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Unraveling the genetic architecture of migraine: exploring the vascular components

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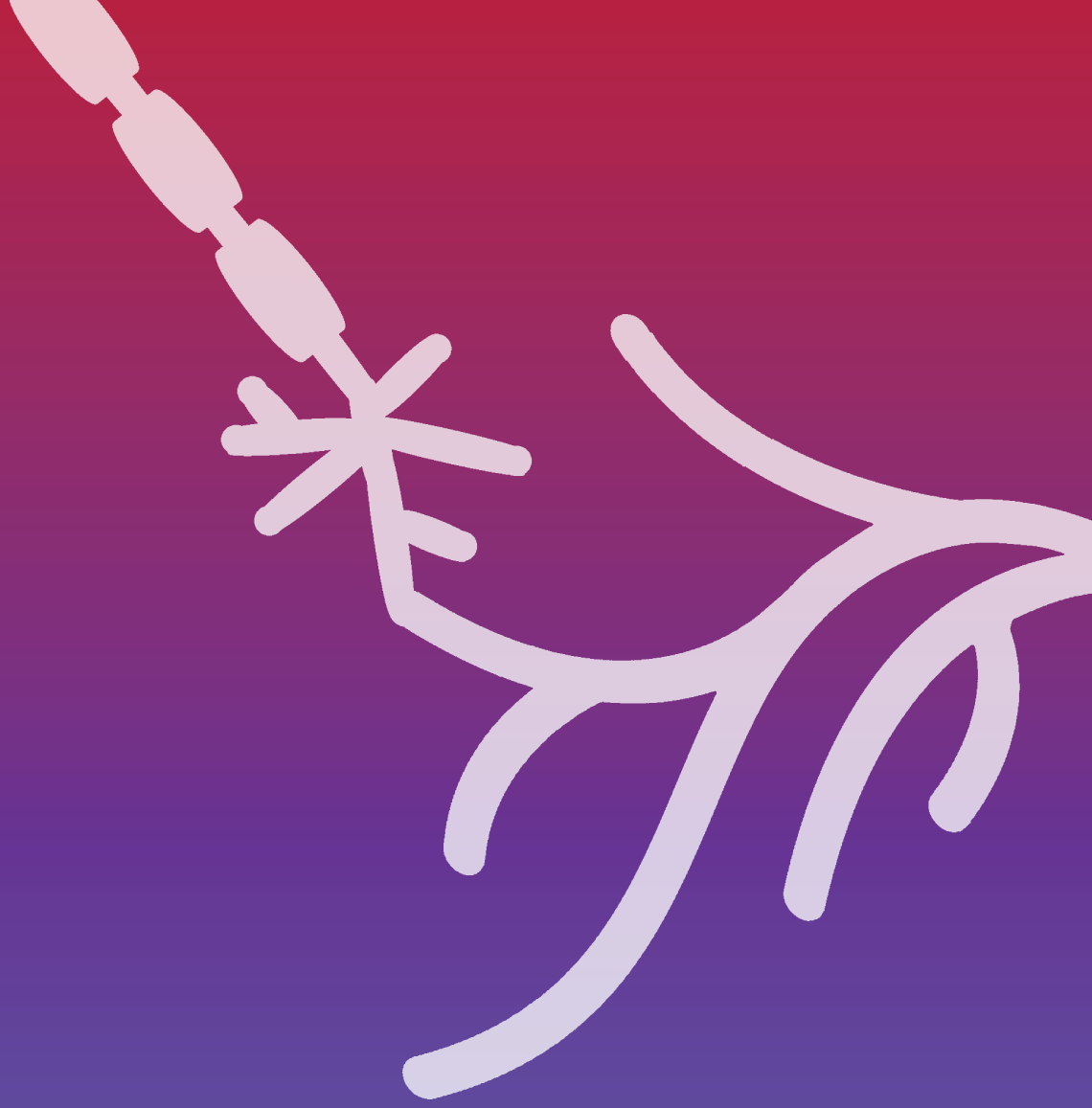

Unraveling the genetic architecture of migraine:

exploring the vascular components

Irene de Boer

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Unraveling the genetic architecture of migraine: exploring the vascular components

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Irene de Boer

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CHAPTER 1

General introduction

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Retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations (RVCL-S). I. de Boer, G.M. Terwindt. Chapter for UpToDate, Wolters Kluwer, last updated: 2022.

Retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations. I. de Boer, N. Pelzer, G.M. Terwindt. Chapter for GeneReviews®, last updated: 2019.

General introduction

Clinical characteristics of migraine

Migraine is a common, debilitating neurovascular disorder with a life-time prevalence of 15-30% worldwide.[1-3] Migraine manifests as recurrent attacks of headache with a range of accompanying symptoms (Table 1). There are two main migraine types depending on the absence/presence of an aura: migraine without aura (MO) and migraine with aura (MA).[4] Auras occur in approximately one-third of migraine patients and are characterized by transient focal neurologic symptoms (i.e., visual, sensory, motor or speech disturbances) that display positive phenomena and a succession or expansion of symptoms.[3-5] In migraine with aura, the headache often also occurs, but the aura phase may also occur without subsequent migrainous headache. Another relevant subtyping of migraine is episodic *vs.* chronic migraine, which is based on number of monthly migraine and headache days. Patients have chronic migraine when they have headache on at least 15 days per month, with eight of these being migraine days, for at least three months.[4]

Table 1. Criteria for migraine with and without aura in accordance with the International Classification of headache disorders (ICHD), third edition.[4]

ICHD-3 criteria for migraine
1.1 Migraine without aura
A. At least five attacks fulfilling criteria B–D
B. Headache attacks lasting 4–72 h (when 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, vomiting, or both
2. Photophobia and phonophobia
E. Not better accounted for by another ICHD-3 diagnosis
1.2 Migraine with aura
A. At least two attacks fulfilling criteria B and C
B. One or more of the following fully reversible aura symptoms:
1. Visual
2. Sensory
3. Speech or language
4. Motor
5. Brainstem
6. Retinal
C. At least three of the following six characteristics:
1. At least one aura symptom spreads gradually over ≥5 min
2. Two or more aura symptoms occur in succession
3. Each individual aura symptom lasts 5–60 min
4. At least one aura symptom is unilateral
5. At least one aura symptom is positive
6. The aura is accompanied, or followed within 60 min, by headache
D. Not better accounted for by another ICHD-3 diagnosis

The diverse and changing nature of migraine challenges adequate comparison of migraine types. Migraine type as well as migraine frequency can change over time.[6-8] This means that attack type and attack frequency are not stationary, but rather dynamic traits. This may especially be problematic when studying migraine pathophysiology. In most large-scale studies, patients are often labeled with a life-time diagnosis, e.g., if a patient had migraine with aura in their twenties, they are still considered migraine with aura patients even if they only suffer from migraine without aura attacks from age 40.

Pathophysiology of migraine

The pathogenesis of the migraine headache involves activation of the trigeminovascular system, which includes the trigeminal ganglion and its peripheral axonal projections that innervate pain-sensitive intracranial structures such as the meninges (Fig. 1).[9] Additionally, axonal projections arise from trigeminal ganglion cells and convey nociceptive impulses to second-order neurons in the brainstem.

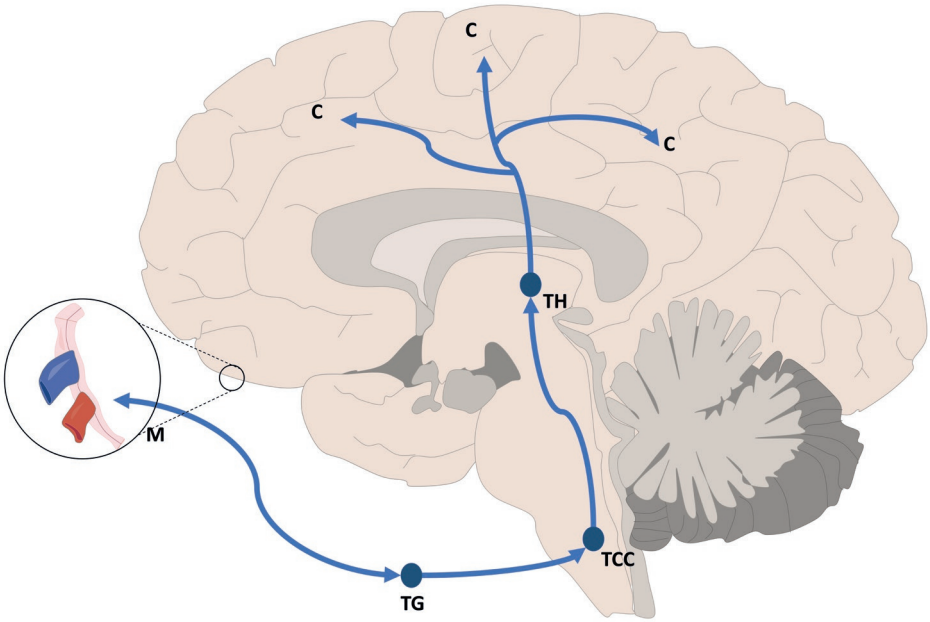

Figure 1. Pathophysiology of migraine.

Peripheral axonal projections of the trigeminal ganglion (TG) innervate pain-sensitive intracranial structures such as the meninges (M). Trigeminal afferents arising from the TG transmit sensory input towards the trigeminocervical complex (TCC). The TCC coordinates trigeminal pain processing between the peripheral and central nervous system. Projecting neurons travel via the trigeminothalamic tracts and synapse on multiple thalamic nuclei (TH). En route collateral projections target several additional nuclei (among which are the locus coeruleus, the periaqueductal gray and the hypothalamus). Thalamocortical neurons convey the sensory input to multiple cortical regions (C).

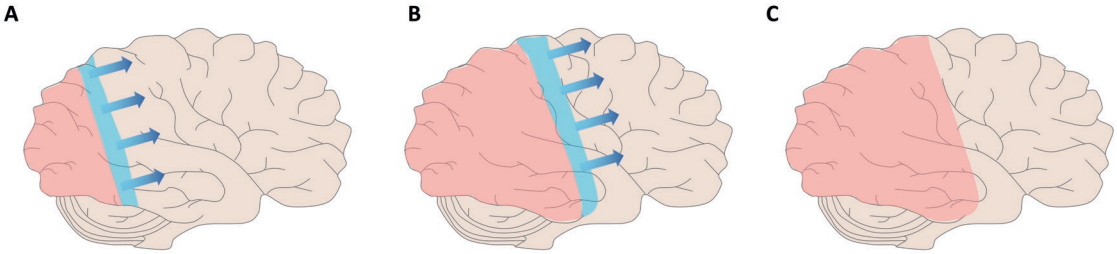

Figure 2. A schematic representation of cortical spreading depolarization.
Cortical spreading depolarization (CSD) is a brief wave of neuronal and glial depolarization that slowly self-propagates across the cerebral cortex (blue) (A and B), followed by long-lasting suppression of brain activity (pink) (A-C).

These neurons, in turn, project to third-order neurons in the thalamus, which then convey nociceptive impulses to a wide array of cortical areas that are involved in pain processing, including the somatosensory cortex (Fig. 1).[9]

Migraine aura is believed to be caused by cortical spreading depolarization (CSD) (Fig. 2). CSD is a wave of electrophysiological hyperactivity followed by a wave of inhibition. Spreading depolarization describes a phenomenon characterized by intense neuronal and glial depolarization that classically starts in the occipital (visual) cortex and slowly (~3 mm/min) propagates frontally through the cortex, until it encounters a less sensitive part of the brain and stops or redirects, thus explaining the wide variety of aura patterns patients report.[10-13] After the initial wave, affected neurons can be depolarized for several minutes up to an hour. Therefore, originally the term 'cortical spreading depression' was used although the term depolarization is now preferred. Genetic factors may lead to a lower CSD threshold and could thereby increase migraine susceptibility.[14-16]

Genetics of migraine

It has long been established that there is a strong genetic component in migraine.[17,18] This is exemplified by the fact that there are several monogenic migraine syndromes in which migraine is either the main symptom or part of a complex of symptoms. With respect to common forms of migraine (i.e., migraine with and without aura), large genome-wide association studies (GWAS) have greatly expanded our knowledge of the genetic variants involved, emphasizing the role of both neuronal and vascular pathways.[19] Examining the genetic structure of prevalent migraine types enhanced

our understanding of the connections among distinct (sub)types, as well as their associations with disorders coexisting with migraine in the same patient. Currently, the acquired knowledge has little utility in diagnosis or tailoring of treatments. This may change in the future with more advanced genetic studies focusing for instance on neuropharmacology (pharmacogenetics). In contrast, the clinical significance of recognizing monogenic migraine syndromes and understanding their underpinning genetic architecture has long been established. Recognizing monogenetic migraine patients is vital for accurate diagnosis, counseling, and treatment.

Complex genetics of common types of migraine

Common migraine is regarded as a complex inherited neurological disorder. Complex traits are typically caused by a combination of multiple genetic factors (each with a small effect size) and environmental factors.[20,21] In migraine family studies, heritability, defined as the contribution of genetic factors to susceptibility of a trait, was estimated to range from 35% up to 60%.[22]

Apart from genetic heterogeneity, the search for gene variants in the common forms of migraine is complicated by clinical heterogeneity. In this regard, it is crucial to note that there are currently no reliable biomarkers for migraine. Therefore, it is impossible to establish a migraine diagnosis based on clear quantifiable (objective) factors. Of note, the current diagnostic criteria of the ICHD-3 [4] are useful to diagnose patients in a clinical setting but may be less suited for pathophysiological research, simply because multiple combinations of symptoms (e.g., pulsating headache, nausea, vomiting) can lead to the same end-diagnosis, but might not necessarily been brought about by the same molecular mechanism.

Genome-Wide Association Studies

Improved technology and the development of cost-effective genotyping platforms has enabled genome-wide association studies (GWAS). GWAS has become the method of choice, to identify gene variants in complex traits. As no prior hypotheses need to be formulated, GWAS can be used to *generate* hypotheses. Typically, in a GWAS, several hundred thousand to millions of single nucleotide polymorphisms (SNPs) are tested for association with a trait (e.g., migraine) by assessing differences in allele frequencies between large numbers (several thousand or even tens or hundred thousand) patients and controls. Of note, in a GWAS typically only variants with a moderate to high minor allele frequency (≥0.01) are genotyped. Therefore, rare variants are overlooked. GWAS is an ideal method when identifying multiple common variants with small effect sizes that play a role in a disorder. The downside of the method is, given the small effect sizes that are observed in a GWAS, the need

for an exceptionally large sample size to obtain a significant association. As such, the direct clinical relevance of these findings may be limited. Furthermore, the most commonly employed method to ascertain the causal gene defect based on the most significant SNP in a locus in GWAS is to identify the gene nearest to that SNP. This does not necessarily mean that the correct gene is picked for each locus. Fortunately, additional bioinformatics tools can be used to determine the correct gene with more validity.[23] The migraine GWAS published in 2016 included 59,674 migraine cases and 316,078 controls; 38 genomic regions associated with migraine were identified, of which 28 were previously unknown.[24]

From a clinical perspective, gene variants with low relative risks may at first sight seem of limited value, but their identification does shed light on possible novel mechanisms that are relevant to migraine pathophysiology. Thus, genetic research has the potential to discover many yet unknown migraine pathways. Insight in these pathways can contribute to the development of new treatments. It has been suggested that selecting genetically supported targets could double the success rate of drug development.[25]

Missing heritability of migraine

All identified migraine-associated SNPs show small effect sizes and thus account for a small part of migraine genetic heritability; when combining all currently known associated SNPs they add up to no more than 14.63% (95%CI = 13.79-15.47) heritability;[24] where the total contribution of genetic factors is estimated to be between 35% to 60% based on family studies.[22,26] This is a common problem in complex traits and is commonly known as the missing heritability problem, which indicates that genetic variants in GWAS may not explain all of the heritability in complex traits. Most likely rarer, medium- to high-risk, alleles that are not captured by a GWAS approach play an important role.[27] Finding an association for rarer alleles is, however, notoriously difficult, as it necessitates even larger cohorts to achieve a sufficiently sizable representation of these rare variants in order to attain statistical significance. Successes of shedding light on missing heritability for any trait are scarce or essentially missing,[28] possibly because the problem of missing heritability was initially ill-defined as it did not take structural variants, variants in the mitochondrial DNA, epigenetic mechanisms and gene–gene and gene-environment interactions into account.

Structural variation and mitochondrial DNA

Unlike SNPs, GWAS cannot assess the contribution of other genetic variations, especially not of structural variations. Copy number variation (CNV) occurs when sections of the genome are repeated and the number of repeats in the genome varies

between individuals. In addition to well-known sporadic chromosomal microdeletion syndromes and Mendelian diseases, many common complex traits can result from CNVs.[29] Within the migraine field this is not a well-researched area. One study has tested whether length variations in the second polymorphic CAG repeat in exon 1 of the *KCNN3* gene, are involved in susceptibility to MA and MO and found no association.[30] It is unclear if and to what extend these repeats or CNV in general have a role in the genetic basis of migraine.

Another part of the missing heritability can be explained by genetic variants in mitochondrial DNA (mtDNA). Mitochondrial DNA is separated from nuclear DNA and inherited exclusively along the maternal lineage. Each cell contains hundreds to thousands of copies of mtDNA, and there is genetic variation between mtDNA molecules.[31] Interestingly, migraine-like headaches have been reported in several mitochondrial disorders, such as mitochondrial encephalopathy, lactic acidosis and stroke-like episodes (MELAS).[32] In addition, structurally abnormal mitochondria and findings indicative of impaired energy metabolism have been demonstrated in migraine.[33] While small candidate gene association studies focusing on mtDNA have reported associations,[34] these could not be replicated in a mitochondrial GWAS.[35] Furthermore, this mitochondrial GWAS found no additional associations with migraine. As only ~4000 cases were included, it seems likely that if mitochondrial DNA is part of the puzzle, a larger sample size is required to find such variants robustly associated with migraine.

Epigenetic Mechanisms

Another explanation for the missing heritability phenomenon can be that the heritability not only resides in the genome, but also in the epigenome. Epigenetics encompasses changes to the DNA structure without changing the genetic code. Epigenetic changes have been implicated in several neurologic disorders.[36] They can be dynamic, be passed on through cell divisions and even to offspring. The main epigenetic modifications are DNA methylation and post-translational modification of histone proteins. So far, small steps have been taken to dissect the epigenetic contribution towards migraine.[37] The first genome-wide analysis of DNA methylation in migraine identified 62 independent differentially methylated regions in blood samples without distinguishing between MO and MA.[38]

Gene-gene and gene-environment interactions

Gene-gene interaction (epistasis) is the modification of the effect of one gene on disease by another gene, or several others. Gene-environment interaction occurs when different genotypes respond to environmental variation in different ways.

This field of research is still in its infancy, also within migraine research.[39-41] It needs mentioning that the interactions are very challenging to study given the enormous possibilities of interactions and computational *force majeure* is needed to find them. Still, in other complex multifactorial diseases, such as multiple sclerosis and breast cancer, epistasis and gene-environment interactions have been suggested to be important in pathophysiology.[42-46]

Shared genetic architecture of migraine with other brain disorders

The comorbidity occurring between two disorders can be spurious due to selection bias or can reflect a unidirectional causal relationship (i.e., migraine causes (or is caused by) the comorbid disorder). It also may be due to shared genetic and/or environmental factors that underlie both migraine and the other disorder.[47] In other words, to certain extent, there may be a shared genetic architecture. It can be an attractive strategy for genetic studies, as it will reduce genetic heterogeneity by selecting those migraine patients that also suffer from the other disorder. There is limited sharing of genetic information *between* neurologic and psychiatric disorders.[48] There was but one exception, which is the observed correlation of migraine, a neurologic disorder, with depression, a psychiatric disorder.

Genetic correlation between Migraine and Psychiatric Disorders

The Brainstorm Consortium study showed that migraine was correlated to several psychiatric disorders, including major depressive disorder.[48] This is in agreement with a GWAS study specifically searching for a shared genetic background for depression and migraine. In that meta-analysis in the combined 8,045,569 SNPs of the migraine GWAS and the top 10,000 SNPs from a major depressive disorder GWAS, three novel risk loci were identified that were associated with both disorders.[49] Furthermore, gene-based association analyses revealed significant enrichment of genes associated with both migraine and major depressive disorder. Pathway analyses suggested several important pathways, especially neural-related pathways of signaling and ion channel regulation, to be involved in this shared etiology.[49] In addition, in a previous study in 2,652 participants of the Dutch Erasmus Rucphen Family Study, a genetic isolate, 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 comparing the heritability scores of depression between migraineurs and controls.[50] Comparison of the heritability scores for depression between patients with migraine and controls showed a genetic correlation between the Hospital Anxiety and Depression Scale-Depression (HADS-D) score and MA. This is in line with epidemiological studies demonstrating

a bidirectional association between depression and migraine.[51] Moreover, a genetic association was found between migraine and anxious depression.[52] This is especially interesting as there is a strong and consistent relationship between migraine and anxiety. The comorbidity of co-occurrence for migraine and anxiety has an average OR of 2.33 (95%CI: 2.20-2.47) among cross sectional studies and an average RR of 1.63 (95%CI: 1.37-1.93) for cohort studies.[53-55] There is limited evidence explaining this comorbidity. Nonetheless, the Brainstorm consortium did demonstrate a possible genetic association between migraine and anxiety disorders.[48]

Genetic correlation between migraine and neurologic disorders

Although the Brainstorm Consortium could not identify significant genetic risk overlap across neurologic disorders, their study suggested genetic sharing between epilepsy and migraine.[48] An explanation for the lack of statistical significance may be that a relatively small epilepsy cohort was used besides the fact that epilepsy represents more heterogenous disorder(s) with a wide variety of underling mechanisms. A genetic connection between migraine and epilepsy is anticipated, as patients with hemiplegic migraine frequently experience concurrent epileptic and (hemiplegic) migraine attacks, as demonstrated by previous research.[56] Additionally, epidemiological studies have revealed comorbidity between common migraine and epilepsy.[57] Of note, the Brainstorm Consortium failed to find a genetic correlation between ischemic stroke and migraine, despite an earlier study using GWAS data revealing substantial genetic overlap between both disorders,[58] and epidemiologic studies repeatedly demonstrating an increased risk of ischemic stroke in migraine patients.[59,60] Future studies with larger cohorts and more detailed clinical and genetic data may be able to further substantiate the genetic correlation for migraine and comorbid neurologic disorders, foremost of epilepsy and stroke.

Genetic correlation between migraine and 'headache'

A study from UK Biobank that had 74,461 cases and 149,312 controls identified 28 genomic loci associated with a broadly-defined headache phenotype.[61] Of note, only 14 of the found loci where previously identified in a migraine GWAS.[24] There are various possible explanations for this finding. These loci are involved in a broader headache pathway, as part of the cases may in fact be patients with tension type headache.[62] However, giving the robustness of the associated SNPs in the migraine GWAS and the fact that six of the loci identified in the headache GWAS were also identified in clinical-based migraine GWAS may suggest that part of the identified SNPs are not only migraine specific but may be related to headache symptoms in general.[24,63]

Monogenic migraine disorders

In rare monogenic migraine syndromes, a single DNA mutation is sufficient to cause disease. The identification of causal gene defects for such syndromes is more straightforward than for complex disorders, already because the hunt is for a single genetic factor. To date, several monogenic migraine syndromes are known (Fig. 3), some in which migraine is the main characteristic while in others it is one among other symptoms. Some of these other symptoms are more easily diagnosed or reported by the families such as stroke or dementia.

Several monogenic disorders are associated with an increased migraine risk. Among these disorders are a disease of the circadian clock, several cerebral small vessel diseases, mitochondrial disorders, and neuronal and glial disorders (Fig. 3). The cause of the higher prevalence of migraine in these disorders is not always understood.

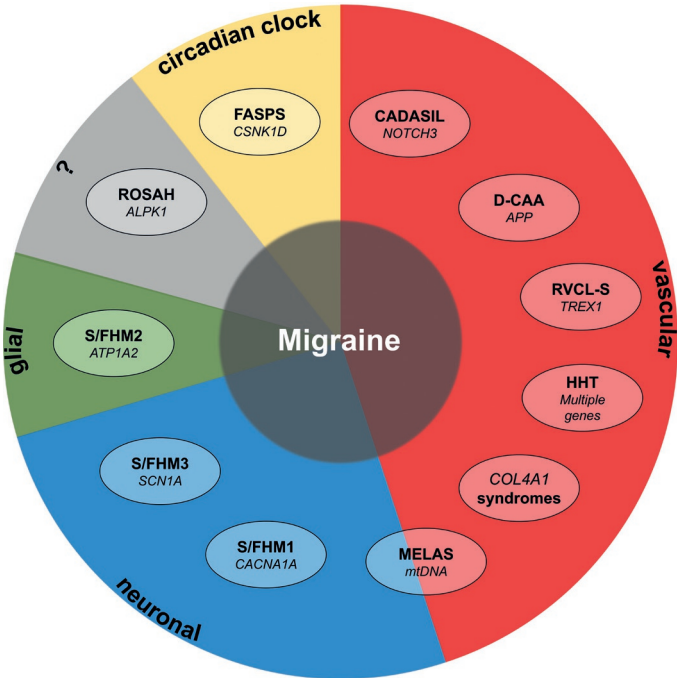

Figure 3. Pathophysiological pathways implicated in migraine and associated monogenic migraine syndromes.

Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL); Dutch-type hereditary cerebral amyloid angiopathy (D-CAA); Retinal Vasculopathy with Cerebral Leukoencephalopathy and Systemic manifestations (RVCL-S); Hereditary hemorrhagic telangiectasia (HHT); *COL4A1*-related disorders; Mitochondrial myopathy with Encephalopathy, Lactic Acidosis, and Stroke (MELAS); Sporadic and Familial Hemiplegic Migraine type 1, 2 and 3 (S/FHM 1, 2, 3); Retinal dystrophy, Optic nerve edema, Splenomegaly, Anhidrosis and migraine Headache (ROSAH); and Familial Advanced Sleep-Phase Syndrome (FASPS).

Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is caused by mutations involving codons of cysteine residues in the *NOTCH3* gene. NOTCH3 plays an important role in vascular smooth muscle cell functioning.[64,65] Patients with CADASIL can experience recurrent ischemic stroke, apathy, mood disturbances and progressive cognitive decline leading to dementia as well as migraine. Disease onset is usually during mid-adulthood. Migraine with aura is the first clinical symptom in almost half of cases.[66] Atypical and typical migraine with aura attacks are reported by more than 50% of patients, and may also include motor aura symptoms. A distinction between a transient ischemic attack (TIA) and migraine aura might be difficult.[66]

Dutch-type Cerebral Amyloid Angiopathy

Dutch-type cerebral amyloid angiopathy (D-CAA, previously called Hereditary Cerebral Hemorrhage with Amyloidosis-Dutch type (HCHWA-D)) is caused by a single missense mutation (E693Q) in the Aβ region of the *APP* (amyloid precursor protein) gene. Patients with D-CAA may suffer from recurrent intracerebral hemorrhages at young age and frequently develop vascular dementia.[67] There is an increased prevalence of migraine with aura among mutations carriers. Strikingly, similar to CADASIL, migraine with aura may be the presenting clinical symptom in up to 80% of D-CAA patients.[68] Importantly, aura symptoms lasting ≥60 minutes signaled acute hemorrhage in 55% of cases.[68]

Hemiplegic migraine

Hemiplegic migraine is a subtype of migraine with aura (Table 2). Hemiplegic migraine can be subdivided into familial hemiplegic migraine (FHM) and sporadic hemiplegic migraine (SHM), depending on the presence or absence of relatives with hemiplegic migraine.[4]

Table 2. Criteria for hemiplegic migraine and its subtypes in accordance with the International Classification of headache disorders, third edition.[4]

ICHD-3 criteria for hemiplegic migraine
1.2.3 Hemiplegic migraine A. Attacks fulfilling criteria for migraine with aura B. Aura consisting of both of the following: 1. fully reversible motor weakness 2. fully reversible visual, sensory and/or speech/language symptoms.
1.2.3.1 Familial hemiplegic migraine (FHM) A. Attacks fulfilling criteria for hemiplegic migraine B. At least one first- or second-degree relative has had attacks fulfilling criteria for hemiplegic migraine
1.2.3.2 Sporadic hemiplegic migraine (SHM) A. Attacks fulfilling criteria for hemiplegic migraine B. No first- or second-degree relative fulfils criteria for hemiplegic migraine

It is characterized by a transient hemiparesis during the aura phase, which may last up to hours or even days. Notably, in hemiplegic migraine the visual and sensory aura symptoms are identical to those seen in common migraine with aura, although the duration is often longer.[69] Nevertheless, several symptoms have been reported to occur more frequently in hemiplegic migraine compared to migraine with aura. Patients with hemiplegic migraine are more likely to experience two or more aura symptoms, to suffer from prolonged aura symptoms (up to days, weeks or even months) and symptomatology indicating to brainstem involvement with dysarthria, vertigo, tinnitus, hypoacusis, diplopia, ataxia and decreased level of consciousness.[70,71] The diagnostic value of these symptoms has not been investigated. Importantly, the majority of patients also experience attacks of migraine with aura without paresis or migraine without aura in addition to their hemiplegic attacks.[72,73]

The chance of finding a causal mutation in hemiplegic migraine patients is higher in those with a lower age of onset, when there are more affected family members, and when attacks exhibit the following characteristics: (1) extensive motor weakness, (2) trigger with mild head trauma, (3) include confusion, (4) manifest symptoms of brainstem pathology, or (5) are associated with brain edema or cerebral spinal fluid (CSF) pleocytosis. Notably, mental retardation and progressive ataxia are only found in patients with a causal mutation in one of the three known hemiplegic migraine genes.[74] As such, phenotypic features in hemiplegic migraine patients may guide physicians in selecting cases for mutation screening and in providing adequate genetic counseling.[74] Physicians should also realize that disease severity may differ based on specific mutations and which of the known hemiplegic migraine genes is involved. As example, the p.Ser218Leu *CACNA1A* mutation is associated with severe attacks and cerebral oedema, seizures and coma, even up to attacks being fatal.[75] Mental retardation and brain atrophy have also been described as a consequence of hemiplegic migraine attacks.[76]

Hemiplegic migraine: pathophysiology

A considerable subset of hemiplegic migraine patients has a monogenic autosomal dominant form that can be caused by a single highly penetrant mutation in genes implicated in FHM1-3. Specific missense mutations in the *CACNA1A* gene, located on chromosome 19p13, cause FHM1. This gene is responsible for encoding the alpha-1A subunit of the $Ca_V2.1$ (P/Q type) calcium channel. FHM2 is caused by distinct missense mutations in the *ATP1A2* gene on chromosome 1q23. *ATP1A2* encodes a catalytic subunit of the alpha-2 subunit of the sodium/potassium ATPase. Finally, FHM3 is caused by specific missense mutations in the *SCN1A* gene on chromosome 2q24. *SCN1A* encodes a transmembrane alpha-1 subunit of the brain $Na_V1.1$ sodium channel.

For all three hemiplegic migraine types of knock-in mouse models have been generated by expressing pathogenic human gene mutations in the respective endogenous mouse gene. FHM1 mutant mice with either the R192Q or the S218L missense mutation revealed increased neuronal calcium influx and enhanced (cortical) excitatory, but not inhibitory, neurotransmitter release.[77-80] FHM2 mutant mice with either the W887R or the G301R mutation demonstrated abnormal glutamate uptake through glial cells.[81-83] Moreover, for the W887R mutant a reduced rate of glutamate and K^+ clearance, and a reduced density of GLT-1a glutamate transporters in astrocytic processes surrounding glutamatergic synapses was shown which facilitated the ignition of experimentally induced CSD.[81,82] In line with these findings, both the FHM1 and FHM2 mutant mice also demonstrate an increased susceptibility for experimentally induced CSD.[14,79,81,84] Recently, the first FHM3 mouse model (expressing the L263V mutation) was generated that demonstrated, for the first time, *spontaneous* CSD events.[85] Interestingly, the waves of CSD consistently propagated from visual to motor cortex, corresponding to the clinical presentation in humans.[69]

Taken together these findings predict increased neurotransmitter and potassium ion levels *in vivo* at the synaptic cleft, which facilitates CSD and explains the aura features in patients with these mutations.[86-91] Moreover, FHM1 mice also demonstrate an enhanced susceptibility to ischemic depolarizations which could be the underlining mechanism predisposing migraineurs to infarction during mild ischemic events, thereby increasing the stroke risk.[92,93]

Several studies have been conducted in humans to further understand the pathological mechanisms causing hemiplegic migraine. As *CACNA1A* is also expressed at the neuromuscular junction, neuromuscular function was evaluated in FHM patients with *CACNA1A* mutations using single-fibre electromyography (EMG). Mean jitter did not differ between patients and control subjects or among patients and no blocking was found.[94] These results suggest that neuromuscular function is normal in FHM. With an exploratory ^{1}H-NMR metabolomics analysis lower levels of 2-hydroxybutyrate were found in patients with hemiplegic migraine.[95] This may indicate a dysregulation of the brain's energy metabolism. Another indication of involvement of energy metabolism came from a study using ^{31}P magnetic resonance spectroscopy (^{31}P-MRS). A higher concentration of adenosine diphosphate in the brains of the patients with familial hemiplegic migraine was found.[96] Another MRS study that used a high-field 7 tesla brain proton MRS (^{1}H-MRS) demonstrated a decreased cerebellar N-acetylaspartate/total creatine ratio in the absence of macroscopic atrophy and regardless of signs of cerebellar dysfunction in hemiplegic migraine patients.[97] This suggests that cerebellar neuronal loss or dysfunction occurs in hemiplegic migraine. The likelihood of inducing a migraine-like

attacks after nitroglycerin and CGRP administration is generally increased in patients with migraine without aura compared with patients with migraine with aura, and in migraineurs with high attack frequency compared with migraineurs with low attack frequency.[98,99] Patients with FHM typically have a much lower attack frequency than patients with migraine without aura, predicting a lower response rate to nitroglycerin or CGRP provocation. Indeed, the incidence of migraine-like headaches after provocation with nitroglycerin or CGRP was lower in patients with FHM than in patients with common types of migraine.[100-104] These results might point to different starting points for attack initiation for hemiplegic migraine compared to common migraine.

Not all hemiplegic migraine patients will be found to have a causal mutation in one of the known FHM1-3 genes.[69,74,105] Several genes have been suggested as the possible fourth hemiplegic migraine gene.[106] Nonetheless, whole exome sequencing (WES) has failed to identify undisputed pathogenic mutations in additional genes that fit a Mendelian inheritance pattern.[74] A major fourth autosomal dominant gene for hemiplegic migraine may therefore be unlikely, although this cannot be fully excluded. Physicians should realize that this means that not all offspring of hemiplegic migraine cases have an *a priori* 50% chance to inherit hemiplegic migraine. Genetic counseling may be helpful to clarify estimated risks.

PRRT2 has been suggested as the fourth hemiplegic migraine gene.[107] While their appears to be an association between hemiplegic migraine and *PRRT2*, the vast majority of *PRRT2* carriers with the presumed causative mutation do not have (hemiplegic) migraine attacks. Moreover, large hemiplegic migraine families in which a *PRRT2* mutation has been found often have many non-penetrant cases or it is later found out that there is an additional mutation in one of the well-established FHM1-3 genes.[74,108] Thus, *PRRT2* more likely seems to act as a genetic modifier of (hemiplegic) migraine risk. Therefore, in clinical practice neurologists and geneticists who find a *PRRT2* mutation in a hemiplegic migraine patient should be careful to claim genetic confirmation of a clinical diagnosis and should be aware the (hemiplegic) migraine will not be inherited in an autosomal dominant manner. Several other genes have been suggested as possible hemiplegic migraine genes (i.e., *SLC1A3* and *SLC4A4* [109-111]) but lack independent confirmation.

Most hemiplegic migraine patients without a mutation in *CACNA1A*, *ATP1A2*, or *SCN1A* display a mild phenotype without additional symptomatology, and may seem to be more like those with common migraine. It is possible that other genetic mechanisms play a key role in these cases. Possibly, a non-autosomal dominant mechanism could be involved in which rare gene variants with intermediate effect sizes lead to the disorder.

This is, however, extremely difficult to study as it is unclear to what extend reduced penetrance is implicated and how many variants might play a role. This can even be up to a point where complex polygenic interactions involving multiple gene variants with small effect sizes may occur, as is the case for the common migraine types. Some evidence for this theory exists. In a large study that used polygenic risk scoring, based on genetic information of common variants from the 2016 migraine GWAS,[24] it was shown that both familial migraine with aura and hemiplegic migraine cases (without a mutation in the known genes) had higher polygenic risk scores compared to cases of familial migraine without aura.[105] This observation implies the likelihood of common variants, and the possibility these variants are playing a significant role in hemiplegic migraine when no mutation is found in the FHM1-3 genes.

Hemiplegic migraine: management

Due to the rarity of hemiplegic migraine, no large randomized controlled trials can be performed in this patient group. There are no targeted treatments for patients with specific mutations, although precision medicine remains an elusive ideal. Treatment largely follows guidelines for the common form of migraine with aura. Preference for certain drugs is mostly based on data from migraine with aura and expert opinion.

In hemiplegic migraine attacks the aura is often more debilitating than the headache. Acute migraine medication may alleviate headache, nausea, photophobia but no acute medication for treating the aura phase is available. First choice in acute treatment usually is acetaminophen or an NSAID. If not effective, triptans can be prescribed. There is no experience with the newer drug treatments, ditans and gepants. But theoretically these will only work on the headache phase similar to the current medication options.

Migraine aura was previously thought to be caused by ischemia, but regional cerebral blood flow studies dispelled that idea.[112] Sumatriptan-induced vasoconstriction occurs only in the dilated vessels, without affecting normal ones.[113] Even so, because hemiplegic migraine patients have severe aura, triptans were officially contraindicated. However, extensive clinical experience has shown that triptans can safely be used in both hemiplegic migraine and migraine with aura and other monogenic small vessel diseases, such as CADASIL. Retrospective studies in hemiplegic migraine patients showed that side effects were rare and minor.[114-116] There are only few reports on preventive treatments in hemiplegic migraine. Abortive treatment with verapamil and acetazolamide was described as incidentally effective.[117] Furthermore, ketamine has been reported to show some improvement as attack treatment in a small sample size. This is especially interesting as NMDA-receptor antagonists blocked CSD in

animal studies.[118] However, as ketamine may cause dependency and tolerance with prolonged use and should therefore not be recommended.

Preventive treatment is mostly prescribed for high-frequency migraine. In hemiplegic migraine it may also be considered for patients with low frequent but severe attacks. Acetazolamide, flunarizine, lamotrigine, sodium valproate and verapamil can be tried in no strictly preferred order.[117] Other migraine prophylactics, such as candesartan, pizotifen, propranolol and topiramate might also be considered, although there is less evidence for efficacy.[117] When deciding on treatment to start it should be taking into account that hemiplegic migraine patients have an increased risk of developing depressive symptoms, so this should be carefully monitored.[119] Hemiplegic migraine attacks can be triggered by (minor) head trauma.[74] Therefore, patients that have experienced trauma-triggered attacks should be advised not to practice contact sports. No evidence-based advice is available on other lifestyle or dietary factors.

Retinal Vasculopathy with Cerebral Leukoencephalopathy and Systemic manifestations

Retinal Vasculopathy with Cerebral Leukoencephalopathy and Systemic Manifestations (RVCL-S) is an autosomal dominant inherited angiopathy caused by mutations in the *TREX1* gene (Table 3).[120,121] Worldwide less than 30 families with RVCL-S are known, so far.[120,122-132] However, this is expected to be an underestimation given that in the Netherlands alone, three unrelated families have been identified.[120] Moreover, RVCL-S is often mistaken for other disorders. With more awareness and new genetic screening techniques, such as whole exome sequencing, new cases and more families will be identified.

Table 3. RVCL-S diagnostic criterium and main features.[133]

Diagnosing and suspecting RVCL-S
Diagnostic criterium A. C-terminal frameshift mutation in TREX1
Main features A. Vascular retinopathy B. Features of focal and/or global brain dysfunction associated on MRI with C. punctate T2 hyperintense white matter lesions with nodular enhancement D. T2 hyperintense white matter mass lesions with rim-enhancement, mass effect, and surrounding edema E. Family history of autosomal dominant inheritance with middle-age onset of disease manifestation
Supportive features A. On CT focal white matter calcifications and/or on MRI nonenhancing punctate T2 hyperintense white matter lesions at an age that non-specific age-related white matter hyperintensities are infrequent B. Microvascular liver disease C. Microvascular kidney disease D. Anemia consistent with blood loss and/or chronic disease E. Microscopic gastro-intestinal bleeding F. Subclinical hypothyroidism

RVCL-S patients often suffer from retinal vasculopathy, focal and global neurological symptoms and systemic manifestations (e.g., impaired renal and liver function).[134-136] In RVCL-S, migraine occurs more frequently than in the general population.[135,136] In affected individuals migraine onset appears later in life (>40 years), which might suggest that migraine is a secondary phenomenon caused by the progressive cerebral vasculopathy.[135,137] As such, the cause of the migraine attacks might be different than in the case of CADASIL or Dutch-type cerebral amyloid angiopathy (D-CAA), where migraine is often an early clinical sign.[66,68] The main causes of morbidity and mortality in RVCL-S are vascular retinopathy, focal neurological complaints including ischemic events and cognitive decline, and kidney failure.[120]

RVCL-S: pathophysiology

RVCL-S is caused by carboxyl terminal (C-terminal) truncating mutations in the *TREX1* gene (encoding 3-prime repair exonuclease 1).[120,121] The underlying lesion is a non-atherosclerotic, amyloid-negative angiopathy involving small arteries and capillaries, primarily in the retina and brain but also in other organs.[120,135]

In mammals TREX1 is a major 3'-5'-exonuclease with high affinity for single-strand DNA and it is responsible for the removal of nucleoside monophosphates from the 3'-ends of DNA.[138,139] The TREX1 protein is highly conserved among species and its N-terminus contains three exonuclease domains while its C-terminal domain is required for its localization to the endoplasmic reticulum.[121,122,138,140,141] The mutations leading to RVCL-S result in a truncated TREX1 protein. The exonuclease function of TREX1 is not affected. However, the subcellular localization is altered.[121,122,141] Additionally, in lymphoblasts of RVCL-S patients an increase in free glycan release was demonstrated, most likely due to altered oligosaccharyltransferase activity.[141] Furthermore, in a transgenic mouse model of RVCL-S (with the V235*fs* mutation) a distinct autoantibody profile was found compared to that of wildtype mice.[142]

Morphological pathological studies have demonstrated thicker multilaminated basement membranes in RVCL-S patients.[120] Also, fibrous thickening of small vessel walls has been demonstrated.[120] Moreover, TREX1 was found to be expressed in a subset of microglia in the normal human brain, often in close proximity to the microvasculature. Interestingly, in ischemic lesions the amount of TREX1 positive microglia was increased.[130,143] This indicates a possible role for TREX1 and microglia in vessel homeostasis and response to ischemic injury. In the undamaged white matter of RVCL-S patients there is also an increased expression of TREX1, suggestive for a widespread ongoing injury that cannot be detected histologically.[130,143] Moreover, TREX1 was found to be expressed in endothelial cells in the brain of a RVCL-S patient.[130]

Experimental studies in in-house generated RVCL-S transgenic knock-in mice (also with the V235*fs* mutation) have shown increased mortality, signs of abnormal vascular function, and increased sensitivity to experimental stroke and can be instrumental to investigate the pathology seen in RVCL-S patients.[144]

RVCL-S: management

There is no specific disease-modifying treatment for RVCL-S. A pilot study for treating RVCL-S with aclarubicin was started but not finished due to serious side effects of the treatment. Limited information is available regarding the management of the major manifestations of the disease. Acute transient ischemic attack and acute stroke in patients with RVCL-S are managed following the general principles of stroke medicine. However, there is no proof that intravenous thrombolytic therapy (IVT) is effective and as RVCL-S is an angiopathy it may lead to increased bleeding risk. Therefore, IVT is not recommended similar to CADASIL. For secondary stroke prevention, we suggest that in patients with RVCL-S and a symptomatic ischemic event which has been confirmed by MRI imaging, available risk reduction strategies should be taken into consideration, including antiplatelet therapy, and statin treatment although specific evidence of effectiveness for RVCL-S is lacking. In addition, smoking cessation may be particularly important. Other suggested lifestyle modifications include limited alcohol consumption, no drugs, weight control, regular physical activity, and a Mediterranean diet that is rich in fruits, vegetables, and low-fat dairy products. We suggest measures to control blood pressure, cholesterol, glucose, and weight for all patients with RVCL-S on a regular basis.

If there are significant signs/symptoms of a specific organ patients should be referred to a specialist for that specific organ. However, experts with knowledge about RVCL-S advisable to avoid unnecessary diagnostics procedures (such as biopsies) as to prevent under- or overtreatment. Furthermore, when a patient is first diagnosed, a consultation with a clinical geneticist with knowledge about RVCL-S is recommended and other family members should be offered the possibility of genetic counseling.

It is advisable to perform a yearly check-up with neurological, ophthalmological, and general evaluation from diagnosis onwards to detect possibly treatable complications and symptoms. If symptoms are occurring and severe, more frequent monitoring may be necessary.

Most symptoms of RVCL-S can be treated with usual care for those specific organs. Retinopathy and macular edema can be treated with retinal laser therapy and intravitreal anti-VEGF. Glaucoma, hypertension, migraine, seizures, hypothyroidism,

anemia, Raynaud phenomenon and psychiatric complaints can be treated according to standard guidelines. For the treatment of anemia in severe cases intravenous blood transfusions may be necessary. In case of renal disease, control of hypertension is especially important. If cerebral vascular edema is present (especially common with pseudotumors) corticosteroid therapy should be considered (intravenous methylprednisolone, followed by oral corticosteroid treatment).

Asymptomatic family members should be offered genetic counseling prior to procedures that could detect signs of RVCL-S. Predictive testing and specific mutation testing is possible, once the *TREX1* pathogenic variant has been identified in an affected family member. Potential consequences of such a test should be discussed (socioeconomic consequences, long term follow-up). However, RVCL-S differs from many hereditary neurological diseases as there are health advantages to early diagnosis. Genetic screenings can identify individuals who will benefit from prompt initiation of treatment to preventive or delay complications of organ damage due to affected small vessels. Evaluation of signs of retinopathy is especially important as treatment can be necessary before onset of ocular complaints and can prevent early blindness at the age of 50 years.

Equality in research

Embracing diversity in the workplace further enhances team well-being and provides a distinct competitive advantage.[145-147] Likewise, a diverse and equal representation of researchers fosters innovation and creativity as different perspectives and experiences contribute to a broader understanding of complex issues.[147] As an example, publications authored by groups with ethnic and gender diverse authors attract significantly more citations than those written by researchers of a single ethnic group or gender.[148] A closer examination of these publications still reveals significant disparities. Despite the noticeable increase in the contribution of women as first authors of original research in high-impact medical journals over the past 20 years, progress has plateaued in recent years.[149]

Outline of this thesis

The research conducted for this thesis is divided in three parts. **Part I** of this thesis focuses on the genetics of migraine. Here migraine is investigated by: (i) reviewing our current understanding of the genetics of the migraine aura, (ii) an untargeted

epigenome-wide approach aimed at understanding migraine chronification, (iii) the prevalent neurological and psychiatric comorbidities and how they are genetically linked to migraine and (iv) how patients with hemiplegic migraine and monogenic migraine syndromes respond to migraine treatment. **Part II** of this thesis focusses on vascular monogenic migraine syndromes, there disease mechanism and possible biomarkers. Finally, **Part III** focusses on the challenges physicians and researchers may face in the headache field and are important to address to ensure that the future of headache research and clinical practices is ensured. This part is aiming to make recommendations for the (inter)national headache field.

Part I: Migraine – insights into genetics, epigenetics, comorbidities, and monogenic factors

In **Chapter 2** an overview of the genetics of migraine aura is given. **Chapter 3** investigates whether DNA methylation changes occur when patients with chronic migraine and medication-overuse respond to treatment. To this end, a longitudinal epigenome-wide association study was conducted where patients with chronic migraine were treated with withdrawal. Baseline methylation status was compared with methylation status after three months of treatment. **Chapter 4** describes neurological and psychiatric comorbidities associated with migraine and how genetics plays a role in this. In **Chapter 5** we investigated how patients with hemiplegic migraine and monogenic migraine disorders (CADASIL and RVCL-S) respond to migraine treatments.

Part II: Monogenic small vessel diseases – understanding vascular migraine models

In **Chapter 6** we discuss the importance of considering safety when treating vascular monogenic migraine, especially given the new migraine treatments targeting the CGRP system. **Chapter 7** explores the hypothesis the RVCL-S and CADASIL are caused by different disease mechanisms involving the endothelium and vascular smooth muscle cells, respectively. The retina is considered an extension of the central nervous system (CNS) and shares structural and functional similarities with the brain. Studying the retina provides a unique opportunity to gain insights into neurological conditions. Additionally, useful biomarkers are currently not available for these disorders. In **Chapter 8** and **Chapter 9** we therefore investigated whether RVCL-S and D-CAA lead to changes in retinal thickness using optical coherence tomography (OCT). In **Chapter 10** we evaluated the vasculature of RVCL-S in more detail using OCT angiography (OCT-A) and in **Chapter 11** we investigated to role of the oxygen metabolism by way of oximetry. These techniques, each non-invasive and user friendly might provide useful biomarkers in the future. In **Chapter 12** we characterized the neuropsychiatric

phenotype of RVCL-S patients and evaluated whether MRI characteristics are related to cognitive impairment, psychiatric morbidity and apathy.

Part III: Equality in headache research – tackling career barriers and harassment

To promote clinical and research opportunities in the headache field based solely on abilities and to remove possible career barriers providing an unfair disadvantage, we performed a global assessment of career barriers (**Chapter 13**) and harassment (**Chapter 14**).

A general discussion of the findings presented in this thesis as well as suggestions for future research are given in **Chapter 15**.

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PART I

Migraine – insights into genetics, epigenetics, comorbidities, and monogenic factors

2

CHAPTER 2

Genetics of migraine aura: an update

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Abstract

Migraine is a common brain disorder with a large genetic component. Of the two main migraine types, migraine with aura and migraine without aura, the genetic underpinning in the former is least understood. Given the evidence from epidemiological studies in cohorts and families that the genetic contribution is highest in migraine with aura, this seems paradoxical. Various genetic approaches have been applied to identify genetic factors that confer risk for migraine. Initially, so-called candidate gene associations studies (CGAS) have been performed that test DNA variants in genes prioritized based on presumed *a priori* knowledge of migraine pathophysiology. More recently, genome-wide association studies (GWAS) tested variants in any gene in an hypothesis-free manner. Whereas GWAS in migraine without aura, or the more general diagnosis migraine have already identified dozens of gene variants, the specific hunt for gene variants in migraine with aura has been disappointing. The only GWAS specifically investigating migraine with aura yielded only one single associated single nucleotide polymorphism (SNP), near *MTDH* and *PGCP*, with genome-wide significance. However, interrogation of all genotyped SNPs, so beyond this one significant hit, was more successful and led to the notion that migraine with aura and migraine without aura are genetically more alike than different. Until now, most relevant genetic discoveries related to migraine with aura came from investigating monogenetic syndromes with migraine aura as a prominent phenotype (i.e. FHM, CADASIL and FASPS). This review will highlight the genetic findings relevant to migraine with aura.

Background

Migraine is a common brain disorder characterised by attacks of severe typically unilateral headache with nausea, vomiting, phono- and/or photophobia.[1] In up to a third of patients, attacks are accompanied by an aura, consisting of transient focal neurological symptoms that are most often visual, but may also include sensory symptoms and/or speech disturbances. If the aura encompasses motor weakness the disease is diagnosed as hemiplegic migraine.[1] The likely underlying mechanism of the aura is cortical spreading depolarisation (CSD), a brief wave of neuronal and glial depolarization that slowly self-propagates across the cerebral cortex, originating in the occipital cortex, that is followed by long-lasting suppression of brain activity.[2-4]

Migraine has a strong genetic component. Epidemiological studies have shown that migraine runs in families and that migraine concordance rates are 1.5- to 2-fold higher in monozygotic compared to dizygotic twins.[5-7] A stronger family history of migraine is associated with migraine with aura, as well as a lower age-at-onset and a higher number of medication days,[8] which suggests a higher genetic susceptibility in this migraine type.[8] Several approaches, discussed below, have been used to identify genetic factors that confer migraine risk. The general assumption is that in common polygenic forms of migraine multiple DNA variants, each with a small effect size, together with environmental factors confer disease risk, whereas in rare monogenic migraine syndromes a single DNA mutation suffices to cause disease in a patient.

Candidate gene association studies in migraine with aura

Initially, candidate gene association studies (CGAS) were performed that test whether specific genetic markers in genes that had been selected based on prior hypotheses that they are implicated in migraine disease pathways, show a different allele frequency in migraine cases vs. controls. CGAS have been performed for many candidate genes for both migraine types (for reviews see [9,10]). Among the largest studies is one that included 841 migraine with aura patients and 884 controls in the discovery cohort and tested several thousand genetic markers in 155 ion transporter genes, but replication in an independent data set was essentially negative.[11] A large CGAS that systematically re-evaluated association findings of the 21 most promising genes from CGAS, but now using a much larger data set of several thousand cases and controls, did not lead to significant results.[12] Hence one may draw the conclusion that many, if not all, of the initial CGAS results may be false-positives. Results are disappointing most likely because of methodological issues, foremost because, almost without exception, CGAS had too small sample sizes (in most cases less than a few hundred cases and an equal number of controls were tested). Another likely explanation is that the diagnosis of aura symptomatology is difficult to obtain through

questionnaires and is often not validated through direct interviews by trained headache physicians.[13,14] In addition, cases and controls may not have been adequately matched for genetic background, gender and age. Finally, without replication of the main results in an independent population in the same study, the risk of reporting false positive results is high. It remains to be tested whether rarer variants (instead of the more common variants usually tested in a CGAS) may in fact elevate risk in specific subgroups of migraine patients. There is also the distinct possibility that the selected genes are not those that confer genetic risk, even if they belong to molecular pathways clearly implicated in migraine pathophysiology (such as genes that play a role in serotonin and dopamine pathways). Perhaps genes regulating (in)directly these pathways would have been better candidates.

Genome-wide association study in migraine with aura

Due to improved technology and the development of cost-effective genotyping platforms genome-wide association studies (GWAS) became feasible in the last decade. Unlike CGAS, in a GWAS no prior hypothesis is formulated about the possible role of a DNA variant. In a GWAS, in one experiment, several hundred thousand to millions of single nucleotide polymorphisms (SNPs), close to equally distributed over the whole genome, are tested for association with a trait (e.g. a disease such as migraine) by assessing differences in allele frequencies between patients and controls. Findings are rigorously corrected for the vast number of tests as an association is considered genome-wide significant only if the p-vale is $< 5x10^{-8}$. In a GWAS typically only variants with a moderate to high minor allele frequency (≥ 0.05) are genotyped; so alternative approaches, foremost next generation sequencing (NGS)-based whole-exome and whole-genome approaches, are used to assess the contribution of rarer variants.

The first migraine GWAS consisted of 2,731 patients with migraine with aura, obtained from three European specialized headache clinics (from Finland, Germany and the Netherlands), and 10,747 population-matched controls in the discovery cohort.[15] Surprisingly, only one single SNP, rs835740, reached genome-wide significance in this sample ($p = 5.38 \times 10^{-9}$, odds ratio = 1.23). The finding was successfully replicated in an independent set of samples from Iceland, Denmark, Germany and the Netherlands encompassing 3,202 cases and 40,062 controls and was highly significant in an overall meta-analysis ($p = 1.69 \times 10^{-11}$, odds ratio = 1.18). The associated SNP is located between *MTDH* (better known as astrocyte elevated gene 1 (*AEG-1)*) and *PGCP* (encoding plasma glutamate carboxypeptidase); both gene products have been implicated in glutamate homeostasis. A subsequent analysis of gene expression in lymphoblastoid cell lines demonstrated that the risk allele correlated with a higher transcript level of the *MTDH*

gene.[15] However, the relation between *MTDH* and migraine remains controversial, also because the SNP did not show significance in subsequent meta-analyses of GWAS data, neither in studies that investigated association with migraine ($p = 5 \times 10^{-3}$,[16] $p = 0.12$[17]) nor migraine with aura (both when the cohorts used to identify the locus were included ($p = 1.49 \times 10^{-4}$) or excluded ($p = 0.59$)).[17]

The most recent migraine GWAS included 59,674 cases and 316,078 controls and identified up to 38 distinct genomic regions associated with migraine.[16] When considering the genes closest to the associated SNPs, which is the typical way SNPs are being linked to implicated genes, it was concluded that current GWAS findings seem to link to neuronal, vascular, metalloproteinase, pain and metal-iron-related pathways.[16-18] Only in sub-analyses the contribution of associated loci to specific migraine types was given further consideration. Whereas seven loci (i.e. near *TSPAN2*, *TRPM8*, *PHACTR1*, *FHL5*, *ASTN2*, near *FGF6*, and *LRP1*) were associated with migraine without aura (in a sample of 8,348 cases and 139,622 controls), none were associated with migraine with aura (6,332 cases vs. 144,883 controls).[16] Of note, the seven loci had already been identified in the initial analysis with migraine in general, not subdivided in its subtypes, as phenotype. Given that two-third of migraineurs suffers from migraine without aura, this finding is perhaps not that surprising as it merely shows that the most abundant migraine type likely seems responsible for the association signals.

Is the genetic component in migraine with and without aura different?

A possible explanation for why GWAS appears more successful in migraine without aura might be that both migraine types are distinct disorders with a different genetic architecture. The suggestion fuels the long-standing debate whether or not both types are different disease entities. It should be noted that the migraine types are diagnosed differently according to the ICHD-III classification as migraine with aura, unlike migraine without aura, does *not* have to fulfil two out of four headache characteristics (moderate/severe pain intensity, unilateral location, pulsating quality, aggravation by routine physical activity) and accompanying symptoms (photophobia and phonophobia and/or nausea or vomiting). Moreover, to be labeled as patient the experienced number of attacks is less in migraine with aura (at least two attacks) compared with migraine without aura (at least five attacks).[1] Notwithstanding, there are long-recognized clinical observations suggestive for a shared etiology of both migraine types, not only because attacks of both types can co-occur in a patient or that the migraine type can change over lifetime (aura attacks may develop in the elderly migraine patient) but also because both types co-occur within families.[19]

It was the hope that GWAS findings could settle the debate. One argument for the distinction of migraine types is the previously mentioned observation that GWAS particularly identified loci for migraine without aura. Another genetic argument is that a polygenetic risk score analysis of 21 migraine-associated SNPs from GWAS showed association only with migraine without aura.[20] Importantly, it should be noted that in that study participants with migraine with aura (152 cases) were fewer than participants with migraine without aura (295 cases), so the study likely lacks statistical power to pick up a signal for migraine with aura. A recent study that took into account all available genetic information, so not just the migraine-associated SNPs ('top hits'), in a much larger data set, however, found statistical evidence for a significant overlap of genes associated with migraine with aura (4,505 cases) and migraine without aura (4,038 cases) and concluded that the genetic architecture of both types is more alike than dissimilar.[21] Likewise, a heterogeneity analysis of SNP effects in migraine with aura and migraine without aura showed that for 12 migraine-associated SNPs the risk-increasing allele was the same.[22] In fact, when more than 23,000 SNPs were analyzed the majority of SNP effects were in the same direction in migraine with aura (5,118 cases) and migraine without aura (7,101 cases).[22] Finally, the international Brainstorm consortium that compared GWAS data of over 20 brain disorders (and investigated genetic information of 265,218 patients and 784,643 controls) found substantial genetic correlation between migraine with aura and migraine without aura.[23] In conclusion, although migraine with aura and migraine without aura seem genetically more alike than dissimilar, the challenge remains to identify more of the underlying causal variants as only a fraction of them have been discovered.

Where is the missing heritability of migraine with aura?

Like what has been proposed for other complex diseases, one can postulate that migraine with aura may be determined by multiple rarer medium- to high-risk alleles that are not captured by a GWAS approach (Figure 1).[24] Finding an association for rarer alleles is notoriously difficult as it requires the analysis of even larger well-defined migraine with aura cohorts and representation of rarer variants in SNP arrays. For rarer variants a more logical approach is to genotype them by Next Generation Sequencing (NGS).

Part of the missing heritability could also be explained by genetic variants in mitochondrial DNA (mtDNA). Mitochondrial DNA is separated from nuclear DNA and inherited exclusively along the maternal lineage. Each cell contains hundreds to thousands of copies of mtDNA, and there may be genetic variation between mtDNA molecules.[25] In fact, migraine-like headache has been reported in several

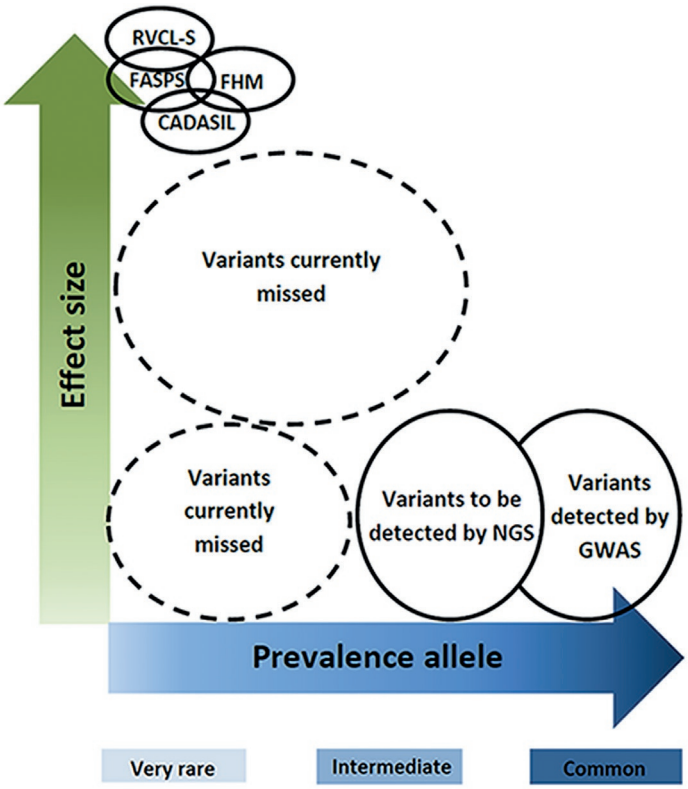

Figure 1. Missing genetic component of migraine with aura.

CADASIL: cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy, FASPS: familial advanced sleep-phase syndrome, FHM: familial hemiplegic migraine, GWAS: genome-wide association studies, NGS: Next generation sequencing, RVCL-S: retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations.

mitochondrial disorders, such as mitochondrial encephalopathy, lactic acidosis and stroke-like episodes (MELAS).[26] Additionally, structurally abnormal mitochondria and findings indicative of impaired energy metabolism have been demonstrated in migraine.[27] While small CGAS focusing on mtDNA have reported associations,[28] these could not be replicated in a recent mitochondrial GWAS (with 4,021 migraine cases included).[29] Furthermore, this mitochondrial GWAS found no additional associations with migraine.[29] It seems likely that a larger sample size is required to find variants robustly associated with migraine.

Another explanation for the missing heritability could be epigenetic processes. Epigenetics encompasses changes to the DNA structure without changing the genetic code and affects gene expression and has also been implicated in neurological disorders.[30] Epigenetic changes can be dynamic, be passed on through cell divisions or transgenerational inheritance. The main epigenetic modifications are DNA methylation and post-translational modification of histone proteins. So far, small steps have been taken to dissect the epigenetic contribution towards migraine.[31] The first genome-wide analysis of DNA methylation in migraine identified 62 independent differentially methylated regions in blood samples without distinguishing between migraine without and with aura.[32] While epigenetic factors might contribute towards migraine pathophysiology, for instance towards migraine chronification, this field is still in its infancy and therefore it is too soon to speculate towards its relevance for migraine with aura.

Genes in monogenetic migraine with aura syndromes

Until now successful identification of genetic factors relevant to migraine with aura almost exclusively came from investigating rare monogenetic syndromes. The identification of causal genes for such syndromes is more straightforward than for polygenic disorders, already because the hunt is for a single genetic factor that is sufficient to cause disease in a patient (Figure 1). Sequencing of the coding regions of the DNA of a patient (and family members) by direct sequencing of exons of possible candidate genes, in the past, and nowadays by sequencing all coding regions in a single experiment by whole exome sequencing has led to the identifications of thousands of disease genes for many disorders;[33] among which a number for migraine with aura syndromes that will be discussed below. Whole exome sequencing is more and more ready to be used in the diagnostic process of migraine with aura syndromes, as shown in a large Finnish sample.[34]

Familial Hemiplegic Migraine

The most investigated monogenetic migraine with aura syndrome is hemiplegic migraine.[1] In patients with hemiplegic migraine, the auras include motor weakness in addition to other focal neurological features commonly seen in migraine with aura patients. Additional clinical symptoms in hemiplegic migraine may include cerebellar ataxia, seizures, cognitive impairment, and decreased consciousness.[35] Of note, many patients also suffer from attacks of migraine with aura and/or migraine without aura.[36] This finding, but also the observation that factors that trigger attacks are the same as for migraine with aura,[37] supports the hypothesis that hemiplegic migraine is part of the spectrum of migraine disorders. DNA sequencing of candidate genes in genomic regions shared by affected family members, have identified causal mutations in three

hemiplegic migraine genes: *CACNA1A* (FHM1), *ATP1A2* (FHM2), *SCN1A* (FHM3).[38] In recent years, whole genome/whole exon NGS was used to identify additional causal genes in those patients in which no mutation in one of the three genes had been found. Surprisingly, this has not led to undisputed additional genes.[38] One important explanation may be that there is clinical heterogeneity among patients with a hemiplegic migraine diagnosis.[38] Chances of finding a causal mutation in hemiplegic migraine patients in one of the known genes is higher in patients with a lower age of disease onset, when there are more affected family members, and when attacks are (1) triggered by mild head trauma, (2) include confusion, (3) have extensive motor weakness, (4) have symptoms of brainstem pathology, or (5) are associated with brain edema; mental retardation and progressive ataxia are only found in hemiplegic migraine patients with a causal mutation in one of the three known genes.

Other monogenetic migraine with aura syndromes

In patients with monogenic cerebral small vessel diseases a high prevalence of migraine with aura can be observed. Almost half of the patients with cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) suffer from migraine with aura, which is often the first symptom of disease.[39] Other symptoms of CADASIL include mid-adult onset of recurrent ischemic stroke, cognitive decline progressing to dementia, mood disturbances and apathy. CADASIL is caused by mutations, typically affecting the number of cysteines, in Notch3 that plays an important role in vascular smooth muscle cell functioning.[40,41] Another small vessel disease in which migraine with (and without) aura is often present is retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations (RVCL-S).[42,43] RVCL-S is caused by carboxyl terminal truncating mutations in TREX1.[44] In addition to migraine (with and without aura), mutation carriers typically suffer from retinal vasculopathy, focal and global neurological symptoms and systemic manifestations including impaired liver and renal function.[42-44] In RVCL-S migraine onset appears later in life (>40 years).[43,45] This might indicate that migraine is a secondary phenomenon due to the progressive cerebral vasculopathy seen in patients with RVCL-S. Of note, in another cerebral small vessel disease, Dutch-type hereditary cerebral amyloid angiopathy (D-CAA) that is caused by a single point mutation (E693Q) in the amyloid precursor protein (*APP*) gene, there also is an increased prevalence of migraine with aura.[46] D-CAA is characterized by intracerebral hemorrhages and vascular dementia.[47] Migraine with aura is the presenting symptom in close to 80% of patients.[46] Patients with specific mutations in *COL4A1* also appear to have an increased prevalence of migraine with aura, in addition to clinical features such as porencephaly type 1, brain small vessel disease with or without ocular anomalies, non-syndromic autosomal dominant congenital cataract, hereditary angiopathy with nephropathy, aneurysms,

muscle cramps syndrome, and/or tortuosity of retinal arteries.[48-50] At present it is not known what causes the higher prevalence of migraine with aura in these genetic vasculopathies. Possible suggested mechanisms are neurovascular changes, including endothelial and vascular smooth muscle dysfunction and amyloid depositions.[40,51-56]

Also in patients with non-small vessel disease familial advanced sleep phase syndrome (FASPS), which is caused by specific missense mutations in the *CSNK1D* gene, migraine with aura is prevalent.[57] Although little over a handful of mutation carriers in two FASPS families have been reported, almost without exception they also suffer from migraine with aura.[57] Given the fact that FASPS is characterized by a profound phase advancement of the sleep-wake, melatonin and temperature rhythms,[57-59] other genes involved in sleep regulation might be implicated in familial migraine. A good candidate is *PER2*, which is already a known cause of FASPS;[60] it is however unknown whether *PER2* mutation carriers also have an increased risk for migraine.

