH ENHANCED ATHEROSCLEROSIS

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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.

INTRODUCTION

Migraine is a prevalent neurovascular brain disorder that is characterized by recurrent disabling attacks of headache associated with nausea, vomiting, and photo- and phonophobia (migraine without aura); in one-third of migraineurs, attacks may be preceded by transient focal neurological aura symptoms (migraine with aura).[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 migraine with aura 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] studies. None of the studies reported on the Framingham Risk Score for ischemic stroke (FRS-Stroke) in migraine patients, probably because the electrocardiogram 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 study.

METHODS

Study population

We performed a case-control study nested within the population-based Erasmus Rucphen Family 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 3,465 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 Center 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 case-finding 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 patient with "definite" migraine with and without aura 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, screen-positives 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 only made after the telephone interview and in consultation with a neurologist specialized 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 is missed using this screening procedure.[15]

Assessment of cardiovascular parameters

Participants (n = 3,465) 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, among which 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 and no. Participants took all prescript 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/m^2) was computed. An electrocardiogram (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 rest using an automated device (OMRON 711, automatic IS; Vernon Hills Illinois, USA). The average of the two measures was used for the brachial blood pressure in the analyses. Plasma concentrations of triglyceride (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 ≥7 mmol/L or both. Total plasma adiponectin was analyzed with the Human adiponectin 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 conform 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 Framingham Risk Scores for coronary heart disease (FRS-CHD)[17] and stroke (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] Intima media thickness (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 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 were 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 at the right arm in sitting position. The Ankle Brachial Index (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 for all migraine, migraine without aura and migraine with aura separately. 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 independent variable, adjusted for age, sex and education. Odds ratios for having a 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 SOLAR 2.1.2 software package (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 increase of risk allele frequencies and this may (falsely) increase associations between traits. Correction for relatedness prevents this and better reflects the association in the general population.

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 (see Chapter 11, Figure 1).[16] In total, 3465 subjects participated in the study, from which we recruited 360 migraine patients (151 with aura, 209 without aura) 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 to 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 those with aura, had lower levels of education. Smoking was more prevalent in migraineurs, in particular in those with aura (49% versus 31% of controls, $p < 0.001$). Migraine with aura 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 migraine with aura patients and controls remained significant. Migraine patients were more likely to have diabetes (5.4% migraine with aura, 5.5% migraine without aura 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 Framingham 10-year risk for stroke or coronary heart disease 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 predicted Framingham 10-year risk for stroke and coronary heart disease were not significantly different between the subjects with and without missing data. No difference was observed in mean Intima Media Thickness (IMT), carotid-femoral Pulse Wave Velocity (PWV), or Ankle Brachial Index (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.[9,23-37] 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 over 100 migraine patients (unselected with regard to cardiovascular risk factors) found slightly decreased values of IMT, which is in line with our findings.

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	0.02	5	4	MA: 0.04 MO: 0.10	9
Medium	64		63	66		65
Lower	31		32	30		26
Body Mass Index, kg/m2	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-Stroke>10 (% of subjects)	12		14	10		15
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 (standard deviation (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. u/w: units (glasses) per week; MA:migraine with aura; MO: migraine without aura; SBP: systolic blood pressure; DBP: diastolic blood pressure; LDL-C: low density lipoprotein cholesterol; HDL-C: high density lipoprotein cholesterol; CRP: 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: Framingham risk score for stroke; FRS-CHD: Framingham Risk Score for coronary heart disease; percentages for these scores represent the 10 year risk of stroke or coronary heart disease.

Table 2. Functional and structural measures of atherosclerosis in migraine patients versus controls

	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
Pulse wave velocity, m/s	9.0 (1.5)	9.0 (1.5)	9.1 (1.5)	9.3 (1.9)	0.55
Ankle Brachial index	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, HDL-C and smoking. MO: migraine without aura; MA: migraine with aura. 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 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.

Compared to previous studies our study has important strengths. We assessed the largest group of migraine patients thus far, including a large group of patients with aura (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 relatively homogenous genetic and environmental background. Finally, data on a large number of cardiovascular risk factors were available and allowed us to calculate both the FRS-CHD as well as 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 due to 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 migraine with aura, we feel this is not a disadvantage of our study as the risk for cardiovascular events is highest among migraine with aura cases

We assessed the FRS-Stroke in migraineurs and showed no increased odds for a higher score, which suggest 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 oral contraceptives and smoking in women. Second, population-based 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 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 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 migraine with aura or ischemia.[34] The latter hypothesis is supported by recent data in migraine (FHM1 $Ca_V2.1$) 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, migraine with aura is associated with strokes with good functional outcome,[36] which seems contradicting with 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 migraine-stroke relationship and to develop prophylactic treatment strategies to prevent cerebro- and cardiovascular events in migraine patients.

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CONFLICT OF INTEREST STATEMENT

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CHAPTER 13

GENERAL DISCUSSION

For this thesis, clinical genetic investigations on migraine, its comorbid diseases and migraine syndromes were performed. The main migraine syndrome studied is monogenic Familial Hemiplegic Migraine (FHM) which is considered a good model for the common forms of migraine because of clinical similarities. The second migraine syndrome is Retinal Vasculopathy with Cerebral Leucodystrophy (RVCL, later termed CHARIOT), a monogenic syndrome with complex migraine as part of the clinical spectrum. Lastly, common migraine was studied by identifying migraine cases in a Dutch genetic isolate to investigate comorbidity with major depression and atherosclerosis. For depression it was assessed whether the disease may share genetic factors with migraine.

PART I: FHM: A MONOGENIC MIGRAINE SYNDROME

13.1 Genetic and clinical spectrum of FHM

Familial hemiplegic migraine (FHM) is genetically heterogeneous with mutations in the *CACNA1A* (FHM1), *ATP1A2 (*FHM2) and *SCNA1* (FHM3) genes.[1-3] Identification of *novel* and *recurrent* mutations in these genes is important to better characterize the clinical and genetic spectrum of the genes, which can have implications for genetic testing. Moreover, increasing the number of mutation carriers with a specific mutation allows for more meaningful genotype-phenotype comparisons. This thesis describes several novel FHM mutations with the corresponding clinical spectrum: i.e., *CACNA1A* missense mutation V1696F in **chapter 3**, *ATP1A2* missense mutation G855R in **chapter 5** and *SCN1A* missense mutation L263V in **chapter 6**. In addition, genotype-phenotype correlation studies were performed on *recurrent CACNA1A* missense mutations R1347Q and S218L (**chapters 2** and **4).** The results do not only have implications for FHM, but also for associated phenotypes such as ataxia, epilepsy, alternating hemiplegia of childhood and the occurrence of lethal brain edema upon mild head trauma (especially in children).

