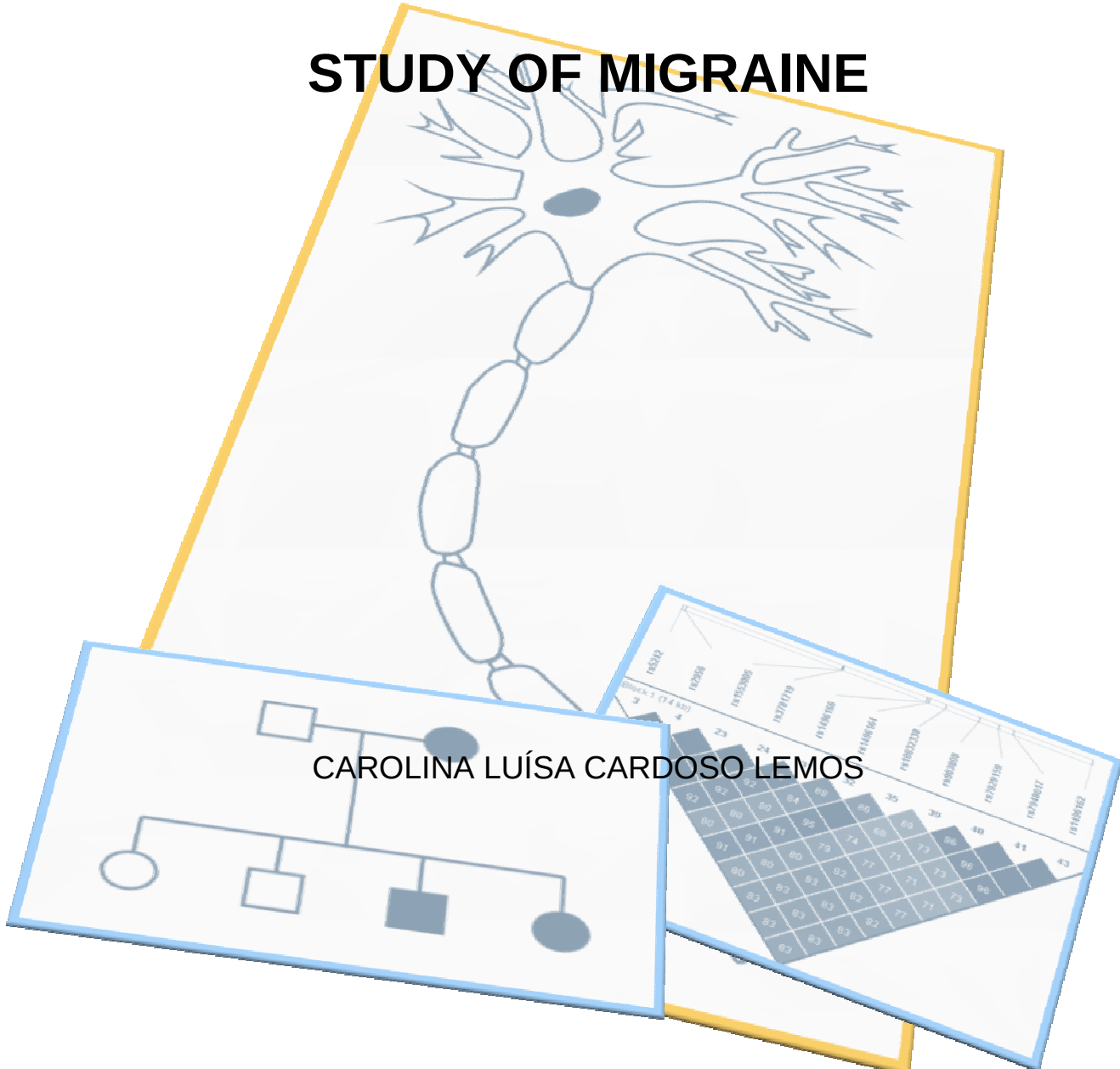


GENETIC AND EPIDEMIOLOGICAL STUDY OF MIGRAINE



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Publications

In this thesis were used results, already published or in preparation, as described below:

Article 1: Lemos C, Castro MJ, Barros J, Sequeiros J, Pereira-Monteiro J, Mendonça D, Sousa A. Familial Clustering of Migraine: Further Evidence From a Portuguese Study. *Headache*. 2009;49:404-11.

Article 2: Lemos C, Pereira-Monteiro J, Sequeiros J, Mendonça D, Sousa A. How useful is the probands' description of family history of migraine? (In preparation)

Article 3: Lemos C, Alonso I, Barros J, Sequeiros J, Pereira-Monteiro J, Mendonça D, Sousa A. Assessing risk factors for migraine: differences in gender transmission? (In preparation)

Article 4: Lemos C, Sousa A, Sequeiros J, Pereira Monteiro J. Headache epidemiological study: do different criteria make a difference? (In preparation)

Article 5: Lemos C, Pereira-Monteiro J, Mendonça D, Ramos EM, Barros J, Sequeiros J, Alonso I, Sousa A. Syntaxin 1A in migraine susceptibility: evidence for its involvement in a Portuguese study. *Arch Neurol* (In press)

Article 6: Lemos C, Neto JL, Pereira-Monteiro J, Mendonça D, Barros J, Sequeiros J, Alonso I, Sousa A. A variant in the endothelin receptor type A is associated with migraine without aura. (Submitted)

Article 7: Lemos C, Mendonça D, Pereira-Monteiro J, Barros J, Sequeiros J, Alonso I, Sousa A. BDNF and CGRP interaction: implications in migraine susceptibility. (In preparation)

Abbreviations

5-HT – Serotonin

AADC – Amino acid decarboxylase

ACE – Angiotensin-converting enzyme

AGRT1 – Angiotensin receptor 1

AGT – Angiotensin

AR – Androgen receptor

ATP1A2 – Na⁺, K⁺ ATPase α_2 subunit gene

BDNF – Brain-derived neurotrophic factor

Ca²⁺ – Calcium

CACNA1A – Voltage-gated P/Q type calcium channel α_{1A} subunit gene

CADASIL – Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leucoencephalopathy

CDCV – Common disease – common variant

CGRP – Calcitonin gene-related peptide

CI – Confidence interval

CNV – Copy number variation

COMT – Catechol-O-methyltransferase

CSD – Cortical Spreading Depression

DAT – Dopamine transporter

DBH – Dopamine β -hydroxylase

DNA – Deoxyribonucleic acid

DZ – Dizygotic twin

EDNRA – Endothelin receptor type-A

eNOS – Endothelial nitric oxide synthase

ESR1 – Estrogen receptor 1

ESR2 – Estrogen receptor 2

ET-1 – Endothelin-1

FHM – Familial hemiplegic migraine

FSHR – Follicle stimulating hormone receptor

GABA-A – Gamma-aminobutyric acid type A

GAT-1 – Gamma-aminobutyric acid transporter

GWA – Genome-wide association

h^2 – Heritability

HSA – Hospital de Santo António

hSERT – Human serotonin transporter gene

HVR – Hereditary vascular retinopathy
 IBD – Identical by descent
 ICHD-I – International Classification of Headache Disorders-I
 ICHD-II – International Classification of Headache Disorders-II
 IHS – International Headache Society
 IL –6 – Interleukin 6
 IL-1 α – Interleukin 1 α
 INSR – Insulin receptor
 IP3 – Inositol-1,4,5-trisphosphate
 LCA – Latent class analysis
 LD – Linkage disequilibrium
 LDLR – Low-density lipoprotein receptor
 LOD – Logarithm (base 10) of the odds
 MA – Migraine with aura
 MAF – Minor allele frequency
 MAO-A – Monoamine oxidase A
 MAO-B – Monoamine oxidase B
 MELAS – Mitochondrial Encephalopathy with Lactic Acidosis and Stroke-like episodes
 MgCl₂ – Magnesium chloride
 MMP-9 – Metalloproteinase-9
 MO – Migraine without aura
 MS – Multiple sclerosis
 mtDNA – mitochondrial DNA
 MTHFR – Methylene tetrahydrofolate reductase
 MZ – Monozygotic twin
 NaCl – Sodium Chloride
 NMDA – N-methyl-d-aspartate
 NO – Nitric oxide
 NPV – Negative Predictive Value
 OD – Optical density
 PCR – Polymerase chain reaction
 PD – Parkinson Disease
 PGR – Progesterone receptor
 PKC – Protein kinase C
 PLA2 – Phospholipase A2
 PPV – Positive Predictive Value
 RR – Relative Risk
 SCN1A – Voltage-gated type-I sodium channel α subunit gene
 SDS – Sodium dodecyl sulphate

Abbreviations

SHM – Sporadic hemiplegic migraine
SNP – Single-nucleotide polymorphism
SPSS – Statistical Package for the Social Sciences
STRs – Short-tandem repeats
Syn1A – Syntaxin 1A
TCA – Trait component analysis
TDT – Transmission disequilibrium test
TGVS – Trigemino-vascular system
TNC – Trigeminal nucleus caudalis
TNFSF7 - Tumor necrosis factor ligand superfamily member 7
TNF- α – Tumor necrosis factor α
TNF- β – Tumor necrosis factor β
TPH – Tryptophan hydroxylase
TS – Thymidylate synthase
TTH – Tension-type headache
WHO – World Health Organization

Abstract

Migraine is a highly prevalent disorder, leading to a diminished quality of life in both migraineurs and their relatives. The two most common subtypes are migraine with (MA) and without aura (MO). Currently, the diagnosis of migraine relies only on clinical criteria, since there are few biomarkers available and the genes involved in these common forms have not yet been identified. An improvement in the diagnosis has been achieved with the classification and the diagnostic criteria of the International Headache Society (IHS) for the different subtypes of headache.

Several studies showed that migraine has a genetic component, with multiple genetic factors involved, in addition to environmental factors, determining the liability for migraine. One of our aims was to assess if, in a large sample of Portuguese families, a genetic component of migraine was also present, in order to continue with subsequent studies. We evaluated familial aggregation of migraine by estimating relative risk (RR), using the prevalence in a previous sample from the general population as reference. We found that first-degree relatives of migraineurs had a 3 to 4-fold increased risk, further suggesting a genetic component. Evidence is provided for both common and specific etiologic mechanisms in either migraine subtype. The report of a family history of migraine by the proband was found to be reliable and, despite a low sensitivity, was highly specific enough and had sufficient high predictive positive value (PPV) to be a valuable ascertainment method, and can be used to maximize the number of informative families for e.g. linkage studies.

Additionally, we evaluated which familial risk factors could contribute to the increased risk observed. Gender of the relatives was a risk factor for MO only, with females having a higher risk. This showed that MA might have a higher genetic load than MO. Mother-to-daughter transmission was more frequent than expected by chance only, what may be explained by mtDNA, X-linked genes or hormonal effects.

Although the new ICHD-II criteria have relevant clinical implications, they did not change significantly our epidemiological results. More importantly, ICHD-II did not present significant changes for MO and MA forms in the clinical sample; therefore, we were able to standardize the diagnosis of the patients ascertained before and after 2004.

Then, we focused on identifying genetic variants that might be associated with susceptibility to migraine in our population. For this we used a case-control approach, analysing 188 cases (111 with MO and 77 with MA) and 287 migraine-free, age-matched

controls. A case-control ratio of 1:1.5 was obtained. Candidate genes were selected based on their possible role in the pathophysiology of migraine. For *STX1A*, we confirmed the involvement of rs941298, and found rs6951030 to be associated also with migraine. These results strengthen the role of neurotransmitter release in migraine pathophysiology.