Other genes implicated in migraine with aura syndromes?

In recent years several genes have been put forward as possible hemiplegic migraine genes (i.e. *PRRT2, SLC1A3* and *SLC4A4*)[61-65], but evaluation of available data should cast doubt on such claims.

PRRT2 - Whereas heterozygous *PRRT2* mutations (typically involving similar deletions in the same cytosine stretch) have been identified in a few patients with hemiplegic migraine,[63,64,66,67] it must be taken into account that many hundreds of patients with such mutation who suffer from paroxysmal kinesigenic dyskinesia, benign familial infantile seizures, or infantile convulsion choreoathetosis syndrome[66] do *not* have hemiplegic migraine. So the presence of such *PRRT2* mutation does not suffice to cause hemiplegic migraine in the Mendelian fashion seen with the three hemiplegic migraine genes.[64] Thus, *PRRT2* mutations may increase the risk of hemiplegic migraine as “add-on” mutations but it seems unlikely that PRRT2 is the FHM4 gene. There is recent functional evidence that mutant PRRT2, which affects the neuronal neurotransmitter release machinery, leads to abnormal neuronal hyperexcitability, as was shown in a knockout rat model.[68]

SLC1A3 - The observation of a missense mutation P290R in *SLC1A3*in a single patient with alternating hemiplegia, episodic ataxia, and seizures, does not qualify *SLC1A3* as a bona fide hemiplegic migraine gene either.[62] *SLC1A3* encodes the excitatory amino acid transporter 1 (EAAT1) that is involved in glutamatergic neurotransmission. More recently, *SLC1A3* missense mutation T387P, shown to diminish potassium binding to EAAT1, was identified in a patient with hemiplegic migraine, strengthening the

believe that EAAT1 might be involved in hemiplegic migraine. Still, doubts concerning causality remains as the mutation did not segregate with the phenotype as the patient's father was a mutation carrier but did not suffer from hemiplegic migraine.[65] At this moment proof for causality based on isolated cases in which the clinical diagnosis is not firmly established or in which the mutation does not segregate in the family seems not sufficient.[62,65]

SLC4A4 - In two patients with proximal renal tubular acidosis and a homozygous frameshift mutation S982NfsX4 in *SLC4A4*, which encodes for a sodium bicarbonate cotransporter NBCe1, hemiplegic migraine was reported, while some heterozygous carriers reported common forms of migraine.[61] Given the notion that NBCe1 dysfunction affects pH and the functioning of astrocytes, the possible involvement of *SLC4A4* in migraine pathophysiology deserves further investigation.

Of note, none of the mutations described in *SLC1A3* and *SLC4A4* above are found in gnomAD, a sequence variant reference database containing the information of over 100,000 exomes.[69] Absence in gnomAD indicates that the mutations must be rare and underscores that the mutations may indeed cause disease albeit that the association with migraine needs more proof.

In addition to the potentially (hemiplegic)migraine genes mentioned above, there is also an interesting report on a gene, *KCNK18*, that was identified in a large multigenerational pedigree suffering from migraine with aura.[70] Thus far, genetic evidence that specific frameshift mutations (such as F139WfsX24) in the *KCNK18* gene, encoding the TRESK channel, cause migraine came from only one *single* family in which the TRESK mutation seemed to segregate perfectly with the phenotype. It was also not reported whether there were other mutations, in the area of linkage, that could equally be disease gene candidates. This scenario is not that unlikely as only a set of 150 ion transporter genes (in the entire genome) were screened for mutations in a set of 110 unrelated migraine patients in the original study.[71] Moreover, loss of one functional copy of TRESK *per se* does not seem to lead to disease as another variant, C110R, was found in both controls and patients.[70] Where functional analyses of cells expressing the frameshift mutation indicated effect on plasma membrane localization leading to hyperexcitability of trigeminal ganglion neurons,[72] this second mutation (C110R) did not increase the excitability of trigeminal ganglion neurons.[73,74] Recent intriguing work sheds some light on the complex mechanism on how TRESK mutations could lead to different functional effects, as frameshift mutations F139WfsX24 and Y121LfsX44, but not C110R, lead to an alternative start codon and consequently production of a second TRESK protein, which co-assembles with TREK1 and TREK2

to downregulate their function leading to increased trigeminal ganglion neuron excitability.[75] This might be the reason why only specific frameshift mutations induce hyperexcitability in trigeminal ganglion neurons. Using CRISPR-Cas9 engineering to correct the F139WfsX24 mutation in induced pluripotent stem cell-based models, a reversal of the neuronal excitability phenotype was demonstrated.[76]

Why shed doubt whether TRESK1 mutations are indeed causal for migraine? Firstly, proof of causality based on one single family, even though segregation is seemingly perfect, is not sufficient, especially when realizing that the linkage region was not thoroughly scanned for other possible causal mutations. Secondly, whereas another frameshift mutation Y121LfsX44 was reported in ClinVar[77] (RCV000490385.1) to be associated with migraine (notably without any record clinical details),[75] for a second case no association with disease was reported. Thirdly, and foremost, frameshift mutations in *KCNK18* in gnomAD are among the most common variants in *KCNK18*.[69] The Y121LfsX44and the F139WfsX24 mutations were found no less than 665 and 123 times, respectively. Although in theory all individuals may have familial migraine, this sheds reasonable doubt on whether TRESK is indeed a migraine gene. The more likely scenario is that TRESK mutations that lead to inactive protein function are well tolerated, which is also suggested by the observation that there 12 homozygous carriers reported in gnomAD. While it thus seems unlikely that a single mutation in TRESK is sufficient to cause migraine with aura, this does not exclude a role for TRESK in migraine pathophysiology. Based on their mode of action TREK1 and TREK2 might qualify as potentially interesting therapeutic targets for migraine.

Functional consequences of gene mutations causing migraine with aura syndromes

To unravel the pathophysiological mechanisms causing hemiplegic migraine, functional consequences of gene mutations have been studied in cellular and animal models (for detailed reviews see [78-80]). First clues came from studies in cellular models in which mutant cDNAs were overexpressed. FHM1 mutations in the α1 subunit of voltage-gated $Ca_V2{\cdot}1$ (P/Q-type) calcium channels result in a gain of channel function.[81] FHM2 mutations in the α2 subunit of sodium-potassium ATPases result in a loss of function in glial cells.[82] The functional consequences of FHM3 mutations on $Na_V1.1$ channels were at first difficult to assess but recent evidence suggest that they are associated with a gain of channel functioning.[83] Together these findings predict increased neurotransmitter and potassium ion levels in vivo at the synaptic cleft, which facilitates CSD and thereby can explain the aura features in patients with the mutations.[82-87]

For all three FHM types knock-in mouse models have been generated by expressing pathogenic human gene mutations in the respective endogenous mouse gene. FHM1 mutant mice with either the R192Q or the S218L mutation reveal increased neuronal calcium influx and enhanced (cortical) excitatory, but not inhibitory, neurotransmitter release.[88-91] FHM2 mutant mice with either the W887R or the G301R mutation showed abnormal glutamate uptake through glial cells.[92-94] For the W887R mutant a reduced rate of glutamate and K^+ clearance, and a reduced density of GLT-1a glutamate transporters in astrocytic processes surrounding glutamatergic synapses, was shown which facilitated the ignition of experimentally induced CSD.[92,93] In line with these findings, both the FHM1 and FHM2 mutant mice also demonstrate an increased susceptibility for experimentally induced CSD.[90,92,95,96] Recently, the first FHM3 mouse model (expressing the L263V mutation) was generated that demonstrated, for the first time, *spontaneous* CSD events.[97] Of note, the waves of SD consistently propagated from visual to motor cortex, in line with clinical observations in humans.

To lesser extent, it has been investigated how gene mutations of the non-hemiplegic migraine genes (*NOTCH3*, *TREX1*, *APP*, *COL4A1*, *CSNK1D*) may cause migraine pathophysiology. Notch3 protein plays an important role in vascular smooth muscle cells of the small blood vessels of the brain.[41] *Notch3* knockout mice and mice that overexpress the R90C mutation specifically in vascular smooth muscle cells were found to have an enhanced susceptibility to experimentally induced SD.[51] In mutant mice that express the FASPS CKIδ-T44A mutation in *CSNK1D*, which encodes CK1D a well-known regulator of circadian rhythms, the susceptibility is also enhanced.[57] It was hypothesized that migraine with aura in FASPS is caused by vascular dysfunction through abnormal astrocytic signaling. There have been no studies yet that investigated the susceptibility of CSD in mutant mice with *TREX1*, *APP* or *COL4A1* mutations. Regardless, there are indirect clues how mutations in these genes may cause a migraine phenotype. RVCL-S mutations in TREX1, the major 3'-5'exonuclease in mammals, were shown to affect the regulation of oligosaccharyltransferase activity,[98] but not the protein's exonuclease function.[44,98] Recently, homozygous knock-in mice that express the RVCL-S V235fs mutation were shown to demonstrate signs of abnormal vascular function and increased sensitivity to experimentally induced stroke.[52] Of note, vasculopathy and endothelial dysfunction have also been demonstrated *in vivo* in patients with RVCL-S.[53] Amyloid precursor protein (APP) also is implicated in regulating endothelial function,[54] in addition to synapse formation[55] and neuroplasticity.[56] In APPDutch mice (overexpressing the E693Q *APP* mutation) neuronal expression of APPDutch leads to cerebrovascular amyloidosis, hemorrhage and smooth muscle cell degeneration,[54] supportive of the idea that vasculopathy may be implicated in migraine. Finally, *COL4A1*, which encodes the procollagen type IV α1, a

basement-membrane protein, causes a pleiotropy of vascular disorders. Mutant mice (expressing the *Col4a1*$^{\Delta ex40}$ mutation) develop a compromised cerebral vasculature (intracellular accumulation of mutant collagen in vascular endothelial cells and pericytes triggers small vessel disease) leading to recurrent hemorrhagic strokes and age- related macroangiopathy.[50,99] Based on the investigations in the mutant mice, it is well possible that vascular changes, such as endothelial dysfunction, may directly or via neuronal pathways increase migraine susceptibility, although this needs to be further investigated.

Are hemiplegic migraine and the common forms of migraine part of one genetic spectrum?

Recently, the close link between hemiplegic migraine (in those that do not have a causal mutation in the known genes) and migraine with aura was further demonstrated in a large Finnish study that used polygenic risk scoring, based on genetic information of common variants from the most recent migraine GWAS.[16,100] It was shown that both familial migraine with aura and hemiplegic migraine cases had higher polygenic risk scores, so to larger extent are explained by the presence of common variants, compared to familial migraine without aura cases. The group of patients with hemiplegic migraine had a higher genetic load than in migraine with (typical) aura but the difference did not reach statistical significance ($p = 0.09$),[100] which could be due to a smaller sample size or because sensory symptoms in some patients with migraine with aura erroneously were mistaken for motor symptoms so these patients ended up in the hemiplegic group. Notwithstanding, the study provides an independent argument that hemiplegic migraine and migraine with aura are part of the migraine spectrum.

Conclusion

Great effort went into unraveling the genetic architecture of migraine, both of migraine with aura and migraine without aura. Although the CGAS approach has been used in many (often very small) studies, findings were not replicated in a much larger GWAS data set. Of note, when taking all genotype information (not just the SNPs that surpass the threshold for genome-wide significance) into account, the genetic architecture of migraine with aura and migraine without aura is more similar than dissimilar. Until now most knowledge of the genetics of migraine with aura came from studies of monogenic syndromes that pointed to roles for neurotransmission and vasculature in migraine pathophysiology. Finally, the genetic load, i.e. the contribution of common genetic variants in families, was particularly high in hemiplegic migraine and migraine with aura compared to migraine without aura re-emphasizing the existence of a migraine spectrum. By increasing patient cohorts,

by testing the contribution or rare(r) variants using recently developed SNP arrays and next generation sequencing approaches and by studying possible epigenetic contributors, it is expected that we will get a better insight of the genetic architecture of migraine, including migraine with aura.

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3

CHAPTER 3

Alterations in DNA methylation associate with reduced migraine and headache days after medication withdrawal treatment in chronic migraine patients: a longitudinal study

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Abstract

Background: Chronic migraine, a highly disabling migraine subtype, affects nearly 2% of the general population. Understanding migraine chronification is vital for developing better treatment and prevention strategies. An important factor in the chronification of migraine is the overuse of acute headache medication. However, the mechanisms behind the transformation of episodic migraine to chronic migraine and vice versa have not yet been elucidated. We performed a longitudinal epigenome-wide association study to identify DNA methylation (DNAm) changes associated with treatment response in patients with chronic migraine and medication overuse as part of the Chronification and Reversibility of Migraine clinical trial. Blood was taken from patients with chronic migraine ($n = 98$) at baseline and after a 12-week medication withdrawal period. Treatment responders, patients with ≥ 50% reduction in monthly headache days (MHD), were compared with non-responders to identify DNAm changes associated with treatment response. Similarly, patients with ≥ 50% versus < 50% reduction in monthly migraine days (MMD) were compared.

Results: At the epigenome-wide significant level ($p < 9.42 \times 10^{-8}$), a longitudinal reduction in DNAm at an intronic CpG site (cg14377273) within the HDAC4 gene was associated with MHD response following the withdrawal of acute medication. HDAC4 is highly expressed in the brain, plays a major role in synaptic plasticity, and modulates the expression and release of several neuroinflammation markers which have been implicated in migraine pathophysiology. Investigating whether baseline DNAm associated with treatment response, we identified lower baseline DNAm at a CpG site (cg15205829) within MARK3 that was significantly associated with MMD response at 12 weeks.

Conclusions: Our findings of a longitudinal reduction in *HDAC4* DNAm status associated with treatment response and baseline *MARK3* DNAm status as an early biomarker for treatment response, provide support for a role of pathways related to chromatin structure and synaptic plasticity in headache chronification and introduce *HDAC4* and *MARK3* as novel therapeutic targets.

Introduction

Chronic migraine is a highly disabling migraine subtype affecting nearly 2% of the general population.[1-3] It is defined by the occurrence of headaches on ≥ 15 days/month for > 3 months, of which ≥ 8 days fulfil migraine criteria.[2] The majority of patients overuse acute headache medications including analgesics, triptans, and/or opioids, which is an important factor in the maintenance of chronic migraine.[1,3,4] Medication withdrawal therapy is effective in reducing headache frequency in 60% of patients with chronic migraine.[4]

The mechanisms behind the transformation of episodic migraine to chronic migraine and vice versa have not yet been elucidated. It has been suggested that epigenetic modifications may be important in this transition.[5] Epigenetic modifications encompass a variety of chemical and structural changes to chromosomal regions with effects of genetic and environmental factors on local DNA transcription. DNA methylation (DNAm) is an important epigenetic modification that involves the covalent binding of methyl groups to CpG (5′-cytosine-phosphate-guanine-3′) sites that are distributed throughout the genome.[6] DNAm status is dynamic but can be inherited from parent to daughter cells. As such, DNAm changes can lead to short- and long-lasting changes influencing disease,[6] with its dynamic nature enabling these changes to be reversible.

The hypothesis that epigenetic changes are implicated in migraine may suggest that altered synaptic plasticity of neurons is accompanied by changes in the epigenome leading to the maintenance of chronification of migraine. If treatment results in conversion back to episodic migraine, these epigenetic processes might also be reverted. Moreover, as some patients do not respond to withdrawal and/or prophylactic therapy, such as monoclonal CGRP-antibodies,[4,7] it is important to understand this clinical variation.

Epigenetic factors have been previously implicated in migraine pathophysiology. For example, one of the migraine genome-wide association study (GWAS) loci, the *PRDM16* locus, encodes a histone H3 lysine methyltransferase that acts as a transcriptional regulator.[8,9] Moreover, in rats, inducing cortical spreading depolarisation (CSD), the underlying mechanism of migraine aura, caused changes in chromatin, including histone H3 lysine methylation, at neuroprotective gene loci and retrotransposon elements.[10,11] Furthermore, depression/anxiety, obesity, stress, and female sex hormones have all been implicated in migraine pathophysiology, and are known to exert their physiological effects partly through epigenetic mechanisms.[11-15]

Some epigenome-wide association studies (EWAS) have indicated suggestive differences in DNAm profiles between patients with migraine and controls, with possible involvement in chronification,[5,16,17] although with limited sample sizes no specific loci were significant. A recent study by Carlsen et al. (2023) examined DNAm in 120 patients diagnosed with medication overuse headache (some with migraine) and found significant differences in immune cell proportions, as well as differentially methylated CpGs localised to three genes (*CORIN*, *CCKBR*, and *CLDN9*) between MOH and controls (which included both episodic migraine and healthy controls).[18] However, the Carlsen et al. (2023) study comprised heterogeneous treatment groups, did not examine chronic migraine patients, and found no CpGs associated with reduced headache or migraine days.

We previously conducted the Chronification and Reversibility of Migraine (CHARM) clinical trial study at Leiden Headache Center, which assessed efficacy of acute medication withdrawal and add-on therapy with botulinum toxin A (botox) for chronic migraine and medication overuse.[4] While withdrawal treatment significantly reduced monthly headache and migraine days in responders, botox did not show any additional benefit. Hypothesising that a reduction in headache and migraine days would be accompanied by specific changes in DNAm, here we utilised our unique CHARM clinical trial resource and performed a novel study to examine longitudinal epigenome-wide DNAm profiles from peripheral blood of patients with chronic migraine before and after medication withdrawal treatment. Furthermore, we determined whether DNAm status at baseline was indicative of treatment response. Our findings identify specific epigenetic changes related to favourable treatment response. Understanding these underlying biological processes will ultimately help identify new treatment targets and preventive opportunities.

Results

Demographics and treatment efficacy

Of the 98 chronic migraine patients with medication overuse (simple analgesics and/or triptans) the average age was 47.0 (standard deviation [SD] ± 10.1). At baseline ($T = 0$) most patients used no prophylactic medication (65.3%). Of the participants who were rapidly tapering off their medication at $T = 0$, patients were using beta blockers ($n = 7$), valproate ($n = 6$), topiramate ($n = 3$), pizotifen ($n = 2$), candesartan ($n = 7$) or a combination or other type of prophylactics ($n = 9$). Characteristics of the cohort are shown in Table 1. The 50% responder rate (% individuals with ≥ 50% reduction from baseline) was 16.3% for MHD and 49.5% for MMD (Fig. 1). Of all patients, 59.8%

converted back to episodic migraine by the end of the 12-week withdrawal period ($T = 1$). In the CHARM study we previously demonstrated that in patients with chronic migraine and medication overuse, botox did not lead to any additional benefits over acute withdrawal alone.[4] As expected, this also applied to the current sample (50% responder rate: MMD 51.9% in the placebo group versus 47.8% in the botox group; 50% responder rate MHD: 19.2% in the placebo group versus 15.2% in the botox group; and rate conversion back to episodic migraine: 55.8% in the placebo group versus 65.2% in the botox group). Finally, as to be expected after randomisation, patients receiving botox or placebo were not significantly different in age, gender, BMI, and MHD, MMD, and monthly medication days at baseline ($p > 0.05$).

Table 1. Patient demographics at baseline

Characteristics	Value
Total, n	98
Female, n (%)	74 (75.5)
Age, mean ± SD	47.0 ± 10.1
BMI, mean ± SD	25.4 ± 4.6
Migraine days ± SD	15.2 ± 5.4
Headache days ± SD	21.2 ± 4.8

BMI, body mass index; MMD, monthly migraine days; MHD, monthly headache days; SD, standard deviation

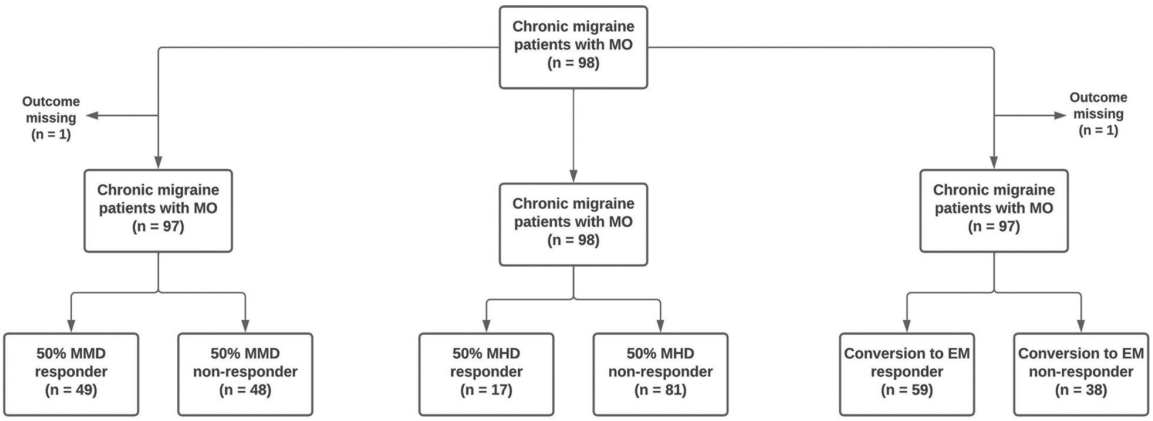

Figure 1. Flowchart treatment effect.

MO = medication overuse, MHD: monthly headache days, MMD; monthly migraine days, EM; episodic migraine.

Longitudinal epigenome-wide DNA methylation differences after treatment

We investigated DNAm levels at two timepoints (baseline [$T = 0$] and after treatment [$T = 1$]) to identify DNAm differences in CpG sites that were associated with treatment response via linear mixed effects models. The Q-Q plot (Additional file 1: Fig. S1) from the epigenome-wide association analysis of changes in DNAm in MHD responders versus non-responders indicated no systematic technical bias or inflation of the test results. The Q-Q plot for MMD produced analogous results (data not shown). At the stringent epigenome-wide *significant* level ($p < 9.42 \times 10^{-8}$), changes in DNAm at an intronic CpG site (probe cg14377273) within the histone deacetylase 4 (*HDAC4*) gene was associated with MHD response following the withdrawal of acute medication ($p = 8.9 \times 10^{-8}$, Table 2, Figs. 2,3). Post-hoc sensitivity analyses showed that this CpG site remained significant even after adjusting for prophylactics use and medication overuse type ($p = 9.4 \times 10^{-8}$). Another 40 CpGs in *HDAC4* were nominally associated with MHD response ($0.05 < p < 3.18 \times 10^{-4}$, Additional file https://clinicalepigeneticsjournal.biomedcentral.com/articles/10.1186/s13148-023-01604-8#Sec14 : Table S1). Using the Blood Brain DNA methylation comparison tool, we observed that several of the *HDAC4* CpG probes had a high correlation between blood and brain prefrontal cortex DNAm patterns (e.g., cg15376007: $r = 0.47$, $p = 2.47 \times 10^{-5}$; and cg10118705: $r = 0.26$, $p = 0.028$). Functional annotation using the Drug-Gene Interaction database (DGIdb) indicated that *HDAC4* is a 'clinically actionable' gene with a range of HDAC inhibitors available.[19] At the epigenome-wide *suggestive* level ($p < 5 \times 10^{-5}$), an additional 40 CpGs across the genome were associated with MHD response ($4.70 \times 10^{-5} < p < 3.93 \times 10^{-7}$, Additional file 2: Table S2). Overall, the CpGs associated with MHD response ($p < 0.05$) were significantly enriched for genes previously associated with migraine and/or headache traits in GWAS (p value of enrichment = 0.011), indicating that the overlap of genes was more than expected by chance.[20-24] Although no CpG sites showed epigenome-wide significant association with MMD response, seven CpGs were associated at the suggestive level ($4.03 \times 10^{-5} < p < 2.30 \times 10^{-6}$, Additional file 2: Table S3). Similarly, no CpG sites showed epigenome-wide significant association when comparing the converted episodic migraine individuals to the remaining chronic migraine individuals, however, ten CpGs were associated at the suggestive level ($4.86 \times 10^{-5} < p < 1.04 \times 10^{-5}$, Additional file 2: Table S4).

Table 2. CpG sites significantly associated with treatment efficacy in chronic migraine

CpG	Gene	Chr	Location in gene	Effect size [SD]	*P* value	Direction (T0 to T1)	Analysis model
cg14377273	*HDAC4*	2	Body	0.02 [0.016]	8.9×10^{-8}	reduced DNAm	longitudinal DNAm ~ MHD
cg15205829	*MARK3*	14	Body	0.02 [0.001]	4.1×10^{-8}	*not applicable*	baseline DNAm ~ MMD

CpG, 5′-C-phosphate-G-3′; Chr, chromosome; SD, standard deviation; MHD, monthly headache days; MMD; monthly migraine days

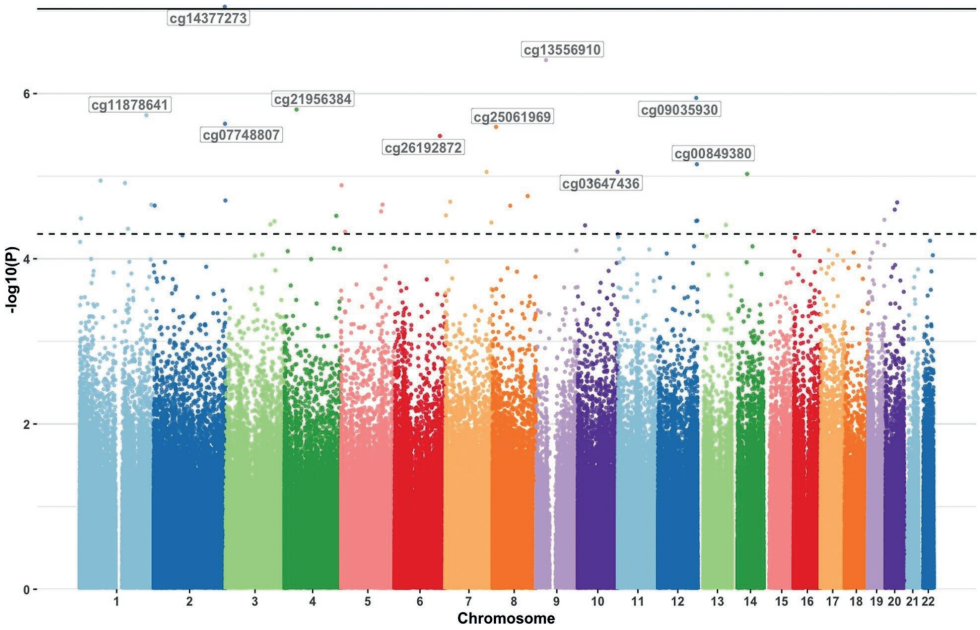

Figure 2. Manhattan plot from the epigenome-wide association study.

Manhattan plot showing the $-\log_{10}(p \text{ value})$ for each CpG site associated with changes in DNA methylation in monthly headache days (MHD) responders versus non-responders. The threshold for epigenome-wide significant association ($p < 9.42 \times 10^{-8}$) is indicated by a solid black line. The threshold for epigenome-wide suggestive association ($p < 5 \times 10^{-5}$) is indicated by a dashed black line

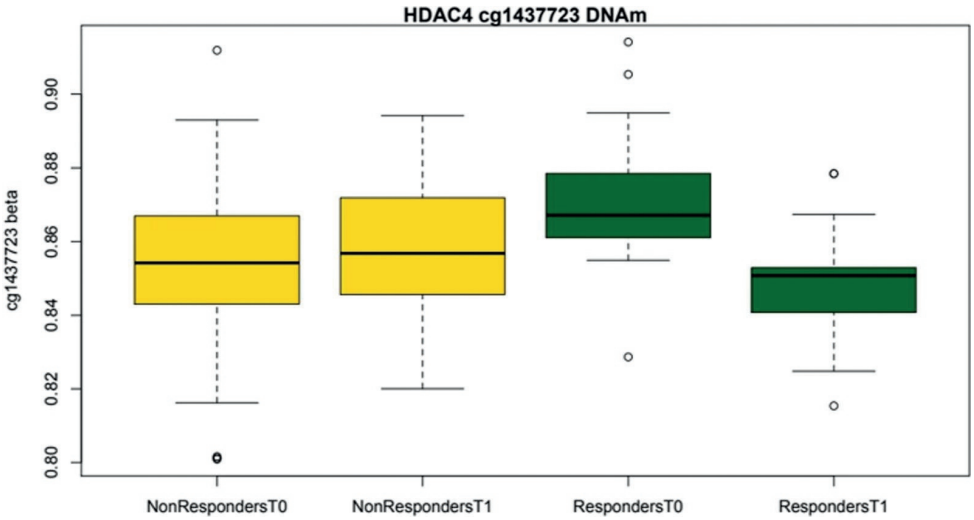

Figure 3. Median of differential *HDAC4* DNA methylation in patients with and without ≥ 50% reduction in monthly headache days (MHD).

Associations between epigenome-wide DNA methylation at baseline and treatment response

Next, we assessed whether DNAm profiles at baseline associated with treatment response after 12 weeks. Analogous to the longitudinal DNAm results, Q-Q plots of the baseline DNAm analysis for MHD and MMD indicated no systematic technical bias or inflation of the test results (data not shown). Methylation levels at one CpG probe (cg15205829) within the *MARK3* gene was associated with MMD response ($p = 4.13 \times 10^{-8}$) at the epigenome-wide significant threshold, with decreased DNAm levels at baseline associated with reduced MMD (Table 2, Fig. 4). At the epigenome-wide suggestive level, an additional 114 CpGs across the genome were associated with MMD response, including a second probe (cg26267011, $p = 4.84 \times 10^{-5}$) in *MARK3* ($4.98 \times 10^{-5} < p < 1.19 \times 10^{-6}$, Additional file 2: Table S5, Fig. 4). *MARK3* is currently not listed as a 'clinically actionable' drug target in DGIdb (i.e., there are no known drug-gene associations for *MARK3*). No CpG sites were associated with MHD response at the epigenome-wide significant threshold; however, 109 CpGs were associated at the suggestive level ($4.82 \times 10^{-5} < p < 6.42 \times 10^{-7}$, Additional file 2: Table S6).

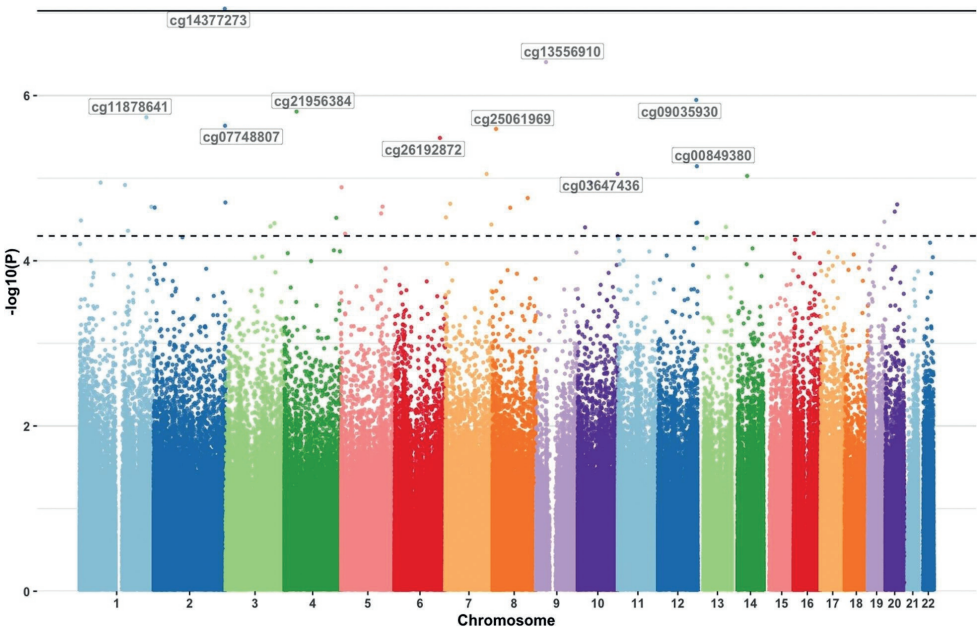

Figure 4. Manhattan plot from the epigenome-wide association study of baseline DNA methylation predicting treatment response.

Manhattan plot showing the $-\log_{10}(p \text{ value})$ for each CpG site associated with baseline DNA methylation in responders versus non-responders at T1. The threshold for epigenome-wide significant association ($p < 9.42 \times 10^{-8}$) is indicated by a solid black line. The threshold for epigenome-wide suggestive association ($p < 5 \times 10^{-5}$) is indicated by a dashed black line

Discussion

In this large longitudinal epigenome-wide association study (EWAS) we identified DNAm differences significantly associated with response to medication withdrawal treatment in individuals with chronic migraine. Our findings of CpG sites associated with improvement of headache and migraine days after treatment are of important clinical relevance. A longitudinal change in DNAm at a CpG site within an intron of *HDAC4* was associated with MHD response, while baseline DNAm levels at a CpG in *MARK3* were associated with MMD response at 12 weeks.

The strongest association with reduced headache days was for an intronic CpG within *HDAC4* (cg14377273, $p = 8.90 \times 10^{-8}$); post-hoc sex-specific analysis indicated this CpG was associated in both sexes ($p = 3.73 \times 10^{-6}$ in females and $p = 0.005$ in males). Nearby SNP rs7581200 is an expression quantitative trait locus (eQTL) for *HDAC4* in the GTEx database suggesting localisation of a regulatory element in this region. Another 40

CpG probes spanning the *HDAC4* gene showed associations at the nominal level (Table S1). *HDAC4* encodes a Class IIa histone deacetylase, and along with co-repressors such as MEF2D encoded by a previously reported migraine GWAS locus,[20,23] its activity targets lysine residues on core histone tails to repress transcription. HDAC4 is highly expressed in the brain and is a major player in synaptic plasticity [25,26] and modulates the expression and release of several neuroinflammation markers, including HMGB1 and NF-κB,[27,28] which have been implicated in migraine pathophysiology.[29] HDAC4 has previously been implicated in chronic pain disorders such as fibromyalgia.[30,31] Furthermore, selective knockout of *Hdac4* in peripheral sensory neurons in two independent mice lines found attenuated development of chronic inflammatory pain, indicating a role for HDAC4 in peripheral sensitization and inflammation-associated thermal hypersensitivity.[32] The conditional *Hdac4* deletions resulted in significant transcriptional dysregulation of genes involved in pain sensitivity, such as *Calca* and *Trpv1*, encoding Calcitonin Gene-Related Peptide (CGRP) and Transient Receptor Potential Cation Channel Subfamily V Member 1 (TRPV1) respectively—both also known to be involved in migraine.[33,34]

Differentially methylated CpGs were also detected in the genes *HDAC1* and *HDAC3* among sites with nominal association ($p < 0.05$) with reduced headache and migraine days in our study. There is evidence for several HDACs being involved in regulating the processes involved in pain chronification. For example, inhibition of HDAC6 decreased cephalic allodynia and reversed cyto-architectural changes in headache-processing brain regions in a mouse model of chronic migraine-associated pain.[35] Thus, non-specific HDAC inhibitors may present a therapeutic avenue for migraine and headache-related disorders via multiple pathways including regulating gene transcription as well as regulating structural proteins. Notably, valproate, a widely prescribed migraine prophylaxis, is a known inhibitor of HDAC activity.[19] Further support for the potential therapeutic utility of HDAC inhibitors in prevention and/or reversal of chronic migraine is provided by a study in a rat model of MOH, that found two pan-HDAC inhibitors (panobinostat and givinostat) reduced expression of the genes coding for calcitonin gene-related peptide (CGRP) and its receptor subunit Receptor Activity Modifying Protein 1 (RAMP1), whose proteins are known to have key roles in migraine pathogenesis and MOH.[36]

The CpG predictive of favourable MMD response after medication withdrawal treatment at baseline was located in *MARK3*, which encodes microtubule affinity regulating kinase 3 (MARK3, also known as CTAK1). MARKs are serine/threonine kinases that regulate numerous cellular functions such as cell polarity, cell cycle progression, glucose metabolism and cytoskeletal dynamics.[37,38] MARKs, including MARK3,

regulate TRESK (TWIK-related spinal cord K^+ channel, *KCNK18*) a major background K(+) channel of sensory neurons,[39] in which mutations can lead to hyperexcitability of trigeminal ganglion neurons.[40] *MARK3* mRNA is specifically transported to and translated in axons of adult dorsal root ganglion neurons,[41] raising the possibility that microtubule dynamics is coupled to the regulation of excitability in neurons.[39] Notably, downstream substrates of MARK3 include several HDACs, of which HDAC4 is one; and in response to specific signals, MARK3 phosphorylates HDACs on their binding sites for the adaptor protein 14–3–3, impairing interactions with 14–3–3, which regulates HDAC nuclear/cytoplasmic localisation and the transcriptional repressor function of HDACs.[42] Therefore, the potential role of MARK3 in migraine chronification may also be via regulation of HDACs.

The female sex hormone estrogen plays important roles in migraine frequency,[43] synaptic plasticity,[44] and in the regulation and recruitment of HDACs.[45,46] With respect to HDAC4, Maddox et al. (2018) identified an estrogen-dependent association of *HDAC4* methylation and expression with fear regulation and PTSD risk in both female mice and women, which may contribute to the increased risk among women for PTSD.[47] Therefore, a pathway involving HDAC4 and estrogen might also partially explain why women have a higher risk for migraine chronification.

Our findings thus provide support for a potential role of pathways related to chromatin structure, and synaptic plasticity that may have relevance to migraine chronification and its reversibility. It remains to be determined whether such changes are a cause or consequence of a decreased frequency of attacks. Interestingly, a previous case–control EWAS comparing 36 female chronic headache patients to 35 female episodic headache patients found the two strongest associated loci were linked to brain-expressed genes (*SH2D5* and *NPTX2*) that are involved in the regulation of synaptic plasticity,[5] although these were not experiment-wide significant. In contrast to our study, longitudinal analysis of MOH patients by Carlsen et al. (2023) did not find any significant DNAm changes associated with reduction in headache frequency over a 6-month period.[18] Importantly, our study was specifically restricted to chronic migraine patients undergoing medication withdrawal, while the Carlsen et al. (2023) study comprised a variety of migraine and tension headache patients, as well as heterogeneous treatment strategies.

We found little overlap between loci at the suggestive level of association between the different endpoints (i.e., MHD response, MMD response, or chronic to episodic migraine conversion). While this could be due to insufficient statistical power (to detect smaller effects), it suggests that while we might expect similarities between migraine

and non-migraine days in patients with chronic migraine from a pathophysiological viewpoint, these days may be influenced by different mechanisms. For example, headache days in patients with chronic migraine with medication overuse might be due to medication overuse mechanisms as opposed to migraine-specific mechanisms. Enrichment analysis of CpGs associated with MHD response ($p < 0.05$) showed significant overlap with genetic loci previously associated with migraine and/or headache traits in GWAS ($p = 0.011$). Although, given that our study only assessed patients with chronic migraine and evaluated treatment, whereas GWAS tests for genetic differences between patients and controls, it is perhaps reasonable to expect only a modest overlap between genes associated with a reduction in MHD and GWAS loci.

Our study had several potential limitations. Firstly, before trial commencement, individuals were on different prophylactic medications, which may influence their epigenetic profile. However, 65% of patients were not using prophylactics at the beginning of the study and the remaining patients were tapered off medications as quickly as possible. Furthermore, we adjusted for prophylactic use in our analyses. Secondly, tissue-specific changes could be missed when analysing DNAm in blood, rather than tissues perceived to be more relevant to headache, such as brain. However, our observation that many of our differentially methylated genes are neuronally expressed and had high correlations between blood and brain DNAm, suggests that blood DNA can indeed reflect methylation changes in the brain. Furthermore, while our longitudinal study design greatly increases our power, direct replication of the findings in an independent cohort may be challenging given the intricacy of our clinical trial and longitudinal study design. Other designs, such as a large case–control study comparing DNAm between several hundred patients with chronic migraine and several hundred patients with episodic migraine, should be able to replicate DNAm associated with headache chronification. However, such cross-sectional studies will not be able to predict response for conversion to episodic migraine, as in the current study. Further work is required to understand the functional impacts of the implicated DNAm sites, such as their impact on gene expression. Lastly, our study only investigated DNAm and did not investigate other epigenetic changes, such as histone modifications.

Despite these limitations, our longitudinal study design is a powerful and robust method to detect within-individual DNAm changes caused by a response to acute medication treatment withdrawal. Other strengths include a well-characterised cohort of patients with chronic migraine and medication overuse, as opposed to studies including medication overuse headache, regardless of what underlying primary headache disorders might be present, and small cohort size.

Conclusions

Understanding migraine chronification is vital for developing better treatment strategies and to prevent chronification. Epigenetic changes in genes represent potential treatment targets and identify mechanisms involved in migraine chronification. In summary, we identified a longitudinal reduction in *HDAC4* DNAm status associated with treatment response and implicated baseline *MARK3* DNAm status as an early biomarker for treatment response. Our findings provide support for a role of pathways related to chromatin structure, gene regulation, and synaptic plasticity in headache chronification and highlight *HDAC4* and *MARK3* as viable therapeutic targets, particularly considering convergence of their pathways and previous studies showing efficacy of general HDAC inhibitors in the treatment of migraine and MOH symptoms.

Methods

Study design and population

This study was conducted as part of the Chronification and Reversibility of Migraine (CHARM) clinical trial study at the Leiden Headache Center, which is described in detail elsewhere.[4] Briefly, consecutive patients aged 18–65 years, diagnosed with chronic migraine and medication overuse according to the International Classification of Headache Disorders (ICHD)-3 criteria,[2] were enrolled. Exclusion criteria were: (i) other primary headache or neurological disorders; (ii) other chronic pain disorders with medium to high pain intensity or requiring pain medication; (iii) major psychiatric disorders, other than depression; (iv) major cognitive, behavioural or oncologic disorders; (v) contraindications for treatment, or inability to adhere to study protocol (vi) (planned) pregnancy or breastfeeding (vii) use of ergots, opioids or barbiturates; (viii) abuse of drugs in the past 12 months. The selected participants were a representative subset of the full CHARM cohort. No significant differences were found when selected participants were compared with non-selected participants on gender, BMI, MHD, MMD, and monthly medication days at baseline ($p > 0.05$). The selected participants were slightly older than the non-selected participants (mean ± SD: 47.0 ± 10.1 versus 43.1 ± 11.2, respectively, $p = 0.02$).

Participants started with a 4-week baseline-assessment period followed by a 12-week withdrawal period, consisting of instruction to withdraw abruptly from all acute anti-headache medications and caffeine ('advice-only'). Prophylactic treatment was tapered off and rescue medication was not allowed. Immediately prior to withdrawal, botulinum toxin A (botox) or placebo injections were administered in a randomised, double-blind manner. In the 12-week withdrawal period, no other prophylactics were

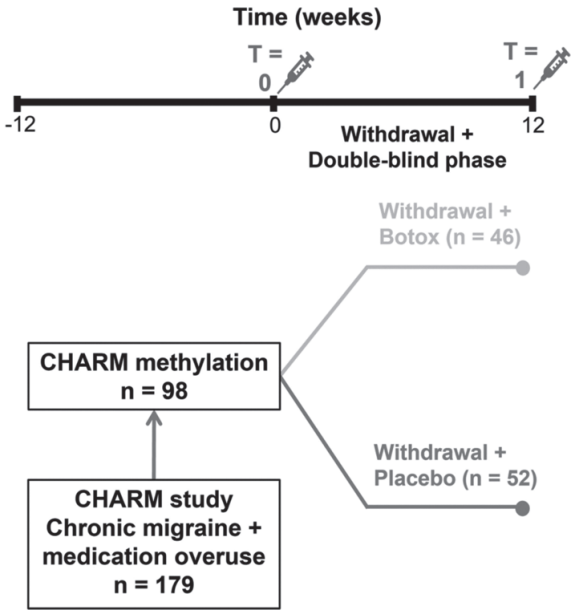

Figure 5. Study protocol.
Botox = Botulin toxin A according to PREEMPT protocol, see Pijpers et al. (2019)[4]

started. Blood samples were taken at baseline ($T = 0$) and at 12 weeks ($T = 1$) (Fig. 5). Patients were selected from the CHARM study based on DNA sample availability, DNA concentration and order of participation. The sample size was well-powered to detect effect sizes equivalent to those found in previous longitudinal studies.[48]

Clinical outcomes

All participants prospectively kept a 4-week diary during the baseline assessment period and the post-treatment period (weeks 9–12). This included daily registration of headache characteristics, accompanying symptoms and acute headache medication used. A migraine day was defined as a day fulfilling criteria for migraine or treated with migraine-specific acute medication.[2] Headache days were defined as a day with a migraine or non-migraine headache. We compared changes in DNAm in monthly headache days (MHD) responders (defined as patients with ≥ 50% reduction in MHD) versus non-responders (patients with < 50% reduction in MHD), and in monthly migraine days (MMD) responders (defined as patients with ≥ 50% reduction in MMD) versus non-responders (patients with < 50% reduction in MMD). We also performed an exploratory analysis with the outcome of reversion from chronic to episodic migraine (i.e., headache no longer fulfilling criteria of chronic migraine).

Samples

Genomic DNA was extracted from peripheral blood leukocytes according to standard protocols. A total of 196 samples were included for DNAm and analysed using the Illumina EPIC DNA methylation arrays (> 860,000 CpG sites at single-nucleotide resolution), using standard manufacturer protocols at the Genomics Research Centre, Queensland University of Technology. Briefly, 500 ng genomic DNA was converted with bisulfite treatment using EZ DNA Methylation Kits (Zymo Research, USA). Then, samples were whole-genome amplified, enzymatically fragmented, and hybridised overnight to locus-specific probes on Illumina Infinium EPIC Beadchips. After a wash step, hybridised probes on the Beadchips underwent a single-base extension reaction followed by a multilayered staining process before scanning on an Illumina HiScan for detection of probe intensities.

Quality control and statistical analysis

The raw values from the Illumina EPIC DNA methylation arrays were imported using GenomeStudio software (Illumina) and uploaded into R (https://www.r-project.org/, version 3.4.0) for further processing. Data were analysed using an established analysis pipeline comprising custom statistical programs and scripts in R.[49] Samples with probe detection call rates < 95%, as well as those with an average intensity value of either < 50% of the experiment-wide sample mean or < 2,000 arbitrary units (AU), were excluded from further analysis. Intensity readouts, normalisation and DNAm beta value (the ratio of the fluorescent signals for the methylated versus unmethylated sites) calculations were performed using the Bioconductor MINFI package version 1.20.2.[50] Subset-quantile Within-Array Normalisation (SWAN) was used to remove technical differences between Infinium I and Infinium II probes available in the MINFI package.[51] The DNAm status for each probe was recorded as a β-value that ranged between 0 and 1, where values close to 1 represent high levels of DNAm and values close to 0 represent low levels of DNAm. Probes with > 50% of the samples with a detection p value > 0.05 and probes with single nucleotide polymorphisms (SNPs) present within 50 bp from the query site were removed. This resulted in a total of 865,823 CpG probes for further analyses.

Using the MINFI package, initial quality checks were performed to predict the sex status of the samples and check for consistency with the true sample sex (predicted sex was consistent with true sex for all samples). Cell type composition was predicted using the DNAm data (CD8T, CD4T, NK, B-cell, Monocytes and Granulocytes) using the Houseman method.[52]

Linear mixed effects models were used to investigate differential DNAm across all 865,823 CpG probes and test its association with phenotypes as repeated measures using the lme4 package in R. Details of the statistical analysis models used, including the R syntax of the analysis models, are provided in Additional file 3: Supplementary Methods. Applying these analysis models in our longitudinal study design is highly powerful, as they retain the within-person structure (repeated measures across a person) while assessing longitudinal differences in DNAm across the timepoints (before and after treatment). That is, one of the major sources of DNAm variability is between-participant variability. Therefore, by repeating measures within participants, each participant acts as its own control, and the between-participant variability is removed. Hence, our paired-test study design is more likely to detect true differences in DNAm between the paired measures and is a powerful approach. Indeed, the within-person Spearman correlations ranged between $r = 0.959–0.989$, with an average and standard deviation (r[SD]) = 0.979[0.009], this is much higher than that observed in MZ twins.[53] Even ignoring this increased correlation, our study design is at least equivalent to comparing 100 unrelated cases to 100 unrelated controls, for which power calculations using the EPIC array power calculator[54] found at https://epigenetics.essex.ac.uk/shiny/EPICDNAmPowerCalcs/, indicate 24.4% of CpG sites have > 80% power to detect an effect of 2%, 53% of sites have > 80% power to detect an effect of 3%, 72.8% of sites have > 80% power to detect an effect of 4%, and 85% of sites have > 80% power to detect an effect of 5%. These estimates of power are conservative given the longitudinal study design and the high within-person correlation we observe in the study. In summary, our study is well-powered to detect small to moderate DNAm differences. Furthermore, we evaluated whether DNAm status at baseline could be used to predict response to withdrawal using the glm function in R. All analyses were corrected for age, sex, smoking, botox injection, body mass index (BMI), and cell type counts.

We also performed post-hoc sensitivity analyses to account for prophylactic use and medication overuse type, and found all results remained significant after adjusting for medication use ($p < 9.42 \times 10^{-8}$). We previously demonstrated in the CHARM study patients with chronic migraine and medication overuse, that botox did not lead to any additional benefits over acute withdrawal alone;[4] however, to ensure that the observed results were not caused by botox injection, we controlled for botox treatment status in our primary analyses and also performed a post-hoc analysis to evaluate whether both the placebo and botox injection group demonstrated an association. None of the presented results were affected by botox injection. Top-ranked CpGs that met a stringent significance level of $p < 9.42 \times 10^{-8}$ were considered *significant* at the epigenome-wide level—the recommended threshold accepted to reduce the rate

of false positives in DNAm studies.[54] The significance level of $p < 5 \times 10^{-5}$ was used to denote *suggestive* sites of relevance at the epigenome-wide level,[55] and sites with $p < 0.05$ were considered nominally significant.

The Blood Brain DNA methylation comparison tool[56] was used to check the correlation between blood and brain DNAm for the significant CpGs. This tool was established for the older 450 K DNA methylation array and therefore does not contain all the CpG probes analysed in this study. To compare results to known genes associated with migraine and/or headache traits, we compiled a list of 184 genes (using the closest gene to a genome-wide significant SNP) identified from five GWASs for migraine and headache,[20-24] of which 118 unique genes corresponding to 4,386 CpGs were present in the current dataset. To test whether the overlap of genes was more than expected by chance, enrichment testing was performed using 1,000 permutations (using random sets) and applying a two-sided Binominal test in R to give a *p* value of enrichment. Functional annotation of the genes corresponding to the CpG sites at the suggestive genome-wide significance level ($p < 5 \times 10^{-5}$) was performed and the Drug-Gene Interaction database (DGIdb, https://www.dgidb.org/, v4.2.0)[57] was used to assess whether these sites were in genes involved in drug-gene interactions—i.e., whether there is a known interaction (e.g., inhibition) between a known drug and a target gene. These genes are targeted by specific known compounds (i.e., it describes whether the gene is 'currently actionable'). We also assessed whether genes were druggable candidates according to the DGIdb. Druggable candidates are genes that are thought to be potentially druggable by various methods of prediction. As such, genes in these categories are 'potentially druggable' and may or may not have existing drugs that target them.

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Ethics approval and consent to participate

The study was performed in accordance with the declaration of Helsinki Ethical Principles and approved by the local and national ethics committees. All patients provided written informed consent. Our cohort was collected at the Leiden University Medical Centre as part of the CHARM study (trial register identifier: NTR3440), a

randomised clinical trial aimed at assessing whether add-on therapy with botox enhanced the efficacy of acute withdrawal in chronic migraine patients.

Competing interests

D. Mehta reports no competing interests. I. de Boer reports grants from Dutch Heart Foundation and the International Retinal Research Foundation (IRRF). H.G. Sutherland, J.A. Pijpers, C. Bron, C. Bainomugisa and L.M. Haupt report no competing interests. L.R. Griffiths reports migraine research funding from the Australian National Health and Medical Research Council (NHMRC-APP1122387), US Migraine Research Foundation, US Dept of Defence and Teva. A.M.J.M. van den Maagdenberg reports grants or consultancy support of Schedule 1 Therapeutics, Praxis Precision Medicine and AbbVie and independent support from Dutch Research Council (NWO and ZonMW) and the European Community. D.R. Nyholt reports migraine research funding unrelated to this study from the Australian National Health and Medical Research Council, US Migraine Research Foundation, US Department of Defence and Teva. G.M. Terwindt reports grants or consultancy support from Novartis, Lilly, Teva, Allergan, Lundbeck and independent support from Dutch Research Council (NWO and ZonMW), European Community, Dutch Heart Foundation, Dutch Brain Foundation, IRRF, and Dioraphte.

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4

CHAPTER 4

Neurological and psychiatric comorbidities of migraine: Concepts and future perspectives

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Abstract

Background: This narrative review aims to discuss **s**everal common neurological and psychiatric disorders that show comorbidity with migraine. Not only can we gain pathophysiological insights by studying these disorders, comorbidities also have important implications for treating migraine patients in clinical practice.

Methods: A literature search on PubMed and Embase was conducted with the keywords "comorbidity", "migraine disorders", "migraine with aura", "migraine without aura", "depression", "depressive disorders", "epilepsy", "stroke", "patent foramen ovale", "sleep wake disorders", "restless legs syndrome", "genetics", "therapeutics".

Results: Several common neurological and psychiatric disorders show comorbidity with migraine. Major depression and migraine show bidirectional causality and have shared genetic factors. Dysregulation of both hypothalamic and thalamic pathways have been implicated as a possibly cause. The increased risk of ischaemic stroke in migraine likely involves spreading depolarizations. Epilepsy is not only bidirectionally related to migraine, but is also co-occurring in monogenic migraine syndromes. Neuronal hyperexcitability is an important overlapping mechanism between these conditions. Hypothalamic dysfunction is suggested as the underlying mechanism for comorbidity between sleep disorders and migraine and might explain altered circadian timing in migraine.

Conclusion: These comorbid conditions in migraine with distinct pathophysiologic mechanisms have important implications for best treatment choices and may provide clues for future approaches.

Introduction

There are prevailing theories about migraine pathophysiology, foremost that cortical spreading depolarization (CSD) is the mechanism underlying the migraine aura and that activation of the trigeminovascular system causes the headache.[1] The exact mechanisms for attack initiation are still unknown, which complicates the identification of novel treatment targets. Several neurological and psychiatric disorders are comorbid with migraine, and some increase the chance of migraine chronification. These comorbidities may provide clues for shared pathophysiological mechanisms, either through direct causal relationships or through shared underlying genetic and environmental factors. For this review we selected highly prevalent neurological and psychiatric comorbidities that have important clinical impact, are well-researched, and have the potential to increase our understanding of migraine. As such, this review explores the most recent advances in understanding the comorbidity of migraine with mood disorders, ischemic stroke, epilepsy and sleep disorders. We highlight clues about shared underlying mechanisms and brain regions (figure 1), with an emphasis on genetic risk factors. While clinical trials on treating

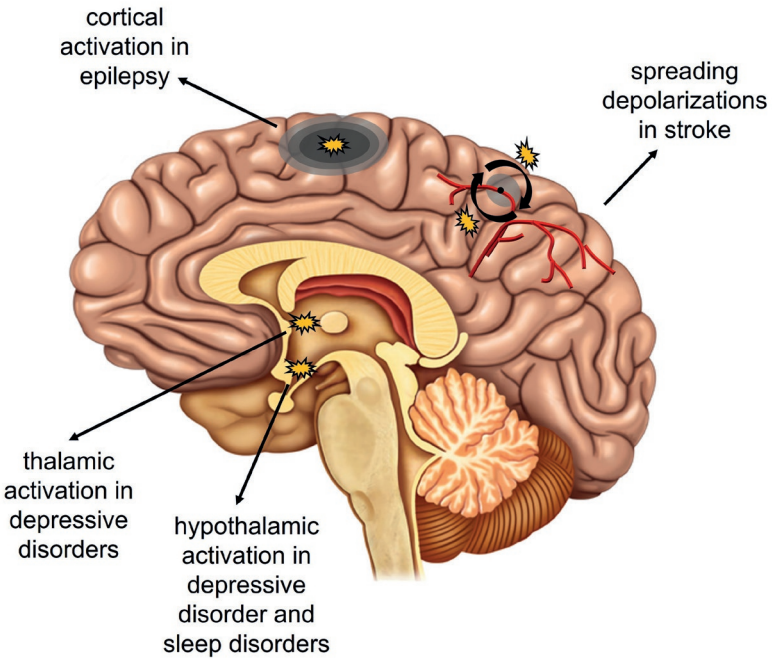

Figure 1. Brain regions most involved in the comorbidity of migraine with depression, stroke, epilepsy and sleep disorders.

migraine with comorbidities are scarcely available, we focus on possible treatment consequences and practical advice that health-care providers taking care of migraine patients should be aware of.

Migraine and mood disorders

Epidemiology

Depression and anxiety are both associated with an increased risk of migraine, which is even higher when both co-occur in a patient.[2] Around 45-65% of patients with a depressive disorder meet the criteria for an anxiety disorder, *vice versa* 30-65% of patients with an anxiety disorder also have depression.[3] Comorbidity of migraine and depression is bidirectional. Lifetime prevalence of depression is three times higher in migraine patients and having migraine constitutes higher risk for onset of depression (relative risk [RR] ~1·5).[4] Migraine prevalence in patients with bipolar disorder is also increased, with estimates ranging from ~30-50%.[5]

A direct causal effect of pain on depressive symptoms seems plausible. However, major depression signals higher risk for first occurrence of migraine (odds ratio [OR] ~3·4), but not for other headaches.[6] Yearly, 3% of patients convert from episodic to chronic migraine (≥15 headache days/month, of which ≥8 migraine days).[7] Depressive symptoms and allodynia during attacks and acute medication overuse are interlinked and lead to chronification.[8]

Pathophysiological mechanisms

Thalamic pathways may be implicated in migraine attack initiation foremost in central sensitization during attacks, which is experienced as allodynia.[9] Decreased thalamic activity has been reported in migraine with comorbid depression, compared to migraine and (major) depression separately, implicating a habituation deficit.[10] Diverse neurochemical pathways converge on thalamic neurons, including noradrenergic, dopaminergic and serotonergic pathways implicated in depression, depicting an intersection of pathways for migraine. A magnetic resonance spectroscopy (MRS) study supported this suggested thalamocortical dysfunction in migraine chronification, as evidenced by bilateral thalamic N-acetyl-aspartate reductions.[11] Although migraine and depression pathophysiology involves monoamine neurotransmitters, how these neurotransmitters contribute towards disease needs further elucidation.[12] In the past most pathophysiological explanations concerning migraine and depression comorbidity focused on serotonin (5-HT). For instance, $5HT_{1a}$ receptor density was increased in migraine on positron emission tomography,[13]

but decreased in depression.[14] Regardless, it is now widely accepted that additional neurotransmitter mechanisms are involved.

Premonitory and ictal symptoms in migraine such as nausea and yawning supposedly involve the hypothalamus. The observation that both migraine and bipolar disorder are associated with an evening chronotype, suggests involvement of the circadian system pointing towards hypothalamic involvement ,[5] and might suggest a role for dopamine. While dopamine is a major neurotransmitter involved in depression, its importance in migraine is still disputed.[12]

Alternatively, depression is hypothesized to involve dysregulation of the adaptive stress response, an innate mechanism aimed at restoring homeostatic level altered by chronic stress. Eventually, this might progress to multiple systemic pathologies such as inflammation and a prothrombotic state potentially resulting in development of co-morbid conditions such as migraine.[15]

Shared genetic susceptibility

Migraine and mood disorders show familial occurrence, with complex inheritance and a combination of genetic and environmental factors together conferring disease susceptibility. In genome-wide association studies (GWAS) many susceptibility loci have been identified for migraine and depression .[16,17] Combining large GWAS provided evidence for genetic overlap for these disorders (cross-disorder genetic correlation (rG = 0·25)),[18] while pointing at neural-related pathways of signaling and ion channel (dys) regulation. A small study found that a family history of bipolar disorder is associated with an increased risk of migraine (OR ~4·4), suggesting a shared genetic basis, but could not establish an increased risk of migraine in those with a family history of major depressive disorder.[19] Several mood disorders, including major depressive disorder and bipolar disorder, show shared genetic etiology.[20] In the Brainstorm Consortium collaboration, the genetic sharing of 25 brain disorders (>250,000 patients, >750,000 controls) were quantified based on GWAS.[21] While neuropsychiatric disorders showed considerable sharing of common variants, neurological disorders appeared more distinct from one another and from the psychiatric disorders, except for migraine that revealed a shared genetic basis with depression (rG = 0·32).[21] A possible explanation for the former is that clinical neuropsychiatric disease features show considerable overlap, more than for neurological disorders.

Epidemiological and genetic studies suggest that migraine with aura patients have higher risk of developing depression compared to patients suffering from migraine without aura (OR ~1·7).[22] Increased risk was found for hemiplegic migraine (OR ~3·7),

a subtype with motor auras.[23] Hemiplegic migraine can be caused by mutations in genes encoding ion transporters subunits, *CACNA1A*, *ATP1A2* and *SCN1A* and functional characterization of these mutations point to neuronal and glial dysfunction, resulting in enhanced glutamatergic neurotransmission.[24] Of note, studies using *in vivo* proton MRS reported decreased levels of glutamate metabolites in regions of the medial frontal cortex in depression,[25] while an increase was demonstrated in the visual cortex in migraine.[26]

Shared environmental factors

Exposure to stress, primarily regulated by corticotropin-releasing factor (CRF)-containing neural circuits and the hypothalamic–pituitary–adrenal (HPA) axis, can precipitate depression. Stress is a frequently self-reported migraine trigger,[27] but there is little proof for abnormal stress response in migraine patients.[12] Perceived acute stress might instead be part of the premonitory phase, which is characterized by other symptoms such as tiredness and food cravings.[28] As such, premonitory symptoms can be misidentified as triggers. Depression is a major determinant of perceived stress in migraine patients.[29] Migraine, depression and medication overuse form a complex triad. Depression is a risk factor for developing medication overuse headache.[8] Withdrawal from medication is an efficacious treatment for patients with chronic migraine and medication overuse.[30] However, worldwide there is debate on whether to withdraw first or add preventive treatment. Patients with comorbid depression are often excluded from such studies as they are more treatment resistant.

Consequences for clinical practice

Medication overuse is often implicated in patients with comorbid depression and chronic migraine. Withdrawal of all acute medications should be considered first choice of treatment, ideally supported by a headache nurse.[31,32] When initializing prophylactic treatment, screening for comorbid depression is recommended. This is because prophylactic drugs such as topiramate, flunarizine and beta-blockers may provoke depressive symptoms,[33] whereas valproate may have mood-stabilizing effects. Anti-depressant drugs may be effective against both depressive symptoms and migraine (Table 1). Amitriptyline might be particularly effective when patients also have tension-type headache; nortriptyline has not been studied systematically. Selective serotonin reuptake inhibitors (SSRIs) or selective norepinephrine reuptake inhibitors (SNRIs) for migraine prophylaxis seem not sufficient.[34] Risk of serotonin syndrome in patients taking SSRI or SNRI and using triptans, serotonin agonists for acute treatment in migraine, is low.[35] Lithium is often the first treatment for preventing mood instability in bipolar disorder. Interestingly, a history of migraine was associated with failure to respond to lithium.[36] Finally it is important to realize that the presence

of depression might influence a patient's response to treatment. One such example is Onabotulinumtoxin A, in which a poorer response was associated with depressive symptoms.[37] Recently, calcitonin gene-related peptide (CGRP) monoclonal antibodies together with small-molecule antagonists have become available as anti-migraine drugs.[38] An increasing amount of studies have suggested that the presence of depression or depressive symptoms was associated with a poorer response to anti-CGRP monoclonal antibodies.[39,40] However, not all studies found this association.[41] Moreover, in a post-hoc analysis anti-CGRP preventive treatment appeared effective in preventive treatment of chronic migraine in patients with comorbid depression compared with placebo.[42]

Table 1. Recommendation regarding anti-migraine treatment and comorbidities

Condition	Acute treatment	Prophylactic treatment	Safety concerns
Depression	- Be warrant of medication overuse (especially in high-frequent migraine) - If medication overuse is present, withdrawal of acute medication should be considered as first-choice treatment	- Valproate (mood stabiliser) - Amitriptyline (especially if tension type headache is also present)	- Risk of serotonin syndrome in patients taking SSRI/SNRI and triptans is low - Flunarizine, topiramate and beta-blockers may provoke depressive symptoms
Stroke	- Triptans are considered safe (even though they have vasoconstrictive effects) - Ditans		- Anti-CGRP treatment is not recommended until (long-term) safety evidence is available - When in doubt whether symptoms are migrainous or ischemic, treatment with IVT seems safe
Epilepsy		- Valproate - Topiramate - Lamotrigine	
Sleep disorders (insomnia*)		- Cognitive behavioural therapy - Amitriptyline no proven efficacy for insomnia	- Benzodiazepines may increase risk of developing migraine

* No specific treatment recommendations for other sleep disorders are available. SSRI= selective serotonin reuptake inhibitors; SNRI= selective norepinephrine reuptake inhibitors; CGRP= calcitonin gene-related peptide; IVT= intravenous thrombolysis

Migraine and stroke

Epidemiology

Migraine doubles the risk for ischemic stroke, most evidently for migraine with aura.[43] Migraine may increase individual vulnerability to ischemic stroke during acute brain ischemia,[44] for hemorrhagic stroke this is controversial.[45] Migraine can be a stroke risk factor or might be an (early) symptom of underlying structural abnormalities.[46] Population-based studies showed increased white matter hyperintensities and posterior lesions in migraine.[47] Although progressive over time, lesion development occurs independently of migraine attack frequency,[47] and total lesion load remains limited.[48]

Pathophysiological mechanisms

The mechanism underlying migraine aura is cortical spreading depolarization (CSD), a wave of neuronal and glial cell depolarization that propagates over the cortex, followed by a prolonged phase of nerve cell depression,[1] associated with hypoperfusion.[49] This neurovascular mechanism may be the link between migraine and stroke. Although CT-perfusion scans are mostly normal in migraine with aura patients that present to the emergency room as a stroke mimic, the aura symptomatology in these patients has often already resolved,[50] Importantly, in patients with prolonged (motor) aura symptoms perfusion defects have indeed been described.[51]

Animal studies have shown that micro-emboli can trigger SDs, even without causing micro-infarctions.[52] This may explain why right-to-left cardiac shunting, often due to a patent foramen ovale (PFO), is a risk factor for stroke, but also associated with migraine with aura.[53] SD has also been shown to affect ischemic stroke development, with peri-infarct depolarizations (the functional equivalent of SD in a migraine aura) circling around and causing expansion of the infarct core.[54] Transgenic hemiplegic migraine mouse models with experimentally induced stroke had larger infarcts due to a higher propensity and more profound impact of peri-infarct depolarizations.[55] This implies an increased tissue sensitivity to a supply-demand mismatch. Interestingly, migraine prophylactics were able to protect mice from ischemic injury.[54] Clinical studies investigating this hypothesis, however, have not shown consistent results.[44,56,57]

Another possibly shared mechanism for migraine and stroke is endothelial dysfunction, which is associated with an increased rate of cerebro- and cardiovascular events.[58] The endothelium is a single layer of endothelial cells that line the interior surface of blood vessels. It acts as a barrier between the blood and extravascular space, but is also a major player in many processes, among which are regulating

immunological processes, inflammation and angiogenesis, controlling blood fluidity, aggregation of platelets and vascular tone, and finally it acts by functioning as a metabolizing and endocrine organ. Endothelial dysfunction has been implicated in migraine, with reduced cerebrovascular reactivity occurring in migraine patients.[59] Markers for endothelium activation, hypercoagulability, and inflammation are also associated with migraine.[58] Systemic inflammation with endothelial involvement may be associated with migraine and stroke, as is seen in cases with systemic lupus erythematosus or other auto-immune disorders.[60] Enhanced platelet aggregation has also been demonstrated in patients with migraine,[61,62] and while interesting, additional well-designed studies are needed to determine if this mechanism, possibly controlled by the endothelium, provides the link between migraine and stroke on the cellular level. Reduced vascular reactivity itself might also (partly) explain the link between migraine and stroke. A recent meta-analysis demonstrated more arterial stiffness and impaired cerebral vasodilator function, demonstrating altered cerebrovascular function.[63]

Shared genetic susceptibility

Several cerebral hereditary angiopathies are associated with migraine and cerebrovascular disease, such as Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL), Retinal Vasculopathy with Cerebral Leukoencephalopathy and Systemic manifestations (RVCL-S), *COL4A1*- and *COL4A2*-associated diseases, Dutch-type of Cerebral Amyloid Angiopathy (D-CAA), and Mitochondrial Encephalopathy, Lactic acidosis, And Stroke-like episodes (MELAS) (figure 2).[64] Less convincing evidence is available for common genetic variants, like the variants described in the *MTHFR* and *ACE D/I* genes.[64] Most clearly a shared genetic susceptibility of migraine and stroke is suggested by GWAS findings,[65] including the identification of a shared locus for migraine and large artery strokes that includes the *LMOD2-WASL* gene region.[65] Of note, *WASL* is implicated in stabilizing endothelial adherens junctions,[66] and fits the hypothesis that endothelial dysfunction seems involved.

