



GENETICS IN THE IDENTIFICATION OF MOLECULAR PATHOGENIC MECHANISMS OF FAMILIAL MIGRAINE

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Dissertação de Doutoramento em Ciências Biomédicas

2008

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Dissertação de Candidatura ao grau de Doutor em Ciências Biomédicas, submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

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Porto, 2008

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- 1: **Castro MJ**, Nunes B, de Vries B, Lemos C, Vanmolkot KRJ, van den Heuvel JJMW, Temudo T, Barros J, Sequeiros J, Frants RR, Koenderink JB, Pereira-Monteiro JM, van den Maagdenberg AMJM (2008). Two novel functional mutations in the Na⁺,K⁺-ATPase α 2 subunit *ATP1A2* gene in patients with familial hemiplegic migraine and associated neurological phenotypes. *Clin Genet* 73: 37-43.
- 2: **Castro MJ**, Stam AH, Lemos C, Barros J, Gouveia RG, Pavão Martins I, Koenderink JB, Vanmolkot KRJ, Mendes AP, Frants RR, Ferrari MD, Sequeiros J, Pereira-Monteiro JM, van den Maagdenberg AMJM (2007). Recurrent *ATP1A2* mutations in Portuguese families with familial hemiplegic migraine. *J Hum Genet* 52: 990-998.
- 3: **Castro MJ**, Stam AH, Lemos C, de Vries B, Vanmolkot KRJ, Barros J, Terwindt GM, Frants RR, Sequeiros J, Ferrari MD, Pereira-Monteiro JM, van den Maagdenberg. First mutation in the voltage-gated Na_v1.1 subunit gene *SCN1A* with co-occurring familial hemiplegic migraine and epilepsy. *Submitted*
- 4: **Castro MJ**, Lemos C, Barros J, Vanmolkot KRJ, van den Heuvel JJMW, Frants RR, Koenderink JB, Maciel P, Pereira-Monteiro JM, van den Maagdenberg AMJM, Sequeiros J. Mutation screening for *CACNA1A* and *ATP1A2* FHM genes in a set of migraine probands: identification of a novel rare R51H variant in *ATP1A2*. *Submitted*
- 5: Lemos C, **Castro MJ**, Barros J, Sequeiros J, Pereira-Monteiro JM, Mendonça D, Sousa A. Familial clustering of migraine: further evidence from a Portuguese study. *Submitted*

- 6: Castro MJ**, Lemos C, Vanmolkot KRJ, Sousa A, Sequeiros J, van den Maagdenberg AMJM, Pereira-Monteiro JM. Linkage analysis of six migraine *loci* in a large set of Portuguese families with migraine with or without aura. *In preparation*
- 7: Ferro A, Castro MJ**, Santos M, Lemos C, Sousa A, Pereira-Monteiro J, Sequeiros J, Maciel P. The C677T polymorphism in *MTHFR* is not associated with migraine in Portugal. *In preparation*
- 8: Castro MJ**, Barros J, Lemos C, Mendes A, Vanmolkot K, Frants R, Sequeiros J, Pereira-Monteiro J, van den Maagdenberg A (**2006**). Nova Mutação Recorrente no Gene *ATP1A2* numa Família Portuguesa com Enxaqueca Hemiplégica Familiar Tipo 2. *Sinapse* 6 (2): 4-10. (Prémio Corino de Andrade/Novartis 2006).
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LIST OF ABBREVIATIONS

ACE - Angiotensin converting enzyme

AD - Autosomal dominant

AHC - Alternating hemiplegia of childhood

AO - Age of onset

ARP - Affected relative pairs

ASP - Affected sib pairs

ATP1A2 - Sodium-potassium ATPase α 2 subunit gene

BM - Basilar migraine

BFIC - Benign familial infantile convulsions

CACNA1A - Neuronal voltage-gated $\text{Ca}_v2.1$ calcium channel α 1 subunit gene

CADASIL - Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy

CD-CV - Common disease-common variant hypothesis

CGRP - Calcitonin gene-related peptide

CNS - Central nervous system

CSD - Cortical spreading depression

CH - Cluster headache

DBH - Dopamine beta hydroxylase gene

DNA - Deoxyribonucleic acid

DZ - Dizygotic

EAAT1 - Excitatory amino acid transporter type 1

EA2 - Episodic ataxia type 2

EEG - Electroencephalogram

FHM - Familial hemiplegic migraine

FHM1 - FHM type 1, linked to chromosome 19p13

FHM2 - FHM type 2, linked to chromosome 1q23

FHM3 - FHM type 3, linked to chromosome 2q24

GEFS+ - Generalized epilepsy with febrile seizures plus

HGSA - Hospital Geral de Santo António

HLOD - Heterogeneity LOD score

HM - Hemiplegic migraine

INSR - Insulin receptor gene

5-HT - Serotonin

IBD - Identical by descent

IBS - Identical by state

IHS - International Headache Society

LCA - Latent class analysis

LD - Linkage disequilibrium

LOD - Logarithm of odds

MA - Migraine with aura

MO - Migraine without aura

MA/MO - Migraine with and without aura

MRI - Magnetic resonance imaging

MTHFR - Methylene tetrahydrofolate reductase gene

MZ - Monozygotic

NMJ - Neuromuscular junction

NPL - Non-parametric linkage

PCR - Polymerase chain reaction

QTL - Quantitative trait loci

RR - Relative risk

RRR - Relative risk ratio

SCA6 - Spinocerebellar ataxia type 6

SCN1A - Neuronal voltage-gated $\text{Na}_v1.1$ sodium channel α 1 subunit gene

SHM - Sporadic hemiplegic migraine

SLC1A3 - Solute carrier family 1, member 3 gene

SMEI - Severe myoclonic epilepsy in infancy

SNP - Single nucleotide polymorphism

SP - Substance P

SSCP - Single-strand conformation polymorphism

TCA - Trait component analysis

TDT - Transmission disequilibrium test

TGV - Trigeminovascular

TTH - Tension-type headache

Aos meus pais, os melhores

TABLE OF CONTENTS

ABSTRACT	15
RESUMO	17
RÉSUMÉ	21
1. INTRODUCTION	25
1.1 Migraine	25
1.1.1 Classification and diagnostic criteria	25
1.1.1.1 Migraine common subtypes:	
Migraine without aura	26
Migraine with aura	26
1.1.1.2 Rare, severe migraine subtypes:	27
Basilar-type migraine	27
Familial hemiplegic migraine	28
Sporadic hemiplegic migraine	28
1.1.2 Epidemiological studies in migraine	29
1.1.2.1 Incidence rates	29
1.1.2.2 Prevalence	29
1.1.2.3 Familial Aggregation:	
Relative Risk	30
Heritability	30
1.1.2.4 Patterns of inheritance in migraine	31
1.1.3 Comorbidity	32
1.1.4 Predisposing factors and triggers	32
1.1.5 Pathophysiology	33
1.1.5.1 Aura	33
1.1.5.2 Headache	33
1.1.6 Biochemical abnormalities	34
1.1.7 Biomarkers	34
1.1.8 Treatment options	34
1.2 Mapping human complex disease genes	35
1.2.1 Main genetic approaches	35
1.2.1.1 Linkage:	35
LOD score	36
Model-based, parametric tests	36
Model-free, non-parametric tests	37
Affected Sib Pairs	37
Affected Relative Pairs	38
1.2.1.2 Association:	38
Case-control studies	39
TDT	39
Candidate versus Genomewide association studies	40
1.2.2 Common disease-common variant versus rare variant hypothesis	40
1.2.3 Models for complex diseases	41
1.2.3.1 Environmental contribution	41
1.2.3.2 Two-locus or multilocus inheritance	41
1.2.4 Recent examples in literature	42
1.3 Genetics of Hemiplegic Migraine	42
1.3.1 FHM as a model	42
1.3.2 Clinical features	43
1.3.3 FHM genes and regions	43
1.3.3.1 FHM1, CACNA1A gene	43
Genotype-phenotype correlations	44
Neuronal Ca _v 2.1 channel: structure and function	45
Functional studies and mice models	45

1.3.3.2 FHM2, <i>ATP1A2</i> gene	46
Na ⁺ ,K ⁺ -ATPase: structure and function	47
Functional studies	48
1.3.3.3 FHM3, <i>SCN1A</i> gene	48
Na _v 1.1 channels: structure and function	49
Major gene for epilepsy	50
Functional data on <i>SCN1A</i> mutations	50
1.3.4 New insights from other candidate gene results	51
1.3.5 Contribution of FHM study for migraine molecular mechanism	51
1.3.6 Novel treatment options from molecular biology	52
 1.4. Genetics of complex migraine subtypes: MA and MO	53
1.4.1 Definition of phenotype	53
1.4.1.1 IHS criteria and genetic studies	53
1.4.1.2 Co-occurrence of migraine subtypes	54
1.4.1.3 Linkage studies in MA	55
1.4.2 Targeting FHM <i>loci</i> and genes	55
1.4.3 Candidate gene studies	56
1.4.4 Mapping susceptibility locus	56
1.4.4.1 Target region: genomewide scans	57
1.4.4.2 Population selection: isolates	58
1.4.4.3 Novel approaches: LCA and TCA	58
1.4.4.4 Other regions	59
1.4.5 Association studies	59
1.4.5.1 Serotonin	59
1.4.5.2 Dopamine	60
1.4.5.3 MTHFR	60
1.4.5.4 Angiotensin	61
1.4.5.5 Other associations	61
 2. AIMS OF THE THESIS	63
 3. PATIENTS AND METHODS	65
3.1 Patients	65
3.1.1 DNA bank	65
3.1.2 Hemiplegic Migraine	66
3.1.2.1 Diagnostic criteria	66
3.1.2.2 Family structure	66
3.1.2.3 Sample selection	67
3.1.3 MO and MA	67
3.1.3.1 Diagnostic criteria	67
3.1.3.2 Sample selection	68
3.2 Molecular methods	69
3.2.1 DNA extraction	69
3.2.2 Genotyping	71
3.2.2.1 Post-PCR Multiplex	71
3.2.3 Mutation detection	71
3.2.3.1 Single-Strand Conformation Polymorphism (SSCP)	72
3.2.3.2 Restriction analysis of PCR products	72
3.2.3.3 Melting curve analysis	73
3.2.4 Sequencing	73
3.2.5 Functional analysis	74
3.2.5.1 cDNA constructs	74
3.2.5.2 Transfection in HeLa cells and ouabain treatment	74
3.2.5.3 Electrophoresis and Western blot analysis	74
3.2.6 Statistical methods	75
3.2.6.1 Relative risk (RR) and familial aggregation calculations	75
3.2.6.2 Mendelian errors and inconsistencies	75
3.2.6.3 Simulations studies	75
3.2.6.4 Linkage analysis	76
3.2.6.4.1 Parametric, model-based	76
3.2.6.4.2 Non-parametric, model-free	77

4. RESULTS	79
4.1 Genetic approaches to study common migraine	79
4.1.1 Evaluate familial aggregation of migraine	79
ARTICLE 1	81
4.1.2 Study the role of migraine susceptibility <i>loci</i> by linkage analysis	89
ARTICLE 2	91
4.1.3 Determine the possible involvement of FHM genes in common migraine	105
ARTICLE 3	107
4.2 Genetics of FHM: a model to study the pathophysiology of migraine	117
4.2.1 To establish the role of <i>ATP1A2</i> in Portuguese FHM samples	117
ARTICLE 4	119
ARTICLE 5	131
4.2.2 Study the involvement of <i>SCN1A</i> in a family with FHM and epilepsy	141
ARTICLE 6	143
5. GENERAL DISCUSSION	153
5.1 Genetic approaches to study common migraine	153
5.1.1 The genetic contribution for migraine	153
5.1.2 MO and MA as part of a migraine spectrum	153
5.1.3 Strategies for studying complex diseases: migraine as an example	154
5.1.3.1 Linkage analysis	154
5.1.3.2 Factors influencing LOD score estimation	155
5.1.4 Candidate gene approaches	156
5.1.5 Genetic characterization of the Portuguese migraine families	157
5.1.6 Involvement of FHM regions and genes in migraine susceptibility	159
5.2 Genetics of FHM: a model to study the pathophysiology of migraine	160
5.2.1 Mutation spectrum in Portuguese FHM families: prioritizing genetic testing	160
5.2.2 Contribution to migraine pathophysiology	161
Concluding Remarks	163
REFERENCES	165
ANNEX A	185
ANNEX B	189

ABSTRACT

Migraine is a common, disabling, episodic disorder of neurovascular origin. The overall disease prevalence exceeds 12 to 15% in most populations. In a Portuguese study, a prevalence of 17% was reported for both migraine and migraine associated with other headache types. Migraine attacks are characterized by throbbing, often unilateral, headache, accompanied by symptoms such as nausea, vomiting, and sensitivity to light and sound (migraine without aura, MO). In about one third of patients, the headache is preceded by an aura phase, consisting of visual and sometimes sensory symptoms (migraine with aura, MA). Migraine is also defined by the recurrence of attacks; at present, it is largely unknown why migraine patients suffer so many attacks. Understanding the pathology of migraine - including the discovery of genetic factors predisposing to disease - will hopefully help design better drugs, which are still very much needed.

Migraine clusters in families and it is well established that it has a strong genetic component. Unravelling the genetic determinants for the common forms of migraine has been challenging, because of its high prevalence and complexity, with both genetic and environmental factors involved. Genetic studies of familial hemiplegic migraine (FHM), a monogenic subtype of MA, however, have been successful and surfaced three genes, all encoding ion transporters: a calcium channel subunit gene, *CACNA1A* (FHM1), a sodium-potassium pump subunit gene, *ATP1A2* (FHM2), and a sodium channel subunit gene *SCN1A* (FHM3). Many susceptibility *loci* have been suggested for the common forms of migraine, underscoring its complex genetic contribution, but causal genes and variants remain to be identified.

We first set out to acquire a large, clinically well-characterized population of Portuguese migraine patients and controls. Our DNA bank now consists of samples of approximately 1200 individuals, belonging to approximately 210 families, which were used in the genetic studies performed. This Portuguese sample will be crucial for future gene identification studies.

The main goal of the work presented here was to study the genetic contribution in familial migraine, in an attempt to further unravel its molecular pathogenic mechanisms. Two main approaches were taken: (1) we used several genetic strategies to study “complex” (common) migraine (i.e. MA and MO), and (2) we performed genetic studies in FHM, as a model for the study of the pathophysiology of migraine.

With respect to the first approach, we investigated familial aggregation, by estimating *relative risks*, showing that relatives of probands have a 3 to 4-fold increased risk of migraine, when compared to the general population, whereas spouses do not show any increased risk. Our results confirmed earlier findings of clustering of migraine in 1st-degree relatives of MA and MO probands; they also confirmed that MA seems to have a stronger familial aggregation than MO and, thus, possibly has a greater genetic predisposition.

A first study of our migraine sample was performed using an “affected-only” linkage analysis. Although simulation studies had shown that this sample would have sufficient power to disclose linkage, if present, no significant results were obtained for any of the tested migraine susceptibility *loci* (including the FHM1-, FHM2- and FHM3- gene regions). Our study showed that phenotypic and genetic heterogeneity in migraine is higher than anticipated, which may hamper linkage studies. In any case, the FHM gene regions do not seem to be major contributors to the overall migraine genetic risk, at least in the Portuguese population. At best, our results reveal a minor role in particular migraine families. This is perhaps illustrated by the identification of a novel FHM2 variant in one of the migraine families. Our studies did not yield a single locus or gene variant that could explain all migraine cases in a family. More likely, migraine is a truly complex disease, with many gene *loci* and variants, each one having only a small contribution to disease susceptibility.

With our second approach, we identified several disease-causing mutations in FHM families. First, we identified two recurrent *ATP1A2* mutations (M731T and T376M), which allowed meaningful genotype-phenotype correlation, showing these mutations to be associated with *pure* FHM (i.e., FHM without associated any neurological disease such as epilepsy or ataxia). In addition, we discovered two novel *ATP1A2* mutations (V362E and P796S), in FHM families with psychiatric phenotypes. For the *ATP1A2* mutations, functional cell survival studies were performed, demonstrating the causality of these mutations. Finally, a novel *SCN1A* mutation (L263V) was identified in a family with FHM *and* epilepsy. Although *SCN1A* is a well-known epilepsy gene, the L263V mutation was the first to be associated with both diseases, thus reinforcing the concept that they share pathophysiological mechanisms. Functional data still have to show how L263V leads to this unique phenotype.

Although we could not find any gene variants for common migraine, our genetic studies, especially in FHM, gave some valuable insights into the pathophysiology of that disease. Future approaches will hopefully reveal new gene variants for common migraine and will further our knowledge on the disease.

RESUMO

A enxaqueca é uma doença episódica, comum e incapacitante de origem neurovascular. A prevalência global da doença excede os 12 a 15% na maioria das populações. Num estudo português, a prevalência atingiu os 17%, incluindo a enxaqueca e a enxaqueca associada a outros tipos de cefaleias. Os episódios de enxaqueca são caracterizados pela presença de uma dor de cabeça (ou cefaleia), pulsátil, na maioria dos casos unilateral, acompanhada por sintomas como náuseas, vómitos, e sensibilidade à luz e ao som (enxaqueca sem aura, MO). Em cerca de um terço dos doentes, a dor de cabeça é precedida de uma fase de aura composta por sintomas visuais e, em alguns casos, sensoriais (enxaqueca com aura, MA). A recorrência dos episódios também caracteriza a enxaqueca; não se conhece ainda o motivo pelo qual os doentes com enxaqueca apresentam tantos episódios. A compreensão da patologia da enxaqueca – incluindo a descoberta dos factores genéticos que predisõem para a doença – irá permitir o desenvolvimento de medicamentos mais adequados para o tratamento da doença.

Sabe-se que a enxaqueca ocorre em famílias, tendo sido demonstrada a existência de uma forte componente genética da doença. Determinar os factores genéticos nas formas mais comuns da enxaqueca tem sido um desafio, quer pela sua elevada prevalência como pela sua complexidade, envolvendo factores genéticos e ambientais. Contudo, os estudos genéticos na enxaqueca hemiplégica familiar (familial hemiplegic migraine, FHM), um subtipo de MA monogénico, tiveram algum sucesso ao identificar três genes que codificam para transportadores iónicos: o gene que codifica para a subunidade do canal de cálcio, *CACNA1A* (FHM1), o gene que codifica para a subunidade de uma bomba de sódio-potássio, *ATP1A2* (FHM2), e o gene que codifica para a subunidade de canal de sódio, *SCN1A* (FHM3). Foram descritos vários *loci* de susceptibilidade para as formas comuns da enxaqueca, o que demonstra a complexa contribuição genética da doença, mas os genes e as variantes genéticas que causam a doença permanecem por identificar.

A tarefa primordial foi a obtenção de uma população bem caracterizada de casos portugueses de doentes com enxaqueca e controlos. Actualmente, o banco de DNA é composto por amostras de cerca de 1200 indivíduos, que pertencem a aproximadamente 210 famílias, e que foram usadas no estudo genético realizado. As amostras portuguesas serão também cruciais para futuros estudos de identificação de novos genes.

O objectivo principal do trabalho apresentado foi estudar a contribuição genética na enxaqueca familiar, na tentativa de elucidar os seus mecanismos moleculares patogénicos. Para isso, foram utilizadas duas abordagens diferentes: (1) foram usadas diferentes estratégias para estudar a enxaqueca “complexa” (comum) (i.e. MA e MO), e (2) foram realizados estudos genéticos na FHM, sendo esta variante rara usada como modelo para estudar a patofisiologia da enxaqueca.

Considerando a primeira abordagem, foi primeiro estudada a agregação familiar, tendo sido estimado o *risco relativo*. Foi demonstrando que os familiares dos probandos com enxaqueca têm um risco aumentado (de 3- a 4-vezes) quando comparados com a população geral; os cônjuges não apresentam um risco aumentado. Os resultados confirmam indicações anteriores de agregação da enxaqueca em familiares de primeiro grau de probandos com MA e MO; os nossos dados confirmam ainda que MA parece apresentar uma maior agregação familiar que MO e, assim, possivelmente uma maior predisposição genética.

Uma primeira análise genética das nossas amostras com enxaqueca foi realizada, utilizando análise de ligação de “affected-only”. Apesar das simulações terem mostrado que a nossa amostra deve ter poder suficiente para detectar ligação, não foram encontrados resultados significativos para qualquer um dos *loci* de susceptibilidade para a enxaqueca testados (incluindo as regiões dos genes de FHM1, FHM2 e FHM3). Este estudo sublinha que a heterogeneidade, fenotípica e genética, nas famílias com enxaqueca é maior do que previsto, o que limita os estudos de ligação. As regiões dos genes da FHM não parecem ter um contributo fundamental para o risco global da enxaqueca, pelo menos na população portuguesa. Na melhor das hipóteses, os resultados apontam para um papel menor em algumas famílias. Isto pode ser talvez demonstrado pela identificação de uma nova variante FHM2 numa das famílias com enxaqueca. Neste trabalho não foi indicado um locus ou uma variante genética que explique todos os casos de enxaqueca numa família. Provavelmente, a enxaqueca é uma doença bastante complexa, com vários *loci* e variantes genéticas a contribuir para a sua susceptibilidade.

Considerando a segunda abordagem, foram identificadas várias mutações causadoras da doença nas famílias com FHM. Primeiro, identificaram-se duas mutações recorrentes no gene *ATP1A2* (M731T e T376M) que permitiram estabelecer uma correlação genótipo-fenótipo, mostrando que estas mutações estão associadas à FHM *pura* (i.e., FHM sem doenças neurológicas, como a epilepsia ou a ataxia, associadas).

Simultaneamente, duas novas mutações no gene *ATP1A2* (V362E e P796S) foram encontradas em famílias com FHM e fenótipos psiquiátricos. Para as mutações no gene *ATP1A2*, foram realizados estudos funcionais de sobrevivência celular, demonstrando-se a causalidade das mutações. Foi ainda identificada uma nova mutação no gene *SCN1A* (L263V) numa família com FHM e epilepsia. Apesar do gene *SCN1A* ser um conhecido gene da epilepsia, a mutação L263V foi a primeira associada a ambas as doenças, reforçando a ideia de que ambas partilham mecanismos patofisiológicos. Dados funcionais terão de mostrar como a mutação L263V conduz a este fenótipo único.

Apesar dos nossos estudos genéticos realizados não terem indicado qualquer variante genética na enxaqueca comum, os estudos na FHM trouxeram informações importantes para a patofisiologia da doença. Novas estratégias futuras poderão indicar as variantes genéticas na enxaqueca comum e apontar para o avanço no conhecimento da doença.

RÉSUMÉ

La migraine est une maladie épisodique, commune et incapacitante d'origine neurovasculaire. Dans la majorité des populations, la prévalence totale de la maladie ronde les 12 - 15%. Dans une étude portugaise, la prévalence a atteint les 17%, notamment la migraine et la migraine associée à d'autres types de céphalées. Les épisodes de la migraine sont caractérisés par la présence d'un mal de tête (ou céphalée), pulsatile, dans la majorité des cas unilatérale, accompagnée par des symptômes comme les nausées, les vomissements et la sensibilité à la lumière et au son (migraine sans aura, MO). Chez environ un tiers des malades, le mal de tête est précédé par une période de aura composée par des symptômes visuels, et dans certains cas, sensoriels (migraine avec aura, MA). C'est la répétition des épisodes qui caractérise la migraine; de moment, il n'est pas connu le motif par lequel les malades avec migraine présentent tant d'épisodes. La compréhension de la pathologie de la migraine – notamment la découverte des facteurs génétiques qui prédisposent à la maladie – permettra le développement de médicaments plus efficaces pour le traitement de la maladie.

Il est su que la migraine se produit en famille, ayant été trouvée un fort component génétique de la maladie. Déterminer les facteurs génétiques sous les formes plus communes de la migraine est une tâche difficile, soit par la prévalence élevée comme par sa complexité en rapport avec les facteurs génétiques et environnementaux. Cependant, les études génétiques en migraine hémiplegique familiale (familial hemiplegic migraine, FHM), un sous-type de MA monogénique, traduisent un certain succès en révélant trois gènes qui codifient pour des transporteurs ioniques: le gène qui codifie pour la sous-unité du canal de calcium, *CACNA1A* (FHM1), le gène qui codifie pour la sous-unité d'une pompe de sodium-potassium, *ATP1A2* (FHM2), et le gène qui codifie pour la sous-unité du canal de sodium, *SCN1A* (FHM3). Plusieurs *loci* de susceptibilité pour les formes communes de migraine ont été décrits, ce qui démontre la complexité de la contribution génétique de la maladie, mais les gènes et les variantes génétiques qui causent la maladie demeurent inidentifiables.

La tâche primordiale a été d'obtenir une population bien caractérisée de cas portugais de malades avec migraine et contrôles. De moment, la banque de DNA est constituée par des échantillons d'environ 1200 individus, qui appartiennent à environ 210 familles, et qui ont été usées dans l'étude génétique conduit. Les échantillons portugais seront cruciaux pour des études futures d'identification de nouveaux gènes.

L'objectif principal du présent travail a été d'étudier la contribution génétique dans la migraine familiale, en tentant élucider ses mécanismes moléculaires pathogéniques. Pour cela, ont été utilisées différents abords: (1) ont été utilisées différentes stratégies pour étudier la migraine 'complexe' (commune) (i.e. MA et MO), et (2) ont été réalisés des études génétiques dans la FHM, étant cette variable usée comme un modèle pour étudier la pathophysiologie de la migraine.

Considérant le premier abord, l'agrégation familiale a d'abord été étudiée estimant le risque relatif. Il a été montré que les familiers des probants avec migraine ont un risque augmenté (de 3 à 4 fois) quand comparés avec la population en général; les conjoints ne présentent pas un risque augmenté. Les résultats confirment des indications antérieures pour l'agrégation de la migraine en familiers du premier degré de probants avec MA et MO; ils confirment aussi que la MA semble présenter une plus grande agrégation familiale que MO, et ainsi possiblement une plus grande prédisposition génétique.

Il a été réalisé une première analyse génétique de nos échantillons avec migraine, utilisant l'analyse de liaison des "affected-only". Malgré le fait que les simulations ont montré que notre échantillon doit avoir le pouvoir suffisant pour détecter une liaison, on n'a pas eu de résultats significatifs pour chacun des *loci* de susceptibilité pour la migraine testés (notamment les régions des gènes de FHM1, FHM2 et FHM3). Cette étude souligne que l'hétérogénéité, phénotypique et génétique, chez les familles avec migraine est plus grande que prévue, ce qui limite les études de liaison. Les régions des gènes de la FHM ne paraissent pas avoir un contributif fondamental pour le risque global de la migraine, du moins chez la population portugaise. Dans la meilleur des hypothèses, les résultats pointent vers un rôle moins important chez des familles particulières. Ceci peut peut-être être démontré par l'identification d'une nouvelle variante FHM2 dans une des familles avec migraine. Dans ce travail, il n'a pas été indiqué un locus ou une variante génétique qui explique tous les cas de migraine chez une famille. Probablement, la migraine est une maladie très complexe, avec d'énormes *loci* et variantes génétiques contribuant pour sa susceptibilité.

Considérant le deuxième abord, plusieurs mutations causant la maladie chez des familles avec FHM ont été identifiées. Tout d'abord, deux mutations dans le gène *ATP1A2* (M731T et T376M) ont été identifiées, permettant établie une corrélation génotype-phénotype, montrant que ces mutations sont associées à la FHM *pure* (i.e., FHM sans maladies neurologiques, comme l'épilepsie ou l'ataxie, associées).

Simultanément, deux nouvelles mutations dans le gène *ATP1A2* (V362E et P796S) ont été découvertes chez des familles avec FHM et phénotypes psychiatriques. Pour les mutations dans le gène *ATP1A2*, des études fonctionnelles de survie cellulaire ont été réalisés, démontrant la cause des mutations. Il a encore été identifié une nouvelle mutation dans le gène *SCN1A* (L263V) chez une famille avec FHM et épilepsie. Malgré le fait que le gène *SCN1A* soit un gène connu de l'épilepsie, la mutation L263V a été la première associée aux deux maladies, renforçant l'idée de que les deux partagent des mécanismes pathophysiologie. Les données fonctionnelles auront à démontrer comment est-ce que la mutation L263V conduit à cet état phénotypique unique.

Malgré le fait que nos études génétiques réalisées n'aient pas indiqué une quelconque variante génétique dans la migraine commune, les études dans la FHM ont ramené des informations importantes pour la pathophysiologie de la maladie. Nouvelles stratégies futures pourront indiquer les variantes génétiques dans la migraine commune et pointer vers l'avance de la connaissance de la maladie.

1. INTRODUCTION

1.1. Migraine

Migraine is a very common type of severe primary headache, ranked by the World Health Organization as one of the most disabling medical illnesses^{1,2}. It is the recurrence of attacks that qualifies it as a disease. Because of the incapability of patients to carry out their day-to-day activities, migraine poses an important Public Health problem, with considerable economic and social implications, with loss of productivity or absence at work, and high direct (medical care) and indirect (examinations and treatments) costs³.

1.1.1 Classification and diagnostic criteria

A systematic approach to headache classification and diagnosis is essential for good clinical management, treatment, and research. The International Headache Society (IHS) published guidelines⁴, which were recently revised (ICHD-II)¹, dividing primary headaches into four main categories: migraine, tension-type headache (TTH), cluster headache (CH) and other trigeminal autonomic cephalalgias (TACs), and other primary headaches. The diagnosis of migraine is currently established according to the ICHD-II criteria¹ (see **Table 1**), based on the headache characteristics and associated symptoms. The main changes introduced by the ICHD-II include the restructuring of these criteria into different groups, modifications for pediatric populations and classification of both chronic and episodic forms.

ICHD-II Classification
1.1 Migraine without aura
1.2 Migraine with aura
1.2.1 Typical aura with migraine headache
1.2.2 Typical aura with non-migraine headache
1.2.3 Typical aura without headache
1.2.4 Familial hemiplegic migraine
1.2.5 Sporadic hemiplegic migraine
1.2.6 Basilar-type migraine
1.3 Childhood periodic syndromes that are commonly precursors of migraine
1.3.1 Cyclical vomiting
1.3.2 Abdominal migraine
1.3.3 Benign paroxysmal vertigo of childhood
1.4 Retinal migraine
1.5 Complications of migraine
1.5.1 Chronic migraine
1.5.2 Status migrainosus
1.5.3 Persistent aura without infarction
1.5.4 Migrainous infarction
1.5.5 Migraine-triggered seizure
1.6 Probable migraine
1.6.1 Probable migraine without aura
1.6.2 Probable migraine with aura
1.6.5 Probable chronic migraine

Table 1 – Migraine subtypes, according to the International Headache Society diagnosis criteria, ICHD-II¹.

1.1.1.1 Migraine common subtypes

Migraine without aura

The ICHD-II diagnosis criteria classify migraine into two main subtypes: migraine without (MO) (1.1; **Table 1**) and with aura (MA) (1.2; **Table 1**)¹. The most common is MO, which is characterized by recurrent attacks of disabling headache lasting between 4 and 72 hours (1.1; **Table 2**)¹. The headache is usually severe, unilateral, pulsating, aggravated by physical activity, and associated with nausea and/or vomiting or photo- and phonophobia¹.

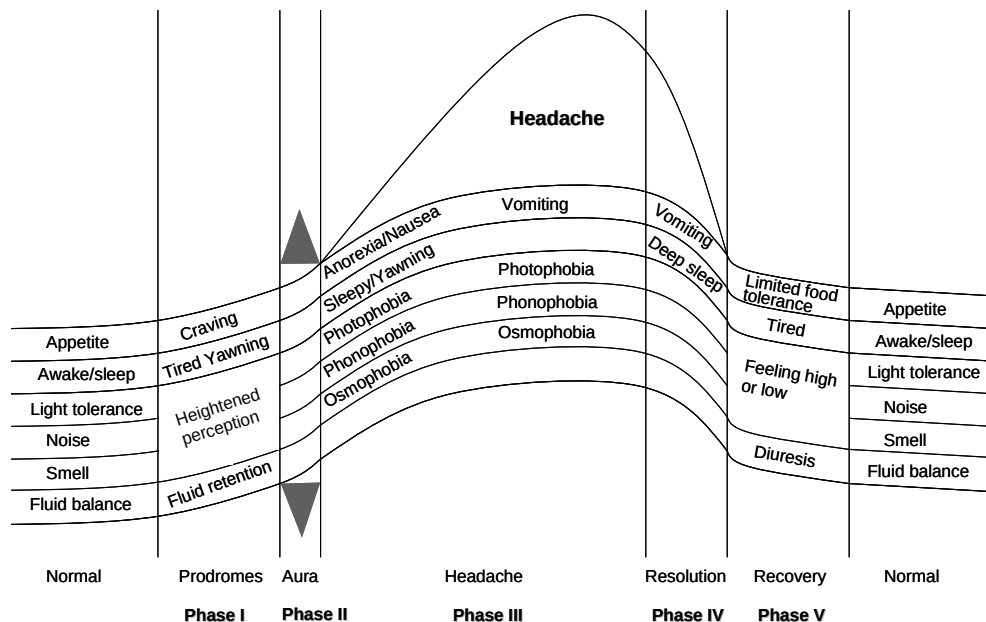
ICHD-II Classification	
1.1 Migraine without aura	
A.	At least five attacks fulfilling criteria B-D
B.	Headache attacks lasting 4-72 h (untreated or unsuccessfully treated)
C.	Headache has at least two of the following characteristics:
1.	Unilateral location
2.	Pulsating quality
3.	Moderate or severe intensity (inhibits or prohibits daily activities)
4.	Aggravation by walking stairs or similar routine physical activity
D.	During headache at least one of the following:
1.	Nausea and/or vomiting
2.	Photophobia and phonophobia
E.	Not attributed to another disorder
1.2.1 Typical aura with migraine headache	
A.	At least two attacks fulfilling criteria B-D
B.	Fully reversible visual, sensory, and/or aphasic aura symptoms (but no motor weakness)
C.	At least two of the following:
	Homonymous positive features and/or unilateral sensory symptoms
	At least one symptom develops gradually over more than 5 min and/or two or more occur in succession
	Each symptom lasts 5-60 min
D.	Headache fulfilling criteria B-D for 1.1 begins during aura or follows aura within 60 min
E.	Not attributed to another disorder

Table 2 – Diagnostic criteria for MO and MA, according to the International Headache Society guidelines, ICHD-II¹.

Migraine with aura

In MA, which occurs in about one-third of the patients, the headache is very similar to that of MO, but the attacks are preceded or accompanied by transient focal neurological, mostly visual benign aura symptoms (1.2.1; **Table 2**)¹. An aura usually develops slowly over 5-20 minutes, and lasts for less than 60 minutes. In 90% of cases, the aura is a visual anomaly, but sensory, motor and mental phenomena, and/or speech disturbances can also be part of the aura. Although benign, and completely reversible in most cases, serious neurological complications have been reported, namely migrainous stroke, migraine-related seizures and persistent auras without infarction, establishing a relationship among MA, stroke and epilepsy⁵.

A typical migraine attack can be subdivided in several phases⁶, as shown in **Figure 1**. The aura phase is absent in MO. Aura can also occur without accompanying headache (1.2.3; **Table 1**).



(Adapted from Blau JN. Migraine: theories of pathogenesis. Lancet 1992 339:1202-7)

Figure 1 – Graphical representation of the stages of a typical migraine attack (Phase I to V), showing most of the associated symptoms.

1.1.1.2 Rare, severe migraine subtypes

Severe migraine variants include basilar-type migraine (BM) (1.2.6) and hemiplegic migraine (HM). Hemiplegic migraine may be sporadic (SHM) (1.2.5) or familial (FHM) (1.2.4) and is characterized by the occurrence of hemiparesis, usually preceding the migraine episodes¹. Based on the number of affected patients identified in a Danish population-based study, the prevalence of HM was estimated to be 0.01%⁷. The male: female sex ratio reported for SHM was 1: 4.5, with age of onset (AO) before 45 years in 97% of the cases⁷, which is not much different from the 1: 2.5 ratio found in FHM⁸. No differences between genders were observed in aura or other headache characteristics⁸.

Basilar-type migraine

Basilar-type migraine (BM) is characterized by aura symptoms, such as dysarthria, vertigo, tinnitus, hypacusia, diplopia, simultaneous bilateral visual symptoms, ataxia, decreased level of consciousness and bilateral paresthesias¹, originated from the brainstem or from both hemispheres simultaneously. The headache meets the criteria for MO. During BM attacks patients can also experience symptoms resembling MA. Diagnosis is restricted to patients without hemiplegic aura, although 60% of the patients with FHM, or with SHM, may have at least two basilar-type aura symptoms during hemiplegic attacks⁹.

Familial hemiplegic migraine

The aura in FHM may be described either as reversible, slowly progressive, or acute, unilateral, motor deficit of the extremities (hemiparesis), with or without hemisensory deficits that can be attributed to cortical motor dysfunction⁹. Visual hemianopsia may be present. Speech and language disturbances (aphasia) are the most frequently reported associated symptoms; dysarthria (slurred speech) occurs in 83% of cases⁹. Some patients have atypical attacks, either with prolonged aura lasting up to five days, or with signs of diffuse encephalopathy, expressed as confusion or coma, fever and, sometimes, seizures^{10,11}. Moreover, about 20% of FHM families show permanent cerebellar symptoms consisting of progressive cerebellar ataxia, with or without nystagmus⁸. In these patients cerebral atrophy may be detectable by brain imaging¹². Epilepsy is less frequent (around 7%)¹³. Minor head trauma is a known trigger of the attacks (9% of the FHM cases)⁹.

In the past, FHM diagnosis was purely based on clinical experience with a small number of patients and families. In the old criteria⁴, FHM was classified as an MA subtype, and hemiplegia was crucial for the diagnosis. The revised guidelines¹ included several changes: 1) relaxing the definition of hemiparesis to motor weakness, 2) obligating the presence of at least one other aura symptom and 3) considering the headache not mandatory. Additionally, the diagnosis is now possible considering the presence of motor weakness in a second-degree - not just first-degree - relative¹. However, some major problems remain, including cases of patients that do not fill all criteria, clinical and genetic heterogeneity, a wide phenotype spectrum for mutation carriers and the presence of phenocopies¹¹.

Sporadic hemiplegic migraine

Hemiplegic patients are not always clustered in families; many are sporadic patients. SHM has clinical symptoms similar to FHM⁸ and different from typical MA¹⁴. Comparing SHM with BM, during the attacks, revealed that 72% of SHM patients fulfilled BM criteria, similar to FHM^{8,9} and unlike MA⁹. Cases with motor weakness should be classified as SHM or FHM⁷. Clinical heterogeneity in SHM is even more pronounced than in FHM and genetically few mutations have been found^{10,15-20}.

MA, FHM and SHM seem all to be different disease entities^{8,14}, but no solid epidemiological or genetic evidence supports that MA and BM are really independent²¹. Recently, a mutation in the *ATP1A2* gene, the FHM2 gene, was reported²², which suggests that BM and FHM may share common genetic pathways.

1.1.2 Epidemiological studies in migraine

1.1.2.1 Incidence rates

Overall incidence rates for migraine reach 6.0 per 1,000 female, and 2.2 per 1,000 male person-years²³ and are comparable between populations. Peak of age at onset (AO) probably is during childhood/adolescence and is earlier for boys, with the peak age for MA being earlier than in MO. However, the particular population survey included only young adults²³. More recently, a higher incidence (8.1 per 1,000 person-years), with a male: female ratio of 1: 6, was reported from a Danish longitudinal study, where TTH presented a 14.2 per 1,000 person-years incidence and a male: female ratio of 1: 3²⁴. The main advantages of the longitudinal method over cross-sectional studies, which may explain the rate differences, is that recall bias is avoided and possible changes in risk over time are considered²⁴.

1.1.2.2 Prevalence

Migraine has an overall prevalence of about 12 to 18% in most populations²⁵⁻²⁷. It has been estimated that 10% of the Western population are active migraineurs²⁸. Prevalence is similar in Europe and North America, although it varies with age, sex, population and socioeconomic background^{29,30}. In fact, Caucasian populations present a higher prevalence, presumably due to differences in genetic susceptibility³¹.

In Portugal, two studies of migraine prevalence were published: one was performed in University students³², a second one was an headache survey in an urban population from the Northern region of Portugal³³. In the latter, the prevalence was estimated at 8.8% for migraine, 7.9% for migraine associated with other types of headaches (i.e., episodic and chronic tension-type headaches and others), which gives a total prevalence for migraine of 16.7%³³.

Migraine is consistently found to be more frequent in women^{25,34,35}. Sex-ratio prevalence can vary across populations, with male: female ratios ranging from 1: 2²⁵ to 1: 4³⁶. In a twin study performed across five European countries and Australia, prevalence of migraine ranged 2 to 17%, in men, and 10 to 34%, in women, showing the typical female excess in adult populations³⁷; prevalence in twins was comparable to that in the general population³⁸.

Migraine is also age-dependent, in both sexes, constantly increasing until the fourth decade of life²⁶, with the incidence peak occurring earlier in men³⁰. The profile of migraine changes over life²⁶, which explains the differences between 1-year and lifetime prevalence rates^{27,35}. Comparison of disease prevalence and incidence values, with their trends overtime, may lead to useful hypotheses on risk factors^{27,39}.

1.1.2.3 Familial Aggregation

Relative Risk

In migraine, the pattern of familial aggregation has been mostly assessed by calculation of relative risk (RR), but twin studies were also reported. Familial clustering is recognized, but methodological differences have provided variations in familial aggregation results, evaluated by calculation of RR, ranging from 1.5 to 11.8⁴⁰⁻⁴³.

RR estimations suggest that familial factors, environmental and/or genetic, account for less than one-half of all cases in the population, and that RR was higher in families of migraine probands⁴⁰. An important study showed that there was an increased disease risk for relatives, with first-degree relatives of probands with MO having 1.9 times the risk of MO and 1.4 times the risk of MA, while first-degree relatives of MA probands had nearly 4 times the risk of MA but no increased risk of MO⁴¹. Another interesting question that was addressed is how proband characteristics (AO, gender, etc.) can influence RR estimation. Earlier onset and greater severity of migraine were associated with higher familial aggregation⁴³. Also, a higher risk was reported among siblings from three-generation families with MA, when compared to families with less generations⁴⁴. One study presented young age, female gender, no education, familial history and high workload as factors of increased risk for migraine²⁴. Thus, migraine is likely to be influenced by different genetic and non-genetic factors; RRs show that it is familial, but cannot provide evidence that is genetic.

Heritability

The quantitative genetic modeling of twin data allows the quantification of the contribution of genes and of the environment, showing the relative importance of genes (heritability) in liability to disease. Spouses can be useful to determine environmental factors, since they partly share the same environment, but differ more in their genetic constitution.

The fact that the concordance in monozygotic⁴⁵ twins does not reach 100% shows that environmental factors play a role in migraine. Twin studies also indicate that up to 50% of the variation in migraine is due to genetic factors, as suggested by the observed concordance rates between dizygotic (DZ) and MZ twin pairs^{38,46}.