The variant rs702757 of *EDNRA* was found to be a susceptibility factor for MO, as previously reported; furthermore, we could not exclude the involvement of this gene in MA in our population, since we found a trend towards an increased risk conferred by rs5333. On its turn, this reinforces the role of the vascular component in migraine. We also explored BDNF, a modulator of the trigeminal nociceptive plasticity and, for the first time, CGRP, a mediator of neurogenic inflammation, and a possible interaction between them, due to their connection in pathophysiology of migraine. No significant results were found, except for an interaction between *BDNF* and *CGRP*.

In conclusion, using different approaches, we were able to characterize a group of Portuguese families, regarding familial aggregation of migraine and risk factors involved, including gene variants associated with susceptibility to migraine, thus expanding our knowledge of the multiple migraine pathways.

Sumário

A enxaqueca é uma doença que apresenta uma elevada prevalência, afectando a qualidade de vida dos doentes e dos seus familiares. Os dois subtipos mais comuns são a enxaqueca com (MA) e sem aura (MO). Até agora, o diagnóstico da enxaqueca tem-se baseado unicamente no diagnóstico clínico dado serem raros os biomarcadores disponíveis e os genes envolvidos nas formas comuns ainda não foram identificados. O diagnóstico sofreu um grande avanço com a classificação e os critérios de diagnóstico da “International Headache Society” (IHS) para os diferentes tipos de cefaleias.

Diversos estudos demonstraram que a enxaqueca tem uma componente genética, com múltiplos factores genéticos envolvidos que, em conjunto com factores ambientais, determinam a susceptibilidade à enxaqueca. Um dos objectivos deste trabalho foi verificar, numa amostra alargada de famílias portuguesas, a presença de uma componente genética na nossa população, de modo a prosseguir com os estudos genéticos. A agregação familiar da enxaqueca foi avaliada, tendo-se estimado o risco relativo (RR), usando a prevalência da enxaqueca de uma amostra da população em geral como referência. Os familiares em 1º grau dos doentes apresentam um risco acrescido 3 a 4 vezes superior, o que é sugestivo de uma componente genética. Para os dois subtipos, parecem existir mecanismos etiológicos comuns e específicos.

A descrição da história familiar da enxaqueca pelo probando foi também avaliada e apesar da baixa sensibilidade, apresentou uma elevada especificidade e um elevado valor preditivo positivo (PPV), sendo assim um método de recolha de informação importante de modo a poder ser usado para maximizar o número de famílias informativas por exemplo para estudos de análise de ligação.

Adicionalmente, foram avaliados os factores de risco que podiam contribuir para o risco aumentado que foi observado nos familiares. O sexo do familiar foi o único factor de risco significativo encontrado e apenas para a MO, apresentando as mulheres um risco superior. Este resultado mostra que a MA tem uma componente genética mais forte que a MO. Verificou-se que a transmissão mãe-filha era mais frequente que o esperado devido apenas ao acaso, o que pode ser explicado por factores ligados ao DNA mitocondrial, pela influência de genes ligados aos cromossomas sexuais ou por efeitos hormonais.

Apesar dos novos critérios da ICHD-II terem importantes implicações clínicas, não modificam significativamente os nossos resultados epidemiológicos. Verificou-se ainda que mesmo para amostras clínicas, a ICHD-II não apresenta diferenças significativas

relativamente à MO e MA. Assim, é possível uniformizar o diagnóstico dos doentes incluídos no estudo antes e depois de 2004.

Em seguida, o nosso objectivo centrou-se na identificação de variantes genéticas associadas com a susceptibilidade para a enxaqueca na nossa população. Para isto, foi utilizada uma estratégia caso-controlo, tendo sido analisados 188 casos (111 com MO e 77 com MA) e 287 controlos, para os quais foi excluída a presença de enxaqueca e emparelhados em termos de idade com os casos. Obteve-se uma razão caso-controlo de 1:1.5. Os genes candidatos foram seleccionados tendo em conta a sua possível função em termos da patofisiologia da enxaqueca. Para o gene *STX1A*, foi confirmado o envolvimento do rs941298 e descobriu-se que o SNP rs6951030 também se encontra associado com a enxaqueca. Estes resultados reforçam o papel dos neurotransmissores na patofisiologia da enxaqueca. Verificou-se que a variante rs702757 do gene *EDNRA* é um factor de susceptibilidade para a MO, tal como anteriormente descrito. Contudo, não é possível excluir o envolvimento deste gene na MA na nossa população, dado ter-se encontrado uma tendência em termos de risco aumentado conferido pelo rs5333 na amostra de doentes com MA. Por seu lado, estes resultados reforçam o papel da componente vascular na enxaqueca. Foi também analisado o *BDNF*, como modulador da plasticidade nociceptiva trigeminal e, pela primeira vez, o *CGRP*, um mediador da inflamação neurogénica, assim como uma possível interacção entre eles, devido à sua ligação na patofisiologia da enxaqueca. Não foram encontrados resultados significativos, exceptuando uma interacção significativa entre o *BDNF* e o *CGRP*.

Em conclusão, usando diversas estratégias, caracterizou-se um grupo de famílias portuguesas, em termos da agregação familiar da enxaqueca e factores de risco envolvidos, incluindo polimorfismos em genes relacionados com a susceptibilidade da enxaqueca, o que permitiu expandir o nosso conhecimento sobre os diversos mecanismos da enxaqueca.

Résumé

La migraine est une maladie qui présente une prévalence élevée, affectant la qualité de vie des malades et de leurs familles. Les deux sous-types plus communs sont la migraine avec (MA) et sans aura (MO). Jusqu'à présent, le diagnostic de la migraine s'appuyait uniquement sur le diagnostic clinique car les biomarqueurs disponibles sont rares et les gènes impliqués dans les formes communes n'ont pas encore été identifiés. Le diagnostic a bien avancé avec la classification et les critères de diagnostic de la "International Headache Society" (IHS) pour les différents types de céphalées.

Plusieurs études ont démontré que la migraine a une composante génétique avec de multiples facteurs génétiques impliqués qui, associés à des facteurs environnementaux, déterminent la susceptibilité à la migraine. Un des objectifs de ce travail était de vérifier, dans un échantillon bien large de familles portugaises, la présence d'une composante génétique de façon à poursuivre des études génétiques. L'agrégation familiale de la migraine a été évaluée, ayant été estimé le risque relatif (RR) utilisant la prévalence de la population en général comme référence. Les membres de la famille en premier degré des malades présentent un risque augmenté 3 à 4 fois supérieur, ce qui suggère une composante génétique. Pour les deux sous-types, il paraît y avoir des mécanismes étiologiques communs et spécifiques.

La description de l'histoire de famille par le probant a été évaluée et malgré la faible sensibilité, elle a présenté une spécificité élevée et une grande valeur prédictive positive (PPV) étant ainsi une méthode de collection d'information importante qui peut être utilisée pour maximiser le nombre de familles informatives par exemple pour des études d'analyse de connexions.

On a aussi évalué les facteurs de risque qui pouvaient contribuer pour le risque augmenté observé chez les membres de la même famille. Le sexe du parent a été le seul facteur significatif et seulement pour la MO, ayant les femmes un risque supérieur. Ce résultat montre que la MA a une composante génétique plus forte que la MO. On a vérifié que la transmission mère-fille était plus fréquente qu'attendu, ce qui peut être expliqué par des facteurs associés au DNA mitochondrial, à des gènes associés aux chromosomes sexuels ou à des effets hormonaux.

Malgré les importantes implications cliniques des nouveaux critères de la ICHD-II, ils n'ont pas introduit des différences significatives dans nos résultats épidémiologiques. On a aussi vérifié que, même pour un échantillon clinique, la ICHD-II ne présente pas de

différences significatives par rapport à la MO et à la MA. Il est ainsi possible uniformiser le diagnostic des malades inclus dans l'étude avant et après 2004.

Notre objectif s'est ensuite centré dans l'identification de variantes associées à la susceptibilité à la migraine dans notre population. Pour cela, on a utilisé une stratégie cas-contrôle, ayant analysé 188 cas (111 avec MO et 77 avec MA) et 287 contrôles, pour lesquels a été exclue la présence de migraine, appariés par âge avec les cas. On a obtenu une raison cas-contrôle de 1:1.5. Les gènes candidats ont été sélectionnés en tenant en compte leur possible fonction liée à la pathophysiologie de la migraine. Pour le gène *STX1A* on a confirmé l'implication du SNP rs941298 ayant aussi trouvé une association entre le SNP rs6951030 et la migraine. Ces résultats renforcent le rôle des neurotransmetteurs dans la pathophysiologie de la migraine.

On a vérifié que la variante rs702757 du gène *EDNRA* est un facteur de susceptibilité à la MO, comme avant décrit. Il n'est, cependant, pas possible exclure l'implication de ce gène dans la susceptibilité à la MA dans notre population, étant donné que l'on a trouvé une tendance se rapportant à un risque augmenté par rapport au SNP rs5333 dans l'échantillon de malades avec MA. Ses résultats renforcent le rôle de la composante vasculaire dans la pathophysiologie de la migraine. On a aussi étudié le *BDNF*, autant que modulateur de la plasticité nociceptive trigémينية et, pour la première fois, le *CGRP*, un médiateur de l'inflammation neurogénique, bien comme une possible interaction entre les deux due à leur connexion avec la pathophysiologie de la migraine. On n'a pas trouvé de résultats significatifs excepté pour l'interaction entre le *BDNF* et le *CGRP*.

En conclusion, en utilisant différentes méthodes, on a caractérisé un groupe de familles portugaises, en ce qui concerne l'occurrence de la migraine, facteurs de risque impliqués, incluant des polymorphismes dans des gènes associés à la pathophysiologie de la migraine, ce qui a permis élargir nos connaissances sur les mécanismes de la migraine.

1. INTRODUCTION

1. Introduction

1.1. Primary Headaches

There are several different headache subtypes, that differ in pain localization, associated symptoms, prevalence, incidence, duration and severity (1). Headaches are described as of one the major reasons to attend a neurologist consultation (2) and have a high impact in patients and their families' life (3, 4).

The "International Headache Society" (IHS) published in 1988 the classification and diagnostic criteria of the different headache subtypes (ICDH-I)(5) and in 2004 this classification was revised (ICHD-II) (6).

Headaches are divided in two major groups: primary and secondary headaches (6). There is also a third minor group constituted by cranial neuralgias, facial pain and other headaches.

Primary headaches are idiopathic and are not caused by other underlying disorder. In contrast, secondary headaches can be caused by an infection, head trauma, stroke, a brain tumor or other diseases (7).

In the 2004 classification (ICHD-II), primary headaches comprise four categories:

- 1) Migraine
- 2) Tension-type headache (TTH)
- 3) Cluster headache and other trigeminal autonomic cephalalgias
- 4) Other primary headaches (e.g. primary thunderclap headache, hemicrania continua, etc)

1.2. Migraine

Migraine is a common disabling primary headache, leading to a diminished quality of life in both migraineurs and their relatives (8). Migraine has also a social and economic impact (1), representing a public health problem (it is the 19th cause of disability on WHO ranking) (9). Migraine is often underestimated or misdiagnosed and many migraineurs are not effectively treated, which makes migraine one of the most untreated neurological diseases (10). Therefore, understanding migraine epidemiology and the mechanisms underlying this disease could lead in the future to the development of new therapeutic approaches.

1.2.1. Classification

Migraine diagnosis has suffered a great improvement when IHS published the classification and diagnostic criteria of the different headache subtypes, since migraine diagnosis is still in general based on clinical diagnosis because there are only few genetic biomarkers available for migraine (5, 6, 11, 12). This classification has contributed to improve migraine diagnosis, by introducing more clear definitions which allowed that clinical heterogeneity could be reduced in subsequent epidemiological and genetic studies (11).