Regardless, no significance at the individual patient level can be attributed to these genetic findings, nor is screening for polymorphisms useful in clinical practice. Still, loci at *LRP1*, a member of the LDL receptor family, were implicated in migraine, stroke and cervical artery dissection, suggesting a shared biology that deserves further study.[67] Moreover, through a GWAS approach single nucleotide polymorphism (SNP) rs9349379 in the *PHACTR1* locus (6p24) was implicated as risk factor for five vascular diseases including migraine.[68] Sophisticated (epi)genetic analyses that also involved induced pluripotent stem cells, indicated a regulatory function of this SNP on the

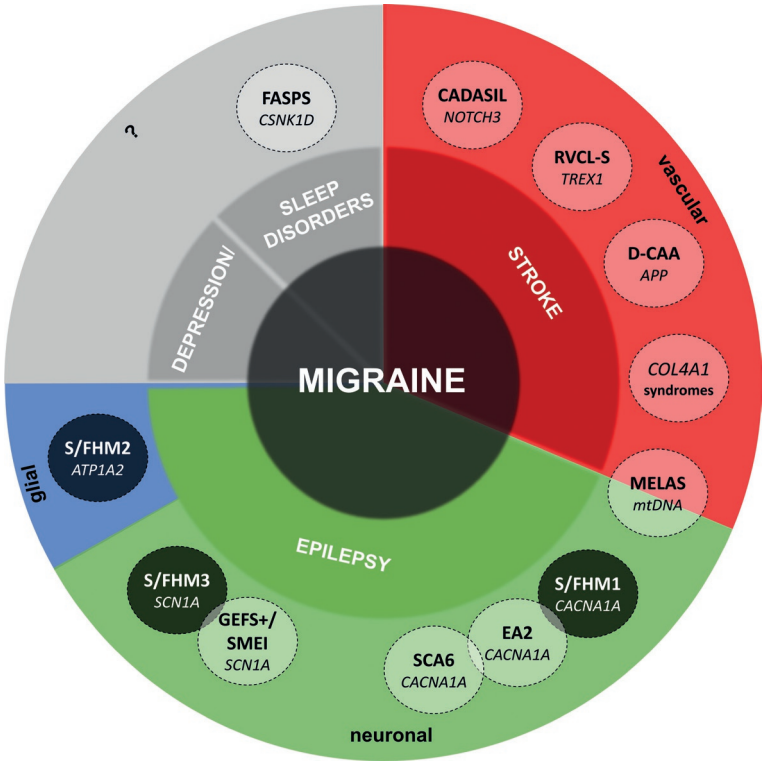

Figure 2. Monogenic syndromes associated with migraine and comorbidity with stroke, epilepsy, depression and sleep disorders.

Depicted are relations of these monogenic syndromes with migraine and each other, causative genes (in italics) and the main associated pathophysiological pathways: Sporadic and Familial Hemiplegic Migraine type 1, 2 and 3 (**S/FHM1, 2, 3**), and allelic disorders Spinocerebellar Ataxia Type 6 (**SCA6**), Episodic Ataxia Type 2 (**EA2**) and Generalized Epilepsy with Febrile Seizures and Severe Myoclonic Epilepsy of Infancy (**GEFS+, SMEI**) affecting glial and neuronal ion channels; vascular syndromes Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (**CADASIL**), Retinal Vasculopathy with Cerebral Leukoencephalopathy and Systemic manifestations (**RVCL-S**), Dutch-type hereditary cerebral amyloid angiopathy (**D-CAA**) and monogenic syndromes associated with ***COL4A1*** mutations, the migraine mimicking disorder Mitochondrial myopathy with Encephalopathy, Lactic Acidosis, and Stroke (**MELAS**, associated with mutations in mitochondrial DNA (mtDNA)) and Familial Advanced Sleep-Phase Syndrome (**FASPS**).

expression of the distantly (600 Kb) located endothelin 1 (*EDN1)* gene. Although it is puzzling that this DNA variant through endothelin increases the risk of cardiovascular disease whereas it decreases migraine risk, this type of analysis illustrates nicely that integration of phenotypic and (epi)genetic data can be a promising avenue to identify potential disease mechanisms.

Mendelian randomization (MR) is a research method that may provide insight of causal relations between risk factors and disease. Recent MR studies did not show direct causal relationships between cardiovascular risk factors and migraine but instead evidence of horizontal pleiotropy, meaning that stroke, migraine, and cardiovascular risk factors share SNPs as genetic factors.[69-71]

Shared environmental factors

There is an increased stroke risk in (older) smokers with migraine (hazard ratio [HR] ~3·2), compared with non-smokers (HR ~0·8).[72] Oral contraceptive use in female migraine patients results in a ~8-fold increased risk of ischemic stroke, which increases to an up to >30-fold increased risk with concurrent smoking in female migraine with aura patients.[73] In many guidelines, migraine with aura is consequently considered a contra-indication for oral contraceptives, although this may not be true for new oral contraceptives with lower estrogen doses. While absolute risks of stroke are still low, smoking and combined use of oral contraceptives should be advised against in women with migraine with aura. Migraine was shown not to be associated with atherosclerosis in large vessels in patients with acute ischemic stroke.[74] In contrast, ^{1}H-NMR metabolite profiling of 225 lipoproteins in plasma samples from large cohorts showed decreased levels of HDL-associated lipoproteins (apoA1, S-HDL-FC ratio, omega-3 fatty acids) to be associated with migraine.[75] Finally, it is suggested that migraine frequency may decrease after a stroke, presumably due to vascular or cortical brain changes, change in medication or lifestyle, or PFO closure.[76]

Consequences for clinical practice

Because of vasoconstrictive effects, triptans have been contraindicated in migraine patients with cardio- or cerebrovascular disease. Notably, in observational studies no increased stroke risk was found, although those prescribed a triptan might have had more favorable vascular risk profiles.[77] Nonetheless, triptan users with cardiovascular medication (as proxy for higher vascular risk) did not show an increased stroke risk.[78] Based on expert opinion, triptan use nowadays is considered safe in stroke patients in many countries, and might even be used in migraine-stroke disorders as CADASIL. The recently developed ditans (selective 5-HT_{1F} receptor agonists) do not cause vasoconstriction, and may have preference over triptans.[79]

CGRP monoclonal antibodies and small-molecule antagonists are also important to consider.[38] CGRP is released from neurons in the trigeminal ganglion and binds to receptors on meningeal vessels, causing vasodilation and neurogenic inflammation.[38] Blocking vasodilation caused by CGRP as escape response to an

ischemic event may potentially lead to a larger area of infarction.[80] So far, subjects with higher vascular risk were excluded from clinical trials,[38] and long-term adverse events will have to be awaited. *In vitro* studies demonstrated that erenumab significantly inhibited CGRP-mediated vasodilation (while not interacting with other vasoactive compounds)[81] and that fremanezumab also had direct functional effects on CGRP-mediated vasodilation.[82] Furthermore, work in mice demonstrated that treatment with small molecule CGRP antagonists led to worsening of cerebral ischemic outcomes.[83] A handful of studies have evaluated the effect of erenumab *in vivo* and while one demonstrated that erenumab did not alter vasomotor reactivity or flow-mediated dilation in migraine patients without aura, another demonstrated that it did affect trigeminovascular reactivity.[84,85] Further large *in vivo* studies are needed to determine in more detail how the vasculature of patients with migraine responds to these drugs. As hypertension is a known risk factor for ischemic stroke, it is important to note that real world data already indicated that treatment with anti-CGRP (receptor) antibodies led to an increase in mean systolic and diastolic blood pressure, and that some of the treated patients required antihypertensive treatment for the increase in blood pressure.[86,87] Blood pressure is a major modifiable risk factor for stroke over a broad range of blood pressure.[88] As such, physicians should monitor the blood pressure of patients when they are treated with anti-CGRP (receptor) antibodies. Furthermore, a report of an ischemic stroke in a 41-year-old woman, using rizatriptan and oral contraceptives, without pre-existing cardiovascular comorbidities following administration of a CGRP receptor blocker,[89] may raise concerns and it seems warranted to exclude patients with significant vascular risk factors from use of anti-CGRP treatment until evidence of long-term safety is available (Table 1). As such, stroke should be considered a contraindication until the long-term use of monoclonal antibodies targeting CGRP or its receptor are further investigated.

Acute stroke treatment increasingly consists of intravenous thrombolysis (IVT). In 10% of patients treated with IVT clinical follow-up reveals stroke-mimics, among them migraine (with aura), in whom IVT treatment fortunately seems safe.[90] PFO closure in stroke patients is debated, although in stroke patients with a migraine history, PFO prevalence may be up to 80%.[91] It is highly debatable whether migraine frequency improves after PFO closure, with studies reporting conflicting results.[92] New onset of migraine has been reported within days after PFO or atrial septal defect closure, with improvement after antiplatelet treatment.[93] Other studies suggested that treatment with acetylsalicylic acid might be effective as migraine prophylaxis for migraine with aura.[94] Most studies were of limited methodological quality and the possibly effective dose is unclear. Therefore, acetylsalicylic acid is not recommended as migraine prophylaxis.

Migraine & epilepsy

Epidemiology

Lifetime prevalence of migraine is increased in epilepsy patients (OR ~1·5) and, *vice versa*, lifetime prevalence of epilepsy is increased in migraine patients (OR ~1·8).[95] The comorbidity is particularly frequent in hemiplegic migraine, with epilepsy occurring in ~40% of hemiplegic migraine patients.[96] Migraine aura-triggered seizures may also develop during or directly following (<60 minutes) a migraine with aura attack, which has not been described in migraine without aura.[7] Ictal epileptic headache is a rare phenomenon during focal seizures that is not necessarily migrainous but may also fit characteristics of tension-type headache.[7] Both entities have been referred to as 'migralepsy', a term advised to avoid because of its unclear meaning. Post-epileptic headache, which may have migrainous features, is more common, most frequently after bilateral tonic clonic seizures.[97] Epilepsy patients also suffering from migraine have a worse prognosis, with lower probability of becoming seizure-free (OR ~0·3) and increased risk of treatment-resistance (OR ~6·2).[98]

Pathophysiological mechanisms

Neuronal hyperexcitability is considered a shared causal mechanism for migraine and epilepsy.[99] This mechanism may cause CSD initiation in migraine, while in epilepsy this may lead to simultaneous firing of populations of cortical neurons. CSD events may even show epileptic-like electrophysiological characteristics, with recordings of CSD evolving into epileptic convulsions.[100] Possibly, CSD causes synchronization in the affected neurons, which may lower the threshold for epileptic seizures.[101] (Hyper) synchronization during epileptic seizures may also prime the brain for CSD. On the other hand, the repolarization phase or depression following epileptic seizures or CSD may also raise the threshold for one another, indicating that co-occurrence of attacks is dependent on timing of pathophysiological processes.

Shared genetic susceptibility

A genetic link between migraine and epilepsy lies in the overlapping involvement of genes encoding ion transporters in hemiplegic migraine and epilepsy, i.e. *CACNA1A*, *ATP1A2*, *SCN1A*, and *PRRT2*.[64] Hemiplegic migraine patients with the p.Ser218Leu *CACNA1A* mutation, for instance, suffer from a particularly severe phenotype, that includes seizures, cerebral edema and coma that can be fatal.[102] Transgenic *Cacna1a*^{S218L} mice exhibit increased susceptibility to induced CSD.[103] Homozygous *Cacna1a*^{S218L} mice display severe attacks, spontaneous (fatal) seizures, and serve as a promising animal model for sudden unexpected death in epilepsy (SUDEP).[104] During seizures, spreading depolarizations in the mutant brainstem are found, which cause

respiratory and subsequent cardiac arrest and death. The beneficial effects of NMDA receptor blockers hold the promise for targets to prevent this dramatic course.[105]

One of the *ATP1A2*-associated familial hemiplegic migraine type 2 (FHM2) mouse models shows increased susceptibility to CSD and epileptiform activity,[106] similar to patients with the same p.Gly301Arg *ATP1A2* mutation.[107] A higher susceptibility to CSD correlates with severe epileptiform activity possibly related to increasing extracellular potassium.[106] Apart from the *ATP1A2*-encoded Na^+K^+-ATPase pump a2 subunit, the excitatory amino acid transporters (EAAT1 and EAAT2) are implicated in this clearance, and constitute therapeutic targets for migraine and epilepsy.

The *SCN1A* gene is implicated in FHM3 and epilepsy syndromes, including Dravet syndrome/ severe myoclonic epilepsy of infancy (SMEI) and generalized epilepsy with febrile seizures (GEFS+).[108] Spontaneous CSD events were shown in transgenic FHM3 mice expressing the p.L263V *SCN1A* mutation.[109] Gain-of-function of $Na_V1.1$ channels seems to result in hemiplegic migraine whereas loss-of-function leads to epilepsy.[108] Possibly, FHM3 *SCN1A* mutations lead to accumulation of potassium, while epileptogenic *SCN1A* mutations facilitate depolarization block of GABAergic neurons, thereby both increasing neuronal hyperexcitability, but through different pathophysiological mechanisms.[110]

Mutations in the *PRRT2* gene are mainly associated with paroxysmal kinesigenic dyskinesia, benign familial infantile seizures and infantile convulsion choreoathetosis syndrome, but have also been found in a few patients with (also) hemiplegic migraine, in which *PRRT2* mutations may act as a genetic cofactor.[111] *PRRT2* encodes a proline-rich transmembrane protein. Mutations likely cause neuronal hyperexcitability by affecting neurotransmitter release.[112]

For common migraine, an increased prevalence of migraine with aura was found in families containing two or more individuals with epilepsy of unknown cause, suggesting shared genetic susceptibility.[113] A GWAS in epilepsy revealed 16 loci, the majority of which are associated with generalized epilepsy,[114] suggesting a role for ion channel genes (*SCN1A*, *SCN2A* and *SCN3A*), and genetic correlations of epilepsy with migraine with aura specifically.

Shared environmental factors

Brain damage due to traumatic head injury or stroke may cause post-traumatic headache resembling migraine and increases the risk of epilepsy.[7] Besides such direct causal factors, there is a large overlap in suggested triggers, such as stress, sleep

deprivation, fatigue, alcoholic beverages, and hormonal changes.[27,115] In rodents, sleep deprivation enhances CSD susceptibility.[116] The influence of stress on CSD appears more complex. In FHM1 mice, corticosterone injection, but not restraint stress, led to enhanced CSD susceptibility,[117] illustrating that corticosterone can enhance CSD susceptibility whereas the rising of corticosterone levels during acute (restraint) stress are not sufficient. Likely, stress gives rise to a more complex biological response with multiple positive and negative modulators. Interestingly, relief after chronic stress did reduce CSD threshold in FHM1 mice.[118]

Consequences for clinical practice

It may be difficult to distinguish migraine aura from epileptic seizures, particularly when diagnosis is based on patient reports, symptoms are focal, or aura is not followed by headache. Positive visual symptoms can occur in both disorders, Todd's palsies may be confused with hemiplegic migraine, and post-ictal headache may have migrainous characteristics. Time interval may be helpful, as aura symptomatology develops more gradually (minutes instead of seconds) in migraine. (Inter)ictal EEGs can be helpful. Routine EEGs cannot detect CSD but direct-current EEG might have a role in the future.[119] Interestingly, patients with migraine and those with epilepsy have different antiepileptic drug adverse events profiles.[120] A retrospective study in women with epilepsy demonstrated that valproate and topiramate are prescribed most frequently in those with comorbid migraine.[121] When in doubt about whether a patient suffers from migraine, epilepsy, or both, prophylactic treatment can be directed at both disorders, with valproic acid, topiramate or lamotrigine (Table 1). Epilepsy patients with a confirmed *SCN1A* mutation should not be treated with sodium channel blockers, as this may worsen their clinical symptoms. Given the postulated gain-of-function of hemiplegic migraine-related *SCN1A* mutations, it is unclear whether effects of sodium channel blockers may in fact be beneficial in FHM3 patients.

Migraine & sleep disorders

Epidemiology

A variety of sleep disorders are suggested to be comorbid with migraine, including insomnia, restless legs syndrome (RLS), and obstructive sleep apnea. Odds ratios for insomnia in migraine patients vary between 1·5-2·5.[122] Insomnia shows a bidirectional relation with migraine, with insomnia preceding worsening of migraines and migraine being a precursor to insomnia (RR ~1·7).[122] RLS prevalence ranges from 9-39% (due to heterogeneity of study designs) with ORs of 1·2-4.[123] *Vice versa*, migraine prevalence in

RLS ranges between 15-63%, independent of sex or migraine type.[123] Comorbidity of sleep apnea syndrome and migraine has been suggested, but evidence was lacking in a large population-based study.[124] Snoring was two-fold more common among individuals with chronic daily headache than episodic headache, independent of traditional sleep apnea risk factors, suggesting an association with snoring rather than sleep apnea.[125] In addition, as obesity is a risk factor for sleep apnea and likely also for migraine, this may also be the cause for the observed comorbidity.[126]

Pathophysiological mechanisms

Migraine and RLS appear to have a temporal association, suggesting that one may trigger the other, with RLS triggering migraine explained by poorer sleep quality.[123] Other mechanisms may involve dysfunction of the hypothalamic dopaminergic system. Dopamine-involvement in migraine is related to symptoms of yawning, food craving, drowsiness, mood changes, nausea and vomiting during either the prodromal, headache or postdromal phase of attacks.[127] Interestingly, prodromal symptoms are more common in patients with comorbid RLS.[127]

Chronobiological mechanisms play a role in migraine pathophysiology as attacks often start in early morning, and patients report being early chronotypes and less flexible in circadian rhythm changes.[128] Serotonin and orexin/hypocretin systems are involved in the pathophysiology of migraine and sleep disorders, and these systems regulate pain perception and sleep–wake rhythm.[129] The locus coeruleus, regulating the sleep-wake-cycle, is also involved in migraine pathophysiology. Dysfunctional locus coeruleus signaling has shown to alter attack susceptibility and to reduce CSD susceptibility threshold.[130]

Sleep disorders can create an imbalance causing accumulation of glycogen during sleep.[131] Glycogen is a vital energy reservoir required to match synaptic demand particularly for re-uptake of potassium and glutamate during glutamatergic transmission. Synaptic metabolic stress caused by insufficient glycogen-derived energy substrate can lower the CSD threshold.[132] Sleep deprivation-induced changes may trigger migraine by reducing glycogen availability. Interestingly, chronic stress is suggested to increase glycogen turnover and decreased glycogen has been implicated in depression-like behavior in rats.[133] Therefore, dysregulation of the glycogen system might link migraine to sleep disorders as well as depression.

Shared genetic susceptibility

Cannabinoid-type-1 receptors (*CNR1* gene) are involved in sleep regulation, and *Cnr1* knock-out mice show sleep alterations.[134] Heterozygous rare coding variants in

CNR1 were associated with headache, including migraine.[135] Endocannabinoids may be a common underlying pathway for migraine and sleep disorders. *MEIS1* variants were associated with increased risk of RLS in migraine, implicating an imbalance in iron homeostasis and the dopaminergic system.[136] Finally, mutations in *CSNK1D* were reported in familial migraine and familial advance sleep phase syndrome (FASPS).[137]

Shared environmental factors

Migraine and sleep disorders may have overlapping trigger factors, such as stressful life events and working night shifts. Sleep deprivation is often reported as a migraine trigger and is associated with more frequent and severe migraine, a risk for migraine chronification.[27,138] An experimental setting in mice showed that insufficient glycogen supply due to an administered glycogen phosphorylase inhibitor and sleep deprivation lowered the threshold for induced CSD.[132]

Consequences for treatment in clinical practice

Use of antidepressants for insomnia is common. While it may seem an attractive 'triple-hit' option for patients with migraine, depression and insomnia, there is no evidence for amitriptyline use for insomnia.[139] A small improvement in sleep quality was found for (short-term) use of doxepin and trazodone, but these drugs are not beneficial for migraine. With the adverse effects of antidepressants, such as daytime sleepiness, these drugs are not recommended for migraine patients suffering from insomnia. Short-acting benzodiazepines for insomnia may increase the risk to develop migraine (Table 1).[140] Cognitive-behavioral insomnia therapy does seem of benefit for (chronic) migraine patients with insomnia.[141] Whether treatment of RLS also relieves migraine is speculative. Sleep apnoea headache,[7] usually recurs daily upon awakening and may be confused with chronic migraine. Screening patients with daily morning headache for sleep apnea is thus warranted, especially when other risk factors such as obesity are present.

Conclusion

Migraine shows comorbidity with several neurological and psychiatric disorders, with strongest and bidirectional relations with mood disorders and epilepsy. Migraine patients also have a higher risk for ischemic stroke. Several sleep disorders, most strikingly insomnia and RLS, also show bidirectional comorbidity with migraine.

Overlapping pathophysiological mechanisms are spreading depolarizations for migraine and stroke and hyperexcitability for migraine and epilepsy. For mood

disorders and sleep disorders shared pathophysiological pathways are less clear, although (hypo)thalamic involvement and locus coeruleus involvement are suggested.

Studies on polygenic risk factors for migraine and its comorbidities have yielded limited results so far. It is not straightforward to link an associated SNP to the actual gene of effect. For GWAS to have true meaning in elucidating pathophysiological mechanisms, efforts should not only aim towards performing larger GWAS but also to designing ways to identify implicated pathways with functional studies. Monogenic syndromes show strong links between migraine and stroke as well as migraine and epilepsy and migraine and sleep disorders. Studying these disorders as models will provide important mechanistic insights. Epigenetic mechanisms, such as DNA methylation and histone modification, likely are also involved in migraine pathophysiology. Only small steps have been taken to unravel their contribution towards migraine,[142] let alone towards understanding its comorbidities. Moreover, gene-environment interaction should also be evaluated as genetic factors modulating sensitivity to environmental exposures, or *vice versa*, might be equally important to understand the relationship between migraine and its comorbidities.

In most studies life-time diagnoses are used, regarding migraine and its comorbidities as traits. However, individuals with depression after the age of 50, are more likely to suffer from headache than younger people with the same risk factors.[143] How differences in migraine subtype and frequency over lifespan may relate to its comorbidities is largely unknown. One can postulate that evolving environmental factors across lifespan as well as biological age, e.g., with changed sex hormone levels, may be important. The elderly also have age-related renal and hepatic function changes that may alter drug metabolism and elimination, and the chances of multiple medication use and interactions are greater, which future studies should take into account.

Lastly, it is important to consider that migraine comorbidities might also be comorbid with one another, which might influence pathophysiological mechanisms and treatment options.[144,145] In this light, it is interesting to realise that many psychiatric disorders display genetic overlap, while this is much less the case for neurological disorders. Here migraine was an exception, which showed significant genetic correlation with several psychiatric disorders.[21]

Simultaneous treatment of comorbid disorders can be beneficial, regarding both drug treatment and tackling shared environmental risk factors, such as acute medication overuse, smoking, and sleep deprivation. Unfortunately, most clinical trials exclude participants with comorbid disorders, limiting evidence for optimal treatment for these patients. Nonetheless, assessing the presence of comorbid disorders may direct physicians towards other choices regarding treatment, not only to prevent harm but aim for positive effects on comorbidities. Altogether, neurological and psychiatric comorbidities do not only provide pathophysiological insight, they also have important implications for clinical practice.

Article highlights

- Overlapping pathophysiological mechanisms between migraine and stroke are spreading depolarizations, while hyperexcitability is a key feature of both migraine and epilepsy.
- For mood disorders and sleep disorders shared pathophysiological pathways are less clear, although (hypo)thalamic involvement and locus coeruleus involvement are suggested.
- Simultaneous treatment of migraine and comorbid disorders can be beneficial, however, dedicated trials are needed.

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CHAPTER 5

Effectiveness of acute and preventive migraine treatment in rare migraine syndromes

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Submitted

Abstract

Background: Monogenic migraine syndromes offer insights into migraine pathophysiology, but treatment is challenging due to low patient numbers and lack of clinical trials. The Leiden Headache Centre is a national referral centre for these disorders, including Hemiplegic Migraine (HM), Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL), and Retinal Vasculopathy with Cerebral Leukoencephalopathy and Systemic manifestations (RVCL-S), providing an opportunity to review treatment effectiveness and side-effects.

Methods: Medical records of patients with HM, CADASIL and RVCL-S were reviewed on prescribed acute and preventive medications, treatment effectiveness and side-effects.

Results: We included n=78 HM patients (median follow up years (FUY):1 IQR 1-5), n=114 CADASIL patients (FUY:1 IQR 0-3) and n=28 RVCL-S (FUY:8 IQR 5-11) patients. Most effective preventive drugs with the least side-effects for HM were lamotrigine, valproate, topiramate, and acetazolamide. Valproate appeared effective in CADASIL, whereas acetazolamide and propranolol showed efficacy in RVCL-S. Triptans were used by 59% of patients (86/148) with migraine without vascular or other severe lasting side-effects.

Conclusion: Preventive treatments aimed at aura symptoms appear to be effective in rare migraine disorders, and triptans appear to be a safe option for acute treatment.

Introduction

Migraine has a strong genetic component in its pathogenesis and is a hallmark feature of several rare monogenic syndromes.[1] Among these are hemiplegic migraine (HM), cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) and retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations (RVCL-S).

HM is a subtype of migraine with aura that is characterized by motor weakness during the aura phase. There are three known hemiplegic migraine genes: *CACNA1A, ATP1A2* and *SCN1A*.[2] Not all patients have a mutation in one of these genes, indicating that there might be undiscovered genes or that hemiplegic migraine might have a multifactorial etiology in some cases, with for instance involvement of co-factor genes such as *PRRT2*.[1] Mean attack frequency is usually lower than in the common types of migraine, but patients with high attack frequency (sometimes including non-hemiplegic migraine attacks) or severe symptomatology still require prophylactic treatment.

CADASIL and RVCL-S are autosomal dominant small vessel diseases. They are caused by heterozygous pathogenic mutations in the *NOTCH3* and *TREX1* gene, respectively.[1] These mutations lead to vasculopathy with cerebral involvement. In both disorders, migraine is part of the phenotype. Migraine prevalence is approximately 40% in RVCL-S and 50% in CADASIL.[3,4]

While these monogenic disorders have contributed greatly to elucidating the pathophysiology of migraine, to date little evidence is available to guide treatment decisions in these patients. Most evidence comes from case reports and series suggesting efficacy of lamotrigine, acetazolamide, verapamil, flunarizine and propranolol.[5-9] A review of migraine treatment in CADASIL indicated that betablockers appeared a poor choice due to unfavorable treatment response.[10]

Receiving adequate acute treatment can also pose challenges for patients diagnosed with rare migraine syndromes. First choice in acute treatment is usually acetaminophen or an NSAID. If common analgesics are not effective, triptans may be prescribed but physicians seem reluctant to start triptans because of presumed vascular side-effects. Migraine with aura was previously thought to be caused by ischemia, but regional cerebral blood flow studies dispelled that notion.[11] Moreover, sumatriptan-induced vasoconstriction occurs only in the dilated vessels, without affecting normal ones.[12] Small retrospective studies in HM and CADASIL patients showed side-effects were

rare and minor.[10,13-15] However, most guidelines still advise against using triptans for patients with hemiplegic migraine or vascular disease.

In this study, we aimed to systematically assess the treatment response and adverse events associated with migraine treatment in patients with HM, CADASIL, and RVCL-S, who were seen at our specialized out-patient clinic.

Methods

Participants and procedures

We included patients diagnosed with HM, CADASIL, and RVCL-S, who were referred to the specialized Leiden Headache Centre (national referral centre for rare migraine syndromes) between 2011 and 2021. Only patients with a proven pathogenic *NOTCH3* or *TREX1* mutation were included in the CADASIL and RVCL-S groups. As HM is not always caused by a mutation in *CACNA1A*, *ATP1A2* or *SCN1A*, the HM patients were not required to be proven carriers of a mutation in one of the three established HM genes. A senior neurologist with expertise in hemiplegic migraine (GMT) was always involved in the diagnosis of these patients.

Medical records were reviewed, and data on demographics, migraine, relevant medical history, mutation status and medication use were extracted. Migraine diagnosis was established by an expert neurologist and based on the ICHD-3 criteria (although the criterium for a minimum number of attacks was not taken into account).[16] Distinguishing between migraine with aura and a transient ischemic attack (TIA) in CADASIL patients can be challenging, even more so because aura symptomatology may be atypical in patients with CADASIL.[3] As such, patients were considered migraine patients when migraine aura was the most probable diagnosis. If symptoms were more consistent with a neurovascular event, patients were treated as such. Dosage, treatment duration, adverse events, and decision for (dis)continuation were documented for each prescribed prophylactic migraine drug. Effectiveness was assessed by shared decision making by the expert neurologist(s) and the patient and based on reduction in intensity of the migraine attacks, shorter duration and/or lower frequency, or a combination of these outcomes. Finally, it was documented whether the patient continued using the medication after three months (as this was the predefined minimal period to assess treatment effect). For acute migraine treatments adverse events and effectiveness were documented based on shared decision making of the treating neurologist(s) and the patient.

This study was approved by the Medical Ethics Committee Leiden The Hague Delft. All analyses were performed with SPSS 25.0 (SPSS Inc., IBM, Armonk, NY). Categorical variables were reported as numbers along with their corresponding percentages. When normally distributed, continuous variables were reported as means with standard deviation, and if not normally distributed, as medians with interquartile range.

Results

Demographics

In total, 78 HM patients, 115 CADASIL patients, and 28 RVCL-S patients were included in this study. Clinical characteristics are summarized in Table 1. Of the 78 HM patients included, 40% (31/78) was diagnosed with familial HM (FHM), 55% (43/78) with sporadic HM (SHM) and for 5% (4/78) of patients it was unknown whether they had first- or second-degree relatives with HM. Twenty-three HM patients carried a

Table 1. Patient characteristics

	HM (n=78)	CADASIL (n=114)[a]	RVCL-S (n=28)
Age (years)	30 (SD 18)	51 (SD 10)	54 (SD 13)
Sex female	49 (63)	65 (57)	18 (64)
Presence of migraine	78 (100)	60 (53)	12 (43)
Type of migraine present[b]			
Hemiplegic migraine	78 (100)	-	-
Migraine with aura	20 (27)	39 (34)	6 (21)
Migraine without aura	8 (11)	18 (16)	11 (39)
Aura without headache	7 (10)	23 (20)	5 (18)
Age of migraine onset (years)[c]	11 (8-16)	32 (20-41)	35.0 (20-52)
TIA/CVA	1 (1)[d]	60 (53)	4 (14)
Cardiac disease	4 (5)	6 (5)	4 (14)
Follow-up duration (months)	17 (IQR 6-57)	6 (IQR 2-36)	94 (IQR 55-132)

TIA: transient ischemic attack; CVA: cerebrovascular accident; CADASIL: cerebral autosomal dominant arteriopathy with subcortical Infarcts and Leukoencephalopathy; RVCL-S: retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations; HM: hemiplegic migraine. Continuous variables are reported as mean (standard deviation) or median (interquartile range), categorical variables as count (percentage). [a] One CADASIL patient had to be excluded due to an uncertain migraine diagnosis. [b] Patients can have multiple migraine diagnoses, [c] CADASIL missing value = 20, Hemiplegic migraine missing value n = 6. [d] One patient (no mutation in *CACNA1A*, *ATP1A2* or *SCN1A*) suffering from linear scleroderma with MRI-proven cerebral ischemia.

Table 2. Effectiveness of preventive migraine medication in hemiplegic migraine, CADASIL, and RVCL-S

Medication	Treated, n	Adverse events, n	Effective, n	Discontinued before last follow-up	Reason for discontinuation
HM					
Lamotrigine[a], 2 x 50mg	22	15/17	12/14	9/22	Ineffective (2/9), side-effects (5/9), Ineffective + side-effects (1/9), low attack frequency + pregnancy wish (1/9)
Valproic acid[b] 3 x 300mg	31	22/23	16/19	25/31	Ineffective (2/19), side-effects (13/19), low attack frequency (4/19)
Topiramate[c] 2 x 50mg	14	7/8	5/6	13/14	Ineffective (2/10), side-effects (5/10), ineffective + side-effects (1/10), low attack frequency (2/10)
Acetazolamide[d], 3 x 250mg	12	10/12	7/9	10/12	Ineffective (2/9), side-effects (2/9), ineffective + side-effects (2/9), low attack frequency (2/9), use of multiple prophylactics (1/9)
Verapamil[e], 3 x 120mg	7	5/5	4/6	5/7	Ineffective (3/5), side-effects (1/5), ineffective + side-effects (1/5)
Flunarizine[f], 1 x 10mg	15	8/9	6/10	13/15	Ineffective (3/12), side-effects (7/12), ineffective + side-effects (1/12), low attack frequency (1/12)
Candesartan[g], 1 x 16mg	11	5/5	4/8	7/11	Ineffective (1/5), side-effects (4/5)
Metoprolol[h], 1x 100mg	7	1/1	3/6	6/7	Ineffective (3/5), side-effects (1/5), low attack frequency (1/5)
Propranolol[i], 1 x 80mg	16	5/5	2/7	15/16	Ineffective (6/11), side-effects (3/11), ineffective + side-effects (1/11), side-effects + pregnancy wish (1/11)
Pizotifen, 1 x 1.5mg	6	2/2	0/3	6/6	Ineffective (2/4), side-effects (2/4)
CADASIL					
Valproic acid, 3 x 300mg	6	3/4	6/6	2/6	Side-effects (1/2), attack free (1/2)
Metoprolol, 2 x 100mg	1		0/1	1/1	Ineffective (1/1)
Propranolol, 1 x 160mg	4	2/2	4/4	3/4	Ineffective (1/3), ineffective + side-effects (1/3), pregnancy (1/3)
RVCL-S					
Topiramate, 2 x 50mg	1	1/1	1/1	0/1	
Azetazolamide, 3 x 250mg	1	1/1	1/1	1/1	Side-effects (1/1)
Propranol 1 x 160mg	1	1/1	1/1	1/1	Side-effects (1/1)[j]
Valproic acid, 3 x 300mg	2	2/2	1/1	2/2	Side-effects (2/2)
Pizotifen, 1 x 2mg	1	0/1	1/1	1/1	No longer available (1/1)

Metoprolol, 1x 200mg	1	0/1	0/1	1/1	Ineffective (1/1)
Candesartan, 1 x 16mg	1	0/1	0/1	1/1	Ineffective (1/1)
Lisinopril, 1 x 20mg	1	0/1	0/0	1/1	Ineffective (1/1)
Amitriptyline, 1 x 40mg	1	1/1	0/0	1/1	Ineffective (1/1)

Medications are ordered based on effectiveness (effective, n (%)). A denominator smaller than the number of patients treated (treated, n) or - indicates missing data. [a] Three patients did not achieve adequate dosage due to discontinuation due to side-effects. [b] Ten patients used less than 900 mg per day, of which two experienced an adequate treatment effect (2 x 300mg and 1 x 300mg, respectively). For the other eight patients no information on effectiveness was available. Of these patients, seven discontinued treatment due to side-effects. [c] Four patients used less than 100 mg per day, of whom one experienced complete remission (1 x 25mg). Two patients did not reach optimal dosage due to side-effects. For the other patients no information on effectiveness was available. [d] Six patients used less than 750 mg per day, of whom two did not achieve adequate dosage due to discontinuation due to side-effects. Two achieved an adequate treatment effect with a dosage of 2 x 250mg. For one patient no information was available on effectiveness and for one effectiveness was unclear as multiple prophylactics were used at the same time. [e] One patient was treated with 1 x 40mg and treatment was discontinued due to side-effects. One patient was treated with a daily dose of 180mg and treatment was effective. Three patients were treated with a daily dose of 240 mg. In one treatment was effective at first. In the end in all these patients treatment was discontinued due to ineffectiveness. [f] Eight patients used 5 mg per day, of whom five discontinued treatment due to side-effects. One patient achieved complete remission. One patient had insufficient effect and for the other patient there was no information on whether there were side-effects or a treatment effect. [g] Four patients did not reach optimal dosage. Two discontinued due to side-effects. One achieved effectiveness with a dosage of 1 x 4mg. For the last patient no information was available on effectiveness or reason for discontinuation. [h] Three patients used less than 100 mg per day, of whom one experienced complete remission (1 x 25mg) but discontinued due to side-effects. One experienced no effectiveness (1 x 50mg) and one had insufficient information to determine effectiveness (1 x 50mg). [i] Three patients used less than 80 mg per day, of whom two discontinued treatment due to side-effects, and one due to ineffectiveness (1 x 40mg). Two patients used 1x 160mg but discontinued treatment due to side-effects. [j] after approximately one year propranolol was tapered off again given the fact that the patient experienced an increase in the severity of already present Raynaud's phenomenon.

pathogenic mutation (*CACNA1A* n = 9, *ATP1A2* n = 14, *SCN1A* n = 0). Two additional HM patients had a variant of unknown significance in *CACNA1A*. There were 60 CADASIL patients with migraine (53%), most experienced migraine with aura attacks (39/60, 65%), 38% (23/60) of the patients had attacks of migraine aura without headache and 30% (18/60) had migraine without aura attacks. Twelve of the 28 RVCL-S patients had migraine (43%), of which most suffered from migraine without aura attacks (11/12, 92%), but 6/12 patients also had attacks of migraine aura without headache (50%) and 5/12 patients had migraine with aura attacks (42%).

Migraine treatment in hemiplegic migraine

Prophylactic treatment

Of the 78 patients with HM, 72 received preventive and/or acute migraine treatment. For one patient it was unclear whether prophylactic medication was started for migraine or for another medical indication. As presence or absence of an effect was also not described, this patient was excluded. Of the included HM patients, 53/78 (68%) were prescribed migraine preventive medication. The preventive drug to be prescribed first was most often valproic acid (n=13) or propranolol (n=11). The treatment responses are summarized in Table 2 and Figure 1. Patients consistently received monotherapy, utilizing one medication at a time, and subsequently used different preventive medications when previous attempts failed.

Valproic acid was prescribed most frequently. Lamotrigine, valproic acid, topiramate and acetazolamide were the most effective medications (effectiveness ranging between 78% and 86%). All medications had a high incidence of side-effects, but these did not always cause patients to discontinue the medication. Severe side-effects (AE grading 3) occurred during use of verapamil and flunarizine. Three patients using verapamil experienced a vasovagal reaction, of whom one fainted. One adolescent on flunarizine gained 25% of his original weight.

Of the HM patients with a proven mutation that were included in this cohort, 16 used preventive medication. When comparing effectiveness between mutation carriers and non-carriers (Supplementary table 1), a few differences are notable. With an effectiveness in 6/6 patients (100%) valproic acid was the most effective prophylactic

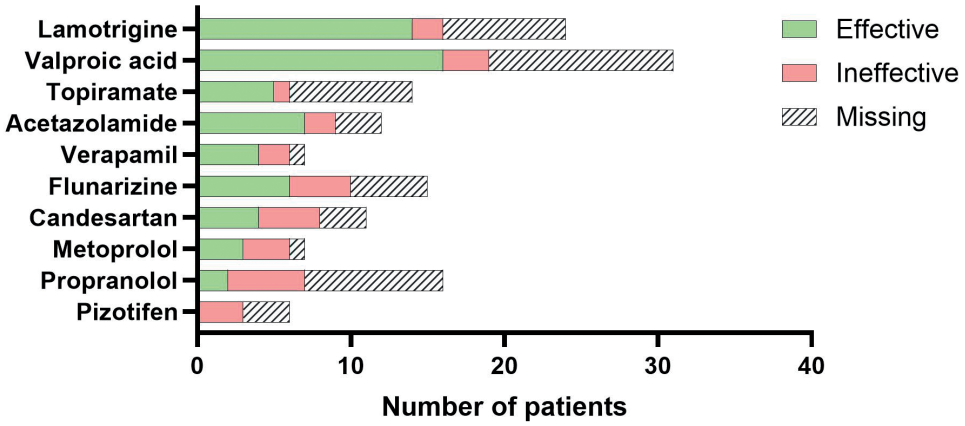

Figure 1. Effectiveness of preventive medication in hemiplegic migraine.

in carriers, where in non-carriers lamotrigine was the most effective treatment (carriers 4/6 (67%) vs. non-carriers 7/7 (100%)).

Acute treatment

A total of 66 HM patients (85%) used triptans. For 20/66 patients (30%) side-effects were reported. One patient (1.5%) experienced a possible severe side-effect (AE grading 3). Shortly after triptan intake the patient felt unwell and had an aggravation of the HM attack, for which he was temporarily hospitalized. This patient had a good experience with using triptans multiple times before, resulting in shorter duration of the migraine headache. For 52 patients information on effectiveness of triptans was available. Triptans were regarded effective in 26/52 patients (50%), had a varying effect in 8/52 patients (15%) and were not effective in 18/52 patients (35%). Of the 20 patients in whom the effect was described in more detail, 15/20 patients (75%) experienced a decrease in pain severity and 5/20 patients (25%) became pain-free shortly after treatment.

Migraine treatment in CADASIL

Prophylactic treatment

Of the 60 CADASIL patients with migraine, two patients were excluded because of insufficient information about medication use. Of the remaining 58 CADASIL patients with migraine, 19 received migraine preventive and/or acute treatment. Ten patients were treated with preventive migraine treatments (17%). The preventive most often prescribed first was valproic acid (n=5). The various treatments and treatment responses are summarized in Table 2. All patients used adequate dosages unless described otherwise. Overall, valproic acid was prescribed most frequently (6/10 patients) and was effective at least to some extent in all patients that used it. Although betablockers are not a preferred choice at our clinic, these were used in 5 out of 10 patients, as the general practitioner or a neurologist elsewhere started the medication before referral to our center. Metoprolol was continued in one patient for six months but was not effective and therefore discontinued. Propranolol appeared effective to some extent in four patients, but in two of them treatment was discontinued due to insufficient effect.

Acute treatment

For two patients it was not clear whether they had used the triptans prescribed to them, and they were, therefore, excluded. Fifteen patients were treated with triptans (27%). One patient (7%) reported mild side-effects (fatigue and weakness of the legs). No severe side-effects occurred. Triptans were effective in 8/14 patients (57%) with available information on effectiveness and possibly effective in 3/14 patients (21%).

Out of the five patients for whom the effect was further specified, 2 out of 5 patients (40%) experienced a shorter attack duration, while 3 out of 5 patients (60%) were headache free after two hours.

Migraine treatment in RVCL-S

Few RVCL-S patients used preventive migraine medication (3/12, 25%). Nonetheless, 9 different treatments were tried. Nonetheless, 9 different treatments were tried. Topiramate, acetazolamide, propranolol and valproic acid were considered effective (Table 2). Interestingly, one patient reported cessation of her migraine attacks after commencing an oral contraceptive. Another patient's migraine frequency increased after stopping oral contraceptive treatment, only to decrease again after restarting the oral contraceptive seven months later. In total, 5 out of 12 patients (42%) used triptans, which were effective in 3 out of 5 patients (60%) and showed some effect in the remaining two patients. No severe side-effects occurred.

Discussion

Rare monogenic migraine syndromes offer valuable insights into migraine's underlying mechanisms, yet effective treatment remains challenging due low patient numbers and lack of clinical trials. We evaluated migraine treatment response in patients with HM, CADASIL, and RVCL-S, focusing on treatment outcomes and adverse reactions to acute and preventive medications. Lamotrigine and valproic acid exhibited high effectiveness in treating HM attacks. For treating migraine in CADASIL, valproic acid was often effective. Most RVCL-S patients with migraine were not treated with preventive medication. As such, our data do not allow strong recommendations on migraine preventives in RVCL-S. We did observe effectiveness of acetazolamide and propranolol in RVCL-S patients. Side-effects were often reported with preventive migraine treatment. No long-lasting vascular and/or otherwise severe side-effects of triptans were reported in any of the patients with monogenic migraine syndromes.

To date, there have been no randomized controlled trials assessing migraine treatment in HM or CADASIL and no information is available on migraine treatment in RVCL-S. For HM and CADASIL, physicians rely on limited data from either small case studies, small retrospective studies, or expert opinion to guide treatment decisions for these patients (Table 3). For preventive treatment, options that have previously been tried include verapamil, acetazolamide, flunarizine, lamotrigine, betablockers, and one case series on a calcitonin gene-related peptide (CGRP) blocking drug. Reports of response are often conflicting. Several reports indicate that acetazolamide may be effective in

CADASIL[6,9,17,18] Unfortunately, none of the patients in our cohort were treated with this drug. The finding that betablockers ranked among the most frequently prescribed preventive treatments in this study aligns with general guidelines, which recommend these drugs as first-line options for common types of migraine but do not take the type of migraine into account. The only systematic review of migraine treatment in CADASIL indicated that treatment with betablockers was associated with an unfavorable response.[10] Our results support this, and also indicate that betablockers are less suitable for HM patients. Therefore, we recommend starting treatment with preventives such as valproic acid, acetazolamide, or lamotrigine.

There are concerns about prescribing topiramate and valproic acid for migraines to women of childbearing age due to their potential to cause birth defects and other pregnancy-related complications. Patients need to be informed about the risks and use adequate contraceptives. Nonetheless, in patient groups where there are limited effective treatment options, these treatments should still be considered, especially since these medications can be discontinued if pregnancy is desired.

The exact mechanisms through which most anti-migraine medications prevent migraine have not been completely established yet. The migraine preventatives discussed in this study are believed to suppress cortical spreading depolarization (CSD) and reduce neuronal sensitization. Valproic acid reduces glutamatergic neurotransmission by blockage of sodium and calcium channels and enhancement of GABAergic inhibition. Moreover, valproic acid reduces CGRP levels. Reduced glutamatergic neurotransmission, enhanced GABAergic inhibition, and reduced CGRP levels contribute to the inhibition of central pain transmission, neurogenic inflammation, and sensitization.[19] In rats, treatment with valproate decreased CSD propagation velocity.[20] Topiramate reduces hyperexcitability of neurons by enhancing the activity of inhibitory neurotransmitters like gamma-aminobutyric acid (GABA) and inhibiting the activity of excitatory neurotransmitters like glutamate. Topiramate also blocks certain types of voltage-gated calcium channels, which are involved in neurotransmitter release and neuronal excitability.[21] Topiramate has been shown in rats to make the cortex more resistant to CSD and to decrease CSD propagation speed.[22] Lamotrigine blocks voltage sensitive Na^+ channels, and by this mechanism of action it is able to block CSD.[23] In rats, lamotrigine decreased CSD frequency.[20] Interestingly, lamotrigine also increased resting motor threshold to transcranial magnetic stimulation in healthy men.[24] Acetazolamide inhibits carbonic anhydrase, leading to pH changes than can influence neuronal excitability.[25] Vice versa, CSD events may cause changes in pH, both extracellular and intracellular.[26] Acetazolamide's ability to alkalize blood and tissues may help counteract these pH fluctuations.

Table 3. Overview of preventive migraine treatment in hemiplegic migraine and CADASIL

Drug	Possible mechanism of action	Dose	Efficacy	Evidence
Hemiplegic migraine				
Acetazolamide	Alterations in local pH surrounding the P/Q Ca2+ channel could lead to enhanced channel function.	250-500 mg twice a day	May be effective in lessening attack burden.	Low; case reports and small studies[33-40]
Valproate	Inhibition gamma-aminobutyric acid (GABA) transaminase, activation glutamic acid decarboxylase and blocking Na+ channels and low-voltage-activated T-type Ca2+ channels, possibly enhancing neuronal inhibition.	1000 mg per day	May be effective in lessening attack burden.	Low, case report and series[7,41,42]
Lamotrigine	blocking voltage-gated Na+-channels and N- and P/Q-type voltage-activated Ca2+-channels inhibits glutamate release.	25 mg a day to max 300mg a day	May be effective in lessening attack burden.	Low; case reports and small studies[43-45]*
Flunarizine	Antagonism of non-selective calcium ion channels, dopamine receptors, 5-HT receptors and antihistamine receptors.	10 mg a day	May be effective in lessening attack burden.	Low; case reports[41,46-49]
Topiramate	Unknown.	25 mg a day	Deterioration of symptoms observed in a single case of hemiplegic migraine. Another case showed absence of attacks for several months.	Low, single reports[35,44]
Verapamil	Antagonism of calcium on L-type calcium channels, blocking calcium influx and diminishing constriction. Effects on P/Q calcium channels are less pronounced.	120 mg twice or three times a day	May be effective in lessening attack burden.	Low, case reports, case series[50-54]
Propranolol	Unknown.	30-50 mg a day	May be effective in lessening attack burden.	Low, case reports and case series[5,39,55]
Galcanezumab	Blockage of the CGRP pathway.	240mg loading dose, followed by monthly 120mg	May be effective in lessening attack burden.	Low, single case series[56]

CADASIL				
Acetazolamide	Alterations in local pH surrounding the P/Q Ca2+ channel could lead to enhanced channel function.	125 to 500 mg a day	May be effective in lessening attack burden.	Low, case report and case series[6,9,17,18]
Valproate	Inhibition gamma-aminobutyric acid (GABA) transaminase, activation glutamic acid decarboxylase and blocking Na+ channels and low-voltage-activated T-type Ca2+ channels, possibly enhancing neuronal inhibition.	1000-1250 mg per day	May be effective in lessening attack burden.	Low, case reports[6,57,58]**
Topiramate	Unknown.	-	May be effective in lessening attack burden.	Low, few cases[58]**
Flunarizine	Antagonism of non-selective calcium ion channels, dopamine receptors, 5-HT receptors and antihistamine receptors.	10 mg a day	May be effective in lessening attack burden.	Low, case report or one case in a larger study[58,59]**
Verapamil	Antagonism of calcium on L-type calcium channels, blocking calcium influx and diminishing vasoconstriction. Effects on P/Q calcium channels are less pronounced.	-	May be effective in lessening attack burden.	Low, two patients in a larger study[58]**
Pizotifen	Serotonin antagonist	-	May be effective in lessening attack burden.	Low, sixteen patients in a larger cohort study[58]**
Propranolol	Unknown.	-	Neutral or unfavorable response	Moderate, systematic review[10]
Metoprolol	Unknown.	100 mg a day	Neutral or unfavorable response	Moderate, systematic review[10]
Erenumab	Blockage of the CGRP pathway.		May be effective in lessening attack burden.	Low, single case report[30]***

*[43] Lampl et al. Authors describe that treatment effect of lamotrigine was not different for different types of aura. Eight patients with paresis were included. No specific statements about hemiplegic migraine were made. **[58] Tan et al. Authors describe a cohort of 98 CADASIL patients in which treatment response is described of flunarizine n=1, verapamil n=2, topiramate n=3, valproate n=3, pizotifen n=16, propranolol n=16. *** Caution against treating patients with small vessel diseases, such as the monogenic disorder CADASIL, with anti-CGRP pathway medication should be taken until relevant safety data and long term follow up results are available.[32]

Regarding acute treatment, medications such as verapamil, nimodipine, ketamine, triptans, pulse steroids, and hypertonic saline have been utilized.[27] If simple analgesics provide inadequate headache relief, triptans should be considered for the treatment of migraine. Triptans are agonists for 5-HT1B/1D receptors and induce vasoconstriction and inhibit the secretion of neuropeptides. Patients with a history of stroke or transient ischemic attack (TIA), or those with a history of hemiplegic migraine or brainstem aura symptoms, are deemed to have a contraindication due to concerns that triptans may increase the risk of stroke. However, in our study only one patient, who had previously benefited from triptan use, experienced a severe HM attack and it remained unclear whether the worsening of this migraine attack was due to triptan intake. Our findings are in line with several previously reported small studies that indicate that triptans may be regarded safe to use in HM and CADASIL.[13-15] As our study and available literature indicates that most patients respond positively to triptans and vascular side-effects are absent, we believe these patients can be offered this treatment safely. With the emergence of 5HT-1F agonists like lasmiditan, there is a non-vasoconstrictive alternative for acute treatment. However, the significant central side-effects of this medication and limited availability in most countries should be taken into account.[28]

Compounds that either inactivate the CGRP molecule by binding to it or its receptor are effective in both acute and preventive treatment of migraine. CGRP might also serve as a protective vasodilator during ischemic events. Therefore, treatments inhibiting the CGRP system might possibly worsen ischemic events.[29] One CADASIL patient was treated with a CGRP receptor monoclonal antibody.[30] A favorable response was reported. Nonetheless, CGRP and its receptor are involved in cardiovascular homeostasis under (patho)physiological conditions. Also, real-word data have shown that blockage of CGRP may lead to an increase in blood pressure, whereas patients with monogenic vascular disorders should strive to control other vascular risk factors.[31] Therefore, we advise physicians to refrain from starting treatment with CGRP blocking agents until long-term vascular safety is proven,[32] at least for CADASIL and RVCL-S patients.

The strength of our study lies in the relatively large cohort of patients with rare monogenic migraine syndromes. Nonetheless, there are limitations to consider. The design could have introduced biases related to selection and recall bias. Assessment of treatment relied on medical records and not a standardized protocol. However, this study was conducted at a clinic specialized in migraine and these monogenic disorders and thus with relatively high-quality documentation in patients' medical records. This precise documentation likely led to a high reported incidence of adverse events in

our study, even if this did not always lead to discontinuation of medication. Most side-effects were mild in nature. Taking these limitations into account, it is important to realize that randomized controlled trials are not expected to be performed in these rare syndromes due to the limited number of patients.

In summary, our results suggest that lamotrigine, valproic acid, topiramate, and acetazolamide are effective preventive treatments for patients with HM. Our findings also indicate that valproic acid may be an effective treatment for migraine in CADASIL patients. As no significant (vascular) side-effects were observed with acute triptan treatment, triptans can be considered in patients with HM, CADASIL and RVCL-S.

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Clinical Implications

- Lamotrigine, valproic acid, topiramate, and acetazolamide are effective in preventing hemiplegic migraine (HM) attacks, while valproic acid appears to be a promising treatment for migraine in CADASIL.
- Despite previous concerns, triptans are crucial for migraine management, showing safety and effectiveness even in high-risk patients.
- Betablockers have an unfavorable treatment response in both hemiplegic migraine and CADASIL.

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Supplementary table 1. Effectiveness of preventive migraine medication in patients with hemiplegic migraine and a pathogenic mutation

Medication	Treated, n	Adverse events, n	Effective, n	Discontinued before last follow-up	Reason for discontinuation
Valproic acid[a] 3 x 300mg	10	9/9	6/6	8/10	Ineffective (1/7), side-effects (6/7)
Acetazolamide[b] 3 x 250mg	6	4/6	4/5	5/6	Ineffective (2/4), ineffective + side-effects (1/4), low attack frequency (1/4)
Topiramate[c] 2 x 50mg	5	3/3	3/4	4/5	Ineffective (1/4), side-effects (1/4), ineffective + side-effects (1/4), low attack frequency (1/4)
Flunarizine[d] 1 x 10mg	7	3/3	3/4	6/7	Ineffective (2/5), side-effects (3/5)
Lamotrigine[e] 2 x 50mg	8	5/6	4/6	4/8	Ineffective (2/4), side-effects (2/4)
Candesartan 1 x 16mg	2	-	1/2	0/2	-
Metoprolol 1x 100mg	2	-	1/2	1/2	Ineffective (1/1)
Verapamil[f] 3 x 120mg	1	1/1	0/1	1/1	Ineffective + side-effects (1/1)
Propranolol[g] 1 x 80mg	5	2/2	0/1	5/5	Ineffective (1/3), side-effects (2/3)
Pizotifen 1 x 1.5mg	3	-	0/1	3/3	Ineffective (1/1)

[a] Four patients used less than 900 mg per day, of whom one experienced effectiveness. For the other three patients information on effectiveness was not available. [b] One patient used less than 750 mg per day (2 x 250mg) and experienced treatment effect. [c] One patient used less than 100 mg per day. Information on effectiveness was not available. [d] Two patients used 5 mg per day, in one of these patients treatment was effective. [e] One patient did not reach adequate dosage due to side-effects. [f] One patient was treated with a daily dosage of 240mg. Treatment was discontinued as it proved ineffective and side-effects occurred. [g] One patient used less than 40 mg per day and discontinued treatment due to side-effects.

PART II

Monogenic small vessel diseases – understanding vascular migraine models

6

CHAPTER 6

The potential danger of blocking CGRP for treating migraine in CADASIL patients

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Abstract

Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL) is an inherited small vessel disease characterized by recurrent ischemic stroke, cognitive decline progressing to dementia, psychiatric disturbances and apathy. More than half of mutation carriers suffer from migraine, most often migraine with aura. Recently a CADASIL patient was treated with a monoclonal antibody targeting the Calcitonin gene-related peptide (CGRP) receptor. Monoclonal antibodies targeting the CGRP system have been demonstrated to be safe, well tolerated, and effective in reducing migraine attacks. There is however, abundant evidence that CGRP is important in maintaining cardiovascular homeostasis under (patho)physiological conditions. CGRP may act as a vasodilatory safeguard during cerebral and cardiac ischemia and blockage of the system could, therefore, potentially worsen ischemic events. Therefore, we caution against treating patients with small vessel diseases, such as the monogenic disorder CADASIL, with these drugs until relevant safety data and long term follow up results are available. Alternative preventive migraine treatments in CADASIL may be acetazolamide, sodium valproate, lamotrigine, topiramate, verapamil, or flunarizine.

Perspective

Until recently migraine management remained relatively unsatisfactory and without mechanistic rationale. Compounds have been developed to either inactivate the calcitonin gene-related peptide (CGRP) molecule by binding to it or its receptor, to prevent migraine from developing. These monoclonal antibodies against CGRP or its receptor are safe, well tolerated, and effective in reducing migraine attacks and headache days.

Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL) is an inherited small vessel disease. CADASIL is characterized by mid-adult onset of recurrent ischemic stroke, cognitive decline progressing to dementia, psychiatric disturbances and apathy, with diffuse white matter lesions and subcortical infarcts on neuroimaging. More than half of mutation carriers suffer from migraine, most often migraine with aura.[1] Migraine is the inaugural manifestation in 40% of patients. Atypical aura symptoms occur in two thirds of mutation carriers who experience migraine.[1]

Recently, a middle-aged CADASIL patient was treated with CGRP-receptor monoclonal antibody erenumab for chronic migraine.[2] This patient suffered from frequent migraine attacks with atypical aura's consisting of visual and confusional symptomatology. The authors reported a favorable response and suggested that this may be a safe treatment for CADASIL patients.

CGRP and its receptor are abundantly present in the vasculature and maintain cardiovascular homeostasis under (patho)physiological conditions. CGRP may act as a vasodilatory safeguard during cerebral and cardiac ischemia and blockage of the system might, therefore, potentially worsen ischemic events. Although CGRP monoclonal antibodies seem safe, three deaths were reported in clinical migraine trials.[3] During treatment with fremanezumab two patients died (suicide and chronic obstructive pulmonary disease).[3] One patient treated with erenumab died due to an 'arteriosclerosis event'.[3] Patients included in these trials were likely considered 'healthy' besides having migraine. In regular clinical practice a 41-year old woman suffered an ischemic stroke after erenumab was started, no vascular risk factors besides combined oral contraceptive use were present.[4]

For patients with increased stroke risk, including those with a monogenetic predisposition such as CADASIL, reports of ischemic events are especially troubling. It should be taken into account that CADASIL is a small vessel disease, and aberrant

CGRP-mediated microvascular responses have been demonstrated in CADASIL patients.[5] Blockade of the CGRP-ergic system may further increase cardiovascular risk in this patient group. Furthermore, the reported suicide is concerning as CADASIL patients are already predisposed to psychiatric illnesses. Lastly, long-term effects of CGRP blockade are still unknown.

Treatment of migraine in CADASIL is based on empirical data and personal experience. Moreover, treatment needs to take into account risk for ischemic events and psychiatric illness. From personal experience, preventive treatment focusing on (atypical) aura is most effective, including acetazolamide, sodium valproate, lamotrigine, topiramate, verapamil, or flunarizine. Depending on other headache comorbidity (migraine without aura, daily "tension-type-like" headache), candesartan or amitriptyline may be effective.

Due to the rarity of CADASIL it is highly unlikely that enough patients can be recruited to perform clinical trials on efficacy and/or complications of CGRP-(receptor)-antibodies. As example, whether CADASIL patients should be treated with antiplatelet medication (a frequently debated subject with great relevance) still remains based on expert opinions. Nevertheless, fundamental research can provide additional information about the effect of blocking the CGRPergic system in this disorder. Recent work in healthy mice already demonstrated that blocking the CGRP system leads to worsened experimental ischemic stroke by inhibiting CGRP-mediated collateral vasodilation.[6] While this may be regarded already as a warning signal that these treatments should not be prescribed to CADASIL patients, work in genetically modified CADASIL mice models will provide additional information about possible risks.

In CADASIL, ischemic events frequently occur, and their outcome could be worsened by blocking the CGRP system. Therefore, blocking CGRP may pose a risk in patients with small vessel diseases such as CADASIL and we strongly advise refraining from CGRP-blocking treatments until their long-term safety is proven.

Clinical implications:

- In CADASIL, cerebral ischemic events frequently occur, and their outcome may be worsened by blocking the CGRP system.
- We strongly advise refraining from CGRP-blocking treatments in patients with small vessel diseases such as CADASIL until long-term safety is proven in case of ischemic events.

- Based on personal experience, specific preventive treatment for (a)typical migraine with aura is effective in CADASIL patients, including acetazolamide, sodium valproate, lamotrigine, topiramate, verapamil, or flunarizine.

Declaration of conflict of interest:

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CHAPTER 7

RVCL-S and CADASIL display distinct impaired vascular function

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Abstract

Objective: We aimed to evaluate the role of endothelial-dependent and endothelial-independent vascular reactivity in Retinal Vasculopathy with Cerebral Leukoencephalopathy and Systemic manifestations (RVCL-S) and Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL), two cerebral small vessel diseases that are considered models for stroke, vascular dementia and migraine.

Methods: RVCL-S (n=18) and CADASIL (n=23) participants with *TREX1* and *NOTCH3* mutations, respectively, were compared with age-, BMI- and sex-matched controls (n=26). Endothelial function was evaluated by flow-mediated vasodilatation and endothelial-independent vascular reactivity (i.e. vascular smooth muscle cell function) was assessed by dermal blood flow response to capsaicin application.

Results: Flow-mediated vasodilatation was decreased in RVCL-S participants compared with controls (2.32 ± 3.83 *vs.* 5.76 ± 3.07 % change in diameter, p=0.023), but normal in CADASIL participants. Vascular smooth muscle cell function was reduced in CADASIL participants compared with controls (maximal dermal blood flow increase at 40 minutes after capsaicin: 1.38 ± 0.88 *vs.* 2.22 ± 1.20 Arbitrary Units, p=0.010), but normal in RVCL-S participants.

Conclusions: We identified endothelial dysfunction in RVCL-S and confirmed impaired vascular smooth muscle cell relaxation in CADASIL. Our findings may prove to be biomarkers for disease progression in both monogenic cerebral small vessel diseases and improve mechanistic insight in their pathophysiology. This may help to understand common neurovascular disorders, including stroke, dementia, and migraine.

Introduction

Retinal Vasculopathy with Cerebral Leukoencephalopathy and Systemic manifestations (RVCL-S) and Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL) are monogenic small vessel diseases with overlapping clinical features, including white matter lesions and stroke. Migraine and depressive symptoms are often presenting symptoms and in many patients apathy and premature cognitive decline are found.[1-7] Because of clinical similarities these monogenic syndromes may serve as a model for common vascular disorders, including stroke, vascular dementia and migraine.

In CADASIL degeneration of vascular smooth muscle cells (VSMCs) with morphologically normal endothelium is observed.[5] In contrast, in RVCL-S muscle cell degeneration is minimal but electron microscopy shows irregular thickening of basement membranes, whose formation is dependent on endothelial cells.[7,8] These morphological abnormalities supposedly affect the dilatation capability of blood vessels, which may lead to hypoxia. Studies in CADASIL showed reduced baseline cerebral blood flow and impaired hemodynamic reserve with an impaired vasodilation response to acetazolamide.[9-11] However, it is unclear whether this reduced vascular reactivity is caused by an endothelium-independent or -dependent mechanism.[12-16] In RVCL-S, functional vascular properties have not been studied. Furthermore, in RVCL-S there is systemic involvement of highly vascularized organs and in CADASIL involvement of peripheral blood vessels has been demonstrated by electron microscopy and staining of NOTCH3.[1,5] Therefore, we investigated whether RVCL-S and CADASIL participants have impaired vascular reactivity and whether this is mediated by endothelial dysfunction or by a VSMCs-mediated mechanism in the peripheral blood vessels.

Methods

Protocol approvals and patient consents

The study was approved by the Ethics Committee of the Leiden University Medical Center (LUMC) and conducted in accordance with the Declaration of Helsinki. All participants were at least eighteen years of age and gave written informed consent.

Participants

RVCL-S and CADASIL participants with a confirmed pathogenic gene mutation in *TREX1* or *NOTCH3*, respectively, were recruited. Controls were recruited through the

families (friends or spouses, not related individuals) and public advertisements. An investigator, different from the operator performing the vascular measurements, obtained medical history and genetic and clinical information. In order to prevent un-blinding, participants were instructed not to reveal their mutation status or medical history to the operator. Patients were age-, BMI- and sex-matched to controls.

Clinical assessment

Participants completed a questionnaire with variables on medical history including migraine, Raynaud's phenomenon, retinopathy and other medical conditions. Additional questions related to current medication, alcohol, caffeine, smoking and drugs intake were asked. During the interview preceding the measurements, diagnoses of migraine and Raynaud's phenomenon were verified, according to established criteria.[17,18] Medication was subdivided in the following categories: anti-hypertensive, cholesterol-lowering, anti-platelet, anti-coagulant, prophylactic migraine medication, analgesics and oral contraceptive. If possible, participants included in the study, were asked to abstain from medication with vascular (side-)effects for one week prior to the measurements. Participants were instructed to abstain from alcohol and caffeine (for 12 hours) and smoking and food (for 6 hours). This was verified during the interview. Weight and height were measured; BMI calculated.

Biochemical measurements

Laboratory parameters (Table 1) were determined according to standard procedures.

Experimental design

One operator, blinded for the participants' status, performed all vascular measurements. Prior to the vascular measurements, participants rested in supine position for 15 minutes in a room with recorded 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 three measurements were analyzed. Subsequently, flow-mediated dilatation (FMD) and capsaicin-induced dermal blood flow (DBF) were measured in a standardized order.

Flow-mediated dilatation (endothelial function of conduit artery)

FMD of the brachial artery was performed to assess endothelium-dependent vasodilatation according to guidelines[19] 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) connected to a

data acquisition and processing unit. Measurements were performed as described earlier.[20] In brief, right brachial artery diameter was measured 5-10 cm proximal to the antecubital crease in a longitudinal plane. At baseline, brachial artery diameter and arterial blood velocity profiles were recorded three times; the mean was used for data analysis. After baseline measurements, a cuff (TMC7, D.E. Hokanson, Bellevue, WA, USA), placed around the forearm, was inflated to 220 mm Hg to temporarily occlude the arterial perfusion. The cuff was released after 5 minutes with recording of the peak blood velocity profile within the first 15 seconds. Brachial artery diameter was measured for 4 seconds at 0.5, 0.75, 1, 1.25, 1.5, 2-5 minutes after cuff release. FMD was expressed as maximal absolute and percentage increase in diameter from baseline. The FMD was corrected for hyperemic stimulus using peak shear rate (=peak flow velocity/baseline diameter) and for blood viscosity using peak shear stress (= peak shear rate*hematocrit), by dividing the FMD by the peak shear rate and shear stress, respectively.

After 15 minutes of recovery, 400 µg sublingual nitroglycerin (NTG) was administered as an exogenous NO-donor and every minute for 6 minutes NTG-induced brachial artery dilatation was assessed. Endothelium-independent vasodilatation capacity after administration of NTG was expressed as percentage increase in diameter from baseline. After one week we asked participants whether a migraine attack was provoked by NTG.