Findings in the FHM1 *CACNA1A* gene

CACNA1A encodes the α1 subunit of voltage-gated neuronal $Ca_V2.1$ (P/Q-type) calcium channels.[1] To date, almost 30 FHM1 (and/or SHM1) mutations have been identified, all involving missense mutations. A spectrum of symptoms exists ranging from pure FHM without additional clinical symptoms to FHM associated with such features like epilepsy and ataxia, and at the extreme end, trauma-triggered FHM attacks that induce cerebral edema, that can lead to death of the patients. **Chapter 2** illustrates well the broad clinical spectrum in FHM in four families carrying the FHM1 R1347Q mutation. R1347Q mutation carriers can suffer from pure FHM or from FHM with slowly progressive ataxia. Notably, attacks can be triggered by head trauma and be accompanied by altered consciousness in some. In addition, focal seizures may occur (with or without secondary generalization) during or independent of hemiplegic migraine attacks. The study also established R1347Q as the fourth recurrent FHM1 mutation, next to T666M,[1,4-8] R583Q[8-12] and S218L.[13-15] **Chapter 3** shows that screening of FHM genes is also worthwhile in patients with symptoms that are less typical for FHM. A monozygotic twin pair was shown to carry a novel *de novo* missense V1696L mutation in *CACNA1A*. The mutation causes an interesting overlap syndrome between FHM and alternating hemiplegia of childhood (AHC)[16]; an early-onset severe neurological disorder characterized by episodes of alternating hemiplegia or quadriplegia and progressive neurological features beginning before the age of 18 months. The study emphasizes that FHM and AHC may share pathophysiological mechanisms that involve calcium channel dysfunction. Previously, screening of FHM1, -2 and -3 genes[17,18] (*SCN1A* unpublished data) revealed no mutations in typical AHC patients. However, recently some 70% of typical AHC patients were shown to have a heterozygous *de novo* missense mutation in the *ATP1A3* gene[19,20] that belongs to the same family as the FHM2 *ATP1A2* gene, implicating indeed related pathophysiological mechanisms between FHM and AHC.

In **chapter 4** the severe end of the FHM disease spectrum was investigated in patients with the *CACNA1A* S218L missense mutation, which exhibit a particularly complex phenotype that is best characterized as 'early seizures and cerebral edema after trivial head trauma', which we consequently termed ESCEATHT. The combination of symptoms was recognized in two S218L patients. By reviewing 11 additional S218L mutation carriers from literature, it became clear that every mutation carrier does not exhibit all clinical features, but certainly is at risk for ESCEATHT. Consequently, clinicians need to be made aware if they see a combination of symptoms in line with ESCEATHT, even when typical FHM attacks have not occurred. Concomitantly with our study, a fourteenth patient with the S218L mutation was published confirming our hypothesis that a specific severe phenotype is indeed associated with the mutation.[13] In addition, in 2011, a fifteenth case was described linking the S218L mutation to hemiconvulsion-hemiplegia-epilepsy syndrome.[21]

Findings in the FHM2 *ATP1A2* gene

ATP1A2 encodes the α2 subunit of sodium-potassium pumps.[2] To date nearly 50 *ATP1A2* mutations have been reported, most of which are associated with pure FHM.[22,23] However, rarely, *ATP1A2* mutations are associated with for instance, cerebellar signs,[24,25] permanent mental retardation,[26] and epilepsy.[22,27] Whether these associations are coincidental or true remains to be seen. For instance, in a family in which both FHM and benign familial infantile convulsions (BFIC) segratated, an *ATP1A2* mutation was described as the causal factor for both diseases.[28] However recently it was shown that mutations in the *PRRT2* gene cause BIFC,[29] which also appeared to be the case in this family (unpublished data).

In **chapter 5** we further established the link between the *ATP1A2* gene and epilepsy by studying an FHM family in which patients carried the *ATP1A2* G855R missense mutation and had febrile seizures. The proband of this family also suffered from frequent unprovoked episodes of severe ataxia since early childhood. Whether cerebellar ataxia in FHM2 is coincidental or based on a functional effect of the underlying *ATP1A2* mutation still needs to be established.

Findings in the FHM3 *SCN1A* gene

SCN1A encodes the α1 subunit of neuronal $Na_V1.1$ voltage-gated sodium channels.[3,30] To date five FHM3 mutations have been identified. Notably, *SCN1A* is a well-known gene for childhood epilepsy, with well over 100 mutations that are associated with severe myoclonic epilepsy of infancy (SMEI) or generalized epilepsy with febrile seizures (GEFS+).[31,32] Besides, elicited repetitive daily blindness can occur in FHM3 patients with mutation Q1489H and F1499.[33,34] In contrast to the FHM1 and -2 genes, no recurrent FHM3 mutation has been reported. In **chapter 6**, for the first time, we describe a novel *SCN1A* mutation (L263V), which is associated with both FHM and epilepsy in multiple mutation carriers. This finding further expands the clinical spectrum associated with *SCN1A* mutations and strengthens molecular evidence that FHM and epilepsy share, at least in part, similar molecular pathways.

13.2 Pathophysiology of FHM gene mutations – a common pathway?

How do FHM mutations cause disease? The functional consequences of FHM1 *CACNA1A* mutations have been studied by various methodologies mainly involving electrophysiology, both in cellular models, i.e., in cell lines or cultured neurons expressing recombinant $Ca_V2.1$ channels, and in transgenic *knock-in* mouse models expressing FHM1 mutations R192Q or S218L. The R192Q mutation causes pure FHM1 without associated neurological features, whereas the S218L mutation causes severe hemiplegic migraine that can be accompanied by (sometimes fatal) brain edema (see below and **chapter 4** on ESCEATH).[35,36] The electrophysiological studies revealed that FHM1 mutations exhibit *gain-of-function* effects, namely an increased opening probability of $Ca_V2.1$ calcium channels and, thereby, an increased calcium influx through these channels[35-38] leading to enhanced neuronal activity with more cortical glutamate release.[39]

In line with the severe clinical consequences observed in S218L patients, electrophysiological studies show that particularly the S218L mutation has the largest *gain-of-function* effect (i.e., inactivation is more slow and recovery from inactivation is faster than with other FHM1 mutation).[37] Corresponding to these more extreme electrophysiological abnormalities on $Ca_V2.1$ channel function, transgenic *knock-in* mice with the S218L mutation, exhibit even a lower threshold for and a higher propagation speed of CSD waves compared with R192Q *knock-in* mice.[36,40] Notably, homozygous S218L mice exhibit a phenotype that closely resembles that in S218L patients.[15,36] Besides a transient hemiparesis after CSD events, these mice show increased mortality that is likely due to the occurrence of generalized tonic-clonic seizures, they respond more dramatic to mild head trauma, and exhibit cerebellar ataxia. The clinical similarities between patients and mice suggest that similar pathways related to an increased neuronal activity and susceptibility to CSD are the underlying cause of ESCEATHT in humans.

In contrast to FHM1, FHM2 *ATP1A2* mutations most often result in *loss-of-function* of the Na+,K^+ ATPase pump protein as was demonstrated by e.g., cell survival assays (showing partial or no cell survival in specific tests).[26] For instance, in **chapter 5** HeLa cells expressing the FHM2 G855R mutant showed a significantly reduced rate of cell survival. This indicates that the expressed mutant protein, which had been made insensitive to Na^+,K^+-ATPase activity blocker ouabain, was not able to compensate adequately for the loss of endogenous Na^+,K^+-ATPase activity, due to the action of ouabain, in the survival assay.

FHM3 *SCN1A* mutations affect the function of neuronal $Na_V1.1$ sodium channels. Electrophysiological studies of wildtype and mutant $Na_V1.1$ sodium channels showed divergent effects.[41] Two mutations, i.e., Q1489K, and L1649Q, which are associated with pure FHM in patients, showed clear *loss-of-function* effects, whereas FHM3 missense mutation L263V, which is associated with FHM and generalized tonic-clonic epilepsy in a number of mutation carriers (see **chapter 6**) exhibited *gain-of-function* features on $Na_V1.1$ channel functioning. It was hypothesized that loss of $Na_v1.1$ channel activity primarily disturbs the functioning of inhibitory neurons, where the $Na_v1.1$ channels normally are expressed[42,43] whereas gain of activity might have its predominant effect on excitatory neurons. This divergent behavior was not reported for mutations in $Na_V1.1$ channels linked to childhood epilepsy, although this has not been investigated thoroughly.