1.2.1.1. Migraine with and without aura

Migraine is divided in two major subtypes:

1.1. Migraine without aura (MO)

1.2. Migraine with aura (MA)

MO occurs in about 70% of the migraineurs and MA in 30% (13). Both subtypes can co-occur in 15%-33% of the patients (10, 14). In this case, both diagnostic codes are filled (6, 7).

The other migraine subtypes are (6):

1.3. Childhood periodic syndromes that are commonly precursors of migraine

1.4. Retinal migraine

1.5. Complications of migraine

1.6. Probable migraine

According to ICHD-II (6), MO is characterized by recurrent headache episodes, lasting 4-72 hours. Typically, pain has a unilateral location, throbbing, with a mild intensity in an early phase, increasing to severe in a later phase. Also, pain can be aggravated by routine physical activity, in association with nausea and/or photophobia and phonophobia (6, 15). However, clinical diagnosis is made according a combination of criteria and migraine can also be characterized, for instance, by a bilateral, not throbbing pain if the other criteria are fulfilled (7).

MA, in addition to these characteristics, is also characterized by transient focal neurological symptoms (the aura), which can be visual (flickering lights, spots, lines or loss of vision), sensory (pins, needles, numbness), aphasic or motor, preceding or accompanying the headache (6). Typically, aura symptoms develop over 5 minutes and last no more than 60 minutes. Additionally, the aura can occur even in the absence of the headache (6, 7).

According to ICHD-II, MA can be divided in:

- 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 (FHM)
- 1.2.5. Sporadic hemiplegic migraine
- 1.2.6. Basilar-type migraine

1.2.1.2. Familial Hemiplegic Migraine

Familial hemiplegic migraine (FHM) is a rare form of migraine with aura characterized by reversible motor weakness (hemiparesis). With the exception of motor weakness, FHM episodes are similar to MA. Typically, at least three or four aura symptoms occur during an FHM attack (frequently in the temporal order: visual, sensory, motor, aphasic) (16, 17).

To be considered as familial, at least one first or second degree-relative must have also hemiplegic migraine. If the cases are sporadic, with no relatives affected, the diagnosis is sporadic hemiplegic migraine (SHM) (6).

1.2.1.3. Migraine and Tension-type headache

Tension-type headache (TTH) is more prevalent than migraine although with less individual impact in the patients (3, 7). Regarding clinical diagnosis, TTH differs from migraine since the pain is typically bilateral, non-throbbing, mild to moderate in intensity and is not aggravated by routine physical activity. Also, nausea is not an associated symptom but the patients can have photophobia or phonophobia (6, 7).

However, migraine is often misdiagnosed as TTH, since some patients have mixed features. The triggers are often the same for migraine and TTH and both are more prevalent in women than men, despite the female:male ratio being higher in migraine (18).

Migraine and TTH frequently co-occur, which also complicates diagnosis (18). There is some controversy if migraine and TTH are distinct entities, however, the coexistence of the two conditions must be due to chance, a consequence of an association between highly prevalent types of headaches and not due to a causal relationship (19-21). It has been described that TTH in migraineurs is not significantly more prevalent than in nonmigraineurs and that more than 80% of migraineurs experience also TTH (19). Migraine and TTH are therefore considered as separate entities with different pathophysiology, risk factors and age and sex distribution (19).

1.2.2. Clinical Episodes

Migraine is in general characterised by attacks of severe headache pain and autonomic and neurological symptoms (10).

Migraine episodes can be divided into five phases (Blau 1984; Silberstein 2004; Blau 2005):

- 1) Premonitory symptoms (prodromes): may occur hours to days before the attack in 20-60% of migraineurs that experience some psychological, neurological, constitutional or autonomic symptoms such as depression, mood changes, food cravings, fluid retention, etc (22-24).
- 2) Aura: occurs in about 30% of the patients (10, 13).

- 3) Headache phase: migraine headache can be divided into a early phase (with a mild intensity) and a late established phase (increasing to severe) (15).
- 4) Resolution phase: often the headache disappears after sleeping, which is called sleep resolution (23, 24).
- 5) Postdromes: after the headache disappears, some symptoms remain; for instance, fatigue, irritability, mood changes. This phase may last until 48 hours (15, 24).

These different phases may not be all present and the phases' characteristics can be different between patients (25).

1.2.3. Pathophysiology

Migraine episodes seem to be initiated by specific triggers, when a certain internal threshold is reached, exceeding the adaptive capabilities of the nervous system. Internal and environmental factors (such as hormonal, emotional, nutritional, physiological or weather changes) may act as modulators of this threshold, leading to the activation of several pathophysiological mechanisms (10, 15, 26). However, pathophysiology of migraine is still being widely discussed, since it is still not well understood (10).

Pathophysiology studies have been focused on the mechanisms and structures underlying pain development (27). During some years, a vascular hypothesis has been accepted, proposing that pain was caused by the dilatation of cranial vessels (27). However, this theory has been abandoned and more recently, theories have been focused on neuronal pathophysiology and neurogenic inflammation (26-29). These theories have been supported by evidences of changes in the brainstem and in the trigeminovascular system (TGVS) and by the involvement of central modulation (26, 29).

A neuronal hyperexcitability seems to be the basis of migraine pathophysiology due to an abnormal activity of the brainstem (with changes in the brainstem cerebral blood flow during migraine episodes) and abnormal activation of the TGVS (28, 30). The abnormal activity of the brainstem would activate structures involved in nociception control and regulation (30). After the activation of brainstem, a neurogenic inflammatory reaction would occur with the release of neuropeptides (such as substance P, neurokinin A or CGRP) at the trigeminovascular nerve endings near the meningeal vessels, leading to vasodilatation, plasma extravasation and release of pro-inflammatory substances (28, 30, 31). The neuropeptides released would activate and sensitize the trigeminal firing,

sustaining the headache and in some cases leading to allodynia (28, 30). The pain impulses would be transmitted to the trigeminal nucleus caudalis (TNC) that subsequently would transmit the pain signals to the higher centres of the brain, including thalamus and cortex (28, 29) (Figure 1).

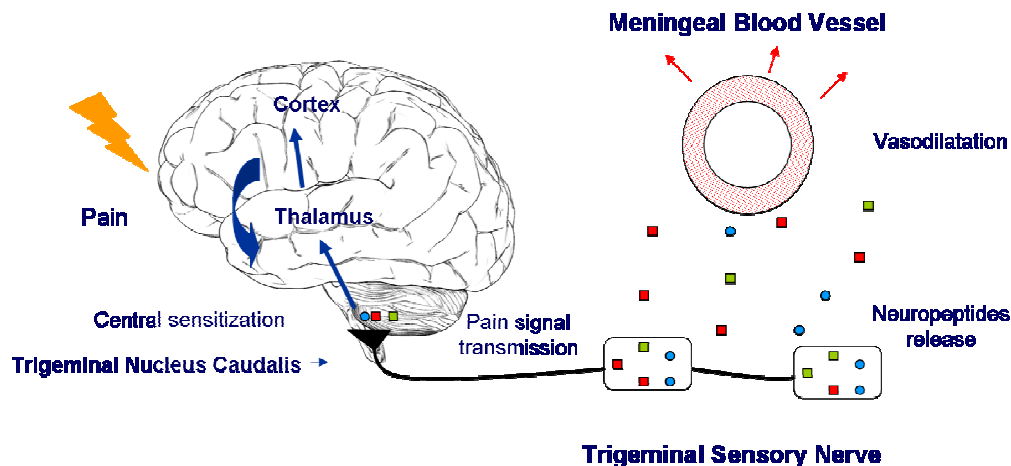


Figure 1. Schematic representation of migraine pathophysiology (Adapted from <http://www.humgen.nl/lab-frants/migraine/Introduction.htm>).

The pain mechanism would then arise from a combination of activation of the nociceptors and abnormal functioning of the pain control mechanisms, leading to altered perception of normal stimulus such as, for instance, normal light (29).

The mechanisms leading to an abnormal activity of brainstem and TGVS are still in discussion (28). In some studies, a hyperexcitability of the cerebral cortex was found in MA and MO patients. Cortical spreading depression (CSD) is a slowly propagating wave of neuronal and glial depolarization spreading across the cortex (at a rate of 3-5 mm/min) followed by long-lasting neural suppression, with cerebral blood flow changes (28, 32, 33). During CSD, it is observed a decrease in brain ion homeostasis and an efflux of excitatory aminoacids that will activate TGVS, leading to neurogenic inflammation and central sensitization (28, 33, 34). Several studies demonstrated that CSD is the underlying mechanism implicated in the neurological changes (visual, sensory or motor aura) observed in MA (34, 35). In MO, some imaging studies suggest that CSD may occur in silent brain areas, leading to pain but not to the other focal symptoms (35, 36).

1.2.4. Epidemiology

1.2.4.1. Incidence Studies

The incidence rate of migraine is estimated by determining the number of new cases developing migraine per unit of time, divided by the total number of individuals studied (37, 38). Migraine incidence studies are not so common as prevalence studies and the different results found are due to different populations studied and different methodologies used (39).

Incidence rate is usually estimated from cross-sectional or prevalence data, whereas longitudinal studies are more scarce (38, 40). A longitudinal study found a migraine incidence of 8.1 per 1000 person-years, with the incidence rate decreasing with age (40).

The peak of migraine incidence is earlier in boys than in girls and is also earlier for MA than MO. Among females, migraine onset may occur after their late twenties while in males it is uncommon (41). Migraine incidence rate is in general higher for females than males (41). A Danish study found an incidence rate of 5.8 per 1000 person-year for females and an incidence rate of 1.6 per 1000 person-year for males (42). In a sample of young adults, migraine incidence rate was 5.0 per 1000 person-years in males and 22.0 per 1000 person-years in females (43). Another study that was based on medical records estimated an average migraine incidence of 3.4 per 1000 person-years, lower than the previous estimates which can reflect some methodological bias since many migraineurs do not seek for medical care (44).

1.2.4.2. Prevalence Studies

The lifetime prevalence of migraine reflects the proportions of individuals who ever had migraine during their lifetime while one-year prevalence represents the proportion of individuals that had migraine in the last year (38). Migraine is a highly prevalent disorder (1), with prevalence values ranging between 11-27.5% (3, 4, 45).

Migraine prevalence has been studied in several countries, however results differ between studies, due to different methodological approaches, ascertainment bias and different diagnostic criteria (46). With the introduction of the IHS classification, clinical diagnosis is now more homogeneous between studies (47). Regarding ascertainment bias, many studies are based in clinical data, which underestimates prevalence values because many migraineurs do not seek medical care. Gender and age distribution of the study samples,

as well as case definition, represents approximately 70% of the variation in the results found (13, 48, 49).

Prevalence increases with age, with the highest values between 25 to 55 years, decreasing after middle age (4, 46). Regarding gender, before puberty, prevalence is higher in boys than in girls, increasing after puberty more rapidly in girls (46). Gender ratio ranges between 4-2 females: 1 male (50-53).

Migraine prevalence values are also different across the continents (3, 46). Migraine is most prevalent in Europe (15%) and lowest in Asia (<10%) and Africa (5%) (3).

In Portugal, there are only two studies of prevalence published: one, in a group of University students, which obtained a migraine prevalence of 6.1% (54); 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) (21, 48, 55).

1.2.5. Comorbidity

Comorbidity refers to an additional coexistent condition in a patient with an index disease or an association not due to chance between two disorders (56, 57). The occurrence of comorbidity is relevant because the clinical course of the index disease can be altered by the co-occurrence of other condition (56). Furthermore, comorbidity can have a impact on quality of life, health care and mortality (58).