In fact, migraine shows high heritability, ranging from 0.28 to 0.65⁴⁷. Heritability is high for both MA and MO^{37,48}. Mulder et al. showed that migraine genetic variance can also be non-additive, showing summed additive and non-additive genetic effects (broad heritability) ranging from 34% to 57%³⁷. This result does not confirm previous studies that supported additive genetic variability with a negligible contribution of non-additive genetic effects⁴⁹. The same study confirmed the contribution of non-shared environmental factors to the liability of the disease, previously indicated as ranging from 35 to 55%, with shared factors having no impact³⁷. This latter fact is in agreement with previous results obtained in MA⁵⁰.

Population-based epidemiological and twin studies have shown that both MO and MA have a genetic component^{41,50,51}. Also, it seems that the genetic contribution is more modest in MO than in MA^{50,51}.

1.1.2.4 Patterns of inheritance in migraine

The mode of transmission of migraine remains a topic of controversy. Several modes have been proposed, such as the autosomal dominant, autosomal recessive and multifactorial⁵². Thus far, segregation analysis studies favors migraine as a multifactorial disorder^{42,53}, but the contribution of a major gene cannot be excluded⁴². Inheritance of the major forms of migraine is probably complex. Environmental factors must play a role, which is in agreement with the heritability estimates (**Figure 2**).

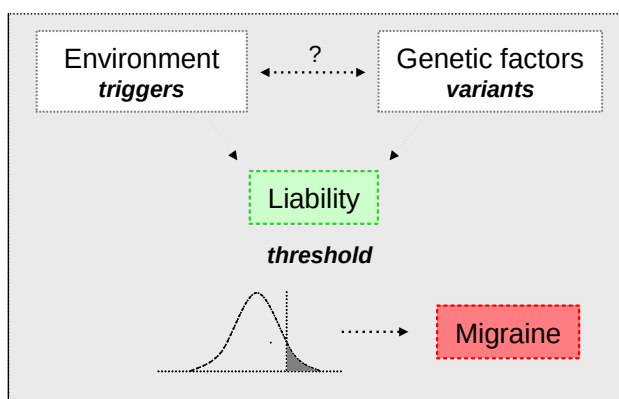


Figure 2 – Schematic representation of migraine as a multifactorial disease: environmental and genetic factors contribute to the overall liability; liability was first defined by Falconer⁵⁴.

1.1.3 Comorbidity

Comorbidity implies more than a coincidental association between two disorders⁵⁵. Particularly in migraine, a number of conditions have been noted to be comorbid, notably psychiatric disorders^{56,57}, epilepsy⁵⁸, stroke, asthma, gastrointestinal disorders, congenital heart defects⁵⁶, and vascular brain diseases, such as CADASIL⁵⁹.

A strong association between migraine and epilepsy has been shown^{58,60} and confirmed by genetic findings⁶¹. Prevalence of epilepsy is lower than that of migraine (around 0.5 to 1%)⁶²; 6% of patients with migraine have epilepsy, whereas 8 to 15% patients with epilepsy have migraine⁶³. Bi-directional association of migraine and major depression has long time been established, as well as association with bipolar and anxiety disorders⁶⁴. MA male cases showed a higher rate of epilepsy⁶⁵, but not of anxiety and depression⁵⁷.

An important message is that migraine patients are at an increased risk for a variety of conditions. Certain approaches should be considered when migraine is present, as prophylaxis of stroke and hypertension, treatment of allergic and psychiatric conditions, as well as careful choice of antiepileptic drugs⁶⁶. The organization of migraine comorbid constellations, to improve diagnosis and disease management, has been suggested⁶⁷.

From a genetic point of view, the main advantage of studying migraine comorbidity is that it provides additional information to locate new susceptibility genes taking into account that both could share metabolic pathways. Recently, a linkage peak was found, for chromosome 9, in a family with epilepsy and migraine⁶⁸.

1.1.4 Predisposing factors and triggers

Risk factors for individual attacks are called precipitating factors, or triggers, and appear before the attacks starts⁶⁴. These include stress, weather changes, suspected food triggers or menstrual cycle; lack of sleep and alcohol abuse can also increase the number of attacks. The abnormal recurrence of attacks suggests that triggers may initiate them when they are particularly strong and frequent or when threshold is reduced⁶⁹. While genetic factors can set the individual threshold, internal and environmental factors may modulate the trigger point⁶⁹.

1.1.5 Pathophysiology

The current hypothesis is that migraine is a neurovascular disease⁷⁰. Migraine is caused by increased excitability of the central nervous system (CNS). The aura in migraine is thought to be caused by neuronal dysfunction, whereas headache starts or follows a cortical blood flow reduction⁷¹.

1.1.5.1 Aura

Cortical spreading depression (CSD)⁷² most likely underlies the visual aura in migraine, based on the slow spread of clinical and electrophysiological events, as seen in animal⁷³ and human⁷⁴ experiments. The aura is associated with an initial hyperaemic phase, followed by reduced cortical blood flow persisting from 30 minutes to 6 hours⁷⁵. Progression of a spreading oligoemia is similar to that of migrainous scotoma and CSD, suggestive of a relation.

Results suggest that migraine aura can be explained by neuronal hyperexcitability because of cortical disinhibition^{71,76}. The cause of (interictal) neuronal hyperexcitability in migraine aura is unknown, but may be linked to instability of cell membrane potential, by impaired oxidative metabolism, the imbalance between excitatory (glutamergic) and inhibitory (gabaergic) systems, or mutations in membrane channels with alterations of neuronal conduction⁷⁷. Aberrant firing of neurons and CSD related elements were shown by functional magnetic resonance imaging (MRI) records of induced and spontaneous migraine aura; vascular alterations followed these changes in neuronal activity⁷⁸.

1.1.5.2 Headache

In brief, headache and associated neurovascular changes are the result from activation of the trigeminovascular (TGV) system, which will induce neurotransmitter release, and subsequent neurogenic inflammation and vasodilatation^{79,80}. Several vasodilator peptides are to be found within the trigeminal neurons that innervate blood vessels, namely substance P (SP), calcitonin gene-related peptide (CGRP) and neurokinin A⁷⁰. Nerve fiber peripheral sensitization will in part cause migraine pain, but central sensitization is also occurring, and may contribute to maintain the headache.

The link between aura and the subsequent headache is still controversial, but recent work in rats showed how a primarily cortical phenomenon, such as CSD, is able to activate trigeminal fibers and trigger brainstem events consistent with the development of

headache⁸¹. CDS activates TGV afferents causing a long-lasting increase in the middle meningeal artery blood flow and plasma protein extravasations within the dura matter.

1.1.6 Biochemical abnormalities

Several biochemical abnormalities seem to support the hypothesis of a hyperexcitable brain in migraine. Low systemic levels of serotonin, mitochondrial defects, magnesium deficiency, dysfunctional ion channels, increased membrane instability, central sensitization of trigeminal fibers and excessive excitation due to abnormal release of excitatory neurotransmitters are just some examples of possible factors intervening.

1.1.7 Biomarkers

A biomarker is defined as a physical sign or laboratory measurement that occurs in association with a pathological process and that can be therefore of use both in putative diagnostic or prognostic processes⁸².

In migraine, several biomarkers (including clinical, genetic, neurophysiologic, and radiological and/or biochemical) may help in improving the diagnosis, knowledge of the pathophysiology and optimize the treatment. Until now, several biomarkers have been used in the clinical practice, but only a part has shown to be reliable⁸³. Functional MRI seems a promising technique⁸⁴.

Genetic biomarkers are restricted to mutations in the FHM genes and polymorphisms in for instance the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene⁸⁵; but, this will likely improve, once that genetic susceptibilities of common migraine forms are discovered.

1.1.8 Treatment options

Pharmacotherapy for migraine can be acute or prophylactic, and depends on the severity and frequency of the episodes, associated symptoms, coexistent disorders, previous treatment response and drug efficacy⁸⁶. There are significant differences across countries in treatment recommendations for migraine. For review on acute and prophylactic treatment options see^{69,87}.

1.2. Mapping human complex disease genes

Complex diseases are thought to be due to combined effects of variations in multiple interacting genes and environment⁸⁸. Heart diseases, asthma, diabetes and psychiatric disorders are well known examples. Mapping susceptibility genes for complex diseases⁸⁹ is still challenging, since straightforward strategies, such as linkage analysis in families and association studies in large population samples, have remained largely unfruitful.

Multifactorial inheritance usually concerns complex, quantitative traits, e.g., traits that vary continuously in a continuous phenotypic range. These traits are influenced by multiple genes, called quantitative trait loci (QTL), each one having a small effect, and interacting with the environment. Migraine is a dichotomous trait, but it is determined by an underlying continuous distribution of its liability, with a threshold above which migraine appears.

1.2.1 Main genetic approaches

1.2.1.1 Linkage

Linkage analysis is based on recombination events that occur during meiosis. Recombination occurs between homologous chromosomes, which will lead to new gametes that may be transmitted to offspring. The rationale of linkage analysis is that the disease gene and a closely linked genetic marker are inherited together, more often than expected, when the distance between them is very small. Over time (i.e. after many generations) they get separated by recombination events.

The identification of disease genes by positional cloning includes genetic mapping of the trait within pedigrees by linkage analysis. Fine mapping and selection of candidate genes are the following steps, which ends by the identification of pathogenic mutations. By using families with multiple affected individuals, many genes for Mendelian diseases inheritance, such as Huntington disease or cystic fibrosis, have been found⁹⁰.

For linkage analysis of complex traits, several methods have been proposed, including maximum likelihood-based methods (LOD scores) and nonparametric approaches, such as affected sib-pairs (ASP) methods and non-parametric linkage (NPL) statistics⁹¹.

LOD score

Likelihood and LOD score⁹² are measures of plausibility of the observed data, with values dependent on assumed values of the recombination fraction (θ), with highest value estimated called maximum likelihood estimate, MLE^{93,94}. In linkage analysis between two *loci*, the basic hypotheses are free recombination (H_0) and linkage (H_1), defined by the corresponding values of $\theta=0.5$ and $\theta<0.5$, respectively. Conventionally, the common (decimal) logarithm of the likelihood ratio (or **odds**), is called LOD score and is used as measure to support for linkage versus absence of linkage for any given value of θ :

$$Z(\theta) = \log_{10} [L(\theta)/L(\theta=0.5)]$$

LOD scores ('lods') are additional over families. If there is linkage, at a given value of θ , the maximum LOD score tends to increase with an increasing number of families. For single-point analysis (two *loci*), linkage is considered to be significant if the LOD score reaches 3 (an odds ratio of 1000: 1 that a marker is linked to a disease locus), whereas if below -2, the marker can be excluded for linkage to the disease; values in between are inconclusive⁸⁸. The threshold for multipoint linkage analysis of Mendelian diseases is 3.3⁸⁸, whereas for complex diseases it can vary from 3.3 to 4.0 depending on the study design and the number of markers used⁹⁵. Results of linkage analysis in complex traits are still controversial, because of the high false-positive rate (type 1 error), especially in multipoint genome-wide approaches⁹⁶ and under heterogeneity⁹⁷.

Model-based, parametric tests

Conventional parametric linkage analysis requires prior knowledge about the genetic model (i.e. the mode of inheritance, the degree of penetrance, gene frequencies, and genetic heterogeneity), and is, therefore, even more difficult to use for gene mapping in diseases with complex inheritance. Parameters include defining the disease allele frequency, the penetrance of the mutant gene, sex differences, and the possibility of genetic heterogeneity and phenocopies⁹³. After obtaining the genotyping data for the target genome region, parametric linkage analysis can be performed, using the LINKAGE program package⁹⁸ or GENEHUNTER⁹¹. These tests can use two-point analysis, where linkage is evaluated between the disease and each marker, or multipoint analysis where multiple markers are tested simultaneously⁹³.

In case several families are analyzed together, genetic heterogeneity may be expected⁹⁹. When several disease *loci* exist, but only one segregates in a given family (heterogeneous traits), an estimate of the proportion (α) of the families in which the trait gene is linked with the marker map can be calculated together with the heterogeneity LOD (HLOD) score, using HOMOG⁹³.

Although it is known that one can legitimately maximize the maximum LOD score with respect to genetic parameters, this approach raises three concerns: (1) multiple testing, (2) effect on power to detect linkage, and (3) adequacy of the model. Commonly, lods are calculated by assuming two simple models, dominant and recessive, each with 50% penetrance, then the higher of the two lods is taken as the raw test statistic and corrected for multiple tests¹⁰⁰.

Model-free, non-parametric tests

The alleles contributing to the disease may be common in the population, which makes linkage very difficult to detect. The non-parametric or allele-sharing methods require no prior knowledge of the disease model⁹¹. Non-parametric analyses have shown to be more efficient when studying common disorders, since parameters such as penetrance, phenocopy rate and gene frequency are often difficult to ascertain.

The NPL method⁹¹ can only use affected individuals from either pairs or all the data set. Family-based NPL study can be used for sib ship analysis, studying ASP¹⁰¹ or affected relative-pairs (ARP)¹⁰². LOD generated in nonparametric analyses represent the log-likelihood ratio of the data under the hypothesis that the allele-sharing proportion has the observed value as compared to the hypothesis of no excess (i.e., random) sharing⁹⁵. Multipoint NPL makes use of information from all markers on a chromosome to estimate the position of a hypothesized gene locus in an interval flanked by two markers. Although this strategy seems more powerful when the model of the disease is unknown, it does not make use of all pedigree information and is therefore more sensitive to heterogeneity when compared with LOD score-based linkage¹⁰³.

Affected Sib Pairs

The sib pair method, introduced by Penrose, is based on the relative frequencies of pairs of siblings being alike or different for two traits¹⁰⁴. In ASP methods, affected siblings are tested on the hypothesis that a marker locus is closely linked to a

disease locus, so that the disease gene is transmitted to the offspring with the same marker allele. It overcomes the problem of incomplete penetrance, but not the occurrence of non-genetic cases (e.g. phenocopies), which can reduce the power of test¹⁰⁵. The rationale is to check how frequently two affected offspring share copies of the same parental marker allele and are said to be identical by descent (IBD); an ASP shares 0, 1 or 2 alleles, IBD that can be computed as IBD sharing probabilities. Thus, in the presence of linkage, one expects a deviation towards a higher number of alleles shared IBD.

In sib pair analyses, it has been recommended that both concordant siblings or discordant pairs provide substantial power¹⁰⁶. Multiple affected siblings can be evaluated with LOD score programs, which are able to analyze independent pairs and correct for related pairs. Increased power of ASP strategies over the LOD score methods remain to be proven, under several complex disease models¹⁰⁷.

Affected Relative Pairs

Pairs of relatives, other than siblings, are suitable for NPL¹⁰². One extension of ASP to sets of ARP was developed in the affected pedigree member method, based on the identity by state (IBS), rather than IBD, which checks if the allele is the same and not if it is coming from a common ancestor. Power of analyzing other, more distantly related, pairs of relatives is set to be higher than closely-related sib pairs, since no information of pedigree members is lost, particularly if the genetic heterogeneity model (no epistasis) is considered¹⁰².

Currently, linkage studies still have limited success in identifying genes for more complex diseases. Association studies for gene mapping in humans are now preferred, both due to the improvement in large-scale genotyping resources and to the increased power and resolution of location of the region mapped, when compared with linkage studies¹⁰⁸.

1.2.1.2 Association

Association between a variation in the human DNA sequence and a disease or trait exists when both occur together in the same person more often than expected just by chance. Association tests compare allele frequencies in cases and controls, to assess the contribution of genetic variants to phenotypes, in a specific population. In principle, for an equivalent number of genotypes and for common disease alleles, population-based tests

offer greater power to detect the presence of genes with minor effects than pedigree-based designs¹⁰⁸, but this is not always the case. In fact, sibship data can be preferred, if multiple genes or common disease alleles are influencing the disease¹⁰⁹⁻¹¹¹.

Case-control studies are particularly useful for late-onset diseases and family-based approaches are indicated to overcome population stratification^{88,112}; for a review see¹¹³. Positive results are increasing, but the number that are also replicated is still rather low¹¹⁴, possibly due to low statistical power, multiple testing, study design variability, phenotype definition and/or statistical modeling and population substructure¹¹⁵. Importantly, family-based genomewide association studies have suggested how to overcome multiple testing problem¹¹⁶.

The term linkage disequilibrium (LD), originally referred to association of alleles at different *loci*, measures the mean association among alleles due to close linkage. The presence of LD does however not imply linkage, but indicates that the marker is tightly associated to the disease region. It is a powerful tool for fine mapping, since it pinpoints a region for the disease susceptibility locus more accurate than in linkage studies¹¹⁷.

Case-control studies

Classic case-control study designs compare allele frequencies or genotypes in a sample of unrelated affected individuals and a sample of matched controls¹¹⁸. Population-based case-control studies are used to test for association, with the advantage of not needing familial ascertainment to detect disease alleles. Isolated populations are a popular target for association studies, because their homogeneity minimizes population structure¹¹². Determinants for the success of this type of study include the significance of candidate genes, the careful selection of cases and control subjects, the previous assessment of the Hardy-Weinberg equilibrium in the markers studied within the control group, and a final correction for multiple comparisons.

TDT

The transmission disequilibrium test (TDT)¹¹⁹ is the simplest family-based design to test for association. It uses genotype data from trios, comparing the observed number of alleles that are transmitted with the number of non-transmitted alleles. The excess of alleles of one type among the affected persons indicates that a disease susceptibility locus for a trait of interest is associated and may be linked with the marker locus.

Initially, TDT was used to test for linkage in the presence of association, but nowadays is used as a test for association¹²⁰. Several extensions to the basic TDT have increased the power of the method, as family-based association tests, which further increase robustness considering the cases of missing parents, general pedigrees, complex phenotypes and haplotypes with missing phase¹¹³.

Population- and family-based methods should be viewed as complementary¹²¹. Statistical power when comparing trios and case-control was not that different¹²², but it actually depends on the allele effect and on disease genetic model¹⁰⁹. One should keep in mind that population stratification is always an issue¹¹⁵ and multiple testing should be overcome.

Candidate versus Genomewide association studies

The characteristics of complex diseases, which are problematic in linkage analysis, including genetic heterogeneity, phenocopies, incomplete penetrance, environment interaction, and multilocus effects, also limit the power of association studies. Linkage is the most powerful method to detect rare high-risk disease alleles, but association studies give more power to detect common disease alleles with low penetrance¹²³. Because “suggestive-linkage” regions can also contain common disease alleles with modest RR, there is some enthusiasm for association analysis of candidate regions of the genome identified with those strategies¹²⁴. Furthermore, the availability of dense SNP maps through the Hap Map Project¹²⁵, is allowing that approaches such as candidate gene association studies are applied effectively, both by candidate/direct SNP analysis (e.g., cSNPs) or an indirect association test (e.g., tagSNPs).

In the not so distant future, genomewide association studies will become feasible, and is thus not so unrealistic as it was thought a few years ago^{126,127}. One should keep in mind that association studies are difficult to perform, give limited results and demands careful interpretation. Large-scale studies with combined population cases and controls resulted in few association variants¹²⁸.

1.2.2 Common disease-common variant *versus* rare variant hypothesis

Rare variants, alone, are not expected to explain common diseases, as they do for monogenic disorders, but a few exceptions have been reported. Two main hypotheses for common diseases are often considered.

(1) The *common disease - common variant* (CD-CV) hypothesis proposes that genetic risk may often be due to predisposing variants relatively common in the population¹²⁹. Based on this, several systematic association analyses of common variants have been performed, such as APOE*4 in Alzheimer's disease¹³⁰. Simultaneously, different common variants conferring susceptibility or genetic modulation of risk have been described, for the sudden infant death syndrome¹³¹, prostate cancer¹³² and type-2 diabetes¹³³. (2) Another theory, however, defends that complex diseases may be attributed to mildly deleterious genes, the overall mutation rate of which is relatively high¹³⁴.

Linkage analysis or association would not be appropriate for predicting rare, mildly deleterious variants¹³⁵. Therefore, direct mutation analysis of candidate genes complements these strategies, overcoming some of its necessary assumptions and limitations.

1.2.3 Models for complex disease

1.2.3.1 Environmental contribution

If a trait “runs in families”, it does not always mean that it has a genetic contribution. Fisher assumed that genes act as risk factors for common traits, in a manner necessarily dominated by an additive polygenic term¹³⁶, but this theory was questioned since phenotypic effects can result also from the synergistic action of alleles¹³⁷. Additionally, the environment must play an important role; both positive and negative gene-environment interactions have also been observed in human diseases¹³⁸. Besides, cultural inheritance, influences from relatives and environments are not easy to distinguish. Classic twin studies (monozygotic (MZ) versus dizygotic (DZ)) are the main tool to dissect the genetic contribution, but are not always suitable¹³⁹.

1.2.3.2 Two-locus or multilocus inheritance

For a quantitative phenotype the effects of environmental and genetic factors are additive and mutually independent, while for a qualitative phenotype a liability multifactorial model with threshold is assumed^{54,93}.

In two-loci¹⁴⁰ and multiloci⁹³ inheritance models, multiple alleles at each locus are contributing to the disease. Three classes of two-loci models are known: 1) the epistatic or multiplicative model (with two dominant *loci* interacting); 2) the heterogeneity model, approximately additive (with susceptibility conferred by a genotype on one of the *loci*); and

3) the threshold model (with the number of susceptibility alleles conferring liability). The threshold liability model is similar to the polygenic inheritance model, but liability is assumed as the quantity needed for an individual to become affected; if it exceeds a certain threshold the disease will occur.

1.2.4 Recent examples in literature

A genome scan, under NPL analysis (ASP) in a large sample set of schizophrenia families, with European and African-American ancestry, showed five potential susceptibility *loci*, two of which in both populations¹⁴¹. Very recently, a genomewide study in a group of families from a Dutch isolate presented significant linkage in four *loci* for late-onset Alzheimer disease, suggesting several potential disease-causing genes that must be under study¹⁴².

Pooling based genomewide association studies that use high-throughput SNP genotyping microarrays were successfully used in common Alzheimer disease and are possibly the logical first step for determining whether major genetic associations exist in complex diseases with higher heritability¹⁴³. A huge genomewide association study, involving 14,000 cases of seven common diseases (bipolar disorder, coronary artery disease, Crohn's disease, rheumatoid arthritis, type 1 and 2 diabetes) produced 24 independent association signals, proving that large sample set demands and appropriate control set are major difficulties for these disorders¹²⁸.

1.3. Genetics of Hemiplegic Migraine

1.3.1 FHM as a model

Much progress in deciphering the genetic background of migraine has been made by studying FHM; for a recent review see¹⁴⁴. Aside from the hemiparesis or motor deficit, the characteristics of the aura and the headache are similar to those of the common forms of the disease⁸. Genetic factors play an important role in the pathophysiology of migraine, probably by lowering the threshold level for migraine attacks.

1.3.2 Clinical features

One of the most important steps for the genetic analysis of a disease like migraine is to define the phenotype, including ascertaining its clinical characteristics such as AO, familial history, the frequency and nature of the episodes, and the presence of other neurological manifestations, such as epilepsy, ataxia or mental retardation. Some mutation carriers in FHM families also present MO or MA attacks. In fact, there is a 7.1 times increased risk of MA in probands with FHM and their relatives, compared to the general population¹⁴⁵. This suggests that the genes causing FHM may also cause part of the symptomatology of MA¹⁴⁵.

FHM attacks resemble those of basilar migraine⁹. Compared with MA, FHM has lower AO (higher genetic load), more prolonged aura and more patients showing basilar-type symptoms (69%) and headache (99%)⁸. Patients can experience atypical attacks with prolonged aura, and, sometimes, epileptic seizures^{10,11}. Around 20% of FHM families show associated cerebellar symptoms⁸. Emotional stress and minor head trauma are among the most common triggers of FHM attacks¹⁰.

1.3.3 FHM genes and regions

FHM is genetically heterogeneous and three genes have been described so far¹⁴⁴. Clinically, there are few differences in symptoms between the different genetic types of FHM, except for the cerebellar symptoms that are almost exclusive of FHM1.

1.3.3.1 FHM1, *CACNA1A* gene

Since, in 1993, linkage of several FHM families to chromosome 19p13 was reported¹⁴⁶, genetic heterogeneity was noticed, as unlinked families were found^{147,148}. Clinically, linked and unlinked families were not different, except for the presence of cerebellar signs, minor head trauma trigger and associated coma that were more usual in the families linked to chromosome 19p13^{10,149}. In around 50% of the FHM families studied, the responsible gene defect maps to the 19p13 locus and mutations have been identified in the *CACNA1A* gene (FHM1), which encodes a brain-specific Ca_v2.1 (P/Q-type) calcium channel subunit (formerly known as α_{1A} , *CACNL1A4* gene)¹⁵⁰. Currently, from more than thirty mutations described in this gene, only nineteen missense mutations have been associated with FHM1¹⁴⁴ (**Figure 3**).

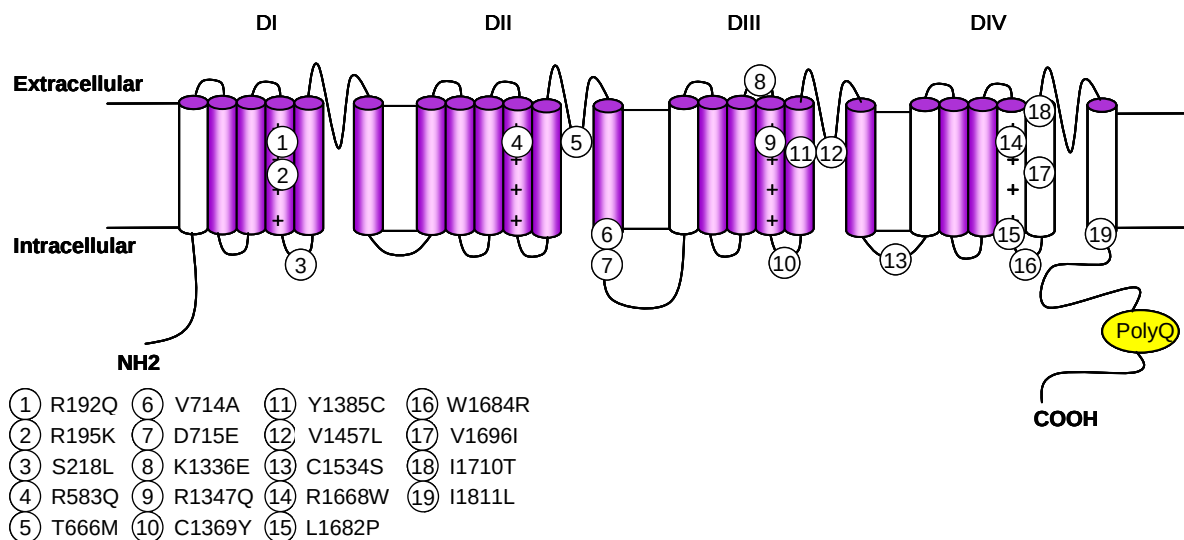


Figure 3 – Ca_v2.1 topology with the location of FHM1 mutations identified.

Mutations in *CACNA1A* gene may cause “pure” FHM or FHM with additional neurological symptoms, including ataxia, epilepsy and/or coma. However, *CACNA1A* has also been involved in other neurological disorders with autosomal dominant (AD) inheritance, namely episodic ataxia type 2 (EA2) and spinocerebellar ataxia type 6 (SCA6)¹⁵⁰⁻¹⁵². This adds further complexity to the role of *CACNA1A* in each of the disease mechanisms, since there is considerable overlapping phenotypes^{150,153}. At the gene level, all three can be distinguished by the nature of mutation, FHM being caused by missense mutations, EA2 by disrupted reading frames or missense mutations, and SCA6 by mild CAG expansions^{154,155}.

The *CACNA1A* gene is also involved in epilepsy, as shown by association and mutation studies¹⁵⁶. *Cacna1a* mutant mice models are also known¹⁵⁷. Interestingly, mutations in *CACNA1A* can cause epilepsy during severe attacks of FHM¹⁶, or occurring independently^{158,159}.

Genotype-phenotype correlations

A considerable number of recurrent mutations have been reported for FHM1: S218L^{18,160}, R583Q^{10,15,153,161}, T666M^{10,15,17,150,162-164}, R1347Q^{165,166}, R1668W¹⁰, I1710T^{158,159}, and I1811L^{13,150}. Even for the two most frequent mutations (T666M and R583Q), however, genotype-phenotype correlations are complex, once that clinical and genetic heterogeneity and considerable symptom overlapping are present^{10,153}.

Neuronal $\text{Ca}_v2.1$ channel: structure and function

Neuronal voltage-gated calcium channels are heteropolymers of the abundant, pore forming, voltage-sensitive α_{1A} -subunit and the smaller α_2 -, β -, and δ -subunits¹⁶⁷. α_{1A} -isoforms play a major role in calcium flux, whereas the other subunits are auxiliary but required for proper channel function¹⁶⁸. The protein has four transmembrane domains, and both the amino and carboxyl terminus are located in cytoplasm¹⁶⁷. These channels are expressed throughout the brain and are present in the motor nerve terminals at the neuromuscular junction (NMJ); their main function is to mediate neurotransmitter release from synaptic nerve terminals¹⁶⁹. A recent review discussed aspects of channel function, structure and regulation¹⁷⁰.

Functional studies and mice models

Several *CACNA1A* mutations have been analyzed with electrophysiological techniques in neuronal and non-neuronal cells. In general, EA2 truncating and missense mutations result in a severe decrease or complete loss of current, whereas FHM1 mutations were shown to cause different effects on conductance, kinetics and expression of the channel¹⁴⁴. All FHM1 mutations are missense mutations. Recent studies have demonstrated that FHM1 mutations result in changes of the biophysical properties of individual mutant channels, namely channel gating, and density of functional channels on the cell surface (and decrease maximal $\text{Ca}_v2.1$ current density in neurons), causing an overall gain-of-function effect¹⁷¹⁻¹⁷⁷. The defect in gating allows the channel to open more easily in response to smaller depolarizations (e.g. enhance calcium influx through single channels), which could contribute to neuronal hyperexcitability. Thus, the mutant channels are expected to modify cortical excitability and susceptibility to CSD, commonly considered the underlying cause of the aura in migraine¹⁴⁴.

Natural mutant mice, such as the *tottering* (tg), with a missense mutation in the mouse orthologous *Cacna1a* gene, showed abnormalities in neurotransmitter release at NMJ¹⁷⁸. *Cacna1a* knock-in mice homozygous for the human FHM1 R192Q mutation have enhanced neurotransmission at NMJ and, most interestingly, increased susceptibility to CSD¹⁷⁹.

1.3.3.2 FHM2, *ATP1A2* gene

The second FHM locus is located on chromosome 1q23. About 20% of FHM families show linkage to this locus^{180,181}. The identity of the second FHM gene (FHM2) was revealed only few years later, when mutations in *ATP1A2* gene were associated with the disease¹⁸². The FHM2 gene, *ATP1A2*, encodes the $\alpha 2$ subunit of Na^+, K^+ -ATPase, which is involved in ion transport across the plasma membrane of glial cells.

Mutations in the *ATP1A2* gene are mostly associated with pure FHM^{182,183} but in rare cases association with cerebellar problems¹⁸⁴, benign familial infantile convulsions (BFIC)¹⁸³, alternating hemiplegia of childhood (AHC)^{185,186}, severe episodic neurological deficits and permanent mental retardation¹⁸⁷ or basilar migraine²² has been reported. A rare and severe case of prolonged hemiplegia in a child suggested a more complex clinical pattern¹⁸⁸. Most of the *ATP1A2* mutations reported are missense mutations (33 out of 35). Lately, interesting particular cases of two *de novo* mutations¹⁸⁹, the first case of a compound heterozygote¹⁹⁰ and several other new mutations^{191,192} were reported.

Meaningful genotype-phenotype studies in *ATP1A2* have not been possible, due to the lack of sufficient carriers for each individual mutation; almost all *ATP1A2* mutations are unique to a particular FHM2 family. Only very few exceptions, still with a small number of mutation carriers, have been reported: T376M¹⁹³, A606T¹⁹³, and R763H¹⁹⁴.

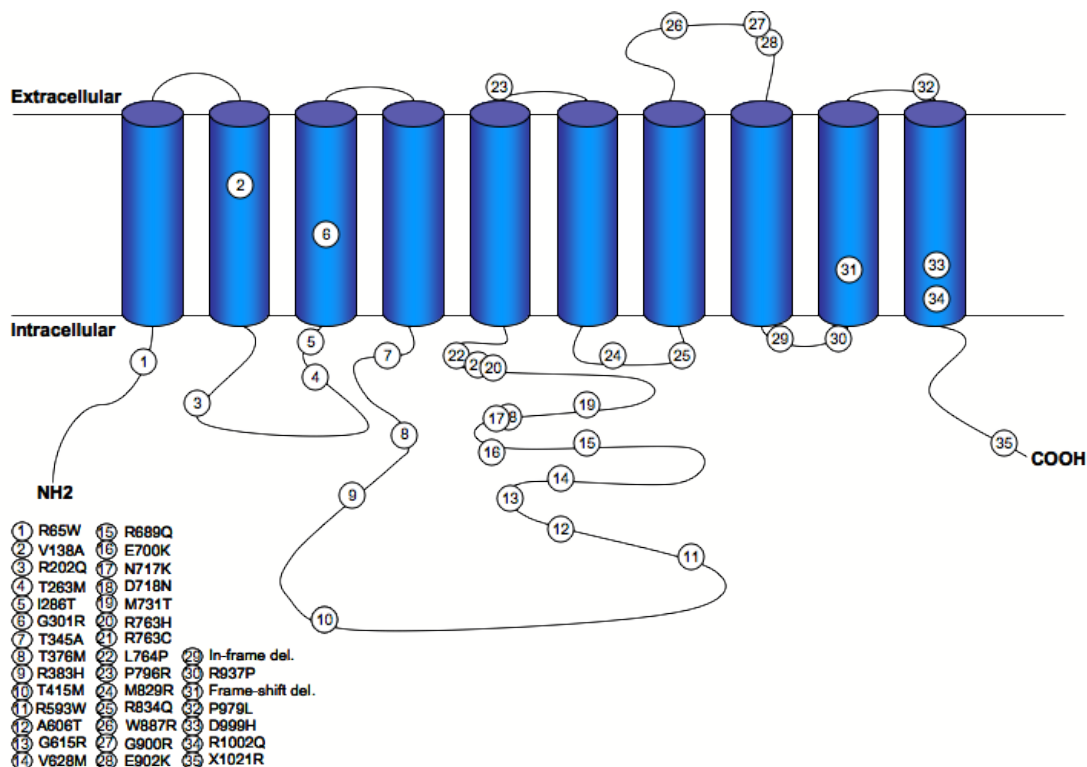


Figure 4 – Schematic structure of Na^+, K^+ -ATPase with FHM2 mutations identified.

Na⁺,K⁺-ATPase: structure and function

P-type transport ATPases comprise an important family of homologous enzymes that are involved in the active pumping of cations across the cell membrane¹⁹⁵. In eukaryotic cells, prominent family members include: (1) Ca²⁺-ATPases that are involved in cellular regulation of Ca²⁺ and present in both plasma membrane and intracellular systems such as the sarco(endo)plasmic reticulum (SERCA-ATPase); (2) Na⁺,K⁺-ATPases that are involved in cellular volume regulation, development of membrane potential, and Na⁺ dependent nutrient uptake; (3) powerful proton pump ATPases, vertebrate H⁺,K⁺-ATPases and K⁺-ATPase are other members of this family. Structurally, the mammalian Na⁺,K⁺- and H⁺, K⁺-ATPases differ significantly from SERCA-ATPase by requiring the presence of a β -subunit, probably to support the native conformation of the catalytic chain in its transport function¹⁹⁵. Homology between SERCA and the Na⁺,K⁺-ATPase is more extensive than with any other ATPase¹⁹⁶.

Na⁺,K⁺-ATPase catalyses exchange of 3 Na⁺ / 2 K⁺ per turnover and is present in different isoforms (α 1-4), expressed by different genes. It combines with its β -glycoprotein subunit before insertion into the plasma membrane¹⁹⁷. The α 2 isoform presents 1012-15 amino acids is predominantly expressed in adult muscle, heart, neurons, adipocytes, as high affinity to ouabain in all species and may be important for K⁺-homeostasis¹⁹⁸. The process of active ion transport across biological membranes is described as a Post-Albers cycle¹⁹⁹.

P-type ATPases have a common structural design: similar amino acid sequence for related ATPases (that in conserved regions can reach 80-90%) and the presence of characteristic sequence motifs and similarities in hydrophilic profiles. Now, that the crystal structure of the calcium pump of the sarcoplasmic reticulum was published, stronger predictions of the impact of the amino acid changes in the pump can be done²⁰⁰; the Na⁺,K⁺-ATPase α 2-subunit has a structure similar to Ca²⁺-ATPases, which comprises 10 transmembrane helices and three cytoplasmic domains: the A-domain (actuator), the P-domain, which contains the motif for phosphorylation, and the N-domain that binds ATP²⁰⁰.

The majority of FHM2 mutations is located in the large M4-M5 cytoplasmic loop of the Na⁺,K⁺-ATPase α 2-subunit (**Figure 4**), but can be also found in for instance the extracellular loops, such as in M7-M8 that binds the β subunit.

Functional studies

Firstly, functional data of *ATP1A2* mutants indicated the possible pathogenic mechanism in FHM2¹⁸². Two mechanisms were then hypothesized to account for an altered excitability of the membrane in mutated cells: 1) an increase in extracellular K^+ , because of impaired clearance of K^+ might induce cortical depolarization and facilitate a spreading depression event; and 2) as the $\alpha 2$ subunit distribution on the plasma membrane is coincidental with the localization of the Na^+/Ca^{2+} exchanger, a local boost in intracellular Na^+ would lead to increased intracellular Ca^{2+} , through the Na^+/Ca^{2+} exchanger, with a mechanism resembling the effect of *CACNA1A* mutations that cause FHM1¹⁴⁴.

Functional studies of mutant Na^+,K^+ -ATPases reveal a unifying theme: mutant sodium-potassium pumps function less well, compared to normal ones. Abnormalities range from complete loss of Na^+,K^+ -pump function, in the case of mutations T378N, L764P, and W887R^{182,201,202}, to specific effects, such as decreased catalytic turnover²⁰³ or reduced affinity for potassium ions²⁰⁴, as for the M731T and T345A mutations, respectively.

Moseley et al. reported that Na^+,K^+ -ATPase $\alpha 2$ isoform is expressed in neonatal neurons and indicated that its absence disrupts neuronal activity in newborn mice²⁰⁵. Heterozygous strains seem to be healthy, although with a fear-anxiety behavior and increased neuronal activity have been observed after conditioned fear stimuli²⁰⁶. The learning and memory deficits observed in the $\alpha 2$ and $\alpha 3$ heterozygous mice reveal that Na^+,K^+ -ATPases may be important in the functioning of pathways associated with spatial learning²⁰⁷.

1.3.3.3 FHM3, *SCN1A* gene

Recently, the third FHM locus was located on chromosome 2q24 (FHM3) and the *SCN1A* gene was for the first time associated with the disease, reinforcing the importance of ion channels and pumps in the pathophysiology of migraine²⁰⁸. The *SCN1A* gene encodes the $Na_v1.1$ neuronal voltage-gated sodium channel $\alpha 1$ -subunit. $Na_v1.1$ channels are expressed in (cortical) neurons and critical for generation and propagation of action potential in brain²⁰⁹.

The majority of mutations reported in *SCN1A* (more than 150) are associated with a wide range of childhood epileptic syndromes, including generalized epilepsy with febrile seizures plus (GEFS+)²¹⁰⁻²¹² and severe myoclonic epilepsy of infancy (SMEI)²¹³⁻²¹⁶. The type of mutation and its location in the protein can sometimes give a hint for the phenotypic severity with mutations that give rise to a truncated protein, as well as

mutations in the regions that constitutes the pore, characterizing the most severe phenotypes²¹⁷. A *SCN1A* R542Q mutation associated with autism²¹⁸ was previously identified in a patient with juvenile myoclonic epilepsy²¹⁹, which may suggest that *SCN1A* susceptibility variants can be mildly deleterious for the phenotype. Only four FHM families, three of them are related, with mutations in *SCN1A* were identified^{208,220}. No cerebellar alterations or language/motor developmental delays were noticed, and epilepsy was only found during childhood²⁰⁸. Remarkably, none of the FHM3 mutations appears to be recurrent, truncating or arise *de novo*.

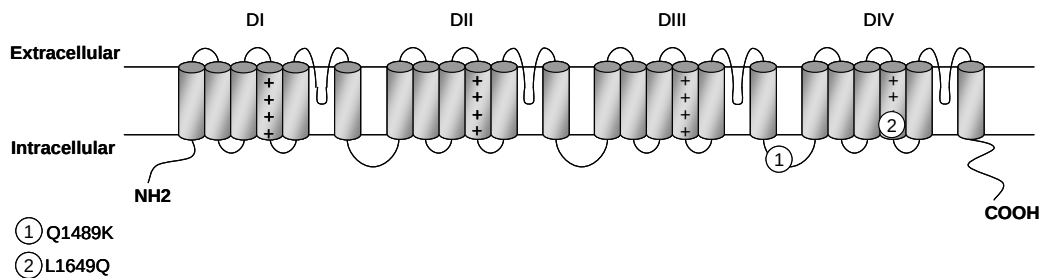


Figure 5 – Na_v1.1 channel topology with FHM3 mutations identified.

Na_v1.1 channels: structure and function

The α_1 subunit of the sodium channel consists of four internally homologous domains (DI-DIV), each containing six highly conserved transmembrane-spanning segments (S1-S6)¹⁷⁰. These four domains are pseudosymmetrically arranged around a central pore, whose structural constituents determine the selectivity and conductance of the channel. S5 and S6 segments and S5-S6 linker line the permeation pathway and are important for ion selectivity and gating kinetics^{221,222}.

Voltage-gated sodium channels are expressed in neurons and glia, throughout the central and peripheral nervous system^{223,224}. Voltage-gated sodium channels are critical for generation and propagation of action potentials, firing and neurotransmitter release, and serve as important pharmacological targets for anticonvulsant agents²²⁵; carbamazepine is actually used in migraine prophylaxis to reduce the number of hemiplegic/epileptic attacks. Voltage changes across the cell membrane control the gating of many cation-selective ion channels. Abnormal ion selectivity in mutated channels would affect several ion concentration gradients and neuronal excitability.

Major gene for epilepsy

Epilepsy is a known, common, neurological condition that reflects neurological hyperexcitability. Epileptic seizures are characterized by abnormal electrical discharges within the brain. Since ion channels are fundamental in membrane potential generation, it is not surprising that mutations of genes encoding some subunits of ion channels may cause epilepsy.

GEFS+ is a benign childhood-onset epileptic syndrome with AD inheritance^{226,227}, characterized by febrile seizures in children and afebrile seizures in adults. Patients with GEFS+ express a variable phenotype combining febrile seizures, afebrile generalized seizures (tonic-clonic, absence, myoclonic or atonic crises) and partial seizures²²⁷. GEFS+ type 1 (MIM 600235) was associated with mutations in *SCN1B*²²⁸, whereas GEFS+ type 2 (MIM 604233) was associated with mutations in *SCN1A* gene²¹⁰.