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

Local application of capsaicin results in binding to the Transient Receptor Potential Vanilloid type I (TRPV1) receptor, which is present at primary sensory neurons (Aδ- and C-fiber nociceptors). TRPV1 receptor binding of capsaicin induces a neurogenic inflammatory response due to predominant release of calcitonin gene-related peptide (CGRP).[21] CGRP is a very potent vasodilator causing a local increase in DBF, which can be quantified by Laser Doppler Perfusion Imaging (LDPI) (PeriScan PIM II®; Perimed, Järfälla, Sweden). Reproducibility of this test was confirmed earlier.[22] After 20 minutes of supine rest the baseline DBF was measured. Participants received single topical doses of 1000 µg per 20 µL capsaicin (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, vehicle was applied; DBF was measured at 10, 20, 30 and 40 minutes after capsaicin application. Almost half of the CADASIL group was measured on a differently calibrated LDPI scanner, for which a separate control group was recruited. Results are expressed as Arbitrary Units (AU) presented as change versus baseline in DBF after capsaicin application.

Statistical analysis

Continuous variables were described by mean ± standard deviation (SD) and discrete variables are presented as counts (percentages) unless indicated otherwise. The Shapiro-Wilk test was used to determine if the data was normally distributed. General characteristics of each group were compared using a one-way analysis of variance (ANOVA) for normally distributed continuous variables (Kruskal-Wallis test for non-normally distributed variables) and the Chi-square test for categorical variables. The results from the FMD were compared using the independent sample T-test for normally distributed variables and the Mann-Whitney U test for non-normally distributed variables. Mean increase in DBF after capsaicin (40 minutes) and mean area under the curve over 40 minutes (AUC_{0-40}) were compared using the one way analysis of covariance (ANCOVA) to take into account the use of two scanners. For all analyses a two sided p-value of alpha <0.05 was considered statistical significant. All statistical analyses were performed using SPSS 23.0 software (IBM Corp., Armonk, NY, USA).

Data availability

Anonymized data not published within this article will be made available by request from any qualified investigator.

Results

Participants

Eighteen RVCL-S and 23 CADASIL participants were compared with 26 matched controls. In Table 1 demographic details, clinical symptoms, and laboratory values are summarized. All tests were well-tolerated. No participant reported an NTG provoked migraine attack. Abstaining from alcohol, caffeine, smoking and food was successful, except for smoking in the CADASIL group (78% abstinence; five participants smoked <6 hours prior to measurements), abstinence from alcohol in the RVCL-S group (94% abstinence; one participant used 2 units alcohol <10 hours) and caffeine in the CADASIL group (96% abstinence; one participant used 1 unit coffee <6 hours). Success for abstaining from medication is depicted in Table 1. Not all participants could be included for all measurements. No significant differences for age, sex, BMI and blood pressure were found between included participants and those excluded.

Table 1. Participants demographics

Variable	Controls (n=26)	CADASIL (n=23)	RVCL-S (n=18)	p-value
Age (years)	49.0 ± 9.1	49.5 ± 12.7	50.1 ± 9.6	*0.94**
BMI (kg/m^2)	25.2 ± 3.6	26.7 ± 4.3	25.2 ± 3.7	*0.17***
Systolic blood pressure (mm Hg)	129.2 ± 20.5	126.2 ± 11.1	132.8 ± 21.7	*0.85***
Diastolic blood pressure (mm Hg)	77.8 ± 10.1	75.4 ± 6.7	75.3 ± 8.8	*0.41***
Female, n (%)	14 (54)	11 (48)	10 (56)	*0.87****
Smoking (Pack years)	3.0 ± 5.0[a]	13.4 ± 16.0	3.7 ± 5.8	***0.029****
Migraine, n (%)	0 (0)	6 (27)[b]	6 (33)	*N.A.*
Retinopathy, n (%)	0 (0)	0 (0)	9 (56)[c]	*N.A.*
Raynaud's phenomenon, n (%)	0 (0)	3 (13)	15 (83)	
Chronic medication use, n (%)[d]	5 (19)	17 (74)	12 (67)	*N.A.*
Oral contraceptive use (% of women)	3 (21)	1 (9)	0 (0)	
Anti-hypertensive drugs	0 (0)	6 (26) *(1)*	6 (33) *(2)*	
Cholesterol lowering medication	0 (0)	9 (39) *(2)*	6 (33) *(2)*	
Antiplatelet drugs	0 (0)	13 (57) *(3)*	8 (44) *(4)*	
Prophylactic migraine medication	0 (0)	1 (4)	0 (0)	
Analgesics (acetaminophen, NSAIDs)[e]	1 (4)	2 (9)	5 (28)	
Other (%)[f]	1 (4) *(1)*	7 (30) *(1)*	10 (56) *(3)*	
Laboratory parameters				
Hb (g/dL)	8.99 ± 0.71	9.15 ± 0.73	8.10 ± 0.65	***<0.001****
Ht (%)	0.43 ± 0.03	0.44 ± 0.04	0.40 ± 0.04	***0.001****

Hb = hemoglobin; Ht = hematocrit; N.A. = not applicable. Values presented are means ± SD or number (percentage). P-values calculated using * the one-way ANOVA, ** the Kruskal-Wallis test, *** the Chi-square test. [a] For one participant pack years was unavailable. [b] For one participant migraine was unclear. [c] Retinopathy status for two participants unknown. [d] Number of participants that abstained from medication 7 days prior to the measurements are depicted in italic. No participant used oral anticoagulants. [e] All participants used these painkillers for acute migraine treatment, none of them used acetaminophen or NSAIDs 24 hours prior to measurements. [f] Other medication: glucose lowering drugs, stomach acid inhibitors, alfuzosin, solifenacin, gabapentin, carbamazepine, pyridoxine, folic acid, valproic acid, alfacalcidol, allopurinol, dorzolamide/timolol and dexamethasone eye drops, baclofen, antihistamine drugs , ursodeoxycholic acid, lynestrenol, levothyroxine, atovaquon, darbepoetin alfa.

Flow-mediated dilatation

FMD was performed in 18 RVCL-S, 14 CADASIL participants and 18 controls but a valid set could only be obtained in 10 participants of each disease group and in 14 controls. When a valid set could not be obtained this was due to technical difficulties with ECG-gating (6) and movement artefacts (10). RVCL-S participants showed a lower FMD compared with controls (2.32 ± 3.83 *vs.* 5.76 ± 3.07, p=0.023) (Figure 1).

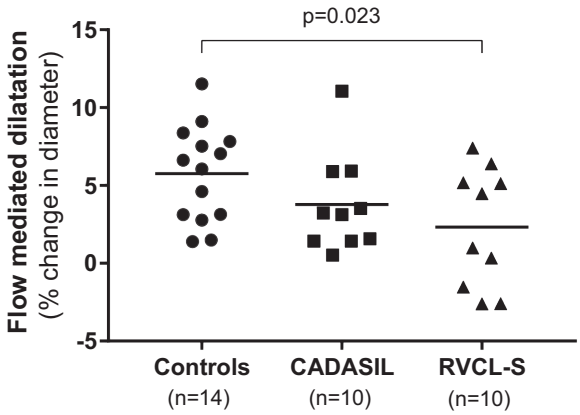

Figure 1. Flow-mediated dilatation expressed as the percentage change in brachial artery diameter after hyperemic stimulus in RVCL-S and CADASIL participants and controls.

Table 2. Vascular parameters of the brachial artery

Variable	Controls (n=14)	CADASIL (n=10)	*p-value*[a]	RVCL-S (n=10)	*p-value*[b]
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**
FMD (absolute increase in diameter) (µm)	332 ± 187	216 ± 169	0.13*	165 ± 256	*0.08**
Pre-occlusion velocity (cm/s)	11.7 ± 4.1	9.5 ± 3.4	0.20**	13.4 ± 6.3	*0.76****
Maximal post-occlusion velocity (cm/s)	82.4 ± 24.9	81.7 ± 20.5	0.94*	88.5 ± 21.4	*0.56**
Peak shear rate (s^{-1})	140.6 ± 33.7	138.4 ± 35.7	1.00**	141.4 ± 34.6	*0.94***
Peak shear rate normalized FMD ($*10^{-3}$) (%.s)	4.35 ± 2.31	2.99 ± 2.33	0.20*	1.32 ± 2.91	***0.015****
Peak shear stress (s^{-1})	61.6 ± 16.2	59.6 ± 14.6	0.78*	56.3 ± 15.3	*0.46**
Peak shear stress normalized FMD ($*10^{-3}$) (%.s)	10.07 ± 5.73	6.96 ± 5.58	0.23*	3.31 ± 7.24	***0.027****
Pre-NTG diameter (µm)	5710 ± 844	6198 ± 1019	0.21*	6190 ± 1034	*0.22**
Maximal post-NTG diameter (µm)	6496 ± 899	6947 ± 891	0.18**	6980 ± 1193	*0.29***
Post-NTG (% change in diameter)	14.13 ± 6.71	12.72 ± 6.61	0.62*	12.99 ± 7.39	*0.70**

NTG = nitroglycerin, FMD = flow-mediated dilatation. Data are presented as mean ± SD. P-values calculated using * the independent sample T-test, ** the Mann-Whitney U test. Due to missing peak shear rate and stress the corrected FMD could not be calculated in 2 controls and in 1 participant of each disease group.
[a] Difference between controls and CADASIL participants and [b] controls and RVCL-S participants.

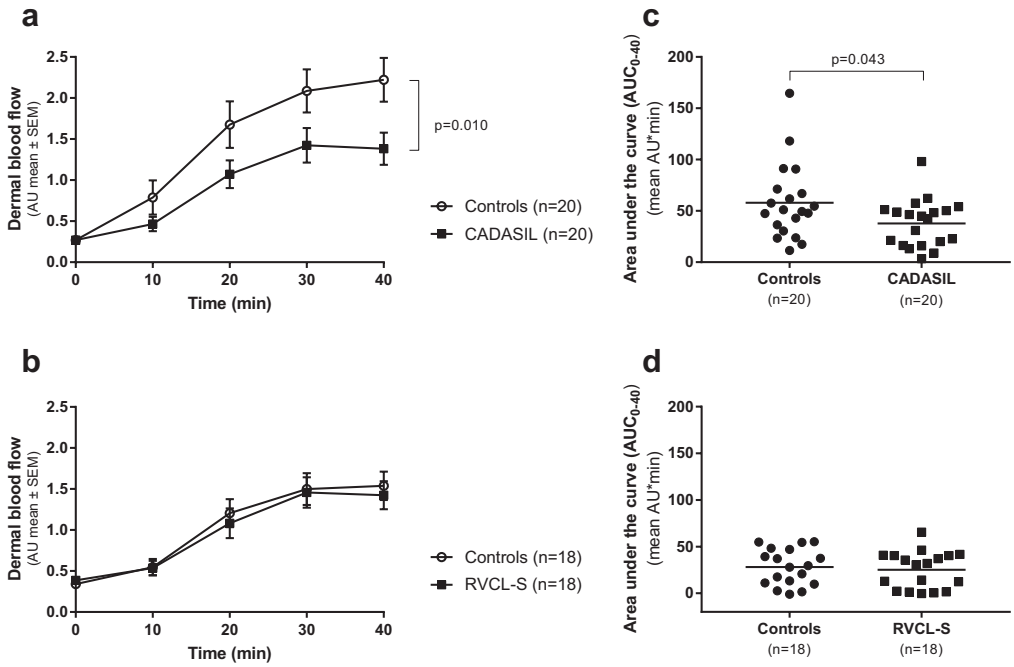

Figure 2. Microvascular reactivity of the skin after topical capsaicin application in CADASIL and RVCL-S participants.

Results shown as mean ± SEM. DBF increase over 40 minutes after capsaicin application in CADASIL **(A)** and RVCL-S **(B)** participants. Total DBF response over a period of 40 minutes after capsaicin application, displayed as AUC_{0-40}, in CADASIL **(C)** and RVCL-S **(D)** participants. The measurements of **(A)** and **(C)** were conducted using two different scanners.

No significant difference in FMD was observed between CADASIL participants and controls (3.77 ± 3.15 *vs.* 5.76 ± 3.07, p=0.14). The decreased FMD in RVCL-S persisted after correction for peak shear rate (p=0.015) and peak shear stress (p=0.027) (Table 2). There was no difference between RVCL-S and CADASIL participants and controls in FMD after the admission of nitroglycerin (Table 2).

Capsaicin-induced dermal blood flow

Capsaicin-induced DBF measurements were performed in 18 RVCL-S participants, 20 CADASIL participants and equal numbers of matching controls. Three participants were excluded because of skin allergy to alcohol containing solutions, colored skin and prophylactic anti-migraine medication (pizotifen). Results are summarized in Figure 2. CADASIL participants compared with controls displayed a lower increase in DBF 40 minutes after capsaicin application (1.38 ± 0.88 *vs.* 2.22 ± 1.20 AU, p=0.010) and a lower AUC_{0-40} (37.8 ± 23.0 *vs.* 57.9 ± 36.6 AU.min, p=0.043). No significant differences were found between RVCL-S participants and controls.

Discussion

This study provides evidence for endothelial dysfunction in RVCL-S patients. In addition, an impaired endothelial-independent vascular reactivity in the microvasculature is confirmed in CADASIL patients.

RVCL-S is caused by heterozygous C-terminal frameshift mutations in the *TREX1* gene[7], which encodes the major 3'-5' DNA exonuclease.[23] How *TREX1* mutations exactly lead to RVCL-S remains an enigma. *TREX1* mutations that cause RVCL-S preserve the enzymatic function of the protein but alter its intracellular localization because the C-terminus that anchors to the endoplasmic reticulum is absent. RVCL-S mutations are hypothesized to lead to a toxic gain-of-function that primarily affects the microvasculature through a mechanism that remains undetermined. Endothelial dysfunction might be such a mechanism. Mutated TREX1 protein may *directly* have detrimental effects on endothelial cells and thereby cause further pathological changes causing clinical symptoms. Alternatively, endothelial dysfunction may be a *consequence* of the pathological changes seen in RVCL-S. Regardless, clinical symptoms that occur in RVCL-S patients, i.e. cerebral infarction[24], Raynaud's phenomenon[25], migraine[20] and subcortical cognitive dysfunction (executive function, processing speed)[26], all have been linked to endothelial dysfunction. It should be noted that we did not measure endothelial function at the level of resistance arteries. Consequently, we are unable to determine whether endothelial dysfunction in RVCL-S can be extrapolated to small resistance vessels, but this seems likely. Iontophoresis or venous occlusion plethysmography with acetylcholine would be of interest as a follow-up study to further explore this. Only minimal degeneration of VSMCs is seen in RVCL-S, which is in accordance with findings of normal VSMC function in RVCL-S in our study.

CADASIL is caused by heterozygous missense mutations (that change the number of cysteine residues) in the *NOTCH3* gene[6], which encodes a cell surface receptor that is expressed on VSMCs. NOTCH3 is involved in a signal transduction pathway critical for development, homeostasis and differentiation of VSMCs.[27] A decreased DBF response to capsaicin was observed in CADASIL participants. Since capsaicin activates pre-synaptic TRPV1 receptors on Aδ- and C-fiber nociceptors and leads to relaxation of VSMCs of the skin microvasculature upon CGRP release, a decreased DBF 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/CGRP-receptors; or iv) a decreased relaxation of VSMCs in response to an endothelium-independent stimulus. Further

studies are required to elucidate the mechanism behind the decreased DBF response observed in CADASIL participants. Given the morphological abnormalities seen in the microvasculature of CADASIL patients, degeneration of VSMCs and a morphologically normal endothelium[5], a decreased relaxation of VSMCs in response to an endothelium-independent stimulus seems most plausible. Thus, our results suggest impaired endothelium-independent VSMC relaxation in CADASIL, and are in line with previous studies showing: i) impaired microvascular reactivity using other techniques without the involvement of CGRP;[13-16] ii) reduced baseline cerebral blood flow; and iii) impaired hemodynamic reserve measured as vasodilation response to acetazolamide.[9-11]

By 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. As such we found no evidence of altered vascular function of conduit vessels, in this case the brachial artery in CADASIL participants. This confirms earlier findings and is consistent with the believe that small arteries are most affected.[13,14] However, in contrast to our findings one study suggested endothelial dysfunction based on L-arginine-induced vasoreactivity of the middle cerebral artery in CADASIL.[15] There is, however, a methodological concern as that study may be biased by baseline differences between CADASIL and controls. Finally, in the microcirculation, forearm *resistance* arteries, impaired endothelium-dependent vasodilatation was reported.[14] Therefore, although we find no evidence for endothelial dysfunction in conduit arteries, our study is limited by not investigating endothelial function in the microvasculature, as there is evidence for dysfunction of endothelium in the microcirculation of CADASIL patients.[14]

The strengths of our study are: i) assessment of vascular reactivity with complementary measurements of endothelial and smooth muscle cell function; ii) investigation of an unique sample of the small vessel disorders RVCL-S and CADASIL that can be considered monogenic models for vascular cerebral disorders in general; iii) use of well-validated techniques[20,22]; and iv) performance of the investigations by one person who was blinded for the health status of the study participants. There are also limitations to our study. Because CADASIL and RVCL-S are rare diseases, sample sizes are relatively small. As a consequence we chose to focus on demonstrating a difference in response to capsaicin or nitroglycerin. Now that a difference in capsaicin-induced dermal blood flow has been demonstrated in CADASIL, it may be interesting to investigate whether there are differences in the dose-response relationship between CADASIL patients and controls. Additionally, not all measurements could be performed in all participants, but there were no differences between included and

excluded participants. Furthermore, due to the different scanners used in the FMD measurements we used two different control groups for the FMD measurements. We did however correct for the effect of the two scanners used. Whereas the effect of hematocrit was corrected via peak shear stress, this was not done for hemoglobin. Hemoglobin absorbs NO, a lower hemoglobin results in an increased FMD.[28] However, RVCL-S participants display slightly lower hemoglobin and thus correction would lead to an even lower FMD in RVCL-S. The slightly (not significant) reduced FMD observed in CADASIL might be the result of the mismatch in cigarette pack years, although only two heavy smokers dramatically influenced the number of pack years. For clinical reasons not all participants were permitted to stop their medication.

The increased prevalence of migraine in both CADASIL and RVCL-S might be related to the altered vascular properties found in the present and previous studies. Vascular dysfunction such as endothelial dysfunction [20] and increased risk for ischemic brain lesions has been linked with migraine, making this hypothesis more amendable.[29-31] In CADASIL altered vasoreactivity of the microcirculation may induce ischemia which may trigger cortical spreading depression (CSD), the underlying mechanism of the migraine aura.[32] In line with this hypothesis, transgenic CADASIL *Notch3* mice show a decreased threshold for CSD.[33] In contrast to CADASIL, RVCL-S patients seem to have mainly migraine without aura. An increased susceptibility for endothelial dysfunction supposedly contributes to the development of migraine without aura in RVCL-S. No migraine attacks were induced by NTG administration, possibly, due to the lower dose nitroglycerin used or sublingual administration.[34]

Future studies need to confirm our findings of endothelial dysfunction in RVCL-S and investigate endothelial dysfunction in microvasculature to further unravel pathophysiological mechanisms behind the distinct vascular impairment in RVCL-S and CADASIL and determine if our findings can be translated to the cerebral circulation. Additionally, further studies may link these vascular functional markers to disease progression and to the individual symptoms of these syndromes, such as stroke, dementia and migraine.

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8

CHAPTER 8

Optical Coherence Tomography Detects Retinal Changes in Hereditary Cerebral Amyloid Angiopathy

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Abstract

Background: Investigating mutation carriers with Dutch-type hereditary Cerebral Amyloid Angiopathy (D-CAA), offers the possibility to identify markers in pre- and symptomatic stages of CAA. Optical Coherence Tomography (OCT) has shown potential to detect retinal changes in several neurodegenerative diseases. We performed an exploratory study on the thickness of retinal layers as possible (early) biomarker in D-CAA mutation carriers.

Methods: D-CAA mutation carriers (n=8 presymptomatic, n=13 symptomatic, median age 50 years) and (n=9, median age 53 years) controls were scanned using Spectral Domain OCT (SD-OCT). Symptomatic mutation carriers were defined as having a history of ≥1 symptomatic intracerebral hemorrhage. D-CAA mutation carriers and controls were recruited from our D-CAA cohort and a healthy control cohort. Total peripapillary Retinal Nerve Fiber Layer (pRNFL) thickness, six regions of pRNFL, total macular volume (TMV), and individual macular regions thickness were measured and analyzed adjusted for age.

Results: The overall median (IQR) thickness of pRNFL was decreased in symptomatic, but not presymptomatic D-CAA mutation carriers compared with controls (91µm (86-95) versus 99µm (87-108),p=0.006). Both presymptomatic (111µm (93-122) versus 131µm (123-143),p<0.001) and symptomatic carriers (119µm (95-128) versus 131µm (123-143),p=0.034) had a thinner temporal-superior quadrant of the pRNFL versus controls. TMV or individual macular layers thickness did not differ between carriers and controls.

Conclusions: Thinning of the Retinal Nerve Fiber Layer may be a candidate marker of disease in hereditary CAA. Further studies are needed to determine whether retinal thinning is present in sporadic CAA and estimate its value as marker for disease progression.

Introduction

Dutch-type hereditary Cerebral Amyloid Angiopathy (D-CAA), also referred to as Hereditary Cerebral Hemorrhage With Amyloidosis-Dutch type (HCHWA-D), is an autosomal dominant neurovascular disease. D-CAA shares a similar pathology with the sporadic form of CAA, both leading to intracerebral hemorrhages (ICH) and vascular dementia. Since D-CAA mutation carriers can be studied also at the presymptomatic stage, they may provide insight in the evolution of CAA-pathology. To date, several neuro-imaging and amyloid biomarkers have been suggested.[1] Recently, the imaging method of Optical Coherence Tomography (OCT) has shown its potential in evaluating structural changes in the retina in patients with confirmed neuro(psycho)logical disorders.[2-4] Given the close relationship between the retina and the brain, studying the retina may provide an interesting predictive biomarker. Optical coherence tomography (OCT) provides high resolution cross-section images allowing quantification and follow-up of structural changes in the retina. We investigated the thickness of retinal and macular layers in D-CAA with an exploratory study using Spectral Domain (SD)-OCT.

Methods

Participants

D-CAA patients were recruited from the *'CAA-Heritage study'* of the Leiden University Medical Center.. This study identifies mutation carriers and follows them over time. Mutation carriers were diagnosed using DNA-analysis of the Glu693Gln mutation in the *APP*-gene. Mutation carriers were considered symptomatic if they suffered at least one symptomatic ICH. Exclusion criteria were age-related macular degeneration, eye trauma, macular dystrophy and primary glaucoma. Controls were included from a healthy ophthalmological control cohort at the LUMC. Controls were excluded if they had any ocular, cerebrovascular, neurodegenerative, metabolic or systemic disease. Participants were ≥18years old**.** All participants provided written informed consent. The study protocol was approved by the Medical Ethical Committe of the LUMC in accordance with the Declaration of Helsinki.

Ophthalmologic examinations

A neuro-ophthalmologist (I.C.N.) performed or supervised ophthalmologic examination of all participants, including slit lamp examination and ophthalmoscopy. Intraocular pressure (IOP) was measured using Goldmann applanation tonometry after applying topical oxybuprocaine monofree 0.4% and fluorescein dye. Afterwards, pupils were dilated with tropicamide monofree 0.5% and phenylephrine monofree

5.0%. Subsequently, color and red-free photographs of optic disc and macula were taken to be assessed for retinopathy (TRC-50DX, Topcon Europe Medical BV). Controls underwent standard ophthalmologic examination and fundus photography.

SD-Optical Coherence Tomography measurements

Optic disc and macula were analyzed in both eyes with dilated pupils using SD-OCT (Heidelberg Spectralis; Heidelberg Engineering) and image alignment eye tracking software (TruTrack ActiveEyeTracking, Heidelberg Engineering). Retinal images were created using software (Heidelberg Eye Explorer v1.9.10.0) with a minimum resolution of 768x768 pixels and field of view of 30° (figure 1A and B). Scans were obtained by blinded examiners. Peripapillary Retinal Nerve Fiber Layer (pRNFL) was measured with a circular scan with a diameter of 3.5 mm centered on the optic disc. Total macular volume (TMV) was measured using nine sectors using a 1-, 3-, and 6-mm grid focused on the fovea. Sectoral analyses of the pRNFL were performed (figure 1A); individual layers were automatically demarcated, qualitatively assessed and objectively quantified by segmentation software to measure thickness (figure 1B) as several neurological diseases have been shown to affect the retina in certain geometric locations and cellular layers.[5,6]

Statistical analyses

Demographics were described as median (interquartile range (IQR)) and count (percentages). SD-OCT-measurements were analyzed using generalized estimating equations (matrix: exchangeable) to account for inter-eye correlations within participants and to correct for age. GEE analysis is an extension of the generalized linear model. A p-value of <0.05 was considered significant. The APOSTEL recommendations for reporting quantitative OCT studies were applied.[7] Symptomatic and presymptomatic mutation carriers were also separately compared with controls.

Data Availability Statement

The data that support the findings of this study are available on reasonable request from the corresponding author. The data are not publicly available due to privacy and ethical restrictions.

Results

We included n=21 D-CAA mutation carriers and n=9 controls (42 eyes and 18 eyes, respectively). Mutation carriers were younger (median 50 years (IQR 47-57) vs. 58 years (IQR 54-64)), sex distribution was similar (women 62% vs. 56%). Of the D-CAA

mutation carriers, n=13 were symptomatic (median age 53 (IQR 49-62)) and n=8 were presymptomatic (median age 49 (IQR 44-52)). Symptomatic carriers developed a symptomatic intracerebral hemorrhage previously with a median of 3.8 years (IQR 0.8-7.8)). Of the eight presymptomatic mutation carriers, six had clinical MRI scans available that were performed after the OCT examination. Of these six, three did not have any microbleeds, little white matter hyperintensities (Fazekas 1) and no cortical superficial siderosis.. The other three had ≤10 strictly lobar microbleeds, varying white matter hyperintensity volumes (Fazekas 1-3), and no cortical superficial siderosis.

Ophthalmologic examinations

Besides slight narrowing of arterioles in one presymptomatic and four symptomatic mutation carriers (n=10 eyes), no retinopathy was observed in D-CAA mutation carriers. One symptomatic mutation carrier had early stage diabetes mellitus type II, but showed no signs of retinopathy. No elevated intraocular pressure was found with a median intraocular pressure in D-CAA mutation carriers of 14.0mmHg (13.0-16.0) and 15.5mmHg (12.8-19.0) in controls.

SD-Optical Coherence Tomography measurements

The pRNFL was thinner in D-CAA mutation carriers compared with controls (median (IQR) 91µm (86-95) vs. 99µm (87-108), p=0.006, figure 1C). This difference was mainly driven by symptomatic mutation carriers who had a thinner pRNFL compared with controls (92µm (87-96) vs. 99µm (87-108) , p=0.006, supplementary table). Presymptomatic mutation carriers showed a similar trend towards a thinner pRNFL (90µm (83-92) vs. 99µm (87-108), p=0.05, supplementary table). A thinner RNFL was also seen in two of the three presymptomatic carriers without presence of lobar microbleeds on MRI.

Sectoral analyses demonstrated that the pRNFL was thinner in the peripapillary temporal-superior sector in mutation carriers vs. controls (113µm (95-126) vs. 131µm (123-143),p=0.009, table 1). This sector was thinner in both symptomatic mutation carriers compared with controls (119µm (95-128) vs. 131µm (123-143),p<0.001, supplementary table) as well as presymptomatic mutation carriers (111µm (93-122) vs. 131µm (123-143),p<0.034, supplementary table). No differences were found in TMV (8.6mm^3 (8.3-8.8) vs. 8.7mm^3 (8.4-8.8), p=0.35, figure 1D) or individual macula layers (table 1 and supplementary table).

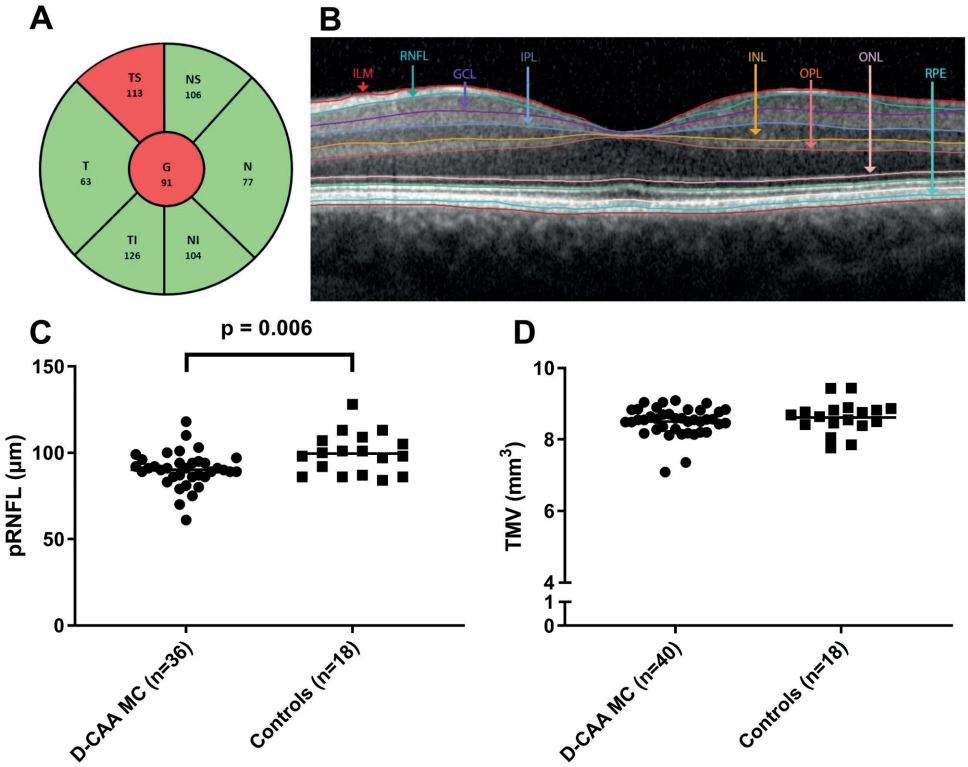

Figure 1. Optical Coherence Tomography measurements.

A circular scan measured the Peripapillary Retinal Nerve Fiber Layer (pRNFL) with a diameter of 3.5 mm centered on the optic disc. Sectoral analysis of the pRNFL was performed. Median of all mutation carriers per sector are reported (figure 1A). The total macular volume was measured by using nine sectors using a 1-, 3-, and 6-mm grid focused on the fovea centralis. Individual layers were automatically demarcated (figure 1B). The mean pRNFL thickness was reduced in D-CAA mutation carriers compared with controls (figure 1C). No difference was found in TMV (figure 1D). Global (G); temporal-superior (TS); temporal (T); temporal-inferior (TI); nasal-inferior (NI); nasal (N); nasal-superior (NS); inner limiting membrane (ILM); retinal nerve fiber layer (RNFL); ganglion cell layer (GCL); inner plexiform layer (IPL); inner nuclear layer (INL); outer plexiform layer (OPL); outer nuclear layer (ONL); retinal pigment epithelium (RPE).

Table 1. Peripapillary retinal fiber layer and macula layer thickness in D-CAA vs. controls.

	D-CAA patients median (IQR) (n=42)	Controls median (IQR) (n=18)	Coefficient	CI	P-value
Thickness of peripapillary sectors of the pRNFL†					
Mean pRNFL (µm)	91 (86-95)	99 (87-108)	-11.4	-19.5;-3.3	**0.006**
nasal-superior (µm)	106 (90-126)	102 (90-114)	-7.4	-25.7;10.9	0.43
nasal (µm)	77 (66-95)	79 (68-104)	-8.1	-26.2:10.1	0.39
nasal-inferior (µm)	104 (91-120)	124 (90-143)	-16.5	-37.0:4.0	0.11
temporal-inferior (µm)	126 (107-135)	135 (120-144)	-15.1	-33.7:3.5	0.11
temporal (µm)	63 (55-71)	62 (57-70)	-3.6	-10.7:3.5	0.32
temporal-superior (µm)	113 (95-126)	131 (123-143)	-23.2	-40.5:-5.8	**0.009**
Thickness of the macula layers ‡					
TMV (mm^3)	8.6 (8.3-8.8)	8.7 (8.4-8.8)	-0.2	-0.5;0.2	0.35
TMT (µm)	311 (305-321)	317 (306-320)	-2.6	-14.3:9.0	0.66
mRNFL (µm)	26 (25-28)	25 (23-27)	1.2	-0.6:2.9	0.19
GCL (µm)	40 (37-41)	40 (36-41)	-0.0	-2.5:2.5	1.00
IPL (µm)	34 (31-35)	34 (32-35)	-0.5	-2.6:1.6	0.65
INL (µm)	35 (34-37)	35 (33-38)	0.2	-1.9:2.4	0.84
OPL (µm)	29 (27-31)	29 (28-29)	-0.9	-2.1:0.3	0.15
ONL (µm)	68 (65-73)	71 (64-74)	-1.8	-8.5:4.9	0.60
RPE (µm)	15 (14-15)	15 (13-15)	0.3	-0.7:1.3	0.56

Interquartile range (IQR); confidence interval (CI); peripapillary retinal nerve fiber layer (pRNFL); total macular thickness (TMT); macular retinal nerve fiber layer (mRNFL); ganglion cell layer (GCL); inner plexiform layer (IPL); inner nuclear layer (INL); outer plexiform layer (OPL); outer nuclear layer (ONL); retinal pigment epithelium (RPE). Analyzes with generalized estimating equations to account for inter-eye correlations within participants and to correct for age. † In three D-CAA patients the scans were not of sufficient quality for analysis. ‡ In one D-CAA patient the scans were not of sufficient quality for analysis.

Discussion

In this exploratory study we demonstrated a thinner pRNFL in symptomatic D-CAA patients. Sectoral analyses showed that the temporal-superior sector of the pRNFL was thinner in both presymptomatic and symptomatic mutation carriers, which might indicate that changes to retinal nerve fibers occur before symptomatic intracerebral hemorrhage.

The pRNFL contains largely unmyelinated axons of the macular ganglion cells, thus mutation carriers demonstrate a loss of intraocular axonal integrity. RNFL thinning of the temporal sector indicates predominant damage of the papillomacular bundle, consisting of smaller, thinly myelinated axons. In several neurological disorders, including other hereditary arteriopathies,[4] it was observed that these thinner axons are more susceptible to injury.[8,9] Thinning of the RNFL is therefore not specific for CAA, although it does add to understanding CAA pathology. The RNFL forms the most inner retinal layer connecting the neuroretina with visual tracts leading to the visual cortex. The visual cortex is of particular interest in CAA, since CAA-pathology predominantly appears in the lobar regions of the brain, and mostly in the occipital lobe in both sporadic CAA[10] and D-CAA.[11] Damage in areas covering the visual tract may lead to retrograde degeneration of the optic nerve causing retinal changes starting with thinning of the RNFL.[3] It will be interesting to investigate if these peripapillary changes correlate with the clinical course as has been demonstrated in other diseases or with other biomarkers.[3,12,13] We investigated a hereditary form of CAA. So far, retinal changes in sporadic CAA have only been reported in case reports.[14,15]

This exploratory study has limitations. No vascular retinopathy was demonstrated in our study, but no invasive fluorescence angiography scans were performed as we aimed to find non-invasive biomarkers. We cannot rule out that invasive fluorescence angiography might have detected vasculopathy in these patients, which is a potential cause of RNFL thinning. However, several neurologic disease without retinal vasculopathy have shown thinning of the RNFL as well.[6] Secondly, our study has a relative small sample size due to the rareness of the disorder and might have been underpowered for subtle retinal changes., Additionally, even though we did multiple statistical tests, the significant level was kept at 0.05 which can be justified by the exploratory nature of this study. Future studies should aim to replicate these findings. Strength of our study are that we adjusted for differences in age and intereye correlation, and used an unique hereditary model for sporadic CAA in which the presymptomatic disease stage can be studied.

In CAA-related research, especially early markers are needed for future prevention trials. To date, several (early) markers in D-CAA have been demonstrated including reduced cerebrovascular reactivity and reduced amyloid-β concentrations in cerebrospinal fluid.[1] This study shows that thinning of the pRNFL measured with OCT might also serve as an early biomarker in CAA, especially since OCT is performed noninvasively in several minutes and with no safety risks.

Conclusion

Thinning of the pRNFL might serve as a marker for early and later phases of CAA-pathology. These results raise the possibility that OCT may be used as a rapid, non-invasive tool to detect early CAA-pathology.

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Supplementary data

Supplementary table 1. **Thickness of peripapillary sectors of the pRNFL in presymptomatic and symptomatic D-CAA mutation carriers and controls.**

	Presymptomatic D-CAA patients median (IQR) (n=16[†])	Symptomatic D-CAA patients median (IQR) (n=26[‡])	Controls median (IQR) (n=18)	Coefficient[§]	CI[§]	P-value[§]	Coefficient[¶]	CI[¶]	P-value[¶]
Thickness of peripapillary sectors of the pRNFL									
pRNFL (µm)	90 (83-92)	92 (87-96)	99 (87-108)	-12.4	-25.0:0.1	0.05	-11.4	-19.4:-3.3	**0.006**
nasal-superior (µm)	104 (88-125)	106 (87-131)	102 (90-114)	-9.3	-28.5:9.8	0.34	-7.5	-28.0:12.9	0.47
nasal (µm)	73 (66-88)	83 (65-97)	79 (68-104)	-3.0	-20.3:14.2	0.73	-7.3	-27.6:13.0	0.48
nasal-inferior (µm)	109 (71-122)	103 (92-116)	124 (90-143)	-13.2	-42.4:15.9	0.37	-17.2	-37.7:3.3	0.10
temporal-inferior (µm)	126 (109-132)	127 (104-137)	135 (120-144)	-22.7	-54.6:9.3	0.16	-15.4	-34.6:3.7	0.11
temporal (µm)	64 (56-76)	62 (55-68)	62 (57-70)	-5.4	-15.2:4.4	0.28	-4.1	-12.2:3.9	0.32
temporal-superior (µm)	111 (93-122)	119 (95-128)	131 (123-143)	-33.3	-51.2:-15.4	**<0.001**	-21.1	-40.5:-1.6	**0.034**
TMV and thickness of the macula layers									
TMV (mm^3)	8.6 (8.5-8.8)	8.5 (8.2-8.7)	8.7 (8.4-8.8)	-0.1	-0.5:0.3	0.53	-0.2	-0.5:0.2	0.41
TMT (µm)	313 (310-322)	308 (303-318)	317 (306-320)	0.8	-12.4:14.2	0.90	-3.0	-15.4:9.4	0.64
mRNFL (µm)	27 (25-28)	26 (25-28)	25 (23-27)	0.9	-1.1:3.0	0.38	1.2	-0.8:3.1	0.24
GCL (µm)	40 (37-41)	40 (36-41)	40 (36-41)	0.0	-2.8:2.9	0.98	0.0	-2.7:2.7	0.99
IPL (µm)	34 (32-35)	34 (31-35)	34 (32-35)	-0.0	-2.4:2.4	0.99	-0.7	-2.9:1.6	0.57
INL (µm)	35 (34-37)	36 (34-38)	35 (33-38)	0.6	-1.7:2.9	0.60	0.5	-1.8:2.9	0.66
OPL (µm)	29 (27-32)	29 (27-31)	29 (28-29)	-0.6	-2.7:1.5	0.57	-0.9	-2.2:0.4	0.19
ONL (µm)	69 (66-74)	68 (61-73)	71 (64-74)	0.9	-5.3:7.1	0.78	-2.4	-9.9:5.0	0.52
RPE (µm)	15 (13-15)	15 (14-15)	15 (13-15)	0.2	-1.1:1.4	0.81	0.4	-0.6:1.4	0.46

Interquartile range (IQR); confidence interval (CI); peripapillary retinal nerve fiber layer (pRNFL); total macular thickness (TMT); macular retinal nerve fiber layer (mRNFL); ganglion cell layer (GCL); inner plexiform layer (IPL); inner nuclear layer (INL); outer plexiform layer (OPL); outer nuclear layer (ONL); retinal pigment epithelium (RPE). Analyzes with generalized estimating equations to account for inter-eye correlations within participants and to correct for age. [†] In one presymptomatic D-CAA patient the scans of the pRNFL were not of sufficient quality for analysis. [‡] Macula scans of one symptomatic D-CAA patient and the pRNFL scans of two symptomatic D-CAA patients were not of sufficient quality for analysis. [§] Analyses of presymptomatic patients and controls. [¶] Analyses of symptomatic patients and controls.

9

CHAPTER 9

Spectral domain optical coherence tomography in RVCL-S, a monogenic small vessel disease

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Abstract

Objective: Retinal Vasculopathy with Cerebral Leukoencephalopathy and Systemic manifestations (RVCL-S) is a monogenic small vessel disease caused by mutations in *TREX1*. Several organs, including retina and brain, are affected. Analyzing retinal anatomy is increasingly used as a biomarker for ophthalmological and neurological disorders (due to their shared embryological origin of retina and brain). Optical coherence tomography (OCT) provides a non-invasive cross-sectional visualization of optic disc and macula. We aimed to use OCT to investigate retinal layer thickness in RVCL-S.

Methods: Cross-sectional, 17 *TREX1* mutation carriers (34 eyes) and 9 controls (18 eyes) underwent comprehensive ophthalmologic assessment followed by spectral domain OCT for measuring peripapillary retinal nerve fiber layer (pRNFL) thickness and total macular volume (TMV). Secondary outcomes included thickness of individual macular retinal layers and peripapillary sectors. Findings were analyzed using generalized estimating equations (GEE) to account for intereye correlation.

Results: *TREX1* mutation carriers had decreased pRNFL thickness (median (IQR) 76 (60-99) vs 99 (87-108)µm, $p<0.001$), and TMV (8.1 (7.4-8.5) vs 8.7 (8.4-8.8)mm^3, $p=0.006$) compared to controls. With the exception of the temporal sector, the thickness of all peripapillary sectors were decreased in *TREX1* mutation carriers. Ganglion cell layer (30 (22-37) vs 39 (36-41)µm, $p<0.001$) and inner plexiform layer (27 (24-34) vs 34 (31-35), $p=0.001$), were thinner in *TREX1* mutation carriers. Notably, in 9 out of 12 eyes with normal fundoscopic examination, retinal thinning was already detected.

Conclusion: RVCL-S, which may serve as a vascular retinopathy model, is associated with retinal thinning in the peripapillary and macular area. OCT findings can potentially serve as early biomarkers for RVCL-S and other vascular retinopathies.

Introduction

Retinal Vasculopathy with Cerebral Leukoencephalopathy and Systemic manifestations (RVCL-S) is a rare monogenic small vessel disease caused by C-terminal truncating mutations in *TREX1*.[1,2] RVCL-S is characterized by progressive vascular retinopathy and focal and diffuse brain dysfunction.

Decreased visual acuity and/or visual field defects due to vascular retinopathy are the most common presenting symptoms of RVCL-S.[2] Despite classic onset of disease between ages 35-50, early signs of vascular retinopathy without symptomatology have been demonstrated in patients in their early twenties.[2,3] Therefore, ophthalmologists play an important role in recognizing this underdiagnosed condition. Vascular retinopathy in RVCL-S is characterized in the early stages by telangiectasia, micro-aneurysms, and cotton wool spots. Later, perifoveal capillary obliterations and neovascularizations can occur. As the disease progresses secondary glaucoma and macula edema can develop as complications of retinopathy.[2] Fluorescein angiography often shows focal and extended areas of ischemia, capillary obliteration and microaneurysms, neovascularization, telangiectasias and fluorescein leaks. These findings are less likely to be due to a vasculitis but show similarity to diabetic retinopathy.[2-4] Ophthalmological treatment is similar to diabetic retinopathy and often involves retinal laser photocoagulation (peripheral retina mostly) and intravitreal anti-vascular endothelial growth factor (anti-VEGF) therapy. Treatment for secondary glaucoma may be required.

Functional tests demonstrated impaired endothelial function, consistent with the wide range of other systemic manifestations, including liver and kidney disease, anemia and Raynaud's phenomenon.[1,2,5,6] Currently, there are only symptomatic treatment options available and symptoms can progress rapidly towards middle age resulting in a premature death.[2,6] RVCL-S serves as a monogenic model for common vascular retinopathies and brain disorders such as diabetic retinopathy and vascular dementia.

Currently, no biomarker is available to predict clinical onset or progression of RVCL-S. Nevertheless, treatment can already be necessary before visual defects occur. Therefore, patients need to be followed up by an ophthalmologist in order to prevent or postpone severe visual complications. With the development of new non-invasive tools to evaluate retinal anatomy in patients at risk of vascular retinopathy this opens promising avenues for ophthalmologists.[7] Additionally, analyzing retinal anatomy has been demonstrated to possibly serve as a biomarker for multiple neurodegenerative

disorders.[8,9] Optical coherence tomography (OCT) provides a non-invasive high-resolution visualization of retinal layers and may thus provide a useful biomarker for neuro-ophthalmological disorders.[10]

We aimed to evaluate spectral domain (SD)-OCT findings in RVCL-S, a monogenetic model for vasculopathy in both retina and brain, by investigating the peripapillary and macular regions by measuring the peripapillary retinal nerve fiber layer (pRNFL) and macular layers.

Methods

Patients and controls

For this cross-sectional cohort study, patients with proven RVCL-S *TREX1* mutations were recruited. Participants were ≥18 years of age and provided informed consent. Controls were selected consecutively from an ophthalmological cohort at the Leiden University Medical Center to obtain a similar age and sex distribution. Exclusion criteria were history of diabetes mellitus, age-related macular degeneration, macular dystrophy, eye trauma, primary glaucoma, other optic neuropathies, and media opacity preventing optimal imaging (severe dry eye, cataract, etc.). Controls were excluded if they had (a history of) ocular, systemic, metabolic or cerebrovascular diseases or were related to RVCL-S patients. To evaluate using SD-OCT measurements as early biomarkers, eyes without signs of retinopathy were also assessed. The study was conducted in accordance with the Declaration of Helsinki and Ethics Committee approval was obtained.

Ophthalmologic examinations

Mutation carriers (MC) underwent an ophthalmologic examination by a neuro-ophthalmologist (ICN) including slit lamp examination, applanation tonometry and ophthalmoscopy. Intraocular pressure (IOP) was measured using Goldmann applanation tonometry after instillation of topical oxybuprocaine monofree 0.4% and fluorescein dye. Subsequently, pupils were dilated (tropicamide monofree 0.5% and phenylephrine monofree 5.0%). Afterwards, color and red-free photographs of optic disc and macula were taken (TRC-50DX, Topcon Europe Medical BV) and assessed for retinopathy. Controls underwent standard ophthalmologic examination and fundus photography.

SD-Optical Coherence Tomography

The optic disc and macula were analyzed in both eyes using SD-OCT (Heidelberg Spectralis, Heidelberg Engineering) with image alignment eye tracking software (TruTrack Active Eye Tracking, Heidelberg Engineering). Software (Heidelberg Eye Explorer v1.9.10.0) rendered retinal images with minimum resolution of 768x768 pixels with field of view set at 30°. The pRNFL was analyzed with a circular scan with a diameter of 3.5 mm centered on the optic disc. Secondarily, sectorial analyses were performed for different peripapillary segments. Macula retinal layers were measured while focusing on the fovea centralis by divided the area into nine sectors, using a 1-, 3-, and 6-mm grid.[11] Individual retinal layers were automatically demarcated, qualitatively assessed and quantified by segmentation software (figure 1B). Primarily, total macular volume (TMV) was measured to evaluate the retina as a whole, and not only macular thinning due to neuronal loss. As secondary outcomes total macular thickness (TMT) and thickness of the individual macula layers were evaluated. Lastly, we evaluated the pattern of retinal thinning. Scans were obtained by blinded examiners in single session. We used APOSTEL recommendations for reporting.[12]

Statistical analyses

Statistical analyses were performed using SPSS 23.0 (IBM Corporation, Armonk, NY). Demographics were compared using Mann-Whitney U test and Fisher's exact test. SD-OCT parameters were analyzed using generalized estimating equations (matrix: exchangeable) to account for intereye correlations within participants. To ensure results were not caused by secondary glaucoma or photocoagulation treatment in the scanned region, we conducted separate analyses for primary outcomes after exclusion of affected eyes. A p-value of <0.05 was considered significant.

Results

Patients and controls

We included 34 eyes of 17 *TREX1* MC and 18 eyes of 9 controls. MC and controls did not differ in age (median age (IQR) 54 (45-62) vs 58 years (54-65), p=0.11) or sex (65 vs 56% female, p=0.69). Retinopathy was observed in 22 eyes (from 12 RVCL-S patients), no controls showed signs of retinopathy. Fourteen eyes of RVCL-S patients had undergone previous photocoagulation treatment, three in scanned region. Five eyes were affected by secondary glaucoma. Median IOP (IQR) in RVCL-S patients was 14mmHg (13-16). Median visual acuity (IQR) in RVCL-S patients was 0.0 LogMAR (0.0-0.2).

Mean peripapillary RNFL and TMV

The pRNFL was significantly thinner in *TREX1* MC compared to controls (median (IQR) 76 (60-99) vs 99 (87-108)µm, p<0.001) (figure 1C). Furthermore, all sectors, with exception of the temporal sector, showed significant thinning in *TREX1* MC (figure 1A, table 1). TMV was decreased in *TREX1* MC compared to controls (median (IQR) 8.1 (7.4-8.5) vs 8.7 (8.4-8.8)mm^3, p=0.006) (figure 1D). After exclusion of eyes affected by secondary glaucoma and photocoagulation treatment in scanned regions pRNFL (median (IQR) 78 (62-99) vs 99 (87-108), p=0.003) and TMV (median (IQR) 8.2 (7.5-8.5) vs. 8.7 (8.4-8.8), p=0.029) remained thinner. We demonstrated that decreased pRNFL thickness and reduction of TMV already occurs as early as age 42 (figure 2).

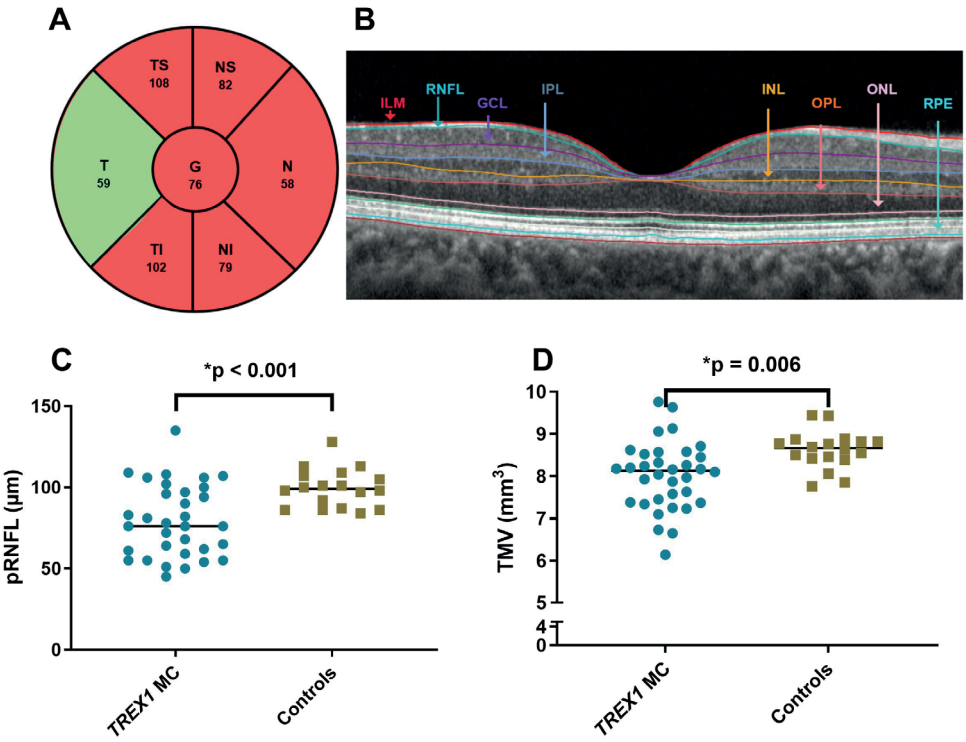

Figure 1. SD-OCT measurements in *TREX1* mutation carriers.

Median of all mutation carriers peripapillary retinal nerve fiber layer (pRNFL) per sector (dark grey indicates decreased thickness) (A). Individual macular layers (B). Median pRNFL (C) and total macular volume (TMV) (D) were reduced in *TREX1* mutation carriers. Global (G); temporal (T); nasal (N); inferior (I), superior (S); inner limiting membrane (ILM); retinal nerve fiber layer (RNFL); ganglion cell layer (GCL); inner plexiform layer (IPL); inner nuclear layer (INL); outer plexiform layer (OPL); outer nuclear layer (ONL); retinal pigment epithelium (RPE).

Table 1. Thickness of peripapillary sectors in *TREX1* mutation carriers.

	TREX1 MC median(IQR) (µm) (n=33#)	Controls median(IQR) (µm) (n=18)	Estimate	CI	P-value
Nasal sup.	82 (63-97)	102 (90-114)	-23.3	-42.0;-4.7	0.014*
Nasal	58 (45-78)	79 (68-104)	-26.5	-42.8;-10.1	0.002**
Nasal inf.	79 (60-109)	124 (90-143)	-34.4	-56.6;-12.2	0.002**
Temporal inf.	102 (64-131)	135 (120-144)	-32.6	-52.7;-12.5	0.001**
Temporal	59 (51-73)	62 (57-70)	-1.5	-12.5;9.5	0.79
Temporal sup.	108 (77-130)	131 (123-143)	-21.6	-39.1;-4.1	0.016*

Mutation carrier (MC); Interquartile range (IQR); confidence interval (CI). #In one patient measurements of one eye were not possible due to the patient's inability to focus. *p<0.05, **p<0.01.

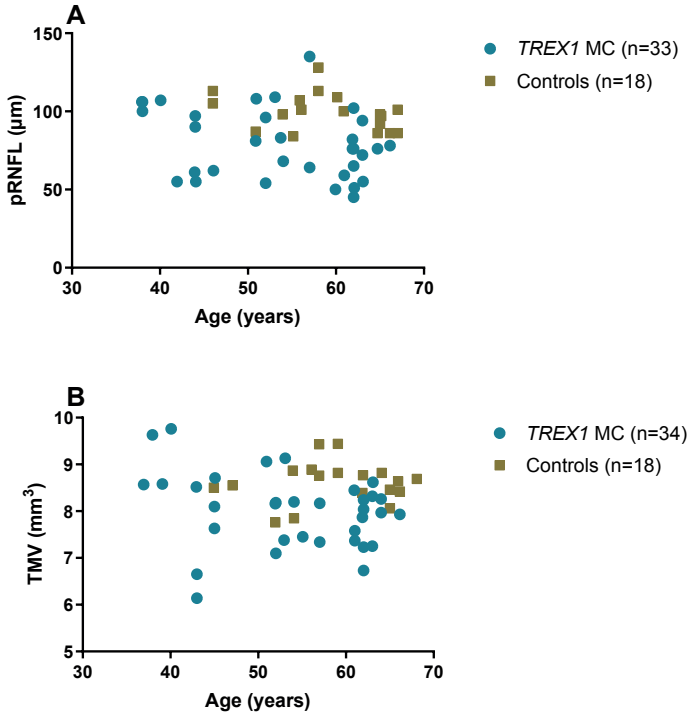

Figure 2. Distribution of peripapillary retinal nerve fiber layer thickness (A) and total macular volume (B) with age in *TREX1* mutation carriers and controls.

Mutation carriers (MC); peripapillary retinal nerve fiber layer (pRNFL); total macular volume (TMV).

Inner retinal layers

Decrease in TMT and individual retina layers thickness was demonstrated for the ganglion cell layer and inner plexiform layer (table 2). The ganglion cell layer showed the most striking reductions. While the majority of patients demonstrated patchy thinning of the GCL, four patients demonstrated sectorial thinning. Three of these patients had no overt signs of retinopathy at the time of scanning.

Table 2. Thickness of macula layers in *TREX1* mutation carriers.

	TREX1 MC median (IQR) (µm) (n=34)	Controls median (IQR) (µm) (n=18)	Estimate	CI	P-value
Total macular thickness	296 (267-314)	316 (308-323)	-21.8	-38.1;-5.6	0.009*
Macular RNFL	23 (20-27)	26 (24-27)	-2.7	-5.6;0.2	0.07
Ganglion cell layer	30 (22-37)	39 (36-41)	-8.7	-13.2;-4.2	<0.001**
Inner plexiform layer	27 (24-34)	34 (31-35)	-5.1	-8.1;-2.2	0.001*
Inner nuclear layer	35 (30-38)	35 (34-39)	-1.8	-4.8;1.3	0.25
Outer plexiform layer	29 (26-31)	29 (28-30)	-0.3	-1.8;1.2	0.70
Outer nuclear layer	69 (65-74)	70 (63-74)	-0.2	-6.3;6.0	0.96
Retinal pigment epithelium	14 (13-15)	14 (13-15)	-0.2	-1.2;0.8	0.68

Mutation carrier (MC); Interquartile range (IQR); confidence interval (CI); retinal nerve fiber layer (RNFL). *p<0.01, **p<0.001.

SD-OCT measurements as early biomarkers for RVCL-S

Importantly, in 9 out of 12 eyes in which funduscopic examination did not detect signs of retinopathy, decreased pRNFL thickness or reduction of TMV could already be detected.

Discussion

We demonstrated decreased pRNFL thickness and reduction of TMV in RVCL-S. The pRNFL contains unmyelinated axons of ganglion cells found in the ganglion cell layer, thus showing loss of intraocular axonal integrity in RVCL-S. Additionally, we found a thinning of the inner plexiform layer, where dendrites from inner nuclear layer neurons are located. Our study findings indicate also an effect of RVCL-S on retinal neurons. Indicating that OCT findings might provide useful biomarkers, as well as pathophysiologic insight in the mechanism of RVCL-S and possibly other vasculopathies.

As very little is known about OCT findings in RVCL-S, we chose one primary outcome focusing on one overall measurement of the peripapillary region (pRNFL thickness),

while the other focused on the macular region (TMV). We deliberately did not choose the GCL as primary outcome as we were not only interested in macular thinning due to neuronal loss. As TMV is frequently used in the literature with similar conditions we chose this outcome to more easily compare the results across diseases.[13] As TMT and TMV are highly correlated, we included TMT as a secondary outcome.

As RVCL-S is both a retinal vasculopathy and a neurodegenerative disorder, it is difficult to determine whether GCL loss is due to retinal disease or retrograde trans-synaptic degeneration. That most patients display a patchy pattern of GCL loss is most consistent with retinal disease. Nonetheless, that GCL loss already is apparent without overt retinal disease, and that this can also occur in a sectorial pattern may suggest that retrograde trans-synaptic degeneration could also play a role. Our findings may suggest that retrograde trans-synaptic degeneration occurs (and might be objectifiable in early disease stages), but also that retinal damage may be the main reason for further GCL thinning when retinopathy becomes more apparent. Studying (pre)symptomatic patients over time and relating OCT findings to MRI-imaging markers will help further elucidate these mechanisms for retinal loss.

There have been inconsistent reports on whether neurodegeneration of the retina occurs in diabetes mellitus. It appears that individual patient characteristics (e.g. diabetes type, control of disease and duration) might favor neurodegeneration. Nonetheless, several studies found thinning of the pRNFL and GCL.[14] Moreover, in early stages of diabetes before retinal vascular damage manifests OCT findings have suggested that retinal neurodegeneration already occurs.[7] Patients with hypertensive retinopathy also have lower central macular thickness and pRNFL thickness compared to controls.[15] Interestingly, patients with end-organ damage had significantly lower SD-OCT measurements, indicating its use as a diagnostic biomarker. Vascular retinopathy seen in RVCL-S is similar to diabetic and hypertensive retinopathy. We now demonstrate that the findings on optical coherence tomography are also similar.

Moreover, pRNFL thinning has also been reported in other hereditary cerebral angiopathies, including Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL) and hereditary Cerebral Amyloid Angiopathy-Dutch type (D-CAA).[13,16] In these disorders, macula thinning was not found. However, in other disorders with multisystemic inflammatory involvement, such as Behçet's disease, an inflammatory multisystemic chronic vasculitis, and systemic lupus erythematosus (SLE) decreased macular and RNFL thickness was demonstrated.[17,18] OCT findings are also promising biomarkers for neurodegenerative

disorders, such as Alzheimer's disease. In patients with Alzheimer's disease a reduction of macular volume correlated with cognitive impairment, and in a population-based study thinner pRNFL was associated with increased risk of developing dementia.[8,9] Additionally, macular and ganglion cell complex thickness were found to associate with the presence of dementia.[19] Taken together, this indicates a promising role for retinal thickness as a diagnostic and prognostic biomarker not only for rare but also for common neurovascular disorders of the eye and brain.

This exploratory study may have some limitations. Firstly, invasive fluorescence angiography was not performed as we focused on non-invasive techniques. Therefore, we cannot report on for instance capillary drop out and the size of foveal avascular zone while this would have been interesting. However, fluorescence angiography is a procedure with possible side effects that only depicts the current vascular status at time of investigation and does not allow for evaluation of retinal tissue. OCT in contrast, captures the permanent scar-like tissue damage, which is still present even in inactive disease stages. Nonetheless, in further studies it may still be useful to systematically evaluate findings on fluorescence angiography and to compare these to non-invasive tools such as OCT techniques. Secondly, we cannot fully exclude an influence of photocoagulation treatment on OCT measurements. As patients with more severe retinal vasculopathy are more likely to receive photocoagulation treatment the distinction of causality for retinal thinning may be difficult. However, the majority of our patients that received laser photocoagulation treatment were treated only in the periphery, and for our secondary analyses only eyes for which the regions of interest were not treated by photocoagulation were selected, thus theoretically decreasing the effect of treatment on our findings. Our findings are further supported by what has been shown in diabetic retinopathy. Laser treatment is used less frequently and thinning of the pRNFL on OCT has still been demonstrated, even before other signs of diabetic retinopathy appear.[20,21] A third limitation of our study is the relatively small sample size due to the rarity of the disorder, this also led to limited availability of individuals aged below 50 years. As such, currently no definite conclusion can be formulated about using SD-OCT findings as very early biomarkers from our exploratory study. Moreover, our study might have been underpowered for subtle retinal changes. Nonetheless, patients in their forties already have retinal thinning while funduscopic examination detected no signs of retinopathy. Finally, as is justified by the exploratory nature of our study, the significance level was kept at 0.05 even though multiple statistical tests were performed. We did perform adjustment for inter-eye correlation. A major strength of our study is the use of a unique hereditary neurovascular model for retinopathy and vascular dementia in which different disease stages can be studied in mutation carriers.

Currently, the retinopathy seen in RVCL-S is classified based on results of fundoscopy (and if indicated fluorescein angiography).[3,22] That almost all eyes without signs of retinopathy already demonstrated retinal thinning highlights the need to update retinopathy criteria with the use of new ophthalmological techniques such as OCT in general and specifically for RVCL-S.

It is vital for RVCL-S patients that they are recognized by ophthalmologists. Not only is ophthalmological monitoring critical, neurological monitoring and treatment for anemia, hypertension and kidney failure might be necessary. If the diagnosis is not recognized this can lead to unnecessary, possibly harmful, biopsies (including, but not limited to brain and kidney biopsies). Furthermore, misdiagnosis can have severe treatment implications. The diagnosis of RVCL-S should be suspected in adult patients of middle age presenting with vascular retinopathy and/or focal or global neurologic deficits, particularly in the setting of a positive family history of retinopathy or neurological diseases.[2,6,23] Given that patients often present with vision loss, the ophthalmologist should be cautious in cases of vascular retinopathy without an apparent etiology. Of note, a negative family history does not exclude RVCL-S, since de novo pathogenic variants can occur.[24] Furthermore, family members with RVCL-S may be misdiagnosed with other conditions (e.g., neurodegenerative disorders, vascular dementia, brain tumor, multiple sclerosis, hypertensive retinopathy and diabetic retinopathy).

This study demonstrated retinal thinning in RVCL-S patients in the peripapillary and macular area, even when funduscopic examination was normal. As OCT is a safe, noninvasive, and precise diagnostic tool, we expect an increasing impact of OCT on the future diagnostic workup of RVCL-S patients. Moreover, SD-OCT measurements may be used as preclinical biomarker in RVCL-S but also in common neurovascular conditions such as diabetic retinopathy and vascular dementia, for which RVCL-S serves as a monogenic model.

Conflict of interest

I. de Boer, S.R. Steenmeijer, N. Pelzer, M. Al-Nofal, G. Dijkman, I.C. Notting report no disclosures. G.M. Terwindt reports independent support from Dutch Heart Foundation, and Dutch Brain Foundation.

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CHAPTER 10

Optical Coherence Tomography Angiography Biomarkers of Microvascular Alterations in RVCL-S

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Abstract

Background: The brain and retina share many neuronal and vasculature characteristics. We investigated the retinal microvasculature in patients with a monogenic vasculopathy using optical coherence tomography angiography (OCT-A). OCT-A is a novel precise noninvasive imaging method that may provide biomarkers suitable for diagnosis and follow-up of small vessel diseases.

Methods: In this exploratory cross-sectional study, eleven RVCL-S patients and eleven age-matched healthy control participants were included. The size of the foveal avascular zone (FAZ) and the vascular density of the superficial capillary networks in the retina were measured by OCT-A.

Results: The symptomatic and presymptomatic patients showed significantly lower vascular density values than controls in the foveal region (median [IQR] 18.2% [15.8 – 18.6] vs. 24.4% [21.5 – 26.8] ($p < 0.001$), 29.8% [29.6 – 30.8] vs. 33.2% [32.0 – 33.6] ($p = 0.002$), respectively). The FAZ was significantly larger in the symptomatic RVCL-S patients than in the control group [13416 square pixels [7529 – 22860] vs. 1405 square pixels [1344 – 2470] ($p < 0.001$)). No significant difference was identified in measurements of FAZ comparing presymptomatic and controls.

Conclusion: Our findings with OCT-A demonstrated that RVCL-S causes an increase in the size of the FAZ in symptomatic RVCL-S patients compared to healthy participants. Moreover, there is a decrease in vessel density in the superficial capillary networks in both symptomatic and presymptomatic patients. In the future, newly developed precise objective instruments such as OCT(-A) may provide important tools in determining disease activity for follow up of common small vessel diseases.

Introduction

Retinal Vasculopathy with Cerebral Leukoencephalopathy and Systemic manifestations (RVCL-S) is a monogenic small vessel disease caused by heterozygous C-terminal truncating mutations in *TREX1*.[1] The best-known features of this disease are neurological manifestations of focal and diffuse brain dysfunction and white matter and intracerebral mass lesions on neuroimaging, and progressive blindness due to vascular retinopathy.[2,3] Besides these major features, RVCL-S can also be accompanied by a wide range of systemic manifestations including renal and liver disease, hypothyroidism and anemia.[4] It is unknown how mutations in the C-terminus of TREX1 cause the multisystem vasculopathy seen in individuals with RVCL-S. Nevertheless, the endothelium appears involved in the pathogenesis of the disorder.[5-7] RVCL-S serves as a monogenic model for common small vessel diseases and vascular brain disorders, such as vascular dementia.

Efficient early diagnostic testing of cerebral small vessel disease is still challenging and difficult. Therefore, the discovery of new non-invasive and economical screening tools for diagnosis is a major goal of current research. Due to the shared embryological origin of the retina and brain, the retina is regarded as an extension of the central nervous system, providing an opportunity to observe both neuronal and vascular changes in the brain. Optical coherence tomography angiography (OCT-A) is a novel non-invasive imaging technique that enables a detailed angiographic view of the retinal vascular network.[8] In addition, as OCT-A does not require intravenous injections of a dye contrast, patients avoid most of the side effects that occur with conventional angiography.