Several studies showed that migraine often co-occurs with other somatic, psychiatric and neurological disorders (57, 59). Epilepsy, stroke, hypertension, depression, anxiety disorders, gastrointestinal disorders and allergic conditions are some of the most frequent disorders associated with migraine (25, 57, 59, 60). White matter abnormalities such as CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leucoencephalopathy) and MELAS (Mitochondrial Encephalopathy with Lactic Acidosis and Stroke-like episodes) can also co-occur with migraine. CADASIL can be associated with MA in 33% of patients (25). In addition, hereditary vascular retinopathy (HVR) can also be associated with migraine (61, 62).

These comorbidities can be useful to study the mechanisms underlying migraine, to ascertain the risk factors involved the aetiology of both disorders and to interfere more efficiently regarding therapeutics and prophylaxis (25, 60).

1.2.6. Migraine: assessment of genetic effects

In the study of a common disorder, like migraine, is important to assess if there is a genetic contribution in the etiology of the disease and which is the disease's mode of inheritance.

1.2.6.1. Familial aggregation studies

Family studies have an important role in assessing the genetic component of a disease. This assessment will depend on the magnitude of the genes' contribution to the disease (63).

In familial aggregation studies, the frequency of the disease in relatives of affected individuals (probands or index cases) is compared with the frequency of the disease in relatives of non-affected individuals or with the disease prevalence in the general population (64, 65). One common measure of familial aggregation is Relative Risk (RR) that is given by the ratio between the frequency of the disease in relatives and the prevalence in the population, providing some insights on the magnitude of the genetic effects underlying the disease. If there is a genetic component underlying the disease, RR should be higher than 1 (65).

The familial aggregation can also be due to environmental factors therefore it is important to assess RR in spouses to exclude possible shared environmental factors and evaluate RR in relatives with different degrees of kinship (65). It is noteworthy that RR may be underestimated if there is incomplete disease penetrance or, on the contrary, overestimated if affected relatives are more ascertained (65).

For migraine, several familial aggregation studies have been performed in different populations. Different approaches were used to estimate familial aggregation; some studies are clinically based (66, 67), while others are population-based (68-70). A twin population-based survey also evaluated RR for first-degree relatives of concordant and discordant monozygotic twin pairs (71).

In all these studies, an increased risk for relatives of migraineurs has been described, with values ranging between 1.4 and 11.8 (66-71). Regarding migraine subtypes, it was found that MA and MO have a different pattern of RR. In a Danish study, first-degree-relatives of probands with MO had a 1.9-fold increased risk of having also MO, while 1.4-fold risk of MA, when compared with the general population (69). However, first-degree relatives of probands with MA had almost a 4-fold increased risk of MA but do not have an increased risk for MO. The results of this study suggested that MA is determined essentially by

genetic factors, whereas MO is a combination of genetic and environmental factors (69). Another study found that relatives of probands with an early migraine age at onset as well as relatives of probands with more severe migraine had higher RR and therefore higher levels of familial aggregation (68).

1.2.6.2. Heritability and twin studies

Heritability (h^2) is the proportion of the total variance that can be attributable to genetic effects (65, 72). Heritability ranges between 0 and 1 and this estimate can be useful to quantify the genetic component of a disease (65, 72).

Although heritability estimates are usually performed for quantitative traits, these estimations can also be applied for discrete traits (72), such as migraine. Twin pairs have been extensively used in migraine heritability studies to disentangle the influence of genetic and environmental factors, using different approaches, although other relatives can be used (65, 73).

Several studies analysed migraine heritability, with values ranging between 0.28 and 0.65 (74-80).

The values obtained for heritability showed that migraine phenotypic variation is mainly due to multiple genetic factors, although these factors are not responsible for all the variance found in migraine, but in addition, there must be specific environmental factors contributing to migraine liability (76, 80). A higher concordance for MA or MO was found between monozygotic (MZ) twins than in dizygotic (DZ) twins, showing that additive genetic factors contribute to migraine susceptibility (73, 81). When comparing twins raised together and apart, it was also found that the environmental effects that may have a role in migraine liability are non-shared (76). Other twin studies also found that essentially both additive genetic factors and an unshared environmental component are involved in migraine etiology although non-additive effects can also influence migraine susceptibility (75, 78, 79). Furthermore, it was found that h^2 do not differ between genders in six countries studied and also in other study no remarkable gender differences were found in the proportion of migraine liability attributable to genetic factors (75, 78).

1.2.6.3. Segregation Analysis

Segregation analysis is a powerful method to establish a model of inheritance for the trait (82). For complex diseases, segregation analysis can also be useful for the establishment of several parameters that can be used in subsequent analyses like linkage analysis (72).

According to some segregation studies performed, there is some genetic heterogeneity in the liability of migraine and most likely this is a complex disease with a multifactorial mode of inheritance. Autosomal dominant or recessive inheritance are more unlikely, also as mitochondrial or X-linked inheritance, although the presence of a major gene can not be ruled out (67, 83, 84).

1.2.6.4. The multifactorial threshold model

All these studies suggest that multiple genetic factors are involved in migraine, in addition to environmental factors, determining liability for this disorder (25). Therefore, as for other common diseases, migraine is a complex trait and individuals are affected when liability to migraine exceeds a certain threshold (Figure 2). Migraine liability seems to be modified according to the multiple genetic and environmental factors involved, being normally distributed. Affected individuals may have different liability thresholds as for instance males have a higher migraine liability threshold than females (71, 72, 85).

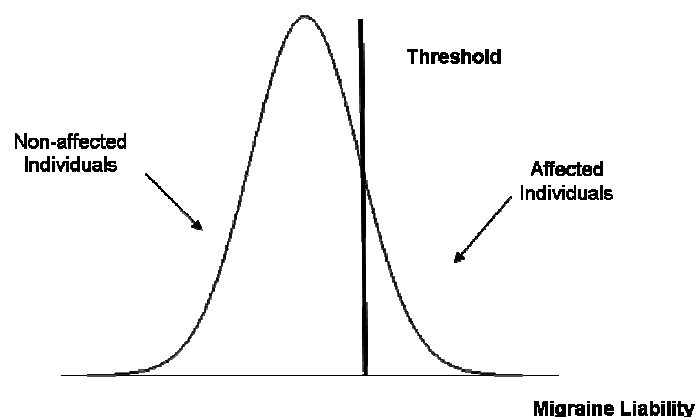


Figure 2. The multifactorial threshold model, with a liability threshold dividing non-affected and affected individuals.

1.2.7. MA and MO: distinct entities or the same?

One question that is still on discussion is whether MA and MO are distinct entities or the same. MA and MO often co-occur in the same individual and within the same families (77). Also, it was found that FHM patients may experience different migraine episodes changing from hemiplegic migraine to severe MO or MA in different times in life as well MO patients can also develop MA episodes later in life (86, 87). The high frequency of the co-occurrence of MA and MO show that this co-occurrence can not be explained only by chance and patients with this mixed phenotype would be liable to both types of attacks, depending on the action of environmental factors (86).

Some authors state that MA and MO do not have the same etiology and that MA can have a particular genetic component regarding aura and a higher genetic load, also evidenced by the different patterns on RR (69, 88, 89). Additionally, some studies identify some differences between MA and MO regarding some epidemiological features, age at onset, age at resolution, patient behaviour during attacks, trigger factors, different correlation with female reproductive events, comorbidity and different therapeutics (90).

Conversely, some studies point out that the co-occurrence of MA and MO in the same individual and within the same families show that these two subtypes are not distinct entities and that there is a continuum of severity, with MA being a more severe subtype but not etiologically different from MO and in the same dimension of liability (77, 78). Also, other study found an overlap of neurological symptoms and nonmutual exclusivity of aura symptoms, not supporting the hypothesis that MA and MO are distinct entities (79). Therefore, when searching for migraine susceptibility genes, it is expected that some genes are involved in both migraine subtypes susceptibility whereas some genetic modifiers and environmental factors may contribute specifically to MA or MO liability (77).

1.2.8. Genetic mechanisms

1.2.8.1. FHM genes

FHM is a Mendelian form of migraine and is the only subtype genetically characterized, presenting an autosomal dominant mode of inheritance. This rare subtype is genetically heterogeneous and mutations in three genes have been found to be involved in FHM: *CACNA1A* on chromosome 19p13 (which is a voltage-dependent calcium channel subunit),

ATP1A2 (a Na^+/K^+ pump) on chromosome 1q21–31 and *SCN1A* (a voltage-dependent sodium channel subunit) on chromosome 2q24 (25).

CACNA1A was identified in 1996 and encodes a pore-forming α_1 subunit of the neuronal voltage-gated calcium channel $\text{CaV}2.1$ (or P/Q type Ca^{2+} channel) (16, 91). Several different missense mutations in this gene have been described as associated with FHM1, in important functional regions such as the pore inside layer and the voltage sensors (16). Mutations in *CACNA1A* are responsible for about 50% of all cases of FHM (25). Mutations in this gene are also involved in episodic ataxia type 2 (EA-2) and Spinocerebellar Ataxia Type 6 (SCA6). Some patients with FHM1 also present epilepsy or cerebellar symptoms (16, 25).

Mutations in *ATP1A2* were found in 2003 in FHM2 families (92). This gene encodes a transmembrane transporter, Na^+/K^+ -ATPase α_2 subunit (25, 92). Mutations in *ATP1A2* can also cause permanent mental retardation (in children belonging to families with FHM), epilepsy, including benign childhood epilepsy (benign familial infantile convulsions) or alternating hemiplegia of childhood (AHC) (25, 93, 94). Rare transient and permanent cerebellar signs can also be found in patients with FHM2 (95).

SCN1A was the third gene to be found to be involved with FHM. This gene was identified in 2005 in a set of FHM families (96). This gene encodes a α_1 subunit of the voltage-gated Na^+ channel $\text{NaV}1.1$ and mutations in this gene have been associated with epilepsy with febrile convulsions, severe myoclonus epilepsy of infancy and some other rare epilepsy syndromes (16, 25, 96).

In some Portuguese families with this rare migraine subtype, mutations in these three genes were also found. In two families with hemiplegic migraine and progressive cerebellar ataxia, mutations in the *CACNA1A* gene were identified (97, 98), four families showed mutations in the *ATP1A2* (99, 100) and one mutation was also found in the *SCN1A* gene in a family with hemiplegic migraine and epilepsy (101).

More recently, a genome-wide scan was performed in a Spanish family with FHM and a locus on 14q32 was found. The family had also had family members with MA and others with MO. Mutation screening in some candidate genes located within this region were negative (102).

1.2.8.2. Searching for migraine susceptibility factors

The identification of the genetic factors involved in migraine susceptibility has been a challenging task, since this is a complex disease with many interacting genetic factors involved, influenced by environmental factors (103).

Linkage and association studies in a complex disorder

The search for susceptibility factors in complex disorders have been improved with the advances in genotyping technologies and the Human Genome Project, unravelling a large number of polymorphisms throughout the genome, such as single-nucleotide polymorphisms (SNPs), short-tandem repeats (STRs) and copy-number variants (CNVs) (104, 105).

Linkage analysis can be useful to identify genome regions where the disease locus is located and can be the first step to search for the causative genes responsible for a disease (106). Linkage analysis has been frequently used to unravel loci responsible for Mendelian disorders (e.g. Huntington), however it has also been used to map loci involved in complex disorders (e.g. bipolar disorder) (104, 107).

