Monogenic models of migraine : from clinical phenotypes to pathophysiological mechanisms

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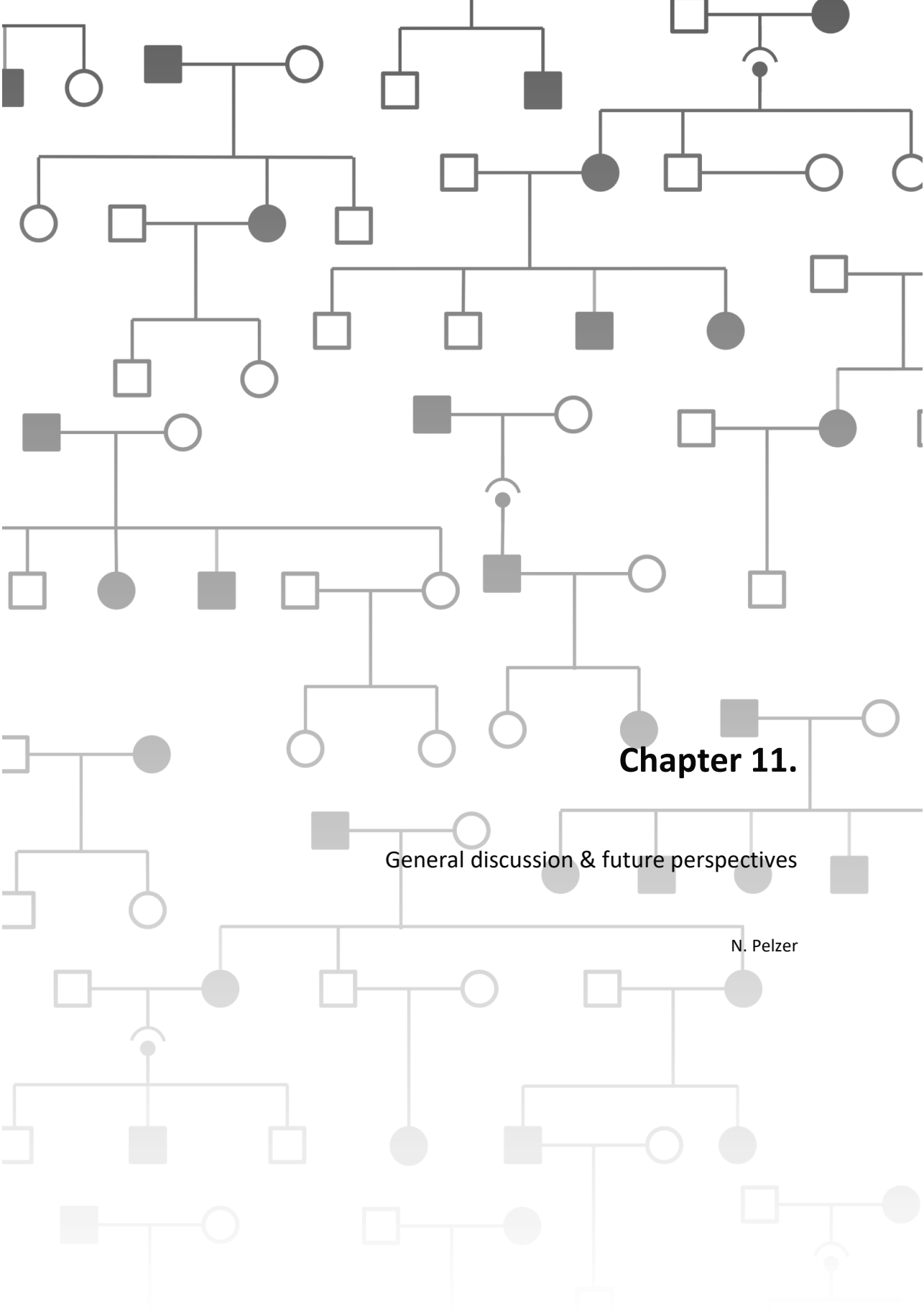

Chapter 11.

General discussion & future perspectives

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In this thesis clinical phenotypes and pathophysiology of rare monogenic and common complex forms of migraine are investigated. The monogenic syndromes may be considered as models for common forms of migraine, as the clinical symptomatology and pathophysiology overlap. The described studies focus on two monogenic syndromes that are associated with migraine: hemiplegic migraine (HM) and retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations (RVCL-S), involving various systemic and cerebral symptoms, including migraine.

Part I: Hemiplegic migraine – a neuronal and glial monogenic migraine model

Clinically, HM is defined as a subtype of migraine with aura that includes motor weakness in addition to visual, sensory and dysphasic aura symptoms.[1] The familial form of hemiplegic migraine (FHM) is characterised by autosomal dominant inheritance. Linkage studies in FHM families have led to the identification of three genes: *CACNA1A* (FHM1), *ATP1A2* (FHM2) and *SCN1A* (FHM3).[2–4] Genetic screening in patients without familial occurrence, sporadic hemiplegic migraine (SHM), revealed mutations in the same genes, many of which arose *de novo*.[1,5,6] Mutation screening of HM genes has diagnostic value in clinical practice, as it allows genetic confirmation of a clinical diagnosis. Moreover, genotype-phenotype correlations may help to unravel disease pathophysiology. In this thesis, the clinical and genetic spectra of HM were studied, focussing on their relevance to common forms of migraine and to implications for clinical practice.