OCT-A data on retinal vasculature may not only serve as an additional diagnostic parameter. A detailed analysis of the retinal vasculature could help understand its role in disease pathophysiology and provide biomarkers for treatment response, not only in retinal but also in neurological disorders. In Alzheimer's disease, vessel density has been shown to be decreased in the superficial capillary networks,[9-11] and an enlarged foveal avascular zone (FAZ) has been described.[10,11] The FAZ is the round, capillary-free zone within the macula. FAZ area has been shown to correlate with cognitive performance, indicating its potential as a useful biomarker.[12] In Parkinson's disease, another neurodegenerative disorder, there also appears to be a decreased vessel density in the retina.[13] In another monogenic small vessel disease, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), a decrease in vascular density in the retina has been demonstrated.[14,15]

Measurements of FAZ size and vascular density using OCT-A have been shown to be highly reproducible.[16,17] Moreover, measurements of the foveal avascular zone area and vessel density have proven to be indicative of microcirculatory problems of the retina. This exploratory study was designed to assess the potential of OCT-angiography in providing non-invasive biomarkers for RVCL-S. Our main aim was to demonstrate whether there were differences between symptomatic RVCL-S patients and healthy controls, as we expected the greatest difference between these groups. Secondly, we evaluated differences between presymptomatic patients and controls to determine the potential of OCT-A measurements as early biomarkers.

Materials and Methods

Participants

This cross-sectional pilot study was conducted at the Leiden University Medical Center (LUMC) in the Netherlands, a tertiary referral center for RVCL-S, where all known families with RVCL-S from the Netherlands are seen. Symptomatic and presymptomatic RVCL-S patients with DNA proven mutations in the *TREX1* gene were recruited. Patients were considered symptomatic if they had any vascular abnormality such as micro-aneurysms, hemorrhages, focal and extended areas of ischemia or neovascular proliferation detected by fundoscopy or fluorescein angiography (FA) of the retina, neurological symptoms or RVCL-S specific findings on MRI (punctate T2 hyperintense white matter lesions with nodular enhancement; and/or larger T2 hyperintense white matter mass lesions with rim-enhancement, mass effect, and surrounding edema). Two age-matched control groups were selected out of an ophthalmological control cohort at the LUMC to achieve a similar age distribution. Exclusion criteria were history of diabetes mellitus, age-related macular degeneration, macular dystrophy, eye trauma or primary glaucoma. Controls were excluded if they had (a history of) ocular, systemic, metabolic or cerebrovascular diseases. All participants were ≥18 years of age and gave informed consent, as documented in their medical file. The study was performed according to the tenets of the Helsinki Declaration and the protocol was approved by the Ethics Committee of the LUMC.

Ophthalmological examination and OCT-A imaging

All patients underwent a detailed ophthalmologic examination by the same physician (MA), supervised by a neuro-ophthalmologist (ICN), including visual acuity measurement, slit lamp examination, applanation tonometry and fundoscopy. Intraocular pressure (IOP) was measured using Goldmann applanation tonometry after instillation of topical oxybuprocaine monofree 0.4% and fluorescein dye. After

achieving a mydriatic effect using topical tropicamide monofree 0,5%, OCT-A scans (3x3mm, focused on the central part of the macula), using NideK RS-3000 (NIDEK, Aichi, Japan) (for symptomatic and age-matched controls) and Heidelberg Spectralis (Heidelberg Engineering, Franklin, USA) (for presymptomatic and age-matched controls), were performed. One OCT-A scan was performed per eye per participant. The superficial capillary plexus was visualized, using the automatic software segmentation algorithm. The superficial capillary plexus was defined as the plexus between the ILM (Internal Limiting Membrane) and IPL (Inner Plexiform Layer) interface. The automatically generated OCT-A images were reviewed manually.

OCT-A imaging processing

OCT-A image processing was performed in the Fiji ImageJ software (developed by Wayne Rasband MD, National Institutes of Health, Bethesda; available at https://imagej.nih.gov/ij/download.html) (Figure 1). The 3x3mm images were adjusted to correspond with 512x512 pixels, setting the scale on 170,6667 pixels/mm. The area of the foveal avascular zone (FAZ) was outlined manually to automatically calculate the surface area. The vessel density of the superficial capillary networks was defined as the proportion of vessel area with blood flow over the total area measured. The vessel density was measured using a plugin feature on ImageJ (available at https://imagej.net/plugins/vessel-analysis), after transforming the image to a binary color code. Eyes were excluded if movement artefacts were present.

Statistical analyses

Statistical analyses were performed using SPSS 25.0 (IBM Corporation, Armonk, NY). Demographics and OCT-A values are presented as median [interquartile range (IQR)]. FAZ size and vascular density were analyzed using generalized estimating equations (matrix: exchangeable) analyses to account for inter-eye correlations within participants. A p-value of <0.05 was considered significant.

Results

Demographics

Six symptomatic DNA proven RVCL-S patients (2 females and 4 males) (11 eyes) and 5 presymptomatic patients (3 female and 2 male) (10 eyes) were included in this study. In the RVCL-S group one patient was monocular due to enucleation following complications of neovascular glaucoma. In the presymptomatic group, in three eyes vascular density could not be determined due to incomplete image acquisition. Two age-matched control groups were included, one for the symptomatic (6 women) (10

eyes) and one for the presymptomatic RVCL-S group (3 females and 2 males) (10 eyes). In total three eyes (one symptomatic patient, two controls) were excluded due to movement artifacts on OCT-A images.

The median age in the RVCL-S group was 62.5 years (IQR 48.0-67.0). The median age in the group of healthy controls was 61.0 years (IQR 55.0-63.0). The median age in the presymptomatic RVCL-S group was 30 years (IQR 29.0-35.0) and the median age in the matched healthy control group was 25.0 years (IQR 23.0-31.0). The median visual acuity of the symptomatic RVCL-S patients was 0.0 in logMAR (IQR 0.0-0.2). Presymtomatic patients and controls had no visual impairments. Intra-ocular pressure was within normal limits in all participants. For a further clinical description of the patient cohort, see table 1.

Table 1. Clinical findings in the RVCL-S cohort

	Symptomatic RVCL-S patients (n=6)	Presymptomatic RVCL-S patients (n=5)
Retinopathy (yes, n(%)	6/6 (100)	0/0 (0)
Features of focal or global brain dysfunction (yes, n(%))	4/6 (67)	0/0 (0)
RVCL-S specific MRI findings (yes, n(%))*	4/5 (80)	0/0 (0)
Internal organ dysfunction (yes, n(%))#	3/6 (50)	0/0 (0)

* Of one patient no MRI was available. # Internal organ dysfunction was defined as organ dysfunction (liver, kidney and thyroid disease and anemia) requiring treatment.

OCT-A imaging in symptomatic RVCL-S patients vs. controls

The area of the foveal avascular zone was greater in symptomatic RVCL-S patients when compared to controls (median [IQR] 13416 square pixels [7529 – 22860] vs. 1405 square pixels [1344 – 2470], $p < 0.001$) (Figure 1 and 2A, Table 2). Moreover, vessel density in the superficial capillary networks was decreased in symptomatic RVCL-S patients (median [IQR] patients: 18.2% [15.8 – 18.6] vs. controls: 24.4% [21.5 – 26.8], $p<0.001$) (Figure 3A, Table 2).

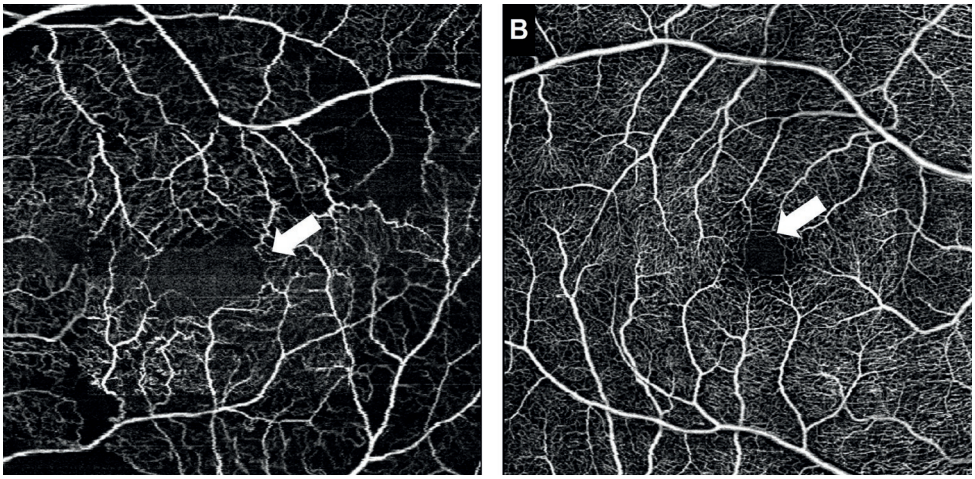

Figure 1. Representative images of the foveal avascular zone (FAZ).

White arrow indicates the outlines of the FAZ in a symptomatic RVCL-S patient (A) and an age-matched healthy control (B)

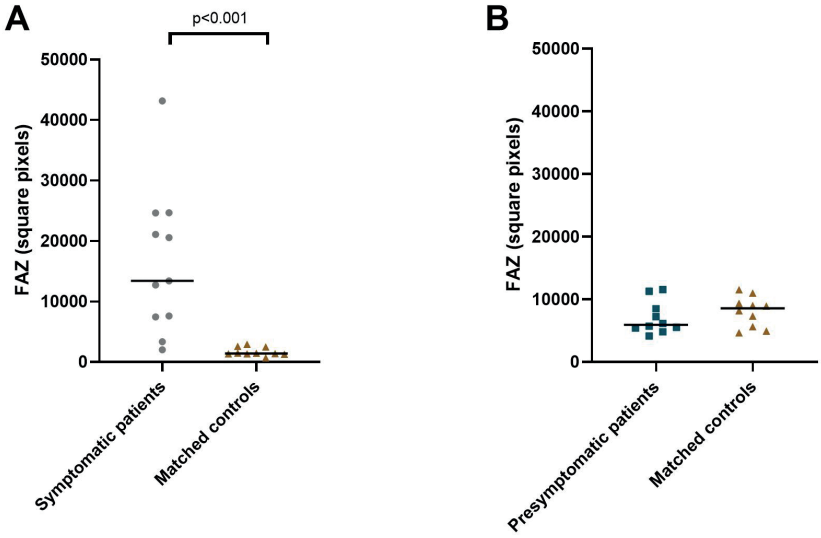

Figure 2. Median foveal avascular zone area in symptomatic RVCL-S patients and controls (A) and presymptomatic RVCL-S patients and controls (B).

Table 2. Foveal avascular zone area and vascular density in RVCL-S patients vs. controls.

	Coefficient	CI	P-value
Symptomatic patients			
Foveal avascular zone area	14734.4	7940.8 – 2.1528.0	<0.001
Vessel density	-7.5	-9.8 – -5.1	<0.001
Presymptomatic patients			
Foveal avascular zone area	-1010	-3.095.9 – 1075.9	0.34
Vessel density	-2.8	-4.6 – -1.1	0.002

OCT-A measurements as early biomarkers for RVCL-S: presymptomatic patients vs. controls

No difference was found in FAZ size between presymptomatic RVCL-S patients and controls (Figure 2B, Table 2). The vessel density did appear to be decreased in presymptomatic RVCL-S patients (median [IQR] 29.8% [29.6 – 30.8] vs. 33.2% [32.0 – 33.6], p = 0.002) (Figure 3B, Table 2).

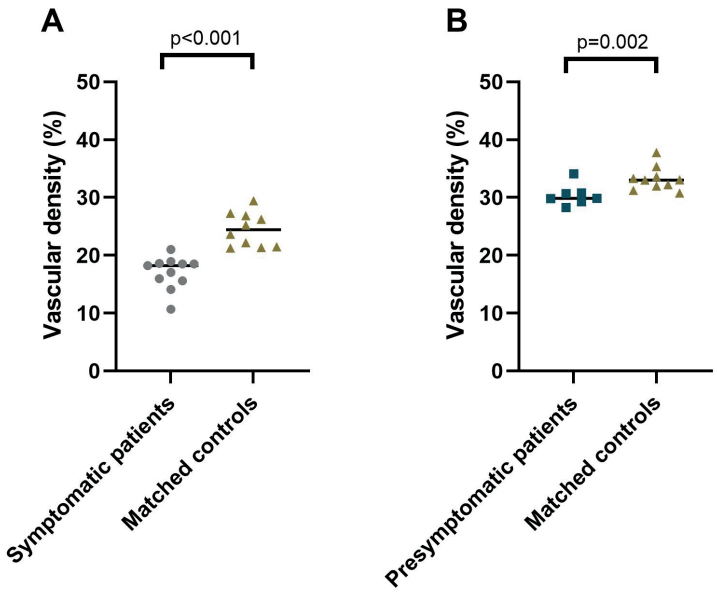

Figure 3. Median vascular density of the superficial capillary networks in symptomatic RVCL-S patients and controls (A) and presymptomatic RVCL-S patients and controls (B).

Discussion

We discovered that the macular vessel density in the superficial capillary networks was lower in symptomatic and presymptomatic RVCL-S patients than in the healthy control groups. Moreover, we found that symptomatic RVCL-S patients had an increase in FAZ size compared to healthy participants. The eye can be used as a window into neurological conditions. As cerebral and retinal arterioles share a similar anatomy and embryology, there is evidence for an association between retinal vessel changes and cerebral small vessel disease.[18,19] OCT-A is a non-invasive technique that can be used as a tool to learn more about these vessels, but also to find biomarkers of disease onset or progression. Furthermore, architecture of retinal vasculature can provide essential clinical information not just in ocular disease but also in systemic vascular disorders. Given the pressing need for improved biomarkers in systemic vascular diseases, non-invasive imaging of retinal vessels appears a promising avenue of research.

In this study we compared manual measurements of the foveal avascular zone and the macular vascular density in the superficial capillary networks in RVCL-S patients with healthy controls. This pilot study already demonstrated retinal abnormalities found on OCT-A images in this small vessel disease. While we could not evaluate whether OCT-A findings correlated with symptomology in this pilot study, in CADASIL, another monogenic small vessel disease, macular vascular density correlated with gait speed, suggesting its use as a biomarker of advanced CADASIL.[15] OCT-A findings are also a promising biomarkers in other neurodegenerative disorders. In preclinical Alzheimer Disease, patients without any visible abnormalities on fundoscopy already demonstrated an increased FAZ.[20] Together, this underlines the possible clinical importance of OCT-A for rare and common neurovascular disorders.

Strikingly, in this pilot study we already demonstrated reduced vascular density in presymptomatic patients. A previous study in patients with cerebral small vessel disease also reported a decrease in vascular density, while no differences in FAZ size were demonstrated.[21] The decrease in vascular density could therefore be the first sign of failure of autoregulation within the retinal circulation. As the blood-retinal-barrier (BRB) shares many similarities with the blood-brain-barrier (BBB) (endothelial cells, basement membranes, pericytes, glial cells and both are crucial for autoregulation),[19] similar pathological processes are likely responsible for both the retinal microvascular changes and the cerebral microangiopathy seen in cerebral small vessel diseases. Ischemic processes, inflammation and endothelial dysfunction have all been implicated to influence BRB and BBB function.[19,22] Interestingly, in patients with RVCL-S, impaired cerebrovascular reactivity, i.e. the ability of the cerebral vessels to dilate and

constrict to vasoactive stimuli, has been demonstrated. While less pronounced, these findings were already present in mutation carriers younger than 40 years.[7] Failure in regulation of blood flow could thus be a major component to the disease pathway and therefore, more research is needed to elucidate the mechanism behind these microvascular changes.

Our exploratory study has several limitations. Due to the small sample size we could not estimate the exact relationship between disease severity and the FAZ. Moreover, confirmatory studies with larger sample sizes are needed. Unfortunately, presymptomatic patients (and their age-matched) controls were included after another OCT-A device had become in use. Due to this use of two different OCT-A devices, we could not compare the processed OCT-A images of symptomatic with presymptomatic patients. In a follow-up study, this should be avoided when possible. Larger sample size will also be required for this comparison to be able to take into account the effect of age on OCT-A parameters. Another limitation was the impossibility to fixate for some participants. Due to the poor eye fixation in both the RVCL-S and the healthy control group (caused by patient related fatigue, poor visual acuity or limited physical condition) movement artefacts on the OCT-A images led to exclusion. However, this non-invasive imaging method could be very useful in patients with limited systemic symptoms.

A major strength of our study is the use of a unique hereditary neurovascular small vessel model for small vessel disease and vascular dementia. In this model we can study different disease stages in mutation carriers, which provides the unique opportunity to evaluate the presymptomatic disease stage. Also, to our knowledge, this is the first study describing the use of OCT-angiography in RVCL-S.

Recently, RVCL-S was shown to be associated with retinal thinning in the peripapillary and macular area.[23] This already demonstrates the possible value of OCT findings as potential biomarkers for RVCL-S. Our pilot study indicates a possible added value of also measuring the foveal avascular zone and vascular density. A more extended study with a larger sample size, as well as a follow-up design, is needed to determine the exact characteristics and cut-off rates needed for the foveal avascular zone and vessel density to be used as biomarkers and to demonstrate their usefulness in clinical practice. Moreover relating OCT-A findings to MRI brain imaging markers will help further determine the role of OCT-A measurements as biomarkers. This promising first step towards achieving reliable retinal vascular biomarkers for RVCL-S, a monogenic small vessel disease, can also help elucidate biomarkers for common (cerebral) small vessel diseases.

Conclusions

Using OCT-A, we demonstrated that RVCL-S leads to an increase in the size of the FAZ. Furthermore, there was a decrease in vessel density in the superficial capillary networks, which was already present in presymptomatic patients. In the future, newly developed precise objective instruments such as OCT(-A) may provide important tools in determining disease activity for follow up of RVCL-S, but possibly also of common small vessel diseases.

Conflict of Interest

M. Al-Nofal, S. Agirman, A.E. Wilms and A.H. Zamanipoor Najafabadi and I.C. Notting report no disclosures. I. de Boer reports independent research support from the international retinal research foundation (IRRF) and Dutch Heart foundation. I.C. Notting reports independent research support by the IRRF and Stichting Dioraphte. G.M. Terwindt reports grants or consultancy support from Novartis, Lilly, Teva, Allergan, and independent support from Netherlands Organization for Health Research and Development (NWO, and ZonMW), NIH, European Community, Dutch Heart Foundation, and Dutch Brain Foundation, IRRF and Stichting Dioraphte.

Author Contributions

MA, IdB, ICN and GMT conceived and designed the study. MA, SA, AW, AHZ collected the data. IdB performed the analyses. MA, IdB, ICN, GMT wrote the first version of the manuscript. All authors interpreted the data, provided intellectual input, revised the manuscript, and approved the manuscript before submission.

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11

CHAPTER 11

Retinal oxygen saturation in patients with Retinal Vasculopathy with Cerebral Leukoencephalopathy and Systemic manifestations

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Submitted

Abstract

Purpose: Retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations (RVCL-S) is an autosomal dominant small vessel disease caused by mutations in *TREX1*. Early in the course of the disease, retinal vasculopathy emerges as a key feature and retinal oxygen metabolism is thought to be affected. We aimed to determine if retinal oxygen saturation differs between RVCL-S patients and controls.

Methods: Retinal oxygen saturation was assessed in 17 eyes from 9 RVCL-S patients and 19 eyes from 15 controls by using a non-invasive technology (Oxymap T1 retinal oximeter). Mean arterial and venular oxygen saturation and arteriovenous difference per eye were measured, as well as vessel calibre.

Results: No differences in arterial oxygen saturation were found between RVCL-S patients and controls (median [IQR]: 94.5% [92.7-98.9] vs 93.4% [87.2-98.3], p-value = 0.12). However, the venular oxygen saturation of RVCL-S patients was considerably higher than in control patients, median of 71.5% [IQR: 55.0-77.2] and 55.5% [IQR: 51.2-59.9] respectively (p-value < 0.001). In line with these findings, the arteriovenous difference was decreased in RVCL-S patients (median [IQR]: 22.6% [18.5-38.3] vs 38.5% [34.1-39.9], p-value < 0.001). Finally, RVCL-S patients exhibited a reduced calibre in both arterial and venular vessels.

Conclusion: Retinal venular oxygen saturation is higher in individuals with RVCL-S compared to controls, likely due to decreased oxygen consumption resulting from retinal atrophy. Retinal oximetry serves as a non-invasive and innovative tool for biomarker identification, aiding in the detection and prediction of complications associated with retinal vasculopathy in RVCL-S.

Introduction

Retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations (RVCL-S) is an autosomal dominant small vessel disease caused by truncating mutations located in the C-terminus of *TREX1*.[1,2] *TREX1* encodes a 3'-exonuclease that removes nucleotides from the 3'ends of DNA molecules. How these mutation lead to RVCL-S is unknown. Patients with RVCL-S mainly present with progressive visual impairment due to vascular retinopathy. Additionally, the disease is characterized by brain white matter lesions, intracerebral tumefactive lesions, migraine and internal organ dysfunction, such as kidney and liver disease.[2] Symptoms advance rapidly towards middle age, resulting in premature death.

Although RVCL-S can cause a broad spectrum of symptoms, visual impairment is often the presenting symptom.[2] As such, ophthalmologists are generally the first to see these patients. Identifying patients with RVCL-S is crucial, as retinopathy and systemic manifestations often necessitate (aggressive) treatment. Retinal vasculopathy exhibits similarities to hypertensive and diabetic retinopathy, possibly contributing to the underdiagnosis of RVCL-S. Early signs of retinopathy in RVCL-S include cotton wool spots, micro-aneurysms and small haemorrhages and can occur prior to onset of symptoms, such as visual loss.[2] Ischemia of the central and peripheral retina and neovascularisation occur in later disease stages.[2] Treatment of retinal vasculopathy in RVCL-S patients is comparable to treatment of diabetic retinopathy, consisting mainly of anti-vascular endothelial growth factor (anti-VEGF) injections for cystic macular oedema and laser photocoagulation for ischemia of the peripheral retina.[2]

No biomarkers are currently available to predict onset and progression of retinal vasculopathy or to monitor treatment response. Therefore, patients have to be strictly followed by an ophthalmologist to evaluate the need for (additional) treatment, sometimes for decades, before signs of retinopathy occur. As fundoscopy alone is not sufficient, invasive procedures such as fundoscopic angiography (FAG) have to be performed to follow disease progression.[2] Identification of predictive biomarkers would enable physicians to effectively screen and timely treat retinal vasculopathy and thereby prevent possible detrimental complications of retinal ischemia, including neovascular glaucoma.

Disturbances in oxygen saturation levels have demonstrated their potential as a biomarker in a wide range of diseases, including diabetic retinopathy, but also central nervous system diseases, such as Alzheimer's disease.[3,4] Diabetic retinopathy shows

similarities to retinal vasculopathy in RVCL-S and thus, retinal oximetry may also be a promising tool to predict and monitor RVCL-S progression.

Retinal oximetry is a non-invasive tool to measure the retinal arterial and venular oxygen saturation. Retinal vascular dysregulation can be part of disease pathophysiology, but also be a consequence of disease. By measuring the retinal arterial and venular oxygen saturation separately, retinal oximetry can provide information on the underlying mechanisms that cause abnormalities in retinal oxygenation. Thus, retinal oximetry might provide new insights into pathophysiology of diseases and might ultimately lead to the identification of new treatment targets.

This study aimed to evaluate whether retinal oxygenation is abnormal in RVCL-S patients by comparing oxygen saturation of the retinal vessels between RVCL-S patients and controls with the goal to elucidate pathophysiological mechanisms involved in small vessel diseases and to identify possible non-invasive biomarkers.

Method

Study population

The Leiden University Medical Centre (LUMC) is a tertiary referral centre for RVCL-S. Confirmed *TREX1* mutation carriers who visited the ophthalmological outpatient clinic between July 2012 and August 2020 were eligible for this cross-sectional study. Controls were selected at random from an ophthalmological cohort at the LUMC. Controls were excluded if they had metabolic, neurological or cerebrovascular diseases. In controls, only healthy eyes were included without abnormalities affecting retinal oxygenation, thus excluding eyes that were affected by present or past retinal or optic nerve diseases, for example choroidal melanoma, papilledema or glaucoma, as this could affect retinal oxygenation. Participants were above the age of 18. The study was approved by the LUMC medical ethics committee and conducted in accordance with the criteria of the Declaration of Helsinki.

Ophthalmological examination

All participants underwent standard ophthalmologic examination by an ophthalmologist consisting of measuring best-corrected visual acuity, slit lamp examination and fundoscopy. Moreover, intraocular pressure (IOP) was measured using Goldmann applanation tonometry after instillation of topical oxybuprocaine monofree 0.4% and fluorescein dye. Prior to performing retinal oximetry, pupils were dilated with tropicamide monofree 0.5% and phenylephrine monofree 5.0%.

Retinal oximetry

The Oxymap T1 retinal oximeter (Oxymap, Reykjavic, Iceland) was used to assess oxygen saturation in retinal vessels. The technique's reliability and reproducibility have been previously established.[5,6] The Oxymap T1 consists of two high resolution digital cameras that are attached to a Topcon TRC-50DX fundus camera (Topcon Corporation, Tokyo, Japan). Images were simultaneously acquired in a 50° field of view at wavelengths of 570 nm (oxygen insensitive) and 600 nm (oxygen sensitive) centred on the optic nerve head.

After combining the images, the oxygen saturation and vessel diameter was automatically calculated by specialised software (Oxymap Analyzer software version

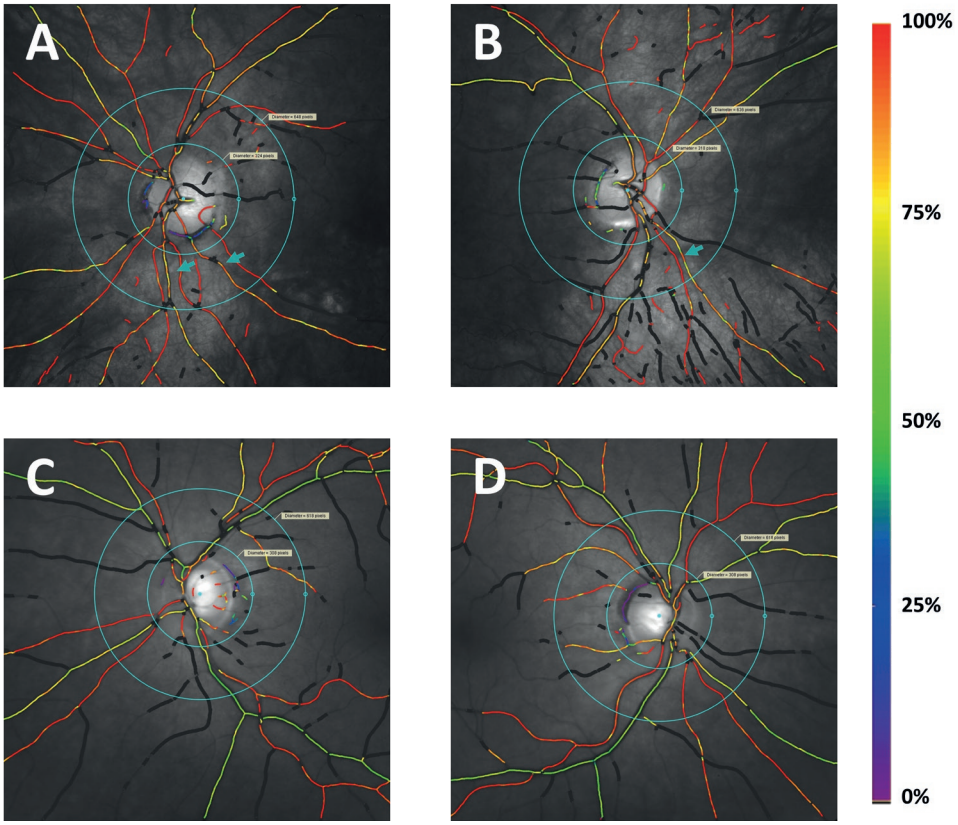

Figure 1. Pseudo colour fundus map of RVCL-S patient and control.

The image is focused around the optic disc. Retinal oximetry images of the left (A) and right (B) eye of a RVCL-S patient and the left (C) and right (D) eye of a control individual are shown. The vessel segments located in the area between the two circles (blue), which have a diameter of 1.5 and 3.0 times the optic disc diameter, were included. Colours reflect oxygen saturation as shown in the colour scale. The blue arrows indicate retinal venules of a RVCL-S patient with increased oxygen saturation.

2.2.1, revision 10927, Oxymap, Reykjavik, Iceland). Images were acquired and analysed in accordance with the protocol for acquisition and analysis of Oxymap T1 oximetry images.[7] Arterioles and venules wider than 8 pixels and longer than 50 pixels were measured (1 pixel corresponds to approx. 9 µm) in a peripapillary annulus within a grid of 1.5 to 3.0 disc diameters from the optic disc centre (fig 1).[7] If needed and available, fundoscopic angiography (FAG) images were used to discern retinal arterioles from venules. An ophthalmologist made the final decision concerning vessel allocation. Images with an overall image quality <5.0, as reported by the Oxymap Analyzer software, were excluded from analysis. Calibration parameters and the vessel detection algorithm are stated in table S1.

To correct for differences in vessel diameter in the retinal circulation, mean arterial saturation and venular saturation per eye were calculated by weighing with the vessel diameter as shown in fig S1. The arteriovenous difference (AV-difference) was calculated by using the mean weighted arterial and venular saturation. Additionally, the mean width of selected arterial and venular vessel segments was reported.

Statistical analysis

Demographics were described as median and interquartile range (IQR) or counts and percentages. They were compared using Mann-Whitney U tests for continuous variables and Fischer's Exact Test for categorical variables. To correct for inter-eye correlations within participants, retinal oximetry parameters were compared between groups using generalized estimating equation (GEE) with an exchangeable correlation structure. All analyses were performed using R version 4.2.1. and RStudio version 2022.02.3+492. P-values of ≤0.05 were considered statistically significant.

Results

Patient characteristics

Retinal oximetry was performed on 20 eyes from 10 RVCL-S patients and 30 eyes from 15 controls. Three eyes from two RVCL-S patients were excluded due to overall image quality <5.0. In the control group 11 eyes from 11 control patients were excluded due to the presence of choroidal melanoma (n = 9) or a peripapillary choroidal naevus (n = 2). As such, 19 eyes, without any (previous) abnormalities, were included in the control cohort. The final study population thus consisted of 17 eyes from 9 RVCL-S patients and 19 eyes from 15 controls. No differences regarding age, sex and IOP were present (table 1). Panretinal laser photocoagulation therapy was performed in 52.9% (9/17) of the eyes of RVCL-S patients.

Table 1. Characteristics study population.

	RVCL-S patients (n = 9)	Controls (n = 15)	P-value
Age (median (IQR))	59.5 (46.8-65.3)	62.0 (54.0-67.2)	0.35
Sex (female, n (%))	4 (44.4)	7 (46.7)	1.00
IOP[a] (median (IQR))	16.5 (10.3-19.3)	14.0 (13.0-16.8)	0.82
Visual acuity (logMAR)	0.0 (0.0-0.1)	-0.1 (-0.2-0.0)	0.001
Retinopathy (yes, n(%))	8 (88.9)	-	-
Neurological manifestations (yes, n(%))[b]	8 (88.9)	-	-
Internal organ dysfunction (yes, n(%))[c]	6 (66.7)	-	-

[a] IOP (in mm Hg) was missing in two eyes of one *TREX1* MC and one eye of one control patient. [b] Neurological manifestations were defined as i) cognitive dysfunction, ii) focal neurological manifestations, iii) MRI lesions typical of RVCL-S. [c] Internal organ dysfunction was defined as organ dysfunction (liver, kidney and thyroid disease and anaemia) requiring treatment. MC, mutation carrier; IOP, intraocular pressure.

Retinal oximetry

No difference in arterial oxygenation was found (table 2, fig 2A). Venular oxygenation was considerably higher in RVCL-S patients than in controls with a median of 71.5% [IQR: 55.0-77.2] and 55.5% [IQR: 51.2-59.9] respectively (p-value < 0.001) (table 2, fig 2B). The AV-difference was found to be lower in RVCL-S patients (median AV-difference [IQR]: 22.6% [18.5-38.3] vs 38.5% [34.1-39.9], p < 0.001) (table 2, fig 2C). In the eyes treated without pan-retinal laser photocoagulation therapy, 3 out of 8 eyes (37.5%) still had a venular saturation of above 70%. Both controls and the patient without retinopathy had no venular saturation values exceeding 65%.

Both arterial and venular vessels were thinner in RVCL-S patients compared to controls. Median [IQR] arterial and venular diameters were 10.4 [9.8-11.2] pixels vs. 12.1 [11.2-12.8] pixels, p < 0.001 and 12.9 [11.8-15.3] pixels vs. 14.9 [14.1-16.8] pixels; p<0.001, respectively (table 2).

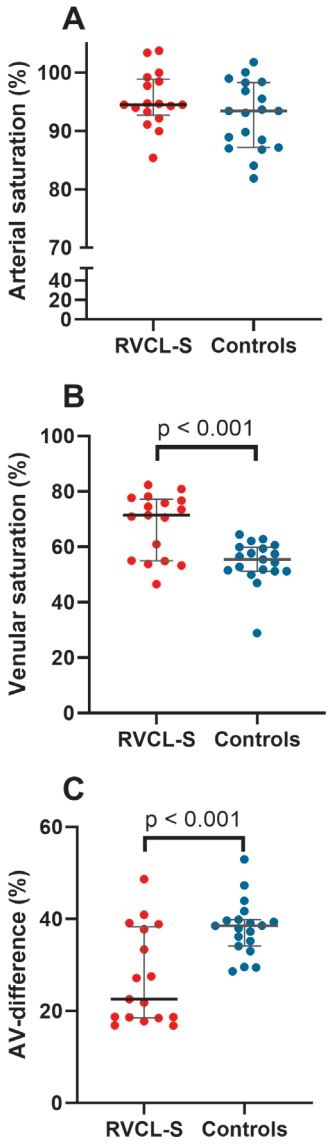

Figure 2. Retinal oxygen saturation in RVCL-S patients and controls.

Arterial oxygen saturation (A), venular oxygen saturation (B) and arteriovenous difference (AV-difference) (C) in RVCL-S patients and controls. AV-difference was calculated using the arterial and venous saturation. Thickened line displays the median and the bars the interquartile range. P-value were calculated using generalized estimating equation (GEE) analyses.

Table 2. **Retinal oxygen saturation in RVCL-S patients.**

	Median (IQR) RVCL-S patients (%) (n = 17)	Median (IQR) controls (%) (n = 19)	Estimate	CI 95%	P-value
ArtSat (%)	94.5 (92.7-98.9)	93.4 (87.2-98.3)	2.8	–0.7 : 6.2	0.12
VenSat (%)	71.5 (55.0-77.2)	55.5 (51.2-59.9)	14.0	6.6 : 21.5	<0.001
AV-diff (%)	22.6 (18.5-38.3)	38.5 (34.1-39.9)	-11.2	–17.7 : -4.7	<0.001
ArtDiam (pixels)[a]	10.4 (9.8-11.2)	12.1 (11.2-12.88)	-1.5	-2.3 : -0.7	<0.001
VenDiam (pixels)[a]	12.9 (11.8-15.3)	14.9 (14.1-16.8)	-2.2	-3.5 : -1.0	<0.001

[a] One-pixel width corresponds to approx. 9 µm. ArtSat, arterial saturation; VenSat, venous saturation; AV-diff, arteriovenous difference; ArtDiam, arterial diameter; VenDiam, venular diameter; MC, mutation carrier; CI, confidence interval.

Discussion

In this study, we demonstrated that retinal saturation is affected in RVCL-S. There was an increase in venular saturation with a decreased AV-difference, but without large differences in arterial saturation. In addition, retinal vessel attenuation was observed in RVCL-S patients.

There are several possible explanations for the elevated venular saturation in RVCL-S. These mechanisms can be divided in the following categories: 1) inadequate distribution of blood flow and oxygen, 2) increased oxygen supply due to increased total retinal blood flow, 3) decreased oxygen consumption, 4) thickening of capillary walls or 5) a combination of the above mechanisms. Poor distribution (mechanism 1) encompasses both closure of retinal capillaries and widening of other capillaries in the retina. When oxygen rich blood passes through wider shunt capillaries, it flows rapidly to the venous side, reducing the amount of oxygen delivered to the tissue per unit volume of blood decreases, causing an increase in venular oxygen saturation. Interestingly, both occluded capillaries and collateral vessels have been seen on fluorescein angiography in RVCL-S.[8] A decreased vascular reactivity has also been demonstrated in both cerebral vessels and in the forearm in RVCL-S.[9,10] Here, we showed a reduction in arteriolar and venular diameter. Together, this renders increase blood flow (mechanism 2) as an improbable explanation. Additionally, a decrease in oxygen consumption, for instance due to less metabolic activity (mechanism 3), could be considered. Thinning of retinal layers, such as the ganglion cell layer which occurs in RVCL-S,[11] which could lead to less metabolic activity, resulting in an increase in venular saturation. Moreover, mechanisms such as ischemia, inflammation and/or neurodegeneration could contribute to cell death, leading ultimately to less oxygen

consumption. Finally, thickening of capillary vessel walls (mechanism 4), can cause decreased oxygen delivery, as the increase in diffusion distance leads to more oxygen being retained in the blood. In RVCL-S, retinal arteries have been shown to have thickened hyalinized walls on histopathological examination, possibly adding to the increase in venular saturation.[8] The mechanisms considered above may also interact in diverse ways to lead to an elevation in venular saturation.

Elevated venular saturation and decreased AV-difference have previously been found in other vascular retinopathies such as diabetic retinopathy and have shown to be related to disease severity.[12,13] Retinal vasculopathy in RVCL-S shows similarities to diabetic retinopathy regarding clinical signs and treatment. Interestingly, this study indicates that retinal oxygenation is also affected in a similar way. Therefore, it could be valuable to determine whether saturation of the retina in RVCL-S also predicts disease severity in a longitudinal study. Besides retinal vasculopathy, RVCL-S is characterised by cerebral white matter disease and accompanying neurological deficits. As the central nervous system and the retina have a shared embryonic origin, it has been hypothesised that changes in the retina potentially reflect abnormalities in the central nervous system. In other diseases that affect or originate from the central nervous system, including multiple sclerosis and Alzheimer's disease, increased venular saturation and decreased AV-differences have indeed been observed.[4,14] In addition, studies indicate that retinal oximetry might serve as an early biomarker for diagnosing Alzheimer's disease.[4,15] If retinal abnormalities indeed reflect changes in the central nervous system, retinal oximetry might therefore not only serve as a biomarker for retinal vasculopathy, but also for overall disease status and disease onset of RVCL-S.

The decreased retinal vessel calibre found in RVCL-S could be due to decreased metabolic demands of the degenerating retina. Prior research employing quantitative assessments of retinal vessel calibre has indicated that narrower retinal vessels are linked to various subclinical and clinical diseases, extending beyond ocular conditions to disorders involving other organs. For example, narrow retinal vessels are associated with lacunar infarcts,[16] predicting incident stroke,[17] and diabetes.[18] Reduced retinal nerve fibre layer thickness, as seen in RVCL-S,[11] is likewise correlated with narrower retinal vessels.[19]

Our study may be subjected to several limitations. To begin, an investigator discerned arteriolar from venular vessel segments based on anatomical characteristics, which could not be fully performed in a blinded manner. Signs of retinopathy and laser photocoagulation could not be blinded, and therefore eyes of RVCL-S patients could be distinguished from eyes from controls. This could potentially have introduced

bias. Uncertainties were, however, very rare (2.3%) and evaluated by a trained ophthalmologist. Another limitation may be the small sample size. However, as RVCL-S is a rare disease, this study already consists of a relatively large cohort. It should be mentioned that laser photocoagulation was performed in a subset of RVCL-S patients. Particularly pan-retinal laser photocoagulation leads to wide-scale atrophy in the retina, thereby reducing oxygen demand and thus potentially increasing venular saturation and decreasing the AV-difference. In practice, however, previous work in diabetic retinopathy demonstrated that only a slight increase in venular and arterial saturation was measured after pan-retinal laser photocoagulation, while the AV-difference remained unchanged.[20] Moreover, several RVCL-S patients in our study that were not treated with pan-retinal laser photocoagulation still had a venular saturation exceeding 70.0%. Finally, it should be noted that oxygen saturation values rely on calibration, and are therefore not absolute, potentially surpassing 100%. However, our method has been shown to give repeatable results, and to be sensitive to changes in oxygen saturation.[6] Several strengths of this study should also be taken into consideration. This was, to our knowledge, the first study to investigate retinal saturation and vessel calibre in patients with RVCL-S. Another strength of our study is the use of a unique study population of RVCL-S patients, a disease that serves as a monogenic model for common vascular retinopathies and small vessel diseases. Our study also provides insights into the pathophysiology of retinal disease in RVCL-S, as we demonstrated that retinal oxygenation is altered in RVCL-S. Monitoring retinopathy in RVCL-S using parameters that indicate the initial metabolic or ischemic phases of the condition, rather than the subsequent morphological alterations, would be preferable. Thus, a next step would be to evaluate the oxygen saturation status in presymptomatic patients.

To conclude, we demonstrated that there is an increase in venular saturation with a decreased AV-difference in RVCL-S. Additionally, we observed retinal vessel attenuation. Our study, also considering similar findings in other related disorders, sheds light on the possible role of retinal oximetry as an objective, non-invasive biomarker for retinal vasculopathy in RVCL-S. Retinal oximetry is a promising non-invasive tool to monitor progression of retinal vasculopathy in RVCL-S and will likely complement conventional evaluation of the retina in the future.

Author contribution

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Irene de Boer, Wendy Winter and Mays Al-Nofal. The first draft of the manuscript was written by Irene de Boer and Wendy Winter and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Statements and Declaration

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Supplementary

$$\text{Mean weighted oxygen saturation} = \frac{Sat_1 * d_1^4 + Sat_2 * d_2^4 + Sat_3 * d_3^4 + Sat_4 * d_4^4}{d_1^4 + d_2^4 + d_3^4 + d_4^4}$$

Figure S1. Calculating the mean arteriolar or venular oxygen saturation by weighing with the fourth power of the vessel diameter.

Sat_x = saturation (in %) of each arteriolar or venular vessel segment; d_x = diameter (in pixels) of each arteriolar or venular vessel segment.

Table S1. Specifications of Oxymap Analyzer software, version 2.2.1

Calibration parameters	
a	–1.28
b	1.24
c	0.01
d	-0.14
Minimum vessel length	50 pixels
Minimum vessel width	8 pixels
Vessel detection algorithm	V2

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CHAPTER 12

Neuropsychiatric and MRI findings in TREX1 mutation carriers: lessons from a small vessel disease

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Abstract

Background and Purpose: Retinal Vasculopathy with Cerebral Leukoencephalopathy and Systemic manifestations (RVCL-S) is caused by mutations in *TREX1* and a model for (early) stages of vascular dementia. We aimed to investigate the neuropsychiatric status of (pre)symptomatic mutation carriers (MC) and whether MRI characteristics (White Matter Hyperintensities (WMH) and Cerebrovascular Reactivity (CVR) in WM or grey matter (GM)) are related to cognitive impairment, psychiatric morbidity and apathy.

Methods: *TREX1* MC (n=29) and matched controls (n=29) underwent a comprehensive set of neuropsychiatric and functional tests, including Cambridge Cognitive Examination-Revised (CAMCOG-R), Mini-Mental State Examination (MMSE), Wechsler Memory Scale (WMS), Word Verbal Learning Test (WVLT), Stroop test, digit symbol substitution test (DSST), Frontal Assessment Battery (FAB), Hospital Anxiety and Depression Scale (HADS), Center for Epidemiological Studies Depression Scale (CES-D), Apathy Evaluation Scale (AES-I), Neuropsychiatric Inventory-Q (NPI-Q), modified Rankin Scale (mRS) and Barthel Index of Activities of Daily Living. The relationship with 3T-MRI findings was assessed. *TREX1* MC were analyzed separately in age groups < 50 and ≥ 50 years.

Results: TREX1 MC showed increased complaints of depression ($p<0.001$), anxiety ($p=0.040$) as well as worse global cognitive functioning ($p=0.008$) and executive functioning ($p=0.022$) compared to controls. MC ≥ 50 years additionally demonstrated worse memory ($p=0.001$) and apathy ($p=0.016$). Young MC demonstrated depressive symptoms ($p=0.047$) which correlated with reduced GM CVR ($p=0.030$). In MC ≥50 years decreased CVR correlated with worse global cognitive functioning (CVR in WM and GM respectively: $p=0.003$ and $p=0.008$). Decreased GM CVR was associated with impaired memory ($p<0.001$) and praxis ($p=0.046$), and higher WMH load with worse executive functioning ($p=0.024$).

Conclusions: TREX1 MC exhibited poorer cognitive function, increased functional disability and higher neuropsychiatric morbidity, including depression and anxiety, particularly in older individuals, with these impairments linked to increased WMH volume and reduced CVR.

Introduction

Retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations (RVCL-S) is an autosomal dominant neurovascular small vessel disease (SVD) caused by C-terminal truncating mutations in *TREX1*.[1,2] The best-known clinical features of RVCL-S are vascular retinopathy and focal and diffuse brain damage with early onset white matter hyperintensities (WMHs), with signs of inflammatory activity with diffusion restriction and gadolinium enhancement.[1] In addition, RVCL-S is a systemic vasculopathy with anemia, Raynaud's phenomenon and liver, thyroid and kidney disease.[1,3]

Additional imaging characteristics include the following findings: on CT brain scans, leukoencephalopathy and focal white matter calcifications and on MRI scans global progressive white matter hyperintensities (WMH) and punctate WMH with nodular enhancement. These might progress into intracerebral mass lesions accompanied by surrounding edema.[1,4] Besides global WMH also other SVD markers, such as subcortical infarcts, lacunes, enlarged perivascular spaces and atrophy can be present in RVCL-S.[1,4] The progression of RVCL-S varies, but symptoms often worsen quickly during middle age, typically resulting in premature death in the fifth decade of life.[1] Cognitive impairment has been shown to manifest as impairment of working memory, processing speed, information progressing and impaired judgement.[1,5] This is based on retrospective evaluation of medical records and a study of 12 RVCL-S patients. There are no studies focusing on the pattern of cognitive decline and psychiatric symptoms in mutation carriers without neurological complaints.[1,6]

Cerebral SVD is characterized by a wide range of focal and global brain changes, which can be made visible on MRI. In SVD there is evidence that WMH and reduced cerebrovascular reactivity (CVR) are associated with cognitive decline and psychiatric disorders.[7-11] For instance, in cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), another small vessel disease affecting cognition, a correlation is found between white matter volume and different cognitive domains.[12] WMH have been described in RVCL-S since the disorder was discovered,[1] and more recently it was demonstrated that RVCL-S is associated with reduced CVR.

In the current study, we aimed to assess the neuropsychiatric status of TREX1 MC compared to controls and to determine whether presymptomatic TREX1 mutation carriers already demonstrated neuropsychiatric complaints. In addition, we conducted exploratory analyses to determine whether specific MRI characteristics (WMH volume

and CVR) correlated with neuropsychiatric symptoms to assess whether WMH and CVR could serve as (early) biomarkers for (pre)symptomatic RVCL-S as monogenic model for SVD in general.

Methods

Participants

Participants, both patients and controls, were included from the RVCL-S ID study, a cross-sectional observational study in *TREX1* MC from three large Dutch families.[3] For this study we included known MC and family members at 25% or 50% risk of being a carrier, who all underwent genetic testing for mutations in *TREX1* and a neuropsychiatric evaluation. Family members without a mutation and unrelated controls were included as controls. We matched controls based on age strata (10 years) and education level (according to the Dutch Verhage classification system).[13] Controls were excluded if they had a (history of) psychiatric disorder or neurological brain disorder. The study was approved by the Medical Ethics Committee of the Leiden University Medical Center, and all participants provided written informed consent. The data that support the findings of this study are available from the corresponding author upon reasonable request.

MRI acquisition and post-processing

A uniform MRI protocol was performed on a 3.0T clinical MRI system (Philips Healthcare, Best, The Netherlands) on the same day as the neuropsychiatric evaluation. WMH volume and CVR data are previously reported by Hoogeveen et al.[14]

Brain WMH volume was assessed using tools of the FSL software package (Functional MRI of the Brain [FMRIB] Software Library v5, www.fmrib.ox.ac.uk/fsl).[15] T1-weighted (MPRAGE, TR=9.8 ms, TE=4.6 ms, flip angle of 8°, voxel size of 0.86 x 0.85 mm, 130 slices with a thickness of 1.2 mm) and FLAIR (TR=4800 ms, TE=291 ms, flip angle of 90°, voxel size of 1 x 1 mm, 310 slices with a thickness of 1.1 mm) images were used. FLIRT was used as brain extraction tool (FMRIB's linear image registration tool). FLAIR images were registered to the MNI152 standard space using FLIRT. WMH were automatically in a conservative MNI white matter mask, identified using a threshold of three standard deviations above mean FLAIR signal intensity.

CVR to hypercapnia (5% carbon dioxide) was measured using dual-echo arterial spin labelling (ASL) MRI, resulting in both cerebral blood flow (CBF; from the first echo) reactivity and blood oxygen level dependent (BOLD; second echo) reactivity.[14] We

used gray matter CBF CVR since CBF is the physiologically easier to interpret parameter, and white matter BOLD CVR due to the too low SNR of ASL in the white matter. To ensure that our results were not impacted by deciding to use the CBF instead of the more traditionally used BOLD CVR gray matter measurement we evaluated if the results were similar for both measurement methods. For detailed description of this method in this patient population see Hoogeveen et al.[14] In brief, a dual-echo pseudo continuous ASL (pCASL) scan was acquired with the following scan parameters: TR = 4100 ms, TE1/TE2 = 13/35 ms, label duration/post-label delay = 1650/1525 ms, multi-slice single shot EPI with background suppression (inversion pulses at 1680 and 2760 ms), 15 slices, voxel size = 3 × 3 × 7 mm, 75 pairs of label and control images. We further acquired a dual-echo M0 scan (TR= 2000 ms, TE1/TE2= 13/35 ms, resolution of 80 x 80, flip angle 90°, voxel size 3 x 3 mm, 15 slices of 7 mm), and a T1blood scan (TR= 150 ms, TE = 11 ms, resolution 240 x 240, flip angle 95°, voxel size 1 x 1 mm, 1 slice of 2 mm). For pre-processing, extraction, smoothing and transforming we refer to Hoogeveen et al.[14]

Neuropsychiatric assessment

A neuropsychiatric test battery lasting 2 hours was administered by trained examiners supervised by an experienced clinical neuropsychologist (H.A.M.M). Global cognitive functioning was assessed using the Cambridge Cognitive Examination-Revised (CAMCOG-R) and Mini-Mental State Examination (MMSE).[16,17] The CAMCOG provides a total score for global cognitive functioning as well as sub scores for specific cognitive functions, i.e. orientation, language comprehension, language expression, memory, praxis, abstract thinking, perception and attention and calculation.[16] We used the sub scores of the CAMCOG-R memory, praxis, gnosis, language and executive functioning domains for analyses. Moreover, memory was elevated by the Wechsler Memory Scale (WMS) and 15 Word Verbal Learning Test (WVLT).[18] Executive functioning was assessed with the Trail-Making Test (TMT), the colour interference section of the Stroop colour and word test (Stroop), the digit symbol substitution test (DSST) of the Wechsler Adult Intelligence Scale–III and the Frontal Assessment Battery (FAB).[19-22] The raw scores of the neuropsychiatric tests were used for analyses and computed into compound scores. Time to complete TMT part A, Stroop interference task, TMT interference time, and Stroop interference score were multiplied minus 1 so lower scores represented poorer performance. Raw scores of each test were converted to standardized Z-scores ((test score - mean)/SD) and a mean Z-score was calculated across the tests tapping cognitive domains of interest, i.e. global memory, language, praxis, gnosis and executive function. The Z-scores were created using the following tests and/or questionnaires: global cognitive functioning: COMCOG-R, MMSE; memory: COMCOG-memory and -orientation, WVLT (total words imprinted, recalled and recognized) and

subtests WMS; language: CAMCOG-expression and -comprehension; praxis: COMCOG-praxis; gnosis: CAMCOG-perception; executive functioning: time to complete TMT part A and Stroop interference task, TMT interference time, Stroop interference score, FAB total score, DSST score and COMCOG-abstract thinking and attention and calculation).

Current anxiety and depression were determined using the Hospital Anxiety and Depression Scale (HADS) and the Center for Epidemiological Studies Depression Scale (CES-D).[23,24] To assess presence of apathy and other psychiatric symptoms, informants were asked to complete the Apathy Evaluation Scale (AES-I) and Neuropsychiatric Inventory-Q (NPI-Q).[25,26] The psychiatric data were also converted to z-scores using the following: global psychiatric functioning: HADS-D, CES-D, HADS-A, AES-I, NPI-Q severity score; depression: HADS-D, CES-D, anxiety: HADS-A, apathy: AES-I. Before calculating the z-scores, time to complete TMT part A, Stroop interference task, TMT interference time, and Stroop interference score were multiplied minus 1 so lower scores represented poorer performance. The degrees of functional disability of all participants was determined by the modified Rankin Scale (mRS) and Barthel Index of Activities of Daily Living (BI).[27,28] mRS scores ≥ 1 and BI scores < 20 were defined as abnormal. Raw MMSE, CAMCOG-R, WMS, HADS, CES-D, BI and MRS data were previously reported by Pelzer et al.[3]

Statistical Analysis

Demographics and general assessments were reported as medians (interquartile range (IQR)) or counts (percentages). Differences in categorical demographics were analysed using Fisher's exact test. Additionally, we evaluated the effect of RVCL-S on different cognitive domains (separately adjusted for psychopathology) and neuropsychiatric symptoms by generalized linear model. We separately adjusted for the effect of neuropsychiatric symptomatology on cognition to insure that the results were not explained by those symptoms.[29] Before assessing the correlation between MRI markers and cognitive performance, neuropsychiatric symptoms, and functional disability, we first examined the strength of the correlations among the MRI markers themselves to determine their potential independent utility. Spearman rank-order correlation correcting for age were used to assess the correlation between WMH volume and CVR in gray and white matter on cognitive performance, neuropsychiatric symptoms and functional disability due to the non-normality of these measurements. *TREX1* MC were also analyzed separately for <50 years of age and ≥50 years based on the age of onset of notable neurological deficits.[1,3] Significance thresholds were set at $p<0.05$. Statistical analyses were performed using SPSS 25 (SPSS inc., IBM, USA).

Results

Demographics

In 29 individuals a *TREX1* mutation was confirmed. These mutation carriers (MC) were matched to 29 controls for age (median age (IQR) 44.0 (27.0-56.0) vs 43.0 (34.5-54.5)) and education (modus both groups: intermediate vocational education). Sex distribution of MC and controls was similar (59% vs 55% female). Despite visual impairments in some MC due to vascular retinopathy, all MC were able to execute the neuropsychiatric tests, and visual acuity did not influence outcomes (data not shown).

Neuropsychiatric and functional assessment

For the raw neuropsychiatric scores see table 1. *TREX1* MC showed worse global cognitive functioning and executive functioning and memory compared with controls (table 2). Across age categories, measures of global and executive functioning and memory were lower for mutation carriers aged ≥ 50 compared with age matched controls, but no differences were found between young MC (< 50 age) and controls (table 3). These findings were independent of depressive symptoms (Supplementary table 1). Overall psychiatric morbidity, depression and anxiety were more often reported in *TREX1* MC (table 2). While most differences appear to be specific to

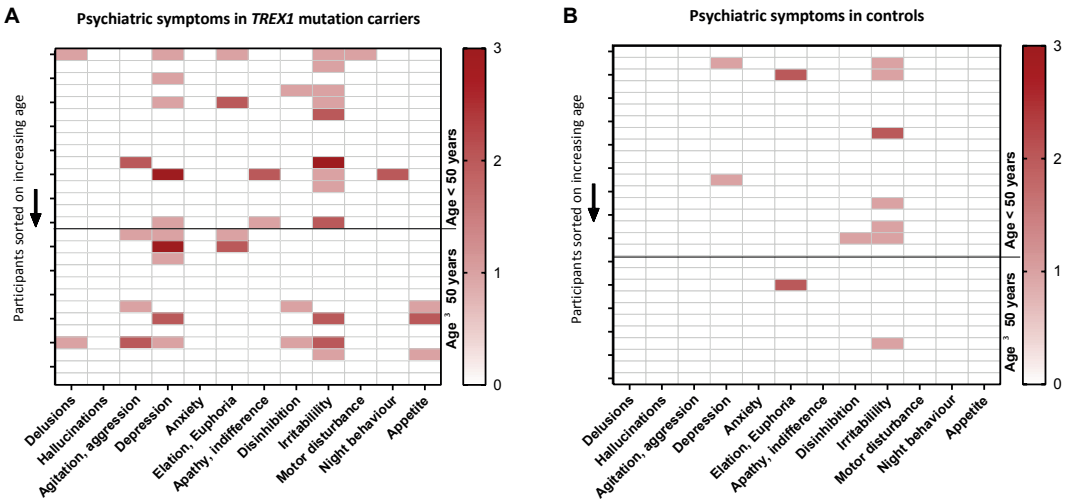

Figure 1. Psychiatric symptoms in *TREX1* mutation carriers and controls.

Mutation carriers (A) demonstrated an overall increase in neuropsychiatric symptoms as assessed with Neuropsychiatric Inventory-Q (ranging 0 to 3) compared to controls (B). Increased color intensity indicates more severe symptoms. In one mutation carrier and one control no informant reported information was available on night behavior.

the elderly, depression already occurred in young MC (table 3). The older patients also suffered from apathy. Moreover, informants indicated more neuropsychiatric symptoms in MC (figure 1). Regarding functional disability, 18% of RVCL-S patients had an abnormal BI score, while 55% had an abnormal mRS score (no controls had abnormal scores) (Fisher exact test, p=0.052 and p<0.001, respectively).

Table 1. Raw scores of cognitive tests and psychiatric surveys.

	RVCL-S n=29	Controls n=29
Global cognitive functioning		
CAMCOG-R, median[IQR]	93.0 [89.0-96.0]	97.0 [91.0-98.5]
MMSE, median[IQR]	29.0 [29.0-30.0]	30.0 [29.5-30.0]
Memory		
CAMCOG-memory, median[IQR]	21.0 [19.0-23.0]	23.0 [20.5-24.0]
CAMCOG-orientation, median[IQR]	10.0 [9.5-10.0]	10.0 [10.0-10.0]
WVLT, median[IQR]	49.0 [44.5-57.5]	50.0 [41.5-57.0]
WMS, median[IQR]	69.0 [60.0-74.3]	71.3 [66.5-76.3]
Language		
CAMCOG-comprehension, median[IQR]	9.0 [9.0-9.0]	9.0 [9.0-9.0]
CAMCOG-expression, median[IQR]	19.0 [17.0-20.0]	20.0 [18.5-20.5]
Gnosis		
CAMCOG-perception, median[IQR]	8.0 [8.0-8.0]	8.0 [8.0-8.0]
Praxis		
CAMCOG-praxis, median[IQR]	12.0 [11.0-12.0]	11.0 [11.0-12.0]
Executive functioning		
CAMCOG-abstract thinking, median[IQR]	7.0 [6.0-8.0]	8.0 [8.0-8.0]
CAMCOG-attention and calculating, median[IQR]	9.0 [8.0-9.0]	9.0 [8.5-9.0]
DSST, median[IQR]	67.0 [52.0-79.0]	83.0 [68.5-91.5]
FAB, median[IQR]	18.0 [17.0-18.0]	18.0 [16.5-18.0]
TMT-A, seconds, median[IQR]	24 [21-34]	20 [18-28]
TMT interference, seconds, median[IQR]	38 [28-55]	28 [16-43]
Stroop time, seconds, median[IQR]	79.5 [72-104]	78 [66-89.5]
Stroop interference, seconds, median[IQR]	37 [29-53]	35 [23-47]
Psychiatric morbidity		
HADS-A, median[IQR]	3.0 [2.0-7.0]	3.0 [1.0-4.0]
HADS-D, median[IQR]	5.0 [1.5-7.0]	1.0 [0.5-3.0]
CES-D, median[IQR]	7.0 [4.0-13.0]	2.0 [0.0-6.5]
AES-I, median[IQR]	27.0 [23.5-33.5]	24.0 [19.5-31.0]
NPI-Q, severity, median[IQR]	1.0 [0.0-4.8]	0.0 [0.0-0.0]

Cambridge Cognitive Examination-Revised (CAMCOG-R), Mini-Mental State Examination (MMSE), Wechsler Memory Scale (WMS), Word Verbal Learning Test (WVLT), Stroop test, digit symbol substitution test (DSST), Frontal Assessment Battery (FAB), Hospital Anxiety and Depression Scale (HADS), Center for Epidemiological Studies Depression Scale (CES-D), Apathy Evaluation Scale (AES-I), Neuropsychiatric Inventory-Q (NPI-Q).

MRI markers and cognitive and functional impairment in *TREX1* mutation carriers

In *TREX1* MC WMH volume showed weak negative correlations with both GM CVR (r_s = -0.37, p= 0.09) and WM (r_s = -0.41, p= 0.06). CVR in both GM and WM also showed a weak correlation with one another (r_s = 0.40, p = 0.07). In older MC (≥ 50 age) impairment of global cognition correlated with lower CVR in GM and WM (r_s = 0.89, p = 0.008 and r_s = 0.87, p = 0.003, respectively) (table 4). Additionally, a correlation was found between lower CVR in GM and worse memory and praxis performance (r_s = 0.96, p < 0.001 and r_s = 0.76, p = 0.046, respectively). Furthermore, a higher WMH volume correlated with worse executive functioning (r_s = -0.64, p = 0.024) and language performance (r_s = -0.74, p = 0.006). Additionally in young MC (< 50 age), a correlation was found with overall neuropsychiatric comorbidity and GM CVR (coefficient: 0.62, p = 0.030) (table 4). Evaluating GM CVR using the BOLD method resulted in similar results (data not shown).

Table 2. Neuropsychiatric assessment of *TREX1* MC compared to controls.

	Beta	Confidence interval	P value*
Cognition			
Global cognitive functioning	-0.65	-1.13:-0.17	**0.008**
Memory	-0.41	-0.91:0.09	0.11
Language	-0.40	-0.90:0.10	0.11
Praxis	0.12	-0.39:0.63	0.65
Gnosis	-0.46	-0.95:0.04	0.07
Executive functioning	-0.57	-1.07:-0.08	**0.022**
Psychiatric morbidity			
Overall psychiatric morbidity	0.81	0.34:1.28	**0.001**
Depression	0.86	0.40:1.32	**<0.001**
Anxiety	0.52	0.02:1.01	**0.040**
Apathy	0.39	-0.11:0.89	0.13

* Analyzed using generalized linear models.

Table 3. Effect of age on neuropsychiatric assessment of *TREX1* MC compared to controls

Age < 50 years	Beta	Confidence interval	P value*
Cognition			
Global cognitive functioning	-0.40	-1.16 - 0.35	0.29
Memory	0.08	-0.62 - 0.78	0.82
Language	-0.22	-0.90 - 0.47	0.53
Praxis	0.14	-0.54 - 0.82	0.69
Gnosis	-0.53	-1.20 - 0.13	0.12
Executive functioning	-0.26	-0.82 - 0.31	0.37
Psychiatric morbidity			
Overall psychiatric morbidity	0.54	-0.09:1.17	0.09
Depression	0.61	0.01:1.21	**0.047**
Anxiety	0.55	-0.16:1.26	0.13
Apathy	0.12	-0.52:0.77	0.71
Age ≥ 50 years	Beta	Confidence interval	
Cognition			
Global cognitive functioning	-1.01	-1.49:-0.53	**<0.001**
Memory	-0.95	-1.52:-0.38	**0.001**
Language	-0.66	-1.39:0.07	0.08
Praxis	0.10	-0.69:0.88	0.81
Gnosis	-0.33	-1.08:0.43	0.40
Executive functioning	-0.81	-1.58:-0.04	**0.040**
Psychiatric morbidity			
Overall psychiatric morbidity	1.11	0.41:1.80	**0.002**
Depression	1.11	0.42:1.80	**0.002**
Anxiety	0.49	-0.18:1.15	0.15
Apathy	0.88	0.17:1.60	**0.016**

* Analyzed using generalized linear models. Age < 50 years *TREX1* mutation carriers (n=15) and controls (n=18). Age ≥ 50 years *TREX1* mutation carriers (n=14) and controls (n=11).

Table 4. Effect of age on association between MRI parameters and neuropsychiatric outcomes in *TREX1* mutation carriers

	WMH volume†				CVR WM BOLD‡				CVR GM CBF‡			
	< 50 years		≥ 50 years		< 50 years		≥ 50 years		< 50 years		≥ 50 years	
	Coefficient	P value*	Coefficient	P value*	Coefficient	P value*	Coefficient	P value*	Coefficient	P value*	Coefficient	P value*
Cognition												
Global cognitive functioning	0.18	0.56	0.50	0.25	0.08	0.80	0.87	**0.003**	0.39	0.19	0.89	**0.008**
Memory	0.10	0.75	-0.30	0.36	0.07	0.82	0.66	0.10	0.14	0.65	0.96	**<0.001**
Language	0.45	0.12	-0.74	**0.006**	-0.09	0.76	0.04	0.93	0.47	0.10	0.43	0.33
Praxis	-0.34	0.26	-0.39	0.21	0.17	0.58	0.47	0.28	-0.17	0.57	0.76	**0.046**
Gnosis	-0.12	0.69	0.33	0.29	0.04	0.89	0.40	0.38	0.30	0.32	0.14	0.76
Executive functioning	-0.05	0.87	-0.64	**0.024**	0.09	0.78	0.06	0.90	0.01	0.99	0.30	0.51
Psychiatric morbidity												
Overall psychiatric morbidity	0.21	0.52	-0.12	0.70	0.01	0.97	-0.04	0.94	0.62	**0.030**	-0.18	0.71
Depression	0.44	0.13	-0.24	0.46	-0.22	0.47	-0.46	0.29	0.41	0.17	-0.23	0.62
Anxiety	0.09	0.77	-0.18	0.58	-0.06	0.84	-0.56	0.19	0.15	0.63	-0.35	0.45
Apathy	-0.05	0.86	-0.20	0.54	-0.09	0.78	0.57	0.18	0.17	0.57	0.43	0.33
Functional disability												
mRS	0.30	0.32	0.065	0.84	-0.19	0.54	-0.17	0.71	0.41	0.17	-0.61	0.14
Barthel index	-0.16	0.59	0.06	0.86	0.08	0.81	0.73	0.06	0.41	0.17	-0.61	0.14

†n=27, ‡ n=32, due to disability or contra indications not all patients could be scanned. * Spearman rank-order correlation correcting for age. Blood oxygen level dependent (BOLD), Cerebral blood flow (CBF), Cerebrovascular reactivity (CVR), gray matter (GM), white matter (WM), white matter hyperintensities (WMH).

Discussion

This study of TREX1 MC revealed poorer global cognitive function, executive functioning, and memory compared to controls, independent of depressive symptoms. Notably, these impairments were not observed in younger individuals (below 50 years of age). Overall neuropsychiatric morbidity, including also depression and anxiety, was more frequently reported in TREX1 MC, with patients above the age of 50 experiencing more apathy. Depressive symptoms appears to be an early neuropsychiatric sign of RVCL-S. Moreover, impaired cognitive function and depression correlated with increased WMH volume and reduced CVR, but this was not the case in young RVCL-S patients.

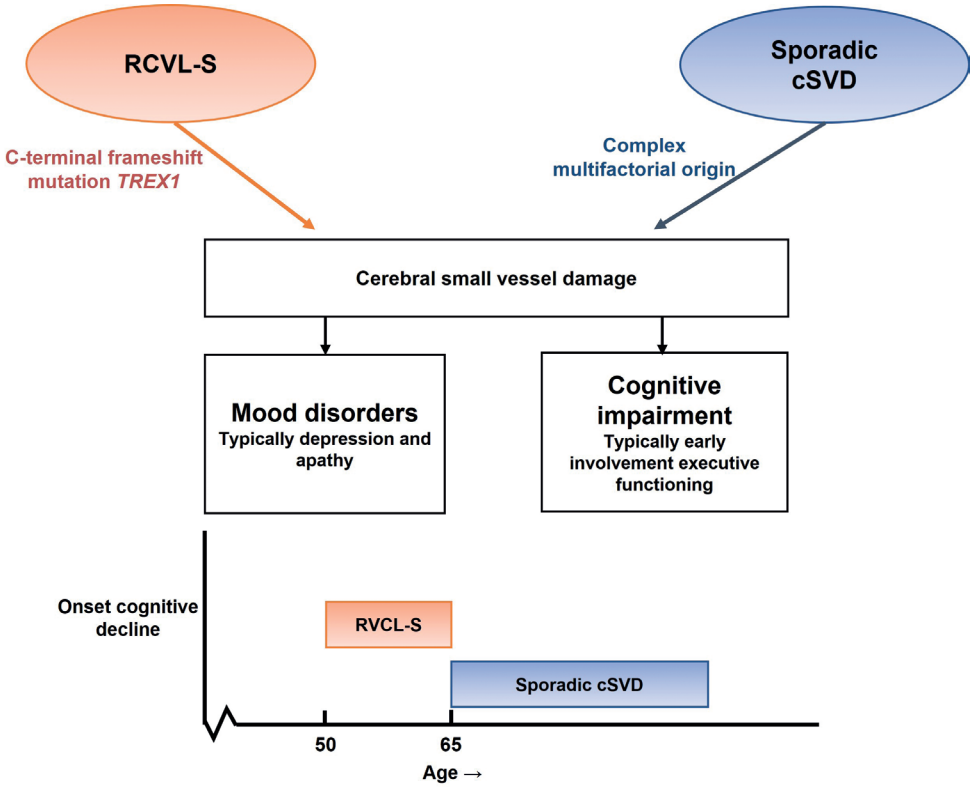

Figure 2. Comparison of cognitive and psychiatric findings in RVCL-S to other cerebral small vessel diseases.

Retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations (RVCL-S), cerebral small vessel disease (cSVD)

In line with our findings, an earlier small study evaluating some cognitive domains demonstrated impaired working memory and processing speed and increased depression in RVCL-S.[5] Due to the relatively young age at which we assessed the MC, other neurodegenerative or neurovascular causes are unlikely. The decreased executive functioning and memory deficits are in line with findings in sporadic cSVD and other monogenic diseases such as CADASIL (figure 2).[30-32]

Similarly, depressive symptoms in young individuals and apathy at older age are commonly reported features of CADASIL and sporadic cSVD.[33-35] While WMH volume is thought to contribute to cognitive decline and dementia in the general population, as it does in our RVCL-S MC, this association is still debated in CADASIL. In another hereditary small vessel disease, Dutch type cerebral amyloid angiopathy (CAA), as well as sporadic CAA, neuropsychiatric symptoms were also a prevalent finding, particularly apathy and irritability.[36] In the presymptomatic stages, before a first bleeding had occurred, this was already apparent. It should be noted however, that overall this CAA cohort was much older than our RVCL-S cohort. In the CAA patients these symptoms were associated with decreased processing speed and executive function but were not linked to the radiological burden of CAA.

While cognitive decline was not observed in young RVCL-S MC, potential subtle declines may have been missed due to the limited number of young participants. In contrast, younger patients did show higher levels of depression. Only six of the young MC were aware of their diagnosis, suggesting that depressive symptoms may be an early indicator of neuropsychiatric involvement in the disease beyond just awareness of their condition. The association between depression and cSVD is well-established, but in typical cSVD,[37,38] distinguishing depression as an early sign or consequence of mild neurocognitive dysfunction may be challenging due to the absence of a clear pre-symptomatic phase. Depression commonly coexists with neurological conditions like stroke and Alzheimer's disease, as well as disorders linked to vascular endothelial dysfunction such as cardiovascular disease and migraine. Recognizing depression as a potential sign of neurovascular involvement in the etiology of cSVD is gaining recognition.[39-42]

Whether WMH and CVR will be useful as a biomarker in the clinic requires further study. Given that RVCL-S is a systemic disorder, a comprehensive approach combining biomarkers such as MRI, ophthalmological assessments, and serum (or CSF) biomarkers may yield valuable prognostic insights for RVCL-S. Non-RVCL-S specific biomarkers may also be used to monitor disease progression, such as serum neurofilament light chain (NfL) and glial fibrillary acidic protein (GFAP) that are associated with decline in cognitive functioning in RVCL-S.[43]

We suggest that RVCL-S is a good model to study one potential important aspect of SVD, the endothelium. Neuropathological studies have identified multilaminated basement membranes on ultrastructural images and endothelial markers were shown to be elevated in serum of patients.[1,44] Furthermore, functional studies demonstrated decreased flow-mediated dilation of the brachial artery (considered an *in vivo* marker of endothelial dysfunction) and reduced CVR (as surrogate measure of endothelial function[45]).[46,47] Interestingly, MRI features of SVD may be a reflection of poor cerebral blood flow regulation, as was also demonstrated in patients with minor ischemic stroke.[48] Further exploration of microvascular dysfunction may uncover new mechanisms and intervention targets to reduce SVD lesion development, cognitive decline, and stroke. Together, this indicates the importance of studying endothelial dysfunction in RVCL-S and common SVD and highlights possible avenues of treatment. RVCL-S is an ideal model for such studies, as there is one cause and a clear pre-symptomatic stage. Furthermore, while the exact mechanism of how aberrant TREX1 leads to a microvascular disease is still under research, their appears to be a clear role for DNA damage and premature senescence pathways.[49] These interesting mechanisms deserve further consideration.

There are limitations to our study to be considered: (i) as RVCL-S is a rare disorder, our sample size is relatively small and as such we could not evaluate all SVD markers; (ii) some MRI data are missing due to patients having contraindications for MRI. This may have led to an underestimation of effect; (iii) the cross-sectional nature of the study prevents statements being made concerning decline and causality, however, this is the largest study in RVCL-S describing cognitive and neuropsychiatric functioning and the first to evaluate association with MRI markers. Strengths of our study include: (i) performing MRI and neuropsychiatric evaluations on the same day; (ii) excluding confounding neurodegenerative or other vascular factors due to the relatively young age of participants; (iii) using an extensive neuropsychiatric test battery to assess various cognitive domains; (iv) the availability of matched patients and controls based on age and education; and (v) separately evaluating the impact of psychiatric symptoms on cognition. It should be noted that the significance level was kept at 0.05 with no correction for multiple comparisons, as is consistent with an exploratory study.

Conclusions

RVCL-S TREX1 mutation carriers exhibit poorer cognitive function and higher neuropsychiatric morbidity, including depression and apathy, particularly above the age of 50. These impairments are linked to increased WMH volume and reduced CVR, though these do not appear as early signs. Importantly, depressive symptoms can be an early indicator of RVCL-S.

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Supplementary

Supplementary table 1. Cognition adjusted for psychopathology.

All mutation carriers	Beta	Confidence interval	
Global cognitive functioning	-0.65	-1.19:-0.12	**0.017**
Memory	-0.41	-0.96:0.14	0.14
Language	-0.46	-1.02:0.09	0.10
Praxis	0.22	-0.34:0.78	0.45
Gnosis	-0.37	-0.92:0.18	0.19
Executive functioning	-0.51	-1.11:-0.01	**0.049**
Age < 50 years	**Beta**	**Confidence interval**	
Global cognitive functioning	-0.40	-1.21:0.42	0.34
Memory	0.02	-0.73:0.77	0.95
Language	-0.31	-1.03:0.41	0.40
Praxis	0.08	-0.64:0.81	0.82
Gnosis	-0.62	-1.33:0.08	0.08
Executive functioning	-0.29	-0.90:0.33	0.36
Age ≥ 50 years	**Beta**	**Confidence interval**	
Global cognitive functioning	-1.06	-1.64:-0.50	**<0.001**
Memory	-0.95	-1.62:-0.28	**0.006**
Language	-0.61	-1.47:0.25	0.16
Praxis	0.53	-0.34:1.40	0.23
Gnosis	0.19	-0.68:1.00	0.63
Executive functioning	-0.85	-1.71:-0.06	0.068

* Analyzed using generalized linear models.

PART III

Equality in headache research – tackling career barriers and harassment

13

CHAPTER 13

Perceived barriers to career progression in the headache field: a global web-based cross-sectional survey

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Abstract

Background: It is well recognized that underrepresented and minoritized groups do not have the same career opportunities. However, there are limited data on the range and specifics of potential barriers that withhold people in headache medicine and science from reaching their full potential. Moreover, people from different geographical regions often perceive different challenges. We aimed to identify world-wide perceived career barriers and possibilities how to promote equality amongst professionals in the headache fields.

Methods: A cross-sectional online survey was conducted among professionals in the field of headache globally. The questions of the survey were aimed to assess perceived career barriers in four domains: professional recognition, opportunities in scientific societies, clinical practice, and salary and compensation. Perceived mentorship was also assessed.

Results: In total 580 responders completed the survey (55.3% women). Gender was the most important perceived barrier in almost all domains. Additionally, country of birth emerged as an important barrier to participation in international scientific societies. Career barriers varied across world regions.

Conclusion: It is essential that longstanding and ongoing disparities by gender and country of origin for professionals in the headache field are globally acknowledged and addressed in areas of recruitment, retention, opportunities, mentor- and sponsorships, and advancement.

Introduction

It is increasingly accepted that diversity in the workplace leads to a competitive edge,[1-3] and also leads to tangible benefits including economic vibrancy[1] and innovativeness.[2] This seems also true for the academic sector.[3] A recent study found that research publications authored by ethnically diverse and gender-diverse groups receive significantly higher numbers of citations than papers written by researchers of a single ethnic group or the same gender.[4] Unfortunately, a closer analysis of the publications unveiled important inequalities. Not only are there less articles written by women as first authors and senior authors in high impact journals compared to men, but these articles also had fewer citations.[5,6] While the contribution of women as first authors of original research in high impact medical journals was significantly higher than 20 years ago, it has plateaued in recent years.[6]

Racial disparity is another important issue that is evident across multiple subspecialties of medicine.[7] In the USA, only about 12% of the neurology workforce is made up of people from underrepresented racial and ethnic groups (Blacks or African Americans, Hispanics or Latinos, American Indians or Alaska Natives, Native Hawaiians and other Pacific Islanders), while women constitute about 30% of this workforce.[8,9] In academics a similar gap is present.[10] While efforts have been made to narrow this gap, disappointingly, over a 35-year period, women physicians in academic medical centers were less likely than men to be promoted to the rank of associate or full professor or to be appointed to department chair. Importantly, there has been no apparent narrowing of this gap over time.[10] Persons of color have also been less likely than Caucasian individuals to be promoted, and women who belong to underrepresented minorities may have double barriers.[10] Additionally, medical professionals in different countries perceive different challenges.[11]

Participation in scientific societies and receiving grant funding are pivotal to the development, growth and success of academic careers. Historically and recently, women have been underrepresented among the American Academy of Neurology (AAN) plenary speakers.[12] While the AAN has taken active steps to address equity, notable gaps remain, especially in specific prestigious plenary sessions.[12] Women are also less frequently recipients of recognition awards,[13,14] and women in academia are awarded less grant funding,[15] which are key factors in progressing their academic careers.[16]These gender gaps in grant funding are at least partially attributable to less favourable assessments of women as principal investigators, and not the quality of proposed research.[17,18]

These differences can have significant repercussions for careers. Nevertheless, the global medical, scientific, and academic communities remain uninformed on the impact of barriers that prohibit full participation by women and people from underrepresented racial, ethnic, and national groups. This lack of awareness and education results in underrepresentation, under acknowledgment and failure to fully mobilize the human potential.

To promote clinical and research opportunities in the headache field based solely on abilities and to remove possible career barriers providing an unfair disadvantage, a global assessment of the problem is necessary. With this international cross-sectional survey, we aimed to define current career barriers amongst the professional headache workforce, specifically in the areas of professional recognition, opportunities in scientific societies, clinical practice, and salary and compensation. Based on the findings obtained regarding perceived barriers, we propose interventions to help strive towards equality.

Methods

Participants

An anonymous and voluntary web-based survey was distributed among people with a proven interest in headache research and clinical care. Members and associate members of the International Headache Society (IHS), delegates of the 2019 International Headache Conference and members of national headache societies affiliated with the IHS were invited to participate. To ensure that all persons working in the headache field had the chance to participate, we contacted the whole membership database of the International Headache Society and allowed national representatives to send out the survey to their members.

Survey instrument and administration

The survey instrument was developed using previously published surveys[19-21] and the experience of authors (IdB, AA, RBH, BB, RHJ, PP, GMT). Consensus was reached with several online rounds and emails. A questionnaire was constructed in English (See supplementary materials). The survey included multiple choice items with some open-ended response options assessing demographics including age, sex, race/ethnicity (combined), country of birth, current country resident status, highest level of education achieved, current position and academic rank if applicable (see Supplementary Methods). Participants were asked about possible career barriers and mentorship opportunities. All responses were kept anonymous, and no personal identifying information was collected. The web-based survey was sent in September

2019 and closed in June 2020 with three reminders to encourage participation and were answered anonymously.

Data Analyses

Data from the questionnaires were analyzed using SPSS 25.0 (IBM Corporation, Armonk, NY). All surveys were examined for inconsistencies and invalid responses were treated as missing values. Questions with open-ended answer options were scanned to look for patterns or common answers, in the absence of these, these answer options were disregarded. Descriptive statistics (frequency counts, percentages) were used to capture demographic data for the study population, as well as perceptions of barriers. Logistic regression was performed to ascertain the effect of gender and workplace location on the likelihood of participants' experiencing career barriers, corrected for age and race/ethnicity during all analyses. Results were considered significant if $p<0.05$.

Results

Participants

In total 580 responders completed the survey. All responders indicated their gender (male, female or non-binary) and the women:men ratio was almost equal to 1, with 320 participants being women (55.3%). One participant identified as non-binary. As all data were analyzed and reported stratifying for gender, this participant was removed from subsequent analyses to ensure anonymity. The highest percentage of responders was from Europe (229, 39.6%), the second most represented region was South East Asia (126, 21.8%) followed by US (78, 13.5%). Only a minority, 23 (4.0%) and 19 (3.3%) were born in African and Eastern Mediterranean countries. In total 556 (96.0%) of the responders were healthcare providers and 480 (82.9%) participated in research activities. Moreover, 458 (79.1%) of responders participated in both clinical and research activities. Most responders were specialists working in academic hospitals. The majority of participants were in a relationship (82.7%), and had children (71.8%). Responders' characteristics are displayed in Supplementary Tables 1 and 2.

Perceived barriers in the headache field

Many participants did encounter barriers affecting professional recognition (33.7-54.0% depending on subdomains), opportunities in scientific societies (47.0-60.8% depending on subdomains) or barriers affecting salary and compensation (39.3%) (Table 1 and Figure 1-3). All barriers surveyed; age, country of birth, ethnicity and gender were reported. In domains reflective of professional recognition, gender was the most frequently reported barrier (13.5-27.8% depending on subdomains) (Table 1 and Figure 1).

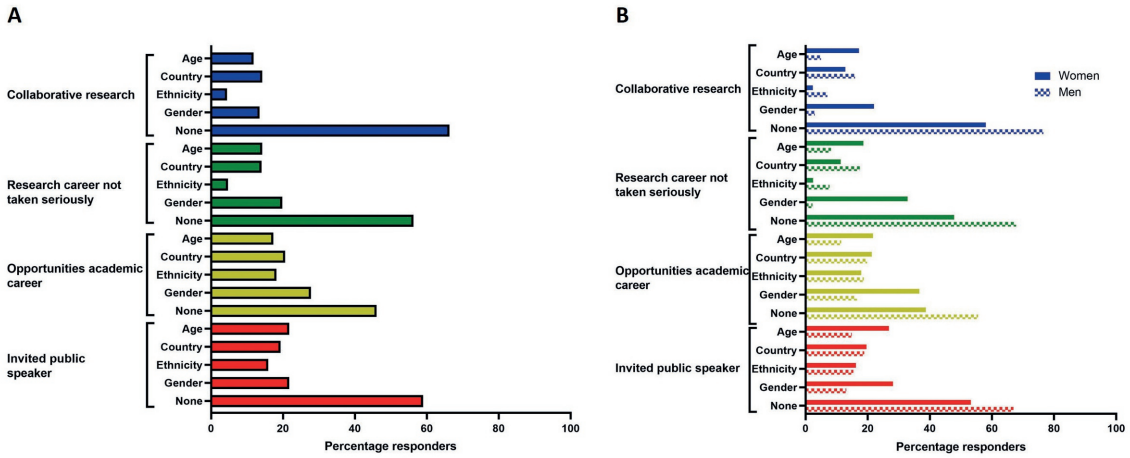

Figure 1. Barriers to a career in headache affecting professional recognition.
Results from all participants (A) and stratified by gender (B). Country is used to indicate country of birth and/or education.

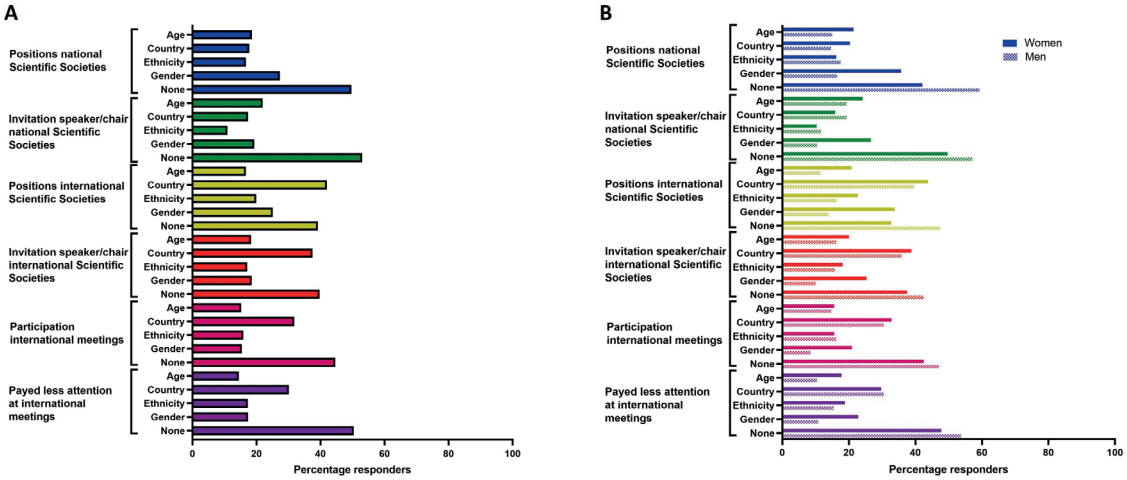

Figure 2. Barriers to a career in headache affecting opportunities in scientific societies.
Results from all participants (A) and stratified by gender (B). Country is used to indicate country of birth and/or education.

In most domains concerning opportunities in scientific societies, country of birth was the most frequently reported to be a barrier in international scientific societies, with 42% indicating that country of birth or education is a barrier to obtaining positions in international scientific societies (Table 1 and Figure 2). For positions within national

societies, gender was the most frequent barrier (27.3%), while age was the most frequent perceived barrier that caused participants not to be invited as a speaker or chair (21.9%) (Table 1 and Figure 2).

Table 1. Perceived career barriers reported by all participants and by gender.

	Breakdown by gender		
	Women (n=320)	**Men (n=259)**	**Total (n=579)**
Work-life balance			
Influence family care			
Yes, positively	49 (15.3)	53 (20.5)	102 (17.6)
Yes, negatively	45 (14.1)	18 (6.9)	63 (10.9)
Both	99 (30.9)	63 (24.3)	162 (28.0)
No	127 (39.7)	125 (48.3)	252 (43.5)
Professional recognition			
Collaborative research impacted by[a]			
Age	43 (17.2)	10 (5.0)	53 (11.8)
Country of birth /education	32 (12.8)	32 (15.9)	64 (14.2)
Ethnicity	6 (2.4)	14 (7.0)	20 (4.4)
Gender	55 (22.0)	6 (3.0)	61 (13.5)
None	145 (58.0)	154 (76.6)	299 (66.3)
Research career not taken seriously/and or not welcome as a colleague due to[b]			
Age	46 (18.6)	15 (8.2)	61 (14.2)
Country of birth /education	28 (11.3)	32 (17.5)	60 (14.0)
Ethnicity	6 (2.4)	14 (7.7)	20 (4.7)
Gender	81 (32.8)	4 (2.2)	85 (19.8)
None	118 (47.8)	124 (67.8)	242 (56.3)
In my country not the same opportunities academic career due to[c]			
Age	51 (21.7)	21 (11.5)	72 (17.3)
Country of birth /education	50 (21.3)	36 (19.8)	86 (20.6)
Ethnicity	42 (17.9)	34 (18.7)	76 (18.2)
Gender	86 (36.6)	30 (16.5)	116 (27.8)
None	91 (38.7)	101 (55.5)	192 (46.0)
In my country not the same opportunities to be invited as public speaker due to[d]			
Age	63 (26.8)	26 (14.9)	89 (21.7)
Country of birth /education	46 (19.6)	33 (18.9)	79 (19.3)
Ethnicity	38 (16.2)	27 (15.4)	65 (15.9)
Gender	66 (28.1)	23 (13.1)	89 (21.7)
None	125 (53.2)	117 (66.9)	242 (59.0)
Opportunities Scientific Societies			
Opportunities relevant positions national scientific societies unequal due to[e]			
Age	57 (21.4)	31 (15.0)	88 (18.6)
Country of birth /education	54 (20.3)	30 (14.6)	84 (17.8)
Ethnicity	43 (16.2)	36 (17.5)	79 (16.7)
Gender	95 (35.7)	34 (16.5)	129 (27.3)
None	112 (42.1)	122 (59.2)	234 (49.6)

Table 1. continued

	Breakdown by gender		
	Women (n=320)	**Men (n=259)**	**Total (n=579)**
Opportunities national scientific society meeting to be speaker/chair unequal due to			
Age	77 (24.1)	50 (19.3)	127 (21.9)
Country of birth /education	51 (15.9)	50 (19.3)	101 (17.4)
Ethnicity	33 (10.3)	30 (11.6)	63 (10.9)
Gender	85 (26.6)	27 (10.4)	112 (19.3)
None	159 (49.7)	148 (57.1)	307 (53.0)
Opportunities relevant positions international scientific societies unequal due to[f]			
Age	54 (20.8)	23 (11.4)	77 (16.7)
Country of birth /education	114 (43.8)	90 (39.6)	194 (42.0)
Ethnicity	59 (22.7)	33 (16.3)	92 (19.9)
Gender	88 (33.8)	28 (13.9)	116 (25.1)
None	85 (32.7)	96 (47.5)	181 (39.2)
Opportunities international scientific society meetings opportunity to be speaker/chair unequal due to			
Age	64 (20.0)	42 (16.2)	106 (18.3)
Country of birth /education	124 (38.8)	93 (35.9)	217 (37.5)
Ethnicity	58 (18.1)	41 (15.8)	99 (17.1)
Gender	81 (25.3)	26 (10.0)	107 (18.5)
None	120 (37.5)	110 (42.5)	230 (39.7)
In international scientific meetings not everyone has the same opportunities to participate in conferences due to			
Age	50 (15.6)	38 (14.7)	88 (15.2)
Country of birth /education	105 (32.8)	79 (30.5)	184 (31.8)
Ethnicity	50 (15.6)	42 (16.2)	92 (15.9)
Gender	67 (20.9)	22 (8.5)	89 (15.4)
None	136 (42.5)	122 (47.1)	258 (44.6)
At scientific meetings/conferences people do not pay the same attention to speakers because of			
Age	57 (17.8)	27 (10.4)	84 (14.5)
Country of birth /education	95 (29.7)	79 (30.5)	174 (30.1)
Ethnicity	60 (18.8)	40 (15.4)	100 (17.3)
Gender	73 (22.8)	28 (10.8)	101 (17.4)
None	153 (47.8)	139 (53.7)	292 (50.4)
Funding			
Receives salary/grant	260 (81.3)	188 (72.6)	448 (77.4)
Salary/grant impacted by[g]			
Age – positively	18 (7.3)	33 (19.0)	51 (12.1)
Age – negatively	22 (8.9)	11 (6.3)	33 (7.9)
Country of birth /education – positively	25 (10.2)	22 (12.6)	47 (11.2)
Country of birth / education – negatively	19 (7.7)	8 (4.6)	27 (6.4)
Ethnicity – positively	6 (2.4)	5 (2.9)	11 (2.6)
Ethnicity – negatively	4 (1.6)	2 (1.1)	6 (1.4)
Gender – positively	4 (1.6)	13 (7.5)	17 (4.0)
Gender – negatively	49 (19.9)	1 (0.6)	50 (11.9)
None	145 (58.9)	110 (63.2)	255 (60.7)
Receive compensation from private practices (including fees from companies)	151 (47.2)	131 (50.6)	282 (48.7)

Table 1. continued

	Breakdown by gender		
	Women (n=320)	**Men (n=259)**	**Total (n=579)**
Compensation from private practices impacted by[h]			
Age – positively	22 (17.7)	20 (19.8)	42 (18.7)
Age – negatively	17 (13.7)	10 (9.9)	27 (12.0)
Country of birth /education – positively	23 (18.5)	17 (16.8)	40 (17.8)
Country of birth / education – negatively	9 (7.3)	11 (10.9)	20 (8.9)
Ethnicity – positively	8 (6.5)	3 (3.0)	11 (4.9)
Ethnicity – negatively	-	5 (5.0)	5 (2.2)
Gender – positively	14 (11.3)	15 (14.9)	29 (12.9)
Gender – negatively	17 (13.7)	3 (3.0)	20 (8.9)
Time to be dedicated – negative	61 (49.2)	38 (37.6)	99 (44.0)
Too few request – negative	14 (11.3)	15 (14.9)	29 (12.9)
Clinical practice			
Contact with patients negatively influenced by[i]			
Age	55 (18.0)	24 (9.6)	79 (14.8)
Country of birth or education	32 (10.5)	20 (8.0)	52 (9.8)
Ethnicity	11 (3.6)	14 (5.6)	25 (4.7)
Gender	65 (21.3)	11 (4.4)	76 (14.3)
None of the above	178 (58.4)	191 (76.1)	369 (69.4)

[a] missing n=29 (15 men, 14 women), [b] missing n=50 (33 men, 17 women), [c] missing n=38 (13 men, 25 women), [d] missing n=45 (20 men, 25 women), [e] missing n=29 (13 men, 16 women), [f] missing n=39 (17 men, 22 women) [g] missing n=28 (14 men, 14 women), [h] missing n=57 (30 men, 27 women), [i] missing n=24 (10 men, 14 women).

Perceived barriers stratified by gender

In the gender stratified analyses, it became clear that women more frequently experienced gender as a barrier (Table 2). In fact, when age, ethnicity and workplace were corrected for, women were two to three times more likely to experience barriers to professional recognition. For instance, women were three times more likely to experience barriers to collaborative research efforts (OR 3.1, 95% confidence interval (95%CI) 1.9-5.0, p<0.001) (Table 2). While they did not indicate a different rate of participation in international conferences, women were two times more likely to experience barriers to holding a position in both national and international scientific societies (respectively OR 2.3, CI 1.5-3.5, p<0.001 and OR 2.0, 95%CI 1.3-3.0, p=0.002). Women in clinical practice also more frequently indicated barriers negatively influencing their interaction with patients (OR 2.8, 95%CI 1.8-4.6, p<0.001).

Table 2. Likelihood of women reporting perceived barriers opposed to men.

	Odds ratio	95%CI	P-value
Professional recognition			
Collaborative research	3.1	1.9 – 5.0	**<0.001**
Being taken seriously and/or welcome as a colleague	2.5	1.6 – 3.9	**<0.001**
Academic career	2.1	1.3 – 3.3	**0.002**
Public speaker	2.1	1.3 – 3.3	**0.003**
Scientific societies			
Position national scientific society	2.3	1.5 – 3.5	**<0.001**
Speaker/chair national scientific society meeting	1.7	1.2 – 2.5	**0.005**
Position international scientific society	2.0	1.3 – 3.0	**0.002**
Speaker/chair international scientific society meeting	1.3	0.9 – 2.0	0.13
Participation in conferences	1.4	1.0 – 2.0	0.08
Attention at meetings/conferences	1.4	1.0 – 2.1	0.08
Clinical practice			
Negative influence on dealing with patients	2.8	1.8 – 4.6	<0.001

Corrected for age, ethnicity and workplace.

The perceived negative effect of gender on salary and/or grants received, as well as on compensation for work in private practices was almost exclusively reported by women (Table 1 and Figure 3). Moreover, men sometimes reported a perceived positive effect of age on salary and/or grants, this was much less often the case for women.

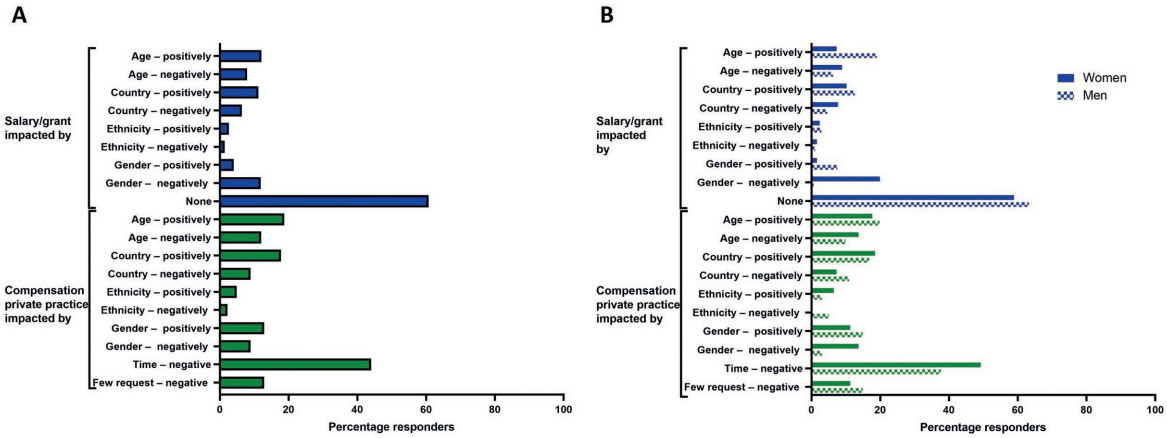

Figure 3. Barriers to a career in headache affecting salary and compensation.
Results from all participants (A) and stratified by gender (B). Country is used to indicate country of birth and/or education.

Perceived barriers by geographical workplace location

Unfortunately, there were not sufficient responders working in the African (3.1%) and Eastern Mediterranean (3.1%) regions to be able to evaluate specific perceived barriers among individuals from these regions. Responders' characteristics by geographic workplace location are displayed in Supplementary Table 2. Supplementary Figures 1-3 illustrate perceived barriers to a career in headache affecting professional recognition, opportunities in scientific societies, salary and compensation by geographic workplace, respectively. These illustrate a certain degree of variability by geographical region in regard to perceived barriers assessed.

As Europe was the most common geographical work region, we examined the likelihood of non-European participants reporting perceived barriers compared to European participants. There were large differences in whether and to what extent barriers are perceived by geographical work location. For instance, North American participants had higher odds of reporting issues of colleagues not taking them seriously or being welcomed by them compared with their European counterparts (OR 2.1, 95%CI 1.0-4.1, p=0.037) (Table 3). The opposite effect was seen in the Western Pacific region where they had lower odds of perceiving these barriers than their European counterparts (OR 0.3, 95%CI 0.1-1.0, p=0.041) (Table 3). North Americans and persons from South America had higher odds of reporting barriers preventing participation in international scientific societies (OR 2.3, 95%CI 1.2-4.4, p<0.015 and OR 3.1, 95%CI 1.3-7.3, p<0.008) compared to persons from Europe (Table 3).

Academic mentorship

The majority of participants has current or experience as a mentee (85.1%) (Supplementary Table 4). For both men and women, most mentors were men. Many positive effects were described, such as fostering a career (85.2%) and promoting participation in external professional activities (79.3%) (Supplementary Table 4). Remarkably, around one third of participants indicated that their mentors used the mentee's work to advance their own career (31.1%).

Table 3. Likelihood of non-European participants experiencing barriers opposed to participants based in Europe.

	Odds ratio	95%CI	P-value
Professional recognition			
Collaborative research			
South East Asia	0.4	0.2 – 1.3	0.13
North America	1.6	0.8 – 3.0	0.20
Western Pacific	0.6	0.2 – 1.7	0.36
South America	0.7	0.3 – 1.4	0.29
Being taken seriously and/or welcome as a colleague			
South East Asia	0.5	0.2 – 1.5	0.23
North America	2.1	1.0 – 4.1	**0.037**
Western Pacific	0.3	0.1 – 1.0	**0.041**
South America	0.7	0.3 – 1.4	0.29
Academic career			
South East Asia	0.3	0.1 – 0.9	**0.027**
North America	1.6	0.8 – 3.3	0.16
Western Pacific	0.3	0.1 – 0.9	**0.028**
South America	1.0	0.4 – 2.2	0.93
Public speaker			
South East Asia	0.7	0.3 – 2.2	0.59
North America	2.7	1.3 – 5.3	**0.005**
Western Pacific	1.1	0.4 – 3.0	0.85
South America	1.1	0.5 – 2.6	0.77
Scientific societies			
Position national scientific society			
South East Asia	0.5	0.2 – 1.1	0.08
North America	2.6	1.4 – 4.9	**0.003**
Western Pacific	0.5	0.2 – 1.4	0.21
South America	2.0	0.9 – 4.1	0.07
Speaker/chair national scientific society meeting			
South East Asia	0.4	0.2 – 0.9	**0.024**
North America	3.5	1.9 – 6.4	**<0.001**
Western Pacific	0.5	0.2 – 1.3	0.16
South America	1.9	1.0 – 3.8	0.06
Position international scientific society			
South East Asia	0.4	0.2 – 0.9	**0.031**
North America	2.3	1.2 – 4.4	**0.015**
Western Pacific	0.5	0.2 – 1.2	0.11
South America	3.1	1.3 – 7.3	**0.008**
Speaker/chair international scientific society meeting			
South East Asia	0.8	0.4 – 1.8	0.57
North America	3.0	1.6 – 5.5	**<0.001**
Western Pacific	1.1	0.5 – 2.6	0.80
South America	3.0	1.4 – 6.3	**0.004**
Participation in conferences			
South East Asia	1.0	0.5 – 2.2	0.97
North America	2.5	1.4 – 4.4	**0.002**
Western Pacific	1.4	0.6 – 3.1	0.43
South America	3.7	1.8 – 7.8	**<0.001**
Attention at meetings/conferences			
South East Asia	0.9	0.4 – 1.9	0.7
North America	2.8	1.5 – 5.0	**0.001**
Western Pacific	1.9	0.8 – 4.5	0.12
South America	1.9	1.0 – 3.7	0.07
Clinical practice			
Negative influence on dealing with patients			
South East Asia	0.2	0.1 – 0.6	**0.005**
North America	1.7	0.9 – 3.2	0.12
Western Pacific	1.0	0.4 – 2.7	0.88
South America	1.0	0.5 – 2.3	0.91

Corrected for age, ethnicity and gender. As the largest group, Europe was taken as the reference category.

Discussion

Our results underline that in the field of headache care and research, women more often experience career barriers as opposed to men. Moreover, gender appears to be one of the most important barriers to a successful career in the headache field. Importantly, country of birth is also an important perceived barrier for active participation in international scientific societies. Lastly, career barriers are perceived differently across world regions. These ongoing disparities need to be acknowledged and addressed in areas of recruitment, retention, and advancement to improve the yield of the headache research field.

Gender bias (unconscious and conscious) and its associated inequity with respect to payment, leadership opportunities, promotion, and retention are universal and permeate nearly all professional and social domains. While women have closed the sex gap with respect to medical school admission in some regions, women remain underrepresented in upper faculty ranks of medical schools .[10] Factors contributing to the sex gap in promotion include a persisting mentality and climate, lack of sex parity in leadership, lack of gender equity in compensation, lack of retention of women (the "leaky pipeline"), and a disproportionate burden of family responsibilities and associated difficulties in achieving work–life balance.[22] However, advising women to better organize their home-responsibilities will not solve the problem of gender inequality. Importantly, perceived barriers most likely lead to fewer women entering and staying in science and medicine, despite their valuable contributions.

UNESCO Institute for Statistics fact sheet released in June 2020 showed that women in research and development represent only 23.1% of the total workforce for South and West Asia, 25% for East Asia and the Pacific region, 31.1% for Sub-Saharan Africa, 32.9% for North America and Western Europe and 39.0% for Central and Eastern Europe.[23] These region-specific differences in the percentage of research scientists who are women are especially interesting considering that perceived barriers varied across regions in this study. While this could be due to local social norms and cultural influences, it might also be in part due to differences in response rate per region or potential response biases. Nonetheless, it is fair to postulate that depending on the region, different barriers to career progression might be more pressing and might require different interventions.

This study has some limitations. Firstly, given that this was a survey study, there is potential for responder bias. As we wanted to give all individuals working in the headache field the chance to participate, we contacted the whole IHS membership database and also

allowed national representatives to send survey links to their members and responders to be anonymous. We contacted all IHS affiliated national societies (the full list can be accessed from the IHS website https://ihs-headache.org/en/about-ihs/affiliate-member-societies/), but we cannot know for sure which representatives actually circulated the survey, as this was totally voluntary. This meant that we were unable to determine the response rate. While unfortunate, we considered the need for all voices to be potentially heard more pressing. Nonetheless, a possible language barrier could have prevented some headache professionals from responding. Additionally, only 2.4% of respondents were Black or African American and 4.0% were Hispanic or Latino, which may make the results of this study less generalizable to these groups. This leads to the discussion regarding underrepresentation of some races and ethnicities in the headache field. Moreover, as very few responders were located in African and Eastern Mediterranean countries, we also had an underrepresentation of individuals from those regions. Underrepresentation based on ethnicity or geographical location led to lack of diversity in the sample. In addition, race and ethnicity were collected together rather than as separate variables, so some specificity may have been lost. We were unable to include all characteristics that could be the base of discrimination in our survey; for instance, we did not study the effect of sexual orientation and disability. Finally, it is a self-report survey of perceived barriers to career achievement. There were no outside supporting data used to substantiate respondent reports; however, the subjective nature of the data are useful to report on the perception and beliefs of the respondents.

While our findings may not be surprising regarding perceived barriers by women and people from specific global regions, it is the first time that these barriers are being surveyed in the professional field of headache, care and research to our knowledge. From our findings we can conclude that there is a continuing need to monitor if enough progress is made for women and people from underrepresented global regions in (academic) headache medicine and healthcare and to improve upon the current gains in equity.

Factors contributing to these career barriers are often multidimensional with potential problems with individual, interpersonal and societal–institutional aspects. Moreover, there are often different nuances at different stages of the career path.[16] There is a need for targeted interventions to counteract social stereotypes, transform "masculine" workplace cultures, end discriminatory behaviors, and promote processes to include women and other underrepresented minorities in a transparent format. The critical question is why there is a lack of retention of women and how the culture at the working place can be changed to promote women to leading positions, hence creating equal opportunities.

Climate and culture must be addressed together. Efforts to build a good climate will be unsuccessful if the policies conflict with the values of an organization.[24] Thus, addressing and improving organizational culture and climate is essential to make underrepresented groups feel welcome, supported, and respected. Non-surprisingly, many advocates are tired of initiatives that focus on the actions of individuals from underrepresented groups as being the problem, and making changes to their actions being the solution. These initiatives may presume a masculine heteronormative and Caucasian homogeneous view of the world and thus automatically require underrepresented individuals to change to fit into the existing dominant system, rather than the system changing to accommodate and nurture the needs of its members. The responsibility of making a cultural shift cannot be made by the

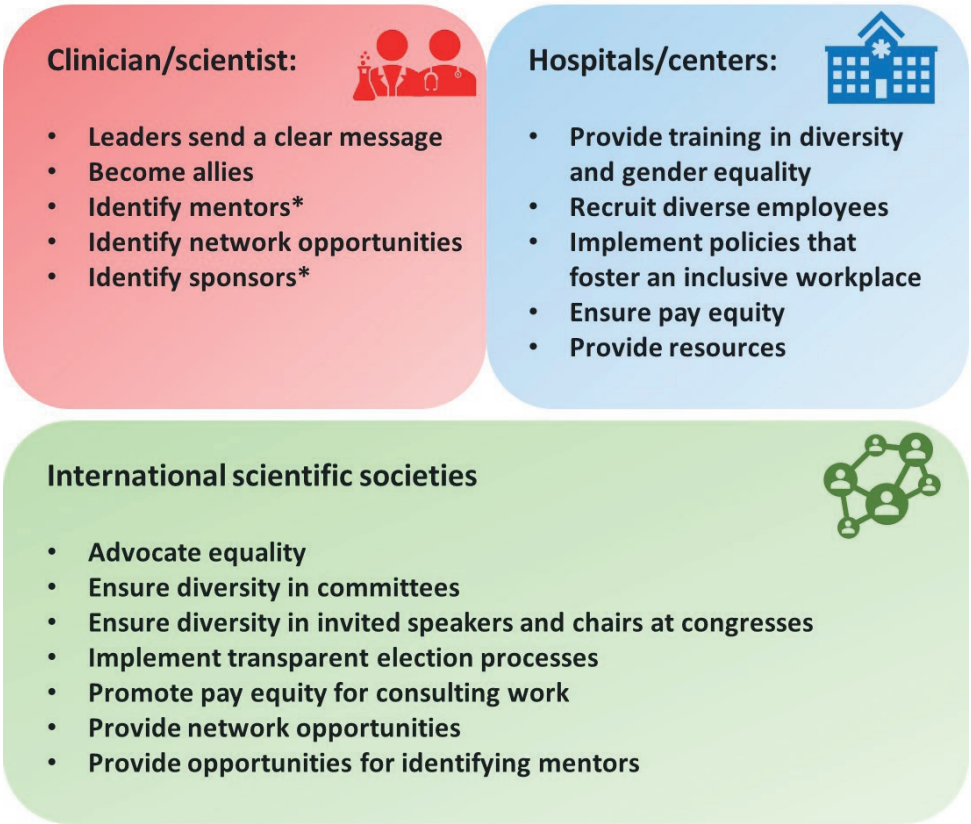

Figure 4. Approaches to help promote inclusivity and diversity in the headache field.

*Both mentors and sponsors can be crucial. Where mentors serve as guides, help solve potential issues and tell mentees that they can do the job, sponsors serve as cheerleaders and speak out about a sponsee publicly and tell others that the sponsee can do the job.

underrepresented individuals themselves as in many cases they do not have the power to affect change. Change must occur at the level of the institution, larger systems and broad shifts in cultural norms and attitudes. To achieve real inclusivity, we propose several approaches on individual, local, and (inter-) national levels (Figure 4).

Providing a safe space for conversations about inclusivity and diversity must be a goal to improve cultures and a responsibility of leadership. Therefore, leaders at all levels should sent clear messages about the value of women and individuals from underrepresented races, ethnicity, religions, sexual orientation, gender identity and countries.[25,26] Successful programs aimed at changing culture and climate require committed resources, for instance sensitivity training of hiring staff, and formal recognition that striving towards diversity is part of the goal and responsibility of leaders. There is also an important role the scientific societies should play in advocating and modeling equality. Explicit statements can change opinions and clear actions can make an even bigger impact. In addition, scientific societies should be committed to organizing inclusive conferences, not only in attendees, but also in terms of speakers, panels and organizing committees.[27,28] The presenting faculty at a meeting should include individuals from underrepresented races, ethnicity, religions, sexual orientation, gender identity and countries, and be a representation of the people working in the field and members of the organization. This should also be the case for society-endorsed conferences. Hybrid conferences and scholarships with both in-person and virtual participants can be helpful to increase participation rates throughout the world, overcoming financial and geographic barriers for participation. Besides voluntary tools for organizational change, legislated approaches such as the use of quotas may also be considered. Quotas are an instrument aimed to accelerate the achievement of diversity by establishing a defined percentage of positions, which are allocated to specific groups of individuals. The concept of quotas often elicits a strong and adverse response, with claims that quality of work will be affected. While there is limited research on the usefulness of quotas, there are indications that they can have positive effects.[29] While they are not the only answer, they are a tool that should be considered.

Another approach to combat career barriers is adequate mentorship. Mentoring is associated with objective and subjective measures of career success.[30] Many participants already indicated that they had mentors, however women less frequently acted as mentors compared to men. Finding a mentor should not solely be the responsibility of underrepresented individuals. There also lies a responsibility for providing adequate mentors within centers and scientific societies. Our data show

that in one third of cases the mentor has a mutual interest, (e.g. doing scientific or clinical work together). Another useful concept that everyone can imply is allyship or sponsorship. In allyship members of organization in a position of privilege or power seek to operate in solidarity with underrepresented groups. As an example, it could involve speaking up and supporting women and calling out discrimination. Some actual instances of sexism experienced by headache healthcare professionals and effective strategies and actions to take to support women were already suggested.[31] Effective allyship requires self-awareness, commitment, respect and hard work.[32,33] However, to inspire cultural change a combination of interventions will likely be necessary.[26,34]

Striving towards an inclusive climate should be a continuous process, not a final destination. Setting and measuring predetermined milestones is important, but any organization should strive to always be diverse and inclusive. Now that we know which barriers are being perceived by healthcare professionals, academics and scientists in the headache field, we can start working together towards establishing an ever more inclusive, nurturing and welcoming domain.

Highlights

- Gender is an important barrier to a career achievement in the headache field.
- Specific perceived career barriers are different across world regions.
- Country of birth is a barrier for participation in international scientific societies.
- (Inter-)national headache organizations should be aware of these barriers and ensure an equal position for all members independent of gender, ethnicity, geographical location or other personal characteristics.

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Supplementary

Supplementary table 1. Demographics responders

	Breakdown by gender		
	Women (n=320)	**Men (n=259)**	**Total (n=579)**
Age[a]			
18-30	32 (10.0)	13 (5.0)	45 (7.8)
31-40	100 (31.3)	90 (34.7)	190 (32.8)
41-50	100 (31.3)	64 (24.7)	164 (28.3)
51-60	61 (19.1)	52 (20.1)	113 (19.5)
>60	27 (8.4)	37 (14.3)	64 (11.1)
Gender[b]			
Women	-	-	320 (55.3)
Men			259 (44.7)
Geographic location – birth[c]			
Africa	5 (1.6)	18 (6.9)	23 (4.0)
Eastern Mediterranean	11 (3.4)	8 (3.1)	19 (3.3)
Europe	147 (45.9)	82 (31.7)	229 (39.6)
North America	42 (13.1)	36 (13.9)	78 (13.5)
South America	33 (10.3)	22 (8.5)	55 (9.5)
South East Asia	55 (17.2)	71 (27.4)	126 (21.8)
Western Pacific	27 (8.4)	22 (8.5)	49 (8.5)
Geographic location – workplace			
Africa	1 (0.3)	17 (6.6)	18 (3.1)
Eastern Mediterranean	11 (3.4)	7 (2.7)	18 (3.1)
Europe	145 (45.3)	81 (31.3)	226 (39.0)
North America	46 (14.4)	37 (14.3)	83 (14.3)
South America	34 (10.6)	21 (8.1)	55 (9.5)
South East Asia	51 (15.9)	70 (27.0)	121 (20.9)
Western Pacific	32 (10.0)	26 (10.0)	58 (10.0)
Ethnicity			
Asian/Pacific islander	67 (20.9)	88 (34.0)	155 (26.8)
Black/African American	0 (0)	14 (5.4)	14 (2.4)
Hispanic	12 (3.8)	11 (4.1)	23 (4.0)
White/Caucasian	215 (67.2)	125 (48.3)	340 (58.8)
Multiple ethnicities	13 (4.1)	2 (0.3)	15 (2.6)
Other	13 (4.1)	19 (7.3)	32 (5.5)
Marital status			
Divorced/separated	21 (6.6)	7 (2.7)	28 (4.8)
Married/long-term relationship	248 (77.5)	231 (89.2)	479 (82.7)
Single	46 (14.4)	20 (7.7)	66 (11.4)
Widow(er)	5 (1.6)	1 (0.4)	6 (1.0)

Supplementary table 1. continued

	Breakdown by gender		
	Women (n=320)	**Men (n=259)**	**Total (n=579)**
Children			
Yes	211 (65.9)	205 (79.2)	416 (71.8)
Education and profession			
Education[d]			
Specialist	113 (35.3)	119 (45.9)	232 (40.1)
Resident / Traineee	16 (5.0)	18 (6.9)	34 (5.9)
PhD	77 (24.1)	43 (16.6)	120 (20.8)
MD	89 (27.8)	58 (22.4)	147 (25.4)
Master's degree	18 (5.6)	15 (5.8)	33 (5.7)
Other	7 (2.2)	6 (2.3)	13 (2.2)
Speciality			
Neurology	243 (88.4)	198 (89.6)	441 (88.9)
Other	32 (11.6)	23 (10.4)	55 (11.1)
Work environment[f]			
Own practice	28 (8.8)	30 (11.6)	58 (10.0)
Private practice	90 (28.1)	76 (29.3)	166 (28.7)
General hospital	86 (26.9)	94 (36.3)	180 (31.1)
Academic hospital	147 (45.9)	124 (47.9)	271 (46.8)
University	95 (29.7)	75 (29.0)	170 (29.4)
Scientific public institution	28 (8.8)	12 (4.6)	40 (6.9)
Scientific private institution	18 (5.6)	9 (3.5)	27 (4.7)
Participation in clinical activities			
Yes	305 (95.3)	251 (96.9)	556 (96.0)
How many days per week do you see patients			
1	11 (3.7)	8 (3.3)	19 (3.5)
2	21 (7.0)	13 (5.3)	34 (6.3)
3	33 (11.1)	26 (10.6)	59 (10.9)
4	50 (16.8)	34 (13.9)	84 (15.5)
5	135 (45.3)	97 (39.6)	232 (42.7)
6	48 (16.1)	67 (27.3)	115 (21.2)
How many headache patients per week			
<10	58 (19.9)	53 (22.1)	111 (20.9)
10-30	139 (47.6)	90 (37.5)	229 (43.0)
31-60	54 (18.5)	62 (25.8)	116 (21.8)
61-80	25 (8.6)	15 (6.3)	40 (7.5)
81-100	6 (2.1)	11 (4.6)	17 (3.2)
>100	10 (3.4)	9 (3.8)	19 (3.4)
How many non-headache patients per week			
<10	69 (23.2)	46 (19.2)	115 (21.4)
10-30	104 (34.9)	57 (23.8)	161 (29.9)
31-60	70 (23.5)	67 (27.9)	137 (25.5)
61-80	26 (8.7)	34 (14.2)	60 (11.2)
81-100	17 (5.7)	11 (4.6)	28 (5.2)
>100	12 (4.0)	25 (10.4)	37 (6.9)
Participation in research activities			
Yes	264 (82.5)	216 (83.4)	480 (82.9)

Supplementary table 1. continued

	Breakdown by gender		
	Women (n=320)	**Men (n=259)**	**Total (n=579)**
Current professional status research[f]			
PhD student	47 (18.4)	13 (6.5)	60 (12.2)
Post-doc	25 (9.8)	22 (11.0)	47 (9.6)
Staff member/Attending	142 (55.6)	121 (60.5)	263 (53.7)
Scientific group leader	57 (22.4)	63 (31.5)	120 (24.5)
Participation in academic career			
Yes	219 (68.4)	157 (60.6)	376 (64.9)
No longer	41 (12.8)	38 (14.7)	79 (13.6)
Never	60 (18.8)	64 (24.7)	124 (21.4)
Current professional status academia[f]			
PhD student/Post-doc	56 (26.9)	24 (16.3)	80 (22.5)
Assistant Professor	43 (20.7)	31 (21.1)	74 (20.8)
Associate Professor	50 (24.0)	27 (18.4)	77 (21.7)
Full Professor	31 (14.9)	40 (27.2)	71 (20.0)
Other	28 (13.5)	25 (17.0)	53 (14.9)
Expects to be promoted in further academic career			
Yes	126 (72.4)	96 (68.6)	222 (59.0)
Involved in scientific societies			
Yes	259 (80.9)	200 (77.2)	459 (79.3)
No longer	23 (7.2)	19 (7.3)	42 (7.3)
Never	38 (11.9)	40 (15.4)	78 (13.5)

[a] Three participants preferred not to disclose their age. [b] One participant reported a non-binary gender identity. As results are separately reported based on gender, we hat to exclude this participant to ensure anonymity. [c] Participants with difference in location birth and workplace: n=38 (6.6%). [d] Made exclusive, order specialist, resident, PhD, MD, MSc, Other, [e] Three residents / trainee reported also a PhD degree, [f] Multiple answers possible.

Supplementary table 2. Demographics responders by workplace.

	Breakdown by workplace				
	Europe n=226	**South East Asia n=121**	**North America n=83**	**Western Pacific n=58**	**South America n=55**
Age[a]					
18-30	31 (13.8)	6 (5.0)	3 (3.7)	1 (1.7)	2 (3.6)
31-40	63 (28.1)	57 (47.1)	24 (29.3)	12 (20.7)	18 (32.7)
41-50	64 (28.6)	27 (22.3)	18 (22.0)	25 (43.1)	17 (30.9)
51-60	40 (17.9)	24 (19.8)	16 (19.5)	17 (29.3)	11 (20.0)
>60	26 (11.6)	7 (5.8)	21 (25.6)	3 (5.2)	7 (12.7)
Gender					
Women	145 (64.2)	51 (42.1)	46 (55.4)	32 (55.2)	34 (61.8)
Education[b]					
Specialist	74 (32.7)	69 (57.0)	19 (22.9)	22 (37.9)	26 (47.3)
Resident / Traineec	15 (6.6)	7 (5.8)	1 (1.2)	2 (3.4)	3 (5.5)
PhD	74 (32.7)	13 (10.7)	7 (8.4)	17 (29.3)	11 (20.0)
MD	57 (25.2)	21 (17.4)	44 (53.0)	12 (20.7)	6 (10.9)
Master's degree	4 (1.8)	10 (8.3)	6 (7.2)	5 (8.6)	8 (14.5)
Other	4 (1.8)	2 (1.7)	6 (7.2)	-	1 (1.8)
Speciality					
Neurology	177 (88.5)	80 (86.0)	60 (84.5)	46 (86.8)	47 (97.9)
Other	23 (11.5)	13 (14.0)	11 (15.5)	7 (13.2)	1 (2.1)
Work environment[d]					
Own practice	14 (6.2)	18 (14.9)	10 (12.0)	-	11 (20.0)
Private practice	50 (22.1)	24 (19.8)	24 (28.9)	15 (25.9)	41 (74.5)
General hospital	60 (26.5)	63 (52.1)	9 (10.8)	26 (44.8)	11 (20.0)
Academic hospital	115 (50.9)	42 (34.7)	47 (56.6)	26 (44.8)	26 (47.3)
University	77 (34.1)	28 (19.0)	21 (25.3)	19 (32.8)	18 (32.7)
Scientific public institution	25 (11.1)	5 (4.1)	2 (2.4)	2 (3.4)	5 (9.1)
Scientific private institution	14 (6.2)	5 (4.1)	1 (1.2)	-	5 (9.1)
Participation in clinical activities					
Yes	206 (91.2)	119 (98.3)	83 (100.0)	57 (98.3)	55 (100.0)
Current professional status clinician[d]					
General practitioner	12 (6.0)	8 (6.9)	2 (2.5)	1 (1.9)	3 (3.7)
Resident	24 (12.0)	9 (7.8)	1 (1.2)	3 (5.6)	1 (1.9)
Consultant/Attending	79 (39.5)	69 (59.5)	51 (63.0)	18 (33.3)	32 (59.3)
Chief consultant/Division chair	27 (13.5)	10 (8.6)	10 (12.3)	15 (27.8)	5 (9.1)
Head of Department	31 (15.5)	16 (13.8)	5 (6.2)	13 (24.1)	9 (16.7)
Other	27 (13.5)	4 (3.4)	12 (14.8)	4 (7.5)	5 (9.1)
Participation in research activities					
Yes	192 (85.0)	92 (76.0)	65 (78.3)	52 (89.7)	50 (90.9)
Current professional status research[d]					
PhD student	40 (21.6)	9 (10.6)	3 (4.7)	4 (8.3)	2 (4.0)
Post-doc	31 (16.8)	12 (14.2)	3 (4.7)	-	-
Staff member/Attending	64 (34.6)	55 (64.7)	56 (87,5)	33 (68.8)	36 (72.0)
Scientific group leader	63 (34.1)	14 (16.4)	5 (7.8)	17 (35.4)	17 (34.0)
Participation in academic career					
Yes	140 (61.9)	69 (57.0)	63 (75.9)	41 (70.7)	34 (61.8)
No longer	34 (15.0)	13 (10.7)	8 (9.6)	11 (19.0)	10 (18.2)
Never	52 (23.0)	39 (32.2)	12 (14.5)	6 (10.3)	11 (20.0)

Supplementary table 2. continued

	Breakdown by workplace				
	Europe n=226	**South East Asia n=121**	**North America n=83**	**Western Pacific n=58**	**South America n=55**
Current professional status academia					
PhD student/Post-doc	50 (37.6)	15 (22.7)	1 (1.7)	4 (11.1)	4 (11.8)
Assistant Professor	17 (12.8)	9 (13.6)	22 (37.9)	3 (8.3)	17 (50.0)
Associate Professor	34 (25.6)	16 (24.2)	14 (24.1)	8 (22.2)	5 (14.7)
Full Professor	15 (11.3)	16 (24.2)	15 (25.9)	18 (50.0)	3 (8.8)
Other	17 (12.8)	10 (15.2)	6 (10.3)	3 (8.3)	5 (14.7)
Expects to be promoted in further academic career					
Yes	79 (66.4)	50 (83.3)	30 (58.8)	24 (80.0)	22 (71.0)
Involved in scientific societies					
Yes	170 (75.2)	80 (66.1)	75 (90.4)	48 (82.8)	54 (98.2)
No longer	22 (9.7)	9 (7.4)	5 (6.0)	3 (5.2)	-
Never	34 (15.0)	32 (26.4)	3 (3.6)	7 (12.1)	1 (1.8)

[a] Three participants preferred not to disclose their age. [b] Made exclusive, order specialist, resident, PhD, MD, MSc, Other, [c] Three residents / trainee reported also a PhD degree, [d] Multiple answers possible

Supplementary table 3. **Career barriers perceived and by workplace.**

	Breakdown by workplace				
	Europe n=226	South East Asia n=121	North America n=83	Western Pacific n=58	South America n=55
Work-life balance					
Influence family care					
Yes, positively	45 (19.9)	16 (13.2)	9 (10.8)	9 (15.5)	12 (21.8)
Yes, negatively	28 (12.4)	9 (7.4)	9 (10.8)	8 (13.8)	8 (14.5)
Both	64 (28.3)	27 (22.3)	35 (42.2)	9 (15.5)	14 (25.5)
No	89 (39.4)	69 (57.0)	30 (36.1)	32 (55.2)	21 (28.2)
Professional recognition					
Collaborative research impacted by[a]					
Age	24 (13.0)	6 (7.1)	10 (17.2)	8 (17.0)	5 (10.4)
Country of birth /education	24 (13.0)	10 (11.8)	5 (8.6)	7 (14.9)	12 (25.0)
Ethnicity	5 (2.7)	3 (3.5)	5 (8.6)	3 (6.4)	1 (2.1)
Gender	28 (15.2)	6 (7.1)	16 (27.6)	6 (12.8)	4 (8.3)
None	119 (64.7)	63 (74.1)	33 (56.9)	32 (68.1)	33 (68.8)
Research career not taken seriously/and or not welcome as a colleague due to[b]					
Age	27 (15.0)	7 (9.7)	11 (19.3)	4 (8.5)	11 (23.4)
Country of birth /education	26 (14.4)	11 (15.3)	5 (8.8)	3 (6.4)	10 (21.3)
Ethnicity	6 (3.3)	3 (4.2)	6 (10.5)	1 (2.1)	2 (4.3)
Gender	45 (25.0)	5 (6.9)	18 (31.6)	9 (19.1)	6 (12.8)
None	91 (50.6)	51 (70.8)	20 (35.1)	35 (74.5)	25 (53.2)
In my country not the same opportunities academic career due to[c]					
Age	26 (16.6)	7 (9.5)	20 (30.8)	11 (22.0)	6 (15.0)
Country of birth /education	31 (19.7)	11 (14.9)	19 (29.2)	6 (12.0)	13 (32.5)
Ethnicity	24 (15.3)	4 (5.4)	28 (43.1)	5 (10.0)	10 (25.0)
Gender	53 (33.8)	9 (12.2)	32 (49.2)	10 (20.0)	7 (17.5)
None	64 (40.8)	42 (56.8)	21 (32.3)	31 (62.0)	15 (37.5)
In my country not the same opportunities to be invited as public speaker due to[d]					
Age	36 (23.4)	6 (7.9)	22 (33.8)	10 (20.4)	12 (31.6)
Country of birth /education	26 (16.9)	13 (17.1)	17 (26.2)	8 (16.3)	10 (26.3)
Ethnicity	21 (13.6)	5 (6.6)	24 (36.9)	5 (10.2)	8 (21.1)
Gender	35 (22.7)	7 (9.2)	25 (38.5)	11 (22.4)	6 (15.8)
None	92 (59.7)	55 (72.4)	25 (38.5)	33 (67.3)	20 (52.6)
Opportunities Scientific Societies					
Opportunities relevant positions national scientific societies unequal due to[e]					
Age	34 (18.8)	10 (12.3)	21 (27.3)	8 (16.7)	14 (26.9)
Country of birth /education	25 (13.8)	13 (16.0)	18 (23.4)	9 (18.8)	14 (26.9)
Ethnicity	27 (14.9)	7 (8.6)	25 (32.5)	5 (10.4)	11 (21.2)
Gender	58 (32.0)	11 (13.6)	35 (45.5)	6 (12.5)	13 (25.0)
None	95 (52.5)	45 (55.6)	28 (36.4)	28 (58.3)	18 (34.6)

Supplementary table 3. continued

	Breakdown by workplace				
	Europe n=226	**South East Asia n=121**	**North America n=83**	**Western Pacific n=58**	**South America n=55**
Opportunities national scientific society meeting opportunity to be speaker/chair unequal due to					
Age	49 (21.7)	27 (22.3)	25 (30.1)	11 (19.0)	14 (25.5)
Country of birth /education	28 (12.4)	32 (26.4)	15 (18.1)	12 (20.7)	11 (20.0)
Ethnicity	24 (10.6)	4 (3.3)	21 (25.3)	5 (8.6)	5 (9.1)
Gender	50 (22.1)	9 (7.4)	34 (41.0)	6 (10.3)	11 (20.0)
None	130 (57.5)	67 (55.4)	27 (32.5)	33 (56.9)	24 (43.6)
Opportunities relevant positions international scientific societies unequal due to[f]					
Age	37 (20.6)	7 (8.6)	17 (23.3)	6 (12.8)	10 (19.6)
Country of birth /education	66 (36.7)	31 (38.3)	29 (39.7)	19 (40.4)	34 (66.7)
Ethnicity	37 (20.6)	12 (14.8)	19 (26.0)	6 (12.8)	12 (23.5)
Gender	60 (33.3)	7 (8.6)	27 (37.0)	7 (14.9)	11 (21.6)
None	76 (42.2)	40 (49.4)	21 (28.8)	22 (46.8)	9 (17.6)
Opportunities international scientific society meetings opportunity to be speaker/chair unequal due to	38 (16.8)	21 (17.4)	26 (31.3)	10 (17.2)	9 (16.4)
Age	63 (27.9)	57 (47.1)	27 (32.5)	21 (36.2)	34 (61.8)
Country of birth /education	30 (13.3)	22 (18.2)	19 (22.9)	11 (19.0)	11 (20.0)
Ethnicity	56 (24.8)	7 (5.8)	29 (34.9)	7 (12.1)	7 (12.7)
Gender	107 (47.3)	47 (38.8)	21 (25.3)	24 (41.4)	13 (23.6)
None					
In international scientific meetings not everyone has the same opportunities to participate in conferences due to	31 (13.7)	21 (17.4)	19 (22.9)	6 (10.3)	10 (18.2)
Age	55 (24.3)	44 (36.4)	24 (28.9)	18 (31.0)	29 (52.7)
Country of birth /education	27 (11.9)	21 (17.4)	18 (21.7)	8 (13.8)	11 (20.0)
Ethnicity	44 (19.5)	5 (4.1)	24 (28.9)	4 (6.9)	10 (18.2)
Gender	122 (54.0)	54 (44.6)	27 (32.5)	25 (43.1)	13 (23.6)
None					
At scientific meetings/conferences people do not pay the same attention to speakers because of					
Age	33 (14.6)	7 (5.8)	24 (28.9)	8 (13.8)	10 (18.2)
Country of birth /education	50 (22.1)	46 (38.0)	19 (22.9)	23 (39.7)	27 (49.1)
Ethnicity	31 (13.7)	24 (19.8)	21 (25.3)	12 (20.7)	9 (16.4)
Gender	43 (19.0)	9 (7.4)	29 (34.9)	11 (19.0)	8 (14.5)
None	130 (57.5)	60 (49.6)	31 (37.3)	24 (41.4)	24 (43.6)
Funding					
Receives salary/grant	179 (79.2)	97 (80.2)	59 (71.1)	47 (81.0)	45 (81.8)

Supplementary table 3. continued

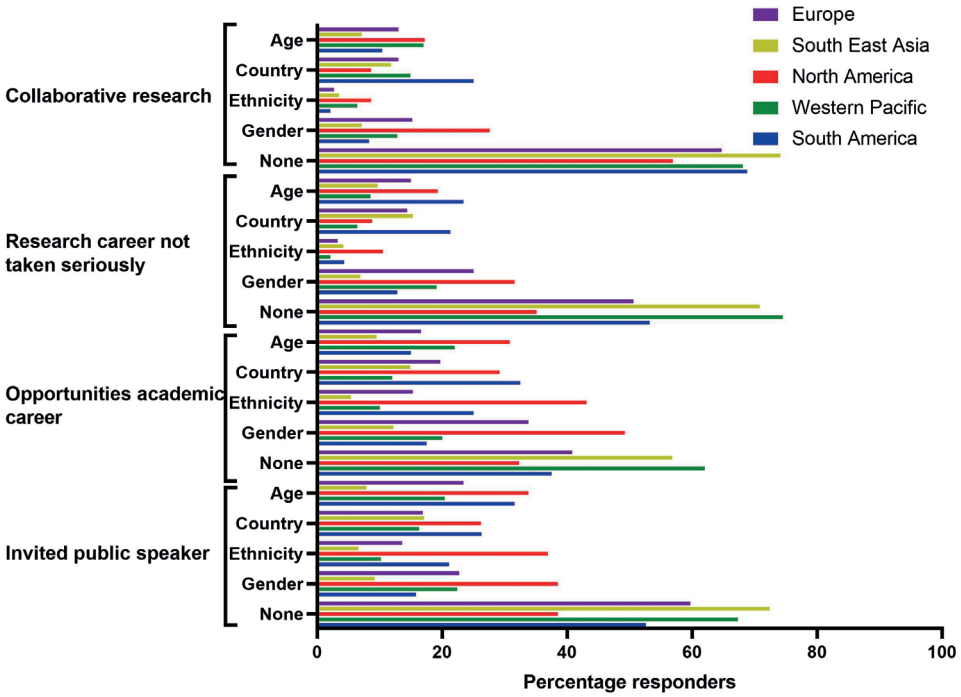
	Breakdown by workplace				
	Europe n=226	**South East Asia n=121**	**North America n=83**	**Western Pacific n=58**	**South America n=55**
Salary/grant impacted by[g]					
Age – positively	8 (4.8)	27 (29.0)	2 (3.7)	7 (16.3)	5 (11.4)
Age – negatively	15 (8.9)	4 (4.3)	6 (11.1)	2 (4.7)	6 (13.6)
Country of birth /education – positively	15 (8.9)	13 (14.0)	5 (9.3)	5 (11.6)	6 (13.6)
Country of birth / education – negatively	11 (6.5)	5 (5.4)	1 (1.9)	3 (7.0)	6 (13.6)
Ethnicity – positively	4 (2.4)	1 (1.1)	1 (1.9)	1 (2.3)	2 (4.5)
Ethnicity – negatively	2 (1.2)	1 (1.1)	-	-	3 (6.8)
Gender - positively	3 (1.8)	-	8 (14.8)	2 (4.7)	2 (4.5)
Gender - negatively	24 (14.3)	3 (3.2)	12 (22.2)	3 (7.0)	7 (15.9)
None	108 (64.3)	51 (54.8)	32 (59.3)	27 (62.8)	25 (56.8)
Receive compensation from private practices (including fees from companies)	98 (43.4)	65 (53.7)	43 (51.8)	30 (51.7)	33 (60.0)
Compensation from private practices impacted by[h]					
Age – positively	16 (21.6)	13 (22.8)	4 (13.3)	2 (8.3)	6 (20.7)
Age – negatively	10 (13.5)	7 (12.3)	3 (10.0)	2 (8.3)	5 (17.2)
Country of birth /education – positively	7 (9.5)	17 (29.8)	5 (16.7)	1 (4.2)	8 (27.6)
Country of birth / education – negatively	3 (4.1)	6 (10.5)	3 (10.0)	3 (12.5)	5 (17.2)
Ethnicity – positively	2 (2.7)	1 (1.8)	2 (6.7)	-	5 (17.2)
Ethnicity – negatively	-	2 (3.5)	1 (3.3)	1 (4.2)	-
Gender - positively	4 (5.4)	7 (12.3)	3 (10.0)	5 (20.8)	8 (27.6)
Gender - negatively	8 (10.8)	2 (3.5)	3 (10.0)	3 (12.5)	4 (13.8)
Time to be dedicated – negative	40 (54.1)	22 (38.6)	14 (46.7)	14 (58.3)	6 (20.7)
Too few request – negative	8 (10.8)	7 (12.3)	1 (3.3)	7 (29.2)	1 (3.4)
Clinical practice					
Contact with patients negatively influenced by[i]					
Age	34 (17.1)	11 (9.6)	9 (11.4)	10 (19.6)	9 (17.0)
Country of birth or education	14 (7.0)	13 (11.4)	6 (7.6)	12 (23.5)	5 (9.4)
Ethnicity	4 (2.0)	3 (2.6)	11 (13.9)	7 (13.7)	-
Gender	24 (12.1)	8 (7.0)	21 (26.6)	11 (21.6)	7 (13.2)
None of the above	140 (70.4)	89 (78.1)	50 (63.3)	29 (56.9)	35 (66.0)

[a]missing n=29 (8 Europe, 7 South East Asia, 7 North America, 5 Western Pacific, 2 South America,), [b]missing n=48 (12 Europe, 20 South East Asia, 8 North America, 5 Western Pacific, 3 South America), [c]missing n=37 (17 Europe, 8 South East Asia, 6 North America, 2 Western Pacific, 4 South America), [d]missing n=41 (20 Europe, 6 South East Asia, 6 North America, 3 Western Pacific, 6 South America), [e] missing n=27 (11 Europe, 8 South East Asia, 3 North America, 3 Western Pacific, 2 South America), [f] missing n=34 (12 Europe, 8 South East Asia, 7 North America, 4 Western Pacific, 3 South America), [g] missing n=25 (11 Europe, 4 South East Asia, 5 North America, 4 Western Pacific, 1 South America), [h] missing n=55 (24 Europe, 8 South East Asia, 13 North America, 6 Western Pacific, 4 South America), [i] missing n=24 (7 Europe, 5 South East Asia, 4 North America, 6 Western Pacific, 2 South America).