SMEI is a rare and malignant childhood epilepsy syndrome, first described by Dravet and therefore also called Dravet syndrome²²⁹. Early manifestations are generalized tonic, clonic, or tonic-clonic seizures, initially induced by fever and benign during the first year of life; seizures are often prolonged, generalized and associated with fever. Later on, patients present afebrile seizures, including myoclonic (myoclonic seizures which are associated with ictal electroencephalogram (EEG) changes), tonic-clonic, absence crises and simple and complex partial seizures. Psychomotor and speech delay becomes evident during the second year of life; patients often become ataxic. Febrile seizures and/or epilepsy are often seen in the family. The rate of *SCN1A* mutations found in GEFS+ reached 6%²¹⁹, whereas in SMEI these can reach 83%²³⁰.

Functional data on SCN1A mutations

By studying the FHM3 Q1489K mutant functional consequences in the highly homologous human channel *SCN5A*, Dichgans et al. showed an accelerated recovery from fast inactivation, therefore promoting higher firing of neurons that result in an increased cortical excitability²⁰⁸. Later, the FHM3 L1649Q mutant showed additional slowed inactivation²²⁰. Thus, both mutations lead to neuronal hyperexcitability (i.e. gain-of-function), supporting the theory of CSD facilitation, likely due to the rise in extracellular K⁺ and release of neurotransmitter glutamate, similarly to the consequence of *CACNA1A* mutations in FHM1¹⁴⁴.

Additionally, functional data of other epilepsy-*SCN1A* mutations revealed that both loss^{231,232} and gain-of-function²³³ of human Na_v1.1 channel defects can occur²¹⁷, supporting the idea that a spectrum of functional sodium channel defects (i.e. channel gating and voltage dependence) is contributing to the pathogenesis of inherited epilepsy²³⁴. This functional heterogeneity may explain part of the clinical variability in the phenotypic severity among families.

1.3.4 New insights from other candidate gene results

Recently, a heterozygous mutation in the *SLC1A3* gene was found in a single patient with a severe phenotype, including alternating hemiplegia, episodic ataxia, and seizures²³⁵. The *SLC1A3* gene encodes the excitatory amino acid transporter type 1 (EAAT1) that is involved in removal of glutamate from the synaptic cleft. Functional testing of mutant EAAT1 showed decreased glutamate uptake. Interestingly, because of its function in glutamate removal, this transporter fits in the same pathway as described above for FHM.

1.3.5 Contribution of FHM study for migraine molecular mechanism

It is not entirely clear how dysfunction in FHM genes may trigger attacks and associated neurological symptoms. FHM1 mutant channels promote an overall gain-of-function effect, through modification of cortical excitability, while FHM2 mutant pumps showed a loss-of-function effect or kinetic alterations, through selective reduction in pump activity that elevates extracellular potassium. FHM3 mutations also lead to neuronal hyperexcitability, through a gain-of-function mechanism; the rise in extracellular K⁺ and release of neurotransmitter glutamate occur and, as a consequence, neurons may depolarize more easily, facilitating CSD (**Figure 5**).

Therefore, all mutants in the three FHM genes support the theory of increasing susceptibility to CSD, the likely mechanism for aura¹⁴⁴. The findings in FHM confirm that dysfunction in ion transport may be also a factor in common migraine pathophysiology and may help in the further elucidation of molecular pathways.

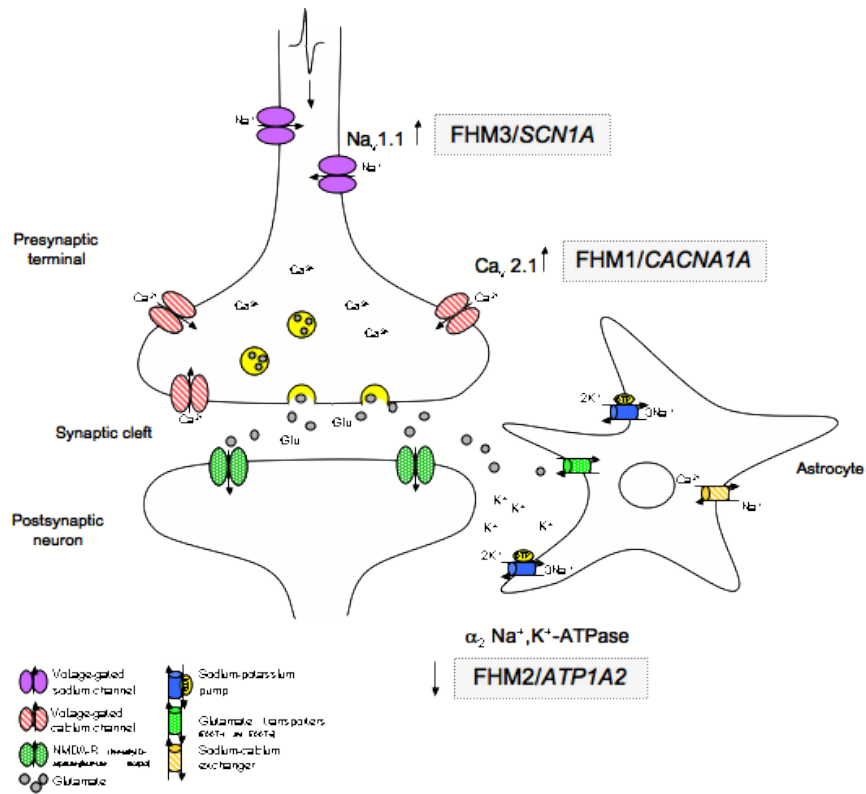


Figure 5 – Schematic drawing of a glutamergic synapse with proteins coded by known FHM genes and consequences of mutations in disease mechanism. Gain-of-function effects caused by Ca_v2.1 and Na_v1.1 channel mutations and a loss-of-function effect caused by α₂ Na⁺,K⁺-ATPase mutations seem to contribute to a common mechanism that involves an increased presence of glutamate and K⁺ in the synaptic cleft during neuronal activity in cortical neurons and consequently a lower threshold for initiation of CSD.

1.3.6 Novel treatment options from molecular biology

Recently, a knock-in mouse model carrying the human pure FHM1 R192Q mutation in the Ca_v2.1 (P/Q-type) calcium channel revealed a pure gain-of-function effect on calcium channels (e.g., increased Ca_v2.1 current density in cerebellar neurons and neurotransmitter release), and a reduced threshold to CSD. No overt behavioral phenotype was observed¹⁷⁹. The NMJ has been also a good synaptic model to study synaptic consequences of *Cacna1a* mutations in single synapses¹⁷⁹. Drugs might shift the activation range of mutated channels to more depolarized voltages, decrease calcium influx through the channels, lower intracellular calcium and, ultimately, raise the CSD threshold.

As mentioned above, a possible common consequence of both mutant Ca_v2.1 channels and Na⁺,K⁺-pumps may be the release of neurotransmitters, such as glutamate. In brain synapses, the release of glutamate from cortical neurons depends on Ca_v2.1 channels that also modulate CGRP. So, promising targets should include CGRP and glutamate receptor antagonists.

1.4. Genetics of complex migraine subtypes: MA and MO

The identification of genes (or more precise allelic variants) predisposing to the most common forms of migraine, i.e., MO and MA, has been a challenging task due to, for instance, the variation in diagnostic criteria, high population prevalence, and sex- and age-dependent differences in the pattern of expression of the disease. Additional clinical difficulties in genetic studies of migraine include inconsistencies in phenotype definition, a variable age at onset, an unknown mode of inheritance, locus heterogeneity, and the still poorly understood role of environmental factors. Two main strategies are usually chosen to determine genetic causes of migraine, linkage and association, as described²³⁶. Each has its own advantages and pitfalls⁸⁹, and none led yet to a clear and direct answer.

Linkage analysis in multifactorial diseases is limited by the lack of clear genetic segregation of any DNA variants in multigenerational family material, and by the modest contribution to disease of individual susceptibility mutations²³⁷. Several lines of research favor a substantial genetic control^{41,238}. Segregation suggests multifactorial inheritance⁵³, but a dominant model could not be ruled out⁴². Though some families appear to have a AD inheritance, this is unlikely to be a single gene disorder²³⁹.

1.4.1 Definition of phenotype

The definition of the “phenotype” is the basis to study the genetic background of any disease. Although diagnosis-based phenotypes have been successfully used for Mendelian disorders, this may be considered as an oversimplified model for a complex, polygenic disease. For these disorders, it has been suggested that clinical data should be dissected in more direct and intermediate clinical traits, commonly referred to as quantitative loci traits (QTLs), trait components or endophenotypes. All characteristics, symptoms or traits observed in the disease group can be used, in order to increase power for genetic analysis²⁴⁰.

1.4.1.1 IHS criteria and genetic studies

In headache disorders, the phenotype is based on the patient’s description of the nature and frequency of the attacks. Together with overlapping symptoms, the lack of biological markers for an unequivocal definition of the affected status hampers genetic studies. The clinical heterogeneity of the disease within families and in the same individual should be taken into account; straightforward cases are rarely observed, even for the rare FHM

phenotype^{10,13}. Recently, two main strategies to dissect migraine diagnosis have been successfully employed to find genetic susceptibility: *latent class analysis* (LCA) and *trait component analysis* (TCA).

LCA analysis is a statistical method, closely analogous to cluster analysis, for finding subtypes of cases (latent classes) from multivariate categorical data²⁴¹ to detect a common genetic susceptibility. In migraine, the same individual often experiences several migraine types, even within the same attack, and the clinical subtype changes over life. Therefore, the dichotomous affected versus unaffected classification, as suggested by IHS, must be complemented by for instance the use of a LCA model-based approach²⁴². The main advantage of this is the lack of assumption of an endpoint diagnosis, thereby making use of all symptom information. Ideally both classifications can be used in migraine research as parallel approaches, hoping that it will decrease clinical (therefore genetic) heterogeneity.

Based on the fact that, in principle, a single symptom (i.e., motor weakness) has lead to the discovery of genes causing FHM and in order to create a more homogeneous study sample for linkage detection, a novel strategy, named TCA, has been suggested²⁴⁰. This method makes use of each individual headache symptom as a trait (for example AO, aggravation by physical exercise or phonophobia) and their sub-groupings (i.e., trait components), to try to reach significant linkage peaks or even to identify migraine susceptibility genes; the majority of the headache clinical symptoms and characteristics are used as individual traits, instead of a MO or MA classification²⁴⁰. These trait-based approaches have been successfully used in other complex diseases, such as asthma, schizophrenia and autism²⁴³⁻²⁴⁵.

Endophenotypes can be used to facilitate the process of gene mapping²⁴⁶ and have been frequently used in psychiatric disorders, such as schizophrenia. Novel endophenotypes were identified in migraine: one for pediatric migraine, characterized by excessive vomiting and abdominal pain during the attack²⁴⁷; and other for high anxiety, both in association with polymorphisms in 5-HT transporter genes²⁴⁸.

1.4.1.2 Co-occurrence of migraine subtypes

Whether MO and MA are etiologically distinct disorders is still a matter of debate: contradictory results have been presented. RR results for MO and MA seem to suggest different etiologies^{41,249,250}. There are also reasons to suggest that they form extremes of a migraine spectrum. First, there is often co-occurrence MO and MA subtypes within

families, as was reported in about 45% of MA Italian families²³⁸. Secondly, up to 33% of migraineurs experience both migraine types during lifetime²⁵. In a more recent study, 42% of individuals with active MA also reported attacks of MO³⁵. Elevated RRs for both MA and MO agree with this finding^{40,242}. There is a tendency of age-dependent expression of variable symptoms within migraine spectrum. Individuals who first present with MO and, later in life, get aura during their attacks, and vice-versa^{148,250}. The current view is that MO and MA may share common underlying genetic and/or environmental factor pathways. Recent studies in Australian and Dutch twins confirmed the existence of a continuum of severity, suggesting MA to be more severe, but not etiologically distinct from MO^{242,251}.

1.4.1.3 Linkage studies in MA

Because of its higher genetic load and less frequent occurrence, MA seems the migraine subtype that should be preferentially studied. A higher genetic load in MA is suggested because of the fact that first-degree relatives of MA probands have nearly 4 times the risk of MA and heritability is estimated at 0.65^{41,252}. Additionally, greater genetic loading in three-generation families corresponds to an increased sibling risk and lower AO⁴⁴, suggesting that stratifying MA families by family history can significantly reduce complexity of MA. Moreover, an AD mode of inheritance with reduced penetrance is likely for this migraine subtype⁴⁴, so parametric linkage analysis can be a fruitful approach for the identification of susceptibility genes, as was shown for early onset breast cancer²⁵³ and Alzheimer²⁵⁴.

1.4.2 Targeting FHM loci and genes

Since the first gene for FHM was found in 1996, several studies have been performed in order to determine the involvement of FHM-related genes in MO and MA. Although there is convincing evidence for the presence of a susceptibility locus for migraine on chromosome 19p13^{255,256}, this issue remains controversial. Some of these studies have reported suggestive linkage²⁵⁵⁻²⁵⁸, but others showed no evidence for linkage and/or association of MA or MO to this region²⁵⁹⁻²⁶¹. Also, genomewide screens performed in migraine samples did not present any evidence of linkage that concur with a major locus in this location. Earlier studies support however that the 19p13 region is likely to be involved in migraine susceptibility and FHM1locus/*CACNA1A* cannot be excluded^{257,258}. As pointed out by some authors, there is no compelling evidence that the *CACNA1A* should be considered a major susceptibility gene for migraine^{255,256,260}.

An interesting finding was the identification of the insulin receptor (INSR) gene, which is located together with *CACNA1A* within the same region (19p13), and has been suggested as of possible major contribution for migraine susceptibility^{256,262}. A first attempt to confirm this finding in 72 multigenerational Finnish migraine families was not successful, so also for this gene the controversy remains²⁶¹.

The involvement of FHM2 locus in common migraine is also unclear. A small number of studies were performed so far, but an excessive-allele sharing in that region pointed to the presence of a susceptibility gene for MA and MO families, although mutations in FHM2 *ATP1A2* gene could not be found²⁶³. Also, there was no evidence for a common contribution of *ATP1A2* variants by association²⁶⁴. It is of course possible that the migraine susceptibility locus may be different from FHM2 locus, and located within 1q23 and 1q31^{263,265}; 1q31 has been a possible candidate region for FHM, reported a few years ago¹⁸¹.

1.4.3 Candidate gene studies

Using a candidate gene approach some groups have (partly) sequenced the *CACNA1A* gene, but no mutations have been found so far²⁶⁶⁻²⁷⁰. Additionally, several groups have failed to find mutations in *CACNA1A* either in cases of familial migraine with vertigo^{271,272}, basilar migraine^{269,270}, AHC²⁷³ and CH²⁷⁴, or to detect FHM1 known mutations in migraine stroke or MA²⁷⁵. Thus, it does not seem likely that the *CACNA1A* gene plays a major role for MA or MO in different patient populations^{145,261,266}, although there still may be high-penetrance alleles contributing to the phenotype in selected families²⁶¹. Similar studies have been performed for the FHM2 and FHM3 genes, but so far no significant results were reported^{263,269,270,272,276}.

1.4.4 Mapping susceptibility locus

Population, family and twin studies implicate a genetic susceptibility for this disorder. The variety of *loci* reported for migraine subtypes probably reflects its genetic heterogeneity. Mapping studies used several different samples and target regions, as well as genetic and statistical methods, which turns validation a primordial step. Even for these apparently simple cases, although significant or suggestive linkage for migraine has been published (**Table 3**), no causative gene alterations were identified yet.

Locus	Origin of families	Disease/Trait or phenotype**	Reference
FHM:			
19p13	European/American	FHM1	Ophoff et al., 1996
1q23	Italian	FHM2	De Fusco et al., 2003
2q24	European	FHM3	Dichgans et al., 2005
Common migraine subtypes:			
1q23	Australian	MA/MO	Lea et al., 2005
1q31	Australian*	MA/MO	Lea et al., 2002
3q-tel	Australian	LCA-severe; in large pedigrees	Lea et al., 2005
4q24	Finnish	MA	Wessman et al., 2002
4q24-28	Finnish	TCA-photophobia, phonophobia, AO or MO	Anttila et al., 2006
4q21	Icelandic	MO	Bjornsson et al., 2003
5q21	Australian	LCA-migraine	Nyholt et al., 2005
6p12-21	Swedish*	MA/MO	Carlsson et al., 2002
6p	Australian	LCA-prohibitive intensity	Nyholt et al., 2005
10q	Australian	LCA-migraine	Nyholt et al., 2005
10q22	Finnish	TCA-photophobia and phonophobia	Anttila et al., 2006
11q24	Canadian	MA	Cader et al., 2003
14q21-22	Italian*	MO	Soragna et al., 2003
14q22	Australian	LCA-severe	Lea et al., 2005
15q11-13	Italian	MA	Russo et al., 2005
15q14	Finnish	TCA-aggravation by physical exercise	Anttila et al., 2006
15q23	Australian	LCA-severe	Lea et al., 2005
17p-tel	Australian	LCA-severe	Lea et al., 2005
17p13	Finnish	TCA-pulsation	Anttila et al., 2006
18p	Icelandic	MO	Bjornsson et al., 2003
18p11	Australian	LCA-severe	Lea et al., 2005
19p13	Australian*	MA/MO	Nyholt et al., 1998
Xq13	Spanish*	MA	Oterino et al., 2001
Xq24-28	Australian	MA/MO	Nyholt et al., 2000

LCA: latent class analysis; TCA: trait component analysis;

*single family study; **phenotype that produced the highest LOD score

Table 3 – Known FHM genes and *loci* with suggestive or significant evidence of linkage identified in common types of migraine.

1.4.4.1 Target region: genomewide scans

Genomewide studies have proved to be efficient, when used to search susceptibility *loci* for common migraine, both by using a single-family strategy^{277,278} or large familial sets²⁷⁹⁻²⁸³. Several *loci* have been found assuming a certain phenotype definition^{240,280,281}. Genetic studies in large families, where MA, MO, or both (MA/MO) segregated as an AD trait, have revealed migraine susceptibility *loci* on chromosomal regions 6p12.2-p21.1²⁷⁷, 11q24²⁷⁹, 14q21.2-q22.3²⁷⁸, 1q31²⁶⁵ and Xq²⁸⁴⁻²⁸⁶. Two novel polymorphisms in the *MEP1A* and *RHAG* genes, located in chromosome 6p12.2-p21.1²⁷⁷, were associated with a disease haplotype²⁸⁷. It was suggested that since the combination of these polymorphisms could not be found in controls, they might be involved in the genetic predisposition to migraine in that family²⁸⁷. Interestingly, by using a completely different functional candidate gene strategy, Russo et al. reported a locus in 15q11-q13 that could hint for a relationship between the gabaergic system and migraine²⁸⁸, which proves that not only genomewide strategies can point susceptibility regions. Though, two recent studies performing association studies in GABA-A receptor cluster with MA were negative, so the linkage results still need confirmation²⁸⁹.

1.4.4.2 Population selection: isolates

Genomewide scans seem to work in isolated populations^{112,290}, which may also hold true for such a complex disorder such as migraine. A locus in 4q21-q24 has been identified in two independent studies in families from genetic isolates from Iceland and Finland^{282,283}. The fact that the 4q24 locus was identified in Icelandic MO and Finnish MA patients, might suggest that the same gene may be involved in both forms of migraine.

1.4.4.3 Novel approaches: LCA and TCA

By using an LCA-based approach that is known to increase power in genetic studies of migraine (see above), recent genomewide linkage scans found significantly increased evidence for linkage in different chromosomal regions. In an Australian family-based study, using 92 pedigrees, Lea et al. found suggestive linkage on chromosome 18p11 (close to the locus reported by Bjornsson et al.²⁸³) and 3q-tel (only in large pedigree subset), using LCA-severe migraine phenotype definition²⁸⁰. Nyholt et al. reported significant linkage for migraine, severe headache on chromosome 5q21 and suggestive linkage on chromosomes 8, 10 and 13 in a large twin sample (families and sib pairs) study²⁸¹. This group further replicated *loci* previously described on 6p12.2-p21.1²⁷⁷ and, most interestingly, the 1q23 FHM2 locus. Additionally, in the former study, a particular association was found between a symptom and a chromosome locus by dissecting migraine symptoms, a chromosome 5 peak predominantly associated with pulsating headache²⁸¹.

The importance of the novel phenotype definition approach was further shown by the Finnish group that - by using affected families - presented TCA as a new powerful tool to detect linkage peaks. They identified positive linkage to chromosomes 4q24, 17p13 (pulsating trait), 18q12 (IHS full criteria) and 4q28 (AO) *loci*, and suggestive linkage to 10q22 (phonophobia) and 12q21, 15q14, and Xp21 (aggravation by physical exercise)²⁴⁰. Interestingly, some of these regions have been also implicated more than once, under different population and study strategies. Examples of these are *loci* in chromosomes: 3q^{240,280}, 4q^{240,282,283}, 6q^{279,282}, 10q^{240,281}, 15q^{240,280,282,288}, 16p^{279,282}, 17p^{240,280,282} and 18q^{240,282,283}, therefore showing that, by stratifying the sample, linkage can be found or confirmed.

1.4.4.4 Other regions

The high prevalence in females³⁵, few cases of male-to-male transmission (except with heterogeneity), and the excess of affected females in migraine families²⁸⁴, all suggested an X-linked dominant component. This was confirmed by finding a locus in chromosome Xq24-28²⁸⁴ and nearby Xq13, in a family with MA and X-linked Charcot-Marie-Tooth (CMTX1) co-segregating²⁸⁶. Besides the possible polygenic mode of inheritance, for both MO and MA, an X-linked susceptibility seems possible, conferred either by a causal defect or interacting component.

1.4.5 Association studies

A large number of association studies have been reported for migraine many focusing on candidate genes belonging to the dopaminergic or serotonergic systems, homocysteine metabolism, and several other metabolic pathways. Firm conclusions could not be drawn, in most cases, because initial positive findings could not be replicated.

1.4.5.1 Serotonin

Brain serotonin (5-HT) has been implicated in migraine pathophysiology, since abnormalities in the serotonergic function were reported both before and during the attacks^{291,292}. Migraine patients show lower plasma levels of 5-HT and higher concentration of its metabolites between migraine episodes.

Association studies of genes involved in the serotonin metabolism gave variable results. A study of polymorphisms in the 5-HT_{2A} and 5-HT_{2C} receptor genes seemed to exclude them as candidate genes for migraine^{293,294}. No or mild evidence for association between 5-HT receptor gene polymorphisms and migraine has been reported²⁹⁵⁻²⁹⁷. Additionally, several genes involved in serotonin biosynthesis and metabolism, namely those encoding for 5-HTT, failed to give convincing results sometimes because of the limited size or type of the sample set²⁹⁸⁻³⁰¹. In other cases, they presented positive results only in certain migraine subgroups^{247,302,303}, or could not be replicated³⁰⁴. Recently, association of a functional promoter polymorphism (5-HTTLPR) in a serotonin transporter gene (*SLC6A4*) with MA was obtained³⁰⁵, but was not confirmed³⁰⁶, showing the complexity of validating association findings.

1.4.5.2 Dopamine

Dopaminergic hypersensitivity in migraine, first described in 1977³⁰⁷, pointed towards a primary role of dopamine receptors in headache and symptoms (such as nausea, hypotension and vomiting). This was further supported by several pharmacological agonists and antagonist assays. Biological data showed that low norepinephrine plasma levels³⁰⁸ and reduced dopamine beta hydroxylase (DBH) activity³⁰⁹ can be observed in patients, but the contribution of this dopaminergic activation to migraine mechanism lacks to be clarified.

Increased frequency of *NcoI* polymorphic allele of dopamine D2 receptor gene (DRD2) was reported in MA patients³¹⁰; a result that was not confirmed and in fact was criticized³¹¹. On the other hand, significant association of another polymorphism in the same gene in selected MO “dopaminergic subgroup”³¹² seems to be sustaining a possible association of DRD2 polymorphisms with migraine. DRD4 allele association with MO also needs further confirmation³¹³. A novel study found significant allelic association of a functional insertion/deletion polymorphism (DBH2) in the promoter region of the *DBH* gene with migraine³¹⁴ that is in strong LD with other *DBH* microsatellite (DBH1) previously associated with typical migraine³¹⁵. Lately, this DBH2 homozygous deletion genotype was reported as a genetic risk factor for the MA migraine subtype, with a significantly increased susceptibility in males³¹⁴. Apparently, allelic variation at the *DBH* locus may predispose some individuals to migraine, especially MA, but this result needs further investigation. As with serotonergic gene alleles, negative results were also frequent; thus, the role of dopamine genes in migraine still remains unclear.

1.4.5.3 MTHFR

The excitatory effect of homocysteine may affect the threshold for migraine manifestation, since the level of homocysteine affects the trigeminovascular (TGV) system, by increasing its firing rate, as a response to pain. Frosst et al. reported an association between a variation in the *MTHFR* gene (the C677T polymorphism), which encodes the enzyme MTHFR involved in folate metabolism, and plasma homocysteine levels³¹⁶.

In migraine association studies, controversial and unclear results also include the C677T variant in the *MTHFR* gene. Association with common migraine or simply MA was replicated in several clinic-based populations³¹⁷⁻³²⁰, even in a younger aged population³²¹. A recent population-based study showed that individuals with the *MTHFR* T/T genotype had two times increased risk for MA, an effect that was independent of other

cardiovascular risk factors³²². Both a German group, which performed TDT and large case-control strategies³²¹ and a Finnish study, that also included another A1298C polymorphism allowing combined analysis³²³, were not able to replicate the initial findings. However, a recent meta-analysis of *MTHFR* association data seems to indicate that this gene may be indeed involved in migraine and should not be discarded as a promising candidate gene³²⁴.

1.4.5.4 Angiotensin

According to the TGV theory⁷⁹, unknown triggers for headache activate perivascular trigeminal axons, which release vasoactive neuropeptides, such as substance P (SP) and CGRP, to promote neurogenic inflammation and spread the inflammation response to adjacent tissues. High interictal levels of plasma SP and CGRP and higher angiotensin converting enzyme (ACE) activity in MA patients were observed and may indicate a preparatory state for attacks, lowering the threshold for triggering events³²⁵

Positive association of the ACE-DD variant in the *ACE* gene was found^{326,327} and, remarkably, this variant seemed to have a slight protective effect against migraine in male patients³²⁸. Moreover, association with the *MTHFR* T/T genotype was increased when in combination with the ACE ID/DD genotype, suggesting that the interaction of both genes can increase MA susceptibility³²⁹.

1.4.5.5 Other associations

Among others, associations between migraine polymorphisms in genes such as the endothelin type A receptor (ETA) gene³³⁰, the *INSR* gene²⁶², the low density lipoprotein receptor (LDLR) gene³³¹, glutathione S-transferase (GST) gene³³² and estrogen receptor (ESR1) gene^{333,334} have been reported. A weak association with monoamine oxidase type A (MAO-A)³³⁵ or the replication of results on tumor necrosis factor alpha (TNF- α)^{336,337}, human leukocyte antigen (HLA)-DRB1³³⁸ and progesterone receptor^{339,340} genes still need confirmation. For a review table see³⁴¹.

2. AIMS OF THE THESIS

The principal aim of this study was to ascertain the contribution of genetics in familial migraine and investigate its molecular pathogenic mechanisms, using a large set of Portuguese migraine patients. For that, two main strategies were followed. (1) One strategy was to investigate common migraine types using different genetic methodological approaches. (2) For the second strategy, FHM was used as a model to unravel migraine genetic mechanisms. The ultimate goal was to identify pathogenic DNA variants that predispose to this disease. Whenever possible, functional consequences studies were performed to try to prove causality of the DNA variants. The following specific aims were addressed in this thesis:

2.1 Genetic approaches to study common migraine

- Evaluation of familial aggregation of migraine, by estimating relative risk in first-degree relatives and spouses
- Investigation of the role of migraine susceptibility *loci* by linkage analysis in the most informative migraine families, using an “affected-only” approach
- Determine the possible involvement of FHM genes in common migraine by mutation screening

2.2 Genetics of FHM: a model to study the pathophysiology of migraine

- Identification of disease-causing mutations in familial hemiplegic migraine (FHM) genes
- Evaluation of the pathogenicity of identified DNA variants, through functional tests
- Perform genotype-phenotype correlations and ascertain atypical migraine phenotypes

3. PATIENTS AND METHODS

3.1 Patients

Since 1999, patients affected by primary and secondary headache forms and respective families have been recruited from the outpatient clinic at Hospital Geral de Santo António (HGSA), in order to participate in our clinical and genetic studies of migraine. The goal was to establish a clinical database to be used for medical follow-up and treatment, and for research purposes.

By 2004, this clinical database included 8415 Portuguese patients with different types of headaches, all classified according to the diagnostic criteria of the International Headache Society (IHS)⁴; namely, there were 2468 patients (29.3%) with migraine, 2402 (28.5%) with tension type headaches, 169 (2.0%) with cluster headaches, and 34 (0.4%) with other primary headaches. Secondary headaches were present in 1856 patients (22.1%), and unclassified headaches in 1486 (17.7%).

A control population of healthy volunteer blood donors, from the HGSA, was also established. Those who did not report any headaches (n=176) were used as controls to test the presence of the DNA variants found in migraine patients (**Articles 3 to 6**).

All participants were evaluated with a clinical interview. After obtaining their written informed consent, peripheral blood was collected and DNA collected for banking. The ethics committee of HGSA approved previously all these procedures.

3.1.1 DNA bank

Subjects and materials for all the experimental procedures performed during these studies originated from this DNA bank of Portuguese families with different types of migraine. This DNA bank consisted of approximately 1200 samples, including probands, relatives and isolated cases, most of them originating from the Northern part of Portugal.

Genomic DNA was collected from several members in more than 210 migraine families. Based on the diagnosis in the proband (migraine with aura - MA, or without aura - MO), we ascertained 119 MO and 56 MA families (**Table 4**).

Number of families/cases	Phenotype*
FE:	
MO	119
MA	56
MA/MO	14
Hemiplegic Migraine	9
Cluster Headache	9
Others	3
Total	210
OH:	
Migraine	2
MO or MA	5
Hemiplegic Migraine	10
Others	5
Total	22

Table 4 – Details of the DNA bank of Portuguese families with primary headaches and other migraine cases used throughout this study.

*Based on proband diagnosis;

FE: "família com enxaqueca";

OH: "outros hospitais"

3.1.2 Hemiplegic Migraine

3.1.2.1 Diagnostic criteria

The clinical diagnosis was established according to the IHS criteria⁴, and involved both a detailed interview and filling a headache questionnaire. In some cases, the diagnosis was based on the most recent classification of ICHD-II¹. Typical attacks include a unilateral motor deficit, associated with paresthesias, speech disturbances or visual signs, but some atypical cases were also object of our interest.

The vast majority of the patients with familial hemiplegic migraine (FHM) were clinically evaluated by either of two neurologists (JB and JMPM) from the same service (**Articles 4 and 6**). Several physicians from other hospitals referred additional families to our hospital in Porto. In some cases, the families were re-evaluated by the same migraine specialists to confirm their diagnosis (**Articles 4 and 5**).

3.1.2.2 Family structure

A total of 27 index cases with hemiplegic migraine are present in our DNA bank: 6 families with FHM, 10 SHM and 11 cases with unknown family history. In 2004, all these families were relatively small; only five FHM families had more than three patients. Additionally, three atypical cases, with migraine running in the family, are included: one with SHM and familial amyloidotic polyneuropathy, other with episodic ataxia (most likely EA2) and third with ophthalmoplegic migraine.

3.1.2.3 Sample selection

Ten Portuguese cases with HM were selected, because of the familial occurrence of the clinical features and the availability of DNA samples, and tested for possible involvement of the 19p13 (FHM1, *CACNA1A*), 1q23 (FHM2, *ATP1A2*) and 2q24 (FHM3, *SCN1A*) *loci*. In total, 66 DNA samples were genotyped for haplotype analysis. Markers selected for genotyping FHM1 were D19S221, D19S1150, D19S226; for FHM2, D1S2624, D1S2707, D1S2844; and for FHM3, D2S156, D2S2330 and D2S2381. Additional markers were tested when these were not informative.

Four of the known and most commonly found FHM1 mutations (R583Q in exon 13, T666M in exon 16, R1347Q in exon 25 and S218L in exon 5) in *CACNA1A* gene were studied in 20 of our hemiplegic migraine cases. Selection of probands was based on the familial information available at the time. Seven probands were selected for *ATP1A2* screening, because haplotypes were compatible with involvement of the FHM2 locus. Three probands, who did not have mutations in *ATP1A2* and had compatible haplotypes for the FHM3 locus, were scanned for *SCN1A* gene mutations. Because of a recent publication²³⁵, four probands, who tested negative for the three FHM genes, were sequenced for mutations in the *SLC1A3* gene.

3.1.3 MO and MA

3.1.3.1 Diagnostic criteria

The majority of migraine families were collected before 2004. Consequently, the clinical diagnosis was based on the first edition of the IHS standardized guidelines⁴. According to these criteria, the most common form is migraine without aura (MO), characterized by recurrent attacks of disabling headache lasting between 4 and 72 hours. The headache is usually severe, unilateral, pulsating, aggravated by physical activity, and associated with nausea, vomiting, photo- and/or phonophobia. In migraine with aura (MA), which occurs in about one-third of the patients, the headache is very similar, but the attacks are preceded or accompanied by transient focal neurological, mostly visual, aura symptoms⁴. The most recent version of these criteria, frequently called ICHD-II, used from 2004 onwards, did not present significant changes for MO and MA forms (see **Table 2**)¹.

3.1.3.2 Sample selection

In a first stage, familial aggregation of migraine was studied in 50 families of MA probands and in 94 families of MO probands (**Article 1**). RR represents the ratio between the frequency of the disease in relatives and its population prevalence³⁴². RR of migraine was calculated for first-degree relatives (with MA, MO or MA/MO), using a population prevalence of 16.7%^{33,343}. In addition, we estimated RR for the probands' spouses, to try to disentangle the influence of genetic and environmental factors. The sample size of spouses (n=48), however, was not robust enough to form sufficiently large subgroups, according to migraine subtype. Thus, proband's spouses (with any form of migraine) were only classified as "affected".

At a second stage, both probands and their first-degree relatives were classified according to their migraine subtype. The risk for MA and MO was evaluated separately, in 50 families of MA-probands and in 94 families of MO-probands. This methodology required that relatives with MA were considered non-affected when analysing the risk for MO, and vice-versa³⁴⁴. Moreover, relatives with MA/MO were considered affected both when analysing the risk for MA and for MO. Prevalence rate in the population sample was 15% for MO and 1.7% for MA^{33,343}.

The study described in **Article 2** was performed in several stages and under different selection criteria (**Table 5**). The first molecular procedure was to study the FHM-related *loci*, FHM1 and FHM2, and to check for a segregation pattern in our sample set (*stage1*). Four 96-well plates were made, including 382 DNA samples from 33 migraine families. The selection criteria were the size (and, thus, potential informativeness) of the families, DNA samples available, and clinical phenotype (MO and/or MA). The same 33 families were studied afterwards for other candidate regions (3p21, 4q24 and 5q21), indicated as migraine *loci*.

Study	FHM <i>loci</i> (stage1)	FHM <i>loci</i> (stage2)	Migraine <i>loci</i>
Proband diagnosis:			
MO	22	55	22
MA	9	27	9
MA/MO	2	4	2
Genotyping:			
DNA genotyped	382	655	382
Migraine patients	240	436	240

Table 5 - Pedigree information of the Portuguese MO and MA families used in the studies described in **Article 2**

Study	FHM <i>loci</i> (stage2)	Migraine <i>loci</i>
Number of:		
Families	86	33
Generations:		
2	28	4
3	41	21
4	17	8
Sibships	237	109
Sib pairs:	581	396
Discordant	254	172
Concordant Affected	266	180
Affected Males/Total	141/390	65/199
Affected Females/Total	302/450	135/213

Later on, additional nuclear families were added to increase sample size for ASP analysis (*stage 2*). The criteria were the presence of sib pairs and/or trios and if parents were available (**Table 6**).

Table 6 - Pedigree information obtained after running PEDINFO, which is an option of the S.A.G.E. program³⁴⁵.

3.2 Molecular methods

3.2.1 DNA extraction

Genomic DNA was isolated from peripheral blood, using a standard salting out extraction method³⁴⁶. In brief, after removal of the red blood cells, lymphocytes are lysed in nuclei lysis buffer, and digested at 37 °C, overnight, in protease K solution and 10% SDS. After protein precipitation with saturated NaCl, the DNA is precipitated with ethanol, retrieved with the tip of a glass pipette and stored in TE buffer, at 4 °C.

DNA concentrations for all samples used were determined using the NanoDrop ND-1000 UV-Vis Spectrophotometer (Nanodrop Technologies). The main advantage of this method is the small sample requirement (1µL) for determination of the DNA concentration. All these DNA samples were gathered in the DNA bank to be available for migraine research.

3.2.2 Genotyping

In the studies described in our articles (**Articles 2, 4 to 6**), several microsatellite markers positioned in different chromosomal regions were analyzed (**Table 7**). Marker selection was made on the basis of its chromosomal location (e.g., FHM *loci* or migraine, candidate region), heterozygosity value and optimal size distribution of the alleles, allowing pooling of markers in panels. The location and distances between markers were mainly collected from the Marshfield Medical Research Foundation Genome Database (<http://research.marshfieldclinic.org/genetics/>).

Chr	Marker	D-number	Genetic position/cM	Het	Physical position/bp	Chr band	UniSTS	Genbank	Primer forward (5'-3')	Primer reverse (5'-3')
1	AFMa123wg5	D1S2624	162.57	0.69	154897940-154898142	1q23.1	53141	Z52151	FAM-GGCGAGCACATCGTTA	gtgtcttTCTGTCACAGAGTCCAA
1	AFMa339wh1	D1S2707	168.52	0.82	158339075-158339223	1q23.2	31815	Z52929	HEX-CCCCCTGGCATAGGGTTCAAGA	gtgtcttAGCCAGGCATCTGCACCTTC
1	AFM344va9	D1S2844	175.03	0.8	161215450-161215632	1q23.3	67016	Z51472	FAM-TCCTGACCTTGGCGATG	gtgtcttAAGAAGTCACTGAGAACCTGGG
2	AFM211yde6	D2S156	164.51	0.85	160357089-160357274	2q24.2	32057	Z16966	HEX-CCCTCTGTTTGCCTGAAATA	gtgtcttCCAGCCTTGATTGTACAC
2	AFMc015yd9	D2S2330	169.41	0.82	166405834-166405990	2q24.3	16131	Z54008	FAM-AGTCATCTTATAGAAAAATGCAAGC	gtgtcttAGTTTACAACCTTAATTNTGGGTG
2	AFMa082f5	D2S2381	175.91	0.77	172366636-172366864	2q31.1	16874	Z51915	FAM-AGTTGCTCAGTAAATATCTCTTATG	gtgtcttTATTGTAAGTCGAGGAATATC
3	AFMa162wd9	D3S3564	67.94	0.78	42393981-42394192	3p22.1	13633	Z52330	HEX-AGCTAAACACAGTCTAACTGCAT	gtgtcttCCACACAGATGATAGGGA
3	AFM273ve9	(D3S1581)	70.61	0.88	48572314-48572405	3p21.31	25421	Z23940	HEX-CAGAACTGCCAAACCA	gtgtcttGGGTAAACAGGAGCGAG
3	AFMb347zg9	D3S3666	72.21	0.85	54627383-54627499	3p14.3	53745	Z53790	HEX-GGCACTGGNCAAGTT	gtgtcttCAAAATACCTGTAAGGCACT
4	AFMa062yc1	D4S3042	83.29	0.84	77124888-77125098	4q21.1	13642	Z51804	FAM-AGCTAACTACTCTCCACCCATAC	gtgtcttCCATGCTAAGTTTATGATGTCTG
4	GATA10G07*	D4S3243	88.45***	0.66	81151398-81151568	4q21.21	5770	G08332	FAM-AATCCAGTAAATGAAATAGTCATCA	gtgtcttATAAGCCAAACATGATGGGA
4	AFMa240zb9	D4S2932	90.28	0.75	83722346-83722560	4q21.22	44255	Z52672	HEX-GAGCAAACTCTGTCTCAAAAATAA	gtgtcttGGCTTACTTGGAAAGTCTCTT
4	ATA2A03	D4S2361	93.48	0.7	85224464-85224619	4q21.23	29902	G08322	FAM-CCACGTGACTTTCTATTAGGG	gtgtcttACACCATCATGCGCGCATG
4	AFMa224wc5	D4S2929	96.16	0.68	89465602-89465706	4q22.1	52937	Z52588	HEX-GGCCAGGAGTTCAAAA	gtgtcttTGCAGCAAGTCCAAACA
4	GATA26B12	D4S2409	96.16	0.71	87137404-87137673	4q21.23	10945	G08342	HEX-ACCTTACCCACCAAGCATTG	gtgtcttCCATCCACCTCAATACTTG
4	AFMa054wg1	(D4S3373)	101.00 (n.a.)	n.a.	94924699-94924875	4q22.3	48417	Z67174	FAM-GCCAGAGAATAAGACTTTTGGCTTG	gtgtcttATGTCTGTATGCTCGGATACC
4	GATA2F11	D4S1647	104.94	0.77	99653996-99654138	4q23	63731	G09195	HEX-TATTTCCAAACCCCTGCTA	gtgtcttAAGCAAAAGAGGATTGAAAGTG
4	ACT3E03	D4S3240	114.04	0.74	110014057-110014375	4q25	62568	G08306	FAM-TAGGTCACTGTGCAATAATGC	gtgtcttCTGCTCTGGCAGATGGATAT
5	GATA12G02	D5S815	101.02	0.8	91026206-91026494	5q14.3	74078	G08429	FAM-TGGTATCTTGTGTAGCAAAATACA	gtgtcttTGCCATGATTGTTAAGTTTCC
5	AFM289va9	D5S644	104.76	0.85	95838500-95838598	5q15	9870	Z24063	FAM-ACTAAGTGTAGATCAATGTGC	gtgtcttTTGGAATTTGCTAAGAGTGTG
5	AFM321yb5	D5S669	112.52	0.81	104003045-104003246	5q21.2	4090	Z24376	FAM-AAGCACATGTGTCTTAAATCAA	gtgtcttCCAGATGGAGCAGAAACA
5	AFM358tb5	D5S2084	114.75	0.71	106628512-106628641	5q21.3	14027	Z51529	FAM-AGCTGCTTACCACGAATGTC	gtgtcttTCAGACTTTGAGCCCTGCTA
5	GATA68A03	D5S2501	116.98	0.75	110064214-110064525	5q22.1	70957	G08469	HEX-TGATTACTCTGAGGAAGAAGGC	gtgtcttTTGAAATGGGCACAGAAATT
5	AFMb304xb5	D5S2027	119.5	0.78	111173443-111173597	5q22.1	10887	Z53452	HEX-ACCTTGCCAGATTTTCCACTC	gtgtcttCACCTCATTGACTGGGAC
5	GATA130E01	D5S2860	124.47	0.78	n.a.	n.a.	76020	G10527	HEX-TGTTCTAGCTGCTGCTGCT	gtgtcttAGTGATGGTCTCTCCAATGA
5	GATA62A04	D5S1505	129.83	0.8	119129545-119129801	5q23.1	60818	G09956	HEX-TAAGTGCCAGAGTCTCCAC	gtgtcttTAAGGCATGCTCGGAGCTA
19	AFM224ye9	D19S221	36.22	0.85	12573793-12573991	19p13.2	46812	Z17017	NED-GCAAGACTCTGACTCAACAAA	gtgtcttCATAGAGATCAATGGCATGAAA
19	n.a.	D19S1150**	39.00 (n.a.)	n.a.	13306310-13306467	19p13.13	473862	n.a.	FAM-GCAAGAAGCATAGAAAAGCCA	gtgtcttCCTGTGAAAACCTCTGACC
19	AFM256yc9	D19S226	42.28	0.84	14494401-14494643	19p13.12	30190	Z17155	HEX-CCAGCAGATTTGGTGTGTCTA	gtgtcttGGTCCAGGATTGAACTAAAGCA

Genetic locations of markers are indicated in Kosambi cM and refer sex-averaged genetic positions from Marshfield map

Physical locations were obtained from Ensembl (<http://www.ensembl.org/>)

Chr-Chromosome; Het-Heterozygosity; n.a.-not available

*-cryptic duplicate GATA168A08; **-CACNA1A intragenic; ***-obtained from deCODE (<http://www.decode.com/>)

Table 7 – Microsatellite marker information, including primer sequences and type of fluorescent tags, used in **Article 2**.