Two loci are linked if they lie physically connected in the same chromosome and do not segregate independently to the offspring. One approach (parametric linkage), used commonly for Mendelian disorders, is based on the co-segregation between a marker and the disease within families, which makes possible to infer the relative position of the disease locus (106, 107). This method is based on LOD score estimation and in the recombination fraction (θ) and requires that several parameters are known such as the mode of inheritance, the gene penetrance and the allele frequency (107). A LOD score is the $\log_{10}$ of the likelihood of the odds ratio for linkage. A LOD score of 3.0 or more show evidence of linkage while with a LOD score of -2.0 or less we can exclude linkage in that region (108).

According to some authors, for complex traits the classical threshold of LOD 3.0 used in Mendelian diseases need to be corrected for 3.3 in order to achieve the genome-wide significance level of 0.05. For suggestive linkage, the authors propose a LOD score of 1.9 as a threshold for mapping loci involved in complex diseases (109).

For complex diseases, there is no clear mode of inheritance and the parameters such as disease allele frequencies or gene penetrance can not be accurately estimated (106, 107). Although is possible to use this approach for complex diseases, simulating those

parameters, non-parametric or model-free linkage analysis has been used for mapping the gene loci involved in complex disorders. This approach is based on the search for an increased sharing of marker alleles identical by descent (IBD) (inherited from a common ancestor) between affected relatives (affected siblings or other relatives) (106, 110).

While linkage deals with a relation between loci in a family level, association refers to a relation between alleles across the population (107, 111). Genetic association studies evaluate the correlation between genetic polymorphisms and a trait or a disease (112).

The association found between a trait and a given polymorphism can have different explanations: 1) the polymorphism studied has a causal role on the disease, with a direct biological action; 2) the polymorphism analysed is in linkage disequilibrium (LD) with the real susceptibility allele located nearby, being transmitted together for many generations, with no recombination occurring over time between them and consequently affected unrelated individuals will have the same allele of the polymorphism genotyped; and 3) the association can be due to population admixture or stratification, due to different background allele frequencies, which will bias the results (112, 113).

Association studies can be performed using two different types of strategies: a case-control or a family-based approach. In a case-control study, allele frequencies of the markers are compared between cases and controls (107). Case-control samples have been extensively used in association studies, with the main advantage of cases being easily ascertained, providing risk estimates of association (104, 112, 114). However, it is difficult to obtain matched control groups and this method is prone to population stratification (112).

In the family-based approach, the typical method employed is called transmission disequilibrium test (TDT), which compares the transmission and non-transmission of marker alleles from parents to affected offspring (115). This method is robust to population stratification, however requires the parental genotypes and heterozygous parents, which reduces the statistical power of this test (107, 114). Others methods have been developed to overcome the need of parental genotypes, such as sib-TDT that assess differences in allele frequencies between affected and non-affected siblings (116). Although a case-control approach is usually preferred, a family-based approach can be an useful complementary strategy (112).

With the identification of a large number of SNPs in the genome and the decrease in the genotyping costs, attention has been turned to association studies, mainly due to fact that many interacting susceptibility genes with small effects are involved in complex diseases

(112, 117). Two methodologies have been employed in the search for susceptibility genes, a candidate gene and a genome-wide approach.

In a candidate gene approach, candidate genes are chosen based upon the biological pathway where they are involved or due to their biological function, therefore, the correct choice of a candidate gene is crucial for the identification of an association between a gene and the disease and by using this approach it is required that the mechanisms underlying the disease are known (117). The alternative approach is to use markers along the entire genome (genome-wide association (GWA)), which is a more expensive approach and due to the high density of markers can lead to some false positives. However, some genome-wide association studies have been capable to identify almost 100 loci for nearly 40 common diseases and traits, unravelling new biological pathways (118).

For complex diseases, association studies seem to be a more promising approach since association can be more powerful to detect common susceptibility alleles with small effects (with $RR < 2$), whereas linkage can be more successful in identifying variants with strong effects for Mendelian diseases (111, 117, 119-121). However, these assumptions are still in debate, in particular because if the “Common Disease-Common Variant” (CDCV) hypothesis has been in general accepted, there are some evidences that rare alleles are also involved in common diseases susceptibility and could explain a larger proportion of the familial risk (122).

FHM genes and common migraine

Several studies focused on FHM to study the genes involved in the common forms, since FHM, MA and MO may share some pathophysiological mechanisms, FHM patients can also have some non-hemiplegic episodes of migraine, in particular MA and mutations in FHM genes have been also found in relatives with non-hemiplegic migraine of FHM probands (25, 87, 89, 103, 123, 124).

However, the results are contradictory. Some studies found that the region around *CACNA1A* is implicated in MA and MO (possibly, with variants in an adjacent gene conferring a higher susceptibility to migraine), other studies showed the involvement of the 19p13 locus in these subtypes, although other authors could not demonstrate these hypothesis (125-135).

The involvement of the FHM2 locus in MA or MO is also not clear. Mutations in *ATP1A2* have been found in families with basilar-type of migraine and in families with MA or MO; however, other studies found no evidence of a major involvement of *ATP1A2* in common forms of migraine (135-140).

Although the connection between the FHM genes and the common forms of migraine is not clarified, the pathways where these genes are involved can be important to understand migraine pathophysiology and susceptibility (25).

Linkage studies

Some loci have already been identified for the common subtypes of migraine by using a genome-wide screen or a candidate region approach as shown in Table 1 (25, 64). Also, a genome-wide scan was performed in a family with occipitotemporal lobe epilepsy associated with MA and found linkage with a region located at 9q21-22, which favours molecular mechanisms shared by epilepsy and migraine (141).

Table 1. Loci identified for common migraine subtypes

Approach	Migraine subtype	Population	Loci	Reference
Genome-wide screen	MA	Finnish	4q24	(142)
Genome-wide screen	MA	Canadian	11q24	(143)
Genome-wide screen	MO	Icelandic	4q21	(144)
Genome-wide screen	MO	Italian	14q21.2-22.3	(145)
Genome-wide screen	MA and MO	Swedish	6p12.2-21.1	(146)
Genome-wide screen	MA and epilepsy	Belgian	9q21-22	(141)
Candidate region	MA	Italian	15q11-13	(147)
Candidate region	Migraine	Australian	1q31	(148)
Candidate region	Migraine	Australian	Xq24-28	(149)

These studies are based on IHS classification, but despite the standardization of diagnosis criteria there are still some limitations due to the absence of well-defined migraine biomarkers, to the changes observed in headache characteristics overtime and

to some artificial distinctions created by the classification since a clinical continuum of headache subtypes can be found (11, 150).

To avoid some heterogeneity between patients groups, two alternative approaches have been used in order to diagnosis migraine based on single traits or symptom components:

- 1) Latent Class Analysis (LCA): an empirical clustering approach, based on the formation of mutually exclusive (latent) classes of patients with a certain pattern of symptom combinations, according to heritability estimates. This approach is free of clinical or biological hypotheses (25, 77).
- 2) Trait Component Analysis (TCA): analyzes individual clinical symptoms, as for instance photophobia, rather than the combination of IHS criteria. Each individual trait is heritable and represents a measurable endophenotype (25, 151).

As shown in Table 2, these approaches have been successful in identifying new migraine *loci*.

Table 2. New migraine *loci* identified by LCA or TCA

Approach	Main phenotype	Loci	Evidence	Reference
LCA	Migrainous headache	Chr 1	Suggestive linkage (Replication)	(152)
LCA	Phonophobia and photophobia	Chr5	Suggestive linkage (Replication)	(152)
LCA	Moderate/severe pain intensity	Chr10	Suggestive linkage (Replication)	(152)
LCA	Pulsating headache	Chr13	Suggestive linkage (Replication)	(152)
LCA	Migrainous headache	Chr20	Suggestive linkage	(152)
LCA	Severe migraine	18p11	Linkage	(153)
LCA	Severe migraine	3q	Linkage	(153)
LCA	Pulsating headache	5q21	Linkage	(154)
LCA	Nausea/vomiting and Moderate/severe headache	Chr 8	Suggestive linkage	(154)
LCA	Phonophobia and photophobia	Chr 10	Suggestive linkage	(154)
LCA	Photophobia	Chr 13	Suggestive linkage	(154)
LCA	Activity-prohibiting headache and photophobia	6p12.2-21.1	Replication	(154)
LCA	Photophobia	1q21-23	Replication	(154)
TCA	Several traits	4q24	Replication	(151)
TCA	Pulsation trait	17p13	Linkage	(151)
TCA	Trait combination (IHS)	18q12	Linkage	(151)
TCA	Age at onset trait	4q28	Linkage	(151)
TCA	Phonophobia trait	10q22	Suggestive linkage	(151)
TCA	Aggravation by physical exercise	12q21 15q14 Xp21	Suggestive linkage (previously reported)	(151)

LCA-Latent Class analysis; TCA-Trait Component Analysis

In a recent study, the locus 10q22-23 was consistently replicated in Australian and Finnish cohorts, using three phenotyping methods (IHS criteria, LCA and TCA). Also in that study, loci 8q21, 14q21, 18q12 and Xp21 were also replicated (155).

Using another alternative approach, a Bayesian latent trait modeling of migraine symptom data, a study found significant linkage to chromosome 7q31-q33 and suggestive linkage to chromosomes 1 and 2 (156).

These studies show evidences that several interacting susceptibility factors with low effects are involved in migraine subtypes and traditional single-locus linkage analyses do not have enough power to detect those genes and therefore, alternative approaches such as the ones described are needed and proven to be very useful (153, 154).

Association studies

In order to identify susceptibility genes for MA and MO several association studies in candidate genes have been performed, using a case-control or a family-based approach. Until now no genome-wide association study has been reported for migraine (157).

Diverse mechanisms have been proposed as contributing for migraine and variants in genes involved in neurotransmitter pathways, vascular and hormonal functions, among other pathways, have been found to be associated with this disorder. However, some results are contradictory and replication is needed to confirm the observed association in other populations (25, 103).

1) Neurotransmitter system

Several neurotransmitters are involved in migraine pathophysiology such as acetylcholine, catecholamines (dopamine and norepinephrine) or serotonin, among others (103).

Serotonergic System

The serotonergic system has been extensively studied in migraine pathophysiology and several association studies have been performed with the different components of this system such as the 5-HT transporter gene and the several 5-HT receptors genes (103, 158).

Changes in 5-HT serum levels during migraine episodes have been observed and 5-HT seems to exert a role in nociception modulation (159-161). More importantly, triptans are

selective serotonin receptor agonists, restoring the effects of 5-HT and have been prove to very useful in migraine therapeutics (162).

A summary of association studies performed in the serotonergic system can be found in Table 3, with contradictory results. Additionally, enzymes involved in the metabolic pathways or in the degradation of 5-HT were also studied, such as monoamine oxidase A (MAO-A), monoamine oxidase B (MAO-B), tryptophan hydroxylase (TPH) or amino acid decarboxylase (AADC) (163-166).

Table 3. Migraine association studies performed in the serotonergic system

Association	Result	References
Serotonin receptors		
5-HT _{1A} , 5-HT _{1B}	Specific clinical symptoms	(167)
5HT _{2C} (Meta-analysis)	n.s	(168)
5-HT _{1A}	n.s	(169)
5HT _{1B-1D} , 5HT _{2C}	n.s	(170)
5-HT _{2C}	MA	(171)
5-HT _{2C}	n.s	(172)
5-HT _{2A}	n.s	(173)
5-HT _{2A}	MA	(174)
5-HT _{2C}	n.s	(175)
5-HT _{2A}	n.s	(176)
Serotonin transporter		
hSERT	n.s	(177)
hSERT	MA	(178)
hSERT	MA	(170)
hSERT	Migraine	(179)
hSERT	MA and MO	(180)
Enzymes involved in serotonergic pathways		
TPH	Migraine	(163)
MAO-A, MAO-B	MAO-A and MO	(166)
MAO-A	n.s	(165)
TPH, AADC and MAO-A	n.s	(164)

Dopaminergic System

Dopaminergic hypersensitivity has been found in migraine patients, which could be the basis for the occurrence of nausea and vomiting during the attacks, with a lower threshold for dopamine receptor activation (103, 158). Also, some antidopaminergic drugs are effective in migraine treatment (158).