Clinical spectrum of HM

Due to the rarity of HM, its clinical spectrum is largely deferred from case reports and (small) family studies. HM is defined in the International Classification of Headache Disorders (ICHD).[1] Recently, the ICHD criteria were adapted, including extension of the 'typical' duration of motor auras from <24 hours in the ICHD-2 to <72 hours (and possibly even longer, lasting weeks) in the ICHD-3, beta version.[1,7] Classification in HM is challenging and important, as an atypical presentation strongly raises the suspicion of differential diagnoses that can be life-threatening and need different treatment, such as stroke, epilepsy or meningitis.[8] **Chapter 2** presents an overview of diagnostic procedures often needed in the acute phase of a (first) HM episode to exclude other conditions. Diagnostic procedures can reveal abnormalities in HM, such as diffuse swelling of the affected hemisphere on brain MRI, asymmetric slowing of background activity on electroencephalography (EEG) or pleocytosis in cerebrospinal fluid (CSF).

FHM/SHM type 1

Additional symptoms may be associated with specific genetic subtypes of HM, leading to a wider HM phenotype. For example, cerebellar ataxia with progressive cerebellar atrophy has been reported in many HM patients with *CACNA1A* mutations.[9,10] Cerebellar atrophy seems almost exclusive to SHM1/FHM1 as it was reported only once in FHM2 (*ATP1A2*) and never in FHM3 (*SCN1A*).[11] Other striking clinical features in FHM1/SHM1 are seizures, coma, and cerebral oedema during HM episodes, which was most severe in patients with the p.Ser218Leu (S218L) *CACNA1A* mutation.[12,13] Although these symptoms, either separately or combined, were subsequently found in some patients with mutations in *ATP1A2* and *SCN1A*, episodes with fatal consequences have only been reported with the S218L *CACNA1A* mutation.[12,14–17]

FHM/SHM type 2

In **chapter 4** the long-term follow-up of an FHM family with a novel *ATP1A2* mutation was described. Patients suffer from recurrent HM episodes with impaired consciousness and in two diffuse swelling of the affected hemisphere was observed on ictal brain MRI, similar to severe episodes associated with the S218L *CACNA1A* mutation, except for the absence of seizures. **Chapter 4** illustrates the difficulties of classifying possible seizures. Several patients reported symptoms suggestive of epilepsy, but ictal EEGs only showed asymmetric slowing of background activity, and no clearly epileptiform abnormalities (except in a patient with possible lissencephaly). It can be hypothesised that seizures in HM are not necessarily epileptic in nature, but represent an entity occurring specifically in relation to cortical spreading depolarisation (CSD), which is considered the phenomenon underlying the migraine aura and shares features with epileptic activity.[18]

The FHM2 family described in **chapter 6** was previously reported to show partial co-segregation of benign familial infantile convulsions (BFIS) and HM with the *ATP1A2* mutation.[19] However, after the discovery of *PRRT2* as causative gene for BFIS,[20] it was concluded that the BFIS phenotype in this family likely is caused by the newly identified *PRRT2* mutation, whereas the HM phenotype is caused by the *ATP1A2* mutation. Thus, we revoked the association between the *ATP1A2* mutation and BFIS.

Altogether it should be encouraged to perform ictal EEGs in HM patients. Unfortunately, routine clinical EEGs cannot detect CSDs, but direct-current EEG or direct-current magnetoencephalography might have a role in clinical practice in the future.[21,22] Such technologies may perhaps also assess occurrence of subcortical spreading depolarisation (towards the brainstem) in HM patients with brainstem auras, as observed in transgenic mice harbouring the S218L *CACNA1A* mutation.[23]

Chapter 4 illustrates issues associated with identifying cerebellar symptoms in HM. In some FHM2 families interictal symptoms suggesting cerebellar dysfunction (such as nystagmus) were found but cerebellar atrophy was not proven with MRI.[24,25] While paroxysmal ataxia has been described more often in FHM2,[11,25–27] symptoms directly caused by hemiparesis, so called ataxic hemiparesis[28] or hemiparaesthesia are possibly misinterpreted as cerebellar symptoms. This likely also occurred in FHM2 family members described in **chapter 4** that reported 'uncontrollable movements of limbs' but only during HM attacks in limbs affected by paresis and paraesthesia. In such cases one should look for other symptoms of cerebellar involvement and describe symptoms precisely (e.g. avoid 'clumsiness' or 'unsteady gait') to allow accurate localisation. The thalamus may also be considered as a localisation of ataxic symptoms,[29] which is interesting given other suggestions of thalamic involvement in migraine.[30]