The study of the functional consequences of FHM genes has been crucial for furthering insight in the pathophysiology of FHM, and migraine in general. FHM1 mutations lead to increased neuronal calcium influx and concomitant increase of neurotransmitters, for instance glutamate in cortical neurons. Mutations in the FHM2 sodium potassium pump gene predict a reduced re-uptake of K^+ and, as a consequence, less uptake of glutamate from the synaptic cleft into glia cells. Mutations in the FHM3 sodium channel gene may also result in hyperxcitability *in vivo*, most likely due to an imbalance of excitatory and inhibitory neurotransmitter release. Thus, FHM mutations all convert to a mechanism of increased cerebral levels of K^+ and glutamate in the synaptic cleft, which would increase neuronal excitability, and thereby can explain the increased susceptibility to CSD. CSD plays an important role in initiating aura symptoms,[44] and, at least in experimental animal models, can activate the TGVS,[45] although it is unclear whether this occurs in humans.

Functional studies may increase insight in pathways that can explain the association of FHM with other diseases, such as in ESCEATHT (as already discussed), epilepsy and ataxia. Like FHM, idiopathic monogenic forms of epilepsy, often result from mutations in voltage-gated ion channels, such as those for sodium, potassium and chloride.[46] Spreading depolarizations occur in epilepsy.[47] Based on this, comorbidity of FHM and epilepsy likely can be explained by the fact that both diseases result from a disturbed balance between excitatory and inhibitory (cortical) mechanisms, which is, at least in a proportion of patients, related to ion channel dysfunction. Overlapping mechanisms may also explain the therapeutic overlap between these diseases. Indeed, for some of the currently available prophylactic migraine treatments that are originally prescribed as anti-epileptic drugs (e.g., valproate and topiramate), experimental evidence in rats has shown that chronic daily, but not acute, administration dose and duration-dependently suppressed KCl-induced CSD frequency by 40–80%, and increased the triggering threshold for inducing CSD.[48]

Why a proportion of FHM1 patients show slowly progressive ataxia is at present unclear. In FHM1 patients that show ataxia, cerebellar atrophy can be seen on MRI and in homozygous S218L mice, which have an ataxic phenotype, alterations in Purkinje cell morphology have been found. It has been suggested, that these morphological alterations may result from changes in intracellular calcium concentration and lead to a disturbed firing pattern of Purkinje cells causing ataxia.[36]

The knowledge of functional consequences could serve as a starting point in the development of new migraine therapies. For example, a drug that shifts the activation of $Ca_v2.1$ channels to more depolarized voltages may inhibit CSD and thereby prevent or abort migraine attacks. In line with the central role of increased release of glutamate in FHM patients and mouse models, a glutamate receptor antagonist may reduce severity and duration of aura symptoms and perhaps headache. Indeed, Ketamine, an N-methyl-D-aspartate receptor antagonist, was shown to reduce severity and duration of aura symptoms in FHM patients,[49] but more effective drugs with less side effects are needed.[50]

13.3 FHM as a model for common migraine

An important reason to study a rare migraine subtype such as FHM is to ultimately be able to translate findings to more common forms of migraine. Clinical similarities suggest that

shared pathophysiological mechanisms may underlie both types of migraine, although there is debate to what extent this is the case. For instance, several genetic studies investigated the role of FHM1 and -2 genes in the common forms of migraine, with inconclusive results; that is they reported some evidence for the involvement of *CACNA1A* [51-53] or *ATPA1A2*[54], while others found no such evidence.[55-57] Admittedly, many of these studies were severely underpowered. Still, a large candidate gene study investigating over 150 ion transporter genes in a large cohort of migraine patients provided no evidence for a major involvement in migraine susceptibility.[58] Recent genome-wide association studies (GWAS) reproduced these genetic data as there are many SNPs that cover the FHM1-3 gene regions that did not reveal significant association with common migraine.[59-62] It shows that genes in rare monogenic and common multifactorial forms of migraine may differ, but this does not imply that the central conclusion of a key role of for instance glutamate, does not apply to common migraine. The fact that the GWAS studies surfaced several genes (i.e., *PGCP, MTDH, LRP1*) that are involved in glutamate signaling, indicates that there may indeed be shared molecular disease pathways in common migraine and FHM.

In a Danish study, two known triggers for migraine without aura, i.e., CGRP (calcitonin gene-related peptide) and GTN (glyceryl trinitrate; an nitric oxide donor), failed to induce more migraine aura or headaches in FHM1 and -2 patients compared to controls. This result was claimed as argument against such shared pathophysiology.[63-65] However, given the fact that nitric oxide *prevents* CSD events, a lack of a clear effect of GTN in FHM patients (in which CSD is very prominent and likely the main trigger of attacks), is not surprising.[66] It merely shows that the triggering paradigm (CGRP and GTN administration) has different effects in common migraine and FHM.

A role for ion channel involvement in common migraine, namely familial migraine with aura, came from a study by Lafreniere and co-workers, who reported an inactivating truncating mutation in TRESK (a TWIK-related spinal cord potassium channel that is encoded by the *KCNK18* gene).[67] However, a successive publication of the same group showed that heterozygous inactivating TRESK mutations also occur in control subjects, casting serious doubt on their original claim of *causality* of a TRESK mutation in familial migraine with aura.[68]

PART II: CHARIOT/RVCL: A MONOGENIC MIGRAINE-ASSOCIATED SYNDROME

13.4 The clinical and genetic spectrum of CHARIOT/RVCL

Migraine can also be part of the clinical spectrum of certain monogenic diseases, which provides unique opportunities to unravel shared pathological molecular mechanisms. The clearest example is the monogenic small vessel disease CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy), where migraine with aura, but not without aura, is present in about 20-40 % of mutation carriers, often as the presenting clinical symptom.[69] A hypothesis for the increased prevalence of migraine with aura in CADASIL could be that vascular changes make the cortex more susceptible to CSD, thereby leading to migraine with aura. This hypothesis is strengthened by the fact that a transgenic *Notch3* mouse model has an increased susceptibility to CSD.[70]

A second example of a monogenic migraine-associated syndrome is cerebral hereditary angiopathy with vascular retinopathy and impaired organ function caused by *TREX1* mutations (CHARIOT). What is now called CHARIOT was originally described in three families under different disease names and abbreviations (i.e., cerebroretinal vasculopathy (CRV)[71], hereditary vascular retinopathy (HVR)[72] and hereditary endotheliopathy, retinopathy, nephropathy and stroke (HERNS).[73] The disease loci were mapped to a sinlge locus on chromosome 3p21.1-p21.3.[74] In **chapter 8** we describe the identification of the disease gene *TREX1,* that encodes the major mammalian 3′-5′ exonuclease trex1, with disease-causing mutations that result in premature truncation of the trex1 protein. At the time of the gene discovery, the disease was termed Retinal Vasculopathy with Cerebral Leukodystrophy (RVCL), based on limited phenotypic knowledge of the disease. Moreover, previous separate clinical descriptions suggested phenotypic variability between families and *TREX1* mutations. For example, stroke en nephropathy were thought to be specific for HERNS[73] and the development of cerebral mass lesions in the end stage of the disease were not described for HVR.[72] Therefore, in **chapter 8** we described the clinical, radiological and pathological data of eleven RVCL families with five different mutations in the *TREX1* gene (in total 78 mutation carriers were investigated). Unlike previously suggested clinical variability, our study shows remarkably shared phenotypic characteristics between different RVCL mutations and between families. Based on our data, updated diagnostic criteria were composed. In addition, because we found no evidence for leukodystrophy, we renamed the disease CHARIOT. Diagnosis of CHARIOT should be considered in families with unexplained autosomal dominant progressive vascular retinopathy accompanied by contrast-enhancing white matter cerebral mass lesions. Signs and symptoms of (mild) liver and/or kidney impairment and white matter hyperintensities can be supportive. In addition migraine, Raynaud's phenomenon, anemia and hypertension can be present.