Several association studies have been performed regarding the dopaminergic system also with contradictory results, as shown in Table 4, including the enzymes dopamine β -hydroxylase (DBH) which regulates dopamine levels and catechol-O-methyltransferase (COMT) which degrades catecholamines including dopamine (181).

Table 4. Migraine association performed in the dopaminergic system

Association	Result	References
Dopamine receptors		
DRD2	MA	(182)
DRD4	MO	(183)
DRD1, DRD3, DRD5	n.s	(184)
DRD2	n.s	(185)
DRD2	n.s	(186)
DRD2, DRD3, DRD4	DRD2 and MO	(187)
DRD2	MA	(188)
Dopamine transporter		
DAT	MA	(182)
DAT	n.s	(183)
Enzymes involved in dopaminergic pathways		
DBH	MA	(182)
DBH	MA	(189)
DBH	n.s	(183)
DBH	MA, MO	(190)
COMT	n.s	(191)
COMT	n.s	(192)

2) Vascular function

In migraine, some abnormalities in vascular function have been described, with vasodilatation of cerebral blood vessels and release of vasoactive neurotransmitters (28, 103, 193). Some candidate genes involved in vascular function have been studied, as shown in Table 5.

One of the genes studied is the methylenetetrahydrofolate reductase gene (*MTHFR*) which encodes the enzyme 5,10-methylenetetrahydrofolate reductase that is involved in the folate metabolism, catalyzing the reduction of 5–10 methyltetrahydrofolate to 5-methyltetrahydrofolate, which in its turn will participate in the re-methylation of homocysteine to methionine (181). Homocysteine is an excitatory amino acid and increased homocysteine levels may produce vascular endothelial injury, modifying migraine threshold (157, 194, 195).

The angiotensin-converting enzyme (ACE) is involved in the synthesis of angiotensin-I, which has a role on vascular tone modulation and blood pressure regulation (103, 196). Besides other association studies based solely on ACE, a study found a positive interaction between ACE and *MTHFR* in migraine susceptibility (197). Also, the association of angiotensin (AGT) and angiotensin receptor 1 (*AGRT1*) with migraine has been assessed (198).

Other candidate genes are endothelin-1 (ET-1) which is a potent vasoconstrictor and a inducer of CSD (199). It has been shown that plasma ET-1 levels were increased during and between migraine attacks (200-202). Endothelin type A receptor (*EDNRA*) mediates the biological effects of ET-1, being expressed on vascular smooth muscle cells, and its activation by ET-1 leads to vasoconstriction (203, 204).

Nitric oxide (NO) has an important role in migraine pathophysiology acting on neurotransmission, smooth muscle motility and nociception modulation (205). In blood vessels, nitric oxide synthase (eNOS), generates NO and has also been studied as a candidate gene.

Table 5. Migraine association studies performed in genes related with vascular function

Association	Result	References
eNOS	n.s	(206)
eNOS	MA	(207)
eNOS	n.s	(208)
EDNRA	MA	(209)
EDNRA (pediatric sample)	n.s	(210)
ET-1, EDNRA, EDNRB	EDNRA	(204)
ACE and MTHFR (interaction)	MA	(197)
AGTR1, AGT	n.s	(198)
ACE	n.s	(211)
ACE	n.s	(212)
ACE	MA	(213)
ACE	MO	(214, 215)
MTHFR	protective effect on MA	(216)
MTHFR	n.s	(217)
MTHFR (meta-analysis)	MA	(218)
MTHFR	n.s	(219)
MTHFR	MA	(220)
MTHFR	n.s	(221)
MTHFR	MA	(222)
MTHFR	↑ frequency MA	(223)
MTHFR	Migraine	(224)
MTHFR	Migraine	(194)

3) Hormonal function

Several studies describe the influence of sex hormones in migraine susceptibility and also it has been described an interaction of ovarian hormones with the neurotransmitter and vascular system (41, 225-227). Candidate genes in this pathway such as estrogen receptor 1 (ESR1) and 2 (ESR2), follicle stimulating hormone receptor (FSHR), androgen receptor (AR) or progesterone receptor (PGR) have also been studied (Table 6).

Table 6. Migraine association studies performed in genes related with hormonal function

Association	Result	References
ESR1, PGR	n.s	(228)
Female Hormones	ESR1, ESR2, FSHR	(229)
ESR1	n.s	(219)
AR, PGR	PGR and migraine	(230)
ESR1	Migraine	(231)

4) Other pathways

Several other pathways have been studied regarding their involvement in migraine susceptibility, but the results are frequently inconclusive. A summary of some of the genes studied in those pathways can be found in Table 7. Genes involved in inflammatory responses have been studied due to the involvement of inflammatory mediators in migraine pathophysiology (31).

Gamma-aminobutyric acid type A (GABA-A) is the main inhibitory neurotransmitter in the brain being involved in migraine pathophysiology and some GABA-A receptors agonists are used in migraine prophylaxis (232). A locus in chromosome X has been linked to migraine and several GABA-A receptor genes which are located within this region, have been studied as candidate genes (232-234).

Regarding the possible involvement of 19p13 locus in migraine common types, some authors also assessed the possible involvement of genes located in that region in migraine susceptibility such as *NOTCH3* gene (which is the gene responsible for CADASIL and has a function in smooth muscle cell survival (235-237) the tumor necrosis factor ligand superfamily member 7 gene (*TNFSF7*) (237), the low-density lipoprotein receptor gene (*LDLR*) and the insulin receptor gene (*INSR*) (196). *TNFSF7* is homologous to the ligands of TNF-alpha and TNF-beta (237) while *LDLR* is involved in cholesterol homeostasis and it has been shown that cholesterol levels seem to be altered in MA patients (238). *INSR* is altered in migraineurs and fasting has been described as a migraine trigger (239).

Recently, an association study was performed in 155 ion transport genes, but no significant results have been found for the common variants analyzed (240).

Table 7. Migraine association studies performed in genes of different pathways

Association	Result	References
Inflammation related genes		
TNF- α , TNF- β	TNF- β and MO	(241)
TNF- α , LT- α	LT- α and migraine	(242)
TNF- α	MO	(243)
TNF- β	MO	(244)
IL-6	n.s	(245)
IL-1 α	MA, MO	(246)
GABA-A receptor system		
GABRA5	n.s	(232, 247)
GABRB3	n.s	(232, 247)
GABRG3	n.s	(232, 247)
GABRE	n.s	(233)
GABRQ	n.s	(233)
Chromosome 19p13		
TNFSF7	MO	(237)
Notch3	n.s	(237)
Notch3	n.s	(248)
Notch3	MO	(249)
INSR	MA	(239)
INSR	Migraine	(250)
LDLR	n.s	(251)
LDLR	MO	(252)
Ion transporter genes	n.s	(240)

The inconclusive results and the differences found between studies can be explained by several effects namely sample size, different clinical criteria or ascertainment, population stratification, differences in allelic frequencies and inappropriate correction for multiple testing (25, 157).

1.2.8.3. Biomarkers

Migraine biomarkers, by linking a certain biological process to a clinical outcome, can be very important in order to improve diagnosis, therapeutics and to better understand migraine pathophysiology (12, 253). In these last years, there has been an extensive search in finding genetic markers such as mutations or polymorphisms that can be used as biomarkers to identify individuals at risk, define more precise therapeutics or to understand migraine susceptibility (12, 254). However, until now only few biomarkers can be used. In FHM, the three genes found to be linked with this rare monogenic form of migraine can be used in molecular diagnosis. For MA, only the *MTHFR* C677T polymorphism has been replicated in several studies (although not in all) and can be a potential biomarker (12).

A recent study found a differential expression of α -fodrin in lymphoblasts of MA patients and an increased expression after CSD in an animal model, although it is not clear the reason for the increased expression in MA patients. Despite this fact, the authors suggest that this gene can be a probable biomarker (255).

Other biomarkers have been suggested such as CGRP, ACE and metalloproteinase-9 (MMP-9) due to the different levels found in the sera of migraine patients when compared to controls (256-258). Some polymorphisms in the serotonergic system have also been proposed as possible biomarkers (256). These evidences need to be validated in order to confirm or not the usefulness of these potential biomarkers.

It is crucial to find more susceptibility variants that can be used as biomarkers and it is important that well-designed linkage and association studies are performed in order to obtain accurate results (12). Also, it would be important to study gene-gene and gene-environment interactions in order to identify risk gene profiles (103).

1.2.8.4. Pharmacogenomics

Migraine therapeutics is not effective for all migraineurs due to the multiple genetic factors and interaction with environmental factors involved. The individual genetic variability can alter the efficacy of a certain drug by influencing the drug target or the drug metabolism (259, 260).

Triptans, as agonists of serotonin receptors are used in acute migraine episodes, although not totally effective. Also, the dopaminergic system has been studied as a target for

migraine therapeutics. There are several migraine preventive drugs such as serotonin antagonists, beta-blockers, calcium blockers, antiepileptics or antidepressants. However, these preventive drugs are also not effective for all patients (260).

Understanding migraine pathophysiology and identifying polymorphisms associated with migraine susceptibility, by creating an individual genetic profiling, could lead to the development of more specific preventive therapeutics (259, 260).

2. OBJECTIVES

2. Major Goals

The aim of this study was the genetic and epidemiological study of migraine. By studying a sample of migraine Portuguese families and using data from a population sample, our goals were to assess familial occurrence of migraine, risk factors, genotype-phenotype interactions and genetic factors involved in migraine susceptibility.

2.1. Specific Objectives

1. Assessment of the genetic component of migraine
 - a) To estimate Relative Risk as a measure of familial aggregation in first-degree relatives of migraineurs and their spouses to try to disentangle the influence of genetic and environmental factors
 - b) To assess if familial aggregation differs between MA and MO
2. Validation of probands' report in a group of migraine families regarding the putative use of that information in subsequent genetic studies
3. To assess which specific factors inside migraine families contributed to migraine risk
4. To compare the ICHD-I criteria with the ICHD-II criteria in order to evaluate whether the new ICHD criteria introduced remarkable differences in epidemiological studies (in particular in what concerns primary headaches)
5. To unravel genetic factors involved in migraine susceptibility by performing association studies in candidate genes
 - a) To confirm the role of Syntaxin 1A and endothelin receptor-A as migraine risk factors
 - b) To assess a possible interaction of brain-derived neurotrophic factor and calcitonin-gene related peptide in migraine susceptibility

3. SUBJECTS AND METHODS

3. Subjects and Methods

3.1. Subjects

Probands and relatives

At the beginning of this study, 187 probands and some of their relatives had been already ascertained from the Neurology out-patient clinic, at Hospital de Santo António (HSA) in Porto, in order to participate in clinical and genetic studies of migraine, comprising a total of 687 individuals.

In the course of this study, further 29 probands and 257 relatives were also included in our DNA bank, involving a total of 973 individuals (probands and their relatives). Additionally, we collected, by telephonic interview, clinical and familial information from more 81 relatives that were unable to come personally to the out-patient clinic.

The probands ascertained had different headaches subtypes (FHM, MA, MO, MAMO or cluster headache).

However, for this study we were only interested in probands with MA, MO or MAMO. Table 8 summarises the type of families enrolled in this study.