PCR reactions were performed using fluorescently labeled primers and conditions available through the Human Genome Database (GDB) (<http://gdbwww.gdb.org/>). PCR amplification was performed using 45 ng of genomic DNA in a final volume of 15 μ L, containing a mixture 1x GeneAmp PCR Buffer II (Applied Biosystems), 250 μ M of each dNTP, 7.5 pmol of each primer, 2.5 mM $MgCl_2$, and 0.25 U of AmpliTaq Gold DNA Polymerase (Applied Biosystems), and under standard conditions (initial denaturation at 94°C for 10 min, followed by 10 cycles of 94°C for 30 sec, annealing at 55°C for 30 sec, 72°C for 40 sec, plus 25 cycles of 89°C for 30 sec, annealing at 55°C for 30 sec, 72°C for 40 sec, and a final extension at 72°C for 10 min). PCR products were detected on an automated sequencer (ABI 3700 or ABI 3730 DNA sequencer, Applied Biosystems). Genotypes were analyzed using Genescan and Genotyper 2.1 Software (Applied Biosystems), and evaluated by two independent persons. The allele sizes were standardized to the Centre d'Etudes du Polymorphisme Humain (CEPH) (<http://www.cephb.fr/cephdb/>), by using two CEPH control samples in each fragment run. In case of conflicting results, the data of that specific marker was not used for further analysis. Haplotypes were constructed for each locus, by inspection of segregation, assuming a minimal number of recombinations.

3.2.2.1 Post-PCR Multiplex

To simplify the genotyping procedures for the MO and MA large sample set (**Article 2**), we developed a post-PCR multiplexing method, by pooling the PCR products of markers, before fragment analysis. The sequential steps of this optimization were mainly based on choosing markers positioned within the same chromosome region, with non-overlapping allele size ranges and different fluorescent label colors.

In practice, the reaction was prepared in 96-well plates, by adding the 10 µL of the internal lane size standard (GeneScan-400HD ROX Standard, Applied Biosystems), 800 µL deionized formamide, and diluted PCR products, for a final 10 µL per well. Pooled PCR products were denatured at 96°C, for 4 min, and then detected on the automated sequencer.

3.2.3 Mutation detection

3.2.3.1 Single-Strand Conformation Polymorphism (SSCP)

For mutation scanning of FHM candidate genes, in a large sample of migraine probands, first SSCP was chosen (**Article 3**). PCR was performed for all exons and flanking intronic regions of *CACNA1A* (Ac. Nr X99897) and *ATP1A2* (Ac. Nr NM_000702) genes, as previously described¹⁵³. PCR was performed using genomic DNA of the probands as a template, and using 1 µM of each primer, 200 µM of dNTPs, 1.0 mM MgCl₂, 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1 U of Taq polymerase (MBI Fermentas, BioPortugal), and 2% formamide, in a final volume of 12.5 µL. Primer sequences for *CACNA1A* were adapted from Ophoff et al.¹⁵⁰; and, for *ATP1A2*, novel primers were designed, using the Oligos Program v.9.5b.

SSCP was performed as described by Orita et al.³⁴⁷. PCR products were analyzed using radioactive labelling¹⁵⁰. For several exons, a non-radioactive procedure was performed, using the Multiphor II apparatus (Amersham Biosciences), with non-denaturing polyacrylamide gels. Final detection of single stranded PCR products was made, using a silver staining protocol with AgNO₃ 0.2%³⁴⁸. PCR fragments with mobility shift variants were analyzed by double-strand sequencing, using standard dideoxy termination methods and ABI PRISM 310 (Applied Biosystems).

3.2.3.2 Restriction analysis of PCR products

Restriction analysis was used to evaluate the presence of a DNA variant in PCR products found in a proband, in the respective family, and in additional patients or controls (**Articles 3 to 6**). Restriction maps were constructed using Webcutter 2.0 (or NEBcutter v2.0) and Infobiogen (<http://www.infobiogen.fr/>). For detection of DNA variants, the respective exon was amplified by PCR, under same conditions as those used for sequencing (see below). PCR products were digested with the appropriate restriction enzyme (i.e. that distinguishes between alleles), using conditions as suggested by manufacturer instructions. Next, digested PCR fragments were electrophoresed on a 1.5 to 3% agarose gel, depending on the sizes of the digested fragments. The concept was that DNA variants cause either a loss or a gain of a restriction site, thus creating distinct electrophoresis patterns for the mutant and the wild-type allele.

3.2.3.3 Melting curve analysis

When performing mutation scanning in a large sample set, SSCP has relative low detection efficiency, whereas direct sequencing is costly. Therefore, to avoid missing important DNA variants and have a cheaper, but still reliable/pre-screening method, we tested and optimized a new method based on melting temperature variations, using a Light Scanner (HR-96 instrument, Idaho Technology). Three fragments in *ATP1A2* and one fragment in *SCN1A* were evaluated and optimized. High-resolution melting curve analysis³⁴⁹ was used to exclude the presence of the target mutation, using a carrier as the reference sample (see **Article 6, Supplementary Figure**).

PCR reactions were performed in 10 μ L total volume, containing 20 ng of genomic DNA, 2.0 mM $MgCl_2$, 200 μ M each dNTP, 3 pmol each primer, 1 U of FastStart Taq DNA Polymerase and 1x PCR Reaction Buffer without $MgCl_2$ (Roche Diagnostics), and 1x LCGreen Plus dye (Idaho Technology), using a 96-well microtiter plate. PCR reactions were overlaid with 15 μ L of mineral oil (Sigma), briefly centrifuged, and subjected to the following PCR-program: initial denaturation of 10 min, at 95° C, 40 cycles of 20 sec at 95° C, 30 sec at 57° C, 40 sec at 72° C, a final extension step of 5 min at 72° C, and a final denaturation of 1 min at 95° C, before cooling down to room temperature.

High resolution melting analysis of the PCR-products was performed with the Light Scanner HR-96 instrument (Idaho Technology), using a ramping from 65 to 95° C at 0.1° C/sec.

Melting curves were normalized and temperature shifted to facilitate melting profile clustering. PCR fragments producing abnormal melting profiles were checked by directly sequencing the purified PCR products.

3.2.4 Sequencing

PCRs were performed for all exons and flanking intronic regions of *CACNA1A*, *ATP1A2*, *SCN1A* or *SLC1A3* genes. For *CACNA1A* fragments, exon-specific primer pairs were designed harboring M13-tag sequences. The use of the M13F (forward) and M13R (reverse) tags allowed sequencing of the PCR product in the forward or reverse direction, respectively. Genomic DNA of one member from selected families (in most cases the proband) was used as template. In general, the PCR reaction was prepared in a 25 μ l reaction volume, containing 15 pmol of each primer, 250 μ M of each dNTP, 1x PCR Buffer A (3 mM Tris-HCl, 75 mM (NH₄) SO₄, 7.5 mM MgCl₂ with a pH of 8.5), 0.5 U AmpliTaq DNA Polymerase (Applied Biosystems) and 75 ng of genomic DNA. This mixture was subjected to 33 cycles of 30 sec at 94 °C, 30 sec at 60 °C, and 40 sec at 72 °C. The PCR was preceded by an initial denaturation step of 3 min at 94 °C and was ended with an extension step of 10 min at 72°C.

After PCR product purification, double-strand sequencing was performed, in both forward and reverse directions, using the dideoxy termination method and an ABI 3730 sequencer (Prism BigDye Terminators Cycle Sequencing kit, Applied Biosystems). Sequences were analyzed using Vector NTi Program software and both forward and reverse sequences were compared with the reference sequence available from the Human Genome Project (NCBI database: <http://www.ncbi.nlm.nih.gov/>).

3.2.5 Functional analysis

Functional consequences of *ATP1A2* variants were investigated using survival assays in HeLa cells (**Articles 3 to 5**). We designed new primers to introduce each mutation in the *ATP1A2* cDNA, in a two-step PCR procedure, using different *ATP1A2* cDNA containing plasmids as a template in the PCR. Two backbone vectors, pcDNA3.1XNaKa human (C602) and/or pcDNA3.1XNaKa human resistant (C603), were used (kindly provided by Dr. Jan Koenderink, Nijmegen): C602 is the wild-type (ouabain-sensitive) vector and C603 has two additional mutations (Q116R and N127D), introduced in the ouabain-binding site of the Na⁺,K⁺-ATPase that abolish the natural ouabain sensitivity.

The ouabain-insensitive vector can be considered the “wild-type”, since the endogenous Na⁺,K⁺-ATPase activity is abolished in ouabain-containing medium. Specific FHM2 mutations were introduced into this cDNA backbone using mutagenesis. In the assay, HeLa cells were transfected with either the “wild-type” or the “mutant” ATP1A2 cDNA constructs, and their ability to grow and survive in an ouabain-containing medium was tested. Depending on the consequences of the mutation, the results show the ability or not of the mutant ATP1A2 to compensate for the loss of inactivated endogenous sodium-potassium pumps.

3.2.5.1 cDNA constructs

Human Na⁺,K⁺-ATPase α 2-subunit cDNA was subcloned into a modified pCDNA3.1 vector (originally from Invitrogen), which also contained the 5'- and 3'- untranslated regions of the Xenopus β -globin gene flanking the multiple cloning sites²⁰². As previously mentioned, to distinguish endogenous Na⁺,K⁺-ATPase activity from that of transfected Na⁺,K⁺-ATPase, mutations Q116R and N127D were introduced in the original α 2-subunit cDNA to express an ouabain-resistant isoform (α 2-WT)³⁵⁰. FHM2 mutations T376M, V362E, P796S and variant R51H were introduced in the ouabain-resistant wild-type construct by site-directed mutagenesis (Quikchange, Stratagene). Thus, α 2-T376M, α 2-V362E, α 2-P796S and α 2-R51H mutants were obtained. All constructs were sequence-verified, using a protocol similar to the one described above.

3.2.5.2 Transfection in HeLa cells and ouabain treatment

For the survival assay, HeLa cells (5×10^5) were transfected with 1.6 μ g of α 2-WT, α 2-mutant (α 2-T376M, α 2-V362E, α 2-P796S and α 2-R51H) plasmid, using Lipofectamine 2000 Transfection Reagent in Opti-Mem medium (Invitrogen) and cultured in DMEM-containing Glutamax and 10% FCS (Invitrogen). Two days after transfection, one-third of the cells (1.7×10^5) were seeded on a 10 cm Petri dishes and subsequently 1 μ M ouabain was added to the culture medium. After 5 days of ouabain challenge, colonies were stained with 1% methylene blue in 70% methanol, scanned and analyzed with Image Pro Plus (MediaCybernetics). Each transfection was performed at least nine times.

3.2.5.3 Electrophoresis and Western blot analysis

Two days after transfection, two-third of the cells (3.3×10^5) were harvested for Western blot analysis. In brief, proteins were resuspended in a mix of protease-inhibitor (Complete

Mini, Roche) and DNaseI. Subsequently, Laemmli-loading buffer and 0.1 M DTT were added and the samples were heated for 10 min at 65 °C. Next, equal amounts of protein as measured by Bio-Rad protein assay (Bio-Rad Laboratories) were separated on 7.5% SDS-polyacrylamide gels for 40 min at 200 V. Proteins were electroblotted to nitrocellulose membranes (Hybond, Amersham), and incubated overnight with the α 2-subunit-specific polyclonal antibody HERED³⁵¹. The primary antibody was detected with goat-anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Sigma). Protein bands were visualized with Super Signal Substrate (Pierce Biotechnology).

3.2.6 Statistical methods

3.2.6.1 Relative risk (RR) and familial aggregation calculations

RR was adjusted for age-at-observation and sex, using the Mantel-Haenszel RR, and its significance analyzed by a Mantel-Haenszel chi-square test³⁵² (**Article 1**). The Breslow-Day test for homogeneity between strata was used³⁵³. Comparisons between the estimates of RR were made using the ratio of relative risks (RRR)³⁵⁴. Values of RR were given 95% confidence intervals. A 5% significance level was used in all analyses. All statistical analyses were performed using STATA (version 9.2) and SPSS (version 14.0 for Windows).

3.2.6.2 Mendelian errors and inconsistencies

In **Article 2**, several strategies and programs were used to analyze genotyping data obtained for a large set of families (n=86) on six migraine *loci*. After pedigree file inspection, errors of Mendelian inheritance and inconsistencies in genotyping data were tested with the UNKNOWN program, an option from the LINKAGE package³⁵⁵, and with MARKERINFO, from S.A.G.E.³⁴⁵. All samples with errors or genotyping incompatibilities were set as having unknown genotype.

3.2.6.3 Simulations studies

After a first analysis of data, that included the first 33 families, it was crucial to evaluate the informativeness of the whole sample set selected (**Article 2, stage 1**). Simulation was used to provide an indication if the pedigrees to be genotyped were sufficient to detect linkage.

With the SLINK program, we could carry out such power calculations, by simulating genotypes at one locus, given the phenotypes at another locus linked with the first one^{355,356}. SLINK generated 500 data replicates that were then analyzed with the programs MSIM, ISIM and ELODHET^{355,356}. We considered a marker locus with equally frequent alleles and the disease model proposed²⁶⁰, and retained the family structure. The expected maximum LOD score (EMLOD), which is the average of the maximum LOD scores for each replicate, and the power, the probability of rejecting the null hypothesis of no linkage when it is false, were evaluated. The power was defined as the proportion of replicates where the LOD score was equal or greater than a given threshold and was calculated with variable proportion of unlinked families, including 0.30, therefore allowing heterogeneity.

3.2.6.4 Linkage analysis

The power to detect linkage depends on a variety of factors, such as the structure of the pedigree, the number of affected individuals, and the informativeness of the markers. Two main strategies can be used to analyze genotyping data: parametric or non-parametric linkage (NPL) analysis^{93,94}. We used both in our set of families (**Article 2**).

3.2.6.4.1 Parametric, model-based

The LINKAGE package for general pedigrees allows linkage analysis with an arbitrary number of *loci*, but the model for the disease needs to be defined³⁵⁵. Two-point parametric linkage analysis was performed in our migraine families. LOD score values were calculated using MLINK (option of LINKAGE), assuming a dominant inheritance, a disease gene frequency of 0.001, penetrance of 0.9 and a phenocopy rate of 0.024²⁶⁰; another more flexible model was tested, assuming a disease gene frequency of 0.05, penetrance of 0.7 and a phenocopy rate of 0.01³⁵. Linkage calculations were performed with equal allele frequencies. An affected-only strategy was used to avoid problems with incomplete penetrance. Only individuals with migraine (MO, MA or both) were considered affected, while all others were considered unknown (set to zero). Affected spouses and respective entire branches were not considered to homogenize sample, except for ASP analysis. A homogeneity test was performed with HOMOG, under the following alternative hypothesis: two family types, one with linkage between a trait (or any gene locus for that matter) and a marker or map of markers, the other without linkage³⁵⁷. At the end, the proportion of linked families in our sample set was indicated together with linkage probability, and its approximate confidence interval under possible heterogeneity (HLOD).

3.2.6.4.2 Non-parametric, model-free

Because the mode of inheritance remains unclear in the family set and no ideal strategy is known, we applied different NPL methods: MERLIN was used for NPL analysis using the family data³⁵⁸; and in the second stage of the study ASP were evaluated using the S.A.G.E. software (version 5.0)³⁴⁵. NLP score calculations were produced *per loci*, for the overall sample set. Allele frequencies were estimated using FREQ and allele-sharing IBD was estimated by GENIBD, both options of S.A.G.E. program³⁴⁵. The SIBPAL program estimated the mean ratio (π) of alleles sharing IBD among ASP at each microsatellite marker. The π obtained was tested against the null hypothesis of no linkage ($\pi=0.5$). P values were considered significant if below 0.05. Both single and multipoint analyses were performed.

4. RESULTS

4.1 Genetic approaches to study common migraine

4.1.1 Evaluate familial aggregation of migraine

A previous headache survey in an urban population from the Northern region of Portugal estimated an overall prevalence for migraine of 16.7%, which is in agreement with estimates of prevalence of about 12 to 18% in other populations.

Age and gender are important factors influencing disease. Risk for both MA and MO subtypes is also increased in relatives of migraine probands. This points to familial factors, which may be environmental and/or genetic. However, different studies have used different methodologies, regarding ascertainment of patients and relatives and analysis of gender, age of relatives and use of control samples. This makes it difficult to have a reliable estimate of the RR, but also hampers meaningful comparisons between studies

Therefore, our first goal was to evaluate familial aggregation of migraine in a group of Portuguese families, and to assess if MA or MO showed distinct patterns of familial clustering (**Article 1**). Since previous reports have shown that migraine in relatives was underestimated by the proband, we decided to proceed first with validation of the family history, including the estimation of its sensitivity and specificity (data not shown). Then, we evaluated familial aggregation by estimating the RR of migraine in first-degree relatives and spouses of 50 probands with MA and 94 probands with MO, using the Portuguese prevalence reported. For this, 196 first-degree relatives of probands with MO and in 143 first-degree relatives of probands with MA were interviewed. In addition, we estimated RR for the proband spouses, to try to disentangle the influence of genetic and environmental factors.

Our results showed that family members of migraineurs have a 3 to 4-fold increased risk, when compared to the general Portuguese population, while spouses do not present a higher risk. We found an increased risk for first-degree relatives of migraineurs, both for 1st-degree relatives of MO-probands (RR=3.7; 95% C.I: 3.2-4.3) and of MA-probands (RR=3.6; 95% C.I: 3.1-4.3), comparatively to the general Portuguese population. The increased risk remained when comparing subtypes of migraine among groups of first-degree relatives. MA showed a stronger familial aggregation and, consequently, may have a greater genetic predisposition than MO.

ARTICLE 1

FAMILIAL CLUSTERING OF MIGRAINE: FURTHER EVIDENCE FROM A PORTUGUESE STUDY

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Running Title: familial aggregation of migraine

Keywords: migraine, familial aggregation, relative risk, family history

Submitted

Abstract

Objective: Our aim was to evaluate for the first time familial aggregation of migraine in a large group of Portuguese families, and to assess if familial aggregation differs between MA and MO.

Background: Migraine is a disabling disease, leading to a diminished quality of life in both migraineurs and their relatives. Migraine with (MA) and without aura (MO) are the most common forms of this disease.

Methods: Familial aggregation was evaluated by estimating relative risk (RR) of migraine in 143 1st-degree relatives of 50 probands with MA, in 196 1st-degree relatives of 94 probands with MO and also in proband's spouses. Probands were enrolled in the study from a clinical sample and a prevalence of 16.7% from a population sample was used as reference.

Results: A significantly increased risk of migraine was found in both 1st-degree relatives of MO-probands (RR=3.7; 95% CI: 3.2-4.3) and of MA-probands (RR=3.6; 95% CI: 3.1-4.3), comparatively to the general population, while risk for spouses was not. We further divided 1st-degree relatives according to their migraine subtype. First-degree relatives of probands with MA had a significantly increased risk of both MA and MO compared to the general population. Similar finding was found in 1st-degree relatives of probands with MO. When comparing the groups among themselves, RR was only higher for MA in 1st-degree relatives of MA-probands (RR=12.2; 95% CI: 7.7-19.5). Also, in the group of MA-probands, RR of MA in 1st-degree relatives reached a 4-fold increase when compared with RR of MO. No differences were found between the other subgroups of relatives.

Conclusions: This is the first study on familial aggregation of migraine in a population with this geographical origin and our results show that MA has a stronger familial aggregation and, consequently, may have a greater genetic predisposition than MO.

Introduction

Migraine is an episodic neurological disorder, with complex inheritance. It is a disabling disease, leading to a diminished quality of life in both migraineurs and their relatives³⁵⁹. Migraine with (MA) and without aura (MO) are the most common forms of this disease⁴.

Migraine has a high prevalence, affecting about 15% of the general population^{26,27}. In Portugal, to our knowledge, there are only two studies of prevalence of migraine published: one, in a group of University students³²; the other, a headache survey, was

conducted in a small urban district of northern Portugal - prevalence for migraine was then estimated at 16,7% (including migraine associated with other types of headache)^{33,343}. An increased risk for relatives of migraineurs has been described, with values ranging between 1.4-11.8^{42,43,344,360-362}. Different approaches were used to estimate familial aggregation; some studies are clinically-based^{42,362} while others are population-based^{43,344,361} and another is a twin population based survey³⁶⁰. Estimates of familial aggregation also differ among studies since some use a control group as reference, while others use migraine prevalence in the general population^{42,43,344,360-362}. Heritability estimates ranged between 0.28-0.65^{37,42,47,48,242,251,252,363,364}. All these studies suggest that genetic factors are involved in both subtypes, in addition to environmental factors, determining liability for migraine. Several susceptibility *loci* have been described for MA and MO, but the genes and mutations involved have not yet been identified³⁶⁵. Our aim was to evaluate familial aggregation of migraine in a large group of Portuguese families, and to assess if familial aggregation differs between MA and MO. It represents the first study on familial aggregation of migraine in a population with this geographical origin.

Methods

Data collection

A sample of probands with MA and MO was sequentially enrolled in the study, from 1999 to 2004, from the outpatient Neurology clinic. Probands were interviewed (by JPM or JB), regardless of their family history. Demographic and clinical information about relatives and spouses was obtained. Family members were then inquired about the occurrence of migraine, either personally or, if not feasible, by telephone, using the same structured questionnaire. To avoid a selection bias towards affected relatives, family members were contacted regardless of the information provided by the proband about their affection status (affected/non-affected). Interviews to 1st-degree relatives (parents, sibs and offspring) and spouses were made by the same neurologist (JPM). Participants were diagnosed according to the first edition of the "International Headache Society" (IHS) criteria⁴, to standardize the diagnostic, given that selection of probands started before 2004. Probands and family members presenting migraine together with other forms of headaches were also included. Only living family members were eligible to be included in the study. Participants gave their informed consent; the Ethics Committee of HGSA approved the project.

Data analysis

First stage

Probands were classified according to their migraine subtype. Familial aggregation of migraine was studied in 50 families of MA-probands and 94 families of MO-probands. Relative risk (RR) represents the ratio between the frequency of the disease in relatives and the prevalence in the population³⁴² and was estimated for first-degree relatives (with MA, MO or MA and MO), using the total prevalence rate of 16,7%^{33,343}. In addition, we estimated RR for the probands' spouses, to try to disentangle the influence of genetic and environmental factors. The sample size of spouses, however, was not robust enough to form subgroups, neither according to migraine subtype nor according age and sex: thus, proband's spouses (with any form of migraine) were classified only as "affected".

Second stage

Both probands and their first-degree relatives were then classified according to their migraine subtype. The risk for MA and MO was evaluated separately, in 50 families of MA-probands and 94 families of MO-probands using the sub-prevalence rates in the population sample for MO (15%) and for MA (1.7%)^{33,343}. This methodology required that, relatives with MA were considered as non-affected when analysing the risk for MO, and vice-versa³⁴⁴. Additionally, relatives with MA and MO were considered as affected both when analysing the risk for MA and for MO.

Statistical analysis

A Student's *t* test was used when comparing quantitative demographic data. For comparisons between probands and the population sample, a log transformation of age-at-observation and age-at-onset was used, since original data did not follow a normal distribution. For categorical variables, comparisons were assessed using a chi-square test. RR was adjusted for age-at-observation and sex, using the Mantel-Haenszel RR, and its significance analyzed by a Mantel-Haenszel chi-square test³⁵². The Breslow-Day test for homogeneity between strata was used³⁶⁶. Comparisons between the estimates of RR were made using the ratio of relative risks (RRR)³⁵⁴. Values of RR are given with 95% confidence intervals. A 5% significance level was used in all analyses. All statistical analyses were performed using STATA (version 9.2) and SPSS (version 14.0 for Windows).

Results

Demographic data

Probands

A total of 144 probands were selected for this study: 116 women and 28 men. Gender differences in age-at-observation ($p=.01$) and age-at-onset ($p<.001$) were found in probands with MA but not MO; no overall differences were found in these variables between both groups of probands (**Table 1**).

Probands	N		Age at observation, mean (SD)		Age at onset, mean (SD)	
	MA	MO	MA	MO	MA	MO
Overall	50	94	32.1 (12.5)	36.0 (12.3)	17.4 (7.1)	17.4 (8.1)
F	35	81	34.9 (10.8) *	36.6 (10.8)	19.2 (7.3) *	17.7 (7.4)
M	15	13	25.6 (14.2) *	31.9 (19.4)	12.9 (3.7) *	15.6 (11.4)

* $p < .05$ for gender differences

Table 1. Demographic and clinical characteristics of migraine with (MA) and without aura (MO) probands.

Comparison between probands and population sample

Among 2008 individuals of the population sample, 334 were migraine-sufferers^{33,343}. Probands were not different from the migraineurs in the population sample, with regard to gender, age-at-observation and age-at-onset (**Table 2**).

Probands	Group	N affected (%)	RR unadjusted (95% CI)	RR age- and sex-adjusted (95% CI)
MA	1st degree	86 (60)	3.6 (3.1-4.3)	3.6 (3.0-4.2)
MO	relatives	125 (64)	3.8 (3.3-4.4)	3.7 (3.2-4.3)
Migraine	Spouses	13 (27)	1.6 (1.0-2.3)	-

* no significant differences were found

Table 2. Demographic and clinical characteristics of probands and affected individuals in the population sample*

Relatives

Interviews were conducted with 143 1st-degree relatives of MA-probands (83 women, 60 men; mean age $\pm$ SD, 41.5 $\pm$ 17.5 years), and with 196 1st-degree relatives of MO-probands (121 women, 75 men; mean age (SD), 40.6 $\pm$ 19.2 years). A similar proportion of affected relatives was found in both groups (**Table 3**).

	Age at observation,	Age at onset,	Sex (n)	
	mean (SD)	mean (SD)	F	M
Probands	34.6 (12.5)	17.4 (7.7)	116	28
Population	38.6 (19.9)	20.0 (12.1)	245	89

*CI, confidence interval. Spouses were not adjusted for age and sex.

Table 3. Relative risk (RR) of migraine in 1st-degree relatives of migraine with (MA) and without aura (MO) probands and in proband's spouses*

Spouses

Interviews were made to 48 (63 %) probands' spouses (24 by direct observation, 24 by telephone): 13 (27%) were migraine-sufferers.

Familial aggregation

First stage

First-degree relatives of both probands with MO and MA had an increased risk for migraine (RR=3.7; 95% CI, 3.2-4.3; and RR=3.6; 95% CI, 3.0-4.2) (**Table 3**), though not significantly different from each other. RR of migraine for spouses was not significantly higher, when compared to the general population (RR=1.6; 95% CI, 1.0-2.3).

Second stage

First-degree relatives were then divided according to their migraine subtype. First-degree relatives of probands with MA had a significantly increased risk of both MA and MO compared to the general population. Similar finding was found in 1st-degree relatives of MO-probands (**Table 4**). When comparing the 4 groups among themselves, RR was only significantly higher for MA in 1st-degree relatives of MA-probands (RR=12.2; 95% CI, 7.7-19.5), whereas the other groups did not differ significantly between themselves. First-degree relatives of MA-probands showed a 2 to 3-fold increase for MA, when compared to 1st-degree relatives of MO-probands. Also in the group of MA-probands, RR of MA in 1st-degree relatives reached a 4-fold increase when compared with RR of MO (**Table 4**).

Proband	1 st degree relatives	No. Affected (%)	RR unadjusted	RR age- and sex-adjusted
MA	MA	32 (22)	13.2 (8.4-20.8)	12.2 (7.7-19.5)
	MO	65 (45)	3.0 (2.5-3.7)	3.1 (2.5-3.8)
MO	MA	19 (9.7)	5.8 (3.3-9.9)	5.3 (3.1-9.2)
	MO	119 (61)	4.1 (3.5-4.8)	4.0 (3.5-4.7)

* CI, confidence interval

Table 4. Relative risk (RR) of migraine with (MA) aura and without aura (MO) in 1st-degree relatives *

Discussion

Our study reports a strong evidence for clustering of migraine in 1st-degree relatives of MA and MO probands. Our results clearly show that family members of migraineurs have a 3 to 4-fold increased risk, when compared to the general population, while spouses do not present a significantly higher risk. Several studies in other populations have shown an increased risk for relatives of migraineurs^{43,344,360-362}. Our study is the first on familial aggregation of migraine in a population with this geographical origin and the confirmation of familial aggregation in our families is important for future studies of migraine in the Portuguese population.

We also found that, in our sample, 1st-degree relatives of MA-probands had a higher risk of MA than MO. Moreover, 1st-degree relatives of MA-probands had a significantly higher risk of MA than all other groups of 1st-degree relatives, reaching a 2 to 4-fold increase, similar to the findings of Nyholt et al., in which they concluded that MO and MA show different levels of severity, but in the same dimension of liability²⁴². Furthermore, MA and MO can co-occur in the same family and in the same individual^{69,250}. While these may not be distinct entities, MA seems to have a stronger familial aggregation and, thus, a greater genetic predisposition²³, as supported by the data presented here.

In our population sample, sub-prevalence of MA was very similar to the frequency of MA in the control group in the study performed in Greece by Kalfakis et al. and also to the study of Cologno et al in a general population of Italy but was smaller than the one found by Russell et al in Denmark^{42,344,361}. These findings made us hypothesize that prevalence of MA in South of Europe is lower than in the North, supported by studies showing population substructure with a possible distinction between northern and southern populations³⁶⁷. Individuals with Portuguese, Italian and Greek ancestry are included in the southern group³⁶⁷, which could explain the similar MA prevalence in that group. The study by Kalfakis et al. is also clinically-based and found a high RR for MA in 1st-degree

relatives of MA-probands (11.8), which is similar to our findings⁴². Although clinical based samples may introduce some selection bias, our sample did not differ from the sample of migraineurs ascertained from an earlier population-based study³³, in particular in what concerns age at onset, which could indicate a bias towards severity.

In our study we had special concern with potential biases, which were removed; and in addition, this study has several other strengths. Interviews to 1st-degree relatives and spouses were made by a single neurologist, reducing diagnostic variability³⁶⁸ and proband's 1st-degree relatives were directly interviewed blinded to their affection-status and to the proband's migraine subtype-status. Also, RR was used as an estimate of familial aggregation, given that migraine is a common disorder and the odds-ratio (OR) method may lead to an overestimation of familial aggregation³⁶⁹. Estimates of RR were adjusted for age and sex, as migraine is an age and sex-dependent trait³⁴⁴. To adjust simultaneously to these variables, we used the Mantel-Haenszel RR, which has been shown to be a valuable method for this type of studies³⁶⁹.

In this study, migraine prevalence rates from the general population were used, estimated from a previous population-based study³³ performed in the same geographical area where our clinically-based study took place and were similar to other studies^{25,26,35,370}. We chose not to use a control group since probands are more likely to identify their affected relatives than controls, leading to an overestimation of RR³⁷¹ and to also avoid some problems in the RR estimations due to sporadic cases or incomplete penetrance in controls³⁷².

Familial aggregation of migraine may be mainly due to genetic factors as it can be assumed from the magnitude of the RR found in our study. Although environmental factors may have an effect, this needs to be very high to account entirely for familial clustering of a given disease^{373,374}. Therefore, the identification of this group of relatives at a higher-risk can bring important insights for the understanding of the aetiology of migraine, namely for developing strategies to search for susceptibility genes in our population.

Acknowledgments

We thank all patients and families for participating in this study. We thank also Isabel Alonso for her helpful suggestions and comments. This study was supported in part by grants of Fundação para a Ciência e Tecnologia, FCT (POCTI-034390/99/FCT) and Sociedade Portuguesa de Cefaleias, SPC. CL is the recipient of a FCT fellowship (SFRH/BD/17761/2004).

4.1.2 Study the role of migraine susceptibility *loci* by linkage analysis

The etiology of migraine remains largely unknown. Different chromosomal *loci* have been identified for MA and MO, but pathogenic mutations in these common forms of migraine remain to be identified. The involvement of FHM *loci* in common migraine gave contradictory results so far. Although there is convincing evidence for the presence of a susceptibility migraine locus on 19p13, it is possible that *CACNA1A* and *INSR* genes are not major contributors. The role of 1q23 FHM2 and 2q24 FHM3 *loci* in common migraine remains unclear and needs further investigation.

In **Article 2**, we investigate the possible involvement of FHM *loci* in a large set of Portuguese families with common migraine. Considering FHM as a model, two distinct strategies were chosen: linkage analysis of candidate regions and gene scanning (see **Article 3**). In addition, we investigated three other migraine susceptibility *loci*: a locus on 3p (previously reported in a large Dutch family with hereditary vascular retinopathy (HVR), Raynaud phenomena and migraine phenotypes), a locus on 4q found in Finnish/Icelandic kindreds (the only migraine susceptibility locus replicated in different populations), and a 5q locus, recently identified by LCA analysis in Australian families.

For this study, we included 86 Portuguese nuclear families with common migraine (i.e., MO, MA and MA/MO), including 33 extended families, with a total of 443 affected individuals. We also estimated the power of detecting linkage, overall and per family, in this sample set.

Parametric and non-parametric linkage analyses showed, overall, no significant LOD scores or excess allele-sharing values. We thus concluded that these *loci* do not play a major role in migraine susceptibility, at least in our sample. None withstanding, slightly positive maximum LODs were found in some families, remaining inconclusive.

Simulations showed that most of the 33 families were informative enough to obtain at least some evidence of linkage: 18 families could reach nominal linkage (LOD>0.59), 5 families could reach suggestive linkage (LOD>1.90) and one family would reach significant linkage (LOD>3.30). Clinical and genetic heterogeneity decrease dramatically the power to detect linkage in a large sample set. However, even for a small α of 0.30, significant linkage to a locus should have been picked up (power=0.53; EMLOD=3.68). Thus, either the tested *loci* do not play a major role in our migraine families, or heterogeneity is much larger than anticipated, suggesting that alternative approaches to linkage should be considered.

ARTICLE 2

LINKAGE ANALYSIS OF SIX MIGRAINE *LOC* IN A LARGE SET OF PORTUGUESE FAMILIES WITH MIGRAINE WITH OR WITHOUT AURA

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Running Title: susceptibility *loci* in Portuguese migraine families

Keywords: migraine, candidate regions, genotyping, linkage analysis, power calculation

In preparation

Abstract

The etiology of migraine remains largely unknown. Different *loci* have been identified for common forms of migraine (i.e., migraine with or without aura), but the gene variants remain to be identified. The rare Mendelian form of the disease, familial hemiplegic migraine (FHM), is frequently used as model to study the common forms of migraine. So far, the involvement of FHM *loci* in common migraine gave contradictory results. Additionally, novel chromosomal *loci*, harbouring migraine susceptibility genes that were identified through genomewide linkage studies, await replication. Our principal aim was to investigate the involvement of six known migraine *loci* by linkage analysis, under the affected-only strategy. We selected a set of 86 Portuguese migraine families, including 33 extended families. Also, we choose the three FHM *loci* (FHM1 on 19p13, FHM2 on 1q23, and FHM3 on 2q24) and three migraine susceptibility regions on chromosomes 3p21, 4q21-q24 and 5q21. Parametric and non-parametric linkage analysis showed, overall, non-significant LOD scores, or allele-sharing deviation values; however, as we tested only a few *loci*, less stringent criteria apply to achieve suggestive or significant linkage, as compared to a whole genome scan. Therefore, the slightly positive maximum LOD scores observed may still be of some relevance. Simulation studies showed that the majority of the 33 families were informative enough to achieve some evidence of linkage (LOD>0.59).

In conclusion, in this Portuguese sample set, we did not find compelling evidence that any of the tested migraine *loci* has a major role in migraine; at least in our migraine sample. One family was large enough, however, to reach significant values (LOD>3.30) in a whole genomewide linkage study. Our results indicate that large migraine families probably suffer from genetic and clinical heterogeneity, which seriously hamper a linkage approach to identify causal gene variants.

Introduction

Migraine is a very common, disabling episodic neurovascular disease, with a prevalence of about 12-18% in most populations^{27,43}. The most common form of migraine is migraine without aura (MO), which, according to the International Headache Society's (IHS) standardized guidelines, is characterized by recurrent attacks of disabling headache, lasting between 4 and 72 hours¹. The headache is usually severe, unilateral, pulsating, aggravated by physical activity, and associated with nausea, vomiting, photo- and/or phonophobia.

In migraine with aura (MA), which occurs in about one-third of the patients, the headache is very similar to that of MO, but the attacks are preceded or accompanied by transient focal neurological, mostly visual, aura symptoms¹.

Whether or not MO and MA are etiological distinct disorders is still a matter of debate. It was suggested that MO and MA have different etiologies^{25,249}. But there are also data indicating that they represent different ends of the migraine spectrum²⁵⁰. For instance both subtypes often occur within the same family²³⁸, or even the same individuals^{25,35}. There is good reason to suggest that MO and MA may share common underlying genetic and/or environmental factors and pathways³⁷⁵. Additional support for this idea comes from a recent study that used latent class analysis (LCA) in an Australian twin population-based study, which showed the existence of a continuum of severity, with MA being more severe, but not etiologically distinct from MO²⁴².

Most progress in deciphering the genetic background of migraine, however, was made by studying families with hemiplegic migraine (FHM) (FHM1, MIM 141500; FHM2, MIM 602481; FHM3, MIM 609634), a rare autosomal dominantly subtype of MA¹. Aside from the hemiparesis, in FHM, the characteristics of the aura and the headache are identical to those of the common forms of migraine. Several studies have been performed to determine the involvement of the FHM genes - *CACNA1A* (FHM1), *ATP1A2* (FHM2), and *SCN1A* (FHM3) - in MO and MA, but without much success³⁶⁵. Although there is convincing evidence for the presence of a migraine susceptibility locus on chromosome 19p13^{255,256}, it can be questioned whether *CACNA1A* is the susceptibility gene^{256,260,261}. Also, most genomewide screens performed did not provide any support that a major locus exists at that location^{279,282,283}. The involvement of the 1q23 FHM2 *locus* in common migraine remains unclear. Only a small number of studies were performed, but an excess of allele-sharing pointed to the presence of a susceptibility gene for MO and MA in that chromosomal area²⁶³. The contribution of the FHM3 region in common migraine subtypes remains to be clarified too.

The etiology of migraine remains largely unknown, although population, family and twin studies clearly revealed a strong genetic component in the pathogenesis of the disorder. Using families segregating MA, MO, or MA/MO as an autosomal dominant trait, different *loci* have been identified on chromosomes 1q31²⁶⁵, 6p21.1-p12.2²⁷⁷, 11q24²⁷⁹, 14q21.2-q22.3²⁷⁸, 15q11-q13²⁸⁸ and Xq²⁸⁴⁻²⁸⁶. Genetic studies in Dutch affected sib-pairs, and in nuclear families from genetic isolates from Iceland and Finland, as well as families from the US, have also provided convincing evidence for migraine susceptibility *loci* on

chromosomes 4q21-q24^{282,283} and 19p13^{255,256}. Using the LCA-based approach, Nyholt et al. reported significant linkage for migraine on chromosome 5q21²⁸¹.

Interestingly, a novel *locus* was found on chromosome 3p21, in a large Dutch family with a complex phenotype including hereditary vascular retinopathy (HVR), Raynaud phenomena and migraine³⁷⁶.

The main goal of this study was to further investigate the involvement of six migraine *loci*. For that, we performed a linkage study in a sample of 86 Portuguese nuclear families with migraine.

Material and Methods

Patients and diagnosis

Since 1999, our group in Porto has been recruiting migraine patients and their respective families from the Outpatient Clinic of Hospital Geral Santo António (HGSA), for clinical and genetic studies. This migraine family set represents mainly families from the Northern region of Portugal. Diagnosis of the probands and family members was mainly performed by one neurologist (JPM), according to the IHS criteria⁴. All participants gave their informed consent. The Ethical Commission of the HGSA approved this study. Clinical and familial information were used for sample selection.

Sample selection

For this study, we selected the most complete 86 independent, multigenerational, migraine families, with an apparently dominant transmission (**Table 1**). Selection criteria were basically the number of DNA samples available and family size (e.g., number of generations with affected persons, number of affected spouses, and number of sib pairs); 33 of these 86 families were first genotyped to check for correct segregation patterns. DNA samples from 655 (out of a total of 1200) family members were selected for genotyping; 436 of these individuals were affected (MA, MO or MA/MO). Most of the DNA samples were received from families with three generations (41 families, out of 86). Of the remaining families, 17 families had four generations and 28 had two generations. When labelling the families, based on the migraine subtype of the proband, 55 were MO families, 27 were MA families and 4 were MA/MO families. Individuals with unclear diagnosis or who were migraine-free were classified as “unaffected”, for the linkage analysis.

Study	FHM <i>loci</i>	Migraine <i>loci</i>
Families:	86	33
No. Generations:		
2	28	4
3	41	21
4	17	8
Sib ships	237	109
Sib pairs:	581	396
Discordant	254	172
Concordant Affected	266	180
Affected Males/Total	141/390	65/199
Affected Females/Total	302/450	135/213

Table 1. Structure of the Portuguese family set used in the FHM *loci* study and the migraine *loci* study. These values were obtained after running PEDINFO, from S.A.G.E. package³⁴⁵.

Genotyping

Genotyping was performed on, (1) 655 individuals, from 86 families, for genetic markers of the FHM1, FHM2 and FHM3 *loci*; and (2) 382 individuals, from 33 families, for the migraine susceptibility *loci* reported on chromosomes 3p21³⁷⁶, 4q21-q24^{282,283} and 5q21²⁸¹. At least three markers were tested *per locus*. Selection of the markers was based on their heterozygosity value, genetic location and allele size range. Detailed information on the genetic markers can be obtained from the Marshfield (<http://research.marshfieldclinic.org/genetics/>) and Ensembl (<http://www.ensembl.org/>) websites (also see **Table 2** for marker information).