As CHARIOT can present with retinal, cerebral, and systemic symptoms a primary consult of patients may be for different medical disciplines, such as ophthalmology, rheumatology, internal medicine and neurology. In the literature several other families with diseases that resemble CHARIOT are reported, i.e., cases with a retinopathy with cerebral mass lesions and systemic involvement. Of them, only the family reported by Niedermeyer et al.[75,76] was screened for *TREX1* mutations, but no mutation was identified, suggesting that there may be genetic heterogeneity. The disease described differs from CHARIOT in the sense that it seems to have a recessive inheritance and the two patients described also had abnormalities characteristic of Fanconi's anemia. Families reported by Gutmann and Winkler[77,78] might also be linked to the *TREX1* gene, unfortunately, no DNA is available for testing this hypothesis.

13.5 RVCL/CHARIOT as a model for migraine?

In RVCL/CHARIOT, the pathophysiological process causes a vasculopathy that affects the integrity of cerebral and systemic small vessels. The mechanism by which this vasculopathy can increase the risk of migraine is unknown. Several explanations can be put forward, such as spurious association, a shared genetic factor, or an increased risk for CSD or endothelial dysfunction; some of which may partly overlap.

Spurious

We observed a migraine prevalence of 59% among *TREX1* mutation carriers (**chapter 9**). This is more than three times higher than what is usually reported as lifetime prevalence of migraine in the Western general population using the same criteria.[79] Even assuming that none of the 35 mutation carriers, for whom no reliable information on their migraine status is available, had migraine, the migraine prevalence in RVCL/CHARIOT would still be 37%, which is still twofold higher compared with the general population. In addition, except for mutation T236fs, migraine occurred in association with every *TREX1* mutation. Therefore a spurious association seems an unlikely explanation for the co-occurrence of RVCL/CHARIOT and migraine.

Shared genetic factor

The co-occurrence with migraine may be causally related to *TREX1* mutations. In this scenario, the gene may be considered to increase the susceptibility for migraine (i.e., serve as genetic modifier). A genetic, family-based, association study demonstrated that the RVCL/CHARIOT locus indeed slightly enhances the susceptibility for migraine in a large Dutch family.[80] On the other hand, in a genetic association study with 5 polymorphisms that cover the *TREX1* gene region using two Dutch samples from the general population (i.e., the Genetic Epidemiology of Migraine study n=860 and the Erasmus Rucphen Family study n=360 cases) no major role of common *TREX1* variants was found (unpublished data). In any case, other risk factors must be involved as well.

Vascular: endothelial dysfunction

There is evidence that migraine attacks are associated with endothelial dysfunction.[81-83] Migraine prevalence seems increased in persons with polymorphisms linked to endothelial function[84-90] and associated with elevated markers of endothelial activation.[91] In **chapter 10** we showed that, in contrast to CADASIL, RVCL/CHARIOT patients have a clear dysfunctional endothelial homeostasis, this may add to the increased risk of migraine. This may also provide a biological link between migraine and the increased cardiovascular risk found in migraine patients.

Neuronal

CSD is thought to be the underlying mechanism of the migraine aura. As RVCL patients mainly suffer from migraine without aura an increased CSD susceptibility causing migraine in RVCL/CHARIOT patients seems less likely.

PART III: MIGRAINE AND COMORBIDITY IN A GENETIC ISOLATE

The study of comorbidity may provide epidemiological or biological novel insights in mechanisms involved in migraine. Various conditions are reported to be comorbid with migraine. Among these are psychiatric disorders (depression, anxiety, bipolar disorder), neurological disorders (epilepsy), vascular disorders (Raynaud's phenomenon, ischemic and hemorrhagic stroke), heart disease (patent foramen ovale (PFO), coronary heart disease) and

others such as asthma and systemic lupus erythematosus (SLE) and comorbid pain disorders (for review see Le et al., 2011[92] and Scher et al., 2005[93]) Some of the reported associations show conflicting results. Conditions that have shown a consistent positive association with migraine include are stroke,[94] depression,[95] and epilepsy.[96]

13.6 Migraine and depression

In **chapter 11** of this thesis we studied the comorbidity of migraine and depression in a genetically isolated population in the southwestern region of the Netherlands. Findings of a bidirectional influence between migraine and depression suggest common neurobiological mechanisms.[95,97-98] There is some evidence of involvement of *similar neurotransmitters* in migraine and depression, such as serotonine, dopamine and glutamate.[99-101] Also, *stress-related mechanisms* have been implicated in both disorders.[102] Finally, the increased prevalence of migraine and depression in women indicates a role for *hormonal* factors in both disorders.[103]

In ERF we identified 360 migraine cases: 209 had migraine without aura (MO) and 151 had migraine with aura (MA). Odds ratios for depression in patients with migraine were 1.29 (95% confidence interval [CI] 0.98-1.70) for MO and 1.70 (95% CI 1.28-2.24) for MA. Heritability estimates were significant for all migraine (0.56), MO (0.77), and MA (0.96).

As overlapping pathophysiological pathways have been suggested for migraine and depression, this would imply that DNA variants involved in these pathways may confer an increased risk to develop both of these diseases. In our study we found some evidence for the existence of shared genetic factors between both traits. That is heritability estimates for migraine decreased after adjustment for symptoms of depression or use of antidepressant medication, in particular for MA. Comparison of the heritability scores for depression between patients with migraine and controls showed a genetic correlation between HADS-D score and MA. This knowledge can be of importance as migraine and depression might be considered an endophenotype, which could facilitate gene identification. The general idea is that an endophenotype may be more robust and uniform than the original trait (i.e., migraine) itself, and perhaps may even reduce locus heterogeneity. This strategy has proven successful in the gene identification of schizophrenia and bipolar disorder.[104,105]

The suggestion of shared genetic factors in migraine and depression that came from our study needs to be interpreted with caution. First, our heritability for migraine with aura is rather high and exceeds prior estimates. Second, confidence intervals in our analyses are very brought, leading to, at best, only marginally significant observations. This is perhaps inevitable, even in a very large sample such as ERF with some 3000 study subject, when analyzing a binary trait (with only 330 cases). Statistical uncertainty might limit the conclusion on the small reduction of heritability after correction for depression. Thus our results clearly should be confirmed in other studies.

Two other twin studies indeed confirmed our results. In parallel with our study, a study of Schur et al estimated that 20% of variability in depression and migraine is due to shared genetic factors.[106] Also a study from Ligthart et al., showed an increased genetic correlation between anxious depression and migraine (*r*G 0.30).[107]

Having statistically established that there are indeed shared genetic factors an important question is *how to identify these factors?* Several approaches can be put forward. One approach is to identify genomic regions with linkage analysis in migraine families and explore positive genomic regions in patients with migraine and depression, or the other way around. Alternatively, an association study can be performed with cases with migraine and depression versus controls, where depression may also be used as a quantitative trait. Two studies did a first attempt to actually identify shared risk variants for migraine and bipolar disease,[108,109] but no such studies were performed with depression thus far.