Table 8. Number of families according to probands' diagnosis

Probands' Diagnosis	Number of families	Family history of migraine (%)
MO	113	101 (89)
MA	56	49 (88)
MAMO	21	14 (67)

Probands were clinically observed mainly for two neurologists of HSA (Professor Pereira-Monteiro and Dr. José Barros), specialized in headaches. During the probands' interview, demographic and clinical information about relatives and spouses was obtained. Interviews to relatives and spouses were made by the same neurologist (Professor Pereira-Monteiro).

Controls

A group of 287 control subjects, with no personal history of migraine, were ascertained among healthy blood donors and from the Obstetrics and Gynecology department of HSA; they were from the same ethnic and geographical origin as the cases, and were age-matched to these. A diagnostic interview was performed in cases and controls, based on the IHS criteria, using the same structured questionnaire.

All the individuals enrolled in the study gave their written informed consent.

3.2. Methods

3.2.1. DNA extraction and quantitation

Genomic DNA for the subsequent genetic studies was isolated from peripheral blood leukocytes using a standard salting out method (261).

In brief, red blood cells were removed after centrifugation and lymphocytes were lysed with a nuclei lysis buffer and digested at 37 °C, overnight, in a proteinase K and a detergent (20% SDS) solution.

Proteins were precipitated with saturated NaCl and afterwards DNA was precipitated by the addition of ethanol 100%, retrieved with the tip of a glass pipette and washed with ethanol 70%. DNA was resuspended in an elution buffer and stored at 4 °C.

When necessary, DNA was isolated from saliva, using ORAGENE kits according to the manufacturer's instructions (DNA Genotek Inc.), resuspended in an elution buffer and stored at 4 °C.

DNA quantitation was performed in a spectrophotometer (GeneQuant *pro* RNA/DNA Calculator). Optical density (OD) was determined at 260 nm, from a DNA dilution of 1:100. DNA concentration was estimated according to the following formula: an OD of 1 corresponds approximately to 50 µg/ml of DNA. For the subsequent studies, all the DNA samples were at a final concentration of 10 ng/µl.

3.2.2. SNP selection

SNPs on candidate genes were selected based on a data dump from The International HapMap Project. Tagging SNP selection methods are based on the presence of high levels of LD between SNPs, allowing to discard redundant information and to improve the

coverage of the region of interest (262). Tagging SNPs can be grouped into three classes according with the following aims: 1) correlate each SNP of interest with a genotyped SNP (pairwise methods), 2) correlate each SNP of interest with a genotyped SNP or a combination of genotyped SNPs (multimarker methods), or 3) explain each haplotype of interest using a set of genotyped SNPs (haplotype-based methods) (262).

Tagging SNPs were selected with Haploview 4.1 (263), using r^2 as a measure of LD, at a threshold of 0.80, with a minor allele frequency (MAF) > 0.10, by an aggressive tagging approach (a multimarker method).

3.2.3. Genotyping

Real-Time Polymerase Chain Reaction and Molecular Beacons

Allelic discrimination was performed using Real-Time Polymerase Chain Reaction (iQ5 Real-Time PCR Detection System, Bio-Rad Laboratories). This technique applies the PCR basis to a real-time detection of the amplified products due to the use of fluorescent probes. In this work we used molecular beacons, which are probes that fluoresce on hybridization to their perfectly complementary targets and are indicated for SNP genotyping by Real-Time PCR (264). Molecular beacons are a single-stranded oligonucleotides with a stem and loop structure. The loop portion is complementary to the target sequence whereas the stem is formed by five to seven basepairs, complementary to each other, located on either side of the sequence (264, 265). A fluorophore and a quencher are linked to the two sides of the stem, and in the close state, the quencher is so close to the fluorophore that prevents that fluorescence is released. However, when annealing with the target sequence occurs, a conformational change takes place, which causes that the fluorophore and the quencher are apart, being fluorescence released. Therefore, for allelic discrimination, molecular beacons can be synthesized with different fluorophores, according to the SNP alleles, allowing multiplex reactions in a single tube (264, 265).

Primers and molecular beacons were designed using Beacon Designer 6.0 (Table 9 and 10).

Table 9. Primers used for association studies

SNP	Forward Primer	Reverse Primer
STX1A		
rs941298	CCTGTCTCAATCAATCAATCAG	GGGCCTTAACCCCTCTGG
rs6951030	AAAATAGATCCAAGTCGCAAGG	TGTGGCTCCCATCACCTG
rs3793243	ACTGGCTTAGTTGGTGTG	GTCAGATTTTCTTCTCACCTC
EDNRA		
rs702757	TCTTCAGCCATCAGAGTGTG	AGTCTACATAGTGTGCTTCAGG
rs5335	CGGAGCAGCCATAAGGACAG	TGGGTGTGGGACTGAATTAAGG
rs5333	TTTTCTCACTTTCCTTTAGCG	AGTAATTCACATCGGTTCTTG
BDNF		
rs6265	CCCAAGGCAGGTTCAAGAGG	ACTACTGAGCATCACCTGGAC
rs2049046	CTAAATCACAATTTTCAGGAGGAG	TCAACCAATTTGTGCAGACC
rs7124442	GGGTTGTTTGTTTTAAATTTGTCC	GAAGACTTTACGTAGTGTAGATTTG
rs11030107	GTCCCTGCATCCAAGAATAAGC	TTCTGGCATGTTCCCAATCCC
CGRP		
rs1553005	CTGGGAGCAAGAACTTTG	GATCCCTGCCTATCTGAGG

Table 10. Molecular Beacons

SNP	Probe 1	Probe 2
STX1A		
rs941298	CGCGATCTCAGCCCTCC<u>C</u>TCCATGCAGCGATCGCG	CGCGATCTCAGCCCTCC<u>T</u>TCCATGCAGCGATCGCG
rs6951030	CGCGATCAACGCCAGCCAG<u>I</u>GCTGCCCCGAAAGGATCGCG	CGCGATCAACGCCAGCCAG<u>G</u>GCTGCCCCGAAAGGATCGCG
rs3793243	CGCGATCACTTTGGGGCTGTG<u>A</u>AAAGATTTACTCGATCGCG	CGCGATCACTTTGGGGCTGTG<u>G</u>AAAGATTTACTCGATCGCG
EDNRA		
rs702757	CGCGATCCTCAAGAAAATAATTA<u>A</u>CCATCTCTGCATTTAGATCGCG	CGCGATCCTCAAGAAAATAATTA<u>I</u>CATCTCTGCATTTAGATCGCG
rs5335	CGCGATCAGGCAACTGTGA<u>C</u>TCCGGGAATCTCGATCTCGATCGCG	CGCGATCAGGCAACTGTGA<u>G</u>CCGGGAATCTCGATCTCGATCGCG
rs5333	CGCGATCGGTTCCCTCTTCA<u>C</u>TTAAGCCGTATAGATCGCG	CGCGATCGGTTCCCTCTTCA<u>T</u>TTAAGCCGTATAGATCGCG
rs10305895	CGCGATCCAATCTTTATAATC<u>A</u>TCTCAGTCTGTGCTGATCGCG	CGCGATCCAATCTTTATAATC<u>G</u>TCTCAGTCTGTGCTGATCGCG
BDNF		
rs6265	CGCGATCACACTTTCGAACAC<u>A</u>TGATAGAAGAGCTGGATCGCG	CGCGATCACACTTTCGAACAC<u>G</u>TGATAGAAGAGCTGGATCGCG
rs2049046	CGCGATCGGAGGTCCAGGG<u>A</u>CTCCAAGTTAAGATCGCG	CGCGATCGGAGGTCCAGGG<u>I</u>CTCCAAGTTAAGATCGCG
rs7124442	CGCGATAAGTTGACATA<u>C</u>AGCAGATATTCCAATCGCG	CGCGATAAGTTGACATA<u>I</u>AGCAGATATTCCAATCGCG
rs11030107	CGCGATCGCAACTGCATT<u>C</u>ATTGTCCCTAAAGGATCGCG	CGCGATCGCAACTGCATT<u>G</u>TTGTCCCTAAAGGATCGCG
CGRP		
rs1553005	CGCGATCTAACTTCACAGAG<u>C</u>CTCAGTGTACTCGATCGCG	CGCGATCTAACTTCACAGAG<u>G</u>CTCAGTGTACTCGATCGCG

In bold: the stem region; in bold and underlined: the localization of the variant 1 and 2 of the SNP

Real-Time reactions were performed using 20 ng/μL of DNA, in 20 μL of final reaction volume. In Table 11, the specific conditions used in Real-Time reactions for each gene are described.

Table 11. Specific Real-Time conditions used for each gene

Gene/SNP	Enzyme (10 μL)	MgCl ₂ [mM]	Primer Forward and Reverse [μM]	Probe 1 [μM] (Fluorophore)	Probe 2 [μM] (Fluorophore)
STX1A					
rs3793243	iQ TM Multiplex Powermix	1.5	0.5	0.08 (Texas Red)	0.02 (HEX)
rs941298	iQ TM Multiplex Powermix	1.2	0.5	0.15 (Texas Red)	0.03 (HEX)
rs6951030	iQ TM Multiplex Powermix	1.5	0.5	0.03 (FAM)	0.25 (Cy3)
EDNRA					
rs702757	iQ TM Supermix	8.0	0.5	0.05 (FAM)	0.15 (Cy3)
rs5335	iQ TM Supermix	8.0	0.5	0.05 (FAM)	0.05 (Cy3)
rs5333	iQ TM Multiplex Powermix	1.5	0.5	0.03 (FAM)	0.30 (Cy3)
BDNF					
rs6265	iQ TM Supermix	7.0	0.5	0.06 (FAM)	0.25 (Cy3)
rs2049046	iQ TM Supermix	8.0	0.5	0.04 (FAM)	0.16 (Cy3)
rs7124442	iQ TM Supermix	8.0	0.5	0.03 (FAM)	0.15 (Cy3)
rs11030107	iQ TM Multiplex Powermix	1.2	0.5	0.03 (FAM)	0.30 (Cy3)
CGRP					
rs1553005	iQ TM Supermix	8.0	0.5	0.03 (FAM)	0.15 (Texas Red)

Cycling conditions used were: an initial denaturing at 95°C for 3 min and 30 sec, followed by 35 cycles of 30 sec at 95°C, 60 sec at 60°C (for annealing, and at this point fluorescence was measured), and 30 sec at 72°C.

Genotypes were determined automatically by the signal processing algorithms of the iQ5 Optical System Software, version 2.0 (Bio-Rad Laboratories), and the results compared with the genotypes visually scored based on the fluorescent emission data depicted (RFUs). To validate the Real-Time PCR reaction, we included three individuals previously

genotyped by sequencing (one heterozygous, one homozygous for variant 1 and one homozygous for variant 2).

Sequencing

To confirm the genotypes of the individuals used as internal controls of the Real-Time PCR reaction or whenever genotypes determined by Real-Time PCR were uncertain, those individuals were genotyped by sequencing.

For sequencing, a conventional PCR amplification was performed, using 25 ng/μL DNA, 10 μL iQTM Supermix or 3.13 μL HotStar Taq Master Mix Kit (Qiagen) and 0.5 μM of each primer. Cycling conditions used were: an initial denaturing at 95°C for 4 min, followed by 40 cycles of 15 s at 95°C, 50 s at 60°C and 35 s at 72°C, followed by a final extension step of 7 min at 72°C. For PCR product purification, incubation with ExoSAP-IT was performed, according to the manufacturer's instructions. This enzymatic purification was performed in two steps: first, at 37°C, to remove remaining primers and nucleotides and, second, at 80°C to inactivate ExoSAP-IT.

Automated sequencing was performed using the Big Dye Terminator Cycle Sequencing v2.0 Ready Reaction (Applied Biosystems), according to the manufacturer's instructions and samples were loaded on an ABI-PRISM 3130 XL genetic analyzer (Applied Biosystems).