FHM/SHM type 3

With only few families and a single sporadic case identified to date, the FHM3/SHM3 phenotype is difficult to define.[4,6,17,31–36] In **chapter 5** two FHM3 families harbouring novel *SCN1A* mutations are described, in which mutation carriers suffer from pure HM, as in five other described FHM3 families.[4,32,35] Two previously identified *SCN1A* mutations were associated with HM and 'elicited repetitive daily blindness' (ERDB), which was suggested to be caused by retinal spreading depolarisation.[31,33] To date, additional ERDB patients have not been reported, not even with the same mutation.[36] Most prominently, FHM3 has been associated with epilepsy. *SCN1A* is a well-known epilepsy gene in which many mutations have been shown to cause Dravet syndrome (also known as severe myoclonic epilepsy of infancy (SMEI)) or the milder generalised epilepsy with febrile seizures (GEFS+).[37] In three FHM3 families patients reported seizures apart from their HM attacks.[16,33,34] The p.Thr1174Ser (T1174S) *SCN1A* mutation was found in a family in which some members had benign occipital epilepsy and others HM.[34] To support occurrence of HM in patients with this *SCN1A* mutation another family was referred to,[38] which, however, did not display motor weakness but an 'ataxic migraine syndrome' and myoclonus in one patient. Another phenotypic discordance was reported for the p.Leu263Val (L263V) *SCN1A* mutation in a family with co-occurring HM and epilepsy but also subjects with HM but no epilepsy.[16,17] Whether the phenotype of *SCN1A* mutation carriers mainly varies within epilepsy and HM or whether ataxia may also be involved remains unclear.

FHM & SHM – other loci

In many patients who fulfil the ICHD-3 criteria[1] for HM no causative mutation is found in *CACNA1A*, *ATP1A2* or *SCN1A*. In **chapter 7**, phenotypes of these patients are explored. Despite limited numbers of patients, phenotypic differences were identified between HM patients with and without a

confirmed mutation in one of the known HM genes. From our study it became apparent that HM patients with a confirmed mutation more often displayed severe motor auras, brainstem auras (most notably impaired consciousness), and triggering of attacks by (minor) head trauma. Other notable features in these patients were confusion and fever during an attack, mental retardation, progressive chronic ataxia, and CSF pleocytosis. Seizures (during or outside HM attacks) and ictal hemispheric swelling on brain MRI were found in >10 patients with a confirmed mutation, but only in one without such mutation. The milder motor auras in patients without a confirmed mutation may raise the suspicion that severe sensory auras were confused for HM. Although most patients are never observed by a physician during an HM attack, we only included HM patients for whom a clear description of motor auras was available. Overall, HM patients without confirmed mutations had a milder phenotype with less additional features, i.e. more similar to migraine with aura, which may constitute a separate clinical subtype between migraine with aura and HM on the hypothesised migraine spectrum.[39]

Genetic spectrum of HM

The genetic spectrum of HM is explored in **chapters 3–5** and **chapter 7**, in which HM patients with novel mutations in *CACNA1A*, *ATP1A2* and *SCN1A* are described.

CACNA1A & HM

CACNA1A encodes the α1 subunit of $Ca_V2.1$ (P/Q-type) voltage-gated calcium channels expressed on neuronal and neuroendocrine cells, especially prominent in the cerebellum.[40,41] This high cerebellar expression of *CACNA1A* likely underlies the cerebellar phenotype of FHM1/SHM1. Approximately 30 different *CACNA1A* mutations for familial HM have been described to date (see the Leiden Open Variation Database: http://grenada.lumc.nl/LOVD2/FHM/home).[42] Like the two novel mutations reported in **chapter 7** (p.Phe1509Tyr (F1509Y) and p.Phe1609Leu (F1609L)), *CACNA1A* mutations in HM are typically missense mutations, for which functional studies are consistent with a gain-of-function effect.[42,43] A rare deletion in *CACNA1A* with a presumed gain-of-function effect has been described in an HM patient with non-episodic progressive ataxia.[44] The gain of $Ca_V2.1$ channel function is hypothesised to cause an increased neuronal Ca^{2+} influx due to an increased channel open probability and channel activation at lower voltages and thereby increased neurotransmission.[43] In transgenic mice harbouring the p.Arg192Gln (R192Q) *CACNA1A* mutation a lowered threshold for CSD was observed, providing more direct evidence of a hyperexcitable state with increased migraine susceptibility.[45]