13.7 Migraine and cardiovascular disease

The association between migraine and cardiovascular disease, including ischemic stroke and subclinical brain lesions is well established for migraine with aura.[110,111] The mechanism for this association, however, is less clear. In **chapter 12** we investigated whether cardiovascular risk factors and atherosclerosis may be involved, but find no evidence for this. The jury is still out on whether this is indeed true as there is discrepancy with other studies.[81,112] Other explanations and mechanisms include CSD, vascular changes that predispose to both migraine and stroke (endothelial dysfunction), migraine-specific medication, or PFO. The latter may predispose to microemboli in the cranial circulation which may lead to triggering of CSD. Notably, in an experimental rat model it was shown that emboli can trigger CSD without causing actual visible infarcts on MRI.[113]

Shared genetic factors between migraine and stroke may also play a role. Indications come from monogenic migraine syndromes that predispose to stroke, such as CADASIL. Another example is MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes)[114] in which stroke-like events are associated with migrainous headache.

Further indications for a shared underlying genetic susceptibility may come from genes that have been implicated to play a role in stroke *and* migraine, including angiotensin converting enzyme (ACE)[115,116] and genes related to endothelial function[117,118] and homocysteine metabolism.[87,115, 119,120] For example the 677C>T variant in the 5',10'-methylenetetrahydrofolate reductase (MTHFR) gene has been investigated for stroke and migraine. The T allele impairs enzyme activity and carriers have modestly increased levels of homocysteine.[121] In a meta-analysis the MTHFR 677TT genotype was associated with a modestly increased risk for ischemic stroke.[122] Similarly, the T allele has been associated to migraine with aura, which was confirmed in two meta-analysis,[115,123] but negative findings have also been reported.[119] Kurth et al. studied the mediating effect of stroke on the association between migraine and MTHFR T allele in the Women's Health Study (WHS). In contrast to other studies, a protective effect of the TT genotype for migraine with aura was observed, however in patients with migraine with aura and the TT genotype an increased risk of ischemic stroke was observed. This might implicate that the MTHFR 677TT genotype is a marker for an increased risk of ischemic stroke in patients with migraine with aura.[120] Similarly, a modulatory effect of the ACE D/I polymorphism was found in the migraine-stroke association in women.[116] Also a GWAS study was published, that provides suggestive evidence for a shared genetic etiology of migraine and ischemic stroke. In women with migraine two SNPs located in the MEPE (matrix extracellular

phosphoglycoprotein) and IRX (iroquois homeobox protein 4) gene associated with ischemic stroke.[124] The function of these variants with regard to the risk of cardiovascular disease is unknown. However no independent replication was performed for the initial association signal so results should be interpreted with caution.

13.8 Future perspectives

The clinical and genetic spectrum of FHM was explored in this thesis. As detailed knowledge on this spectrum is now available, sequence analysis of known FHM genes remains mainly relevant for genetic diagnosis. As not all FHM families are linked to FHM1-3, it is still worthwhile identifying additional FHM genes as they can provide novel and valuable insights in the pathogenesis of FHM. Therefore, the collection of novel - and extension of existing - FHM families remains important. When clinical material is available, identification of genes will be relatively 'effortless' compared to the past with currently available dense SNP arrays designed for linkage studies and the *"Next Generation Sequencing"* technology which allows for high-throughput sequencing in increasingly large samples.

Transgenic mouse models can be used as tools to study the functional consequences of mutated FHM genes *in vivo*, which may refine genotype-phenotype relations and may increase our understanding of the actions of anti-migraine drugs. These studies can be complemented by investigating biochemical compounds in Cerebrospinal Fluid (CSF) and/or with MR brain spectroscopy (MRS) of FHM and migraine patients to correlate genetic findings to biochemical changes.

The identification of the *TREX1* gene for RVCL/CHARIOT opened new routes to obtain insight in the syndrome itself and in its relation with migraine. Future investigations of progression of the disease by detailed follow-up of mutation carriers will be important. How vascular changes in RVCL/CHARIOT arise is an unanswered question, but endothelial dysfunction may be involved, as was apparent from studies in **chapter 10**. Further studies of endothelial function may include assessment of markers for endothelial function in blood and CSF, and assessment of endothelial function in cerebro by functional imaging. On a molecular level a developed Trex1 antibody could be used in immunohistochemical and electron microscopy studies, which may shed more light on the exact location of (mutated) Trex1 in cells and tissues. In a recently generated RVCL/CHARIOT *Trex1* knock-in mouse model the functional consequences of mutated Trex1 can be assessed *in vivo*. Together these initiatives hopefully contribute to the development of a treatment option for this devastating disease.

In recent years, many GWAS have been performed for complex neurological disorders,[125,126] and identification of common genetic variants for migraine has also been successful.[59-62] Most identified loci (12 in total) are located in or immerdialtely outside genes with a known function in synaptic or neuronal regulation, pain pathways and some interact with each other. Further functional studies of identified variants and correlation with endophenotypes will be of great importance to translate the genetic findings to detailed pathways and potential treatment options for patients. It is unlikely that common genetic variability entirely explains the heritability of migraine and in the coming years much is expected from exome and whole genome sequencing, as these techniques could enable the identification low frequency

intermediate and rare high risk disease alleles.[127] Finally, it is expected that a large proportion of heritability of migraine may not be accounted for by the (combined) effect of identified genomic variants, but that epigenetic, post-genomic and/or regulatory events are also involved.[128] It will be a future challenge to capture these factors.[127]

The efficient web-based diagnostic process for migraine and evolving, interactive, biobank LUMINA (Leiden University MIgraine Neuro-Analysis program),[129] that combines clinical material, DNA and CSF and imaging data of migraine patients, will be of great relevance for future large-scale multidisciplinairy studies. Whether diagnostic categories defined in clinical practice have a common underlying genetic cause remains to be seen. Yet (unknown) endophenotypes may exist which are composed of a combination of disease features, as was suggested for migraine and depression in this thesis. Therefore, collecting data on comorbidity will remain a potentially very rewarding research activity.

In conclusion, the studies in this thesis improved our understanding of migraine and comorbid conditions and provide examples of how migraine syndromes and comorbidity can be used as a tool to learn more about the genetic factors involved in migraine. Together these findings contribute to a better understanding on how migraine attacks start. It remains vital to finally translate molecular genetic findings to novel treatment options for migraine patients.

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ADDENDUM

SUMMARY

In this thesis genetic aspects of migraine and related migraine syndromes were investigated. The research can be divided in three parts: the study of syndromes associated with Familial Hemiplegic Migraine (FHM), a monogenic variant of migraine *(***Part I***)*; the study of a migraine-associated syndrome termed Retinal Vasculopathy with Cerebral Leukodystrophy (RVCL; later renamed CHARIOT) *(***Part II***)*; and the study of migraine and comorbidity in a Dutch genetic isolate *(***Part III***)*.

In *part one*, clinical genetic studies on FHM are described. FHM can present as pure FHM (only hemiplegic migraine attacks), or be accompanied by additional clinical features. Further insight in the associated clinical symptoms of FHM1 (caused by mutations in the *CACNA1A* gene) came from a study of four families with the R1347Q mutation (**Chapter 2)**. The study reinforces the wide clinical spectrum of FHM1 including (trauma-triggered) hemiplegic migraine with and without ataxia, loss of consciousness and epilepsy. A direct implication of the study is that genetic screening in hemiplegic migraine patients should focus on screening for recurrent mutations, such as R1347Q, first.