Chromosome	Marker	Genetic position (cM) ^a	Heterozygosity value	Chromosomal region ^b
1	D1S2624	162,57	0,69	1q23.1
1	D1S2707	168,52	0,82	1q23.2
1	D1S2844	175,03	0,80	1q23.3
2	D2S156	164,51	0,85	2q24.2
2	D2S2330	169,41	0,82	2q24.3
2	D2S2381	175,91	0,77	2q31.1
3	D3S3564	67,94	0,78	3p22.1
3	D3S1581	70,61	0,88	3p21.31
3	D3S3666	72,21	0,85	3p14.3
4	D4S3042	83,29	0,84	4q21.1
4	D4S3243	88,45**	0,66	4q21.21
4	D4S2932	90,28	0,75	4q21.22
4	D4S2361	93,48	0,70	4q21.23
4	D4S2929	96,16	0,68	4q22.1
4	D4S3373	101,00***	n.a.	4q22.3
4	D4S1647	104,94	0,77	4q23
4	D4S3240	114,04	0,74	4q25
5	D5S815	101,02	0,80	5q14.3
5	D5S644	104,76	0,85	5q15
5	D5S669	112,52	0,81	5q21.2
5	D5S2084	114,75	0,71	5q21.3
5	D5S2501	116,98	0,75	5q22.1
5	D5S2027	119,50	0,78	5q22.1
5	D5S2860	124,47	0,78	n.a.
5	D5S1505	129,83	0,80	5q23.1
19	D19S221	36,22	0,85	19p13.2
19	D19S1150*	39,00***	n.a.	19p13.13
19	D19S226	42,28	0,84	19p13.12

Table 2. Marker information, including genetic information content and chromosomal location.

^aGenetic location of the genetic markers is indicated in Kosambi cM and refers to sex-averaged the genetic position from Marshfield map;
^bPhysical locations were obtained from Ensembl;

n.a.: not available;
 *CACNA1A intragenic;
 **obtained from deCODE;
 ***estimated.

Oligonucleotide primer sequences were obtained from the Human Genome Database (GDB) (<http://www.gdb.org/>). All genomic DNAs were extracted using a standard salting-out method³⁴⁶. DNA concentrations were determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

PCR amplification was performed using 45 ng of genomic DNA in a final volume of 15 μ L containing a mixture 1x GeneAmp PCR Buffer II (Applied Biosystems, Foster City, CA, USA), 250 μ M of each dNTP, 7.5 pmol of each primer, 2.5 mM $MgCl_2$, and 0.25 U of AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA, USA), and under standard conditions (initial denaturation at 94 °C for 10 min, followed by 10 cycles of 94 °C for 30 sec, annealing at 55 °C for 30 sec, 72 °C for 40 sec, plus 25 cycles of 89 °C for 30 sec, annealing at 55 °C for 30 sec, 72 °C for 40 sec, and a final extension at 72 °C for 10 min). After DNA amplification, PCR products were genotyped, using a post-PCR multiplexing pooling method. In 96-well plates, the reaction was prepared by adding the 10 μ L of the internal lane size standard (GeneScan-400HD ROX Standard, Applied Biosystems), 800 μ L deionized formamide and diluted PCR products for a final 10 μ L per well. Pooled PCR products were denatured at 96 °C for 4 min, being then detected on an automated sequencer (ABI 3700 DNA sequencer, Applied Biosystems, Foster City, CA). All genotypes were analyzed and independently scored by M-JC and KRJV, using Genescan and Genotyper 2.1 software (Applied Biosystems, Foster City, CA). In case of conflicting results, the data of that specific marker was not used for further analysis. Control genotypes were obtained from DNA samples of two CEPH control individuals in each fragment run. Allele sizes for these control samples were available from the CEPH Genotype database (<http://www.cephb.fr/cephdb/>) and were used for standardization of allele labelling. In total, 28 highly polymorphic microsatellite markers, from the six chromosomal candidate regions, were genotyped. Haplotypes were constructed for each locus by inspection of segregation, while assuming a minimal number of recombinations.

Statistical analysis

Familial segregation of alleles obtained for the first two *loci* (FHM1 and FHM2) was checked, and statistical analysis was performed. After pedigree file inspection, errors of Mendelian inheritance and inconsistencies in genotyping data were tested with the UNKNOWN program, an option of the LINKAGE software package³⁵⁵, and with the MARKERINFO option of the S.A.G.E software³⁴⁵. All samples with errors or genotyping incompatibilities were set as having unknown genotype.

Informativity of our selected sample set was evaluated and power calculations were carried out with the SLINK program^{355,356}. SLINK generated 500 data replicates, which were analyzed with MSIM and ELODHE^{355,356}. The following model was used for linkage analysis: a marker *locus* with five equally-frequent alleles and a disease model with characteristics as proposed by Hovatta et al.²⁶⁰. The *expected maximum LOD score* (EMLOD), which is the average of the maximum LOD scores for each replicate, and *power* (the probability of rejecting the null hypothesis of no linkage when it is false) were analyzed. The power was defined as the proportion of replicates where the LOD score was equal or greater than a given threshold (LOD>3.3) in the presence of linkage admixture⁹⁵, and was calculated for a variable proportion of unlinked families.

Two-point parametric linkage analysis was performed, using the LINKAGE package³⁵⁵. LOD score values were calculated using MLINK, assuming dominant inheritance, a disease gene frequency of 0.001, a penetrance of 0.9, and a phenocopy rate of 0.024²⁶⁰. Another, more flexible, model was tested, assuming a disease gene frequency of 0.05, penetrance of 0.7 and a phenocopy rate of 0.01³⁵. Linkage calculations were performed with equal allele frequencies. An “affected-only” analysis was performed to avoid problems with incomplete penetrance. Only individuals with migraine (MO, MA or MO/MA) were considered affected, while all others were considered unknown (i.e., set to zero). Considering that *locus* admixture (heterogeneity) might be occurring, a homogeneity test was performed using HOMOG^{93,357}. Finally, the proportion of linked families in our sample set was indicated together with linkage probability and its approximate confidence interval under possible heterogeneity (HLOD).

As the disease mode of inheritance is not exactly known, non-parametric linkage (NPL) methods were also applied: MERLIN was used for NPL analysis of the family data³⁵⁸. The S.A.G.E. software (version 5.0)³⁴⁵ was used to perform an analysis in affected relative pairs (ARP) and affected sib pairs (ASP). NLP score calculations were produced *per locus* for the overall sample set. Allele frequencies were estimated using FREQ, and IBD allele-sharing was estimated by GENIBD, programs of the S.A.G.E. package³⁴⁵. LODPAL performs an analysis based on the LOD score formulation for ASP, and the SIBPAL program estimated the mean ratio of IBD alleles shared among ASP at each microsatellite marker. Weighing for multiple affected sib pairs was considered by the same program³⁴⁵. *P*-values below 0.05 were considered significant. Both single- and multipoint analyses were performed. The Bonferroni correction was carried out only for positive results.

Results

Two-point linkage analysis, using an “affected-only” strategy, was performed on genotype data of 33 (later extended to 86) families with a broad migraine phenotype. The information content of the genetic markers studied varied from 0.66 to 0.88 (**Table 2**). None of the genotyped markers showed any compelling evidence for linkage to any of the respective *loci*, either under homogeneity or heterogeneity, when the total set of families was analyzed.

Stage 1

Two-point parametric linkage

Analysis of the FHM *loci* produced the highest LOD score of 0.35 (at $\theta=0.37$) with marker D19S221 (FHM1), but gave negative LODs for all FHM2 markers (**Table 3a**). Under the more flexible model, similar results were produced; the LOD score for the D19S221 increased slightly to 0.39 (at $\theta=0.36$), but for other markers LODs decreased (data not shown). Nevertheless, family FE67 gave a maximum LOD score of 1.43, at $\theta=0.00$ with D19S226 (**Table 4**). Results from HOMOG showed that under heterogeneity, the HLOD scores increased slightly for D19S221, now reaching 0.33 (at $\theta=0.38$), with $\alpha=1.00$ (where α is the proportion of linked families); for D1S2624, HLOD reached 0.20 (at $\theta=0.00$), with $\alpha=0.10$, although there were no significant differences from the results under homogeneity.

(a)

Chr.	Marker	Position (cM) ^a	LOD	θ^b	HLOD	α	θ^b	NPL LOD	<i>P</i> value	ARP* LOD	<i>Beta 1</i> ^c
19	D19S221	36	0.35	0.37	0.33	1.00	0.38	0.03	0.3	0.12	0.084
19	D19S1150	39	0.16	0.38	0.08	0.28	0.30	0.04	0.3	0.11	0.081
19	D19S226	42	0.25	0.33	0.18	0.15	0.12	0.00	0.4	0.03	0.004
1	D1S2624	163	-	-	0.20	0.10	0.00	-	-	0.32	0.180
1	D1S2707	169	-	-	-	-	-	-	-	-	-
1	D1S2844	175	-	-	-	-	-	-	-	0.54	0.162

(b)

Chr.	Marker	Position (cM) ^a	LOD	θ^b	NPL LOD	P value	ASP** Mean sharing (alleles; pairs)	P value
19	D19S221	36	0.01	0.48	0.38	0.1	-	-
19	D19S1150	39	0.18	0.39	0.40	0.1	-	-
19	D19S226	42	<u>1.02</u>	0.31	0.44	0.1	0.5657 (0; 61)	<u>0.0434</u>
1	D1S2624	163	-	-	0.04	0.3	-	-
1	D1S2707	169	-	-	-	-	-	-
1	D1S2844	175	-	-	-	-	-	-
2	D2S156	165	-	-	0.21	0.2	0.5310 (2; 261)	<u>0.0429</u>
2	D2S2330	169	-	-	0.19	0.2	-	-
2	D2S2381	176	0.21	0.37	0.09	0.3	-	-

(c)

Chr.	Marker	Position (cM) ^a	LOD	θ^b	NPL LOD	P value
3	D3S3564	68	-	-	-	-
3	D3S1581	71	0.03	0.45	0.02	0.4
3	D3S3666	72	-	-	-	-
4	D4S3042	83	-	-	-	-
4	D4S3243	88	-	-	-	-
4	D4S2932	90	0.04	0.47	-	-
4	D4S2361	93	-	-	-	-
4	D4S2929	96	<u>0.59</u>	0.28	-	-
4	D4S3373	101	0.25	0.36	-	-
4	D4S1647	105	0.01	0.47	-	-
4	D4S3240	114	-	-	-	-
5	D5S815	101	0.45	0.33	0.19	0.2
5	D5S644	105	0.14	0.37	0.06	0.3
5	D5S669	113	0.15	0.36	-	-
5	D5S2084	115	-	-	-	-
5	D5S2501	117	-	-	-	-
5	D5S2027	119	-	-	-	-
5	D5S2860	124	-	-	-	-
5	D5S1505	130	-	-	-	-

^a Sex-average genetic positions of the genetic markers based on the Marshfield map and indicated in Kosambi cM; ^b The maximum recombination fraction for which the maximum LOD score was found; ^c Parameter that reflects overall allele sharing among affected relative pairs; Chr.: chromosome; LOD: logarithm of odds; "-": means that no significant or positive (>0.00) value was reached; *ARP was tested with LODPAL, assuming different disease models, with highest LOD represented here; **ASP analysis, significant deviations in mean sharing values were observed, under multipoint approach.

Table 3. Results of linkage analysis obtained for the total set of families, with indication of highest positive LOD scores and deviations in mean IBD allele sharing. (a) Stage 1 was performed in FHM1 and FHM2 loci (n=33); and Stage 2 was performed in (b) the three FHM loci (n=86) and on (c) chromosome 3p21, 4q21-q24 and 5q21 regions (n=33).

Non-parametric linkage (NPL)

Non-parametric LOD scores were also non-significant (with a highest NPL LOD of 0.04, with $P=0.3$, for D19S1150), and were smaller than in the parametric tests, even when analyzed *per* family. Of the 10 families with a LOD score larger than 0.59, which corresponds to a nominal P value of 0.05⁹⁵, after NPL, only marker D19S221 in family FE67 still gave (now marginal) evidence for linkage (**Table 4**).

Next, ARP was tested with LODPAL, assuming different disease models, with maximum LOD score of 0.54, for D1S2844 (under a “dominant single point” model) and of 0.12, for D19S221 (under a “recessive multipoint” model). ASP showed no excess of allele sharing for two alleles, since no deviations were observed from the expected IBD proportions (0.25, 0.5 and 0.25, for sharing 0, 1 or 2 alleles, respectively).

Marker	FE17		FE28		FE36		FE62		FE67	
	LOD	θ	LOD	θ	LOD	θ	LOD	θ	LOD	θ
D19S221	0.15	0.16	0.79	0.00	0.28	0.00	-	-	0.36	0.11
D19S1150	0.22	0.00	-	-	0.85	0.00	-	-	0.13	0.19
D19S226	-	-	0.81	0.00	-	-	0.60	0.00	1.43	0.00
D1S2624	0.61	0.00	0.55	0.00	0.28	0.00	-	-	0.60	0.00
D1S2707	-	-	0.11	0.21	0.07	0.32	-	-	0.10	0.21
D1S2844	-	-	0.76	0.00	0.07	0.32	-	-	0.08	0.22
<i>Simulations:</i>										
EMLOD	0.81		0.46		0.43		0.72		1.09	
Max LOD	1.98		0.85		0.85		1.44		2.31	

Marker	FE68		FE88		FE108		FE110		FE179	
	LOD	θ	LOD	θ	LOD	θ	LOD	θ	LOD	θ
D19S221	-	-	-	-	-	-	0.10	0.24	0.87	0.11
D19S1150	-	-	0.50	0.00	0.71	0.00	0.10	0.24	0.87	0.11
D19S226	-	-	0.77	0.00	0.81	0.00	0.01	0.36	0.88	0.11
D1S2624	0.46	0.00	-	-	-	-	0.97	0.00	0.22	0.00
D1S2707	-	-	-	-	-	-	-	-	0.13	0.22
D1S2844	0.72	0.09	-	-	-	-	-	-	-	-
<i>Simulations:</i>										
EMLOD	1.53		0.55		0.65		0.57		1.19	
Max LOD	3.42		1.15		1.70		1.16		2.58	

Table 4. Maximum two-point LOD scores for *individual* families that reached nominal evidence of linkage, with LOD score ≥ 0.59 (presented in **bold**)⁹⁵ in at least one marker *per locus*. EMLOD estimations were performed at $\alpha=0.70$.

Haplotypes and power calculations

Importantly, haplotype analysis of the 33 migraine families confirmed correct segregation patterns within our families; two possible non-paternity cases were excluded.

SLINK, MSIM and ELODHET were used to estimate power of finding linkage in our set of 33 families. The EMLOD and the power to detect linkage were calculated for several α values. The results showed that, for this data set, it should be possible to detect significant linkage even for $\alpha=0.30$ (power=0.53; EMLOD=3.68); a power of 1.00 was reached at a threshold of 3.3, with $\alpha=0.70$. Analysis *per* family showed that some evidence of linkage can be achieved, even when we considered the restrictive thresholds described by Lander⁹⁵: 18 families reached nominal linkage (LOD>0.59), 5 families reached suggestive linkage (LOD>1.90) and family FE 67 could even reach a significant linkage value (LOD>3.30). Notably, six families would give significant values in two-point parametric analysis (see **Table 4**; underlined).

Stage 2

Two-point parametric linkage

In the second stage we performed the analysis of (1) the FHM1 and FHM2 *loci* with additional genotyped samples (for ASP analysis; n=86 families), and the FHM3 *locus* (**Table 3b**), and (2) the three candidate regions (3p21, 4q21-q24 and 5q21) for the 33 families (**Table 3c**). The highest LOD score of 1.02 (at $\theta=0.31$) was obtained for D19S226, under *locus* homogeneity. Slightly positive LOD of 0.59 (at $\theta=0.28$), for marker D4S2929 was observed.

Non-parametric linkage

Also the NPL results gave no significant LOD values. The highest, overall, non-parametric LOD scores were obtained for the FHM1 *locus*: 0.44 ($P=0.1$), with D19S226; the FHM2 *locus*, 0.04 ($P=0.3$), with D1S2624; the FHM3 *locus*, 0.21 ($P=0.2$), with D2S156; the 3p21 *locus*, 0.02 ($P=0.4$), with D3S1581; the 5q21 *locus*, 0.19 ($P=0.2$), with marker D5S815; and for the 4q21-24 *locus*, none of the markers gave positive LOD scores.

In the ASP analysis, significant deviations in mean sharing values were observed only for D19S226 (0.5657; $P=0.04$; with 61 pairs, sharing zero alleles), and for D2S156 (0.5310; $P=0.0429$; with 261 pairs, sharing two alleles), both under multipoint analysis.

All additional analyses gave no significant results. None of the ASP results remained significant, after applying the Bonferroni correction.

Discussion

Here we report on the linkage analysis of a set of 86 Portuguese families with common migraine forms (MO, MA or MO/MA), to study the involvement of three FHM regions in common migraine. In addition, we present analyses on three migraine susceptibility *loci*, in a subset of 33 extended families. Based on the overall non-significant LOD scores and allele sharing values, it is very unlikely that these *loci* confer a significant contribution to migraine susceptibility, at least in this Portuguese sample set. In addition, although slightly positive LOD scores were observed in several families, it remains elusive whether these results are relevant.

One of the main advantages of studying candidate regions is that thresholds for evidence of linkage should be set lower than reported for genomewide studies, even under heterogeneity⁹⁵. Additionally, in this case no rigorous corrections for multiple testing are required. We conservatively set a threshold for significant LOD scores at a level larger than 3.3.

The majority of linkage and association studies support the possible involvement of the 19p13 region in common migraine^{255-258,260}. However, the role of the FHM1 *locus* and genes, such as *CACNA1A* and *INSR*, remains unclear. Most likely, the 19p13 region is important for the susceptibility of migraine, through one or more *loci/genes*^{256,259,260,266}, though its impact will not be a major one²⁶¹. Interestingly, our results for intragenic *CACNA1A* marker (D19S1150; LOD score of 0.18, at $\theta=0.39$) provided no evidence that this gene is involved in common migraine types, as suggested from studies in other populations^{259,261}. An excess allele sharing pointed towards the presence of a susceptibility gene for MO and MA in the 1q23 region²⁶³, but that the *locus* may be different from FHM2, and located within 1q23 and 1q31^{263,265}. Our results also did not provide strong evidence that FHM2 is involved in migraine. This study investigated, for the first time, the possible involvement of the FHM3 *locus*, on 2q24, in common migraine; as we have shown for the FHM1 and FHM2 *loci*, also for the FHM3 *locus* our data do not support its involvement in migraine.

Comparing parametric and NPL approaches resulted in minor changes in LOD score values when individual families were considered indicating that for certain families, under

a certain model, parametric linkage may be more appropriate. Comparing LOD score results with simulations, ten families produced LOD scores with a significance level higher than 0.59 for at least one marker, six of which were predicted by the simulations. Interestingly, larger families provided lower LOD scores, which indicate that the true problem was the high clinical and genetic heterogeneity of migraine. These results have implications for future ascertainment of migraine families and subsequent selection of families for genetic analyses.

From our results, we were not able to confirm the involvement of the three *loci* on 3p21, 4q21-q24 and 5q21 in our family set. The 4q21-q24 *locus*, that was identified in two independent genomewide screens in genetic isolates for MO²⁸³ and MA²⁸², hinted that the same gene could be involved in both forms of migraine. Unfortunately, we could not confirm this hypothesis in the Portuguese set. Perhaps this region can only typify isolated populations. The 5q21 *locus* found by Nyholt et al.²⁸¹ could not be confirmed either. Possible explanation can be simply that, indeed, LCA-based phenotype classification analysis has more power to detect linkage peaks than IHS-based phenotype.

Several factors could have influenced the low LOD score results. First, phenotypic misclassification can have negatively influenced the analyses. The affected status of our selected samples was based on familial information that has been previously validated³⁷⁷. All efforts should be made to obtain a more homogeneous clinical phenotype, as this increases the power to detect linkage³⁷⁸. Second, marker allele frequencies may have influenced the LOD scores. In general, two options could overcome this problem: estimate allele frequencies in our population and consider allele frequencies to be similar to the general population and simultaneously genotype family members. Third, the disease model chosen for linkage analysis could also have influenced LOD score calculations. Although, a different model was tested, we cannot discard its limitations compared with the real disease model.

By simulation studies we have confirmed that our family set has enough power to detect significant linkage. Only one of the families is large enough to reach genomewide significance by itself (FE68), but the majority of families (73%) are large enough to obtain at least suggestive linkage. The nine families that could not contribute even with nominal values should either be extended or removed in further linkage analysis, if one investigates them as single families.

In order to increase the power to detect linkage peaks, new approaches, such as trait component analysis (TCA) analysis, have been successfully applied to complex traits.

Basically, TCA analyzes for linkage of individual traits of the disease, thereby stratifying the study sample. In a recent publication of Anttila et al., new migraine *loci* have been reported using this approach²⁴⁰. We might consider this a fruitful approach, in the future, for our migraine sample set, using e.g. vomiting or photo/phonophobia, not just MO or MA.

Here we first evaluated and characterized the Portuguese migraine material, which will be crucial for future genetic analyses. We conclude that neither the FHM-related, nor three other known migraine susceptibility *loci* showed any compelling evidence for linkage. For the first time, migraine susceptibility *loci* on chromosomes 3p21, 4q21-q24 and 5q21 were evaluated in a large set of clinic-based migraine families. We conclude that the FHM-related regions are not major contributors to the overall migraine genetic risk, at least not in the Portuguese sample set. We cannot exclude, however, that these *loci* may still play a role in particular migraine families.

Acknowledgements

We would like to thank to all Portuguese families for their participation in this study. We thank the European Commission for having provided most of the financial support for this work, namely by a Marie-Curie grant for PhD students (MJC, RRF; Contract nº QLGA-CT-2000-60005), and by the "EUROHEAD" grant (LSHM-CT-2004-504837; RRF, MDF, AMJM, vdM), complemented by Fundação para a Ciência e Tecnologia (FCT) (POCTI-034390/99/FCT). CL is the recipient of a FCT fellowship (SFRH/BD/17761/2004). Some of the results of this paper were obtained by using the software package S.A.G.E., which is supported by a U.S. Public Health Service Resource Grant (RR03655), from the National Center for Research Resources.

4.1.3 Determine the possible involvement of FHM genes in common migraine

The *common disease - common variant* (CD-CV) theory is very popular in modern-day genetics. It proposes that genetic risk may be due to predisposing variants that are relatively common in the population. An alternative theory defends that complex disease, at least in part, may also be attributed to moderately high penetrant variants in genes of specific importance to the disease. **Article 3** aims at addressing this, through two studies that were performed in a set of our families: (1) the first intended to scan FHM *CACNA1A* and *ATP1A2* genes for mutations, while (2) the second involved very large-scale sequencing of more than 500 ion channel genes that is still ongoing.

(1) We scanned the entire coding region and intronic sequences flanking the exons of the FHM1 and FHM2 genes, in a subset of our migraine probands (31 for *CACNA1A* and 50 for *ATP1A2*). We identified several polymorphisms and a novel *ATP1A2* R51H variant in a large family with MO (i.e. FE17). Based on segregation analysis of the R51H variant with the disease and functional data for this variant, we concluded that this is most probably not disease-causing. This rare novel R51H variant still may have some effect, but it does not seem to be a major contributor to migraine susceptibility.

(2) The functional gene high-throughput mutation scanning project is being performed by a Canadian company and includes 50 of our migraine probands (the whole project investigates 368 patients affected by paroxysmal neurological diseases, i.e., bipolar disorder, essential tremor, Tourette syndrome, migraine, and epilepsy). Some 150 CNS-expressed ion channel genes were chosen as candidates and are now being scanned for mutations. Preliminary results showed that none of the rare DNA variants identified segregated with migraine in our families (data not shown).

In conclusion, we provide additional evidence that rare variants in the two FHM genes are not major contributors to susceptibility of common migraine. One might argue that, at least with respect to migraine, rare evolutionarily conserved variants need not always be pathogenic. Incomplete co-segregation of such variants in large families may be expected because of clinical and genetic heterogeneity, especially in frequent disorders such as migraine, and will make it very difficult to draw definite conclusions about their pathogenicity. Importantly, exhaustive functional studies are needed to address whether such variants may have small functional consequences. We propose that well-powered association studies may be a more promising approach to identify genetic variants for migraine.

**MUTATION SCREENING FOR *CACNA1A* AND *ATP1A2* FHM GENES IN
A SET OF MIGRAINE PROBANDS: IDENTIFICATION OF A NOVEL
RARE R51H VARIANT IN *ATP1A2***

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Running title: FHM genes in common migraine

Keywords: candidate genes; familial hemiplegic migraine; mutation screening;
rare variants; Na⁺,K⁺-ATPase

Submitted

Abstract

Common migraine has a strong genetic component, although genes and mutations remain to be identified. Familial hemiplegic migraine (FHM), a rare and severe autosomal dominant subtype of migraine with aura, was used as a model to study migraine. The involvement of the FHM *loci* and genes in migraine remains, nevertheless, controversial. Our aim was to study the possible involvement of *CACNA1A* and *ATP1A2* (FHM1 and FHM2) genes in migraine, through mutation analysis, in a set of Portuguese probands. Among 149 families with primary headache, those with migraine segregating in several generations were selected for mutation scanning of the *CACNA1A* gene (n=31) and/or the *ATP1A2* gene (n=50). SSCP revealed no abnormal electrophoretic patterns in *CACNA1A*. A novel R51H variant was found in the *ATP1A2* gene in the proband of a three-generation family with migraine without aura (MO). R51H is evolutionarily conserved, and was not present in 346 control chromosomes or in other migraine patients. Although we expected R51H to be pathogenic and as it co-segregated well with MO in the proband's nuclear family, no single haplotype seemed to explain all disease cases in the larger kindred. Furthermore, cell survival assays did not suggest any functional consequence, leading us to conclude that the R51H is a rare variant that most likely is not disease-causing. In conclusion, our results reinforce the idea that rare variants in FHM genes do not appear to be major contributors for common migraine, and suggest that well-powered association studies may be a more promising approach.

Introduction

Migraine is the most common cause of chronic, episodic, severe headache; it affects around 18% of the general population. Diagnostic criteria of the International Headache Society (IHS) distinguish two major subtypes: migraine without aura (MO) and with aura (MA)⁴. MO is characterized by moderate to severe attacks of pulsating, unilateral headache, with nausea, vomiting, and photophobia and phonophobia, typically lasting 4-72 hours. In MA, attacks are accompanied by transient focal neurological symptoms, usually visual and/or sensory abnormalities.

Familial hemiplegic migraine (FHM) is a rare, severe, autosomal dominant, subtype of migraine, characterized by hemiparesis during the aura⁴. But, apart from these, aura and headache are very similar in FHM and common forms of migraine. Moreover, a high percentage of FHM patients also have episodes of common migraine. FHM may, thus, be considered a valuable model to study migraine.

Three genes for FHM have been identified (*CACNA1A*¹⁵⁰, *ATP1A2*¹⁸² and, recently, *SCN1A*²⁰⁸), but little progress has been made in elucidating the genetic component of common migraine. In addition to the clinical overlap, some genetic findings support the concept that FHM and common migraine may be etiologically related. Although the involvement of the FHM *loci* in migraine remains controversial^{255,261}, mutations in *CACNA1A* and *ATP1A2* have been reported in non-FHM patients from FHM families^{13,182}. On the other hand, the screenings for FHM genes have been negative in common migraine^{269,270}.

Complex disorders are thought to be due to the combined effects of variation at multiple (often interacting) genes with the environment. Mapping studies are still challenging, since linkage and association studies have been largely unfruitful. Only few *loci* have been reportedly mapped for migraine, although genes and mutations remain to be identified²⁴⁰.

Single-rare variants alone are not expected to explain common diseases, as they do in disorders, but a few exceptions have been reported. Two main hypotheses that susceptibility alleles contribute for common diseases are often followed. (1) The common disease-common variant (CD-CV) hypothesis proposes that genetic risk may often be due to predisposing variants relatively common in the population¹²⁹. (2) Another theory defends that complex disease may be attributed to mildly deleterious genes, which overall mutation rate is relatively high¹³⁴.

Linkage analysis is most effective in identifying rare genetic variants with high penetrance, whereas association studies are most suitable to detect common variants with low penetrance; thus, none of these would be appropriate for predicting rare, mildly deleterious variants¹³⁵. Therefore, direct mutation analysis of candidate genes complements these strategies, overcoming some of its assumptions and limitations.

The aim of this work was, thus, to determine the possible involvement of *CACNA1A* and *ATP1A2* FHM genes in common migraine, through mutation analysis in a set of probands with migraine. At the same time, we wanted to study the contribution of rare variants for overall migraine susceptibility.

Material and Methods

Sample selection

A set of 149 Portuguese migraine families was available for mutation analysis of known FHM genes. All families were diagnosed according to the criteria of the IHS⁴, by the same team of neurologists (JB and JPM). Only families with migraine (mainly MA and MO) segregating in several generations were selected; some family members had aura with motor symptoms or hemiplegia. Selection criteria also included the availability of clinical data and DNA samples. In a first phase, 82 families were screened for the exons of the *CACNA1A* gene with mutations previously reported, since *CACNA1A* is a large gene; the 31 most informative families were screened for the remaining exons. Then, 50 probands were re-selected for mutation analysis of all coding exons in the *ATP1A2* gene. In total, 26 probands were entirely screened for both genes. A panel of 173 blood donors from HGSA, with no history of headache, was used as controls. All participants gave their informed consent.

Genetic analysis

Genomic DNA was extracted from peripheral blood samples, using a standard salting-out method³⁴⁶. For mutation analysis, PCR was performed for all exons and flanking intronic regions of *CACNA1A* (Ac. Nr X99897) and *ATP1A2* (Ac. Nr NM_000702) genes, using PCR conditions described¹⁵³. Primer sequences for *CACNA1A* were adapted from Ophoff et al.¹⁵⁰; and, for *ATP1A2*, novel primers were designed, using the Oligos Program v.9.5b. Mutation screening was performed using SSCP³⁴⁷. PCR products were analyzed using radioactive labelling¹⁵⁰; for some exons, a non-radioactive procedure was performed, using the Multiphor II apparatus (Amersham Biosciences, Lisbon, Portugal) with non-denaturing polyacrylamide gels. Final detection used a silver staining protocol. PCR fragments with mobility variants were analyzed by double-strand sequencing, using standard dideoxy termination methods and ABI PRISM 310 (Applied Biosystems, Foster City, CA). Haplotypes in family FE17 were constructed with three polymorphic markers at 1q23, encompassing *ATP1A2* (for primers, see <http://gdbwww.gdb.org/>), as described¹⁸³. For detection of the R51H mutation (G>A, nt position 256; Ac nr NM_000702), exon 3 was amplified using primers “exon 2+3F” (5'-TTGAACACACACCCCACT-3') and “exon 2+3R” (5'-GCATGGTTTTCTTGCTCTT-3'). PCR products were digested with restriction enzyme *Acil* (New England Biolabs, Ipswich, MA) as suggested by the manufacturer, and electrophoresed on a 3% agarose gel; the R51H variant causes a loss of an *Acil* site.

Functional analysis

Human Na⁺,K⁺-ATPase α 2-subunit cDNA was subcloned into a modified pCDNA3.1 vector (originally from Invitrogen, Carlsbad, CA), which also contained the 5'- and 3'-untranslated regions of the Xenopus β -globin gene, flanking the multiple cloning sites. An ATP1A2 cDNA was used, in which the ouabain-binding site was inactivated by two additional mutations, rendering wild-type ouabain-resistant (α 2-WT)²⁰². R51H was introduced in this wild-type construct by site-directed mutagenesis (QuikChange, Stratagene, La Jolla, CA). All constructs were sequence-verified. HeLa cells were transfected with α 2-WT and α 2-R51H plasmids, for survival assays and Western blot analysis, as published¹⁸⁹. By transfecting ouabain-insensitive wild-type (α 2-WT) or mutant (α 2-R51H) Na⁺,K⁺-ATPase α 2-subunits in these cells it can be determined whether recombinant protein is able to compensate for the loss of Na⁺,K⁺-ATPase activity after ouabain treatment. If rescue is compromised this indicates pathogenicity of the DNA variant that was tested.

Results

Mutation screening

No abnormal electrophoretic patterns were found by SSCP, in any of the *CACNA1A* exons, for both sets of probands. SSCP and subsequent sequencing analysis of the *ATP1A2* gene revealed a heterozygous nt 256 G >A substitution in exon 3, resulting in an Arginine-to-Histidine change in codon 51 in the proband of a three-generation MO family (**Figure 1**). Several other known and new polymorphisms were also identified for both genes (**Table 1**; for *CACNA1A*, data not shown).

Location	Nucleotide Change			Frequency	Consequence
Exon 3	nt 233	G→A	Lys ₄₃	0.01	-
Exon 6	nt 713	C→T	Ile ₂₀₃	0.02	-
Exon 9	nt 1223	G→A	Ser ₃₇₃	0.37	-
Exon 13	nt 1808	C→T	Phe ₅₆₈	0.04	-
Exon 16	nt 2363	C→T	Ala ₇₅₃	0.16	-

Table 1 - Polymorphisms detected in the coding sequence of the *ATP1A2* gene in the patients that were scanned for mutations.

(a)

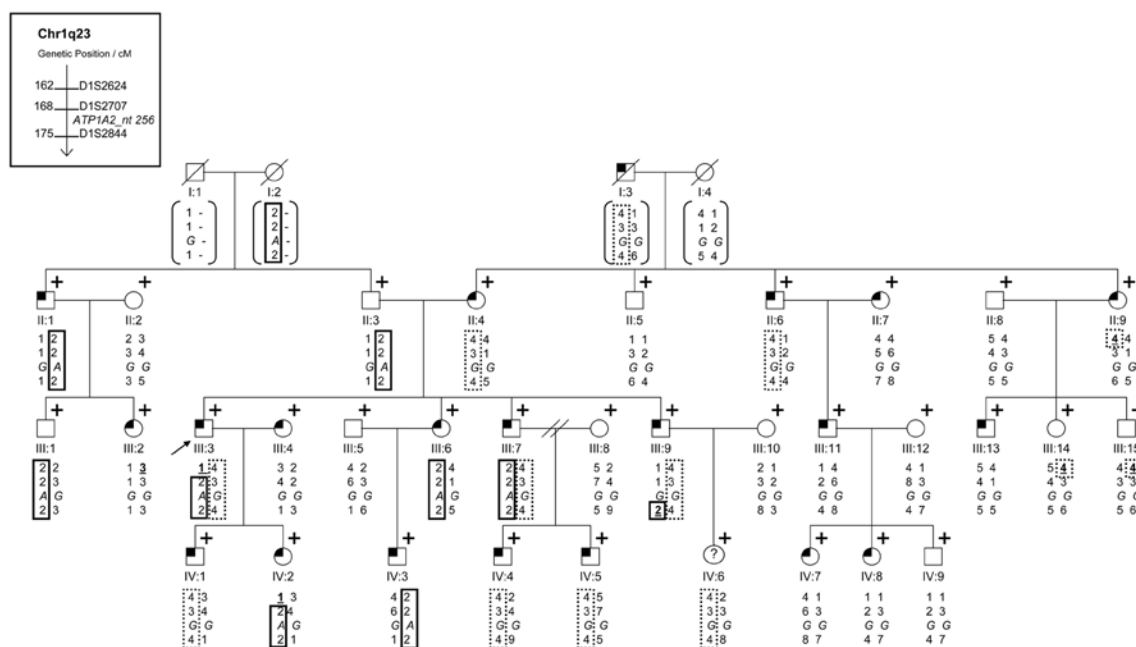
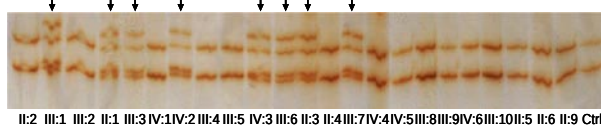


Figure 1. Pedigree of the Portuguese MO family (FE17), with genetic information of markers in the 1q23 *ATP1A2* gene region.

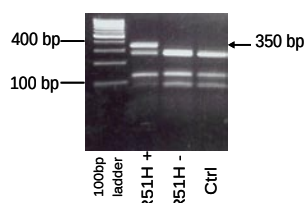
(a) Clinical diagnosis is represented, with upper-left black quarter symbols indicating individuals with migraine without aura (MO), and question mark symbol pointing individuals with probable migraine. A plus sign indicates individuals that were clinically examined. Two haplotypes are marked; R51H variant segregating in the fully boxed haplotype. Possible recombinations are underlined. Genetic markers and their position relatively to the *ATP1A2* gene are indicated on the top; genetic positions were collected from Marshfield maps (<http://www.marshfieldclinic.org/research/>).

(b)



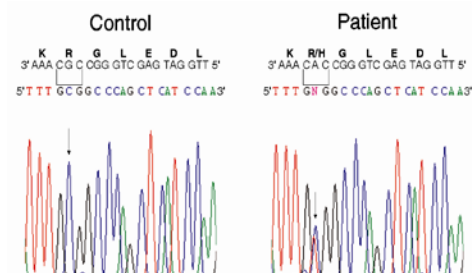
(b) SSCP analysis of exon 3 in some family members, obtained in 15% polyacrylamide gel (run at 15°C followed by silver staining detection), with positive pattern indicated by arrows.

(c)



(c) Restriction analysis to detect R51H carriers, using *Acil* digestion analyzed in 3% agarose gel, with 350 bps band indicating its presence.

(d)



(d) Sequencing of exon 3 fragment with electropherograms of a control versus an heterozygous carrier resulting in R51H substitution; changed nucleotide is indicated by an arrow.

FE17 family and R51H studies

This MO family was further extended and re-interviewed by two independent clinicians (JPM and JB). In this 33-member family, a wide clinical heterogeneity was observed. Subject II:1 had cluster headache and cervicogenic pain, in addition to attacks of MO. Episodic tension-type headache was found in subjects III:4 (who also suffered from MO), III:5 and III:15. Unclassified headaches were reported in III:8, III:10 and III:14, and in IV:6 (with probable MO). Migraine associated with menstrual cycle was described in II:9, III:4 and III:8. A wide variation of age at onset (8-60 years) was observed in MO patients (n=20). Their attacks of migraine were characterized by unilateral, pulsating, severe pain, aggravated by physical exercise, and accompanied with nausea and photo and phonophobia, lasting 6 to 72 hours. R51H mutation carriers in family FE17 were verified by direct sequencing and restriction enzyme digestion. R51H was absent from all 346 control chromosomes and it was also not present in 234 migraineurs from 181 additional families. This novel variant replaces an Arginine by a Histidine at position 51 and is located within the cytoplasmic N-terminal of the Na^+, K^+ -pump $\alpha 2$ subunit (**Figure 2a**). Sequence alignments showed strong evolutionary conservation of this part of the protein sequence, including the mutated residue (**Figure 2b**).

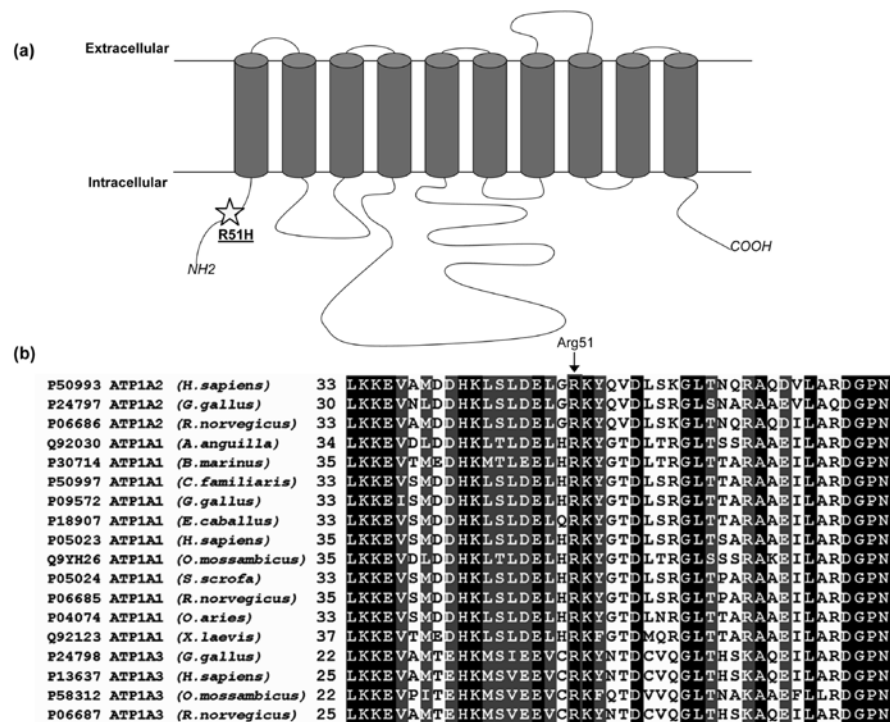


Figure 2. Topography of the Na^+, K^+ ATPase $\alpha 2$ -subunit and location and conservation of ATP1A2 mutant.

- (a) Scheme of the Na^+, K^+ -ATPase $\alpha 2$ subunit, showing the location of mutation R51H in the pump subunit.
(b) Conservation of amino acid Arg⁵¹ among several α -subunits of the P_2 -type ATPase subfamily and within different species.

Haplotype analysis

In family FE17 (**Figure 1**) the haplotype with the R51H variant (boxed) co-segregated with MO in the nuclear family of the proband. Subjects II:3 and III:1 are apparent non-penetrance, while III:2 could represent a phenocopy. Unfortunately, no single haplotype associated with all migraine patient cases in the whole kindred. Moreover, family members with the haplotype indicated in dotted-boxes seemed to inherit the disease from the other branch of the family; although with MO, they did not carry the R51H variant. Therefore, it was concluded that there was no clear segregation of R51H with migraine in this family. In line with this conclusion, parametric and non-parametric linkage analysis did not support involvement of this gene region (data not shown).

Functional data

Survival assays were performed to evaluate possible functional consequences of the R51H on pump functioning in HeLa cells, in which endogenous Na^+, K^+ -ATPase activity was inhibited by ouabain. Western blot analysis revealed that wild-type and mutant proteins were expressed at levels comparable to wild-type (**Figure 3a**). Cells expressing the mutant $\alpha 2$ -R51H construct were able to survive ouabain treatment, comparable to the wild-type (**Figure 3b**). Although there may be functional consequences that do not result in decreased cell survival, our results did provide any evidence in favour of a functional consequence for this rare variant.

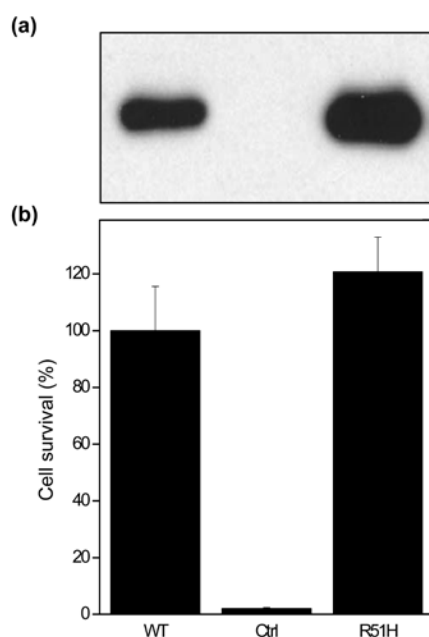


Figure 3. Western blot analysis and ouabain survival assay in transfected HeLa cells.