ATP1A2 & HM

The majority of HM mutations has been found in *ATP1A2*, with more than 60 (mostly missense) mutations in *ATP1A2* reported so far.[42] *ATP1A2* encodes the α_2 subunit of a Na^+/K^+-ATPase, which creates a steep sodium gradient.[42] FHM2 mutations appear to result in a loss of function of the Na^+/K^+-ATPase, causing increased potassium and glutamate concentrations in the synaptic cleft, leading to a hyperexcitable state.[46] An increased susceptibility to CSD and an increased velocity of CSD propagation were demonstrated in FHM2 knock-in mice carrying the human p.Trp887Arg (W887R)[47] or the p.Gly301Arg (G301R) mutation,[48] caused by increased glutamatergic neurotransmission due to reduced glial ATPase function.[48,49] The overview in **chapter 4** of *ATP1A2* mutations reported so far shows that there are neither clear hotspots for mutations in ATP1A2, nor evident clustering of mutations associated with severe phenotypes (as displayed by the FHM2 family in **chapter 4**). *ATP1A2* has rarely been associated with disorders other than HM. A novel *ATP1A2* missense variant was identified in a family with progressive sensorineural hearing loss and migraine without aura.[50] Despite its poor co-segregation with the phenotype, it is questionable whether this variant is pathogenic, also because *in vitro* studies did not reveal functional effects of the mutation. In another family an *ATP1A2* mutation was associated with Alternating Hemiplegic of Childhood (AHC).[51] As AHC is clinically similar to HM and its association with *ATP1A2* has not been replicated, these patients may in fact suffer from FHM2.[52] Moreover, with the discovery of *ATP1A3* mutations in >80% of patients in international AHC cohorts, one wonders if these AHC patients may carry an *ATP1A3* mutation.[53,54]

SCN1A & HM

Chapter 5 describes the discovery of novel *SCN1A* mutations (p.Ile1498Met (I1498M) and p.Phe1661Leu (F1661L)) in two FHM3 families, which were only the 6th and 7th FHM3 *SCN1A* mutations. *SCN1A* encodes the α1 subunit of voltage-gated $Na_V1.1$ channels.[4] Experiments in heterologous expression systems suggested that dysfunctional channels lead to neuronal hyperexcitability and thereby increased susceptibility to HM.[4,55] However, both reduced activity (for *SCN1A* mutations p.Gln1489Lys (Q1489K) and p.Leu1649Gln (L1649Q)) and increased activity (for *SCN1A* mutation L263V) of $Na_V1.1$ channels have been reported.[4,33,55] Functional effects of the novel F1661L *SCN1A* mutation cannot be clearly predicted but the particular location of the I1498M *SCN1A* mutation points towards a loss-of-function effect. Amino acid Ile^{1498} is located in the so-called IFMT motif that encodes a hydrophobic latch, which is hypothesised to delay $Na_V1.1$ channel activation in a dysfunctional state.[56,57] It is hypothesised that reduced activity of the $Na_V1.1$ channels mainly affects inhibitory neurons, where the $Na_V1.1$ channels are suggested to be primarily expressed, whereas increased activity would primarily affect excitatory neurons.[58] Given the discordant phenotypes

linked to FHM3 *SCN1A* mutations, even with the exact same mutation, additional genetic or environmental factors seem to determine which phenotypes are expressed.[17] As a first clue, an *in vitro* study suggested that modulating factors, e.g. injecting depolarising currents of increasing amplitude and increasing K^+ currents, may cause effects of the same mutation to switch from gain-of-function to loss-of-function.[34] In that study, HM was linked to gain-of-function effects and epilepsy to loss-of-function effects of $Na_V1.1$ channels, which the same group subsequently also demonstrated functionally.[34,59,60] Further studies are needed to confirm whether functional effects and phenotypes of *SCN1A* mutations can truly be segregated as such. Mouse models are currently not available for FHM3, so most *in vivo* knowledge at the moment comes from extrapolation of findings in available mouse models for other *SCN1A*-associated (severe) epilepsy syndromes.[61–64]

Novel HM genes

The search for novel HM genes has been ongoing for years. In 2012, *PRRT2* was proposed as the 4th HM gene.[65–69] The functional consequences of *PRRT2* mutations largely remain to be determined but loss of function of PRRT2 has been suggested to cause increased glutamate release and neuronal hyperexcitability, similar to the postulated effects of mutations in the known HM genes.[70,71] A critical analysis of the proposed *PRRT2*-HM association is described in **chapter 6**. First of all, it was noticed that mutations in *CACNA1A*, *ATP1A2* and *SCN1A* were not always fully excluded in *PRRT2* mutation carriers with HM.[66–69] While occurrence of multiple rare mutations in one family may at first appear unlikely, a *PRRT2* mutation was encountered in addition to an *ATP1A2* mutation in the FHM-BFIS family described in **chapter 6**. Also for two families described in **chapter 7** in addition to a *CACNA1A* mutation a *PRRT2* mutation was identified. Caution is therefore warranted when attributing phenotypes in one family to a particular mutation. The same applies to the many *PRRT2* mutation carriers diagnosed with HM who also suffered from other *PRRT2*-associated conditions (e.g. BFIS, paroxysmal kinesigenic dyskinesia (PKD) or infantile convulsion choreoathetosis (ICCA) syndrome).[65,67–69,72,73] Conspicuously, the vast majority of subjects with a *PRRT2* mutation does not have HM, even with the exact same (highly recurrent) mutation c.649dupC. Hence, most importantly, the *PRRT2*-HM association appears to be different from associations observed in FHM1, -2 and -3, as large families showing a clear autosomal dominant inheritance of HM with a *PRRT2* mutation are still lacking.

