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Unraveling the genetic architecture of migraine: exploring the vascular components

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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*, *MRV11*, *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- κ B,^{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 sparse 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 disorders¹⁸⁻²⁰, 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} endometriosis²⁵ 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-HT_{1B/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.

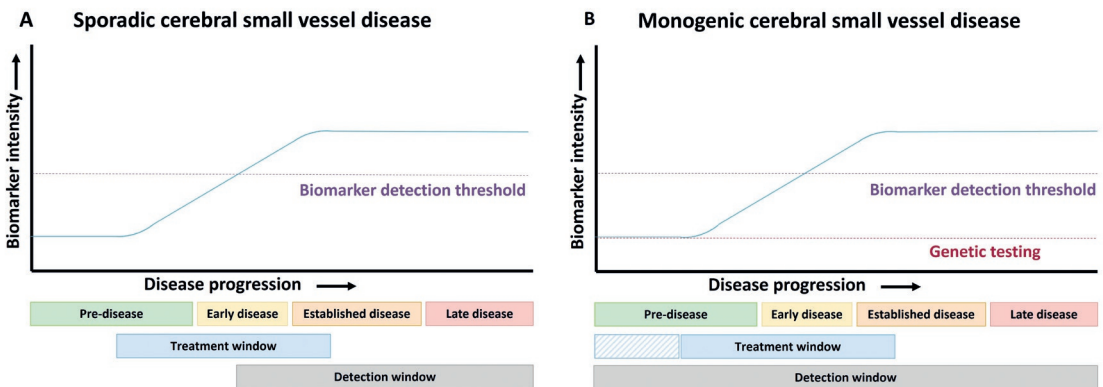


Figure 1. Studying mechanisms causing cerebral small vessel diseases.

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 certainly 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 vasodilatation 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 CO₂ cerebrovascular reactivity testing demonstrated impaired vascular reactivity.⁷⁰ CO₂ 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 than 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 ($A\beta$) 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 A β 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 A β is initially deposited within the outer basement membranes surrounding VSMCs, the basement membranes of the endothelium remain largely unaffected.⁷⁴ Clearance of A β 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 $A\beta$, 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 affected inevitably will affect other elements within the neurovascular or gliovascular unit. Monogenic disease models offer a unique opportunity to study these sequential processes.

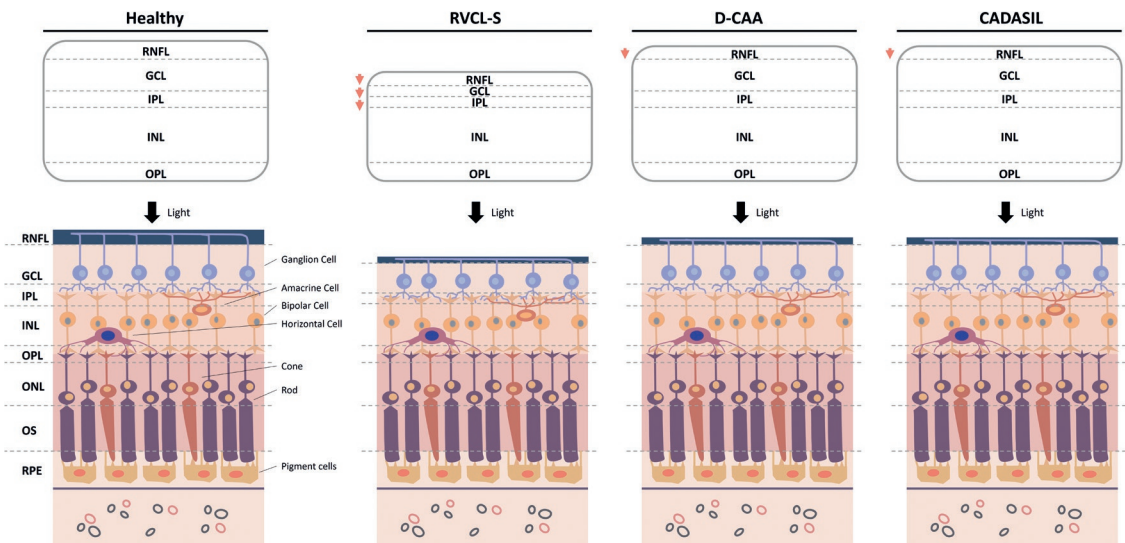


Figure 2. Schematic of the layers of the healthy retina and the hypothetical changes in the retina in RVCL-S, D-CAA and CADASIL.