In **Chapter 3** a monozygotic twin is described with clinical features of AHC (alternating hemiplegia of childhood) and FHM, which lead to difficulties in the differential diagnosis. By identifying a novel *de novo* mutation it became evident that FHM and *atypical* AHC may share pathophysiological mechanism that involves calcium channel dysfunction. It builds on a previous observation that a family with atypical AHC was shown to have an FHM2 (i.e., *ATP1A2*) mutation. Recently it was shown that the majority of AHC patients have de novo missense mutations in the related *ATP1A3* gene.

The clinical consequences of the S218L mutation in the *CACNA1A* gene were investigated in **Chapter 4**. *CACNA1A* S218L mutation can cause "early seizures and cerebral edema after trivial head trauma" (ESCEATHT), at least in three of thirteen mutation carriers. These clinical features are at the extreme severe end of the FHM disease spectrum. Cerebellar ataxic symptoms and hemiplegia, the latter often being triggered by trivial head trauma, were present in the far majority of cases; seizures were reported in seven patients. The importance of the study is that *all* S218L mutation carriers are at *risk* for developing ESCEATHT with its devastating consequences. In knock-in mice the S218L mutation increases the propensity for CSD and increases the risk of epilepsy and mild head trauma-triggered edema. This indicates that the same pathway, i.e., increased susceptibility for CSD, may cause the ESCEATHT phenotype in humans.

In **Chapter 5** an *ATP1A2* mutation is described that causes FHM2 associated with febrile seizures. This study strengthens the concept of shared molecular pathways in migraine and epilepsy. Additional support for this link comes from the study described in **Chapter 6.** In the well-known "epilepsy gene" *SCN1A*, a third FHM3 mutation (i.e., L263V) was found in a Portuguese family, in which three out of five mutation carriers exhibited generalized epilepsy, in addition to FHM. The findings support the association of a wide continuum of episodic brain disorders with this gene.

In **chapter 7** the role of the *SCL1A3* gene, encoding the glutamate transporter EAAT1, in episodic ataxia (EA) families without a *CACNA1A* gene mutation, is explored. The EAAT1 protein is responsible for removal of glutamate from the synaptic cleft and herewith is involved in the same pathway as the FHM1-3 genes. Reason to perfom this study was given by publication of a sporadic patient with a P290R mutation in *SCL1A3* with severe episodic and progressive ataxia, seizures, alternating hemiplegia and migraine headache. A missense C186S mutation was identified in one of 20 tested EA probands. This mutation segregated in three affected family members. Clinical symptoms in these patients were much milder compared to the previously reported sporadic patient. This milder phenotype could be explained by functional analysis of the mutation, which showed only a modest dysfunctional glutamate transporter compared to the P290R mutation.

In *part two*, genetic and clinical studies on *TREX1*, the gene for Retinal Vasculopathy with Cerebral Leukodystrophy (RVCL) are described. The original three published RVCL families were known under different names: CerebroRetinal Vasculopathy (CRV), Hereditary Vascular Retinopathy (HVR), and Hereditary Endotheliopathy, Retinopathy, Nephropathy and Stroke (HERNS). The identification of *TREX1* mutations in all of them, indicated that the original names were reflections of the broad clinical spectrum of the RVCL syndrome (**Chapter 8**). RVCL mutations in the *TREX1* gene, encoding a 3'-5'-exonuclease, result in C-terminal truncated proteins that retain their exonuclease activity, but lose their normal perinuclear localization. The clinical spectrum of 78 mutation carriers of eleven RVCL families carrying five different *TREX1* mutations is presented in **Chapter 9**. RVCL was renamed CHARIOT (Cerebral Hereditary Angiopathy with vascular Retinopathy and Impaired Organ Function caused by *TREX1* Mutations) because this acronym better reflects the clinical spectrum of the disease and because leukodystrophy (indicated by the "L" in RVCL) is not the right term for the radiological and pathological cerebral abnormalities seen in this syndrome. It was demonstrated that the CHARIOT syndrome consisted of a broad range of clinical manifestations, without an apparent genotype-phenotype correlation, that include vascular retinopathy, focal neurological symptoms, cognitive and psychiatric complaints, migraine (mainly without aura) and systemic symptoms (renal and liver impairment, Raynaud's phenomenon). Neuroradiological abnormalities include punctate white matter lesions, contrast-enhancing intracerebral mass lesions and calcifications. Histopathology revealed a stenotic vasculopathy mainly in the brain and retina. In addition basement membrane abnormalities in the small vessels of the cerebral white matter and other organs were found, however the significance of this finding is unclear. How mutated truncated TREX1 causes disease is unknown. In **chapter 10**, we investigated vascular properties and endothelial function in CHARIOT, compared to controls and patients with a second neurovascular migraine syndrome CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy). The latter syndrome is pathologically characterized by degeneration of arteriolar vascular smooth muscle cells, which in CHARIOT is only marginally present. From the study it became clear that CHARIOT patients have endothelial function in conduit arteries, in contrast to CADASIL where vascular impairment seems to be due to defect in smooth muscle cell functioning in resistance arteries. Because of the overlapping clinical features, CHARIOT may serve a model for various common neurovascular disorders, such as vascular dementia and migraine.

The *third part* investigates comorbidity of common migraine with other diseases and describes how comorbidity may be helpful for genetic studies of migraine. **Chapter 11** describes the identification of migraine cases in a genetically isolated Dutch population; the Erasmus Rucphen Family (ERF) study. Genetic isolates may help to identify new genes because of reduced genetic and clinical heterogeneity. The use of endophenotypes may further reduce heterogeneity as it will result in more robust phenotypes.

In **Chapter 11** the co-occurrence of migraine and depression in ERF was investigated. There is a bidirectional association between migraine and depression which suggest a common etiology. Therefore it was also assessed whether shared genetic factors may underlie both diseases. In total, 360 migraine cases were ascertained: 209 of them had migraine without aura (MO), 151 had migraine with aura (MA). Odds ratios (OR) for depression in migraine patients were significantly increased, in particular for migraine with aura. Heritability estimates in migraine (subtypes) were significant and decreased after adjustment for symptoms of depression or use of antidepressant medication, in particular for MA. This confirms migraine to be a highly genetic disorder and indirectly that the bidirectional association between depression and migraine (and particularly in MA), at least in part, can be explained by shared genetic factors.

Migraine patients have an increased risk for ischemic stroke en possible also for other major cardiovascular diseases. This could be explained by a predisposition to atherosclerosis caused by an unfavorable cardiovascular risk profile. Therefore, in **chapter 12** various markers for atherosclerosis (i.e., Pulse Wave Velocity, Intima Media Thickness, and Ankle Brachial Index) were assessed in migraine patients and controls from the ERF study. It was concluded that migraineurs have no increased risk for central or peripheral atherosclerosis, making it an unlikely explanation for the relation between migraine and ischemic cardiovascular events. Other suggested mechanisms, such as a general endothelial dysfunction, increased susceptibility to CSD or a shared genetic susceptibility, should be considered.

Chapter 13 places clinical and genetic findings from the previous chapters in a broader perspective. Pathophysiological mechanisms for the various migraine syndromes and its value as a model for common migraine are discussed, as well as the potential for comorbidity of common migraine (endophenotypes) in the identification of novel migraine genes and pathways.