An obvious explanation for the findings is that the association of *PRRT2* with HM is false, and that the *PRRT2* mutation may actually cause another phenotype (e.g. BFIS) that may have been missed or is non-penetrant in the few patients with HM alone. This explanation became less plausible considering the rather high frequency of reported *PRRT2* mutations in HM patients,[70] and the sheer absence of

the recurrent c.649dupC mutation in very large cohorts of controls.[72,74–76] Of note, in **chapter** 7 we identified a *PRRT2* mutation in yet another HM family that also included BFIS patients.

Second, if there is an association between *PRRT2* and HM, many mutation carriers appear non-penetrant for HM, which suggests that a *PRRT2* mutation may, at best, moderately increase chances to suffer from HM, but on its own is not sufficient to cause HM and needs another mutation in an additional gene. As none of the HM families in which we identified a *PRRT2* mutation (in **chapters 6 and 7**) showed clear autosomal dominant inheritance of HM with the *PRRT2* mutation a different genetic mechanism may thus be involved that is more similar to complex or polygenic inheritance. While we could not demonstrate a role for *PRRT2* in our cohort of HM patients without a confirmed mutation in *CACNA1A*, *ATP1A2* or *SCN1A*, as it was only found in the FHM-BFIS family described in **chapter 7**, it may act as a genetic modifier (cofactor) in a proportion of HM patients.

Finally, *PRRT2* may be linked to HM indirectly. Suffering from BFIS (or e.g. PKD or ICCA syndrome) could make an individual more susceptible to HM, similar to other neurological or neuropsychiatric symptoms reported later in life in some *PRRT2* mutation carriers with BFIS, ICCA or PKD.[77,78]

Besides *PRRT2* other genes have been associated with 'monogenic migraine', but none have been proven as undisputed HM genes. *KCNK18* and *CSNK1D* have only been linked to familial migraine without motor auras[79,80], and other genes were found in very few HM cases, often with comorbid disorders (*SLC1A3*[81,82], *SLC4A4*[83]). Some of these genes, in theory, fit nicely the hypothesis of increased concentrations of glutamate in the synaptic cleft in migraine, for example *SLC1A3* encoding glial glutamate transporter EAAT1, in which mutations were shown to cause reduced glutamate uptake.[84] Nonetheless, more evidence is needed to establish a role for these genes in HM.

A novel technique that appears promising to identify additional HM genes is next-generation sequencing (NGS),[85,86] which was successful for many autosomal dominant disorders, e.g. AHC.[87,88] NGS is very powerful in identifying large numbers of genetic variants in each individual in a single experiment. Depending on the type of variant that is sought after, data sets are filtered, allowing the identification of identical or different variants in the same gene in multiple patients with the disease of interest. **Chapter 7** describes a NGS study in HM, for which HM patients without a mutation in *CACNA1A*, *ATP1A2* or *SCN1A* were investigated by whole exome sequencing (WES). Besides *PRRT2* mutations, the presence of mutations in *KCNK18*, *CSNK1D*, *SLC1A3,* and *SLC4A4* was checked among the identified list of variants from WES. Analysing the data of no less than 47 exomes did not result in novel HM genes, as we did not identify any gene in which mutations showed (near) full co-

segregation with HM, or occurred in independent HM patients. We encountered various challenges when filtering and analysing the WES data, foremost that mutations in novel HM genes may show reduced effect size compared to mutations in the known genes (i.e. *CACNA1A*, *ATP1A2* or *SCN1A*). If HM indeed is caused by multiple gene variants with a smaller effect size, it will be a daunting task to identify which combination of variants identified by NGS is causal in a patient. In fact, this would require a statistical, association-based type of approach, similar to what is employed in genome-wide association studies (GWAS). Likely genetic data of hundreds to thousands of HM cases will be needed to obtain sufficient evidence for involvement of a gene, which is not a feasible scenario given the rarity of HM. An additional challenge is that it is unclear how much evidence can be deferred from *in silico* pathogenicity predictions or how many repeated findings are needed to establish true pathogenicity of a genetic variant. Although this may shed ultimate light on whether a variant is pathogenic or not, functional *in vitro* testing of all variants of interest will simply not be feasible.

HM in clinical practice

Clinicians struggle to correctly diagnose and effectively treat HM patients. **Chapter 2** describes a review of literature on diagnostic and therapeutic options for HM. Genetic screening of the HM genes often takes months, but this will be more efficient now NGS is implemented in the practice of clinical geneticists, for instance by screening patients with a neurological disorder for mutations in a large panel of genes. In **chapter 7** it is shown that WES appears a reliable method for screening for mutations in the known HM genes that will certainly provide a rich data source for the evaluation of (future) candidate genes. From a therapeutic viewpoint, unfortunately, a (long-lasting) trial-and-error process is still the only option in HM. In **chapter 3** an FHM family is described in which a dramatic and sustained therapeutic effect of prophylactic treatment with sodium valproate and lamotrigine was observed. This case report illustrates that combining several migraine prophylactics can be considered when a single drug is insufficient, although strict monitoring of side effects is advised. Recommendations for clinical practice are summarised in Table 1.