Supplementary table 4. Academic mentorship.

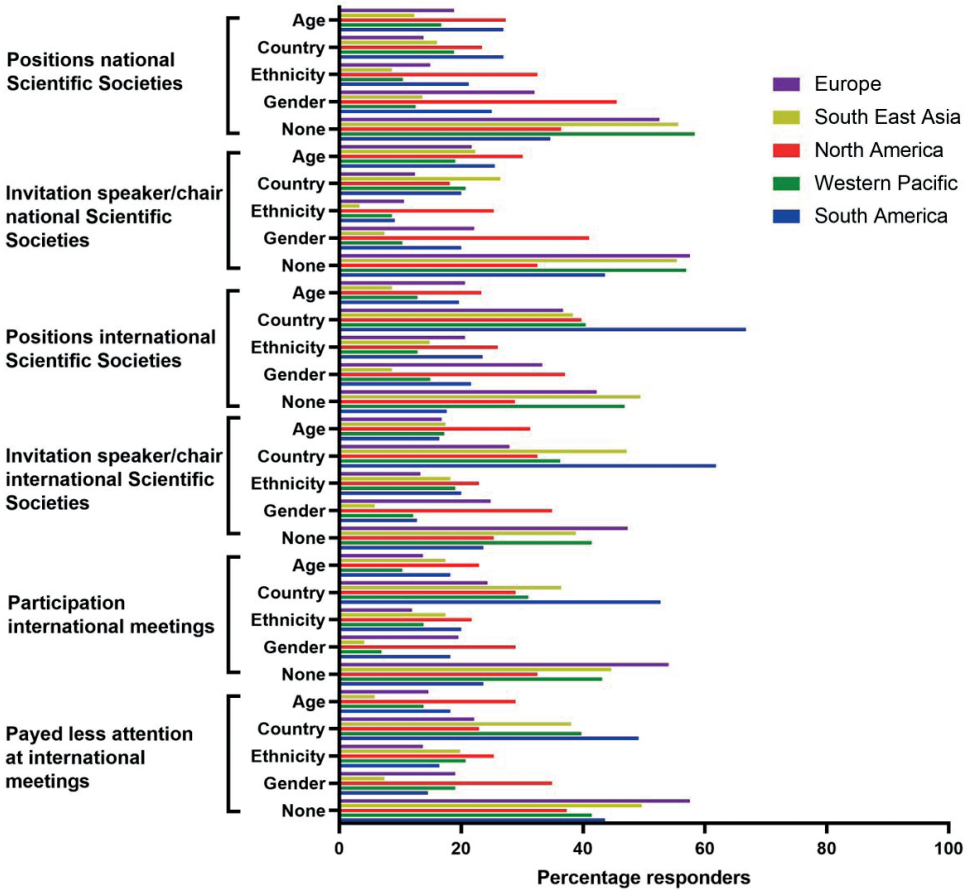
	Breakdown by gender		
	Women (n=252)	**Men (n=183)**	**Total (n=435)**
Currently or in the past mentor[a]			
Yes	211 (83.7)	165 (90.2)	376 (85.1)
Current or past mentor has			
Critiqued my scientific work[b]	121 (57.9)	92 (56.1)	213 (57.1)
Fostered my career[c]	168 (83.2)	143 (87.7)	311 (85.2)
Promoted participation in external professional activities	159 (75.4)	139 (84.2)	298 (79.3)
Facilitated participation in chairing conference	106 (50.2)	98 (59.4)	204 (54.3)
Facilitated participation in invited manuscripts	139 (65.9)	115 (69.7)	254 (67.6)
Used my work to advance their own career instead of mine	59 (28.0)	58 (35.2)	117 (31.1)
Sex of current/past mentors was			
Women	42 (19.9)	19 (11.5)	61 (16.2)
Men	87 (41.2)	109 (66.1)	196 (52.1)
Both	75 (35.5)	37 (22.4)	112 (29.8)

[a] missing n=20 (12 men, 8 women), [b] missing n=3 (1 men, 2 women), [c] missing n=11 (2 men, 9 women),



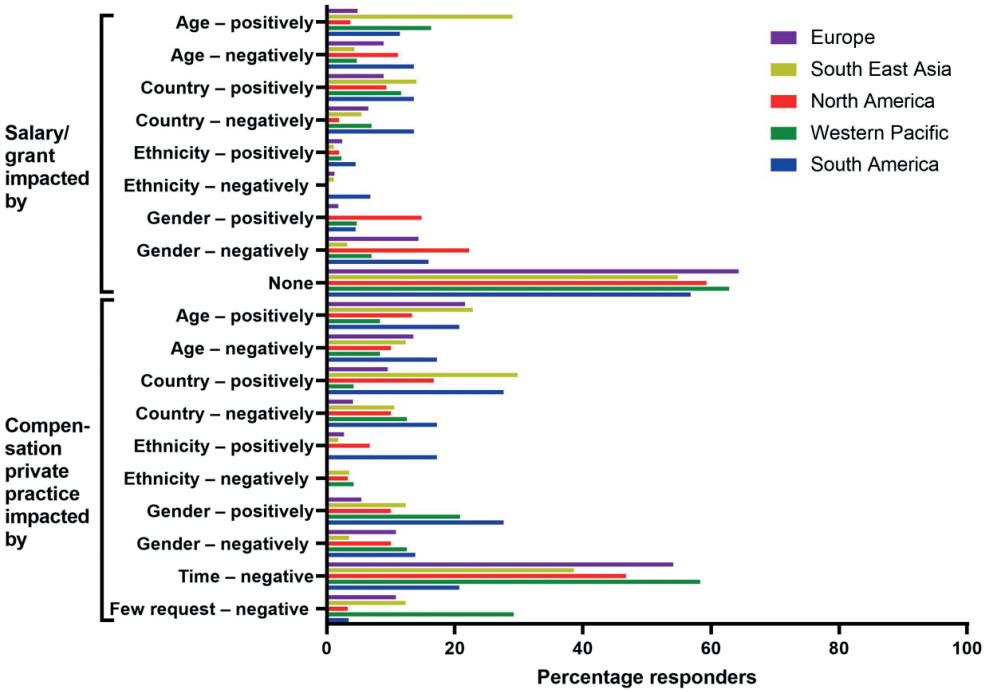
Supplementary figure 1. Barriers to a career in headache affecting professional recognition by workplace.

Country is used to indicate country of birth and/or education.



Supplementary figure 2. Barriers to a career in headache affecting opportunities in scientific societies by workplace.

Country is used to indicate country of birth and/or education.



Supplementary figure 3. Barriers to a career in headache affecting salary and compensation by workplace.

Country is used to indicate country of birth and/or education.

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CHAPTER 14

Harassment in the headache field: a global web-based cross-sectional survey

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Abstract

Background: Matters of workplace harassment are an important issue. This issue needs to be recognized and studied to prevent occurrences. These important sensitive areas of effective workplace management are increasingly gaining more interest. We aimed to identify the prevalence of workplace sexual, verbal and physical harassment among headache professionals.

Methods: We adopted a cross-sectional exploratory survey approach with quantitative design. The survey was distributed electronically among headache healthcare and research professionals globally through the International Headache Society (IHS).

Results: Data were obtained from 579 respondents (55.3% (320/579) women). A large percentage of respondents (46.6%, 270/579) had experienced harassment, specifically, 16.1% (93/578) reported sexual harassment, 40.4% (234/579) verbal harassment and 5.5% (32/579) physical harassment. Women were almost seven times more likely to experience sexual harassment compared to men (Odds ratio: 6.8 [95%CI 3.5-13.2]). While women did also more frequently report other types of harassment, this was not statistically significant (Odds ratio: 1.4 [95%CI 1.0-2.0]).

Conclusion: Lifetime exposure to workplace harassment is prevalent among headache professionals, especially in women. This study uncovers a widespread issue and calls for strategies to be implemented for building a healthy and safe workplace environment.

Introduction

Although not always recognized, healthcare as an industry is predisposed to harassment.[1-4] In some cases, this may be related to differences in economic status, prestige and power.[5-7] Not only can this problematic behavior affect victims severely,[8,9] but it may also influence patient outcomes. A consistently positive association has indeed been found between a positive workplace environment and positive patient outcome across multiple studies.[10,11]

Harassment in the workplace can take many forms, including behaviors which affect an individual's employment, cause employee discomfort, impact work environment, or interfere with work performance. Verbal harassment in the workplace includes insults, disrespectful or unpleasant jokes, name calling and unjustified and/or public criticism. Physical harassment encompasses behavior such as damaging property, threats and assaults. Sexual harassment includes requests for sexual favors, and other undesired or unsolicited verbal or physical behavior of a sexual nature.

Excluding quid pro quo cases of sexual coercion and other behavior with obviously bad intent, to a certain degree, the problem of harassment can result from differences in perception. In a proportion of cases, the perpetrator has different beliefs on the offensiveness of their behavior. What is considered innocuous by one individual is not perceived that way by another. This difference in threshold concerning acceptable behavior inevitably leads to disputes. To be deemed unlawful, harassing conduct must objectively be viewed as creating a hostile work environment; however, the perspectives of the person being harassed are still very important and should always be considered. In the American court system, often the 'reasonable person' standard is used. With this standard, in addition to the behavior being unwelcome and affecting the work environment of the victim, an objective reasonable person's perspective would also view the behavior as harassment.[12] Of course, this remains a subjective matter, which is greatly influenced by who this reasonable person is.[12,13]

Harassment is prevalent in the medical profession.[5,14-17] In academic and workplace environments in medical institutions and practices, harassment can for instance occur both in teacher–student relationships and in manager–employee relationships, involving students and trainees, medical personnel, managers, and academic faculty. Shockingly, a meta-analysis of 51 studies demonstrated that nearly 60% of medical students and trainees had experienced harassment or discrimination during their training, with female students and trainees being targeted more often than men.[16]

In an academic sample of clinician-researchers, one-third of women reported sexual harassment compared to four percent of men.[17]

Being subjected to workplace harassment has been associated with many negative effects, such as increase of depressive symptoms, lower levels of vitality, employee discomfort, lower levels of job satisfaction and sense of safety at work, higher ethical or moral distress, increased turnover intentions and suicidal behavior.[8,9,18-20]

Efforts to curtail this type of behavior are particularly challenging. Perpetrators often hold senior leadership and supervisory positions. This contributes towards the fact that people who experience work-based sexual harassment seldom file complaints or report their experiences.[21] Moreover, other factors such as stigma, fear of consequences and difficulties in proving allegations all play important roles in this.[22,23]

Discussing these behaviors in our own field is often uncomfortable. Nonetheless understanding what happens, where, when, and to whom, is essential for efforts to transform culture and to eradicate problematic behaviors. We therefore aimed to determine the prevalence and potential risk factors of workplace sexual, verbal, and physical harassment among headache professionals.

Method

Design and setting

Using a nonexperimental crosssectional exploratory survey approach with a quantitative design, we conducted a global-wide survey aimed at headache professionals, including medical doctors, other healthcare providers, and all those that are involved in the field of headache research. This study was part of a previously published effort to determine relevant career barriers for headache professionals.[24] In short, (associate) members of the International Headache Society (IHS), and delegates of the 2019 International Headache Congress (IHC) were sent the web-based survey by email. Additionally, associate national headache societies affiliated with the IHS were requested to send out the survey to their members. The survey was conducted anonymous and voluntary.

Survey development

The survey was developed in English and used previously published surveys,[25,26] and the experience of authors (IdB, AA, RHS, BB, RHJ, PPR, GMT). Consensus on the content of the questionnaire was reached by online discussion and email communication. The survey collected basic demographic information including country of birth and information on whether respondents had experienced harassment, the severity and perpetrator of harassment, and on if/how they responded as well as other information which has been analyzed and reported separately.[24] Finally, participants were asked how they responded to witnessing sexual harassment/being a bystander to sexual harassment. The web-based survey was first sent in September 2019 and closed in June 2020 after three reminders were sent to motivate partaking.

Data Analyses

Data was analyzed using SPSS 25.0 (IBM Corporation, Armonk, NY). All questionnaires were examined for invalid responses as well as inconsistencies, which then were considered missing values. Descriptive statistics were used to capture demographic data. Primary outcomes were reported as percentages. To identify risk factors for experiencing sexual harassment and other types of harassment, we conducted logistic regression analyses on the following demographic characteristics as independent variables: age at time of survey completion, gender, race/ethnicity, region of employment and marital status. Risk factors are reported with odds ratios (ORs) with associated 95% confidence intervals (CIs). Results were considered significant if $p<0.05$.

Table 1. Demographics respondents

	Breakdown by gender		
	Women (n=320)	**Men (n=259)**	**Total (n=579)**
Age[a]			
18-30	32 (10.0)	13 (5.0)	45 (7.8)
31-40	100 (31.3)	90 (34.7)	190 (32.8)
41-50	100 (31.3)	64 (24.7)	164 (28.3)
51-60	61 (19.1)	52 (20.1)	113 (19.5)
>60	27 (8.4)	37 (14.3)	64 (11.1)
Gender[b]			
Women	-	-	320 (55.3)
Men			259 (44.7)
Geographic location – birth[c]			
Africa	5 (1.6)	18 (6.9)	23 (4.0)
Eastern Mediterranean	11 (3.4)	8 (3.1)	19 (3.3)
Europe	147 (45.9)	82 (31.7)	229 (39.6)
North America	42 (13.1)	36 (13.9)	78 (13.5)
South America	33 (10.3)	22 (8.5)	55 (9.5)
South East Asia	55 (17.2)	71 (27.4)	126 (21.8)
Western Pacific	27 (8.4)	22 (8.5)	49 (8.5)
Geographic location – workplace			
Africa	1 (0.3)	17 (6.6)	18 (3.1)
Eastern Mediterranean	11 (3.4)	7 (2.7)	18 (3.1)
Europe	145 (45.3)	81 (31.3)	226 (39.0)
North America	46 (14.4)	37 (14.3)	83 (14.3)
South America	34 (10.6)	21 (8.1)	55 (9.5)
South East Asia	51 (15.9)	70 (27.0)	121 (20.9)
Western Pacific	32 (10.0)	26 (10.0)	58 (10.0)
Ethnicity			
Asian/Pacific islander	67 (20.9)	88 (34.0)	155 (26.8)
Black/African American	0 (0)	14 (5.4)	14 (2.4)
Hispanic	12 (3.8)	11 (4.1)	23 (4.0)
White/Caucasian	215 (67.2)	125 (48.3)	340 (58.8)
Multiple ethnicities	13 (4.1)	2 (0.3)	15 (2.6)
Other	13 (4.1)	19 (7.3)	32 (5.5)
Marital status			
Divorced/separated	21 (6.6)	7 (2.7)	28 (4.8)
Married/long-term relationship	248 (77.5)	231 (89.2)	479 (82.7)
Single	46 (14.4)	20 (7.7)	66 (11.4)
Widow(er)	5 (1.6)	1 (0.4)	6 (1.0)
Children			
Yes	211 (65.9)	205 (79.2)	416 (71.8)
Participation in clinical activities			
Yes	305 (95.3)	251 (96.9)	556 (96.0)
Participation in research activities			
Yes	264 (82.5)	216 (83.4)	480 (82.9)
Participation in academic career			
Yes	219 (68.4)	157 (60.6)	376 (64.9)
No longer	41 (12.8)	38 (14.7)	79 (13.6)
Never	60 (18.8)	64 (24.7)	124 (21.4)

[a] Three participants preferred not to disclose their age. [b] One participant reported a non-binary gender identity. As results are separately reported based on gender, we hat to exclude this participant to ensure anonymity. [c] Participants with difference in location birth and workplace: n=38 (6.6%).

Results

Participant demographics

A total of 580 participants completed the survey. One respondent identified with the non-binary gender identity. To ensure anonymity, this participant was removed from subsequent analyses. Just over half of participants were women (55.3% 320/579). The highest percentage of responders was from Europe (39.6% 229/579), the second most represented region was South East Asia (21.8% 126/579) followed by North America (13.5% 78/579). Only a minority, 4.0% (23/579) and 3.3% (19/579) were born in African and Eastern Mediterranean countries, respectively. In total 556 (96.0%) of the respondents were healthcare providers and 480/579 (82.9%) participated in research. Moreover, the majority of respondents participated in both clinical and research activities (79.1% 458/579). The majority of respondents have or had an academic career path (78,5% 455/579). The majority of participants were in a relationship (82.7% 479/579), and had children (71.8% 416/579). Additional respondents' characteristics can be found Table 1. Almost half of respondents indicated that they had experienced harrasment in the workplace (46.6% 270/579). For participants who considered themselves healthcare providers this was 46.6% (259/556), while for those participating in research or academics it was 41.0% (197/480) and 46.8% (213/455) respectively.

Prevalence, severity and perpetrators of sexual harassment

Overall, the prevalence of sexual harassment was 16.1% (93/578) (Figure 1A). In women, the prevalence of sexual harassment was 25.6% (82/320), compared to 4.3% (11/258) in men. For participants working in healthcare, research, and academics the prevalence of sexual harassment was similar, 15.9% (88/555), 14.8% (71/479), and 15.6% (71/454) respectively. Most frequently harassment was subjectively characterized as mild (62.4% 58/93). Nevertheless, 8.6% (8/93) of respondents indicated the harassment as severe. Of note, 18.2% (2/11) of men reported sexual harassment they were subjected to as severe (Figure 1B). The most frequent reported perpetrator was a boss, supervisor, attending or another senior employer (68.8% 64/93), followed by patients (39.8% 37/93) and colleagues (29.0% 27/93). Respondents indicated sexual harassment by subordinates in 8.6% (8/93) of the time and in 2.2% (2/93) the victim did not know the harasser.

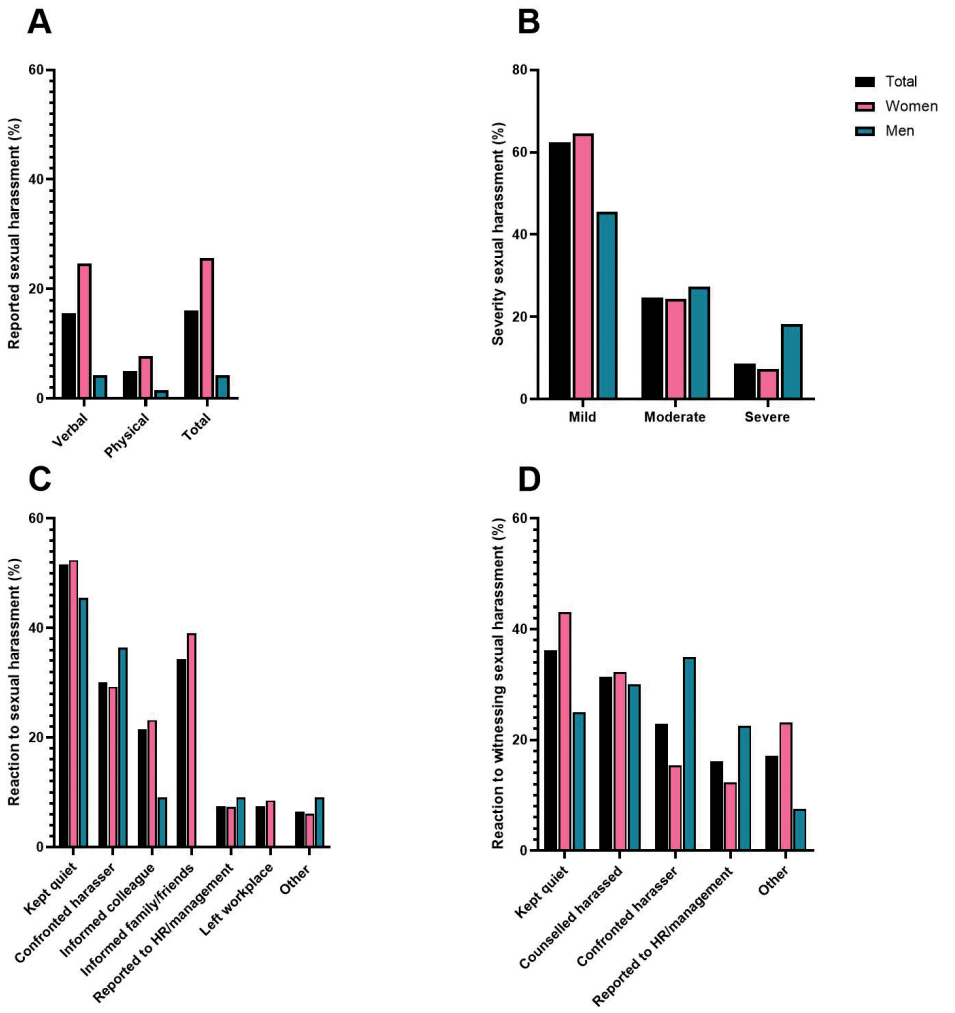

Figure 1. Reported sexual harassment in a work-based setting.

In all questions, multiple responses were allowed. Proportion of respondents reporting at least one experience of work-based sexual harassment (A). Severity of the reported sexual harassment defined as mild, moderate or severe (B). Individuals' reaction to the occurrence of the sexual harassment affecting them in a work-based setting (C). Reaction to witnessing sexual harassment in a work-based setting (D).

Reporting of sexual harassment and bystander behaviour

After incidences of sexual harassment, 51.6% (48/93) of respondents did not report or share information about the incident and only 7.5% (7/93) reported the harassment to human resources or management (Figure 1C). Of all respondents, 18.1% (105/579) indicated that they witnessed sexual harassment in the workplace. Interestingly, when sexual harassment was witnessed, 16.2% (17/105) of respondents indicated that

they reported this to human resources while 36.2% (38/105) kept quiet (Figure 1D). Interestingly, victims reported more frequently that they confronted the harasser compared with what bystanders reported, 30.1% (28/93) vs. 22.9% (24/105) (Figure 1C and 1D).

Risk factors for sexual harassment

Women were more likely than men to have experienced sexual harassment (OR 6.8 [95%CI 3.5-13.2]) (Table 2). Being Caucasian was also associated with an increased risk of experiencing sexual harassment (OR 2.8 [95%CI 1.4-5.6]). Additionally, respondents that were single were more likely to experience sexual harassment (OR 2.3 [95%CI 1.2-4.7]).

Table 2. Multiple logistic regression analysis of risk factors for sexual harassment.

	OR	95% CI	p-value
Age at time of survey completion			
18-30	Ref		
31-40	1.4	0.6 – 3.5	0.45
41-50	1.3	0.5 – 3.4	0.61
51-60	2.5	0.9 – 6.7	0.07
>60	1.2	0.4 – 3.8	0.75
Gender			
Male	Ref		
Female	6.8	3.5-13.2	<0.001
Race/ethnicity			
Non-white/Caucasian	Ref		
White/Caucasian	2.8	1.4 – 5.6	0.003
Marital status			
Married	Ref		
Single	2.3	1.2 – 4.7	0.016
Other	0.4	0.1 – 1.3	0.14
Region of employment			
Other regions Europe	Ref		
and North America	1.5	0.8 – 2.7	0.20

OR: Odds ratio, CI: Confidence interval.

Prevalence and severity of other verbal and physical harassment

Among all headache professionals, the prevalence of verbal harassment was 40.4% (234/579). The prevalence of physical harassment was 5.5% (32/579) (Figure 2A). While harassment was mostly indicated by the respondent to be mild (40.1% 95/237) or moderate (33.3% 79/237), almost one out of ten reports it indicated as severe (9.7% 23/237) (Figure 2B).

Risk factors for other verbal and physical harassment

While women appeared to experience also other verbal and physical harassment more frequently compared with men, this was not statistically significant (OR 1.4 [95%CI 1.0-2.0]) (Table 3). Moreover, no other significant risk factors were identified.

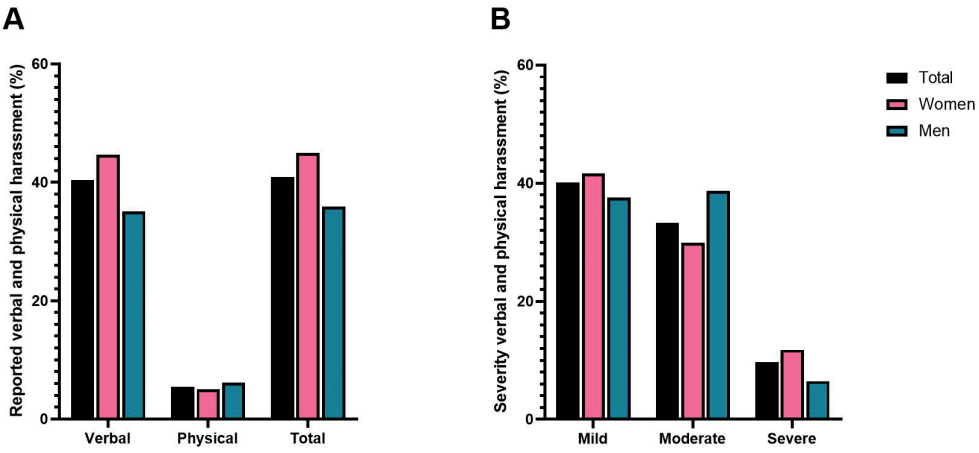

Figure 2. Reported verbal and physical harassment in a work-based setting.

In all questions, multiple responses were allowed. Proportion of respondents reporting at least one experience of work-based harassment that was not sexual in nature (A). Severity of the reported harassment defined as mild, moderate or severe (B).

Table 3. Multiple logistic regression analysis of risk factors for verbal or physical harassment that was not sexual in nature.

	OR	95% CI	p-value
Age at time of survey completion			
18-30	Ref		
31-40	0.9	0.5 – 1.8	0.80
41-50	0.6	0.3 – 1.2	0.13
51-60	0.7	0.3 – 1.4	0.29
>60	1.0	0.4 – 2.3	0.99
Gender			
Male	Ref		
Female	1.4	1.0 – 2.0	0.06
Race/ethnicity			
Non-white/Caucasian	Ref		
White/Caucasian	1.5	0.9 – 2.3	0.10

Marital status			
Married	Ref		
Single	0.9	0.5 – 1.5	0.60
Other	1.0	0.5 – 2.0	0.90
Region of employment			
Other regions Europe and North America	Ref 1.1	0.7 – 1.7	0.70

OR: Odds ratio, CI: Confidence interval.

Discussion

In this survey, workplace harassment emerges as a common incident in the headache field, with almost half of respondents experiencing one or more type of harassment. Weak governance, and an underappreciation of the impact of the misconduct, can all contribute towards an environment in which conductors are not held accountable. As the global demand for equity within the workplace intensifies, it is also crucial to explore the possibility of sexual harassment as a contributor to enduring inequities, and its impact on career progression. We need to understand its occurrence to be able to enact advantageous interventions. We found a high prevalence (16.1%) of sexual harassment in the global headache field. Women were seven times more likely than men to experience sexual harassment. Sexual harassment is a deprivation of fundamental rights, as well as unprofessional and illegal, and should be treated as such. We also found a high prevalence of other types of harassment (40.4%). We could not identify specific risk factors for this type of harassment.

Almost 40% of respondents that experienced harassment indicated to have been harassed by patients. Harassment perpetrated by a patient or their family is a serious problem that the healthcare system has been experiencing for quite some time.[27-30] Physicians report inadequate training in dealing with sexual harassment by patients and there are barriers for reporting incidents or even discussing this with colleagues or supervisors. We need ways to better address patients' inappropriate behaviors towards healthcare providers.[28] Employees should be attended to institutional policies and patients should be educated on behavioral standards. For an overview of suggested recommendations to prevent and address harassment see Table 4. Suggested recommendations could help foster a culture in which headache professionals feel more prepared to address sexual harassment and where they are prepared to prioritize their own safety and wellbeing. However and importantly, the majority of perpetrators were not patients. More than two-third of the respondents that experienced sexual harassment indicated to have been harassed by their boss or

superior. This is especially shocking as we know that perpetrators accused of sexual harassment are rarely held to account. A United States based study of 125 faculty members in biomedical and medicine who had been accused of sexual misconduct and received media attention (affecting at least 1668 individuals) showed that as many as 50 remained in academia, of whom 40% held positions at a different institution.[27] As these are cases in which victims reported behavior and there was media attention, this is likely the tip of the iceberg. We need to consider that several factors might impact an employers' response to complaints of sexual harassment. Fear of defamation claims is one of those. Academic employers might find it easier to let harassers leave, resign, or limit their contact with specific groups, in the hope of limiting public scrutiny and reputational damage. In this we need to recognize that human resource departments and other individuals involved often represent the institution and not the individual reporting the harassment. As such, they have other incentives influencing how they respond to the report. This of course only becomes an issue when harassment is reported.

We need to recognize that reporting workplace harassment is incredibly important, but also exceedingly difficult. Often extreme power asymmetries are at work. This means that speaking out and trying to enact change within the profession is extremely hard. Frequently reported reasons for underreporting also include belief that no action will be undertaken, fear of consequences and negative impact on own career, stigma, fear of worsening the situation, and not knowing how to report.[22,23,28] Another contributing factor is the lack of a clear definition. While some behaviors are obviously harassment (e.g., inappropriate unwanted physical contact), many behaviors are more subtle. This lack of a clear definition has been a barrier for reporting by individuals experiencing sexual harassment.[29] It is important to realize that some of these unwanted behaviors have been "normalized". Women in particular may not only downplay harassment, but may feel shame, guilt or stigma themselves and also second guess their own feelings and actions.[29] Moreover, the lack of 'evidence' or witnesses may stop victims from reporting harassment, as they might fear a 'their word against mine' situation. In our cohort, more than half of respondents kept quiet after harassment, while more than one-third of witnesses/bystanders did the same. Shockingly, 7.5% of victims felt compelled to leave their workplace. Less than 10% sought assistance from institutional resources to address the harassment. The low utilization rate is very worrisome. It is vital that we create an environment in which victims feel safe and empowered to report harassment. Healthcare and research institutions need policies clearly underlining the unacceptability of these behaviors. They also need to facilitate an appropriate investigation and protection for those who report harassment. They need to ensure accountability and transparent

responses to unprofessional behaviors, including sexual harassment. They should work on fostering a safe and supportive environment that can encourage witnesses to come forward and victims to report incidents of harassment. The bare minimum that all organizations and societies can do is to ask potential hires or committee candidates whether they have violated harassment or any ethics policies or if they left during active investigations. They should demand permission to gather information about such behaviors, investigations, warnings or suspensions from previous employers.[30]

In addition to the high prevalence of sexual harassment, our data demonstrate that nearly half of headache professionals experience harassment that was not of a sexual nature. Unfortunately, a hostile work environment is frequently reported across different medical and healthcare disciplines.[16,23,26,31] Nonetheless, these data come at a crucial timepoint, as harassment and the 'toxic hierarchical' culture in medicine are being openly challenged. Our findings indicate the need for a culture change and raise a call to action. A complicating factor is that what may work in one country and culture, may not be relevant elsewhere. The effectiveness of proposed strategies needs to be meticulously evaluated in each environment and results should be communicated.[32]

The limitations of the present study include the absence of a response rate. We prioritized giving all headache professionals the chance to participate over calculating a response rate. We therefore allowed national societies and representatives to send out the survey and could as such not calculate a response rate. Additionally, it is important to acknowledge that, due to limitations in our outreach efforts, we were only able to reach out to IHS (associate) members, IHC delegates, and affiliated headache societies. Therefore, we recognize that not all headache professionals may have had the opportunity to participate. Moreover, a possible language barrier, outdated contact information or institutional blockage of emails may have potentially prevented part of the headache professionals from responding. Finally, while we contacted all IHS affiliated national societies (full list to be accessed from the IHS website https://ihs-headache.org/en/about-ihs/affiliate-member-societies/), we were unable to determine which representatives actually circulated the survey, as this was on a voluntary basis. While unfortunate, we still consider this an adequate first approach for this sensitive topic. To prevent responder bias we strongly emphasized in our correspondence with prospective respondents that the survey was aimed at all headache professionals. Nonetheless, we cannot exclude that those respondents that strongly emphasized with this topic or the topic of our first manuscript (gender bias and challenges in career achievement) or who had experienced harassment were more likely to participate. While an important result on its own, several ethnic groups were underrepresented, which made it impossible to evaluate to which

extent different ethnicities are a risk factor for sexual harassment. As such, while our finding of a reduced risk of experiencing sexual harassment for non-Caucasian respondents is interesting, this should be interpreted with caution. Moreover, we had an underrepresentation of individuals from African and Eastern Mediterranean countries. In addition, we did not collect data on age, job position, years of experience and workplace culture at the time of harassment incident(s). These may be important predictor variables if we had those data and should be included in future research projects. Furthermore, delving deeper into the circumstances surrounding incidents of harassment is likely to yield crucial insights into this challenging matter. To further investigate this issue, it is important to conduct a more comprehensive analysis to determine the specific types of harassment that occur (such as verbal harassment encompassing bullying, name-calling, criticism). Additionally, it is important to examine the severity and duration of the harassment (whether it is a single occurrence, repetitive behavior by one individual, or involves multiple perpetrators) and its impact (such as increased employee absenteeism, heightened anxiety, diminished self-confidence). Also, determining the role of workplace culture, the organizational culture and leadership, both on the occurrence and attitude towards harassment is likewise important. Finally, we need to consider that recall bias and social desirability bias might have prevented victims from reporting harassment in our survey. By ensuring anonymity we hoped to prevent the second. If these biases occurred, this likely led to an underestimation of the prevalence of harassment. Likewise, individuals that experience severe or traumatic incidents might have been less likely to participate, ensuring a further underestimation of the issue. Another important matter that should be recognized is that we determined how many professionals had dealt with harassment. We did not examine how often victims were harassed. This likely leads to a further underestimation of the problem.

The following approaches, several which were previously discussed in more detail, might significantly reduce (sexual) harassment in the workplace.[2] Professionals and organizations should strive towards creating a diverse, inclusive, and respectful environment. Institutions should focus on diffusing the power structure, on reducing isolation, on improving transparency and accountability and on having working available supportive structures. If no working supportive structures are available, a system should be developed, afterwards, its effectiveness should be evaluated. Furthermore, they should ensure diverse and accountable leadership that is clear about its dedication to reducing and eliminating all harassment. Institutions and leaders should clearly convey that reporting (sexual) harassment is honorable and courageous. Finally, effective sexual harassment training should be given. Harassment training should not be given out of 'symbolic compliance', as to often its primary aim is to protect from liability.

The effectiveness of training aimed at preventing sexual harassment is often not evaluated. Many training programs are aimed at providing information (for instance about anti-harassment policies) and on changing attitudes.[2,33] Research, however sparsely, has often demonstrated that a person's believes and attitude are very resistant to change. It is important to realize that thoughts are in the end not harmful. The actions and behaviors of a person lead to harm of the victim. Given this, targeting behavior (even amongst those with sexist attitudes and those that do not see the harmful nature of their actions) seems better justified.[2] Another important type of training is bystander intervention training.[2,33] Bystander intervention training has long been used, especially in colleges and high schools. It focuses on creating awareness (recognizing problematic behavior), creating a sense of collective responsibility, providing bystanders with the skills and confidence needed to intervene (direct or indirect) and on educating where available resources can be found. Simply put, bystander education equips bystanders with the skills necessary to recognize and take actions when problematic behavior occurs.[33] Evaluating the effectiveness of these trainings is important and one should realize that what is helpful in one setting, might not necessarily be part of the solution in another setting.

We are not the only medical field facing these challenges. Among emergency medicine residents, mistreatment was reported to occur in 29.9% a few times per year.[34] While in the vascular workplace, with a vast majority working in vascular surgery, the 43% of responders indicated bullying, undermining behavior or harassment to have occurred in the last 12 months.[35] Another example comes from the field of cardiology. Here, 44% of responders indicated that the worked in a hostile work environment.[31] While these types of studies are difficult to compare due to different endpoints and differences in study population, they do indicate that this issue is widespread in the medical community and that we might learn from one another when facing these challenges.

The prevalence of sexual harassment and other harassment is high in the headache field and women are more likely to experience sexual harassment than men. Understanding mistreatment within our professional field is an important first step in creating and maintaining a safe work environment. Our leadership can help create a culture in which everyone can speak out when perceiving or experiencing harassment. Our efforts might provoke other specialties and societies to also put this issue on their agendas. Moreover, our results may provide the impetus for headache societies, headache research groups and hospitals to develop action plans and to re-examine and enforce policies addressing these damaging behaviors. We owe it to our (future) colleagues, ourselves, and our patients that we all strive towards implementing such policies.

Key findings:

- Workplace harassment is common in the headache field, with almost half of respondents experiencing some type of harassment.
- Sexual harassment was reported by 16% of respondents, and women are seven times more likely to experience this.
- The Women's Leadership Forum of the IHS recommends anti-sexual harassment training programs that focus on changing behavior, not on changing beliefs.

Table 4. Recommendations for preventing and/or dealing with incidents of harassment.

Target of recommendations	Recommendations
Head of department/ Principle investigator/ Supervisor	- Review institutional policies with employees. - Remind employees that experiencing harassment does not reflect on work performance. - Support employees after an incident. - Present the topic to leadership to increase awareness. - Normalize harassment, also sexual harassment, as a topic for supervision. - Be mindful of power dynamics when discussing sensitive issues with employees. - Instruct employees to seek safety if they do not feel safe. - When an incident occurs, first ensure the victims safety. - Carefully take on a non-judgmental stance to help victims and alleged perpetrators.
Hospitals/ Organizations/ Institutions	- Create clear, visible, usable policies for reporting and dealing with harassment. - Create harassment reporting procedures that are clear, visible, usable, and not overly time-consuming - Create harassment procedures where victims are protected. - Review policies often to ensure they are up-to-date. Collect feedback from employees. - Educate professionals, students and patients about behavioral standards. - Provide training on harassment, power differentials and implicit bias. - Train supervisors/leaders on the importance of addressing harassment. - Provide bystander intervention training. These equip bystanders with the skills necessary to recognize and take actions when problematic behavior occurs. - Healthcare providers should be able to transfer patients after incidents or if they do not feel safe. - Phone numbers for security, supervisors, colleagues and the human relations office should be easily accessible. - Results of reported incidents should be transparent. Methods to prevent future incidents should also be reported. - Create taskforces/working groups to address harassment and collect employee feedback. - Ensure diverse and accountable leadership that is clearly dedicated to reducing and eliminating harassment.
Individual leaders/ leadership of institutions/societies	- Clearly convey that reporting harassment is honorable and courageous. - Strive towards creating a diverse, inclusive and respectful environment - Ask potential hires whether they have violated harassment or any ethics policies. - Build a safe culture where harassment is addressed and taken seriously. - Increase awareness and advocate addressing systemic issues. - Encourage team debriefing and (personal) reflection after an incident. - Diffuse power structure and reduce isolation of employees.

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Conflict of Interest

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Research ethics and patient consent:

All responses were kept anonymous and no personal identifying information was collected. No ethical approval was required.

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CHAPTER 15

General discussion

In this thesis migraine and monogenic small vessel diseases, where migraine can manifest as a symptom, were investigated. The aim of **Part I** was to gain a better understanding of the common forms of migraine by (1) evaluating our understanding of the genetics of migraine with aura, a topic that has long remained elusive, (2) performing an untargeted epigenome-wide association study to understand response-to-treatment in chronic migraine with medication overuse (headache), (3) evaluating prevalent neurological and psychiatric comorbidities and the genetic architecture they share with migraine, and (4) to what extent patients with hemiplegic migraine and vascular monogenic migraine syndromes respond to acute and preventive migraine treatment. Findings of the studies and how they relate to the headache field are discussed. **Part II** of this thesis describes studies performed in vascular monogenic migraine syndromes. The main objective was to discover new treatment targets and biomarkers for the disorders, which are urgently needed. Here the findings of the studies and how they are related to other small vessel diseases are discussed. In **Part III** it was determined what challenges physicians and researchers are experiencing in the headache field, as well as the frequency of workplace harassment in this field. The final goal of this section of the thesis was to address inequalities in this field of research. Finally, potential avenues for future research are examined.

Part I: Migraine – insights into genetics, epigenetics, comorbidities, and monogenic factors

Migraine is a highly prevalent neurovascular disorder marked by episodes of intense, usually one-sided, headache accompanied by symptoms like nausea, vomiting, and sensitivity to light and/or sound.[1] In as many as one-third of patients, migraine attacks are accompanied by an aura, which involves temporary focal neurological symptoms, typically visual disturbances, although sensory symptoms, speech disturbances or motor weakness may also occur.[1] If motor weakness occurs, the disease is diagnosed as hemiplegic migraine. The probable mechanism behind the aura symptoms is cortical spreading depolarization (CSD), a transient wave of neuronal and glial depolarization that gradually spreads across the cerebral cortex, starting in the occipital (visual) cortex. The wave of excitation is followed by a prolonged suppression of brain activity.[2-4] Genetics plays a large role in migraine susceptibility. The common polygenic forms of migraine are thought to arise from the cumulative impact of multiple DNA variants, each with small to modest effect, combined with the impact of environmental factors. Conversely, in rare monogenic migraine syndromes only a single DNA mutation is sufficient to cause disease.

Genetic and Epigenetic architecture of migraine

As hypothesized in **Chapter 2**, by increasing the cohort size to over 100,000 patients with migraine (and an even larger number of control individuals), the 2022 migraine GWAS identified 123 genetic variants, all with small effect sizes.[5] Moreover, it was demonstrated that when the 123 lead index single nucleotide polymorphisms (SNPs) identified were evaluated in clinically well-phenotyped cohorts, three risk variants (in *CACNA1A*, *HMOX2* and *MPPED2*) seemed specific for migraine with aura, two specific for migraine without aura (near *FECH* and near *SPINK2*), and nine associated with both migraine subtypes (i.e., *DLST*, *FHL5*, *LRP1*, *MRVI1*, *PLEC1*, *PRMD16*, *SUGCT*, near *FGF6*, and near *TRPM8*).[5] The detection of risk variants linked to *CACNA1A* for migraine with aura is especially interesting as mutations in this gene are known to cause hemiplegic migraine.[1]

The contribution of the epigenome to chronic migraine and medication overuse, evaluated in **Chapter 3**, identified DNA methylation differences that associated with response-to-medication withdrawal in patients with chronic migraine and overuse of acute (headache) pain medication using a longitudinal epigenome-wide association approach. A change in DNA methylation in intron of *HDAC4* was found associated with monthly headache day (MHD) response, while baseline DNA methylation levels in *MARK3* associated with monthly migraine day (MMD) response. Together with co-repressors, such as *MEF2D* (previously reported migraine as a locus in a migraine GWAS)[5], *HDAC4*, a Class IIa histone deacetylase, targets lysine residues on core histone tails to repress transcription. HDAC4 is an important contributor to synaptic plasticity[6,7] and modifies the expression and release of neuroinflammation markers, including HMGB1 and NF-κB,[8,9] that are both suggested to be involved in the pathophysiology of migraine.[10] Additionally, differentially methylated CpG sites were also detected in the genes *HDAC1* and *HDAC3*, albeit showing only nominal association with reduced headache and migraine days ($p < 0.05$). As such, it thus seems that even non-specific HDAC inhibitors might serve as useful therapeutics in migraine. One such HDAC inhibitor, valproate acid, is already used in clinical practice as preventive for migraine attacks.[11] *MARK3*, encoding microtubule affinity regulating kinase 3, is also known as *CTAK1*. MARKs are serine/threonine kinases that control various cellular processes, such as cell polarity, cell cycle advancement, glucose metabolism, and cytoskeletal activity.[12,13] MARK3 and other MARKs also regulate TRESK (TWIK-related spinal cord K^+ channel, *KCNK18*) a major potassium channel.[14] Mutations in the *TRESK* gene have been shown to cause hyperexcitability of trigeminal ganglion neurons.[15] It also has been suggested that mutations in *TRESK* may cause migraine with aura in an autosomal dominant manner, but evidence supporting this claim is sparce and debatable, as mentioned in **Chapter 2**. Remarkably, downstream substrates of MARK3 include

several HDACs, among which HDAC4.[16] Therefore, it was suggested that MARK3 might influence migraine chronification through regulation of these HDACs. Taking all this epigenetic findings together, it indicates the importance of pathways related to chromatin structure and synaptic plasticity in migraine chronification and its reversibility. The possibility of involvement of synaptic plasticity was also previously suggested by a small methylation study that researched headache chronification.[17] Although the findings of that study did not reach statistical significance, the strongest associated CpG sites were related to *SH2D5* and *NPTX2*, two genes involved in synaptic plasticity.

Comorbidities of migraine and the role of genetics

The term comorbidity refers to two or more diseases co-occurring in the same individual, either subsequently or at the same time. When addressing comorbidities of migraine, diseases are referred to whose prevalence is elevated among patients with migraine compared to the prevalence among healthy controls. In **Chapter 4**, four of the most prevalent neurological and psychiatric disorders, namely mood disorders, stroke, epilepsy, and sleep disorders, are discussed. Understanding comorbidities can be vital for clinical care. Additionally, studying comorbidities can provide valuable insights into the pathophysiology of the diseases involved.

Comorbidities may arise from shared underlying pathophysiological mechanisms. By studying the co-occurrence of certain diseases, common pathways, genetic factors, molecular mechanisms, and physiological processes can be identified that contribute to the development and progression of both conditions. Furthermore, compounding effects might be identified, where comorbidities exacerbate pathophysiological processes of one another.

Genetic research and comorbidities: lessons learned

By estimating their genetic correlation using GWAS data, shared genetic architecture between migraine and other disorders was investigated. Numerous studies utilized GWAS data to evaluate the presence of shared genetic predisposition between migraine and various disorders, including psychiatric disorders[18-20], sleep disorders,[21] ischemic stroke (as well as stroke outcomes),[22,23] hypertension,[24] and endometriosis.[25] By assessing causal relationships between two conditions, using genetic variants as proxies for exposure, a method known as Mendelian Randomization, it becomes possible to investigate whether disorders are causally linked. Mendelian randomization is increasingly used in migraine research. Several examples include the relationships between migraine and stroke,[23,26-28] hypertension,[24,29] endometriosis[25] and sleep disorders,[21] but the results need to be interpreted with caution. For instance

the causal link between migraine and stroke has been examined in three studies, with two of them failing to substantiate a causal association between both disorders,[26,27] while the third demonstrated a protective influence of large artery stroke on migraine.[28] Potentially clinically relevant is also the finding that using Mendelian Randomization, migraine appeared associated with a poor functional outcome after ischemic stroke.[23] While, migraine might not be casually related to stroke, they do share risk factors or a common disease pathway. For example, susceptibility to cortical spreading depolarization might play a role altering cerebral blood flow causing brain tissue (in migraine and stroke) to be more sensitive to supply-demand mismatch.[30] Nevertheless, clinical studies investigating this hypothesis have not shown consistent results.[31-34] To mention another example; despite a genetic correlation, no causal relationship was found between migraine and endometriosis.[25] A possibly shared mechanism for migraine, endometriosis and stroke is endothelial dysfunction. Endothelial dysfunction has been implicated in migraine and altered cerebrovascular function has been demonstrated.[35,36] Functional studies have shown conflicting results, possible due to age effects, differences in migraine subtypes and/or the research modality used in the study.[36,37] Interestingly, Mendelian Randomization showed that an increase in blood pressure led to an increased migraine risk.[24,29] The mechanism for this remains, however, unknown, but endothelium dysfunction seems a likely candidate.[38]

As discussed in **Chapter 4**, there is a bidirectional comorbidity between migraine and major depression disorder. Recently, using bivariate causal mixture model analysis, migraine variants were demonstrated to influence depression and schizophrenia, suggesting that these variants might affect susceptibility to multiple brain disorders.[20] Furthermore, Mendelian randomization supported a causal relationship between major depression disorder and an increased risk of migraine, while finding no evidence for the reverse relationship being causal.[39] While these findings still need to be replicated, they underscore the importance of monitoring migraine symptoms in people with depression and vice versa. Notably, Mendelian Randomization can only be applied to prove causality between exposure and outcome (disease) when the studied SNPs (exposure) fulfill three assumptions. The first assumption is that the SNPs (instrumental variables) are associated with the exposure; the second assumption is that the SNPs are not associated with potential confounders; and the third assumption is that the SNPs are independent of the outcome (migraine), meaning they are not implicated in both exposure and outcome. If the SNPS are implicated in both exposure and outcome, this is called horizontal pleiotropy. Violation of the assumptions can lead to biased estimates and incorrect conclusions. It is also crucial to acknowledge that the power of a Mendelian Randomization study relies on the

scale of the initial GWAS and the strength of the instrumental variables. Replication of Mendelian Randomization findings after expanding GWAS size is therefore required.

Clinical implication of migraine genetics

While monogenetic disorders have contributed greatly to elucidating the pathophysiology of migraine (**Chapter 2**), to date little evidence is available to guide treatment decisions in patients. In **Chapter 5**, we aimed to determine which preventive migraine treatments are most successful for patients with hemiplegic migraine, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), and retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations (RVCL-S). For treating migraine in patients with CADASIL, valproic acid showed the highest efficacy. It is important to note that in our patient cohort none of the patients were treated with acetazolamide. Other reports indicated that this might also be effective in treating migraine in CADASIL.[40-43] Additionally, the fact that beta blockers ranked among the most frequently prescribed preventive treatments not only aligns with current general migraine guidelines but also highlights the importance of studying this type of medication in rare migraine disorders, such as CADASIL, where beta blockers appear to be less effective. This is in line with the only systematic review of migraine treatment in CADASIL that indicated that beta blocker treatment was associated with unfavorable responses.[44] Alternative preventive treatment should, therefore, be considered as first-line treatment.

If over-the-counter analgesics failed to provide sufficient relief from migraine headaches, triptans are the next step in the acute treatment of migraine. Triptans are agonists for the 5-HT1B/1D receptors, leading to vasoconstriction and inhibition of neuropeptide secretion. However, for patients with a history of stroke or transient ischemic attack (TIA), as well as those with a history of hemiplegic or basilar migraine, triptans are considered contra-indicated according to current guidelines. Findings in **Chapter 5**, in conjunction with existing literature,[45-47] suggest that most patients respond favorably to triptans with minimal side effects, advocating that offering this treatment to these patients is justified. While triptans appear to be safe, compounds that inactivate the calcitonin gene-related peptide (CGRP) pathway should be used with caution in vascular monogenic migraine syndromes, as CGRP is involved in cardiovascular homeostasis under (patho)physiological conditions. Therefore, physicians should refrain from starting treatment with CGRP blocking agents until long-term vascular safety is proven, as discussed in **Chapter 6**.

In **Chapter 3**, an association was found between DNA methylation status and treatment success of withdrawal in patients with chronic migraine with medication overuse (headache). Epigenetic information can potentially be used to guide treatment decisions. First steps that need to be undertaken are to demonstrate predictive value and clinically relevant outcomes differences based on epigenetic information. Hence, more studies are needed before (epi)genetic testing can be of added value in clinical practice for common forms of migraine. More important, elucidating biological pathways through (epi)genetics can help to identify novel therapeutic targets. Examples of a recent proof-of-principle are two risk loci discovered in the latest migraine GWAS that are related to genes encoding for already known new migraine treatment targets (i.e., calcitonin gene-related peptide (*CALCA/CALCB*) and serotonin 1F receptor (*HTR1F*)).[5] Furthermore, approximately two-thirds of FDA-approved drugs in 2021 are indicated to have targets supported by genetic evidence demonstrating an association with the target and the disease necessitating treatment (or a closely associated phenotype).[48] This clearly demonstrates the potential of genetic studies to identify new treatment targets, which remains essential given that many patients do not respond to currently available treatments.

Part II: Monogenic small vessel diseases – understanding vascular migraine models

Patients affected by several monogenic cerebral small vessel diseases frequently also suffer from migraine (with aura). One of such diseases is RVCL-S, where both migraine with (and without) aura co-occur.[49,50] RVCL-S is caused by truncating mutations in the carboxyl terminal region of the *TREX1* gene.[51] First signs of disease occur from approximately 35 years onwards and patients are afflicted not only with retinal vasculopathy, but also focal neurological complaints, cognitive decline, anemia, impaired liver and renal function, and hypertension.[49-51] Interestingly, in RVCL-S, migraine onset appears later in life (>40 years), while in other cerebral small vessel diseases, such as CADASIL and Dutch-type hereditary cerebral amyloid angiopathy (D-CAA), migraine often is the first presenting disease symptom.[50,52-54] This implies that migraine may be a secondary phenomenon resulting from progressive cerebral vasculopathy in RVCL-S, whereas in the other cerebral hereditary angiopathy (CHA) disorders it may be a primary sign of disturbed vascular reactivity. Almost half of patients with CADASIL have migraine with aura.[54] Additional symptoms of CADASIL include recurrent ischemic stroke typically manifesting in mid-adulthood, cognitive deterioration leading to dementia, as well as mood alterations and apathy, though the onset is highly variable and determined partly by the type of variant causing the

disorder.[55] CADASIL is caused by mutations in *NOTCH3*, characteristically leading to loss or gain of a cysteine residue in one of the 34 epidermal growth factor-like repeat (EGFr) domains.[56,57] In D-CAA, migraine with aura presents as the initial symptom in nearly 80% of mutation carriers. D-CAA is caused by a single point mutation (E693Q) in the amyloid precursor protein (*APP*) gene. This leads to an accumulation of amyloid in blood vessel walls, resulting in brain hemorrhage, with the first signs usually occurring between the age of 45 and 65 years.

Studying these monogenic diseases where migraine is part of the clinical spectrum has several advantages. Unlike in common disease, in a monogenic disease one mutation is causing the phenotype. Another important advantage is that the presymptomatic stage of disease can be studied (Figure 1). This is especially interesting as it seems logical that treatment will be more effective in the presymptomatic stage and may be able to prevent secondary damage of the underlying vasculopathy. Animal models, such as transgenic mice carrying specific gene mutations, can be engineered to mimic human monogenic diseases. By studying how these mutations disrupt molecular, cellular, tissue processes, as well as behavior, valuable insight can be obtained into the underlying biology of disease, such as in migraine. This understanding can be crucial for developing targeted therapies and interventions.

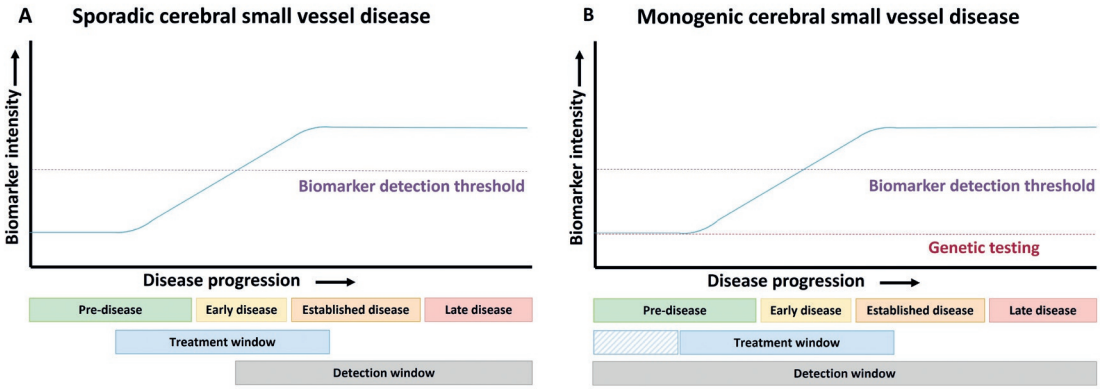

Figure 1. Studying mechanisms causing cerebral small vessel diseases.

The asymptomatic interval between initial molecular alterations (blue solid line) and reaching the detection limit (purple dash line) is a key problem in the investigation of cerebral small vessel diseases (A). Not only does this effect research (e.g., initial mechanism of disease might no longer be visible at advance disease stage), this also delays treatment. By studying monogenic small vessel diseases, genetic testing can identify patients before molecular alterations occur (red dash line) (B). As such, a clear presymptomatic population can be studied that will certainty develop the disease. Furthermore, the treatment window is distended in these patients giving the possibility for early interventions.

Pathophysiology of cerebral hereditary angiopathies with migraine

In mammals, TREX1 is a major 3'-5'-exonuclease with a high affinity for single-strand DNA.[58,59] TREX1's N-terminus contains three exonuclease domains while its C-terminal domain is required for its localization to the endoplasmic reticulum.[58,60-63] Frameshift mutations cause RVCL-S and lead to a truncated TREX1 protein. The most prevalent mutation is the V235fs mutation. Hence, *TREX1* mutations preserve the enzymatic function of TREX1 but alter its intracellular localization, as the C-terminus that anchors to the endoplasmic reticulum is absent.[61-63] The pathophysiological mechanisms of RVCL-S remain elusive. TREX1 is expressed in many cell types. Interestingly, it was also found to be expressed in a subset of microglia in the normal human brain, often nearby the microvasculature,[64,65] as well as in endothelial cells in the brain.[65] RVCL-S patients are found to have thicker multilaminated basement membranes and the walls of small vessels demonstrate fibrous thickening.[66] Transgenic RVCL-S knock-in mice (carrying the V235fs mutation) have shown increased mortality, signs of abnormal vascular function, and increased sensitivity to experimental stroke.[67]

In **Chapter 7**, functional vascular measurements were performed suggesting an impairment of endothelial function in RVCL-S.[68] Vascular function was investigated by endothelium-dependent flow-mediated vasodilatation and endothelial-independent vascular reactivity determined by measuring dermal blood flow responses to capsaicin application (i.e., vascular smooth muscle cell (VSMC) function). While in RVCL-S flow-mediated vasodilation was impaired, no differences in dermal blood flow response to capsaicin were demonstrated, indicating that endothelial dysfunction plays a role in the disease. Moreover, elevated markers for chronic endothelial activation (von Willebrand factor and angiopoietin-2) were found in RVCL-S,[69] and CO_2 cerebrovascular reactivity testing demonstrated impaired vascular reactivity.[70] CO_2 cerebrovascular reactivity is considered a surrogate marker of endothelial function in the vasculature of the brain.[71] Interestingly, a difference in cerebrovascular reactivity could already be found in patients younger than 40 years. Currently, it remains uncertain whether mutated TREX1 protein has a direct detrimental effect on endothelial cells or whether endothelial dysfunction is a consequence of the pathological changes seen in RVCL-S. Nevertheless, given the importance of the endothelium, it is a promising target for possible therapies aimed at alleviating symptoms caused by small vessel diseases.

Additionally, we did not find a clear indication of abnormal vascular smooth muscle cell (VSMC) function in RVCL-S, differentiating RVCL-S from CADASIL (**Chapter 7**). To further investigate the pathophysiological mechanism involved in RVCL-S, in **Chapter 11**, retinal saturation levels and retinal vessel thickness were determined.

Venular saturation increased while the arteriovenous difference decreased, with no significant variances noted in arterial saturation. Furthermore, retinal vessel narrowing was observed in patients with RVCL-S. The most likely mechanisms for these findings include inadequate distribution of blood flow and oxygen, thickening of retinal arteries, and decrease in oxygen consumption (supported by the fact that in **Chapter 9**, thinning of the retinal layers,[66] such as the ganglion cell layer was demonstrated, possibly leading to less metabolic activity (Figure 2)). It is important to realize that retinal changes can be due to retinal vasculopathy and retrograde trans-synaptic degeneration, as seen with neurological conditions. As RVCL-S is both a retinal vasculopathy and a neurodegenerative disorder, the pattern of ganglion cell layer loss in the retina might shed light on which mechanisms are predominantly involved. Our cohort described in **Chapter 9**, predominantly suffered from patchy ganglion cell layer loss, consistent with retinal disease. However, ganglion cell layer loss already became apparent in some patients that did not suffer from quantifiable retinal disease. This most often occurred in a sectorial pattern, which suggests that retrograde transsynaptic degeneration seems to play a role. It is, therefore, not unlikely that while retrograde transsynaptic degeneration occurs at first, retinal damage is likely the most important contributor to further ganglion cell layer thinning when the disease progresses and (severe) retinopathy occurs.

Notch3 is critical for VSMC differentiation and development.[56] *NOTCH3* mutations causing CADASIL are pathogenic variants located in the extracellular region of the Notch3 transmembrane receptor within epidermal growth factor (EGF)-like repeat domains. Similar to other Notch receptors, the Notch3 receptor undergoes proteolytic processing. This processing yields a large extracellular fragment and a small intracellular fragment containing the transmembrane region. In CADASIL, the extracellular domain of the Notch3 receptor accumulates within blood vessels, particularly at the cytoplasmic membrane of VSMCs and pericytes, closely associated with the granular osmiophilic material (GOM) deposition, a hallmark characteristic of the disease.[72] In **Chapter 7**, patients with CADASIL displayed a lower increase in dermal blood flow after capsaicin application then controls, while no difference in flow-mediated dilatation was demonstrated. This further implicates impaired endothelium-independent VSMC relaxation in CADASIL.

As of 2022, the number of identified amyloid fibril proteins capable of causing human disease is 42.[73] The term CAA is, however, near synonymously used for the disease caused by deposition of amyloid beta (Aβ) in the walls of small arteries, arterioles, and capillaries of the brain.[74] All mutations identified to primarily cause amyloid β CAA are associated with the APP gene. Several mutations are confirmed to be

pathogenic, but with the exception of Dutch-type CAA, also known as hereditary cerebral hemorrhage with amyloidosis of the Dutch type, only a limited number of cases have been described for each mutation.[75] Dutch-type CAA is, therefore, the most widely recognized and thoroughly studied variant of the hereditary CAAs.[74] Severe Aβ deposition is present in the small arteries, arterioles, and capillaries of the leptomeninges, cerebral cortex, and cerebellar cortex accompanied by loss of VSMCs and thickening of the vessel walls, indicating a severe vasculopathy.[76] While neuropathological findings indicate that Aβ is initially deposited within the outer basement membranes surrounding VSMCs, the basement membranes of the endothelium remain largely unaffected.[74] Clearance of Aβ can occur through both enzymatic and non-enzymatic pathways, with a role for neuronal and glial cells, the blood-brain barrier (including pericytes and the endothelium), perivascular drainage, and cerebrospinal fluid absorption pathways in the brain.[77] In **Chapter 8**, thinning of the retinal nerve fiber layer (RNFL) in CAA is demonstrated (Figure 2). The RNFL is the most inner retinal layer and connects the neuroretina with visual tracts that lead to the visual cortex. CAA pathology predominantly appears in the lobar regions of the brain, specifically the occipital lobe in D-CAA.[78] Therefore, retrograde degeneration of the optic nerve causing retinal changes starting with thinning of the RNFL is likely the mechanism responsible for the thinning of the RNFL.

Integrating various models of monogenic small vessel diseases

The foundation of our understanding regarding the molecular background of many common diseases predominantly stems from insight garnered from rare familial manifestations of the disorders. Even now, investigating rare monogenic diseases may be the most effective approach for uncovering the essential pathways that are impaired in human disease. By combining research into different monogenic small vessel diseases, potentially this strategy might even be expanded upon.

Demonstrating whether vascular changes already occur in presymptomatic mutation carriers or early-stage disease patients can be extremely difficult with (neuro) pathological examination alone. These changes are only observable in deceased patients eligible for autopsy or when a biopsy is taken from living patients. As discussed in **Chapters 10 - 12**, the retinal vasculature can now be visualized and examined in great detail *in vivo*. In RVCL-S, a reduction in vessel density was observed and the vessels had thickening of their vessel wall. Also in CADASIL, vessel thickening and decreased vascular density was demonstrated,[79,80] indicating similarities between disease phenotypes, while in **Chapter 7** evidence was provided that distinct vascular mechanisms likely play a role. Similarly, by evaluating retinal thickness in both patients with D-CAA (**Chapter 8**) and RVCL-S (**Chapter 9**), differences in disease mechanisms

became apparent (Figure 2). Where in RVCL-S, thinning of the peripapillary RNFL (pRNFL) and a decrease in total macular volume were demonstrated, in D-CAA, only changes in the pRNFL were demonstrated. These differences cannot simply be explained by the fact that RVCL-S is a systemic small vessel disease, whereas D-CAA only affects the blood vessels in the brain. In Alzheimer's disease, another brain disorder associated with Aβ, both thinning of the pRNFL and the total macular volume have been reported.[81] The investigation into which signs are attributed to retrograde degeneration and which are caused by a more widespread vasculopathy is still pending.

The mechanisms leading to (monogenic) small vessels disease, with or without migraine, are complex. Likely an intimate interaction exists between endothelial cells, pericytes, smooth muscle cells, and other cell types, such as microglia. Disorders in which one cell type is primarily is affected inevitably will affect other elements within the neurovascular or gliovascular unit. Monogenic disease models offer a unique opportunity to study these sequential processes.

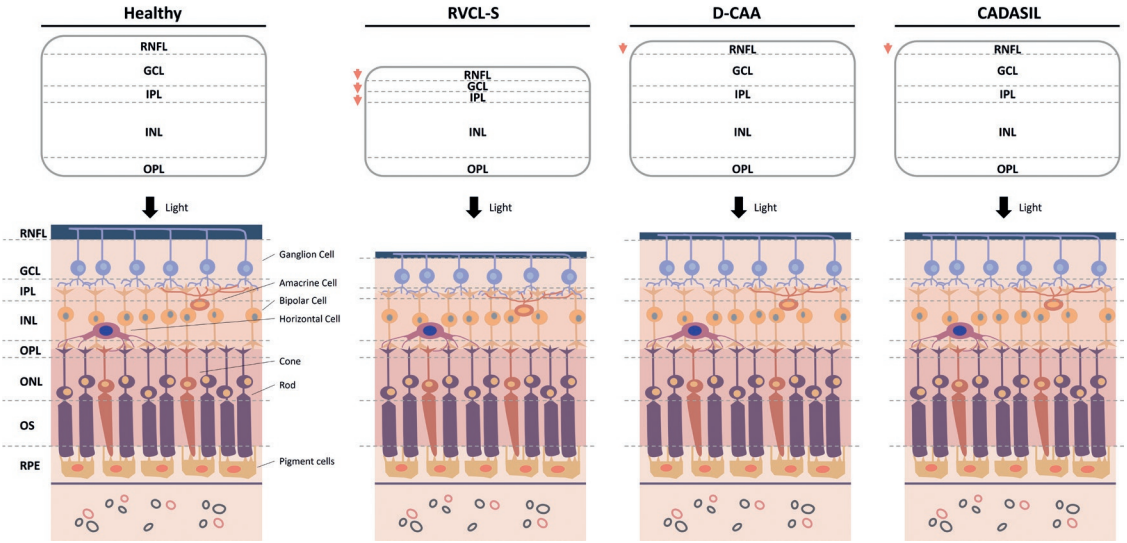

Figure 2. Schematic of the layers of the healthy retina and the hypothetical changes in the retina in RVCL-S, D-CAA and CADASIL.