In RVCL-S, thinning of the RNFL, GCL and 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.

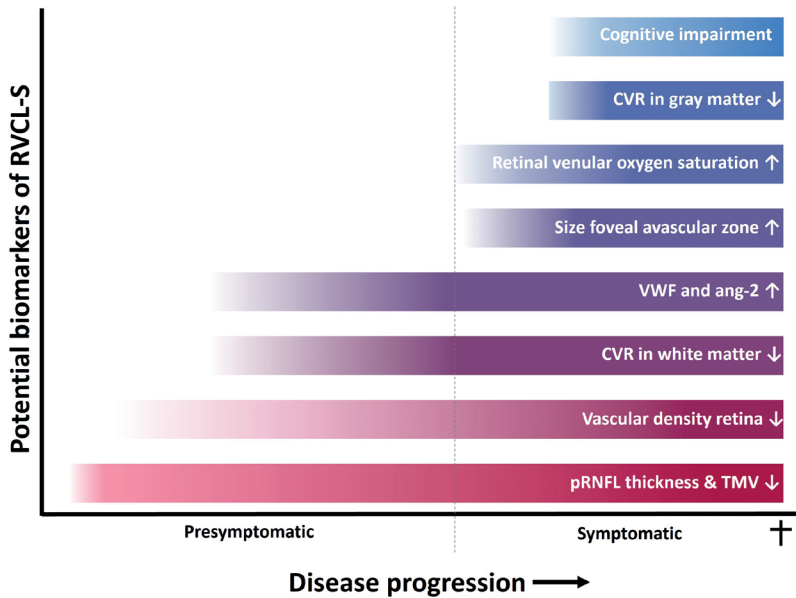


Figure 3. Hypothesis of biomarker progression in RVCL-S.

Angiopietin-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.

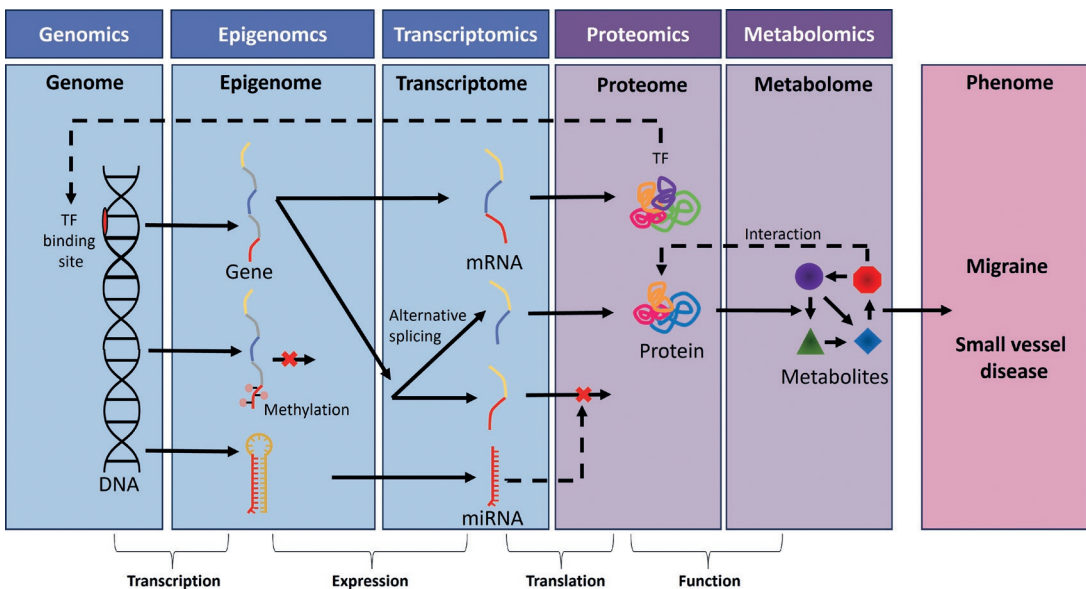


Figure 4. Overview of omics fields.

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 mononuclear 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 underlying 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 A β 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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