Table 1: Recommendations for clinical practice when suspecting hemiplegic migraine (HM) in a patient.

Patient interview
• **Confirm the presence of motor aura symptoms and distinct these from sensory aura symptoms**
• **Identify additional features during attacks that point towards HM:** epilepsy, confusion, fever, decreased consciousness, other brainstem auras, prolonged aura symptoms
• **Identify additional features outside attacks that point towards HM:** epilepsy (including during infancy or childhood), ataxia
• **Obtain family history:** interview 1st and/or 2nd degree relatives to investigate familial occurrence of HM
Additional diagnostics
• **Molecular genetic testing:** mutations in *CACNA1A, ATP1A2* or *SCN1A*, future: next-generation sequencing
• **MRI:** progressive cerebellar (and cerebral) atrophy, diffuse one-sided (cortical) oedema during attacks
• **Cerebrospinal fluid analysis:** pleocytosis during attacks
• **Electroencephalography:** diffuse one-sided slow waves (theta- and/or delta-activity) during attacks
Treatment
• **Acute treatment:** conform treatment of common migraine types, including triptans
• **Prophylactic treatment (in no strictly preferred order):** lamotrigine, flunarizine, sodium valproate, verapamil and acetazolamide

Table adapted from *Hemiplegic Migraine and Other Monogenic Migraine Subtypes and Syndromes.* N. Pelzer, T. Freilinger, G.M. Terwindt. Book chapter for *Oxford Textbook of Headache Syndromes*, Oxford Textbooks in Clinical Neurology, Oxford University Press. *in preparation*

Part II: Retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations – a vascular monogenic migraine model?

A second approach applied to study migraine in this thesis is the investigation of monogenic (vascular) syndromes in which a higher prevalence of migraine is observed in carriers of a pathogenic mutation than can be expected based on the population risk of migraine. Retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations (RVCL-S) is such a disease that has been associated with migraine. In 2007 it was discovered that C-terminal truncating mutations in the *TREX1* gene were present in a collection of hereditary small-vessel neurovascular syndromes. As a result, hereditary vascular retinopathy (HVR),[89] cerebroretinal vasculopathy (CRV),[90] and hereditary endotheliopathy with retinopathy, nephropathy and stroke (HERNS)[91] were, initially, renamed as retinal vasculopathy with cerebral leukodystrophy (RVCL).[92] A review of the 16 available RVCL families world-wide revealed that systemic symptoms are frequently present,[93] resulting in renaming the syndrome to RVCL-S. Due to a lack of uniformity in the clinical information that was collected via non-systematic methods, RVCL-S symptoms could not be described in great detail. In this thesis we aimed to give a more detailed description of clinical symptoms of RVCL-S during different disease stages and to identify pathophysiological mechanisms of this incurable disease for which we used patients of three Dutch RVCL-S families.

Clinical spectrum of RVCL-S

A systematic cross-sectional study, the RVCL-ID study, was performed in the Dutch RVCL-S population, with a focus on the occurrence and severity of internal organ disease and the nature of neurological deficits during different stages of disease. The main results of the RVCL-ID study are described in **chapter 8**. Notable findings were that we confirmed, but more accurately quantified, the presence of systemic symptoms, such as liver and kidney disease, Raynaud's phenomenon and anaemia, and added presence of subclinical hypothyroidism to the RVCL-S syndrome. We could not confirm hypertension as part of the RVCL-S phenotype. In addition, we found that neurological symptoms were generally very mild, or even unnoticeable, until the age of ~50–55 years, after which symptoms progressed rapidly during the last stage of the disease. We envisage that our findings will create more awareness and better recognition of RVCL-S, also among clinicians in the field of internal medicine, avoiding unnecessary and possibly harmful diagnostics as is currently the case.

Despite previous reports,[89,93] we did not find an increased lifetime prevalence of migraine in RVCL-S patients compared to family members without the *TREX1* mutation. A prevalence of 27% (n=9/33) was found in RVCL-S patients, which is lower than the 59% (n=24/41) that was reported in the largest review of RVCL-S patients to date.[93] The majority of RVCL-S patients in our study suffered from migraine with aura. Atypical aura symptoms, as described in another small-vessel disease associated with migraine, i.e. cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL),[94] such as prolonged auras (i.e. >60 minutes), motor auras or auras with acute onset were not reported. In our study one family, which was previously described by Terwindt et al.,[89] contributed majorly to the migraine prevalence in both RVCL-S patients and family members without the *TREX1* mutation. This might suggest that a high migraine prevalence, as described before, in this family is specifically due to other factors and less to the presence of the *TREX1* mutation. The estimation of prevalence of migraine is influenced by the fact that this RVCL-S family, by far, is the largest family described to date, and majorly contributes to the total number of RVCL-S patients with migraine. At first sight, these findings seem to cast doubt on whether migraine is really part of the clinical spectrum of RVCL-S. However, the observation that the age at onset of migraine in patients with RVCL-S was high (40 years), whereas onset of disease normally is in adolescence (<25 years[95]), suggests that migraine may occur *secondary* to the vasculopathy. Of note, in CADASIL, also a rather high mean age at onset of migraine with aura (~30 years) was observed, which was higher in men.[94] Although in general onset of migraine after age 35 years is considered atypical, a large population-based study found an onset of disease in 'normal' migraine patients after age 35 in 25% of cases.[95] Altogether, later age at onset of migraine in RVCL-S may be a good explanation for not finding a high prevalence in the last study as many young mutation carriers (<40

years) were included. Furthermore, it is of interest, as it might shed light on vascular mechanismsnvolved in migraine. A prospective study of these younger RVCL-S mutation carriers may be informative to see whether they will develop migraine.