In RVCL-S, thinning of the RNFL, GCL en IPL is observed (Chapter 9). In D-CAA, thinning of the RNFL layer occurs (Chapter 8). Also in CADASIL thinning of the RNFL layer has been demonstrated.[82] Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), Dutch-type hereditary cerebral amyloid angiopathy (D-CAA), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer nuclear layer (ONL), outer plexiform layer (OPL), photoreceptor outer segments (OS), retinal nerve fiber layer (RNFL), retinal pigment epithelium (RPE), retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations (RVCL-S).

Biomarkers for cerebral hereditary angiopathies with migraine

Biomarker research in cerebral hereditary angiopathies is important as biomarkers will be helpful to diagnose conditions earlier, monitor disease progression, and/or predict patient outcomes. Moreover, biomarkers can be used as surrogate endpoints in clinical trials and can help monitor the effectiveness of treatment, leading to treatment decisions. Finally, biomarker research can enhance our understanding of biological processes underlying the cerebral hereditary angiopathies. Studying patients with small vessel disease resulting from a monogenic cause offers the advantage of knowing who will develop the disorder. This provides the advantageous opportunity of identifying a clear presymptomatic disease stage in patients who will subsequently become symptomatic (Figure 1). It eliminates the need for large cohorts of individuals that may or may not develop small vessel disease. Testing biomarkers identified in cerebral hereditary angiopathies can also be used to assess disease risk and identify individuals at higher risk of developing sporadic (non-monogenic) small vessel diseases. Early identification of at-risk individuals allows for preventive measures to be implemented, potentially reducing the incidence and burden of cerebral small vessel diseases. The most ideal biomarker techniques should be safe, non-invasive, repeatable, reproducible, and inexpensive.

In this thesis, several potential biomarkers of small vessel diseases were investigated, including RVCL-S (Figure 3). In **Chapter 7**, it was demonstrated with *in vivo* measurements that vascular function in RVCL-S and CADASIL is affected. Furthermore, in **Chapter 12**, an association between cognitive functioning and vascular reactivity in RVCL-S was demonstrated. While useful for our understanding of the disease, the techniques used in these studies are difficult to assimilate in clinical practice as they are sensitive to change and time-consuming. For example, while flow-mediated dilation is a non-invasive method that enables the evaluation of endothelial dysfunction in the brachial artery in response to reactive hyperemia, there are several drawbacks of using this method. The absence of standardization and discrepancies in cuff positioning on wrists/arms, but also variations in vessel diameters, changes in blood vessel structures and compromised dilation, as well as challenges such as lower resolution relative to artery size, limited reproducibility, and reliance on operator skill, constitute the main drawbacks of this methodology. Currently, routinely accessing endothelial function is challenging, technically demanding, and not generally available in the clinical practice.

Another technique discussed in **Chapters 8 - 11** can be coined as “oculomics”, e.g., using the eye as a biomarker. Imaging of the eye holds the vast potential as a (noninvasive) biomarker for systemic and neurological conditions, given that the eye

allows for straightforward visualization of both the vasculature and neuronal tissue, unlike in any other part of the body. The eyes and brain share the same embryological origin. During early embryonic development, both the eyes and the brain arise from the neural tube, which is a structure that forms from the ectoderm layer of the developing embryo. As development progresses, specific regions of the neural tube give rise to the different parts of the central nervous system, including the brain and the retina of the eyes. This common developmental origin contributes to the close relationship between the eyes and the brain in terms of function and pathology.

In D-CAA it was found that overall thickness of pRNFL was decreased in symptomatic but not presymptomatic patients compared with controls. Interestingly, in both a thinner temporal-superior quadrant of the pRNFL was found, indicating that it might be a useful early disease marker (**Chapter 8**). In RVCL-S, not only the pRNFL was thinned, but total macular volume was decreased (**Chapter 9**). In addition, the foveal avascular zone was enlarged in symptomatic but not presymptomatic patients (**Chapter 10**). In the same study, a decrease in vessel density already had occurred in presymptomatic mutation carriers. Finally, in **Chapter 11**, using retinal oximetry, an increase in retinal venular oxygen saturation and a decreased arteriovenous difference was found in RVCL-S. Together, these studies not only improve our pathophysiological understanding of RVCL-S, but they also demonstrate that oculomics is a promising avenue of research for finding non-invasive biomarkers. Presymptomatic individuals already exhibit retinal changes that are more pronounced in symptomatic patients. This suggests that employing these tools may enable (early) disease tracking. Many of these ophthalmological techniques are increasingly investigated and have demonstrated promising results indicating that they can serve as clinical biomarkers in other neurological disorders. In both retinal vasculopathies and diseases affecting the central nervous system, retinal oximetry and retinal morphology have been shown to be able to serve as early and clinical relevant biomarkers.[83-87] Interestingly, in patients with CADASIL, both macular vessel density and inner retinal thickness were positively correlated with gait speed and were inversely correlated with the number of lacunae.[88] The expression “the eye is the window to the brain” does indeed apply for cerebral small vessel diseases, and suggests that examination of the eyes can provide valuable insights into the health and function of the brain in patients with these disorders.

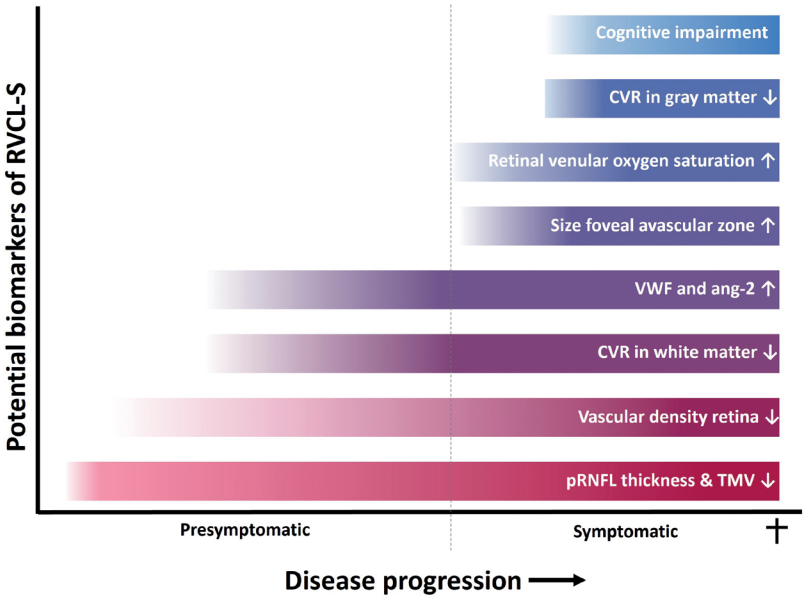

Figure 3. Hypothesis of biomarker progression in RVCL-S.

Angiopoietin-2 (ang-2), cerebrovascular reactivity (CVR), peripapillary retinal nerve fiber layer (pRNFL), retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations (RVCL-S), total macular volume (TMV), Von Willebrand factor (vWF).

Clinical implication monogenic small vessel diseases

The objective of this thesis is to enhance comprehension of the pathophysiological mechanisms underlying monogenic small vessel diseases, while also identifying potential biomarkers suitable for monitoring disease progression and treatment outcome. Importantly, here it was demonstrated that before signs visible on fundoscopy appear, non-invasive ophthalmological examination can already demonstrate alteration in retina morphology in RVCL-S (**Chapters 9 and 10**), likely requiring monitoring. Furthermore, our ophthalmological evaluations demonstrated that patients require frequent and extensive follow up to treat signs of retinopathy. Nevertheless, currently no validated biomarker is available to predict disease progression in RVCL-S. Additionally, we evaluated the efficacy of migraine treatment for patients with RVCL-S and CADASIL (see the section *Clinical implications migraine genetics*). Here, it is important to note that therapeutic uncertainty should not translate to therapeutic nihilism.

Part III: Equality in headache research – tackling career barriers and harassment

Crucial for the success of scientific research and clinical care for migraine and its comorbid conditions is the establishment of a healthy and safe work environment. In this context, the imperative emerges to educate and guide future experts in the field. In **Chapters 13 and 14** the prevalence of career barriers and harassment in the headache field is studied in a large global cohort. Women encounter career barriers and sexual harassment more frequently than men in the field of headache. Notably country of birth also emerged as a notable barrier to active involvement in international scientific societies.

These findings are important, but unfortunately, not surprising. Although progress in achieving parity in medical school admissions based on gender in certain areas has been made, women continue to be underrepresented in senior faculty positions within medical schools.[89] Contributing factors to the gender disparity include persistent cultural attitudes and workplace climates, the absence of gender parity in leadership roles, disparities in compensation based on gender, challenges in retaining women in the workforce (often referred to as the "leaky pipeline" phenomenon), and the unequal distribution of family responsibilities, making it difficult for women to achieve a satisfactory work-life balance.[90] Moreover, although not always acknowledged, the healthcare industry is prone to instances of harassment.[91-94] This tendency may stem from disparities in economic status, prestige, and power.[95-97] This problematic behavior affect victims severely,[98,99] and may affect patient outcomes.[100,101] Addressing this behavior poses significant challenges, especially considering that perpetrators often occupy senior leadership and supervisory roles. Consequently, individuals experiencing workplace sexual harassment often refrain from filing complaints.[102] Additionally, other factors such as stigma, apprehension of repercussions and difficulties in substantiating allegations all contribute to this.[103,104]

Targeted interventions are necessary to challenge social stereotypes, reshape workplace cultures that favor traditionally masculine traits, eliminate discriminatory practices, and establish transparent processes to ensure the inclusion of women and other underrepresented minorities. However, these interventions should take into account that career barriers may be perceived differently across world regions as we demonstrated in **Chapter 13**. An important finding that is supported by other medical fields.[105,106] It is reasonable to suggest that varying regions may face distinct challenges regarding career advancement, necessitating tailored interventions accordingly. It is important to note that a language barrier may have prevented professionals from

responding in these studies. Additionally, underrepresentation of individuals from African and Eastern Mediterranean countries is an important limitation. This finding on itself indicates that additional resources are needed to involve practitioners and researchers from these regions in the international headache field.

Working towards an inclusive and safe work environment should be an ongoing endeavor, rather than a fixed endpoint. Establishing and assessing predetermined milestones is crucial, yet every organization should aspire to maintain continual diversity and inclusivity. Ensuring equality in research is not just a matter of ethical responsibility; it also enhances the quality, validity, and impact of research findings.

Further perspectives

Migraine genetics

Although we have garnered valuable insights from GWAS on migraine, future research remains needed to uncover more about the genetic factors contributing to migraine. Genetic research in migraine should focus on: i) generating larger-scale studies, particularly within non-European populations, also to increase transferability of results, ii) collecting genetic data of large cohorts with high-quality phenotype data, iii) determining the mechanism behind the association between loci and migraine risk, and iv) identifying sources of missing heritability in migraine.

Transferability of GWAS across populations

Demographic and cultural history have resulted in human groups that on average exhibit genetic and environmental differences. These genetic variances impact how allelic variants are distributed among these groups, while environmental distinctions can modify their influences within groups on susceptibility to disease. Therefore, findings from GWAS cannot always be transferred to a group of different ancestry, even though there is sufficient statistical power. For example, in a GWAS of depression in 194,549 patients with East Asian ancestry, only 11% of previously identified depression loci showed limited evidence for transferability.[107] As GWAS findings might not necessarily replicate in different cohorts, replication cohorts of similar ancestry should be preferable before findings are dismissed. Another consequence of lack of transferability is that genetics instruments such as polygenic risk scores might underperform in different populations,[108,109] as there is often an overrepresentation of European ancestries participants in GWAS.[110] Several GWAS have been performed in Asian populations, although all with small sample sizes. For example, in the Han Chinese population (1042 patients) SNPs in *KCNK5* and *FHL5* were evaluated,[111] while

in a separate study including 715 patients susceptibility loci associations with age of onset of migraine were determined.[112] Facilitating collecting more GWAS data from diverse populations should be a focus of the GWAS field going forward.

While currently there appears to not be a place for genetics in the clinical care of common migraine, this might not remain so. For example, in other diseases, such as breast cancer, prostate cancer, multiple sclerosis, and type 1 diabetes, polygenic risk scores can be used to predict risk in patients of European descent with more accuracy than current clinical models.[113-116] As such, including patients from diverse genetic ancestries in GWAS is not only beneficial for understanding pathophysiological mechanism, but also important ensuring that future clinical applications are not limited to patients from European ancestry. However, despite the large number of polygenic risk scores described in literature, prospective studies of their clinical utility, are rare and needed before clinical gain can be expected.

In-depth phenotyping

There is an important dilemma in GWAS, whether broad phenotyping leading to maximization of sample size (and statistical power) is preferential over in-depth phenotyping, which frequently leads to smaller sample sizes is preferable. While the first approach has been successful in increasing the number of significant genetic hits, it has also been criticized for a possible lack of specificity. By only including minimal phenotyping strategies, a part of the genetic architecture of a disorder might be missed. Sufficient high-quality phenotype data in combination with genetic data is required for these analyses. In migraine, (sub)type, comorbidities and treatment efficacy are all interesting avenues of research. It is important to realize that some of these traits are not stationary and might change over time (e.g., episodic vs. chronic migraine), or could still be developed over time. It, therefore, remains very important that adequate data on a cohort is collected and published, so that different cohorts can be compared to one another. Even the genetic contribution towards sex difference has not been studied sufficiently. In migraine, there are clear phenotypic sex-based differences, for instance in risk of chronification.[117,118] But even when phenotypes are similar, the underlying biology between sexes may differ. Therefore, recent methods for incorporating sex-specific differences should be employed in the future genetic studies.[119]

From SNP to gene

It is important to determine how genetic loci influence migraine risk. The challenge of experimental validation of causal variants, genes and DNA functional elements associated with disease remains. Furthermore, identifying involved gene networks,

biological pathways and determining the implicated cell types and tissues are also needed to learn more about pathophysiology. First-off, linked SNPs are usually situated in intronic or intergenic regions, serving merely as genomic markers in linkage disequilibrium with the actual causal variant.[120] Efficiently integrating data from GWAS, expression quantitative trait loci (eQTL) analyses, enhancer-gene predictions and 3D chromosomal interactions (as well as other omics data) is crucial for prioritizing potential causal genes.[120,121] This way, meaningful functional analysis following GWAS discoveries can be conducted. Even so, to functionally characterize GWAS findings, a number of high-throughput strategies in both cell-based and whole-animal models are likely required.[122] Nonetheless, while these steps might prove difficult, they are vital if we want to further our understanding of migraine pathophysiology.

Missing heritability of migraine – beyond GWAS

Despite advances in genome-wide association studies (GWAS) and other genetic research methods, genetic variants identified through these studies account for only a small fraction of the total heritability of complex traits or diseases, such as migraine; this gap is referred to as 'missing heritability'. Due to small effect sizes, the identification of genetic factors might require larger cohorts. Rare variants may also contribute significantly to heritability but are not captured by the genotyping arrays typically utilized in GWAS. Such variants can, however, be identified using other approaches, such as next-generation sequencing (NGS). Therefore, NGS approaches, such as whole-exome and whole-genome sequencing, can be used to assess the contribution of rarer variants. Additionally, other approaches, such as transcriptomic approaches (evaluating RNA by RNA-seq methods) can also provide insights into gene expression patterns and regulatory mechanisms. Additionally, addressing gene-gene interactions (epistasis), gene-environment interactions, mitochondrial DNA variants and structural variants and non-coding regions will likely help understand the missing heritability problem.[123-125] Innovative statistical methods for analyzing complex genetic data, and integration of genomic information with other omics data (e.g., transcriptomics, epigenomics) and environmental factors will be required to identify the 'missing heritability' of migraine (Figure 4).

Beyond genomics

Additionally, while genetic data on large populations of migraine patients is widely available, almost no epigenetic data has been collected. Epigenetics is an especially promising avenue of research as epigenetic processes are important for programming enduring cellular responses to environmental triggers. As such, epigenetic mechanisms may explain how both endogenous and exogenous factors (e.g., sex hormones and stress) may influence migraine attack frequency. Studying different

epigenetic changes (e.g., DNA methylation, histone modification, non-coding RNA), has therefore the potential of elucidating why certain patients are sensitive to certain exogenous factors, while others are not. One example of an interesting avenue of research are microRNAs (miRNAs). miRNAs are small noncoding RNAs, approximately 18-25 nucleotides in length, and are recognized as major gene regulatory mechanism. miRNAs, as epigenetic modulators, modulate protein levels of the target mRNAs either by inhibiting translation or by promoting degradation (Figure 4). Preliminary findings indicate that miRNA levels might change with migraine frequency, ictal and interictal phase of migraine and between migraine patients and controls.[126-128] Replication of these findings in larger cohorts are still needed. Nonetheless, this avenue of research might provide useful biomarkers for migraine and related disorders. Developing treatment aimed at epigenetic mechanisms may lead to new avenues of migraine treatment. However, first more research is required to identify additional changes and these need to be replicated.

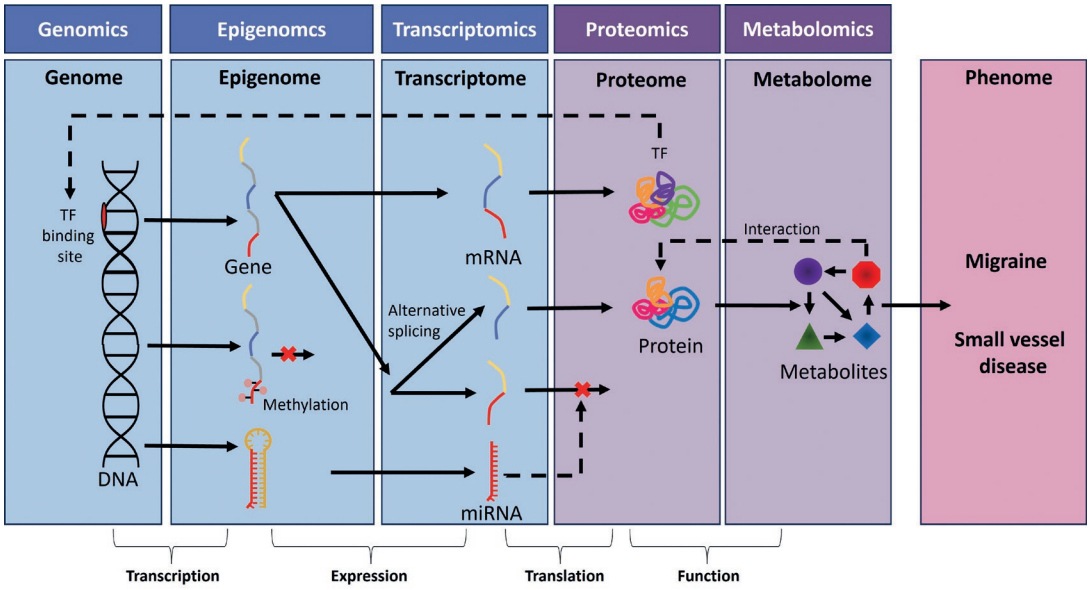

Figure 4. Overview of omics fields.

Interactions between the different levels of omics fields are frequent (interactions represented in the figure are a simplification). These interactions need to be addressed with multi-omics approaches. TF = transcription factor.

The integration of genomics research with other "-omics" approaches (e.g., transcriptomics, proteomics, and metabolomics) will offer a more comprehensive understanding of biological systems (Figure 4). By combining these approaches, new insights into the pathways involved in migraine will likely be gained. This will facilitate the identification of biomarkers, elucidation of disease mechanisms, and discovery of potential therapeutic targets.

Monogenic small vessel disease

Rare, inherited cerebral small vessel diseases can drastically impact individuals harboring disease-causing mutations. Typically, targeted therapies specific to these diseases are non-existent. The primary obstacles to gathering credible evidence for rare disorders include limited statistical power and, in certain cases, disease heterogeneity. However, dedicated international consortia and the establishment and utilization of biomarkers may present a viable road forward. In the meantime, for rare monogenic disorders it should remain possible to publish novel phenotypes and attempted treatment trails (e.g., n=1 studies) in scientific journals with rigorous peer review. This is not only vital for combatting diagnostic delay and for assisting in finding treatments for these disorders, lessons learned from rare monogenic diseases can also help understand common disorders.

Furthermore, by studying monogenic models in combination with continuous improvement in the field of image techniques important key problems that have hampered elucidating small vessel disease can be addressed. These key problems include (1) the inherent difficulty in visualizing human small vessels *in vivo*, (2) the asymptomatic interval between initial molecular alterations and clinical manifestation, (3) the variability in clinical manifestations, such as stroke and dementia. The ophthalmological department has been visualizing vessels and the nerve fiber layer of the retina for a long time. The field of small vessel diseases and neurology should continue to learn from their efforts. Moreover, easy to use techniques with high spatial (and temporal) resolution are needed to study the circulation (in the brain). Tools, such as functional ultrasound and ultra-high-resolution 7 Tesla should be further developed,[129,130] and appropriate tools for visualization and interpretation of their signals should be build.

Biomarkers and Natural history studies

This thesis identified several (early) biomarkers for monogenic small vessel diseases. A crucial next step would be to assess how these biomarkers behave as these disorders progress. Natural history studies (longitudinal cohort studies) should be performed to assess that these markers can be measured consistently and whether they show

changes as a disease course progresses. Figure 3. shows a proposed simplified model for how biomarkers in RVCL-S might behave. Biomarkers likely develop in a sequential order. Finding a marker that shows improvement when pathways involved in monogenic small vessel diseases are targeted and can be used as a surrogate endpoint is the ultimate goal. Natural history studies are not only needed for identifying biomarkers. These type of studies for rare disorders are required for observing natural disease course, variability in symptom severity and factors influencing disease progression. This knowledge is essential for predicting disease progression and for finding effective treatment strategies and designing robust treatment trails.

Pathophysiology and future treatments

Understanding the pathophysiology of small vessel disease, even monogenic ones, requires further study. The exact contribution and temporal sequence of compromised blood-brain barrier (BBB) integrity, reduced blood flow and reduced vascular reactivity, endothelial dysfunction, inflammation (especially chronic, sterile, low-grade inflammation) and the glymphatic system remain unknown.[131,132] Longitudinal cohort studies are urgently needed to determine the contribution of these factors.

While a lot of work remains to be done to elucidate the disease mechanism, it is important to consider possible treatment choices. For RVCL-S, a suggested potential treatment option is the Janus kinase (JAK) inhibitor ruxolitinib.[133] Ruxolitinib leads to disruption of both cytokine and growth factor signaling pathways, among which is CXCL10. In human lymphoblasts of RVCL-S patients elevated CXCL10 has been demonstrated. CXCL10 modulates angiogenesis and is involved in chemoattraction. Therefore, after elevated CXCL10 transcripts were demonstrated in peripheral blood monocular cells treatment was started in one patient.[133] After treatment, the CXCL10 concentration lowered. This is, however, a consequence of treatment and not proof of (clinical) improvement and further evidence is required before this treatment is systematically prescribed. Importantly, as patients with RVCL-S can suffer from severe anemia, if treatment with ruxolitinib is started, this should be monitored closely. Dose-dependent anemia has been observed with treatment, though Hb levels have been shown to returning to baseline by week 24.[134] Another suggested treatment is crizanlizumab. Crizanlizumab is monoclonal antibody that binds to P-selectin.[135] Treatment prevents leukocyte adhesion to the vascular endothelium, thereby limiting risk of microvascular occlusion. P-selectin is mobilized to the surface of activated endothelial cells and there it can lead to leukocyte adhesion. A trial was started in the USA in 2021 (ClinicalTrials.gov Identifier: NCT04611880). The endpoint of this trial was volume increase in lesions of FLAIR MRI between baseline and one year. Whether this

is an adequate endpoint can be disputed. As disease progression can fluctuate over time and as no control group (historical or otherwise) was included interpreting the results may prove challenging.

Gene-editing, the process of precisely modifying DNA sequences within an organism's genome, is an intriguing modality in the realm of genetic medicine that has garnered significant attention in recent years. Gene-editing treatment, exemplified by techniques like CRISPR-Cas9, offers immense potential for tackling a range of monogenic diseases. CRISPR/Cas, an RNA-guided engineered nuclease, can target virtually any genomic sequence for modification. Traditional CRISPR gene editing involves creating site-specific double-strand breaks using a single guide RNA (sgRNA) to guide the Cas endonuclease. Double-strand breaks trigger cellular DNA repair pathways: non-homologous end joining (NHEJ) introduces indels for efficient gene knockout, while homologous repair (HR) can introduce mutations or insertions in the presence of an exogenous template. In 2023, the first therapy based on Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated nuclease 9 (CRISPR/Cas9) gene editing was approved by the FDA (however *ex vivo*).[136] Utilizing the precise DNA-cutting capability of CRISPR–Cas9, this therapy targets *BCL11A*, a suppressor of the fetal hemoglobin gene, within harvested hematopoietic stem and progenitor cells (HSPCs) from patients. Typically silenced in the months post-birth, the reactivation of the fetal hemoglobin gene yields proteins capable of compensating for faulty or absent beta-globin in sickle cell disease and beta-thalassemia respectively (both caused by mutations in HBB). While an extremely valuable prove of principle, this success story is not readily applicable to monogenic migraine syndromes for two important reasons. Firstly, blood based disorders can be treated by harvested hematopoietic stem and progenitor cells that are re-administered to the patient. In case of hemiplegic migraine and cerebral hereditary angiopathies, altering affected cells must be done in vivo. Therefore, safe and effective delivery systems have to be created. While this is currently a field in which progress is being made, for instance by a delivery system targeting endothelium cells,[137] a lot of work remains to be done to insure high target specificity. Subsequently, gene editing efficiency needs to be high enough while off target effects should be negligible. Secondly, by silencing a suppressor gene of *HBF* it does not matter what underling mutation affects the *HBB* gene, therefore, this one approach can be used to treat many patients with different mutations. Using this technique for hemiplegic migraine or one of the cerebral hereditary angiopathies would require mutation specific approaches, as gene silencing is not an option. The exception being D-CAA where one point mutation causes the disorder. There are still significant challenges, including improving delivery of the CRISPR components and minimizing off-target effects to ensure safety.

Another avenue of treatment are antisense oligonucleotides (AONs), short strands of modified nucleotides (typically between 15-30 nucleotides) that target RNA in a sequence-specific manner. AONs can be used to I) decrease levels of toxic protein by causing degradation of mRNA transcripts or by blocking translation or II) to modulate the splicing process to restore (non-faulty) protein production (e.g., causing exon skipping or by blocking intronic splice silencers for exon inclusion). In CADASIL, proof of concept of the exon skipping approach was both demonstrated in vitro and by a family in which a mutation led to exon skipping of exon 9 of *NOTCH3*.[138,139] However, before transitioning to human trials, it is necessary to conduct in vivo studies using animal models. Exon skipping was also evaluated in D-CAA. AON assisted skipping of exon 17 was not only successful in induced pluripotent stem cells but also in wild-type mice. Exon skipping led to a shorter APP isoform that lacks part of the Aβ domain that contains the D-CAA mutation.[140] Evidence of treatment effect of APP protein modification and its safety is however still lacking. As *TREX1* contains a single exon, exon skipping is not a promising avenue for RVCL-S. Likewise, as hemiplegic migraine is caused by missense mutations that do not effect splicing but does effect ion channel function, a defect not readily amenable to AON therapy, this makes the successful implementation of AON therapy less likely.

Other less-sophisticated therapeutics of possible interest for cerebral small vessel disease include candesartan, minocycline and nicotinamide mononucleotide. Candesartan was demonstrated to modulate pathological extracellular matrix accumulation in a mice model for cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL).[141] Minocycline has been shown to reduce white matter damage, improve cerebral blood flow, and let to prolonged survival in hypertensive rats.[142] Finally, nicotinamide mononucleotide (a key NAD+ intermediate) has been shown to not only benefit cerebrovascular endothelial function and neurovascular coupling responses, but to also improve cognitive function in aged mice.[143] Other possible interesting avenues of research should focus on the communication between endothelial cells and pericytes (for instance targeting the TGF-β pathway) and vascular muscle cells and on how to preserve this communication when damage occurs.

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ADDENDUM

Summary
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Summary

This thesis explores the genetic architecture of migraine, including familial hemiplegic migraine (FHM), as well as related monogenic neurovascular disorders such as cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations (RVCL-S) and Dutch-type hereditary cerebral amyloid angiopathy (D-CAA), their clinical phenotype, and their pathophysiology. The ultimate goal of the thesis is to contribute towards the understanding of migraine and small vessel disease pathophysiology and identify novel treatment targets. Finally, the thesis aims to not only contribute towards the knowledge accumulated in the respective research fields, but also strives towards achieving gender equality in research, as this is not merely a matter of ethical responsibility but also enhances the quality, validity, and impact of research findings. The research is divided in three parts. **Part I** describes studies focusing on the genetics of migraine and how this may influence treatment (**Chapters 2-5**). **Part II** describes studies on monogenic vascular syndromes that have been associated with migraine (**Chapters 6-12**). Finally, **Part III** aims at identifying challenges that physicians and researchers face in the headache field (**Chapters 13 and 14**).

Part I: Migraine – insights into genetics, epigenetics, comorbidities, and monogenic factors

Considering the data derived from epidemiological studies in both cohorts and families it has become clear that the genetic influence in migraine with aura is more pronounced than that of migraine without aura. In **Chapter 2**, the ground was laid for studies into monogenic migraine by writing a review on the genetic underpinning of migraine with aura. Several genetic approaches have been employed to identify genetic factors conferring migraine with aura risk. Genome-wide association studies (GWAS) tested variants across the genome in a hypothesis-free manner. Whereas GWAS for the diagnosis migraine has already identified dozens of gene variants, the search for gene variants specific for migraine without aura or migraine with aura has been disappointing. Investigating all genotyped single nucleotide polymorphisms (SNPs) together, so not focusing on individual significant hits, had already proven more successful and led to the notion that migraine with aura and migraine without aura are genetically more alike than different. Importantly, most relevant genetic discoveries related to migraine with aura came from investigating monogenetic syndromes with migraine aura as a prominent phenotype (i.e., FHM and CADASIL) that pointed to roles for neurotransmission and vasculature in migraine pathophysiology. By increasing cohort size, by testing the contribution or rare variants and by studying

epigenetic factors, we are expected to increase our understanding of the genetic architecture of migraine, including migraine with aura.

In **Chapter 3** it was investigated whether DNA methylation (DNAm) changes occur when patients with chronic migraine and medication overuse (headache) respond to treatment and whether there are already differences between responders and non-responders at baseline. To this end, a longitudinal epigenome-wide association study was conducted as part of the CHronification And Reversibility of Migraine (CHARM) clinical trial. Blood was taken from patients with chronic migraine (n = 98) at baseline and after a 12-week medication withdrawal period. Treatment responders, defined as patients with ≥ 50% reduction in monthly headache days (MHD), were compared with non-responders to identify DNAm changes associated with treatment response. Likewise, patients with ≥ 50% versus < 50% reduction in monthly migraine days (MMD) were compared. The results revealed that at the epigenome-wide significant level a longitudinal reduction in DNAm at an intronic CpG site within the *HDAC4* gene was associated with MHD response following withdrawal of acute medication. HDAC4 is highly expressed in the brain, plays a major role in synaptic plasticity, and modulates the expression and release of several neuroinflammation markers that are implicated in migraine pathophysiology. Investigating whether baseline DNAm status associated with treatment response identified lower baseline DNAm at a CpG site within *MARK3* that associated with MMD response at 12 weeks. *MARK3* encodes microtubule affinity regulating kinase 3. MARKs are serine/threonine kinases that regulate numerous cellular functions such as cell polarity, cell cycle progression, glucose metabolism and cytoskeletal dynamics.

Patients with migraine often suffer from other disorders, such as epilepsy, stroke, depression and sleep disorders, but their relationship with migraine is not well understood. Especially, a clear overview of neurological and psychiatric disorders that are comorbid with migraine with an overview of the role of genetics in this was lacking. Therefore, in **Chapter 4** a literature review was conducted on common neurological and psychiatric disorders that show comorbidity with migraine. To this end, the overlap was evaluated with respect to pathophysiological mechanisms and genetic architecture. It was found that depression and migraine show bidirectional causality and share genetic factors. Dysregulation of both hypothalamic and thalamic pathways have been suggested as a possible cause. Migraine patients have an increased risk for ischaemic stroke that likely involves spreading depolarization. Epilepsy and migraine exhibit a bidirectional relationship, where neuronal hyperexcitability serves as a crucial overlapping mechanism between the two conditions. Finally, concerning the

association between sleep disorders and migraine, hypothalamic dysfunction was suggested as the underlying mechanism.

In **Chapter 5** it was investigated how patients with hemiplegic migraine and monogenic migraine disorders (CADASIL and RVCL-S) respond to acute and preventive migraine treatment. In total n=78 patients with hemiplegic migraine (n=78), mutation carriers with CADASIL (n=114) and mutation carriers with RVCL-S (n=28) were included in the study. Migraine prevalence was 53% in CADASIL and 43% in RVCL-S mutation carriers. The most effective preventives with the least side effects for hemiplegic migraine were lamotrigine, valproate, topiramate, and acetazolamide. Valproate appeared most effective in CADASIL. Acetazolamide and propranolol showed efficacy in RVCL-S. Therefore, in hemiplegic migraine and CADASIL an alternative first-line treatment should be considered comparable with treatment that is more effective for migraine with aura. Regarding acute treatment, medications such as verapamil, nimodipine, ketamine, triptans, pulse steroids, and hypertonic saline have been utilized. If over-the-counter analgesics provide inadequate headache relief, triptans may be considered for the treatment of migraine. Patients with a history of stroke or transient ischemic attack (TIA) or a history of hemiplegic or basilar migraine are often not prescribed triptans out of fear of a stroke. In our cohort, triptans were used by 86 patients from these populations without vascular side effects.

Part II: Monogenic small vessel diseases – understanding vascular migraine models.

Calcitonin gene-related peptide (CGRP) plays a crucial role in migraine headaches. CGRP is a neuropeptide found in the nervous system, and its level increases during a migraine attack. The development of CGRP-targeted therapies represents a significant advancement in migraine treatment and has provided new options for individuals who do not respond well to traditional migraine medications. Anti-CGRP(-receptor) antibodies are designed to prevent migraine attacks. However, as CGRP is also involved in cardiovascular regulatory mechanisms, there have been concerns whether these drugs might give cardiovascular side-effects. Therefore, in **Chapter 6**, the importance of safety was discussed when treating migraine as part of a vascular monogenic syndrome. Treating patients with small vessel diseases such as the monogenic disorder CADASIL with these drugs until relevant safety data and long-term follow up results are available was deemed unwise. Alternative preventive migraine treatments in CADASIL may be acetazolamide, sodium valproate, lamotrigine, topiramate, verapamil, or flunarizine.

In **Chapter 7**, the role of endothelial-dependent and -independent vascular reactivity in the pathophysiology of RVCL-S and CADASIL, two cerebral small vessel diseases that are considered models for stroke, vascular dementia, and migraine was evaluated. Participants with either RVCL-S or CADASIL were investigated and compared with age-, BMI-, and sex-matched controls. Endothelial-dependent vascular reactivity was evaluated by flow-mediated vasodilatation and endothelial-independent vascular reactivity (i.e., primarily vascular smooth muscle cell function) measurement that assessed dermal blood flow in response to capsaicin application. Flow-mediated vasodilatation was decreased in participants with RVCL-S compared with controls, while no such difference was found for participants with CADASIL. Contrastingly, where vascular smooth muscle cell function was reduced in participants with CADASIL compared with controls, there appeared no large difference in vascular smooth muscle function in participants with RVCL-S. This discovery suggests that endothelial dysfunction is implicated in RVCL-S, while impairment in vascular smooth muscle relaxation is more likely to play a role in CADASIL.

The retina is considered an extension of the central nervous system and shares structural and functional similarities with the brain. Therefore, by studying the retina one can obtain unique insight into neurological conditions. Optical Coherence Tomography (OCT) is a non-invasive, user- and patient-friendly method that has shown potential to detect structural retinal changes in several neurodegenerative diseases. It provides a cross-sectional visualization of the optic disc and macula in which the different macular layers can be analyzed separately. In **Chapters 8 and 9** it was investigated whether D-CAA and RVCL-S lead to changes in retinal thickness using OCT. In **Chapter 8**, carriers of an *APP* mutation carriers, a mutation that leads to D-CAA, and controls were compared. Symptomatic mutation carriers were defined as having a history of ≥1 symptomatic intracerebral hemorrhage. The overall thickness of the peripapillary retinal nerve fiber layer (pRNFL) was decreased in symptomatic but not presymptomatic D-CAA mutation carriers compared with controls. Both pre- and symptomatic carriers had a thinner temporal-superior quadrant of the pRNFL compared to controls. Total macular volume or the thickness of the individual layers of the macula did not differ between mutation carriers and controls. **Chapter 9** explores the retinal structure in RVCL-S. As patients with RVCL-S do not only suffer from neurological complaints, but also develop visual impairment due to vascular retinopathy, this is especially an interesting avenue of biomarker research. For this study, *TREX1* mutation carriers, mutation leading to RVCL-S, and controls were examined by spectral domain OCT. *TREX1* mutation carriers had a decreased

peripapillary retinal nerve fiber layer and total macular volume compared with controls. With the exception of the temporal sector, the thickness of all peripapillary sectors was decreased in *TREX1* mutation carriers. Finally, the ganglion cell layer and inner plexiform layer were also thinner in *TREX1* mutation carriers. Notably, in nine out of twelve eyes with normal fundoscopic examination, retinal thinning was already detected. So, in both D-CAA and RVCL-S there is a thinning of the peripapillary retinal nerve fiber layer already before symptom onset. Therefore, OCT findings can potentially serve as early biomarkers for D-CAA and RVCL-S and other vascular retinopathies.

Next, the vasculature of the retina was examined in RVCL-S *in vivo*. In **Chapter 10**, OCT angiography (OCT-A) was used. OCT-A is precise noninvasive imaging method that can be used to evaluate the vascular density of the superficial capillary networks in the retina and the size of the foveal avascular zone (FAZ). Our findings demonstrated that RVCL-S causes an increase in the size of the FAZ in symptomatic patients with RVCL-S. Moreover, there is a decrease in vessel density in the superficial capillary networks in the foveal region of the retina in both pre- and symptomatic patients. In the future, newly developed precise objective instruments such as OCT(-A) may, therefore, provide important tools in determining disease activity for follow-up of (common) small vessel diseases.

Next, not only the morphology of the retina and the retinal vessels, but also their function was evaluated. In **Chapter 11**, it was determined whether retinal oxygen saturation differs between patients with RVCL-S and controls. Oxygen saturation and vessel caliber was assessed in patients with RVCL-S and controls using non-invasive technology (Oxymap T1 retinal oximeter). No differences were found in arterial oxygen saturation. However, the venular oxygen saturation of patients with RVCL-S was considerably higher than in control patients. In line with these findings, the arteriovenous difference was decreased in patients with RVCL-S. Moreover, patients with RVCL-S had a reduced vessel caliber in both arterial and venular vessels. The changes in venular oxygen saturation are likely due to decreased oxygen consumption resulting from retinal atrophy. Retinal oximetry may serve as a non-invasive and innovative tool for biomarker identification, aiding in the detection and prediction of complications associated with retinal vasculopathy in RVCL-S.

In **Chapter 12,** it was investigated the neuropsychiatric status of (pre)symptomatic mutation carriers of RVCL-S and whether MRI characteristics, white matter hyperintensities (WMH) and cerebrovascular reactivity (CVR), were related to cognitive impairment, psychiatric morbidity, and functional disability in RVCL-S. *TREX1*

mutation carriers and healthy controls were asked to conduct a comprehensive set of neuropsychological and functional tests. In addition, an MRI scan was performed on each participant. Additional to T1-weighted and FLAIR sequences, CVR was determined by CO_2 inhalation and simultaneous dual-echo arterial spin labeling (ASL) MRI acquisition. Patients with RVCL-S were analyzed separated into age groups above and below 50 years of age, as this is the assumed median age of onset of notable neurological deficits. Patients with RVCL-S aged <50 years demonstrated no cognitive deterioration compared to controls. However, they already reported more depressive symptoms in absence of other signs of psychopathology. An increase in WMH load was associated with worse executive functioning and decreased cerebrovascular reactivity (CVR) in gray and white matter with overall cognitive functioning in patients older than 50. Moreover, decreased CVR in gray matter was associated with impaired memory. Therefore, increased WMH volume and decreased cerebrovascular reactivity associate with cognitive impairment in RVCL-S but cannot be considered early markers of disease. Depressive symptoms, however, do occur early in the disease course.

Part III: Equality in headache research – tackling career barriers and harassment

While it is extremely important that researchers take diversity, inclusion and equality into account when designing their research, one should also take these topics into account when looking at the work environment. Career barriers and harassment are known to withhold people in medicine and science from reaching their full potential. The research performed in **Chapters 13 and 14** was aimed at assessing perceived career barriers holding back professionals in the headache field (**Chapter 13**) and whether harassment a frequently occurring problem in the headache field (**Chapter 14**). To this end, a global cross-sectional online survey was performed among professionals in the field of headache. In total 580 responders completed the survey. The first part of the survey was aimed at assessing perceived career barriers in four domains: (1) professional recognition, (2) opportunities in scientific societies, (3) clinical practice, and (4) salary and compensation. Gender was to be the most important perceived barrier in almost all domains. Furthermore, the participant's country of birth surfaced as a significant obstacle preventing their engagement in international scientific societies. Finally, it was noted that career barriers varied across world regions. Our conclusion emphasizes the critical need for global recognition and action regarding persistent and ongoing gender and country-of-origin disparities among headache professionals. This recognition should extend to areas such as recruitment, retention, opportunities, mentorships, sponsorships, and career advancement. The second part of the survey focused on workplace harassment. It was demonstrated that almost half of respondents had experienced harassment;

16.1% reported sexual harassment, 40.4% verbal harassment, and 5.5% physical harassment. Importantly, women were almost seven times more likely to experience sexual harassment than men. Headache professionals, especially women, commonly experience workplace harassment throughout their careers. Our study revealed the widespread nature of this issue and underscores the urgency of implementing strategies to cultivate a healthy and safe work environment.

Finally, **Chapter 15** provides a general discussion of this thesis with suggests possibilities for future research.

Nederlandse samenvatting

Dit proefschrift onderzoekt de genetische architectuur van migraine, waaronder familiaire hemiplegische migraine (FHM) en verwante monogene neurovasculaire aandoeningen zoals cerebrale autosomaal dominante arteriopathie met subcorticale infarcten en leukoencefalopathie (CADASIL), retinale vasculopathie met cerebrale leukoencefalopathie en systemische manifestaties (RVCL-S) en Dutch-type cerebrale amyloïde angiopathie (D-CAA). Dit onderzoek richt zich op de pathofysiologie en het klinische fenotypen van deze aandoeningen. Het uiteindelijk doel van dit proefschrift is bij te dragen aan de kennis over de pathofysiologie van migraine en ziekte van de kleine hersenvaten en om nieuwe behandelopties te identificeren. Dit proefschrift streeft er ook naar om gendergelijkheid in onderzoek te bevorderen. Deze gelijkheid is niet alleen een ethische verantwoordelijkheid, maar verhoogt ook de kwaliteit, validiteit en impact van onderzoeksresultaten. Het onderzoek is opgedeeld in drie delen. **Deel I** beschrijft studies die zich richten op de genetica van migraine en hoe genetica de behandeling van migraine kan beïnvloeden (**Hoofdstukken 2-5**). **Deel II** beschrijft studies over monogene vasculaire aandoeningen die geassocieerd zijn met migraine (**Hoofdstukken 6-12**). Tot slot richt **Deel III** zich op het identificeren van uitdagingen waarmee artsen en onderzoekers in het hoofdpijnveld te maken hebben (**Hoofdstukken 13 en 14**).

Deel I: Migraine – inzichten in genetica, epigenetica, comorbiditeiten en monogenetische factoren

Epidemiologisch onderzoek toont aan dat de genetische invloed bij migraine met aura groter is dan bij migraine zonder aura. **Hoofdstuk 2** geeft een overzicht van de genetische basis van migraine met aura. Diverse genetische benaderingen, waaronder genoomwijde associatiestudies (GWAS), hebben genetische factoren geïdentificeerd die het risico op migraine met aura vergroten. GWAS hebben varianten over het hele genoom getest zonder vooraf vastgestelde hypothesen. Hoewel GWAS voor de diagnose migraine al tientallen varianten had geïdentificeerd, was de zoektocht naar specifieke genvarianten voor migraine zonder aura of migraine met aura teleurstellend. Het onderzoeken van alle gegenotypeerde single-nucleotide polymorphisms (SNPs), zonder alleen de significante resultaten mee te nemen van eerdere studies, toonde aan dat migraine met en zonder aura waarschijnlijk genetisch meer op elkaar lijken dan dat zij van elkaar verschillen. Belangrijk is dat de meeste relevante genetische ontdekkingen met betrekking tot migraine met aura voortkwamen uit het onderzoeken van monogenetische syndromen waar migraine met aura een belangrijke uiting van de aandoening vormt (zoals FHM en CADASIL), wat wees op de rol van neurotransmissie en de vasculatuur in de pathofysiologie van

migraine. Door de cohortgrootte te vergroten, de bijdrage van zeldzame varianten te testen en epigenetische factoren te bestuderen, verwachten we ons begrip van de genetische architectuur van migraine, inclusief migraine met aura, te vergroten.

In **Hoofdstuk 3** werd onderzocht of veranderingen in DNA-methylatie (DNAm) optreden wanneer patiënten met chronische migraine en medicatieovergebruiks-hoofdpijn op behandeling reageren en of er al verschillen zijn tussen responders en non-responders voor aanvang van de behandeling. Daartoe werd een longitudinale epigenoomwijde associatiestudie uitgevoerd als onderdeel van de CHronification And Reversibility of Migraine (CHARM) studie. Bloed werd afgenomen van patiënten met chronische migraine (n = 98) bij baseline en na een periode van 12 weken zonder medicatie. Responders, gedefinieerd als patiënten met ≥ 50% vermindering in het aantal maandelijkse hoofdpijndagen (MHD), werden vergeleken met non-responders om DNAm-veranderingen geassocieerd met behandelingsrespons te identificeren. Tevens werd de methylatiestatus van patiënten met ≥ 50% versus < 50% vermindering in maandelijkse migrainedagen (MMD) vergeleken. De resultaten toonden aan dat een vermindering in DNAm op een intronisch CpG-locus binnen het *HDAC4* gen geassocieerd was met de MHD-respons na het stoppen van acute medicatie. HDAC4 wordt sterk tot expressie gebracht in de hersenen, speelt een belangrijke rol in synaptische plasticiteit en reguleert de expressie en afgifte van verschillende neuro-inflammatiemarkers die betrokken zijn bij de pathofysiologie van migraine. Methylatiestatus bij baseline hield liet een associatie zien met behandelrespons. Minder methylatie op een CpG-locus binnen *MARK3*, was geassocieerd met de MMD-respons na 12 weken. *MARK3* codeert voor microtubule-affiniteit regulerende kinase 3. MARKs zijn serine/threonine-kinasen die verschillende cellulaire functies reguleren, zoals cel polariteit, celcyclusprogressie, glucosemetabolisme en cytoskeletdynamiek.

Patiënten met migraine lijden vaak ook aan andere aandoeningen, zoals epilepsie, beroerte, depressie en slaapstoornissen, maar de relatie tussen deze aandoeningen en migraine is vaak nog onduidelijk. Een duidelijk overzicht van neurologische en psychiatrische stoornissen die samen voorkomen met migraine en de rol van genetica daarin ontbrak in de literatuur. Daarom werd in **Hoofdstuk 4** een literatuuronderzoek uitgevoerd naar veelvoorkomende neurologische en psychiatrische aandoeningen die comorbiditeit vertonen met migraine. In dit onderzoek werden de overeenkomsten in pathofysiologische mechanismen en genetische architectuur geëvalueerd. Depressie en migraine vertonen een bidirectionele causaliteit en delen genetische factoren. Dysregulatie van neuronale pathways, zowel hypothalamische als thalamische, is een mogelijke gezamenlijke oorzaak. Patiënten met hebben een verhoogd risico op ischemische beroerte, waarbij waarschijnlijk een spreidende depolarisatie betrokken is. Epilepsie en migraine hebben ook een bidirectionele relatie, waarbij neuronale

hyperexciteerbaarheid als cruciaal overlappend mechanisme wordt gezien. Tot slot werd, met betrekking tot de associatie tussen slaapstoornissen en migraine, hypothalamische disfunctie gesuggereerd als onderliggend mechanisme.

In **Hoofdstuk 5** werd onderzocht hoe patiënten met hemiplegische migraine en monogenetische migraine-aandoeningen (CADASIL en RVCL-S) reageren op acute en preventieve migrainebehandelingen. In totaal werden 78 patiënten met hemiplegische migraine, 114 patiënten met CADASIL, en 28 patiënten met RVCL-S geïncludeerd. Migraineprevalentie was 53% bij CADASIL en 43% bij RVCL-S mutatiedragers. De meest effectieve preventieve behandelingen met de minste bijwerkingen voor hemiplegische migraine waren lamotrigine, valproaat, topiramaat en acetazolamide. Valproaat bleek het meest effectief bij CADASIL, terwijl acetazolamide en propranolol werkzaam waren bij RVCL-S. Daarom zou bij hemiplegische migraine en CADASIL een alternatieve eerstelijnsbehandeling moeten worden overwogen. Voor acute behandelingen werden geneesmiddelen zoals verapamil, nimodipine, ketamine, triptanen, pulssteroïden en hypertonische zoutoplossing gebruikt. Echter, wanneer vrij verkrijgbare pijnstillers onvoldoende hoofdpijnverlichting bieden, kunnen triptanen worden overwogen voor de behandeling van migraine bij deze patiënten. Patiënten met een voorgeschiedenis van beroerte, of een voorgeschiedenis van hemiplegische of basilaire migraine krijgen vaak geen triptanen voorgeschreven uit angst vanuit de behandelaar voor een beroerte. In onze cohorte gebruikten 86 patiënten uit deze groepen triptanen zonder vasculaire bijwerkingen.

Deel II: Monogenetische ziekte van de kleine bloedvaten – het begrijpen van vasculaire migraine modellen

Calcitonine-gen-gerelateerd peptide (CGRP) speelt een cruciale rol bij migraine. CGRP is een neuropeptide en tijdens een migraine aanval is de CGRP-spiegel verhoogd. De ontwikkeling van therapieën gericht op CGRP waren een doorbraak in de behandeling van migraine en leiden tot nieuwe behandelopties. Anti-CGRP(-receptor)-antilichamen zijn ontworpen om migraineaanvallen te voorkomen. Omdat CGRP echter ook betrokken is bij de regulatie van cardiovasculaire mechanismes, bestaat er bezorgdheid over mogelijke cardiovasculaire bijwerkingen. Daarom werden in **Hoofdstuk 6** zorgen over de veiligheid van CGRP-gerichte behandelingen besproken wanneer migraine wordt behandeld bij een patiënt met een vasculair monogenetisch syndroom. Het werd onverstandig geacht om patiënten met ziekten van de kleine bloedvaten, zoals de monogenetische aandoening CADASIL, met deze geneesmiddelen te behandelen totdat relevante veiligheidsgegevens en resultaten van lange termijn studies beschikbaar zijn. Alternatieve preventieve behandelingen werden daarom aanbevolen.

In **Hoofdstuk 7** werd de rol van endotheel-afhankelijke en endotheel-onafhankelijke vasculaire reactiviteit in de pathofysiologie van RVCL-S en CADASIL, twee ziekte waarbij de kleine bloedvaten van de hersenen aangedaan zijn en die worden beschouwd als modellen voor beroerte, vasculaire dementie en migraine, onderzocht. Deelnemers met RVCL-S of CADASIL werden geïncludeerd en vergeleken met leeftijd-, BMI- en geslacht-gepaarde controles. Endotheel-afhankelijke vasculaire reactiviteit werd onderzocht door middel van flow-gemedieerde vasodilatatie, terwijl de endotheel-onafhankelijke vasculaire reactiviteit (d.w.z. voornamelijk de functie van vasculaire gladde spiercellen) werd gemeten door de dermale bloedstroom te beoordelen als reactie op capsaïcine. De flow-gemediëerde vasodilatatie was verminderd bij RVCL-S deelnemers in vergelijking met de controles, terwijl er voor deelnemers met CADASIL geen verschil werden gevonden. Daarentegen was de functie van vasculaire gladde spiercellen verminderd bij deelnemers met CADASIL in vergelijking met de controles, maar leek er geen groot verschil te zijn tussen deelnemers met RVCL-S en controles. Deze ontdekking suggereert dat endotheel disfunctie betrokken is bij RVCL-S, terwijl de functie van vasculaire gladde spiercellen aangedaan is bij CADASIL.

De retina wordt beschouwd als een voortzetting van het centrale zenuwstelsel en vertoont structurele en functionele overeenkomsten met de hersenen. Door de retina te bestuderen, kan men unieke inzichten verkrijgen in neurologische aandoeningen. Optische Coherentie Tomografie (OCT) is een niet-invasieve, gebruiksvriendelijke methode die structurele veranderingen in de retina heeft gedetecteerd bij verschillende neurodegeneratieve ziekten. Hiermee creëert men een visualisatie via dwarsdoorsnede van de papil en de macula, waarin de verschillende lagen van het netvlies afzonderlijk kunnen worden geanalyseerd. In **Hoofdstukken 8** en **9** werd onderzocht of D-CAA en RVCL-S leiden tot veranderingen in de retinadikte met behulp van OCT. In **Hoofdstuk 8** werden dragers van een APP-mutatie, de mutatie die leidt tot D-CAA, en controles vergeleken. Aangedane patiënten werden gedefinieerd als personen met ≥1 symptomatische intracerebrale bloeding. De peripapillaire zenuwvezellaag (pRNFL) in zijn geheel beschouwd was dunner bij symptomatische *APP*-mutatiedragers in vergelijking met de controles. Dit verschil werd niet gevonden presymptomatische mutatiedragers. Zowel presymptomatische als symptomatische dragers hadden een dunner temporale-superieure kwadrant van de pRNFL vergeleken met de controles. Het totale maculaire volume of de dikte van de individuele lagen van de macula verschilden niet tussen mutatiedragers en controles. **Hoofdstuk 9** onderzocht de structuur van het netvlies in RVCL-S. Aangezien patiënten met RVCL-S niet alleen lijden aan neurologische klachten, maar ook visuele beperkingen ontwikkelen als gevolg van vasculaire retinopathie, is dit potentieel een interessante biomarker voor ziekte. Voor deze studie werden TREX1-mutatiedragers,

mutatie die leiden tot RVCL-S, en controles onderzocht met behulp van spectrale domein OCT. TREX1-mutatiedragers hadden een dunnere pRNFL en een verminderd totaal maculair volume vergeleken met controles. Met uitzondering van de temporale kwadrant was de dikte van alle peripapillaire kwadranten verminderd bij TREX1-mutatiedragers. Ten slotte waren de ganglioncel laag en de innerplexiforme laag ook dunner bij TREX1-mutatiedragers. Opmerkelijk is dat in negen van de twaalf ogen met een normaal fundoscopische onderzoek al een dunnere retina werd vastgesteld. Dus, zowel bij D-CAA als bij RVCL-S is er een dunnere peripapillaire zenuwvezellaag al vóór het begin van de symptomen. Daarom kunnen de OCT- bevindingen mogelijk dienen als vroege biomarkers voor D-CAA, RVCL-S en andere vasculaire retinopathieën.

Vervolgens werd de vasculatuur van de retina in vivo onderzocht in RVCL-S patiënten. In **Hoofdstuk 10** werd gebruik gemaakt van OCT-angiografie (OCT-A). OCT-A is een nauwkeurige niet-invasieve beeldvormingsmethode die kan worden gebruikt om de vasculaire dichtheid van de oppervlakkige capillaire netwerken in de retina en de grootte van de foveale avasculaire zone (FAZ) te onderzoeken. Onze bevindingen toonden aan dat RVCL-S leidt tot een toename van de grootte van de FAZ bij symptomatische patiënten. Bovendien is er een afname van de vaatdichtheid in de oppervlakkige capillaire netwerken in de foveale regio van de retina, zowel bij presymptomatische als bij symptomatische patiënten. In de toekomst kunnen nieuw ontwikkelde nauwkeurige objectieve instrumenten zoals OCT(-A) waarschijnlijk bijdragen bij het vinden van biomarkers van ziekteactiviteit voor de follow-up van (veel voorkomende) kleine vaatziekten.

Vervolgens werd niet alleen de morfologie van de retina en de retinale bloedvaten onderzocht, maar ook hun functie. In **Hoofdstuk 11** werd bepaald of de zuurstofsaturatie van de retina verschilt tussen patiënten met RVCL-S en controles. De zuurstofsaturatie en het vaatkaliber werden beoordeeld met behulp van niet-invasieve technologie (Oxymap T1 retinale oxymeter). Er werden geen verschillen gevonden in de arteriële zuurstofsaturatie. De veneuze zuurstofsaturatie van patiënten met RVCL-S was echter aanzienlijk hoger dan bij controle patiënten. In lijn met deze bevindingen was het arterioveneuze verschil verlaagd bij patiënten met RVCL-S. Bovendien hadden patiënten met RVCL-S een verminderd vaatkaliber in zowel arteriële als veneuze vaten. De veranderingen in de veneuze zuurstofsaturatie zijn waarschijnlijk het gevolg van een verminderde zuurstofconsumptie als gevolg van retinale atrofie. Bevindingen van retinale oxymetrie kunnen waarschijnlijk dienen als niet-invasief en innovatief biomarkers, wat kan helpen bij de detectie en voorspelling van complicaties die verband houden met retinale vasculopathie bij RVCL-S.

In **Hoofdstuk 12** werd onderzocht wat voor neuropsychiatrische klachten (pre) symptomatische RVCL-S patiënten ervaren en of de MRI markers, witte stof hyperintensiteiten (WMH) en cerebrovasculaire reactiviteit (CVR), gerelateerd waren aan cognitieve achteruitgang, psychiatrische morbiditeit en functionele beperkingen bij RVCL-S. Dragers van de TREX1-mutatie en gezonde controles werden gevraagd een uitgebreide reeks neuropsychologische en functionele tests uit te voeren. Daarnaast werd er bij elke deelnemer een MRI-scan gemaakt. Naast T1-gewogen en FLAIR-sequenties werd de CVR bepaald door CO_2-inhalatie en gelijktijdige dual-echo arteriële spin labeling (ASL) MRI-acquisitie. Patiënten met RVCL-S werden geanalyseerd in leeftijdsgroepen boven en onder de 50 jaar, aangezien dit de veronderstelde mediane leeftijd van het begin van merkbare neurologische klachten is. Patiënten met RVCL-S jonger dan 50 jaar vertoonden geen cognitieve achteruitgang vergeleken met controles. Ze rapporteerden echter al meer depressieve symptomen in afwezigheid van andere tekenen van psychopathologie. Een toename van de WMH werd geassocieerd met slechtere executieve functies en een verminderde cerebrovasculaire reactiviteit (CVR) in de grijze en witte stof met een algehele impact op de cognitieve functie bij patiënten ouder dan 50 jaar. Bovendien werd een verminderde CVR in de grijze stof geassocieerd met een aangetast geheugen. Daarom zijn een verhoogd WMH-volume en verminderde cerebrovasculaire reactiviteit geassocieerd met cognitieve achteruitgang bij RVCL-S, maar kunnen ze niet als vroege markers van de ziekte worden beschouwd. Depressieve symptomen komen echter wel vroeg in het ziekteproces voor.

Deel III: Gelijkheid in hoofdpijnonderzoek – aanpakken van carrièrebarrières en pesterijen en intimidatie

Hoewel het van uiterst belang is dat onderzoekers diversiteit, inclusie en gelijkheid in overweging nemen bij het ontwerpen van hun onderzoek, moeten deze onderwerpen ook worden meegenomen bij het evalueren van de werkomgeving. Loopbaanbelemmeringen en intimidatie kunnen mensen in de geneeskunde en wetenschap weerhouden van het volledig benutten van hun mogelijkheden. Het onderzoek uitgevoerd in **Hoofdstukken 13** en **14** was gericht op het beoordelen van de ervaren loopbaanbelemmeringen die professionals in het hoofdpijnveld tegenhouden (**Hoofdstuk 13**) en of intimidatie een vaak voorkomend probleem is in het hoofpijnveld (**Hoofdstuk 14**). Hiertoe werd een wereldwijde cross-sectionele online enquête uitgevoerd onder professionals op het gebied van hoofdpijn. In totaal voltooiden 580 respondenten de enquête. Het eerste deel van de enquête was gericht op het beoordelen van ervaren loopbaanbelemmeringen in vier domeinen: (1) professionele erkenning, (2) kansen in wetenschappelijke verenigingen, (3) klinische praktijk en (4) salaris en compensatie. Geslacht bleek de belangrijkste

ervaren belemmering te zijn in bijna alle domeinen. Verder bleek het geboorteland van de deelnemers een significante belemmering te zijn die hun betrokkenheid bij internationale wetenschappelijke verenigingen verhinderde. Tenslotte werd opgemerkt dat loopbaanbelemmeringen varieerden tussen wereldregio's. Onze conclusies benadrukken de noodzaak van wereldwijde erkenning en actie met betrekking tot aanhoudende en voortdurende ongelijkheden op basis van geslacht en land van herkomst onder hoofdpijnprofessionals. Deze erkenning moet zich ook uitstrekken tot gebieden zoals werving, kansen, mentorschap, sponsoring en loopbaanontwikkeling. Het tweede deel van de enquête richtte zich op intimidatie op de werkvloer. Er werd aangetoond dat bijna de helft van de respondenten intimidatie had ervaren; 16,1% meldde seksuele intimidatie, 40,4% verbale intimidatie en 5,5% fysieke intimidatie. Vrouwen hadden bijna zeven keer meer kans om seksuele intimidatie te ervaren dan mannen. Hoofdpijnprofessionals, vooral vrouwen, ervaren vaak intimidatie op de werkvloer gedurende hun carrière. Onze studie onthulde de omvang van dit probleem en onderstreepte de urgentie van het implementeren van strategieën om een gezonde en veilige werkomgeving te cultiveren.

Tenslotte bevat **Hoofdstuk 15** een algemene discussie over de resultaten van dit proefschrift en suggesties voor toekomstig onderzoek.

List of abbreviations

AES	Apathy Evaluation Scale
Aβ	Amyloid beta
AON	antisense oligonucleotides
Ang-2	Angiopoietin-2
ArtSat	Arterial saturation
ArtDiam	Arterial diameter
ASL	Arterial spin labelling
AV-diff	Arteriovenous difference
AU	Arbitrary unit
BBB	blood-brain-barrier
BI	Barthel Index of Activities of Daily living
BMI	Body mass index
BOLD	Blood oxygen level dependent
BRB	Blood-retinal-barrier
CADASIL	Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
CAMCOG-R	Cambridge Cognitive Examination-Revised
CARASIL	Cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy
CBF	Cerebral blood flow
CES-D	Center for Epidemiological Studies Depression Scale
CGAS	Candidate gene associations studies
CGRP	Calcitonin Gene-Related Peptide
CNS	Central nervous system
CNV	Copy number variation
CSD	cortical spreading depolarization
CSF	Cerebral spinal fluid
CVR	Cerebrovascular reactivity
cSVD	Cerebral small vessel disease
DBF	Dermal blood flow
D-CAA	Dutch-type hereditary cerebral amyloid angiopathy
DGIdb	Drug-Gene Interaction database
DNAm	DNA methylation
DSST	Digit symbol substitution test
eQTL	Expression quantitative trait locus
EMG	Electromyography
EWAS	Epigenome-wide association studies

FAB	Frontal assessment battery
FA(G)	Fluorescein angiography
FASPS	Familial advanced sleep-phase syndrome
FAZ	Foveal avascular zone
FHM	Familial hemiplegic migraine
FMD	Flow-mediated dilatation
GABA	Gamma-aminobutyric acid
GCL	Ganglion cell layer
GEE	Generalized estimating equations
GEFS+	Generalized epilepsy with febrile seizures
GFAP	Glial fibrillary acidic protein
GM	Grey matter
GOM	Granular osmiophilic material
GWAS	Genome-wide association studies
HADS-D	Hospital anxiety and depression scale-depression
HCHWA-D	Hereditary cerebral hemorrhage with amyloidosis-dutch type
HHT	Hereditary hemorrhagic telangiectasia
HM	Hemiplegic migraine
HPA	Hypothalamic–pituitary–adrenal
HR	Homologous repair
HSPCs	Hematopoietic stem and progenitor cells
ICH	Intracerebral hemorrhages
ICHD	International Classification of Headache Disorders
IHS	International Headache Society
ILM	Internal limiting membrane
INL	Inner nuclear layer
IOP	Intraocular pressure
IPL	Inner plexiform layer
IVT	Intravenous thrombolytic therapy
MA	Migraine with aura
MC	Mutation carriers
MELAS	Mitochondrial encephalopathy, lactic acidosis and stroke-like episodes
MHD	Monthly headache days
MMD	Monthly migraine days
MMSE	Mini-Mental State Examination
MO	Migraine without aura
MR	Mendelian randomization
mRNFL	Macular retinal nerve fiber layer
MRS	Magnetic resonance spectroscopy

mRS	modified Rankin Scale
mtDNA	Mitochondrial DNA
NfL	Neurofilament light chain
NGS	Next generation sequencing
NHEJ	Non-homologous end joining
NPI	Neuropsychiatric Inventory
NTG	Nitroglycerin
OCT	Optical coherence tomography
OCT-A	Optical coherence tomography - angiography
ONL	Outer nuclear layer
OPL	Outer plexiform layer
pRNVL	Peripapillary retinal nerve fiber layer
PVO	Patent foramen ovale
RLS	Restless leg syndrome
ROSAH	Retinal dystrophy, optic nerve edema, splenomegaly, anhidrosis and migraine headache
RPE	Retinal pigment epithelium
RVCL-S	Retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations
SD-OCT	Spectral Domain optical coherence tomography
sgRNA	Single guide RNA
SHM	Sporadic hemiplegic migraine
SLE	Systemic lupus erythematosus
SMEI	Severe myoclonic epilepsy of infancy
SNP	single nucleotide polymorphism
SNRI	Selective norepinephrine reuptake inhibitors
SSRI	Selective serotonin reuptake inhibitor
SVD	Small vessel disease
SUDEP	Sudden unexpected death in epilepsy
TIA	Transient ischemic attack
TMT	Trail-Making Test
TMT	total macular thickness
TMV	Total macular volume
VEGF	Vascular endothelial growth factor
VenSat	Venous saturation
VenDiam	Venular diameter
VSMC	Vascular smooth muscle cell
vWF	Von Willebrand factor
WES	Whole exome sequencing

WM	White matter
WMH	White matter hyperintensities
WMS	Wechsler Memory Scale
WVLT	Word Verbal Learning Test

List of Publications

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Curriculum vitae

Irene de Boer was born on the 30th of January 1991 in Blaricum. She graduated secondary school at "Laar en Berg" in Laren (cum laude). In 2009, she started her study Medicine at Leiden University. As part of the Leiden University "Program for Excellent Students", she combined her study of medicine with a Biomedical Science degree starting in 2011 and completed the Honours college program during her Medicine Bachelor. In 2016 she obtained her Master degree in Medicine and in 2017 her Master degree in Biomedical Science (cum laude). From 2017 to 2024 she worked as a PhD candidate and clinical research fellow at the department of Neurology of Leiden University Medical Center (LUMC), supervised by Prof. dr. G.M. Terwindt, Prof. dr. A.M.J.M. van den Maagdenberg and dr. I.C. Notting. The results of this research are described in this thesis. Here, she combined her research with her work at the headache outpatient clinic and the cerebral hereditary angiopathy outpatient clinic. She has been involved in several successful grand applications (International Retinal Research Foundation (2019), Clayco foundation (2023)) and received a personal Dekker grant from the Dutch Heart Foundation in 2021. This opportunity allowed her to extend her time at the department of Neurology at the LUMC. She is passionate about education and in 2023 she received her University Teaching Qualification. Finally, she is not only a fervent advocate of research, she also strongly believes in the importance of diversity and inclusion, and creating equal opportunities in the workforce. The passion led to her joining the International Headache Society as an active member of the Juniors Group, of whom she is now the chair, and the Women's Leadership Forum and Science and Research committee. In addition to her work as a research fellow, she started in 2024 as a resident at the department of Clinical Genetics of the LUMC where she is currently still working and now training to become a clinical geneticist.

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