9

General discussion

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

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

Mutation analysis in monogenic syndromes with relevance to migraine

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

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

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

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

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

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

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

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

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

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

Validation of questionnaires for genetic studies of primary headache disorders

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

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

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

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

Genetic studies in complex migraine and cluster headache

Recent findings from genetic studies in migraine

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

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

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

Linkage and candidate gene association studies in common migraine

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

Genome-Wide Association Studies in Common Migraine

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

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

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

Future genetic studies in migraine

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

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

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

Recent findings from genetic studies in cluster headache

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

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

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

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

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

Investigating Comorbid Depression in Migraine Genetics

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

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

Investigating Comorbid Cardiovascular Disease in Migraine Genetics

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

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

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

Future perspectives

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

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

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

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

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

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

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