Pathophysiology of RVCL-S

To investigate the hypothesised endothelial involvement in RVCL-S,[96] we assessed several circulating endothelial markers in blood samples of RVCL-S patients who participated in the RVCL-ID study. These patients, aged 19 to 65 years, represent all stages of disease. As described in **chapter 9**, we observed a strong correlation between increased levels of Von Willebrand Factor (VWF) antigen, VWF propeptide and angiopoietin-2 and presence of a *TREX1* mutation. Levels of all three markers clearly surpassed a threshold in RVCL-S patients from approximately age 40 years onwards, when clinical symptoms are known to clinically manifest, as confirmed in **chapter 8**. An important finding is that VWF antigen and angiopoietin-2 were also increased in RVCL-S patients aged <40 years compared to unrelated healthy controls, suggesting that VWF and Ang-2 may server as the first biomarkers able to identify disease when it is clinically still silent. Altogether, our findings confirm that activation of the endothelium is part of RVCL-S pathophysiology, and that VWF and angiopoietin-2 appear promising (early) biomarkers of disease activity that may predict clinical progression and may even constitute future treatment targets.

Although our study in **chapter 9** shows a strong association with RVCL-S, increased levels of VWF and angiopoietin-2 were also found in diabetes mellitus[97,98] and hypertensive complications such as hypertensive retinopathy.[99] High VWF levels also increase the risk of stroke,[100] and an increase in VWF, although less pronounced, was recently reported for CADASIL.[101] Notably, we were able to exclude a role for many factors that may influence levels of VWF and angiopoietin-2, including ABO-blood type, sex, age, blood pressure, diabetes mellitus, hypercholesterolemia, smoking and alcohol use.[98,102] On the contrary, we did not find increased levels of VWF and angiopoietin-2 in our cohort of patients with migraine without aura (age- and sex-matched with RVCL-S patients described in **chapter 9**), who did not suffer from (cardio)vascular comorbidities.(Pelzer et al., *unpublished data*) These findings suggest that VWF and angiopoietin-2 are markers of (micro)vascular damage, *per se*, and not specific to RVCL-S. One may conclude that RVCL-S, foremost, may serve as a monogenic model to study more common neurovascular disease, such as stroke and vascular dementia.

To investigate a possible broader role of *TREX1* mutations in cerebrovascular disease and identify possible new RVCL-S patients, we screened the coding part of *TREX1* in 100 subjects with clinical

symptoms suggestive of CADASIL, but in whom the presence of a CADASIL-causing *NOTCH3* mutation had been excluded. The results of this study are described in **chapter 10**. Two separate heterozygous missense *TREX1* mutations (p.Tyr305Cys (Y305C)) and p.Arg114His (R114H)) were identified in two patients with early-onset cerebrovascular disease. Mutation Y305C is not described in control subjects and affects a highly conserved amino acid residue,[103,104] so well may be the cause in the respective patient. For mutation R114H it is rather difficult to determine whether it causes disease in our patient as this mutation is also found in patients with Systemic Lupus Erythematosus (SLE),[103–105] healthy controls,[104] and in parents of patients with Aicardi-Goutières Syndrome (in whom homozygous mutations are present).[106] Although presence of one copy of the mutation does not seem to cause disease, an *in vitro* study has suggested altered enzymatic activity of the mutation also in the heterozygous situation. Therefore, in heterozygous form, the R114H mutation may act as a genetic modified (cofactor) increasing the risk of (early-onset) vascular disease. The lack of RVCL-S-associated C-terminus frame-shift *TREX1* mutations in this study indicates that RVCL-S does not appear to be missed. The fact that only 16 RVCL-S families have been identified world-wide, and that three of them originate from a small country like the Netherlands, strongly suggests that there must be many RVCL-S families that currently remain unidentified.[93]

RVCL-S in clinical practice

From our RVCL-ID study, described in **chapter 8**, we learned that symptoms of internal organ disease, such as kidney and liver disease and anaemia, may deteriorate quickly to a level requiring treatment. Therefore, we now advise patients to have annual check-ups from approximately age 35 years onwards, and more frequent (for example twice a year) check-ups when symptoms become apparent. Although neither the exact aetiology of the symptoms nor the triggering factors for worsening of symptoms are known, simple symptomatic treatment such as prescription of antihypertensive drugs or iron supplements can be beneficial. The newly observed hypothyroidism in RVCL-S usually appears to remain subclinical and does not require treatment, but can be a signal of systemic involvement and should certainly warrant further screening of internal organ disease.