(a) For the Western blots, the detection of protein was performed using the polyclonal antibody (HERED3) that is specific for $\alpha 2$ -subunit Na^+, K^+ -ATPase protein.

(b) Ouabain sensitivity of cell transfected with either WT or mutant ATP1A2 cDNA constructs. Graphic representation of cell survival, after 5 days of ouabain challenge (n=9); Lane 1: $\alpha 2$ -WT construct; lane 2: Ctrl: mock-transfected cells; lane 3: $\alpha 2$ -R51H construct.

Discussion

Here we describe a novel R51H variant in the *ATP1A2* gene in a large Portuguese family with migraine without aura. The absence of this variant in a large control sample and its evolutionary conservation suggests that this variant may be a pathogenic mutation. Nevertheless, we could not establish a clear segregation with migraine in the family (**Figure 1**). In addition, functional assays of mutant R51H $\alpha 2$ -subunit also did not reveal obvious consequences on Na^+, K^+ -ATPase functioning. Therefore, it must be concluded that the R51H DNA variant most likely is not a disease-causing rare DNA variant. However, it cannot be excluded that the R51H variant still plays a minor role in conferring migraine susceptibility in this family.

Although cell survival assays are good predictors of pathogenicity of *ATP1A2* mutations in case these tests show decreased cell survival, this does not mean that they detect all functional consequences of *ATP1A2* mutations. For instance, mutation M731T has normal cell survival but has abnormal catalytic turnover²⁰³. Other functional tests are necessary to detect possible subtle functional consequences of the R51H variant. That such a “hidden” causal effect may be present comes also from the fact that Arg⁵¹ is well conserved and located at the border between the variable and the homologous region from the N-terminal tail, which represents a consensus-splicing site for ATPases¹⁹⁵. In *ATP1A4*, the equivalent position is within a cation ATPase transporter domain³⁷⁹. Proteolysis cleavage at these sites reduces, but does not abolish, enzyme activity. The N-terminal provides targeting information for insertion in plasma membrane as well, which might interfere in the regulation of the rate of conformational transitions, both in agreement with functional predictions.

No other potentially disease-causing mutations were identified in *CACNA1A* and *ATP1A2*, in this set of Portuguese migraine families. Although SSCP is a commonly used method to detect mutations in large sample sets, it has relatively low efficiency. Thus, DNA variants may have been missed. Additionally, promoter and splicing sites of these two genes should also be evaluated.

The hypothesis that *ATP1A2* variants might be involved in common forms of migraine has been previously considered, but no pathogenic mutations were found^{269,270}. Two novel rare variants in *ATP1A2* were apparently associated with migraine, but segregation patterns and functional data could also not support it³⁸⁰.

In conclusion, although this rare novel R51H variant may still have some very mild functional consequence, it does not seem to be a major contributor. This study also illustrates that, in complex diseases such as migraine, rare and evolutionarily conserved variants are not always pathogenic. Hence, functional studies should always be performed. Although co-segregation studies in large families will offer some valuable indications, complex inheritance and genetic heterogeneity often make conclusions difficult to be drawn.

Acknowledgements

We are particularly grateful to all Portuguese families for their participation in this study. We thank Fundação para a Ciência e Tecnologia (FCT) for having provided most of the financial support for this work (POCTI-034390/99/FCT), complemented by European Community funding, namely a Marie-Curie grant for PhD students (MJC, RRF; Contract nº QLGA-CT-2000-60005). We report no conflicts of interest.

4.2 Genetics of FHM: a model to study the pathophysiology of migraine

4.2.1 To establish the role of *ATP1A2* in Portuguese FHM samples

Because of its clinical similarities with the common forms of migraine, familial hemiplegic migraine (FHM) may be considered as a useful model to unravel the molecular mechanisms underlying the pathophysiology of migraine.

We aimed to study the involvement of the *CACNA1A* (FHM1), *ATP1A2* (FHM2) and *SCN1A* (FHM3) genes in our Portuguese families with FHM. First, haplotype analysis allowed us to determine which FHM gene(s) might be involved in each respective family. Next, we performed extensive mutation scanning, by direct sequencing, in *ATP1A2* (**Article 4** and **5**) and *SCN1A* (see **Article 6**); for *CACNA1A*, the presence of most known mutations was evaluated. Additionally, the *SLC1A3* gene, previously shown to be involved in a sporadic case with a severe, complex, neurological phenotype of (alternating) hemiplegia and seizures, was entirely scanned in a selected subset of FHM probands.

No mutations were identified in the *CACNA1A* and *SLC1A3* genes in our set of FHM patients. However, four mutations were found in *ATP1A2*: two had previously been reported and are now associated with a pure FHM phenotype (**Article 4**), the other two are novel mutations, associated with FHM and, possibly, psychiatric disturbances (**Article 5**). For this study, cDNA constructs were generated with the novel mutations that were analyzed for functional anomalies using cell survival assays in HeLa cells. Western blot analysis showed that all constructs, α 2-T376M (**Article 4**), and α 2-V362E and α 2-P796S (**Article 5**), were expressed at a level comparable to the wild-type levels. Although the wild-type construct was able to rescue cell death, hardly any survival was observed for the V362E and P796S mutants (**Article 5**). Also, the T376M mutant gave only very reduced level of survival (<10%) (**Article 4**). This clearly indicates that all three mutants have functional consequences on sodium-potassium pump functioning and can be considered pathogenic.

Here, we present meaningful genotype-phenotype correlations for the *ATP1A2* gene, and show that recurrence of *ATP1A2* mutations is more frequent than thought. Additionally, a possible link was established with “novel” mental illness phenotypes and *ATP1A2* mutations. This implies that such mutations should be first screened in the genetic study of Portuguese FHM families. It also shows that, with complex phenotypes, it may be particularly interesting to screen for *ATP1A2* mutations.

RECURRENT *ATP1A2* MUTATIONS IN PORTUGUESE FAMILIES WITH FAMILIAL HEMIPLEGIC MIGRAINE

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Running Title: recurrent *ATP1A2* mutations

Keywords: cell survival assay, FHM2, genotype-phenotype correlations,
recurrent mutations, Na⁺,K⁺-ATPase

J Hum Genet. 2007; 52 (12): 990-998

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Recurrent *ATP1A2* mutations in Portuguese families with familial hemiplegic migraine

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Received: 13 June 2007 / Accepted: 27 September 2007 / Published online: 19 October 2007
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Abstract Familial hemiplegic migraine is a rare autosomal dominant subtype of migraine with aura. Three genes have been identified, all involved in ion transport. There is considerable clinical variation associated with FHM mutations. Genotype–phenotype correlation studies are needed, but are challenging mainly because the number of carriers of individual mutations is low. One exception is the recurrent T666M mutation in the FHM1 *CACNA1A* gene that was identified in almost one-third of FHM families and showed variable associated clinical features and severity, both within and among FHM families. Similar studies in the FHM2 *ATP1A2* gene have not been performed because of the low number of carriers with individual mutations. Here we report on the recurrence of *ATP1A2* mutations M731T and T376M that affect sodium–potassium pump functioning in two Portuguese FHM families. Considerably increasing the number of mutation carriers with these mutations indicated a clear genotype–phenotype correlation: both mutations are associated with

pure FHM. In addition, we show that recurrent mutations for *ATP1A2* are more frequent than previously thought, which has implications for genotype–phenotype correlations and genetic testing.

Keywords Cell survival assay · FHM2 · Genotype–phenotype correlations · Recurrent mutations · Na⁺,K⁺-ATPase

Introduction

Familial hemiplegic migraine (FHM) is a rare autosomal dominantly inherited subtype of migraine with aura (MA), in which attacks are accompanied by the presence of motor weakness during the aura phase that is followed by headache (ICHD-II 2004). FHM is a genetically heterogeneous disorder and three genes have been identified thus far. The FHM1 gene, *CACNA1A*, located on chromosome 19p13,

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encodes the pore-forming $\text{Ca}_v2.1\text{-}\alpha1$ subunit of neuronal voltage-gated $\text{Ca}_v2.1$ (P/Q-type) Ca^{2+} channels. Mutations in *CACNA1A* cause a broad clinical spectrum of FHM and associated features such as ataxia, recurrent coma, or absence or generalized epilepsy (Ophoff et al. 1996; Ducros et al. 2001; Jouvenceau et al. 2001). The FHM2 gene, *ATP1A2*, on chromosome 1q21–23 (FHM2) (Ducros et al. 1997) encodes the $\alpha2$ subunit of Na^+, K^+ -ATPases, and is involved in ion transport across the plasma membrane. Mutations in the *ATP1A2* gene are associated with pure FHM (De Fusco et al. 2003; Vanmolkot et al. 2003; Jurkat-Rott et al. 2004; Kaunisto et al. 2004; Riant et al. 2005; Pierelli et al. 2006) and in rare cases, cerebellar problems (Spadaro et al. 2004), benign familial infantile convulsions (BFIC) (Vanmolkot et al. 2003), alternating hemiplegia of childhood (AHC) (Bassi et al. 2004; Swoboda et al. 2004), severe episodic neurological deficits and permanent mental retardation (Vanmolkot et al. 2006) or basilar migraine (Ambrosini et al. 2005). Recently, a third FHM gene, *SCN1A*, located on chromosome 2q24, encoding the pore-forming subunit of voltage-gated $\text{Na}_v1.1$ sodium channels, was identified in three German FHM families that share a common ancestry (Dichgans et al. 2005).

Straightforward genotype–phenotype correlation studies for FHM are difficult, because of the clinical variation and low number of mutation carriers. However, for certain FHM mutations that are found more frequently, especially the FHM1 mutation T666M, which was reported in about one-third of hemiplegic migraine probands, such detailed studies could be performed (Ducros et al. 2001). T666M mutations were identified on many different haplotypes, indicating they occurred as independent mutational events. For instance, Ducros and colleagues reported nine families and one isolated case that showed eight different genetic backgrounds (Ducros et al. 1999). Interestingly, there is considerable phenotypic variation described with the T666M mutation, ranging from persons with non-hemiparetic migraine episodes, pure hemiplegic migraine, and patients with associated permanent cerebellar ataxia (Ophoff et al. 1996; Ducros et al. 1999; Friend et al. 1999; Ducros et al. 2001; Terwindt et al. 2002; Kors et al. 2003). A second recurrent FHM1 mutation R583Q was mostly reported in patients suffering from FHM and associated ataxia (Battistini et al. 1999; Ducros et al. 2001; Alonso et al. 2003). A homogeneous clinical phenotype also seems apparent for FHM3 mutation Q1489K as all 15 mutation carriers had pure FHM, although three mutation carriers reported benign epileptic seizures during infancy (Dichgans et al. 2005).

However, meaningful genotype–phenotype studies in *ATP1A2* have not been possible, due to the lack of sufficient carriers for individual mutations. The main reason is

that almost all *ATP1A2* mutations are unique to a particular FHM2 family. Few exceptions with a very small number of mutation carriers have been reported (Jurkat-Rott et al. 2004; Riant et al. 2005). Here, we describe the recurrence of two *ATP1A2* mutations in two Portuguese FHM families without ataxia or epilepsy and, for the first time, were able to perform genotype–correlation studies for *ATP1A2* on a large number carriers with specific mutations. Our findings clearly show that recurrence of *ATP1A2* mutations is more frequent than described until now. This not only implies that such mutations should, perhaps, be screened with priority in genetic screening, but that identification of additional mutation carriers will also facilitate meaningful genotype–phenotype correlations.

Materials and methods

Clinical description

Two Portuguese families were interviewed and the clinical diagnosis was established according to the criteria of the International Headache Society (IHS) as mentioned in the 2004 revision of the International Classification of Headache Disorders (ICHD-II 2004). A panel of 176 blood donors with no headache history from the Hospital Geral de Santo António (HGSA) was selected as a control group. All participant subjects gave their informed consent for this study. The HGSA ethics committee approved this study.

Family 1

Six of the FHM patients (I:2, II:3, II:5, II:6, III:1 and III:2) from this family (Fig. 1a) had typical hemiplegic migraine attacks, in many cases precipitated by head trauma (details on clinical features of patients with a gene mutation are listed in Table 1a). The onset of these attacks ranged from 9 to 16 years old, and attack frequency varied from less than one (i.e. II:3) to six per year. Auras in some cases were prolonged, and started with visual and sensitive symptoms that preceded motor disturbances and dysphasia. In some cases the duration of the headaches was shorter than 4 h. Visual hallucinations and neuropsychological aura were reported by patient II:3. Neurological evaluation in FHM patients was normal, except for patient III:1 that showed minor cerebellar dysarthria. Interictal neurological examination revealed no nystagmus. At the initial examination, an MRI was made that revealed “unspecific periventricular white matter lesions” (data not available). Unfortunately, patients II:3, III:1 and III:2 refused genetic testing. Although very likely, it remains (somewhat) unclear whether these additional patients have the same gene mutation as identified

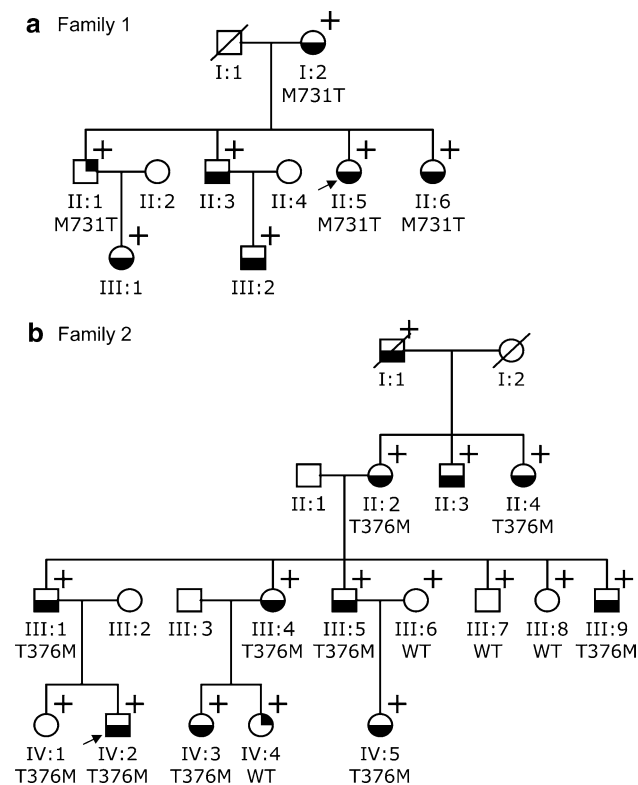


Fig. 1 Pedigrees of Portuguese FHM families 1 (a) and 2 (b). Clinical diagnosis is represented by different symbols: *black lower half symbols* indicate individuals with hemiplegic migraine; *black upper right squares* represent migraine with aura. *Arrows* indicate probands. *Plus signs* indicate individuals that were clinically evaluated. Heterozygous mutation carriers are indicated *M731T* or *T376M*. Tested non-mutation carriers are indicated *WT* (wild-type)

in the other hemiplegic migraine patients in this family. No history of ataxia, seizures, or abnormalities in mental or physical development was reported.

Family 2

In this four-generation family, eleven individuals affected by pure hemiplegic migraine were clinically evaluated (Fig. 1 b) (details of the clinical features of patients with a gene mutation are listed in Table 1b). Patients I:1, II:2, II:3, II:4, III:1, III:4, III:5, III:9, IV:2, IV:3, and IV:5 all suffered from attacks of hemiplegic migraine, with a frequency ranging from four in total to once a month. Attacks were associated with visual and/or sensory aura symptoms, hemiparesis and speech disturbances (varying in duration between 10 min and 10 h). Patient IV:2 had one atypical attack lasting 1 week, during which he had generalized slowing over the left hemisphere, on EEG, and normal brain MRI. Interictal EEG was normal. Of note, all four attacks of hemiplegic aura in patient II:2 were not associated with headache. Ataxia or epilepsy was not reported for any of the affected family

members. Except for patient III:9 who showed abnormal aggressive behavior (not further specified episodes of aggressive and inappropriate or dangerous behavior after minor frustration) and “some learning difficulties”, all affected individuals had a normal physical, cognitive, and social development, and their interictal neurological examination was also normal. In this family only one patient (II:3) refused genetic testing.

Genetic analysis

Peripheral blood samples were collected from all family members willing to participate in this study. Genomic DNA was extracted using a standard salting-out method (Miller et al. 1988). To evaluate the involvement of the FHM2 locus in the Portuguese FHM families, microsatellite markers D1S2624, D1S2707 and D1S2844 were tested (data not shown). Oligonucleotide primer sequences were obtained from the Human Genome Database (GDB) (<http://www.gdb.org/>). After DNA amplification, PCR products were detected by use of an automated sequencer (ABI 3700 DNA sequencer, Applied Biosystems, Foster City, CA, USA). All genotypes were analyzed and independently scored by M-JC and KRJV, using Genescan and Genotyper 2.1 software (Applied Biosystems). Haplotypes were constructed by evaluating the segregation and assuming a minimal number of recombinations.

For mutation analysis, PCR was performed for all 23 exons and flanking intronic regions of the *ATP1A2* gene, using genomic DNA of patients I:2 and III:1 from families 1 and 2, respectively, as a template. All PCR products were directly sequenced, using the Big Dye Terminator Ready Reaction Kit (Applied Biosystems) and an ABI 3730 sequencer apparatus (Applied Biosystems). For detection of the M731T mutation (T > C, nt position 2,296; Ac nr NM_000702), standard PCR followed by digestion with restriction enzyme *NcoI* was performed, as described elsewhere (Vanmolkot et al. 2003) (data not shown). For detection of the T376M mutation (C > T, nt position 1,231, Ac nr NM_000702), exons 9 and 10 were amplified by PCR, using primers “exon9–10F” (5′-GCCACGGTCTAGGGT AAGGT-3′) and “exon9–10R” (5′-GCAAGAGGCTTTGG AGACAC-3′), resulting in a 571-bp product. Carrier status for this mutation was evaluated by direct sequencing of the PCR products.

Functional analysis

Functional analysis of *ATP1A2* mutation T376M was performed by survival assays. Human Na⁺,K⁺-ATPase α 2-subunit cDNA was subcloned into a modified pCDNA3.1

Table 1 Summary of the clinical features of gene mutation carriers from FHM families 1 and 2

Clinical features of M731T <i>ATP1A2</i> mutation carriers (family 1)									
Patient	I:2	II:1	II:5	II:6					
Age/onset (in years)	72/15	46/10	31/11	28/9					
Hemiplegia attacks									
Auras ^a	+ / + / + / +	+ / + / - / -	+ / + / + / +	+ / + / + / +					
Duration	15 min–1 h	15 min	1 h–5 days	15 min–1 h					
Prolonged episodes	–	–	+	–					
Frequency (per year)	2	1	6	2					
Head trauma triggering	–	–	–	–					
Headache	Unilateral	Bilateral	Unilateral	Unilateral					
Pain character	Non-throbbing	Throbbing	Throbbing	Non-throbbing					
Severity	Mild	Moderate	Severe	Mild					
Nausea/vomiting	+ / exuberant	+ / –	+ / +	NA					
Photophobia/phonophobia	+ / +	NA	+ / +	NA					
Other phenotypes									
Psychomotor development delay	–	–	–	–					
Neurological exam (interictal)	Normal	Normal	Normal	Normal					
Imaging	Normal MRI	–	Normal MRI	–					
Clinical features of T376M <i>ATP1A2</i> mutation carriers (family 2)									
Patient	II:2	II:4	III:1	III:4	III:5	III:9	IV:2	IV:3	IV:5
Age/onset (in years)	76/13	62/5	51/7	49/14	48/12	36/8	15/10	28/14	17/6
Hemiplegia attacks									
Auras ^a	– / + / + / +	+ / + / + / +	+ / + / + / +	+ ^b / + / + / +	– / + / + / +	– / + / + / +	+ / + / + / +	+ / + / + / +	– / + / + / +
Duration	15 min	15 min	1–2 h	10 h	15 min	1 h	1.5–2 h	20 min	10 min
Prolonged episodes	–	–	–	+	–	–	+ (once) ^d	–	–
Frequency (per year)	4 episodes in life	6 (from age 5–8)	4	12 (since age 25)	5 episodes in life	1	4–12	12	12 (since age 9)
Head trauma triggering	–	–	–	–	–	–	–	–	–
Headache	^c	Bilateral	Unilateral	Unilateral	Bilateral	Bilateral	Uni/bilateral	Bilateral	Bilateral
Pain character	^c	Throbbing	Throbbing	Throbbing	Throbbing	Throbbing	Non-throbbing	Throbbing	Non-throbbing
Severity	^c	Severe	Severe	Severe	Severe	Severe	Severe	Severe	Severe
Nausea/vomiting	^c	+ / –	+ / rarely	+ / +	+ / –	+ / –	+ / +	+ / +	+ / +
Photophobia/phonophobia	^c	+ / +	+ / +	+ / +	+ / +	– / –	+ / +	+ / +	+ / +
Other phenotypes									
Psychomotor development delay	–	–	–	–	–	+	–	–	–

Table 1 continued
Clinical features of T376M *ATP1A2* mutation carriers (family 2)

Patient	II:2	II:4	III:1	III:4	III:5	III:9	IV:2	IV:3	IV:5
Neurological exam (interictal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Imaging	–	–	Normal CT and MRI	Normal CT	–	Normal CT	Normal CT and MRI; altered EEG (once) ^d	Normal CT and EEG	Normal CT and EEG

For genotype–phenotype correlations, it is important to only include the patients with mutations. Patients II:3, III:1, and III:2 (from family 1) and patient II:3 (from family 2) refused genetic testing and were not included in the table

NA not available; – absent; + present

^a Characteristics of auras: visual/sensitive/hemiplegic/aphasic

^b Visual patterns trigger attacks

^c Never had headache

^d Generalized slowing over the left hemisphere

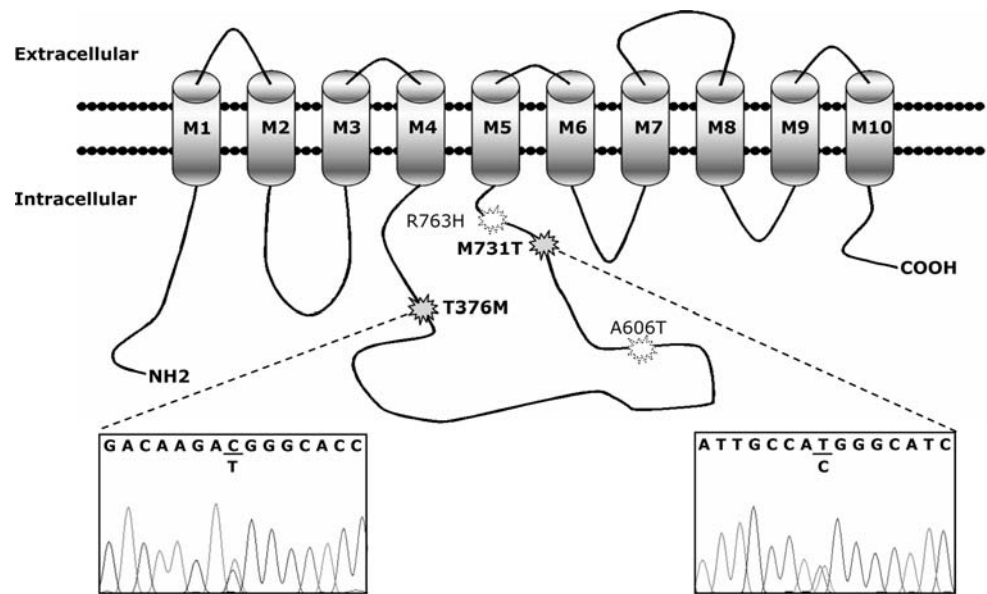
vector (Koenderink et al. 2005). To distinguish endogenous Na^+ , K^+ -ATPase activity from that of transfected Na^+ , K^+ -ATPase, we used a cDNA encoding ouabain-resistant wild-type (*ATP1A2*-WT) (Price et al. 1990). Mutation T376M was introduced in the ouabain-resistant wild-type $\alpha 2$ -subunit construct by site-directed mutagenesis (Quikchange; Stratagene, La Jolla, CA, USA). HeLa cells (5×10^5) were transfected with plasmid DNA of either *ATP1A2*-WT or *ATP1A2*-mutant (*ATP1A2*-T376M) using Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA). Two days after transfection, two-thirds of the cells were harvested for immunoblotting. In brief, proteins were resuspended in a mix of protease-inhibitor (Complete Mini, Roche, Basel, Switzerland) and DNaseI. Subsequently, Laemmli-loading buffer and 0.1 mol L^{-1} DTT were added and the samples were heated for 10 min at 65°C . Next, equal amounts of protein as measured by the Bio-Rad protein assay (Bio-Rad Laboratories, Munich, Germany) were separated on 7.5% SDS-polyacrylamide gels for 40 min at 200 V. Proteins were electroblotted to nitrocellulose membranes (Hybond, Amersham, Buckinghamshire, UK) and incubated overnight with the $\alpha 2$ -subunit-specific polyclonal antibody HERED (Pressley 1992). The primary antibody was detected with goat-anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Sigma, St Louis, MO, USA). Protein bands were visualized with Super Signal Substrate (Pierce Biotechnology, Rockford, IL, USA).

For the survival assay itself, the remaining one-third of the cells (1.7×10^5) was seeded on 10 cm Petri dishes and subsequently $1 \mu\text{mol L}^{-1}$ ouabain was added to the culture medium. After 5 days of ouabain challenge, colonies were stained with 1% methylene blue in 70% methanol, scanned and analyzed with Image Pro Plus (MediaCybernetics, Silver Spring, MD, USA). In these assays, untransfected cells do not produce colonies. Each transfection was performed 7–15 times and the average values of visible colonies were calculated for each construct. The average number of colonies obtained with the wild-type construct was used as a reference (i.e. 100% cell survival). Next, the relative percentage of cell survival was calculated for the mutant construct. Statistical significance was determined using a Student's *t* test ($P < 0.05$).

Results

In both Portuguese families the phenotype was pure FHM, although in some patients some variation in additional clinical features was observed, such as prolonged, atypical auras and abnormal behavior with learning difficulties. In both families, haplotypes were compatible with the involvement of the chromosome 1q23-linked FHM2 locus

Fig. 2 Scheme of the Na^+, K^+ -ATPase $\alpha 2$ subunit, showing the location of all FHM2 recurrent mutations: *gray-filled stars* depict the mutations reported here; *white-filled stars* represent mutations previously reported in more than one family, namely R763H (Jurkat-Rott et al. 2004) and A606T (Riant et al. 2005). *Inserts* Electropherograms of heterozygous mutations resulting in substitutions M731T and T376M



(data not shown). Mutation analysis of the *ATP1A2* gene revealed missense mutations M731T and T376M in *families 1* and *2*, respectively.

The M731T mutation in exon 16 (nt 2,296 T > C) causes a substitution of a threonine for a methionine, at position 731 of the $\alpha 2$ subunit of the Na^+, K^+ -ATPase pump (Fig. 2). The mutation co-segregated with FHM in all three mutation carriers for which DNA was available, and in an obligate carrier diagnosed with “non-motor auras” (Fig. 1a). The T376M mutation is located in exon 9 (nt 1,231, C > T) and leads to a threonine for methionine change at position 376 of the pump protein (Fig. 2). This mutation co-segregated in all nine FHM patients of which DNA was available, and was also identified in one asymptomatic young girl (Fig. 1b).

Neither mutation was observed in a panel of 352 Portuguese control chromosomes.

Conservation of both amino acids Met⁷³¹ and Thr³⁷⁶ was very high among several α -subunits of the P_2 -type ATPase subfamily and within different species, as previously shown (Vanmolkot et al. 2003; Riant et al. 2005). Functional analyses of mutant M731T already revealed dysfunction of the Na^+, K^+ pump (Capendeguy and Horisberger 2004; Segall et al. 2005). However, dysfunction of sodium–potassium pumps that contained the $\alpha 2$ subunit with the T376M mutation has not been reported before. Here functional consequences of *ATP1A2* mutation T376M were investigated using survival assays in HeLa cells. Survival assays test for the ability of mutant alleles to compensate for the loss of endogenous Na^+, K^+ pump function (achieved by ouabain treatment). In the assay, transfected wild-type (*ATP1A2*-WT) and mutant (*ATP1A2*-T376M) Na^+, K^+ -ATPase $\alpha 2$ subunits are

ouabain-insensitive, because of mutations in the ouabain-binding site (Price et al. 1990). Western blot analysis showed that the wild-type and mutant constructs were expressed at comparable levels (Fig. 3a). In the survival assay, cells expressing the wild-type construct survived ouabain treatment (set as reference to 100%). In contrast, *ATP1A2* mutant T376M gave only very reduced level of survival (<10%), indicating, for the first time, a clear functional consequence for this mutant (Fig. 3b).

Discussion

Here we describe two Portuguese families with pure FHM, presenting with mutations M731T and T376M in the Na^+, K^+ -ATPase *ATP1A2* gene and, for the first time, present meaningful genotype–phenotype correlations for this gene. Both mutations can be considered disease-causing because they:

- 1 co-segregated with the affected phenotype in the respective families (with only one case of incomplete penetrance in *family 1* (II:1) and in *family 2* (IV:1));
- 2 were described in previous FHM studies (Vanmolkot et al. 2003; Riant et al. 2005);
- 3 were not found in a large Portuguese control panel;
- 4 affected highly conserved, important, amino acid residues in the $\alpha 2$ Na^+, K^+ pump protein;
- 5 mutation M731T had already been shown to cause dysfunction of the Na^+, K^+ pump (Capendeguy and Horisberger 2004; Segall et al. 2005); and
- 6 mutation T376M was shown here to result in dysfunctional Na^+, K^+ pumps as shown by survival assays.

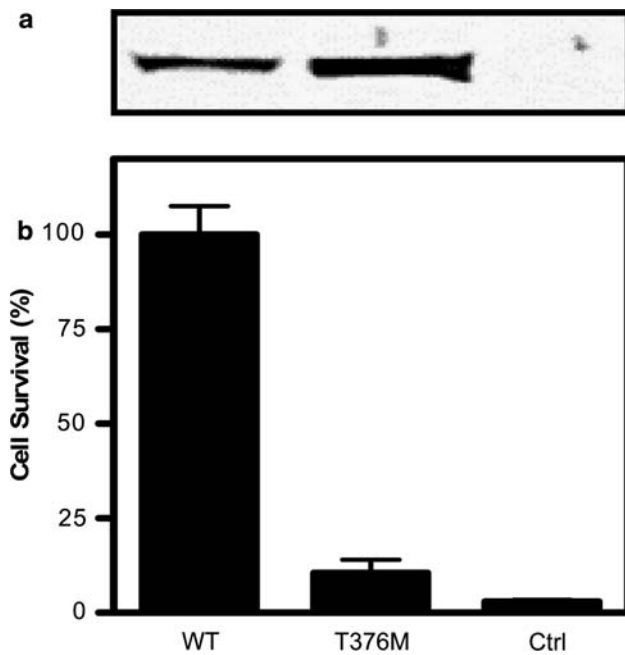


Fig. 3 Ouabain survival assay of *ATP1A2* mutant T376M. **a** Western blot analysis of HeLa cells transfected with WT or mutant *ATP1A2* cDNA. Detection of Na^+, K^+ -ATPase $\alpha 2$ -subunit protein was performed using a specific polyclonal antibody (anti-HERED). Specificity of the antibody is also shown from the absence of signal in the Ctrl lane (containing untransfected HeLa cells that express primarily the $\alpha 1$ -subunit) **b** Ouabain sensitivity of cells transfected with either wild-type or mutant *ATP1A2* cDNA. Bars represent cell survival after 5 days of ouabain treatment (error bars: SEM). Ctrl control; represents mock-transfected HeLa cells

Both mutations are located in the large M4-M5 cytoplasmic loop of the sodium–potassium pump subunit. Met⁷³¹ is located in the conserved part of the domain P of the Rossman fold that is next to the phosphorylation site (Toyoshima et al. 2000). Interestingly, the pig kidney Na^+, K^+ -ATPase homologous methionine is located in close proximity to a segment reported as essential for the overall folding and maturation of the pump (Jorgensen et al. 1998). Thr³⁷⁶ is located only two amino acids away from Asp³⁷⁴ that is equivalent to Asp³⁵¹, a crucial amino acid in a phosphorylation site of Ca^{2+} -ATPases (Toyoshima et al. 2000). Studies of yeast PMA1 H^+ -ATPase revealed that residues surrounding the equivalent aspartate residue Asp³⁷⁸ are critical for ATPase biogenesis, appropriate folding, and interaction with the secretory machinery (DeWitt et al. 1998). Functional consequences of mutation M731T, introduced in the human *ATP1A2* cDNA, have been analyzed previously, revealing reduced catalytic turnover (Capendeguy and Horisberger 2004; Segall et al. 2005). The functional consequences of human mutation T376M have yet not been reported, but studies of yeast PMA1 H^+ -ATPase mutant T380A (e.g., the equivalent of human residue Thr³⁷⁶) revealed serious functional consequences as the mutant

ATPase protein was retained in the endoplasmic reticulum and did not reach the plasma membrane in sufficient quantity (DeWitt et al. 1998).

Although recurrent mutations are rather common in FHM1, before this study, only three FHM2 mutations had been found in more than one family: T376M (Riant et al. 2005), A606T (Riant et al. 2005), and R763H (Jurkat-Rott et al. 2004). One important reason to identify recurrent mutations is that a larger number of mutation carriers allow better evaluation of the associated clinical phenotype (including variation in clinical features and associated symptoms). Secondly, recurrence of a mutation adds weight to the evidence that it is indeed the mutation that causes the disease phenotype—the mutation is present on different haplotypes.

Before this study, five carriers of mutation M731T and nine carriers of mutation T376M had been identified in three families (Vanmolkot et al. 2003; Riant et al. 2005). Four M731T mutation carriers in a Dutch family (Vanmolkot et al. 2003) and the three new Portuguese carriers in this study suffer from pure FHM, indicating that this mutation is clearly associated with this phenotype. Since haplotype analysis revealed different disease haplotypes for both families, the M731T is a recurrent mutation (data not shown). The three published T376M families originated from different countries (France, Italy, and Portugal) (Riant et al. 2005 and this study) and the mutation, is most probably recurrent, although no haplotyping data were reported. Ten additional T376M mutation carriers are presented here, increasing the total number of mutation carriers to nineteen, allowing better genotype–phenotype correlations. The T376M mutation is associated with pure FHM in this large set of mutation carriers.

Recurrence of a mutation is more likely when the mutation is located in a CpG dinucleotide that is methylated on its cytosine, because it is more prone to deamination causing a C to T mutation event. Recurrent FHM1 mutations S218L, R583Q, T666M, and R1667W and FHM2 mutations T376M and R763H affect a CpG dinucleotide, and these mutations thus probably have occurred via this mechanism. Mutation M731T, now also a recurrent mutation, does not affect a CpG dinucleotide and occurred through a deamination-independent mutation event.

In conclusion, we demonstrated by considerably expanding the number of carriers of *ATP1A2* mutations M731T and T376M that the phenotype associated with FHM2 mutations is homogenous (i.e. pure FHM). Of note, the clinical phenotype associated with *ATP1A2* mutations seems more homogenous than reported for FHM1 mutations with a large number of mutation carriers. The fact that recurrence of FHM mutations was observed in our two Portuguese FHM families gives unique opportunities for genotype–phenotype correlations. In addition, this suggests

that recurrent mutation sites in these FHM genes should be analyzed with priority when performing molecular diagnosis.

Acknowledgments We would like to thank Dr A. Levy Gomes for referring the proband of *family 2* to us. We are particularly grateful to both Portuguese families for their participation and contribution for this study. The European Community provided financial support for this work, through a Marie-Curie grant for PhD students (M.-J.C., R.R.F.; contract no QLGA-CT-2000-60005), the “EUROHEAD” grant (LSHM-CT-2004-504837; R.R.F., M.D.F., A.M.J.M.vdM.), and Center of Medical System Biology (CMSB) established by The Netherlands Genomics Initiative/Netherlands Organisation for Scientific Research (NGI/NWO).

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**TWO NOVEL FUNCTIONAL MUTATIONS IN THE Na^+/K^+ -ATPASE $\alpha 2$
SUBUNIT *ATP1A2* GENE IN PATIENTS WITH FAMILIAL HEMIPLEGIC
MIGRAINE AND ASSOCIATED NEUROLOGICAL PHENOTYPES**

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Running Title: novel functional mutations in *ATP1A2*

Keywords: borderline personality disturbance, cell survival, FHM2, mental retardation, migraine

Clin Genet. 2008; 73: 37-43

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Original Article

Two novel functional mutations in the Na^+,K^+ -ATPase $\alpha 2$ -subunit *ATP1A2* gene in patients with familial hemiplegic migraine and associated neurological phenotypes

Castro M-J, Nunes B, de Vries B, Lemos C, Vanmolkot KRJ, van den Heuvel JJMW, Temudo T, Barros J, Sequeiros J, Frants RR, Koenderink JB, Pereira-Monteiro JM, van den Maagdenberg AMJM. Two novel functional mutations in the Na^+,K^+ -ATPase $\alpha 2$ -subunit *ATP1A2* gene in patients with familial hemiplegic migraine and associated neurological phenotypes.

Clin Genet 2008; 73: 37–43. © Blackwell Munksgaard, 2008

Mutations in the *ATP1A2* gene, encoding the $\alpha 2$ -subunit of the Na^+,K^+ -ATPase, are associated with familial hemiplegic migraine type 2. The majority of *ATP1A2* mutations were reported in patients with hemiplegic migraine without any additional neurological findings. Here, we report on two novel *ATP1A2* mutations that were identified in two Portuguese probands with hemiplegic migraine and interesting additional clinical features. The proband's of family 1 (with a V362E mutation) had mood alterations, classified as a borderline personality. The proband in family 2 (with a P796S mutation) had mild mental impairment, in addition to hemiplegic migraine; more severe mental retardation was observed in his brother, who also had hemiplegic migraine and carried the same mutation. Cell-survival assays clearly showed abnormal functioning of mutant Na^+,K^+ -ATPase, indicating that both *ATP1A2* mutants are disease causing. Additionally, our results suggest a possible causal relationship of the *ATP1A2* mutations with the complex clinical phenotypes observed in the probands.

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Key words: borderline personality disturbance – cell survival – FHM2 – mental retardation – migraine

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Received 24 August 2007, revised and accepted for publication 24 September 2007

Familial hemiplegic migraine (FHM) is a rare autosomal dominant subtype of migraine, characterized by hemiparesis as part of the aura (1).

Three genes have been identified so far in FHM, all involved in ion transport. FHM1 is caused by mutations in the *CACNA1A* gene, encoding the

$\text{Ca}_v2.1$ -subunit of neuronal voltage-gated P/Q-type calcium channels (2, 3). FHM2 is caused by mutations in the *ATP1A2* gene, encoding the Na^+, K^+ -ATPase $\alpha 2$ -subunit (4, 5). Recently, a third FHM gene (*SCN1A*) was identified, encoding a subunit of neuronal voltage-gated $\text{Na}_v1.1$ channels (6).

Considerable clinical variation in symptoms and severity has been reported for *CACNA1A* and *ATP1A2* mutations (7). However, for mutations in the *ATP1A2* gene, the clinical spectrum is less well established, as most patients have 'pure' hemiplegic migraine, without any associated neurological features. Most strikingly, when compared with FHM1, ataxia is a less-prominent symptom in FHM2 patients, and has only been reported in association with G301R *ATP1A2* mutation (8). Other clinical features that may be associated with FHM2 mutations are childhood epilepsy (5), atypical alternating hemiplegia (9, 10), and, rarely, seizures at a later age (4, 11) and mental retardation (12).

Functional studies of mutant Na^+, K^+ -ATPases reveal a unifying theme: mutant sodium-potassium pumps function less well compared with normal pumps. Abnormalities range from complete loss of Na^+, K^+ -pump function (4, 13, 14) to specific effects, such as decreased catalytic turnover (15) or reduced affinity for potassium ions (16).

Here, we describe two novel *ATP1A2* mutations in unique Portuguese patients, who in addition to hemiplegic migraine also have neurological and/or psychiatric symptoms. Both mutants result in almost complete failure to compensate for loss of endogenous Na^+, K^+ -ATPase activity in cell-survival assays, clearly demonstrating that both mutations have a severe impact on sodium-potassium pump functioning.

Materials and methods

Families

Family members of both Portuguese families underwent neurological examination and peripheral blood was collected. The diagnosis of migraine was based on the International Classification of Headache Disorders-second edition criteria (1). Psychological phenotypes were diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders - fourth revision classification. All subjects gave written informed consent. This study was approved by the Hospital Geral de Santo António Ethics Committee.

Family 1

The proband (II-2) was a 40-year-old male, who came to our attention in 2001 when experiencing aphasia and right-sided hemiparesis, very intense headache, nausea and confusion (Fig. 1a). He mentioned having frequent bifrontal headaches, only 3–4 years preceding this attack. Thereafter, he developed monthly episodes of migraine without aura (MO). He was given 10 mg/day flunarizine until complete remission of all migraine attacks. In 2003, the patient suffered from one episode of severe vertigo, ataxia and a very discrete right motor paresis, accompanied by headache. Vertigo disappeared soon after the

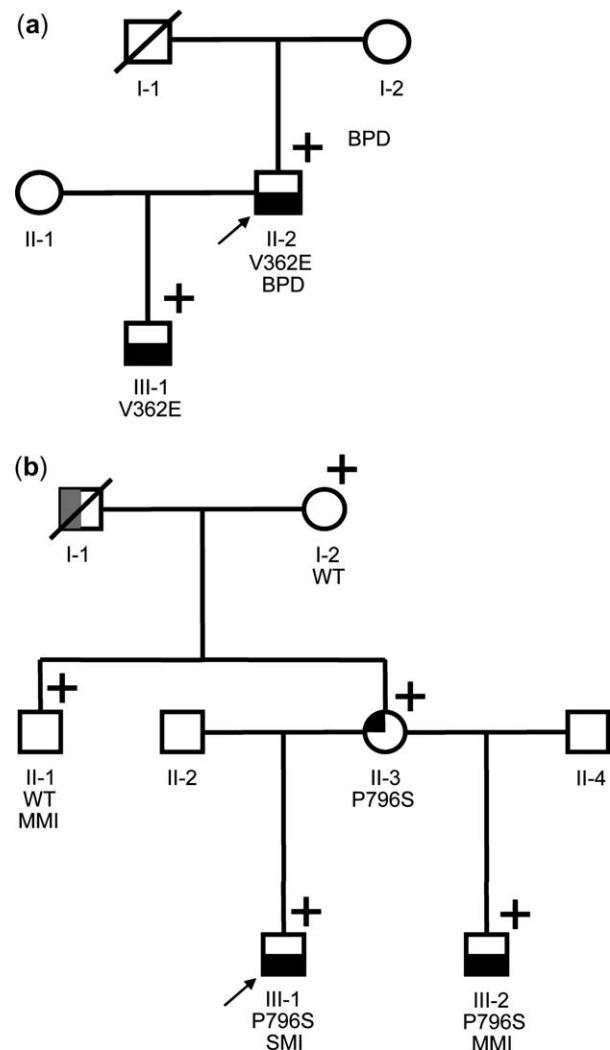


Fig. 1. Pedigrees of the two Portuguese familial hemiplegic migraine (FHM) families: (a) family 1 and (b) 2. FHM, Black-filled lower half; migraine without aura, left upper quadrant; epilepsy, gray-filled left half; arrows, probands; BPD, borderline personality disturbance; SMI, severe mental impairment; MMI, mild mental impairment; plus signs, individuals clinically examined; V362E or P796S, heterozygous mutation carriers; WT, wild-type individuals.

reintroduction of flunarizine. Imaging Angio-Magnetic Resonance Image (angio-MRI), Electroencephalogram and anti-thrombotic studies did not reveal interictal abnormalities (data not shown). In 2006, while still taking flunarizine, he suffered sporadic attacks of migraine, typically related with sleep deprivation and overworking. At that time, he was diagnosed as having borderline personality disturbances (BPD) and admitted expressing sometimes aggressive behavior. His 20-year-old son (III-1) reported two episodes (in 2000 and 2001) of migraine with left hemiparesis; no follow-up was possible since then. The proband's mother (I-2) was also diagnosed with BPD and had been under psychiatric care. Other family members were not available for clinical or genetic investigation.