NEDERLANDSE SAMENVATTING

Dit proefschrift onderzoekt genetische aspecten van migraine en gerelateerde migraine syndromen. Het onderzoek is opgedeeld in drie delen: onderzoek naar syndromen geassocieerd met Familiaire Hemiplegische Migraine (FHM), een monogenetische migraine variant (**deel 1**); onderzoek naar een syndroom gepaard gaande met migraine genaamd Retinale Vasculopathie met Cerebrale Leukodystrofie (RVCL, later genoemd CHARIOT) (**deel 2**); en onderzoek naar migraine en comorbiditeit in een Nederlands genetische isolaat (**deel 3**).

Deel 1 behandelt klinisch genetisch onderzoek naar FHM. FHM kan zich uiten als puur FHM (alleen aanvallen van hemiplegische migraine) of kan gepaard gaan met bijkomende klinische verschijnselen. Meer inzicht in de klinische symptomen die samengaan met FHM1 (veroorzaakt door mutaties in het *CACNA1A*-gen) werd verkregen door bestudering van vier families met de mutatie R1347Q (**hoofdstuk 2**). De studie benadrukt het brede klinisch spectrum van FHM1, inclusief (door hoofdtrauma uitgelokte) aanvallen van hemiplegische migraine met of zonder ataxie, stoornis in het bewustzijn en epilepsie. Een directe implicatie van deze studie is dat bij genetische screening van hemiplegische migraine patiënten eerst gescreend moet worden op terugkerende, zogenoemde *recurrent* mutaties, zoals R1347Q, alvorens het gehele gen te screenen.

In **hoofdstuk 3** wordt een monozygote tweeling beschreven met een combinatie van klinische verschijnselen van AHC (alternerende hemiplegie op de kinderleeftijd) en FHM, waardoor de differentiële diagnostiek lastig was. Door identificatie van een nieuwe *de novo*-mutatie werd duidelijk dat FHM en deze atypische AHC een gedeeld pathofysiologisch mechanisme lijken te hebben waarbij dysfunctie van calciumkanalen een rol speelt. Het onderzoek borduurt voort op voorgaand onderzoek waarbij een familie met atypische AHC een FHM2-(*ATP1A2*-)mutatie bleek te hebben. Recent werd aangetoond dat de meerderheid van AHC patiënten mutaties in het, aan *ATP1A2* verwante, *ATP1A3*-gen hebben.

De klinische consequenties van de S218L-mutatie in het *CACNA1A*-gen worden onderzocht in **hoofdstuk 4.** De *CACNA1A*-S218L-mutatie veroorzaakte in tenminste 3 van de 13 mutatiedragers "vroege convulsies en cerebraal oedeem na triviaal hoofd trauma" (ESCEATHT). Deze klinische kenmerken vormen de meest ernstige variant van van het FHM-ziektespectrum. In de meerderheid van de gevallen was cerebellaire ataxie en hemiplegie aanwezig, dit laatste vaak uitgelokt door triviaal hoofdtrauma; in zeven patiënten bleken convulsies voor te komen. Het belang van de studie is dat alle S218L-mutatie dragers risico hebben op de ontwikkeling van ESCEATHT met uitermate ernstige consequenties. *cacna1a*-S218L-knock-in-muizen hebben een verhoogde gevoeligheid voor CSD (cortical spreading depression) en een verhoogd risico op epilepsie en cerebraal oedeem uitgelokt door hoofdtrauma. Dit duidt erop dat eenzelfde mechanisme, nl. een verhoogde gevoeligheid voor CSD, ESCEATHT kan veroorzaken in mensen.

Hoofdstuk 5 spits zich toe op een *ATP1A2*-mutatie die FHM2 en koortsconvulsies veroorzaakt. Dit onderzoek draagt bewijs aan voor het concept van gedeelde moleculaire mechanismen tussen migraine en epilepsie. In **hoofdstuk 6** wordt de link tussen migraine en epilepsie verder bekrachtigd. In het *SCN1A* 'epilepsie' gen werd een derde FHM3 mutatie

(i.e., L283V) gevonden in een Portugese familie, waarin 3 van de 5 mutatie dragers, naast FHM ook gegeneraliseerde epilepsie aanvallen hadden. De bevindingen bevestigen dat een breed continuüm van episodische aandoeningen met dit gen geassocieerd zijn.

In **hoofdstuk 7** wordt de rol onderzocht van het *SCL1A3*-gen bij episodische ataxie (EA) families die geen mutatie hebben in het *CACNA1A*-gen. Het *SCL1A3*-gen codeert voor de glutamaat transporter EAAT1. Dit EAAT1-eiwit is verantwoordelijk voor de verwijdering van glutamaat uit de synaptische spleet en past daarmee in eenzelfde mechanisme als de drie FHM-genen. Aanleiding tot de studie was publicatie van een sporadische patiënt met een P290R mutatie in *SCL1A3* met ernstige episodische en progressieve ataxie, convulsies, alternerende hemiplegie en migraineuse hoofdpijn. Een missense C186S mutatie werd geïdentificeerd in 1 van de 20 geteste episodische ataxie patiënten. Deze segregeerde in drie familieleden met EA. De klinische symptomen in deze patiënten zijn veel milder dan die van de eerder gerapporteerde sporadische patiënt. Functionele analyse van de P290R mutatie liet een beperktere dysfunctie van de glutamaattransporter zien, dan de eerder beschreven P290R-mutatie, correlerend met de mildere klinische verschijnselen.

Deel 2 behandelt genetisch en klinisch onderzoek naar *TREX1*, het gen voor retinale vasculopathie met cerebrale leukodystrofie. Eerder gepubliceerde RVCL families waren bekend onder verschillende ziekte namen: CerebroRetinale Vasculopathie (CRV), Hereditaire Vasculaire Retinopathie (HVR) en Hereditaire endotheliopathie,Retinopathie, Nefropathie en Beroerte (HERNS). De identificatie van *TREX1* mutaties in deze drie families liet zien dat de originele ziekte namen het brede klinische spectrum geassocieerd met het RVCL syndroom reflecteerden (**hoofdstuk 8**). RVCL-mutaties in het *TREX*-gen, coderend voor het 3'-5' exonuclease, resulteren in C-terminaal getrunceerde eiwitten die hun exonuclease activiteit behouden, maar hun normale perinucleaire lokalisatie verliezen. In **hoofdstuk 9** wordt het klinisch spectrum van 78 mutatiedragers uit 11 RVCL families onderzocht. RVCL werd CHARIOT (cerebrale erfelijke angiopathie met vasculaire retinopathie en disfunctionerende andere organen veroorzaakt door *TREX1* gen mutaties) genoemd omdat dit acronym een betere reflectie geeft van het klinisch spectrum van deze aandoening en omdat leukodystrofie (de "L" in RVCL) niet de juiste beschrijving geeft van de afwijkingen die gezien worden bij beeldvorming en pathologische anatomisch onderzoek van de hersenen. Er werd aangetoond dat het RVCL syndroom bestaat uit een breed spectrum van klinische manifestaties, zonder een duidelijke genotype phenotype correlatie, namelijk vasculaire retinopathie, focaal neurologische symptomen, cognitieve en psychiatrische klachten, migraine (grotendeels zonder aura) en systemische symptomen (nierfunctie stoornissen, verhoging van leverenzymen, het fenomeen van Raynaud). Neuroradiologische afwijkingen bestaan onder andere uit witte stof laesies, intracerebrale ruimte innemende aankleurende processen en calcificaties. Histopathologisch onderzoek laat een stenoserende vasculopathie zien, met name in de hersenen en retina. Tevens wordt een afwijkende basaal membraan in vaten in het brein maar ook in andere organen (waaronder de nier) gevonden, echter de betekenis hiervan is onduidelijk. In **hoofdstuk 10** werd onderzoek gedaan naar vasculaire eigenschappen en endotheel functie van CHARIOT patiënten en vergeleken met gezonde controles en een tweede neurovasculair migraine syndroom, namelijk CADASIL (cerebrale autosomaal dominante arteriopathie

met subcorticale infarcten en leukoencephalopathie). Het laatst genoemde syndroom wordt pathologisch ondermeer gekarakteriseerd door degeneratie van gladde spiercellen van arteriolen, dit in tegenstelling tot CHARIOT waar dit maar marginaal aanwezig is. Uit de studie werd duidelijk dat CHARIOT patiënten endotheliale dysfunctie van grote vaten hebben, in tegenstelling tot CADASIL patiënten waarbij de stoornis in vasculaire functie lijkt te worden veroorzaakt door een defect in functioneren van gladde spiercellen in kleine vaten. Vanwege overlappende klinische symptomen, zou CHARIOT een model kunnen vormen voor meer voorkomende neurovasculaire ziekten zoals vasculaire dementie en migraine.