Future perspectives

1) Hemiplegic migraine

In this thesis, the clinical and genetic spectra of HM were investigated. Thorough phenotyping of HM patients should remain a continuous effort, as this may reveal novel endophenotypes within the migraine spectrum, which may involve different underlying pathophysiological mechanisms and may benefit from different treatments. For such a rare disease as HM, it should remain possible to

document such novel phenotypes and treatment options, also in scientific journals, even though the scientific value of case reports or series appears to decrease. Alternatively, such information could (and should) be made available on online platforms, but only when these platforms are able to follow the same strict guidelines with regard to safeguarding patient privacy and quality of the data.

To be able to expand on genotype-phenotype correlations, sequencing the known HM genes remains important. NGS has already been implemented for genetic screening in clinical practice, in clinical genetics departments, by the use of gene panels that allow for cost-effective screening of mutations in targeted patient groups. However, for WES or whole genome sequencing (WGS) to become a success in clinical practice, some hurdles have to be taken.[107] First, sequencing someone's entire exome or genome has ethical issues. It has to be clear which areas of the genome are analysed, and which are not, and this has to be controlled by an informed consent procedure. After all, the risk is considerable that genetic risk factors are identified for diseases that were not subject of the patient's initial request for help, and patients have the explicit right not to be informed on genetic findings, especially on risk factors for untreatable diseases. Second, NGS does not make the interpretation of genetic variants any easier, now more variants are detected. To some extent *in silico* prediction programs can aid in determining a variant's pathogenicity, but many will remain 'unclassified' without functional tests - that are often not feasible -, resulting in an unclear (genetic) diagnosis. Internationally shared registration of detected genetic variants and the associated phenotypes is vital to allow better classification of variants.

The hope remains that the identification of novel HM genes will become a reality when NGS is performed on large numbers of HM patients and pipelines for the analysis of data have improved. Even when to be identified genes are found in only a handful of HM patients, still, their discovery opens opportunities to learn more of the molecular mechanisms of HM, beyond current belief that increased concentrations of potassium and glutamate in the synaptic cleft and increased cortical excitability solely underlie the pathophysiology of hemiplegic migraine.

For HM patients that can be categorised at the mild end of the phenotypic spectrum it may be worthwhile to adapt techniques used in complex diseases (i.e. genome-wide association studies (GWAS)). Although GWAS already led to the discovery of over 40 gene variants for common forms of migraine,[108–112] including migraine with aura that is closest to HM, it will be challenging to use this approach to identify HM genes as one would need thousands of patients. Still, it not only has been shown very challenging to accurately assign associated single nucleotide polymorphisms (SNPs) to

genes (and pathways they are involved in), but also there are no efficient ways to investigate the true functional consequences of these SNPs to further our understanding in disease pathology.

With regard to the treatment of HM, physicians should be advised to monitor the development of novel treatment options for common forms of migraine. Expectations are rather high for antibodies that inhibit Calcitonin Gene-Related Peptide (CGRP) or its receptor function, which may be useful for prophylactic treatment of migraine,[113, 114] and perhaps hemiplegic migraine. Non-invasive and invasive neuromodulation approaches may also become therapeutic options in migraine. As future clinical trials will likely not include HM patients, clinicians are highly encouraged to keep reporting about the efficacy and tolerability of any novel (off-label) treatment in those patients.

2) Retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations

As part of the RVCL-ID study, we extracted a pseudo-longitudinal disease course from a cross-sectional study of RVCL-S patients of a wide age range. Logically, prospective studies are needed to investigate the true clinical course. Such follow-up studies should include standardized measurements of blood count, kidney, liver and thyroid function, and brain imaging at regular intervals. Monitoring of onset of additional symptoms, like migraine, will be instrumental to assess whether and/or how these symptoms are linked to RVCL-S.

With the postulated involvement of oligosaccharyltransferase activity, a new mechanism in RVCL-S pathophysiology has been suggested.[115] Systematic investigation of glycans and glycosylation and the suggested immune reactions would therefore be an interesting future research objective. Other studies that focussed on (auto)immune responses in RVCL-S so far have not provided much evidence in this direction. For instance, IFNα levels (in cerebrospinal fluid and serum) were very low in RVCL-S patients; in serum much lower when compared to allelic disorders such as Aicardi-Goutières syndrome and Systemic Lupus Erythematosus.[116]

Angiopoietin-2 is a therapeutic target in diabetes mellitus and cancer research.[117] Given the elevated levels of angiopoietin-2 in RVCL-S patients, restricting angiogenesis and endothelial damage may limit (or even halt) disease progression in RVCL-S. To assess efficacy of such treatments, one could perhaps measure VWF and angiopoietin-2 in serum, as monitoring changes of e.g. kidney function would require too much time. Although such treatment may tackle secondary rather than primary pathophysiological pathways, any therapeutic option in RVCL-S would be revolutionary.

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