Family 2

The proband (III-1) was a 15-year-old boy, who was admitted for the first time to our hospital at age 7, presenting with headache and vomiting, impaired consciousness and hemiparesis, in a febrile context (Fig. 1b). At age 9, he was again admitted with a similar attack. This time, severe learning difficulties were observed. In 2002, several headache attacks, 1 week apart, were reported. In at least three of the attacks, hemiparesis was present, and one of the attacks was precipitated by head trauma. Consequently, a diagnosis of hemiplegic migraine was made, and treatment with flunarizine 10 mg/day was started. His younger half-brother (III-2) came to the hospital when he was 4 years old, with a severe attack of right-sided hemiparesis and with a lowered level of consciousness and speech disturbances, also suggesting hemiplegic migraine. He had started this episode 4 days before, with fixed look and hypotonic limbs and then somnolence, agitation and confusion. Left hemiparesis with febrile status (39.6°C) began the second day after admittance; only MRI was abnormal, revealing possible cortical edema in the right hemisphere and slight atrophy in the left one (data not shown). The same results were obtained a week later. Treatment with flunarizine 10 mg/day was started. When he started going to school, learning disabilities were noticed, and Ritalin 20 mg/day was added to his treatment regime. In 2005, cognitive evaluation showed mild mental retardation with an Intelligence Quotient of 53 using Wechsler Intelligence Scale for Children and/or Griffith's scales). No additional headaches were reported. His mother (II-3) mentioned that he had insomnia and a slightly dystonic posturing when 'eating with his left (dominant) hand'. After having had a head trauma, his mother had

episodes of loss of consciousness with or without headache, about two to three times a week. She was diagnosed with MO. Her brother (II-1) had (mild) learning disabilities. Proband's grandfather (I-1) could not be clinically investigated by us, but had been diagnosed with epilepsy at the age of 5. He also suffered from frequent headaches. Subject I-2 was normal. Subjects II-2 and II-4 were not available for clinical or genetic follow-up.

Genetic analysis

Peripheral blood was collected, and genomic DNA was isolated using a standard salting-out extraction method (17). PCR was performed for all 23 exons and flanking intronic regions of the *ATP1A2* gene using genomic DNA of the probands as template. After amplification, direct sequencing was performed by Cycle Sequencing (Prism Big Dye Terminators Cycle Sequencing Kit; Applied Biosystems, Foster City, CA) using the dideoxy termination method and an ABI3700 automated sequencer (Applied Biosystems) as described (5).

Detection of the P796S mutation (c.2490C>T; exon 17; NM_000702) was performed by amplification of exon 17 using primers 'exon17F' (5'-CTGATGCCCTCAGAATCTCC-3') and 'exon17R' (5'-CTGTGGGGGTTACAGGAGAC-3') that resulted in a 266-bp fragment. PCR products were digested with restriction enzyme *FokI* and electrophoresed on a 10% polyacrylamide gel. The wild-type allele resulted in several bands of 168, 42, 29 and 27 bp, whereas the presence of the P796S mutation resulted in the loss of a *FokI* site at position 210, yielding an extra band of 69 bp (combining the products of 42 and 27 bp). For the detection of the V362E mutation (c.1189T>A; exon 9; NM_000702), exons 9 and 10 were amplified by PCR using primers 'exon9-10F' (5'-GCCACGGTCTAGGGTAAGGT-3') and 'exon9-10R' (5'-GCAAGAGGCTTTGGAGACAC-3'), resulting in a 571-bp product that was evaluated by direct sequencing as described above. For both mutations, a panel of 100 non-migrainous Portuguese controls were screened.

Functional analysis

cDNA constructs

Human Na⁺,K⁺-ATPase α 2-subunit cDNA was subcloned into a modified pCDNA3.1 vector (originally from Invitrogen, Carlsbad, CA), which also contained the 5'- and 3'-untranslated regions of the *Xenopus* β -globin gene flanking the

multiple cloning sites (13). To distinguish endogenous Na^+, K^+ -ATPase activity from that of transfected Na^+, K^+ -ATPase, two additional mutations were introduced in the original $\alpha 2$ -subunit cDNA to express an ouabain-resistant isoform ($\alpha 2$ -WT) (18). Next, FHM2 mutations V362E and P796S were introduced into the ouabain-resistant wild-type $\alpha 2$ -subunit construct by site-directed mutagenesis (Quikchange; Stratagene, La Jolla, CA). Thus, $\alpha 2$ -V362E and $\alpha 2$ -P796S mutants were obtained. All constructs were sequence verified.

Transfection and ouabain treatment

HeLa cells (5×10^5) were transfected with 1.6 μg plasmid DNA of $\alpha 2$ -WT, $\alpha 2$ -V362E or $\alpha 2$ -P796S using Lipofectamine 2000 Transfection Reagent in Opti-Mem medium (Invitrogen) and cultured in DMEM-containing Glutamax and 10% FCS (Invitrogen). Two days after transfection, one-third of the cells (1.7×10^5) were seeded on 10-cm petri dishes and subsequently, 1 μM ouabain was added to the culture medium. After 5 days of ouabain challenge, colonies were stained with 1% methylene blue in 70% methanol, scanned and analyzed with IMAGE PRO PLUS (MediaCybernetics, Silver Spring, MD). Each transfection was performed nine times.

Electrophoresis and Western blot analysis

Two days after transfection, two-thirds of the cells (3.3×10^5) were harvested for Western blot analysis. In brief, proteins were resuspended in a mix of protease inhibitor (Complete Mini; Roche, Basel, CH) and DNaseI. Subsequently, Laemmli-loading buffer and 0.1 M DTT were added and the samples were heated for 10 min at 65°C. Next, equal amounts of protein as measured by Bio-Rad protein assay (Bio-Rad Laboratories, Munich, Germany) were separated on 7.5% SDS-polyacrylamide gels for 40 min at 200 V. Proteins were electroblotted to nitrocellulose membranes (Hybond; Amersham, Buckinghamshire, UK) and incubated overnight with the $\alpha 2$ -subunit-specific polyclonal antibody HERED (19). The primary antibody was detected with goat-anti-rabbit horseradish peroxidase-conjugated secondary antibody (Sigma, St Louis, MO). Protein bands were visualized with Super Signal Substrate (Pierce Biotechnology, Rockford, IL).

Results

Genetic analysis

Two novel missense mutations were identified in the FHM2 *ATP1A2* gene in two probands of Portuguese FHM families. Mutation V362E in exon 9 (c.1189T>A) causing valine to glutamate

substitution was identified in the proband (II-2) of family 1 as well as in his son III-1 (Fig. 1a). Mutation P796S in exon 17 (c.2490C>T) resulting in a proline to serine substitution was identified in proband (III-1) of family 2 and relatives II-3 and III-2, but was absent in I-2 and II-1 (Fig. 1b). Both mutations were absent in 100 Portuguese controls. Mutation V362E is located in the large cytoplasmic loop between M4 and M5 ahead of the phosphorylation site, whereas P796S mutation is located in the highly conserved 'PLPL' putative turn region between M5 and M6 transmembrane domains (Fig. 2a). Taxonomy analysis showed a strong evolutionary conservation of both Val³⁶² and Pro⁷⁹⁶ amino acids among different α -subunits of P2-type ATPases (Fig. 2b).

Functional data

In survival assays, the endogenous Na^+, K^+ -ATPase activity is abolished by ouabain challenge. Ouabain-insensitive wild-type and mutant Na^+, K^+ -ATPase α -subunits were transfected into HeLa cells. Transfected wild-type Na^+, K^+ -ATPase is able to compensate for the loss of endogenous enzyme activity; the cells survive the ouabain treatment. The actual readout of the assay is to see whether or not transfected mutant protein is able to rescue cells from loss of endogenous Na^+, K^+ -ATPase activity as well. Western blot analysis showed that our constructs were expressed at comparable levels (Fig. 3a). Although wild-type construct was able to rescue cell death, hardly any survival was observed for the V362E and P796S mutants (Fig. 3b). This clearly indicates that both mutants have functional consequences on sodium-potassium pump functioning and can be considered pathogenic.

Discussion

Here, we describe two novel *ATP1A2* missense mutations in two Portuguese probands with FHM. Of particular interest was the fact that one proband (family 1) had mood alterations and was diagnosed as having BPD and aggressive behavior. The other proband from a family with FHM (family 2) had also severe mental impairment.

Genetic analysis revealed *ATP1A2* mutations V362E and P796S in the probands of families 1 and 2, respectively. Both mutations were identified in one relative with hemiplegic migraine. The son of the proband from family 1 had hemiplegic migraine without the psychiatric phenotype seen

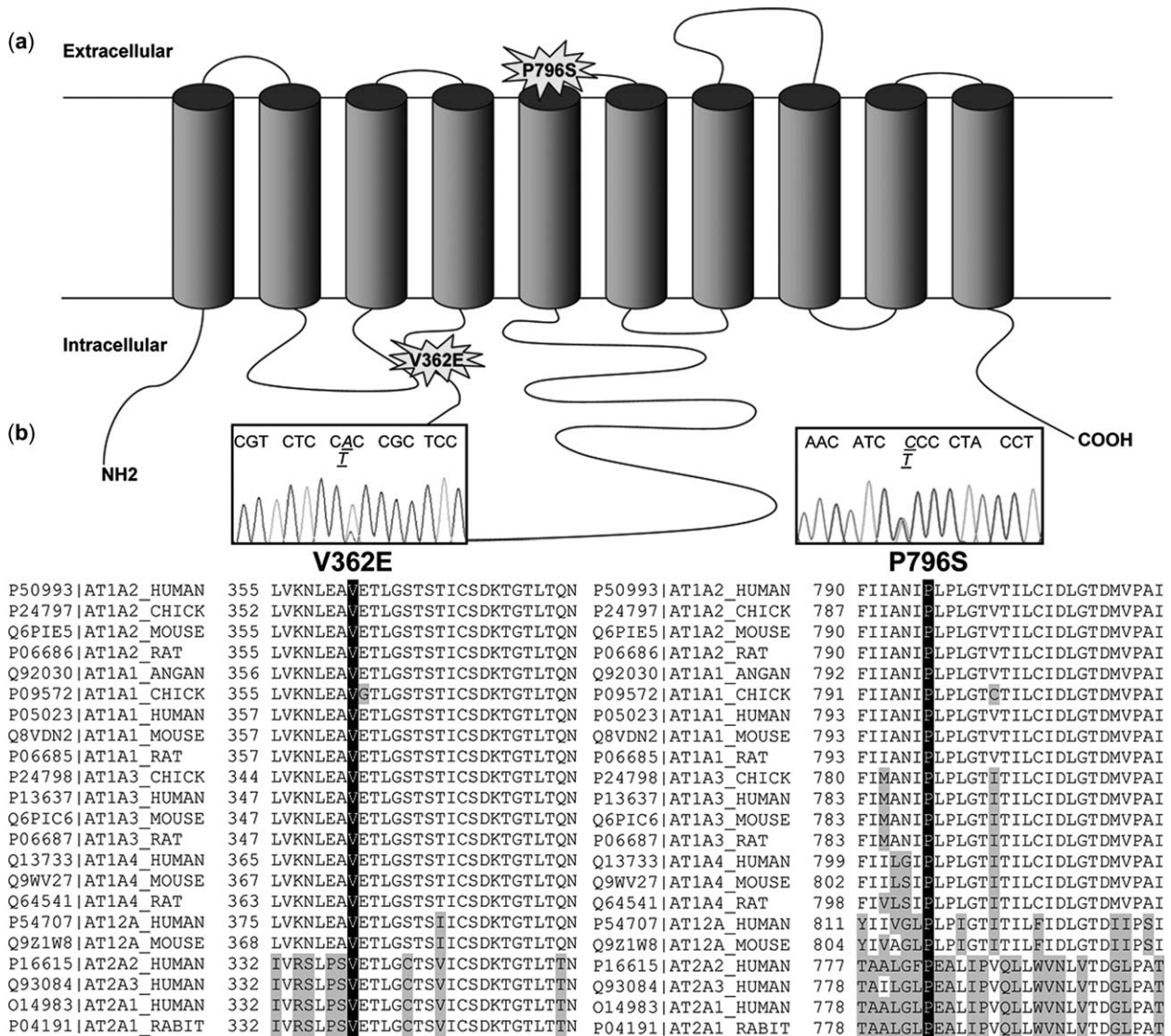


Fig. 2. Topography of the Na^+,K^+ -ATPase α_2 -subunit and location and conservation of *ATP1A2* mutants. **(a)** Schematic drawing of the *ATP1A2* gene product with V362E and P796S locations. **(b)** Electropherograms showing heterozygous mutations and alignments of amino acid sequence of several vertebrate sodium-potassium ATPase α -subunits, with Val³⁶² and Pro⁷⁹⁶ residues represented in black boxes. V362E or P796S, heterozygous mutation carriers.

in the proband. In family 2, the brother of the proband, in addition to hemiplegic migraine, had mild mental retardation. Clinical heterogeneity is further exemplified by the fact that P796S mutation carrier II-3 did not have hemiplegic migraine (thus showing reduced penetrance) but did have MO and episodes of loss of consciousness.

We present good evidence that both novel V362E and P796S mutations are disease causing as neither could be found in a large set of Portuguese controls without history of headache and functional studies, using cell-survival assays, showed that both mutants were virtually unable to rescue cell death after blocking of endogenous Na^+,K^+ -ATPase activity. Moreover, both muta-

tions are highly conserved and affect amino acids in important functional domains of the protein.

Mutation V362E adds a negative charge to a region of the protein that requires structural integrity to ensure for functionality of the phosphorylation domain (20). Mutations in this region of Ca^{2+} -ATPase often affect Ca^{2+} transport (21). Of note, changing Val³³⁹ to an alanine, in the homologous sarcoplasmic reticulum Ca^{2+} -ATPase, resulted in profound impairment of both transport and catalytic functions of pump (20). Mutation P796S affects the same amino acid that was found mutated to an arginine in a patient with pure FHM (11). Both mutants Ser⁷⁹⁶ and Arg⁷⁹⁶ may contribute to destabilizing the putative PLPL

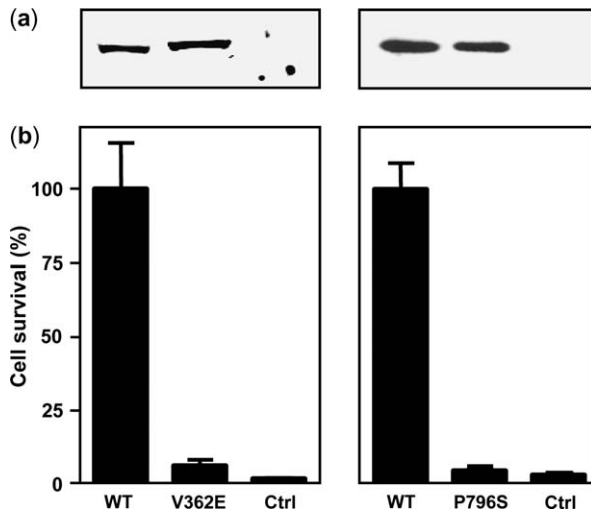


Fig. 3. Ouabain-survival assay in transfected HeLa cells. (a) Western blot analysis of HeLa cells transfected with wild-type or mutant *ATP1A2* cDNA. Detection of Na^+/K^+ -ATPase $\alpha 2$ -subunit protein was performed using a specific polyclonal antibody (anti-HERED). (b) Ouabain sensitivity as determined by cell survival of cells transfected with either wild-type or mutant *ATP1A2* cDNA constructs. Bars represent cell survival after 5 days of ouabain treatment ($n = 9$). Error bars show standard error of the mean. Ctrl, control; V362E or P796S, heterozygous mutation carriers; WT, wild-type.

turn, which is located within the M5–M6 region of the protein. Apparently, it matters what amino acid replaces the original proline as the phenotypes of P796S and P796R are rather different. Hence, identifying several mutations affecting the same amino acid proved of particular interest to investigate genotype–phenotype relationships. Unfortunately, for the original paper describing P796R the functional consequences were not investigated.

As International Headache Society criteria require that two patients with hemiplegic migraine in a core family are enough to make the diagnosis of FHM, both families qualify as such (1). In the literature, there are multiple examples of equally small FHM2 families exhibiting reduced penetrance; however, both our families are too small and segregation does not provide convincing evidence that these mutations also cause the psychiatric–mental problems. Nevertheless, mild or more severe mental retardation associated with FHM2 *ATP1A2* mutations has been described before (4, 11, 12).

Interestingly, several psychiatric conditions, such as depression, anxiety and mood disorders, have been associated with migraine and chronic headache (22). It was hypothesized that these may share a common etiological factor (e.g. genetic), increasing susceptibility to both diseases sepa-

rately (22). However, a bidirectionally increased risk was reported for migraine and depression (23). BPD, the associated clinical feature in the proband of family 1, are frequently characterized as angry, impulsive, unpredictable individuals, with features that overlap those of bipolar disorder (24). Although the prevalence of migraine and severe headache may be higher in patients with BPD, than in general population (22, 24), the exact relation between them is still far from being understood.

In conclusion, we found two novel *ATP1A2* mutations in two Portuguese probands with hemiplegic migraine and associated psychiatric–mental problems. Although there is sufficient evidence to state that the mutations have caused hemiplegic migraine, it can only be suggested that the additional clinical features were also due to dysfunction of sodium–potassium pumps.

Acknowledgements

We are particularly grateful to both Portuguese families for their participation and contribution for this study. The European Community provided financial support for this work through a Marie-Curie grant for PhD students (M.-J. C. and R. R. F.; contract QLGA-CT-2000-60005) and the ‘EUROHEAD’ grant (LSHM-CT-2004-504837; R. R. F. and A. M. J. M. van den M.).

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4.2.2 Study the involvement of *SCN1A* in a family with FHM and epilepsy

Epilepsy has been reported in several FHM1 mutation carriers. In most cases, seizures occur only during severe hemiplegic migraine attacks. Seizures can also be present in FHM2 mutation carriers. Rather surprisingly, epilepsy is only rarely reported in the limited number of patients with FHM3 *SCN1A* mutations, despite this gene being a well-known epilepsy gene. Based on these observations, the hypothesis was postulated of a possible clinical continuum between migraine and epileptic syndromes, caused by altered neuronal excitability with a similar underlying genetic basis.

In our genetic studies in FHM families, we investigated both the *ATP1A2* (**Article 4** and **5**) and the *SCN1A* gene (**Article 6**). In one Portuguese family, haplotype analysis was fully compatible with both the FHM2 (1q23) and FHM3 (2q24) locus for those patients with DNA available at the time of the investigation. Sequence analysis of the *ATP1A2* gene was tested first and did not reveal a causative mutation; in the *SCN1A* gene, a novel heterozygous missense mutation was identified, changing a single nucleotide in exon 6 (nt 787 C>G). This C to G transversion causes the replacement of a Leucine for a Valine, at position 263 of the Na_v1.1 α 1 subunit. Leu²⁶³ shows strong evolutionary conservation among several orthologous and homologous voltage-gated sodium channels, while its location in the transmembrane domain DIS5 of the protein suggests also an important functional role.

This Portuguese family with a L263V mutation is the first one with co-occurring hemiplegic migraine and epilepsy. Seizure attacks, either tonic-clonic or partial and complex, were described in the majority of mutation carriers and as occurring independently, at all times, from the hemiplegic migraine attacks. Carbamazepine, which is recommended when migraine and epilepsy co-occur, was the best therapeutic option for patients in this family, but it was not always effective. It is not clear how this mutation can cause epilepsy, in certain episodes, but hemiplegic migraine in others. A detailed functional analysis of the functional consequences of the L263V mutation is necessary; such studies are now underway.

In summary, these results extend the *SCN1A* phenotype and further strengthen the molecular evidence that FHM and epilepsy may share, at least partly, the same genetic pathways.

**FIRST MUTATION IN THE VOLTAGE-GATED $Na_v1.1$ SUBUNIT
GENE *SCN1A* WITH CO-OCCURRING
FAMILIAL HEMIPLEGIC MIGRAINE AND EPILEPSY**

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Running Title: novel *SCN1A* mutation in FHM and epilepsy

Keywords: comorbidity, FHM3, migraine, seizures, voltage-gated sodium channel

Submitted

Abstract

Objective: Almost all mutations in the *SCN1A* gene, that encodes the $\alpha 1$ subunit of neuronal voltage-gated $\text{Na}_v1.1$ sodium channels, are associated with severe childhood epilepsy. Recently two mutations were identified in patients with pure familial hemiplegic migraine (FHM). Here we test the involvement of the *SCN1A* gene in a family with partly co-segregating hemiplegic migraine and epilepsy.

Methods: DNA from a large Portuguese FHM family was tested for segregation of genetic markers of FHM *loci*. Mutation analysis was performed for the *ATP1A2* (FHM2) and *SCN1A* (FHM3) genes by direct sequencing.

Results: A novel L263V mutation was identified in the *SCN1A* gene that segregated with FHM in five patients. Three of the L263V mutation carriers in addition to FHM also have epilepsy attacks independent of the hemiplegic attacks.

Interpretation: We report the first *SCN1A* mutation in a Portuguese family with co-occurring FHM and epilepsy in several mutation carriers. Our results underscore the complex relation of migraine and epilepsy and provide further molecular evidence that both diseases share common pathways.

Introduction

Both migraine and epilepsy are complex episodic neurological diseases with genetic and environmental factors playing a role in their pathogenesis^{365,381}. Epidemiological studies have indicated that there is a clear bidirectional increased risk of migraine and epilepsy³⁸². This suggests that migraine and epilepsy, at least in part, may share pathophysiological mechanisms⁶⁰.

Most genes in rare monogenetic forms of epilepsy encode subunits of channels transporting sodium, potassium or chloride ions (for a recent review see³⁸¹). Gene identification in migraine has only been successful for familial hemiplegic migraine (FHM), a rare, monogenic, autosomal dominant subtype of migraine with aura with unilateral motor weakness during the aura phase¹. All three FHM genes identified thus far are involved in ion transport¹⁴⁴. The FHM genes encode subunits of $\text{Ca}_v2.1$ calcium channels (*CACNA1A* (FHM1)¹⁵⁰), sodium-potassium ATPases (*ATP1A2* (FHM2)¹⁸²), and $\text{Na}_v1.1$ sodium channels (*SCN1A* (FHM3)²⁰⁸).

Despite their co-morbidity, co-occurring migraine in epileptic mutation carriers has not been mentioned; likely because migraine in a patient or family with epilepsy will often be regarded coincidental because of the high prevalence of migraine in the general population. Epilepsy, however, has specifically been reported in many FHM mutation carriers³⁸³. For FHM1 mutation carriers, seizures may occur during severe hemiplegic migraine attacks¹⁰, but have also been described with a particular mutation (i.e. I1710T) in one family *independently* of hemiplegic attacks¹⁵⁸ and causing *status epilepticus* during FHM attacks in another family¹⁵⁹. Several carriers of FHM1 mutation S218L, which is associated with severe FHM attacks triggered by trivial head trauma, had generalized epileptic seizures^{18,160}. In FHM2 mutations carriers various types of childhood and adult epilepsy have been reported with different FHM2 mutations^{182,183,193,194}. In rare cases, epilepsy is part of particularly severe phenotypes with alternating hemiplegia, coma and permanent cerebellar signs or mental retardation^{184,185,187}.

A recent molecular link between migraine and epilepsy came from the identification of the first two FHM mutations in *SCN1A*; a well-known epilepsy gene^{208,220}. Over 150 mutations in the *SCN1A* gene have been reported with generalized epilepsy with febrile seizure plus additional symptoms (GEFS+) and severe myoclonic epilepsy of infancy (SMEI)^{217,384}. Both are severe epileptic phenotypes occurring in childhood. The majority of SMEI mutations occur *de novo*, and are either nonsense or frameshift mutations resulting in protein truncation and consequent loss-of-function or missense mutations with a wide range of functional consequences³⁸⁵. The milder GEFS+ phenotype is mostly caused by missense mutations, showing either loss- or gain-of-function effects³⁸⁶. Up to now, no migraine was reported for any of the epilepsy mutations.

It is puzzling why most *SCN1A* mutations cause childhood epilepsy whereas only two cause hemiplegic migraine. Perhaps a clinical continuum exists in *SCN1A* mutations carriers between migraine and epileptic syndromes resulting from altered neuronal excitability³⁸⁷.

Here we describe the first *SCN1A* mutation with several mutation carriers with partly co-segregating FHM and epilepsy, which provides additional support for the clinical continuum hypothesis. Not only, do the results extend the clinical spectrum associated with mutations in *SCN1A*, but they further strengthen the molecular evidence that FHM and epilepsy share, at least in part, similar molecular pathways.

Material and Methods

Clinical description

We investigated a Portuguese family with partially co-segregating familial hemiplegic migraine and epilepsy (**Fig. 1A**). All subjects were interviewed and the clinical headache diagnoses were established according to International Headache Society criteria¹ whereas seizures were classified according to the criteria of the International League Against Epilepsy³⁸⁸. Diagnoses were made prior to genetic analyses by a neurologist experienced in migraine and epilepsy. This study was approved by the ethical committee of the Hospital Geral de Santo António (HGSA), in Porto. All individuals gave written informed consent.

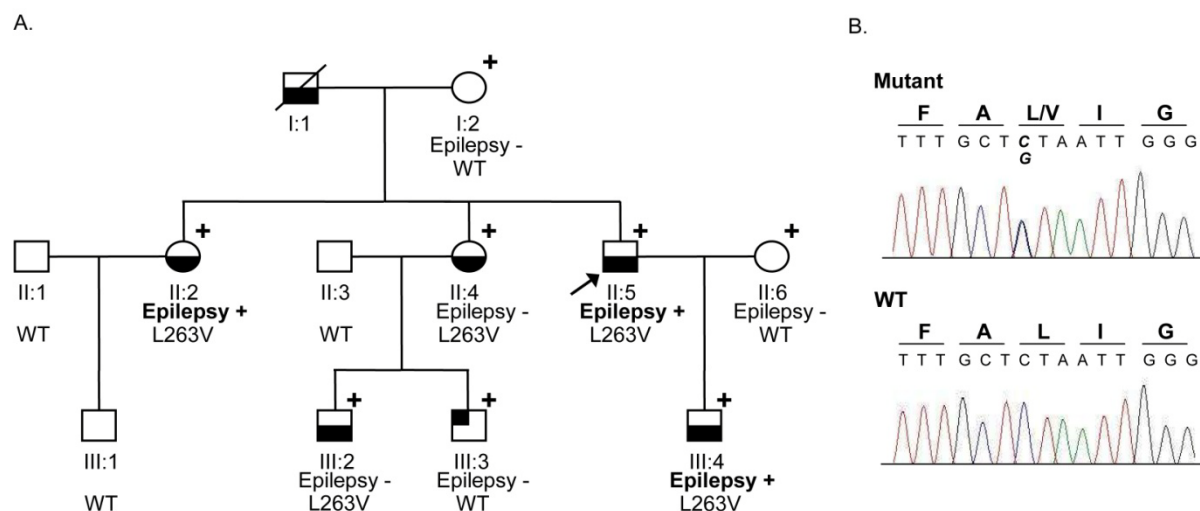


Figure 1. P Pedigree of the Portuguese family with FHM and epilepsy.

A: Symbols filled in lower half represent the individuals with FHM and in upper-left quarter represent individuals with MO; the arrow indicates the proband. The "+" shows individuals that were clinically evaluated; "Epilepsy+" indicates with epileptic seizures, whereas "Epilepsy-" shows individuals without epileptic seizures. The presence of mutation is indicated by L263V and the absence by WT (wild-type).

B: Electropherogram of the relevant part of exon 6 showing the heterozygous C>G nucleotide change (L263V) in the proband (Mutant) and healthy subject (WT) are shown.

Clinical details of affected family members are shown in **Table 1**. Six patients suffered from typical hemiplegic migraine attacks, with an age of onset varying from 10 to 18 years and an attack frequency between of 1-3 times per year. None of the patients reported (inter)ictal cerebellar abnormalities. Generalized tonic-clonic seizures or complex partial seizures were co-segregating with hemiplegic migraine in three family members (i.e. II:2, II:5, and III:4), and likely in one deceased person (I:1) and were reported by his wife (I:2).

Interictal EEGs only of patients II:2 and III:4 were available and did not show abnormalities. Febrile convulsions were not reported for any of the family members. Epileptic seizures occurred independently from FHM attacks and the age at onset was generally later than for the FHM attacks. Treatment with carbamazepine (400 mg/day) was successfully with respect to both epileptic and hemiplegic attacks for all four patients that took medication, as patients remained attack-free. Psychomotor development of all individuals was normal.

Haplotype analysis

Genomic DNA was isolated from peripheral leukocytes using a standard salting-out extraction method³⁴⁶. The involvement of the FHM1 (19p13) locus was excluded on the basis of genetic markers D19S221, D19S1150, D19S226 as described before¹⁵⁰. In addition, involvement of the FHM2 and FHM3 *loci* was investigated by analyzing genetic markers D1S2624, D1S2707, D1S2844 (FHM2; 1q23)¹⁸³ and D2S156, D2S2330 and D2S2381 (FHM3; 2q24)²⁰⁸, respectively (data not shown). Oligonucleotide primer sequences were obtained from the Human Genome Database (GDB) (<http://www.gdb.org/>). PCR amplification was performed using standard conditions. PCR products were detected on an automated fragment run analyzer (ABI 3700 DNA sequencer; Applied Biosystems, Foster City, CA). All genotypes were analyzed and independently scored by MJC and KRJV using Genescan and Genotyper 2.1 software (Applied Biosystems, Foster City, CA). Haplotype was constructed evaluating the segregation and assuming a minimal number of recombinations.

Mutation screening

All exons and flanking intronic regions of the *ATP1A2* and *SCN1A* genes were amplified by PCR, using genomic DNA of the proband as a template. Direct sequencing was performed on PCR products using Cycle Sequencing (Prism Big Dye Terminators Cycle Sequencing kit, Applied Biosystems, Foster City, CA) and an ABI3700 DNA sequencer (Applied Biosystems, Foster City, CA). Sequencing was performed in the forward and reverse direction. For detection of the *SCN1A* mutation L263V (C>G, nt position 787, Acc nr NM_006920), exon 6 was amplified by PCR using primers “E6F” (5'-TTGCTTCTCCACTAGCGTTG-3') and “E6R” (5'-ACTTGAGGGGCTGGATATCC-3'), resulting in a 489 bps product that was subjected to direct sequencing. Some 150 healthy controls (i.e. Portuguese blood donors without migraine history) were screened for the *SCN1A* mutation by direct sequencing.

	II:2	II:4	II:5	III:2	III:4
Hemiplegia attacks:					
Age/Onset (<i>in years</i>)	59/14	57/16	52/18	35/12	12/10
Auras*	+/+/-	+/+/+	+/+/+	-/+/+	+/+/+
Duration	15 min-1 h	1 h	1 h	1 h	15 min-1 h
Frequency (<i>per year</i>)	< 1	2-3	1	1-2	> 1
Impaired consciousness	Frequent; Once with convulsions	Once without convulsions	Once with convulsions	-	Frequent; Twice with convulsions
Headache:	Bilateral	Bilateral	Bilateral	Unilateral	Unilateral
Pain character	Throbbing	Throbbing	Non- throbbing	Throbbing	Non- throbbing
Severity	Severe	Severe	Mild	Moderate	Moderate
Nausea/Vomiting	+/+	+/+	-/-	+/-	+/+
Photophobia/Phonophobia	+/+	+/+	+/+	-/-	+/+
Seizures/epilepsy:					
Type of epilepsy	CP and GTC	-	GTC	-	GTC

*Characteristics of auras: visual/sensitive/hemiplegic/aphasic; "-": absent; "+": present; GTC, generalized tonic-clonic seizure; CP, complex partial seizure.

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

Results

Haplotype analysis in the Portuguese family was compatible with both the FHM2 (1q24) and FHM3 (2q24) locus as single haplotypes co-segregated in all five FHM patients for which DNA was available (data not shown). Sequence analysis of the *ATP1A2* gene did not reveal a causative mutation. In contrast, in the *SCN1A* gene a novel heterozygous missense mutation was identified changing a single nucleotide change in exon 6 (nt 787 C>G) (**Fig. 1B**). This variant was not present in the healthy control samples when compared with a positive control, but additional polymorphisms were found (data not submitted; **Supplementary Figure** and **Table** at the end of the Article). The C to G nucleotide transversion replaces a Leucine for a Valine at position 263 of the Na_v1.1 α 1 subunit (**Fig. 2A**). The L263V mutation co-segregated in all five FHM individuals. Notably, three of the five FHM patients with the L263V mutation also had epileptic seizures. Patient I:1 most probably is the fourth patient from this family suffering from hemiplegic migraine and epilepsy, but no DNA is available to confirm the presence of the L263V mutation. No other associated neurological symptoms such as ataxia were reported for any of the family members. Taxonomy analysis showed a strong evolutionary conservation of Leu²⁶³ amino acid among different voltage-gated sodium channels α subunits (**Fig. 2B**).

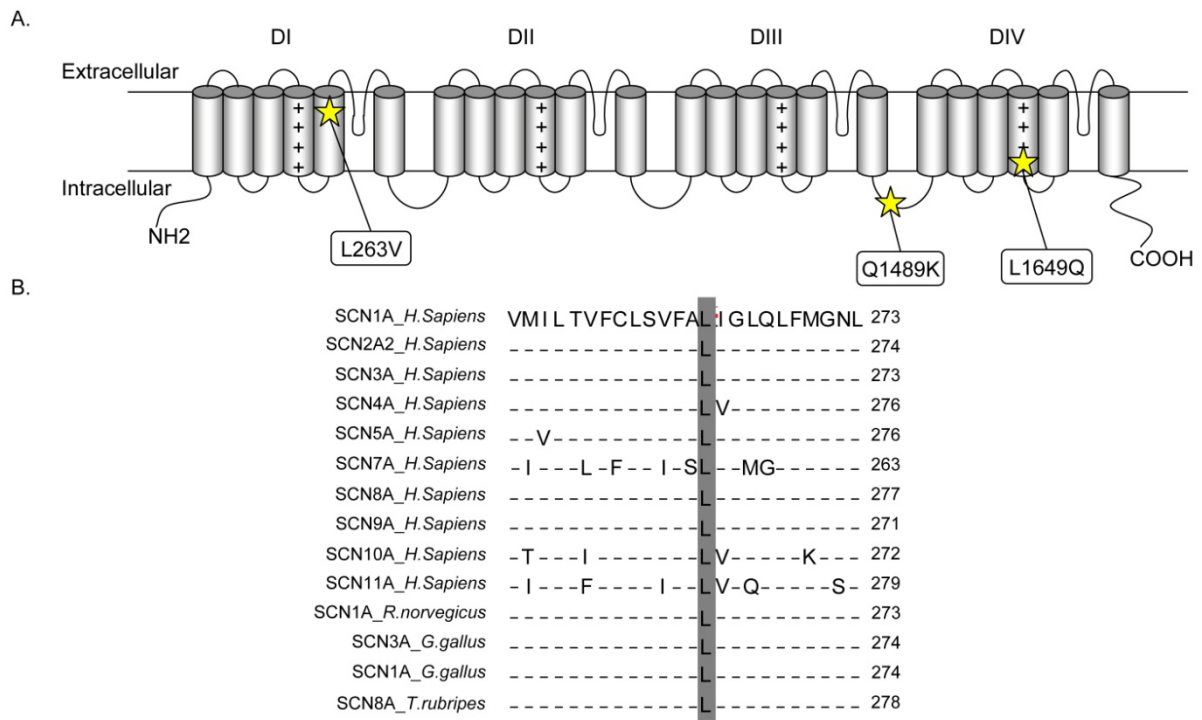


Figure 2. Location of FHM3 *SCN1A* mutations.

A: Schematic representation in the $\text{Na}_v1.1$ $\alpha 1$ subunit with the location of the FHM3 mutations.

B: Evolutionary conservation of the relevant part of the S5 transmembrane segment. Amino acid numbering for each sequence is presented. The gray box represents conservation of residue Leu²⁶³. Accession numbers are as follows: *Homo sapiens*: SCN1A (P35498), SCN2A2 (Q99250), SCN3A (Q9NY46), SCN4A (P35499), SCN5A (Q14524), SCN7A (Q01118), SCN8A (Q9UQD0), SCN9A (Q15858), SCN10A (Q9Y5Y9), SCN11A (Q9UI33); *Rattus norvegicus* SCN1A (P04774); *Gallus gallus*: SCN1A (ENSGALP00000017793) and SCN3A (ENSGALP00000017960); and *Takifugu rubripes* SCN8A (NEWSINFRUP00000180009). All sequences were retrieved from NCBI or Ensembl databases.

Discussion

Here we report a novel L263V missense mutation in the *SCN1A* gene encoding the $\alpha 1$ -subunit of voltage-gated sodium $\text{Na}_v1.1$ channels in a Portuguese family with FHM and partially co-segregating epilepsy. Several reasons indicate that L263V is the causative mutation in this family with FHM and epilepsy: i) L263V co-segregates in all five patients with hemiplegic migraine, three of them also have epilepsy (a fourth deceased person likely has epilepsy and the mutation); no phenocopies or cases of incomplete penetrance were observed, ii) L263V is absent in 300 control chromosomes, iii) Leu²⁶³ shows strong evolutionary conservation among several voltage-gated sodium channel subunit homologues across species, and iv) its location in transmembrane domain DIS5 of the protein suggests an important functional role.

Alpha1-subunits of $\text{Na}_v1.1$ sodium channels consist of four homologous domains (DI-DIV), each containing six highly conserved transmembrane segments (S1-S6), which form the central pore of the sodium channel¹⁷⁰. Mutation L263V is located in the highly conserved S5 segment of the first domain (DIS5).

S5 and S6 segments and S5-S6 linkers line the permeation pathway and are important for ion selectivity and gating kinetics^{221,222}. No particular functional role has been established for DIS5, but closely located mutation I252N was found associated with SMEI²¹⁶. Various other missense *SCN1A* mutations have been identified in S5 segments and are associated with different epileptic phenotypes of GEFS+^{211,212}, SMEI^{213,389} or SMEB^{213,230,390}.

The L263V mutation is the third FHM3 mutation. The first two FHM3 mutations, Q1489K and L1649Q, were identified in large German and US families with FHM without additional neurological features such as epilepsy or ataxia^{208,220}. Only in three of the eighteen Q1489K mutation carriers with hemiplegic migraine, seizures were reported that occurred *only* during infancy. The Portuguese family with the L263V mutation however is the first family with clearly co-occurring hemiplegic migraine and generalized tonic-clonic epilepsy. It is not clear how this mutation can cause epilepsy in certain attacks and hemiplegic migraine in other attacks. A detailed functional analysis of the functional consequences of the L263V mutation is necessary; such studies are now underway.

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

Acknowledgements

We thank our Portuguese FHM family for their dedicated participation in this study. Main financial support for this work was provided by the European Community, through a Marie-Curie grant for PhD students (M.-J. Castro; Contract n° QLGA-CT-2000-60005). Additional support was obtained from grants of the Netherlands Organization for Scientific Research (903-52-291, M.D.F, R.R.F., and Vici 918.56.602, M.D.F), The Migraine Trust, (R.R.F., M.D.F), the EU "EUROHEAD" grant (LSHM-CT-2004-504837; M.D.F, R.R.F, A.M.J.M.v.d.M), and the Center of Medical System Biology (CMSB) established by the Netherlands Genomics Initiative/Netherlands Organisation for Scientific Research (NGI/NWO).

Supplementary Figure

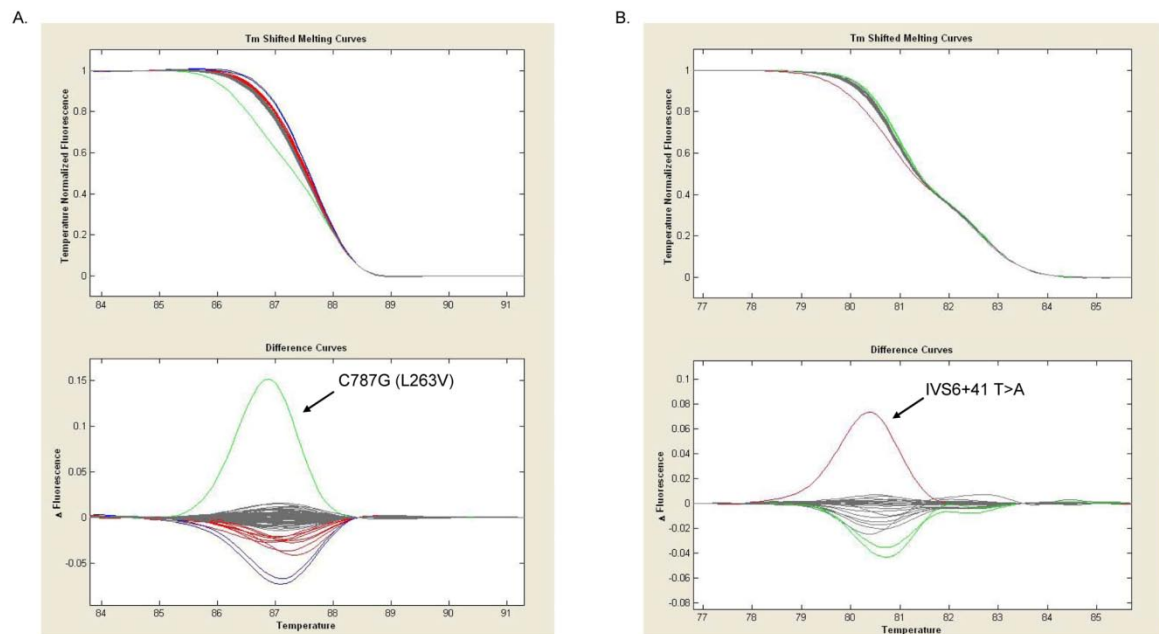


Figure - Melting analysis of *SCN1A* exon 6 PCR fragment from samples of 175 controls and 24 additional FHM patients. Results are represented by melting plots (top) and subtractive difference plots (bottom) with subtractive differences obtained by mathematically subtracting each curve from that of a normal control. From 12 different patterns sequenced two were not wild-type, corresponding to heterozygous changes: C>G (L263V; positive control sample; **A**) and T>A (IVS6+41; control sample; **B**).