Deel 3 onderzoekt comorbiditeit van migraine met andere ziekten en beschrijft hoe comorbiditeit behulpzaam kan zijn bij genetisch onderzoek naar migraine. **Hoofdstuk 11** beschrijft de identificatie van migraine patiënten in een Nederlands genetisch isolaat, het Erasmus Rucphen familieonderzoek (ERF). Genetische geïsoleerde populaties zouden kunnen helpen bij de identificatie van genen omdat zowel de heterogeniteit van genen als omgevingsfactoren kleiner is. Het gebruik van endophenotypen kan de heterogeniteit verder kunnen reduceren omdat het resulteert in meer robuustere fenotypen.

Hoofdstuk 11 gaat over de comorbiditeit van migraine en depressie in ERF. Er is een bidirectionale relatie tussen migraine en depressie, wat een gedeelde etiologie suggereert. Daarom werd onderzocht of er gedeelde genetische risicofactoren zijn tussen beide ziekten. In totaal werden er 360 migraine patiënten geïdentificeerd: 209 hadden migraine zonder aura, 151 migraine met aura. De odds ratios (OR) voor depressie in migraine patiënten was significant verhoogd, in het bijzonder voor migraine met aura. Heritabiliteits-schattingen voor migraine (subtypen) waren significant en verminderden na correctie voor depressieve symptomen of gebruik van antidepressiva, in het bijzonder voor migraine met aura. Dit bevestigt dat migraine een grote genetische component heeft en indirect dat de bidirectionele associatie tussen depressie en migraine (in het bijzonder MA), tenminste ten dele, kan worden verklaard door gedeelde genetische factoren.

Migraine patiënten hebben ook een verhoogd risico op het krijgen van een herseninfarct en mogelijk op andere cardiovasculaire aandoeningen. Een predispositie voor het ontwikkelen van atherosclerose door een ongunstig cardiovasculair risicoprofiel zou een mogelijke verklaring kunnen zijn. Daarom werden in **hoofdstuk 12** diverse markers voor atherosclerose (pulse wave velocity (PWV), Intima Media dikte (IMT) en Enkel arm index (ABI)) onderzocht in migraine patiënten en controles uit ERF. Hieruit bleek dat migraineurs geen verhoogd risico hebben op centrale of perifere atherosclerose, waardoor het een onwaarschijnlijk verklaring wordt voor de relatie tussen migraine en ischemisch cardiovasculaire ziekten. Andere mechanismen, zoals endotheel dysfunctie, verhoogde gevoeligheid voor CSD of een gedeelde genetische gevoeligheid moeten worden overwogen.

Hoofdstuk 13 ten slotte plaatst de klinische en genetische bevindingen uit voorgaande hoofdstukken in een breder perspectief. Pathofysiologische mechanismen voor de verschillen migraine syndromen en de waarde als model voor het veel voorkomende migraine met en zonder aura worden besproken, evenals het potentieel voor de comorbiteit van complex genetische migraine (endophenotypen) bij de identificatie van nieuwe migraine genen en mechanismen.

LIST OF ABBREVIATIONS

AIx	augmentation Index
AHC	alternating hemiplegia of childhood
AGS	Aicardi-Goutière syndrome
ATP	adenosine tri-phophate
BFIC	benign familial infantile convulsions
CADASIL	cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
CGRP	calcitonin-gene related peptide
DNA	deoxyribonucleic acid
CNS	central nervous system
CRV	cerebrovascular retinopathy
CSD	cortical spreading cepression
CHARIOT	cerebral hereditary angiopathy with vascular retinopathy and impaired organ function caused by *TREX1* mutations
DBF	dermal blood flow
EA	episodic ataxia
EAAT1	excitatory amino acid transporter 1
EEG	electroencephalogram
ERF	Erasmus Rucphen Family (study)
ESCEATH	early seizures and cerebral edema after trivial head trauma
FMD	flow-mediated dilatation
FHM	familial hemiplegic migraine
FP	fluorescent protein
GEFS+	generalized epilepsy with febrile seizures plus
GWAS	genome-wide association study
HERNS	hereditary endotheliopathy, retinopathy, nephropathy and stroke
HVR	hereditary vascular retinopathy
IHS	International Headache Society
ICHD	international classification of headache disorders
KI	knock-in
LDPI	laser doppler perfusion imaging
LD	linkage disequilibrium
LOD	logarithm of odds
MA	migraine with aura
MO	migraine without aura
MRI	magnetic resonance imaging
NTG	nitroglycerin
NO	nitric oxide
PCR	polymerase chain reaction
PWA	pulse wave analysis

PWV	pulse wave velocity
RVCL	retinal vasculopathy with cerebral leukodystrophy
SCA6	spinocerebellar ataxia type 6
SHM	sporadic hemiplegic migraine
SMEI	severe myoclonic epilepsy of infancy
SNP	single nucleotide polymorphism
TGVS	trigeminal vascular system
TREX1	three prime repair exonuclease 1
TNC	trigeminal nucleus caudalis
TRPV1	transient receptor potential vanilloid type I receptor
VSMCs	vascular smooth muscle cells
WMH	white matter hyperintensities
WT	wild type

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CURRICULUM VITAE

Anine Henrike Stam was born on November 7, 1979 in Den Helder. She attended secondary school at "Scholengemeenschap Nieuwediep". In 1998 she started to study Biomedical Sciences and she passed her propaedeutic level examination in 1999 cum laude. As part of the Leiden University "Program for Excellent Students", she combined this study with a Medicine study since 2000. In 2002 she obtained her Master degree in Medicine, in 2004 her Medical degree (cum laude) and in 2005 her Master degree in Biomedical Sciences. As part of her studies she participated in a research project concerning the role of the thalamus in the trigeminovascular system in migraine at the Institute of Neurology, Queen Square, London, supervised by prof. P. J. Goadsby. During this project she also learned about the clinical aspects of headache disorders at the outpatient's headache service of The National Hospital for Neurology and Neurosurgery. From 2005 to 2009 she worked as a research-physician at the departments of Neurology and Human Genetics of Leiden University Medical Center (LUMC). The results of this research are described in this thesis. During her PhD training she received an AGIKO-stipendium from the Netherland Organization for Scientific Research (NWO) entitled "TREX1 in migraine and other neurovascular disorders". In 2009, she started as a resident in neurology at the department of Neurology of LUMC (Prof.dr. R.A.C. Roos). She hopes to qualify as a neurologist by April 2015.