Supplementary Table

Location	Polymorphism	Genotype	SNP ID	Panel	Allele frequencies /per population (in database)			
		of II:5	(in database)	(in database)	A	G	C	T
Intron 2	IVS2+65T>C	T/C	rs8191987	North American	0	0	0.188	0.812
Intron 5	IVS5-106G>T	T/T	rs3812719	-	-	-	-	-
Intron 5	IVS5-91G>A	G/A	rs3812718	-	-	-	-	-
Intron 6	IVS6+41T>A	^a	^b					
Intron 7	IVS7-21C>T	T/T	rs994399	-	-	-	-	-
Intron 7	IVS7+21T>C	T/C	rs1542484	-	-	-	-	-
Intron 8	IVS8+75C>A	C/A	rs11690962	-	-	-	-	-
Exon 9	A1212G (V404V)	G/G	rs7580482	-	-	-	-	-
Intron 11	IVS11-47T>G	G/G	rs6753355	-	-	-	-	-
Intron 12	IVS12+11A>C	A/A	^b					
Exon 13	T2292C (V764V)	C/C	rs6432860	-	-	-	-	-
Intron 14	IVS14-37A>C	C/C	rs2126152	European	0.358	0	0.642	0
Intron 16	IVS16-41C>T	T/T	rs7601520	North American	0.521	0.479	0	0
Exon 16	G3199A (A1067T)	A/A	rs2298771	European	0.642	0.358	0	0
				North American	0.521	0.479	0	0
3'-UTR	3'UTRA>G	A/G	rs4667859	-	-	-	-	-
3'-UTR	3'UTRT>C	T/C	rs10497275	European	0.875	0.125	0	0
				North American	0.812	0.188	0	0
3'-UTR	3'UTRC>T	C/T	rs1813502	^c				

SNP, single nucleotide polymorphism; "-", not available yet; ^aonly in one control sample; ^bnot in Ensembl database (<http://www.ensembl.org/>); ^conly determined for other populations; North American from PERLEGEN:AFD_EUR_PANEL and European from CSHL-HAPMAP:HapMap-CEU.

Table - Polymorphisms found in *SCN1A* gene screening.

5. GENERAL DISCUSSION

5.1 Genetic approaches to study common migraine

5.1.1 The genetic contribution for migraine

The first major achievement coming out from this thesis was the establishment of a very large DNA collection of Portuguese migraine patients and controls, with detailed familial information and completed questionnaires, to be used for genetic studies. In this clinic-based sample set, the majority of individuals came from the Northern region of Portugal. The overall prevalence of migraine in this region was estimated to be 16.7% (8.8% for migraine, and 7.9% for migraine associated with other types of headaches). This is in agreement with previously reported estimates for overall prevalence of migraine (about 12 to 18%) in various populations²⁵⁻²⁷.

As our sample is based on clinical practice, there is a general underestimation of headache and migraine cases; the main reason for this is that only the more severe cases (i.e., MA or HM) usually require medical care at outpatient headache clinics. In this context, the importance of extensive validation of familial history needs to be emphasized. According to our research - although probands were fairly able to correctly identify their affected relatives - migraine was still underestimated by relatives³⁷⁷ (see **Annex A**). We are confident that our clinic-based sample will be very important for future epidemiological and genetic studies.

Familial aggregation may be studied by calculating *relative risk* (RR) in relatives of migraine probands. The RRs in our sample are in line with existing evidence of clustering of migraine in 1st-degree relatives of MA and MO probands (**Article 1**). These relatives have a (3 to 4-fold) increased risk of migraine, when compared to the general population, whereas spouses do not show any increased risk at all (**Article 1**). Based on our results, we expect that familial aggregation of migraine may be mainly due to genetic factors, as was previously suggested³⁷³, and in line with the magnitude of the RRs found in our study.

5.1.2 MO and MA as part of a migraine spectrum

One of the current theories suggests that MO and MA should be regarded as belonging to a same migraine spectrum, since an increased risk was found for both subtypes. Most

MA patients also have attacks of migraine without aura, but this fact is usually underestimated. It is defensible that our MA patients should be (re)classified according to the numbers of MA and MO attacks. This might improve the accuracy of their MA diagnosis. In that case, patients with both MO and MA might be considered belonging to an intermediate disease class; that is, between MO (with more disabling headache) and MA (with additional neurological symptoms such as aura). This would support the existence of a migraine spectrum.

In this work, we first assessed whether MO and MA have distinct patterns of familial clustering. In our sample, 1st-degree relatives of MA-probands had a higher risk of MA than of MO, which is in agreement with previous reports^{242,344}. Moreover, we found that 1st-degree relatives of MA-probands had a significantly higher risk of MA than all other groups of 1st-degree relatives, reaching a 2 to 4-fold increase when compared to each other (**Article 1**). Our data show that MA seems to have a stronger familial aggregation and, thus, possibly a greater genetic predisposition (**Article 1**), but the environmental contribution needs to be ascertained. Unfortunately, we could not get a genetic confirmation of this hypothesis, since our sample set was too small for such studies.

A common genetic background is supported by the clinical overlap between MA and MO³⁹¹, but also by the linkage peaks found with patients with either MA or MO^{282,283}. Nyholt et al. concluded that MO and MA may share a few genes²⁴². Considering the possible existence of life-long reduced and age-dependent penetrance, an “affected-only” strategy was used for linkage analysis in our family set (**Article 2**). The MO vs. MA dilemma supports the view that the present classification¹ is not particularly suited for genetic research, and leaves room for a new level of diagnosis optimally designed for genetic studies.

5.1.3 Strategies for studying complex diseases: migraine as an example

5.1.3.1 Linkage analysis

Complex diseases are thought to result from a combination of different gene variants with variable penetrance interacting with each other and with environmental factors. Linkage analysis, in theory, is a reasonable approach to find susceptibility genes. Although there are many examples of linkage studies yielding novel chromosomal *loci* for complex diseases, such as migraine^{279,282}, diabetes³⁹² and asthma³⁹³, only very few genes have been identified.

Prospects are slightly better using a candidate gene region approach, as exemplified by, for instance, the identification of the *CLCN2* gene in a susceptibility region of childhood absence epilepsy³⁹⁴. However, also for this approach the majority of studies did not live up to its high expectations, also because the needed replication of initial positive findings is still lacking. Moreover, the *a priori* chances of finding the DNA variant involved in the disease, using the candidate gene strategy, are rather low. We started by studying the FHM regions. In order to optimize the strategy to use for our sample set, we divided the linkage study in two parts: the first was to characterize our sample and the second was to analyze all genotyping data (**Article 2**). The study of six known migraine *loci* in the Portuguese family set showed overall nominal evidence of linkage to marker *loci* D19S226, in 19p13 (FHM1), and D4S2929, in 4q22, both using a parametric linkage approach. No significant or even suggestive evidence of linkage was obtained. These observations indicate that no major gene is located in the regions studied that can explain migraine susceptibility in our sample set. The results obtained per family also support this conclusion.

Both parametric and NPL analyses can be regarded as complementary strategies. In our case, linkage analysis, especially using NPL, could not provide sufficient evidence of linkage in the studied regions (**Article 2**). In fact, only genomewide NPL analyses have been used successfully in detecting linkage, such as in prostate cancer³⁹⁵ and, more recently, in bipolar disorder³⁹⁶. Another important observation is that the *affected relative pairs* (ARP) strategy seems appropriate for common diseases, since it is less sensitive to clinical/genetic heterogeneity and requires only nuclear families.

5.1.3.2 Factors influencing LOD score estimation

Important factors for success in parametric analysis include correct assumptions of the disease model and adequate selection (and number) of markers for genotyping. The values for the distance between markers and their heterozygosity are also factors to be considered, as they may give rise to false findings of linkage. Also, conservative thresholds for linkage should be used, to obtain significant results. Including more genetic markers within FHM *loci* for the families with nominal evidence of linkage, in theory, was a reasonable next step; however, even the use of eight markers in our study of chromosome 4 or 5 *loci* (**Article 2**) did not change these results; so, expectations should not be too high, in case more markers are tested in other chromosomal regions with relatively low LOD values.

In our study (**Article 2**), we conservatively set a threshold for significant LOD scores at a level larger than 3.3, as referred for a genomewide using 400 markers; for multipoint LOD score calculations, evidence of linkage should follow Lander and Kruglyak criteria⁹⁵. Since we tested only a few *loci* (**Article 2**), less stringent criteria could have been applied to achieve suggestive or significant linkage, even under heterogeneity. Also, because the expected size effect of susceptibility *loci* in complex diseases is small, our slightly positive maximum LOD scores observed may still be of some relevance. This must be followed up in future studies.

5.1.4 Candidate gene approaches

Advantages of choosing candidate regions or genes are the following: (i) single genetic targets are directly tested, when there is *a priori* knowledge of its possible involvement in the disease, (ii) genetic testing of the linked polymorphisms is relatively straightforward, and (iii) if properly done, multiple testing is less of an issue, compared to genomewide association studies with hundreds of thousands of genetic markers.

Searching for rare variants in smaller genetic regions (e.g., genes) can directly yield the disease cause. In our high-throughput study, scanning for mutations in a large set of candidate genes has shown that, so far, none of the identified rare variants segregated with migraine³⁹⁷. These observations are in line with our *CACNA1A* and *ATP1A2* results (**Article 3**). Clearly, the search for rare variants with a large effect in migraine is a very appealing strategy. Future experiments will have to show whether such variants exist in migraine.

Until a few years ago, SSCP was a method often used to detect mutations (**Article 3; Figure1b**), though admittedly has relatively low-detection efficiency. Direct sequencing is far more reliable, but the costs involved are also much higher, especially when scanning large genes and sample sets. In **Article 6 (Supplementary Figure)**, we used a novel promising alternative method that makes use of variations in melting temperatures of PCR fragments with DNA variations.

The *common disease – common variant* (CD-CV) hypothesis, which proposes that genetic risk may be due to predisposing DNA variants that are relatively common in the general population, is very attractive for migraine research (**Article 3**). Susceptibility alleles, under a certain disease model (additive, multiplicative or epistatic) may be contributing for migraine susceptibility. Of course the CD-CV hypothesis does not exclude the presence of

rare, high-penetrant, alleles; it merely suggests that there may be different genetic mechanisms leading to disease. One approach to test the CD-CV hypothesis in our migraine set was performing a case-control association study; for results on this study see **Annex B**. Because of the many reports on the involvement of the *MTHFR* gene in migraine^{317,318}, the *MTHFR* C677T polymorphism was investigated in our sample: we did not find any evidence that the C677T polymorphism was associated with migraine susceptibility. The *MTHFR* gene may play a small or no role at all, in the Portuguese population (**Annex B**). Other case-control association studies, testing polymorphisms in different pathways, are ongoing, and hopefully will give a positive outcome.

5.1.5 Genetic characterization of the Portuguese migraine families

Two main achievements came from our genetic studies of complex forms of migraine. First, haplotype analysis made us feel confident that our family material was collected in a proper way. Since no segregation analysis had been performed, the fact that we observed Mendelian segregation of haplotypes for several chromosomal *loci* was reassuring. Secondly, simulation studies showed that our sample set had sufficient power for finding linkage, if present (**Article 2**).

In genetic studies, next to Mendelian errors, genotyping errors are an important possible confounder (**Article 2**). An additional type of error is mislabeling after genotyping or sample switch (before or during genotyping). In most laboratories the error rate is said to be smaller than 1%, but notably an average of 3% was found in the reanalysis of CEPH data³⁹⁸. The genotyping data from our 655 samples was very accurate and can be the basis of future statistical approaches, since the error rate was well below 3%.

The selection of three-generation families with a high genetic load has proven useful for both genomewide²⁸² and functional candidate gene approaches²⁸⁸. Another way to increase the chances of finding relevant linkage peaks, in common diseases, is stratifying large families into small nuclear families; thus, intra-familial genetic and perhaps clinical heterogeneity can be removed. But a disadvantage would be that this may introduce unwanted bias, as the families are no longer independent. In order to increase the power in our sample set (*stage 1* to *stage 2*; **Article 2**), especially for NPL, we increased our sample set with small families, but we did not do this by splitting larger families into smaller ones.

For this work, the first independent data sets were obtained using a large set of clinical-based migraine families, for migraine susceptibility *loci* on chromosomes 3p21, 4q21-24 and 5q21. Neither the FHM-related (see above), nor these three migraine susceptibility *loci* showed compelling evidence for linkage (**Article 2**). Other *loci* might be causing migraine in the Portuguese families, which suggested that genomewide genetic strategies should be applied to find the underlying genes. In other words, the Portuguese migraine material is now ready for a scan to identify novel migraine *loci*.

Importantly, the number of Portuguese migraine families collected is constantly increasing; however, due to the genetic complexity of migraine a potential downside of adding more families is that additional genetic heterogeneity is introduced. Genomewide studies are promising, especially in large informative families. Given genetic heterogeneity, we studied also each family independently. After simulation, six families showed power for suggestive (FE17, FE45, FE61, FE67, and FE179) or significant (FE68) evidence of linkage (**Article 2**).

Phenotypic and genetic heterogeneity in migraine families is a major obstacle for linkage studies. Defining family-specific phenotypes could help reducing some of the heterogeneity, thereby increasing the chances of finding chromosomal regions harbouring migraine susceptibility genes. Stratifying our migraine sample set in subgroups (i.e., endophenotyping) may generate better evidence for linkage. This strategy has been efficiently used in other complex disorders such as opioid dependence³⁹⁹ or rheumatoid arthritis⁴⁰⁰, where improvement in significance over the full data set were primordial to detect susceptibility *loci*.

Accurate phenotype definition is the basis to study the genetic background of any disease. In the case of migraine, Lea et al. found suggestive linkage on chromosomes 3 and 18, using *latent class analysis* (LCA), by reclassifying migraine characteristics²⁸⁰. Nyholt et al. reported significant linkage results for migraine on chromosome 5q21 and suggestive linkage on chromosomes 8, 10 and 13, using a similar approach²⁸¹. The importance of novel endophenotyping approaches became also apparent from studies by a Finnish group that used *trait component analysis* (TCA) and identified linkage peaks, on chromosomes 4q24, 4q28, 17p13, and 18q12 and suggestive linkage on chromosomes 10, 12, 15 and X²⁴⁰, in their migraine families. Therefore, TCA might be considered a fruitful approach for our sample set as well; especially if using traits such as pulsating pain, vomiting and/or photo/phonophobia.

5.1.6 Involvement of FHM regions and genes in migraine susceptibility

Although the FHM gene *CACNA1A* has been proposed to be the susceptibility gene of chromosomal region 19p13 in common forms of migraine, this remains a controversial issue^{255,256}. Our results (**Articles 2 and 3**) indicate that FHM gene regions and their genes do not seem to be major contributors to the overall migraine genetic risk in the Portuguese population. However, these *loci* may still play a small role in particular migraine families, and this needs further investigation. Our results, however, are in line with the most recent findings from a Finnish study in MA families, which also could not find evidence for a role of the *CACNA1A* gene in common migraine²⁶¹.

It is an unlikely scenario that a single susceptibility allele would explain disease susceptibility. In our gene screening on the *CACNA1A* and *ATP1A2* genes (**Article 3**), none of the polymorphisms found in probands from 141 families, seems obviously pathogenic: nucleotide changes were frequently found in controls or did not involve an amino acid change.

One novel *ATP1A2* variant, R51H, was selected for follow up, but showed a complex segregation with migraine in the family. Also, functional assays did not support its pathogenic role. Mutation analysis of the *CACNA1A* gene in our families was negative. No rare variants with a potential large effect were identified that might explain susceptibility to migraine. However, polymorphisms can have functional consequences too, or detect nearby, still undetected, causal variants (i.e., variants that are in *linkage disequilibrium*), and may thus exert a mild but significant effect on gene expression. A detailed genetic analysis of the *CACNA1A* gene is still needed to search for or exclude such “hidden” causal variants.

Considering that common DNA variants are contributing to common disease susceptibility, future approaches such as candidate gene association studies of genes involved in ion transport (see also below), for instance, may be useful. The feasibility of performing genomewide, hypothesis-free association studies more and more will replace the hypothesis-driven candidate gene association studies^{126,127}. Central to the discussion on which genes may confer migraine susceptibility is that rare FHM is usually regarded a useful monogenic model for common forms of migraine, such as MO and MA.

5.2 Genetics of FHM: a model to study the pathophysiology of migraine

5.2.1 Mutation spectrum in Portuguese FHM families: prioritizing genetic testing

Since our FHM families were too small for genomewide linkage approaches, haplotype analysis was performed to investigate whether one of the known FHM *loci* might be involved. In parallel, also because of the well-known clinical variability among carriers of the same *CACNA1A* mutation¹⁶⁴, we selected and tested four mutations in our FHM probands (S218L, R583Q, T666M, and R1347Q). These were selected because they were previously reported in Portuguese families (R583Q and R1347Q)^{153,165} or were shown to occur in more than one family in non-Portuguese families^{18,160}. All four mutations were excluded in our FHM probands; none of our patients had ataxia, which has been described with all these four mutations. These results do not exclude, of course, the presence of other *CACNA1A* mutations in our sample set.

For the first time, we present a meaningful genotype-phenotype correlation for several *ATP1A2* gene mutations, as we have identified additional gene mutation carriers. The observation of recurrent *ATP1A2* mutations in our sample indicates that “family-specific” *ATP1A2* mutations occur less frequently than reported (**Article 4**). *ATP1A2* mutations M731T and T376M were (i) both proven to be recurrent, (ii) functional mutations, and (iii) associated with a pure FHM phenotype. Notably, in some of the mutation carriers, prolonged episodes and psychomotor development delay were reported. Mental illness phenotypes might also be associated with novel functional *ATP1A2* mutations, V362E and P796S (**Article 5**). Aggressive behaviour and borderline personality, and mild to severe mental impairment, were observed in some mutation carriers and deserve further attention. Our mutation scanning implies that recurrent mutations should be screened, with priority, in Portuguese patients.

One of our most important findings in FHM was obtained with the *SCN1A* gene (**Article 6**). Well over 150 mutations have been found associated with several forms of childhood epilepsy³⁸⁴. Recently, the first *SCN1A* mutation (Q1489K) was identified in three related German FHM families and the *SCN1A* gene designated as FHM3²⁰⁸. Independent confirmation was recently obtained, after the identification of a second FHM3 mutation in a large North-American family²²⁰. The identification of the novel L263V mutation in one of our Portuguese FHM families is interesting, as several mutation carriers had also epilepsy (**Article 6**).

The L263V mutation is the first *SCN1A* mutation clearly linking migraine and adult epilepsy. A more appropriate treatment is required when both episodic disturbances occur in the same person. Testing for the IVS5-91G>A polymorphism could offer a good indication on the best carbamazepine dosage for those cases⁴⁰¹.

Some of our FHM families remain to be characterized. Only a few were tested for FHM1, FHM2 and FHM3, as well as for *SCL1A3*, the gene encoding the glutamate-transporter EAAT1 (see also below), for which a mutation was reported in a sporadic patient with a complex severe phenotype, including alternating hemiplegia, seizures and headache²³⁵. For the families already studied, after collecting additional members, we aim to search for a new FHM locus, using a genomewide approach. At the same time, familial and sporadic hemiplegic migraine cases should be screened for mutations in FHM genes¹⁹, in order to improve the genotype-phenotype correlation and establish genetic testing priorities.

5.2.2 Contribution to migraine pathophysiology

What is the possible contribution of the three FHM genes, *CACNA1A*, *ATP1A2* and *SCN1A* to the pathophysiology of migraine? The common effect of the functional consequences seems to be an increased concentration of K⁺ and glutamate in the synaptic cleft, both being facilitators of CSD, which may be considered the underlying cause of aura (see **Figure 5**, in **Introduction**). The propensity for CSD is lowered in patients and may trigger FHM attacks, as also suggested by a knock-in mouse model that harbours a pathogenic *CACNA1A* mutation¹⁷⁹. Animal experiments have shown that CSD can activate the trigeminal vascular system⁸¹, providing a link between the aura and headache-pain symptoms. Furthermore, glutamate is also implicated in trigeminovascular activation and central sensitization. In case the aura is absent, e.g., in MO, other mechanisms should be involved. One possibility is that spreading depression occurs in regions of the brain outside the visual cortex, but this attractive hypothesis lacks experimental evidence.

Na⁺,K⁺-pumps that are expressed in astrocytes help clearing of extracellular K⁺ and create a gradient of Na⁺, used for the uptake of glutamate from the synaptic cleft. Functional studies of *ATP1A2* mutations, including our Portuguese mutations, predict a loss of function that would result in a slowed removal of extracellular K⁺ and glutamate from the synaptic cleft^{182,201,203,204}. As proposed for *CACNA1A* mutations, this could translate in increased susceptibility to CSD.

The identification of our novel FHM gene mutations allows some speculations on how mutated FHM gene products may cause specific clinical phenotypes (**Articles 3, 4 and 5**). With respect to the Na⁺,K⁺-pump mutations we identified, we showed that Pro⁷⁹⁶ can be mutated either to a Ser (the P796S mutation in our family, **Article 5**), or to an Arg (P796R¹⁹⁴). Due to lack of a functional characterization of P796R and available clinical data, we cannot make a detailed comparison of the phenotypes in both families and speculate why a Ser at this position causes a seemingly more severe phenotype with psychiatric and mental problems.

Our studies reinforced the idea that specific protein regions of the Na⁺,K⁺-pump are very important for protein functioning, and that its dysfunction causes hemiplegic migraine. There are hotspots for mutations including the large M4-M5 cytoplasmatic loop, as highlighted by our Portuguese mutations (**Articles 4 and 5**). Only a few mutations are found in the transmembrane domains, and all are associated with ataxia. The importance of the N-terminal region for pathogenesis is still uncertain, but Arg⁵¹ represents a consensus-splicing site for ATPases¹⁹⁵, predicting a mild effect for the mutant of the variant R51H that we identified in an MO family (**Article 3**). Interestingly, two novel mutations were recently detected in the N-terminal, i.e., Y9N (with SHM) and R65W (with FHM)²⁰, suggesting that this region is indeed important. Unfortunately, no functional assays were performed to support causality. Such assays are necessary, because the N-terminal region is not entirely conserved.

With respect to our genetic findings in *SCN1A*, we can also make some additional remarks. Na_v1.1 channels are essential for generation and propagation of action potentials. Mutations in these channels are thought to exert gain-of-function effects^{208,220}, resulting in an increased neuronal firing rate and consequent increased release of neurotransmitters, such as glutamate. These predicted functional consequences fit well the hypothesis of increased neuronal hyperexcitability in migraine, as suggested from functional studies of *CACNA1A* and *ATP1A2* mutations. The position of Leu²⁶³ on the voltage-gated sodium channel domain (DIS5) predicts an important functional role for the L263V *SCN1A* mutation (**Article 6**). Based on the literature, a mutation in the same *SCN1A* DIS5 region caused classical severe myoclonic epilepsy (SMEI)²¹⁶, while mutations in other S5 segments can cause GEFS+^{211,212}, SMEI^{213,389} or SMEB^{213,230,390}. Moreover, mutations in S5 segments of homologous sodium channel proteins can cause hyperkalemic periodic paralysis (*SCN4A*)⁴⁰² or Brugada syndrome (*SCN5A*)⁴⁰³.

However, both the type of the mutation and its location in the protein cannot predict its exact functional consequences. Therefore, functional studies on L263V mutation are now underway. Mutagenesis and functional tests will be obtained on the original SCN1A, instead of the homologous human channel SCN5A as in previous studies^{208,220}, which will produce more accurate data on the consequences of this mutant to migraine mechanisms. The relevance of this specific mutation to our understanding of the relation between migraine and epilepsy will be of particular interest since neuronal hyperexcitability (i.e. gain-of-function)^{208,220} is so far indicated as the key process on both disorders. A detailed comparison of functional consequences of the various FHM3 mutations might reveal how different phenotypes (i.e., migraine and epilepsy) can occur in the same person and shed light on the underlying pathophysiology of both episodic disorders.

Other “hemiplegia” genes also fit in this mechanism; this is the case of the *SLC1A3* gene, encoding the glutamate transporter EAAT1, important for glutamate uptake. No mutations were found in *SLC1A3* gene in our set of FHM patients. Together with the negative results reported by De Vries et al. in alternating hemiplegia of childhood⁸⁵, this reinforces the idea that this gene perhaps may not be important in other hemiplegic patients.

It is obvious, that novel genes will provide better insights in migraine pathophysiology, which is why more genes for FHM, and definitely for MA and MO, must be found. Increased knowledge of the pathophysiology of migraine will certainly help designing strategies to identify new treatment options for patients with migraine.

CONCLUDING REMARKS

Unravelling the genetic component of migraine is challenging because of the complexity of the disease, with genetic and environmental factors involved. Our results contributed to the understanding of the genetics of migraine. Using several methodological approaches, we concluded that, most likely, no single strategy allows the identification of the genetic susceptibility factors in migraine.

Rare, severe, monogenic forms of migraine (in this case, familial hemiplegic migraine), were used as a model to study pathogenic mechanisms that may be extrapolated, at least to a certain extent, to complex, non-Mendelian, forms of migraine.

A well-characterized population of Portuguese migraine patients and controls was acquired, which will be valuable for gene identification initiatives. A first genetic analysis of this sample was presented, but no significant migraine susceptibility *loci* were obtained.

Specific phenotypic criteria and sub-group analysis (i.e., endophenotyping) may reduce clinical and genetic heterogeneity and lead to positive results.

Candidate gene association studies have been a popular strategy for genetic studies until now, but, because of the low probability of finding positive results, which hardly ever get replicated, *state-of-the-art* genetic studies in other complex diseases have turned to genomewide, hypothesis-free, association studies involving hundreds of thousands of genetic markers. Such studies are now also planned for migraine and our Portuguese migraine samples will be useful as a replication sample, when positive findings are identified.

The identification of genetic DNA variants, conferring susceptibility to the common forms of migraine, will be the challenge in the coming decade. Knowledge that will come from such studies will be instrumental for patient diagnosis and to guide drug discovery.

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ANNEX A

P1126. What is the best way to break large genealogies for the genetic study of complex traits?

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Large genealogies provide many individuals for linkage analysis. However, the software available for nonparametric multipoint linkage analysis is limited in the complexity of the families it can handle. Partition into subfamilies is necessary.

Two methods have been proposed to partition pedigrees automatically, one based on factor analysis¹, and the other on a maximum-clique partitioning approach². Both were proposed for the study of quantitative traits.

We adapted these methods to qualitative trait analysis and assessed the sensitivity of subsequent NPL analysis to these approaches and their parameters. Better results were obtained when the pair relationship measure on which the breaking is based is highly correlated with the linkage informativity of the pair. Additionally, results were appreciably improved if partitioning was first based on affected individuals only.

Our results are illustrated with a genome-wide NPL analysis of hypertension in a 2636-member pedigree from the isolated village of Campora, South Italy. We show that the genome-wide significant linkage detection of a new locus on 8q22-23 as well as the replication of two known loci (1q42-43 and 4p16) are conditioned on the quality of the genealogy partition.

Our results illustrate the great sensitivity of linkage analyses in large genealogies to the quality of the genealogy partition. They also show that, partitioned in an optimal way, large genealogies from population isolates offer a good power to detect genetic risk factors for complex diseases.

1 Pankratz and Iturria (2001) Genet Epidemiol ; 21 Suppl 1:S258-63
2 Falchi et al (2004) Am J Hum Genet ; 75(6):1015-31

P1127. Use of isolated population to understand genetic bases of late onset progressive hearing impairments

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Hearing impairment is a frequent disorder affecting one in 1,000 children, whose prevalence increases with age. At least 60% of cases are due to genetic causes; and may manifest itself as syndromic (20% of all cases) or non-syndromic (80% of all cases) form. Despite the fact that a number of loci has been identified for hearing loss, a poor knowledge exist on the genetic bases of late onset progressive hearing impairments (starting around the age of 40), the most common cause of adult auditory deficiency, resulting from yet unidentified interactions between environmental and strong genetic factors. The study of late-onset forms in isolated populations is particularly useful because of both genetic and environmental homogeneity. We have selected 3 isolated Italian villages for which genotypes and audiological phenotypes have been collected: Campora (600 inhabitants), Carlantino (1400) and Stoccardedo (400). In Campora 51% of the whole population has been diagnosed as suffering from late onset severe hearing impairments. In Carlantino 54% of the whole population is affected by bilateral sensorineural hearing loss (10% in people aged less than 40). Data from Stoccardedo are still under evaluation. A first known locus between markers D1S104 and D1S466 (DFNA7) has been already identified in Campora. Since a huge number of information has been collected for each individual (biochemical, instrumental and lifestyle data) it would be much easier to understand the relationships between genes involved in hearing loss in these populations, including modifier genes, and to define the interplay role between genetic and environmental factors.

P1128. Familial risk for migraine with aura and migraine without aura among first-degree relatives of migraineurs

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Migraine is a primary, chronic, headache, affecting 11%-20% of the general population. Familial clustering of migraine with aura (MA) and without aura (MO), the two most common subtypes, was described in several studies.

Probands and their first-degree relatives were classified according to their migraine subtype, to evaluate familial risk for MA and MO. The use of family history to classify relatives as migraine sufferers was also validated; though probands were able to correctly identify their affected relatives, migraine was still underestimated: only relatives with a direct interview were thus included.

Familial risk was estimated using the relative risk (RR) in first-degree relatives. Prevalence in our general population has been estimated previously at 1.4% for MA, and 6.0% for MO. The risk for MA and MO was evaluated separately in 51 families of probands with MA and in 94 families of migraineurs with MO.

The RR of MA and MO among first-degree relatives of probands with MA was 10.58 (95% CI: 6.24-17.93) and 6.51 (95% CI: 4.98-8.51), respectively. The RR of MO of first-degree relatives of probands with MO was 8.82 (7.10-10.95). The risk for MA in relatives of MO probands was not possible to estimate, as only a few parents were affected by MA. Our findings show that, in our sample, MA is less frequent than MO as previously described. The RR for first-degree relatives of migraineurs with MA is highly suggestive of a genetic contribution. For MO, the results also point out to familial clustering.

P1129. Mitochondrial DNA polymorphism in Evenks and Buryats from Chita region of Russia

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Chita region is administrative unit of Russian Federation situated in the Transbaikal Region, north to Mongolia and China. Now most of the population are Russians who started to arrive there since the 17th Century. Indigenous peoples of the region are Evenks living in the north of the region (about 1000) and Buryats in the south (about 42000). We have studied mtDNA polymorphism (by HVS-I sequencing and high-resolution RFLP) in 81 Evenks and 115 Buryats from the area. In total, 33 and 75 different HVS-I haplotypes were found in Evenks and Buryats, respectively. The most frequent haplogroups in Evenks were C and D; A, G, N9 and Z haplogroups were also present. Buryats are more heterogeneous, with predominance of C, D, and G, while A, B, F, Z and M7 were found to be less frequent. West-Eurasian derived haplogroups (H, J, U and a few others) encompassed about 8% of samples in Evenks and 11% in Buryats. Interestingly, one Buryat individual carrying haplogroup X was identified. The two Buryats with 16325 substitution in haplogroup C and two Evenks encompassing 16111 change in haplogroup A may mark Native American founder haplotypes. The two ethnic samples had only 8 mtDNA haplotypes in common, indicating quite isolated gene pools of these populations. In contrast to that, studied by us Buryats had 23 lineages in common with Buryats from the Republic of Buryatia (Derenko et al. 2003). The study was supported by RFBR grant 04-04-48792.

P1130. Manganese superoxide dismutase (MnSOD) polymorphism and breast cancer risk

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Oxidative damage induced by the generation of reactive oxygen species (ROS) by exogenous and endogenous exposures is believed to be implicated in breast cancer. MnSOD is the primary antioxidant enzyme in the mitochondria with a function of the detoxification of superoxide free radicals and thus protecting cells from oxidative stress. MnSOD is a nuclear-encoded protein that is transported into the mitochondrion through the mitochondrial targeting sequence. A polymorphism in this sequence with a valine to alanine substitution is believed to alter transport of the enzyme into mitochondria, has been

ANNEX B

POSTER 13**Molecular and Genealogical Analyses of The BRCA1 R1443X Founder Mutation in High Risk French-Canadian Breast/Ovarian Cancer Families**

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In the 17th century, about 5 000 immigrants, coming mostly from France, settled in Canada. Among them, 3 500 to 4 000 have descendants in the contemporary Quebec population and they account for the major part of the gene pool of the 5 million Quebecers of French descent. The goal of this study is to understand the role of settlement patterns and demographic history in the spread of the BRCA1 R1443X mutation, representing nearly 70% of all BRCA1 mutations observed in our French-Canadian families, and to identify the founders who are the most likely to have introduced this mutation. We performed haplotyping analysis of this BRCA1 mutation (R1443X) in our carriers using seventeen microsatellite markers spanning a 9.4 cM region on chromosome 17 surrounding the BRCA1 locus. The highly conserved haplotype for this mutation confirmed that it represented a founder mutation in the Quebec population. Ascending genealogies of one carrier individual per family (n=17) and of controls (n=51) were reconstructed using the BALSAC population register. These genealogies have an average depth of 11.0 generations, and a number of 12 866 distinct ancestors were traced. We identified the two founders with the highest probability of having introduced the mutation in the Quebec population. These founders who migrated from France and Portugal married in Quebec City in 1671. Analysis of the genealogical paths linking this founding couple to the contemporary carriers points toward geographical clustering of the mutation in some specific regions of Quebec.

POSTER 14**Identification of genetic risk factors for cervical cancer**

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Cervical cancer is the second most common cancer worldwide among women, with 471,000 cases in year 2000 (Parkin et al. 2001). Almost 80% of all cases occur in developing countries, where it is the leading cause of cancer mortality in women (Pisani et al.1999). Infection with oncogenic types of human papillomavirus (HPV) is considered the major risk factor for developing cervical cancer and the virus is found in almost 100% of all excised cervical tumours (Walboomers et al.1999). We have shown that first-degree relatives with a sib or mother affected by cervical cancer have an almost 100% increased risk of developing cervical cancer, indicating the existence of genetic susceptibility for cervical cancer (Magnusson et al.1999). In order to understand the development of cervical cancer there is a need to focus on the genetic factors in the host that has the potential to affect the risk for cervical cancer development. In theory, there are several genes that might impact the outcome of cervical cancer. The genes encoding components of the cell-mediated immune response are among the many candidate genetic factors. We previously found an association of HLA class II alleles and increased risk of developing cervical cancer through infection with oncogenic forms of HPV (Beskow et al. 2001)

The purpose of this project is to identify genetic risk factors for cervical cancer using a genome scan analysis. By cross-matching the Swedish family registry and the Swedish cancer registry we identified over 1800 sibpairs affected with cervical cancer in the Swedish population. Blood samples were then collected and 576 samples were selected for the initial phase of the genetic analysis. A set of 400 microsatellite markers distributed across the genome with an, on average, 10 cM distance has been analysed. HLA class I and class II genes have also been typed in these sibpairs and this information will be used to stratify the data in the linkage analysis.

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POSTER 15**Study of CTG-trinucleotide repeats polymorphism in the myotonic dystrophy gene in Yakut and Evenk populations**

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CTG trinucleotide repeats length polymorphism at the 3'-end of myotonic dystrophy gene was investigated in two ethnic groups living in north-eastern Siberian region of the Russian Federation (Yakuts and Evenks).

In population of Yakuts (n=64) 11 alleles were observed within range from 5 to 35 CTG-repeats. The most of alleles were within range from 11 to 14 repeats (88%); alleles containing 12 and 13 repeats were most frequent in this group (37% and 35% respectively). The frequency of (CTG)₅ allele which is predominant in European populations (40%) was only 5% in Yakuts.

All the 13 allelic variations with CTG-repeats from 5 to 27 were found in population of Evenks (n=45). The allele frequency distribution had two peaks corresponding to alleles with 5 and 11-14 CTG-repeats (amounting 15% and 73% respectively).

We executed exact test of population differentiation comparing populations of Yakuts and Evenks with populations of Volga-Ural region (Tatars, Bashkirs, Chuvashes, Maris, Mordvinians, Udmurts, Komis) and other human populations from various regions of the world. The significant differences basing on alleles frequencies were revealed comparing Yakuts and Evenks with Volga-Ural populations. The lowest level of the genetic diversity was observed in Yakuts. So we suggest that the specific unimodal distribution of CTG-alleles with highest prevalence of the two alleles containing 12 and 13 repeats and the high frequency of myotonic dystrophy in population of Yakuts (about 1 per 5000 individuals) might be conditioned by isolation of not large Yakut population over a long period.

POSTER 16**The C677T MTHFR polymorphism is not a genetic risk factor for migraine in the Portuguese population**

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Migraine is one of the most frequent types of episodic and chronic headache. Several studies suggest the influence of genetic and environmental factors in this complex neurovascular disorder. The molecular mechanisms underlying migraine remain largely unknown. Dilatation of cerebral vessels with the concomitant release of vasoactive neurotransmitters may be one of the mechanisms involved. The methylenetetrahydrofolate reductase (MTHFR) is an enzyme involved in folate metabolism. The C677T polymorphism has been described as causing mildly reduced enzyme activity. This genetic variant, which encodes a thermolabile form of MTHFR, is associated with hyperhomocysteinemia and implicated in several neurological and vascular pathologies. It has been proposed that the abnormally high level of the excitatory aminoacid homocysteine may influence the threshold of migraine, acting as a predisposing genetic factor for this disorder.

To test this hypothesis in the Portuguese population, we genotyped a sample of 124 unrelated migraine patients (75% female; mean age 37±16); among all, 43 had migraine with aura, 71 without aura, and 10 with and without aura; patients with hemiplegic migraine were excluded from this study. A control sample of 198 anonymous Guthrie cards from the national programme of phenylketonuria screening was also studied. Genotyping was performed by PCR-RFLP, as described previously. After PCR amplification, products were separated on a 8% non-denaturing polyacrilamide gel and visualized with ethidium bromide.

Both populations were in Hardy-Weinberg equilibrium; the CC, CT and TT genotype frequencies were 44%, 47% and 10% in patients, and 45%, 41% and 14% in controls, respectively. We did not find an enrichment of the homozygous state for the C677T variant in our migraine group ($\chi^2=1.66$ p=0.437; OR (TT)=0.678, 95% CI: 0.3301–1.3948), suggesting that the C677T variant is not a factor associated with susceptibility to migraine in the Portuguese population.

POSTER 17**Association of the human *Adiponectin* (*Apm1*) gene and insulin-resistance.**

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Insulin-resistance is considered the core factor in the pathogenesis of common disorders such as type 2 diabetes, atherosclerosis and the metabolic syndrome, and is often associated with obesity. Genetic and epidemiological studies strongly suggest that insulin-resistance is, at least in part, genetically determined. The *adiponectin* gene, also known as *APM1* gene, has been suggested as a potential candidate gene for insulin-resistance. Adiponectin, a protein secreted by adipocytes, has been shown to modulate insulin-sensitivity, and a strong relationship between plasma adiponectin levels and insulin-resistance has been demonstrated in several epidemiological and experimental studies.

In the present study we have analysed the prevalence of genotypes identified by two SNPs (+45T>G and +276G>T), previously shown to be associated with insulin-resistance, in 280 normal subjects, looking for association with features of insulin-resistance.

The SNPs +45T>G and the SNP +276G>T of the human *adiponectin* gene were detected in real-time with LightCycler hybridization probes, using fluorescently-labelled nucleotides. No association was found with the +45T>G SNP; the T/T genotype of the +276G>T SNP was associated in univariate analysis with a higher BMI (p<0.01), a higher plasma insulin (p<0.02) and a higher HOMA_{IR} (p<0.02).

In order to analyse the possible interaction between BMI and the *adiponectin* gene on insulin resistance, the study group was divided in

AGRADECIMENTOS

Nestas últimas linhas dedico uma palavra de apreço a todas as pessoas que de alguma forma contribuíram para esta dissertação. A todos, os meus mais profundos e sinceros agradecimentos.

Ao Professor Doutor Pereira Monteiro. Pelo interesse que fomentou pela área das cefaleias, área que tão bem domina, e na qual se tornou um excelente especialista. Pela abertura de espírito ao abraçar este projecto da genética da enxaqueca, mesmo sabendo que seria uma tarefa árdua e nem sempre fácil. Pela prontidão em resolver questões burocráticas, mediar colaborações e pelo empenho e entusiasmo na comunicação e divulgação de todos os nossos resultados. Pelo exemplo de vida que é, e principalmente pela extrema dedicação ao trabalho, sem dúvida um dos seus verdadeiros prazeres.

Ao Professor Doutor Jorge Sequeiros. Por me ter acolhido no seu laboratório e por tanto me ter ensinado na genética humana aplicada a doenças. Pela sua sabedoria e extremo domínio no assunto. Por conseguir tornar um conjunto de resultados, uma história realmente interessante, de forma simples e directa. Pelo incentivo à participação nas reuniões e congressos, e em tudo o que contribui para evoluir cientificamente. Por ser uma das maiores referências da genética em Portugal, e por ter marcado esta minha breve passagem pela área.

To Doutor Arn van den Maagdenberg. To my “Professor” in Migraine Genetics Research. Most of this work and acknowledges go directly to him, as the real guru on migraine genetics related issues. To accept me on his lab, sharing data, supervising most of the lab work, and writing (a lot). For all the debates, talks and work discussions and for understanding me so well, thank you very much, dear “Boss”.

A toda a equipa da UnIGENE. A todo(a)s o(a)s colegas, actuais e anteriores, pelo apoio, ajuda e partilha de conhecimento. Em especial, agradeço à Tia Paula Magalhães, Teresa Matamá, Anabela Ferro, Isabel Alonso, Andreia Perdigão, Cláudia Santos, Ana Seixas, Sandra Martins, Bárbara Guimarães, Teresa Almeida e Marisa Ramos. Ao Victor, por toda a paciência nos “problemas” informáticos, e por todas as dívidas perdoadas. À Carolina Lemos por tudo o que temos partilhado, pela amizade e paciência, enfim, pela ajuda preciosa em quase tudo o que fiz neste trabalho...Muito obrigada, Carolzita.

To all colleagues at LUMC, Leiden. To “lab Frants” and migraine group. A special thanks to Barbera, Boyan, Boukje, Corrie, Eelke, Ludo, Rob and to dear Kaate. It was very nice sharing those days with all of you. *Tot ziens en bedankt*.

Á Karine e à D. Carolina Lemos, pela pronta ajuda nas traduções do texto.

Á Fátima Ferreirinha e à Milena Paneque, pela amizade e carinho que me têm dedicado nesta recta final.

A todos os meus amigos de sempre, e eles sabem quem são, pelo enorme carinho e dedicação com que partilham esta viagem comigo. Ao Duarte. À Cristina, Sofia, Patrícia, Vanessa, Pedro e Zito, um abraço especial. Ao João, obrigada pelo novo caminho.

A toda a minha família. Por tão bem me compreender, apoiar e sempre aceitar todas as escolhas que faço. Em especial aos meus pais, pela confiança que em mim depositaram e acima de tudo pelo apoio incondicional.