Restriction Enzyme analysis

For confirmation of rs6951030 (*STX1A*) genotypes, a restriction enzyme analysis was performed with *BsrI*, after PCR amplification, and digestion products were electrophoresed in a 2% agarose gel, stained with ethidium bromide. Bands were visualized and image acquired in a Typhoon imager (Amersham).

3.2.3. *In silico* analysis of SNPs functional effects

In order to assess a putative functional role of the SNPs studied, a PupaSuite database (266) search was performed. It was assessed if each SNP had regulatory properties (influencing transcription factor binding sites, microRNA targets, promoter flexibility and conserved regions) or structural properties (influencing splice sites, exonic splicing enhancers or silencers).

3.2.4. Statistical Analysis

Throughout this work, several statistical approaches have been used, specific for each of our objectives. The statistical analyses used for each of our aims are described below in order to have a broader view of the methods employed in the analysis of our results.

A Student's t-test was used when comparing quantitative demographic data. For comparisons between probands and the population sample, since the data do not followed a normal distribution, a log transformation was used **[article 1]**.

Categorical variables comparisons were assessed using a chi-square test, as the case of comparisons between the subgroups of probands and between probands and the population sample **[article 1]**. A chi-square test was also used to assess parents-offspring transmission of migraine **[article 3]**.

Unless specific software was required, all the analyses were performed in SPSS (version 16.0 for Windows).

3.2.4.1. Familial aggregation

Relative risk represents the ratio between the frequency of the disease in relatives and the prevalence in the population (65). RR was used as a measure of familial aggregation given that migraine is a common disorder and the odds-ratio (OR) method may lead to an overestimation of familial aggregation (267).

RR was adjusted for age at observation and sex using the Mantel-Haenszel RR, and its significance analyzed by a Mantel-Haenszel chi-square test (268). The Breslow-Day test for homogeneity between strata was used (269). Comparisons between the estimates of RR were made using the ratio of relative risks (RRR) (270). Values of RR are given with 95% confidence intervals. A 5% significance level was used in all analyses **[article 1]**.

3.2.4.2. Validation of familial history

In addition to the relatives observed and included in the familial aggregation study, probands also described the affection status of relatives which were never clinically observed. In order to assess if proband's description of migraine family history was reliable, sensitivity (which measures the ability of probands to identify affected relatives) and specificity (which evaluates the capacity of probands to report non-migraineur relatives) were calculated (271, 272) as well as positive predictive value (PPV, proband's

identification of a migraineur relative) and negative predictive value (NPV, proband's identification of a healthy relative). Cohen's Kappa statistic was used to assess the extent of agreement between family history reported by probands and their relatives' clinical interview; values of Kappa were given with 95% confidence intervals and P values are one-sided. Statistical analyses used STATA 9.2 software (StataCorp LP, College Station, Texas) [article 2].

3.2.4.3. Risk factors

Our aim was to assess which specific risk factors contributed to the increased risk observed in the familial aggregation study. For that, a multilevel approach was used to account for the dependency among members from the same family. We included in the model relative's gender, the proband's gender and age-at-onset, to evaluate if any of these variables were associated with relative's affection status in our families. We also included in the model proband's migraine subtype. The model was adjusted for relative's age-at-observation.

A 5% significance level was used in all analyses. The models were fit using MLwiN 1.10 software [article 3].

3.2.4.4. IHS classification

Since our probands were enrolled in the study since 1999, we aimed to standardize the diagnosis criteria used. Therefore, in order to compare the ICHD-I and ICHD-II criteria, the chance-corrected agreement rate (Cohen's Kappa) was estimated. We estimated Cohen's Kappa to evaluate the agreement between the two classifications regarding 1) primary and combined headaches, and 2) frequency of migraine and tension-type headache in the population. Since our aim was to focus on the possible changes in the two classifications regarding primary and combined headaches, we estimated the Cohen's Kappa, excluding secondary headaches from the analyses. A 5% significance level was used in all analyses [article 4].

3.2.4.5. Association studies

Power sample estimations were performed using the Genetic Power Calculator (<http://pngu.mgh.harvard.edu/~purcell/gpc/>). Analysis of Hardy-Weinberg equilibrium was performed using HWE software (<http://linkage.rockefeller.edu/ott/linkutil.htm>).

To compare SNP allele frequencies between cases and controls, the chi-square test was used and odds ratios (OR) were estimated, with 95% confidence intervals (CI).

A multivariable-logistic regression was performed (with the most frequent homozygote as the reference category), in order to evaluate the association between the SNPs' genotypes and the occurrence of migraine, by including the SNPs analysed in the model and adjusting for gender. To evaluate the involvement of the SNPs studied in the migraine subtypes, all the analyses were performed in the total sample, as well as in the MA and MO subsets. The P-values obtained in the logistic regression were corrected for multiple comparisons using a Bonferroni correction; considering the number of logistic regressions performed. Haplotype frequencies were compared between cases and controls, using Haploview 4.1 (263) with all parameters set at the default values. Haplotypes were estimated by the software using an accelerated EM algorithm similar to the partition-ligation–expectation-maximization algorithm described by Qin et al. and case-control counts were obtained by summing the fractional likelihoods of each individual for each haplotype (263, 273). Frequencies of haplotypes analysed were above >1%, according to the Haploview threshold; to correct for multiple comparisons, regarding the estimation of allelic and haplotype frequencies, permutations tests were performed using 10,000 permutations [articles 5, 6, 7].

4. RESULTS

4. Results

4.1. Genetic component in migraine

We studied familial aggregation of migraine in a group of 1st-degree relatives of probands with MA and probands with MO, by estimating RR, in order to assess a possible genetic component for migraine. We also estimated RR in probands' spouses in order to disentangle genetic and environmental factors. We found that, in our group of Portuguese families, family members of migraineurs have a 3 to 4-fold increased risk, when compared with the prevalence of migraine in general population. We also found that spouses did not show a significantly increased risk, which is another finding in favour of a genetic component of migraine **[article 1]**. Importantly, we found that while MA and MO may not be distinct entities, sharing common mechanisms, evidenced by the similar risk of migraine in first-degree relatives of MA and MO probands, MA may have a stronger familial aggregation and, thus, a greater genetic predisposition, as supported by the data found in this study **[article 1]**.

In this first study we only studied the first-degree relatives that were clinically observed. However, probands had also reported affection status of other first-degree relatives that were not observed. Therefore, we wanted to assess if the proband's report was reliable in order to include that information in subsequent studies. We found that MO probands showed a higher sensitivity when identifying their affected relatives; MA probands may undervalue less well-defined migraine episodes in their relatives, when they have just MO. But, despite the low sensitivity, we found that reports were highly specific and had a high PPV so this could be a valuable ascertainment method to maximize the number of informative families for e.g. linkage studies **[article 2]**.

After estimating familial aggregation, our aim was to assess specific factors contributing to the increased risk for this disorder. We found that relative's gender is a migraine risk factor, with females at a higher risk. We also found that mother-to-daughter transmissions were more frequent than expected by chance alone which could be explained by hormone, mtDNA or sex chromosome effects **[article 3]**.

Article 1. Familial clustering of migraine: further evidence from a Portuguese study

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Abstract

Objective: 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.

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 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. Risk for spouses was not increased. First degree relatives of MA-probands and MO-probands had a significantly increased risk of both MA and MO compared to the general population. In the group of MA-probands, RR of MA in 1st-degree relatives reached a significant 4-fold increase when compared with RR of MO ($RR_{MA|MA}=12.2$, 95%CI: 7.7-19.5; $RR_{MO|MA}=3.1$, 95%CI: 2.5-3.8), while, in the group of MO-probands, RR of MA was not significantly increased when compared with RR of MO ($RR_{MA|MO}=5.3$, 95%CI: 3.1-9.2; $RR_{MO|MO}=4.0$, 95%CI: 3.5- 4.7).

Conclusions: The present study focus on familial aggregation of migraine in a Portuguese population. Our results demonstrate a substantial familial risk of migraine with evidence of both common and specific etiologic mechanisms for either migraine subtypes.

Keywords: Migraine, Familial Aggregation, Relative Risk, Family History

Abbreviations: MA-migraine with aura, MO-migraine without aura, RR-relative risk, CI-confidence interval, IHS-International Headache Society, RRR-ratio of relative risk

Introduction

Migraine is a chronic neurological disorder with episodic manifestations, with complex inheritance (25, 274). It is a disabling disease, leading to a diminished quality of life in both migraineurs and their relatives (8). Migraine has a high prevalence, affecting about 15% of the general population (4, 45). In Portugal, to our knowledge, there are only two studies of prevalence of migraine published: one, in a group of University students (54); 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) (21, 275).

An increased risk for relatives of migraineurs has been described, with values ranging between 1.4-11.8 (66-71). Heritability estimates ranged between 0.28-0.65 (67, 74-80, 276). Estimates of familial aggregation differ among studies since some use a control group as reference, while others use migraine prevalence in the general population (66-71). The different studies also used different methodologies and sample sizes which can make difficult to compare results obtained (66, 277). Differences in family risk can also be due to variation in allele frequencies among different populations (75).

Different approaches were used to estimate familial aggregation; some studies are clinically-based (66, 67) while others are population-based (68-70) and another is a twin population based survey (71). Clinically-based studies are described as more influenced by selection bias than population-based studies but, on the other hand, may have more power when large samples are studied (14, 278). Both clinical and population studies, combined, can be useful when assessing familial aggregation (14). All these studies suggest that multiple genetic factors are involved in this disorder, in addition to environmental factors, determining liability for migraine (25).

Migraine with (MA) and without aura (MO) are the most common forms of this disease (279). Several susceptibility loci have been described for MA and MO, but the genes and mutations involved have not yet been identified (25).

Some studies point out that co-occurrence of MO and MA in the same individual and within the same families show that these two subtypes are not distinct entities and that there is a continuum of severity. However, some authors state that MA and MO do not have the same etiology and that MA can have a particular genetic component regarding aura and a high genetic load (77, 88, 89, 280).

Our aim was to evaluate familial aggregation of migraine in a large group of Portuguese families, and to assess whether familial aggregation differs between MA and MO.

Methods

Data collection

A sample of probands with MA and MO was sequentially enrolled in the study, from 1999 to 2004, from the out-patient Neurology clinic, at HGSA, Porto. Probands were interviewed (by JPM or JB), regardless of their family history. Demographic and clinical information about relatives and spouses was obtained. Interviews to 1st-degree relatives (parents, sibs and offspring) and spouses were made by the same neurologist (JPM). Family members were then inquired about the occurrence of migraine, either personally or, if not feasible, by telephone. In both cases, a diagnostic interview was performed using the same structured questionnaire based on the operational diagnostic criteria of the “International Headache Society” (IHS), avoiding recall bias. Inaccuracy in the diagnosis of MA can introduce some bias both in clinical and population-based studies (14). However, the assessment of MA in the population-sample was carefully performed following the same IHS criteria, with questions regarding all types of aura, including the duration of aura episodes, with no differences to the MA-assessment in the clinical sample.

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).

Participants were diagnosed according to the first edition of the IHS criteria (279) to standardize the diagnostic, given that selection of probands started before 2004. Probands and family members presenting other forms of headaches in addition to migraine were also included.

Similarly to other studies (281, 282), relatives were included independently of their age. 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 population (65) and was estimated for first-degree relatives (with MA, MO or MA+MO), using the total prevalence rate of 16,7% in the population sample (21, 275). 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, probands'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 to assess if familial aggregation differs between MA and MO, evaluating the hypothesis of a specific etiology either for MA or MO.

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%) (21, 275). This methodology required that, relatives only with MA were considered as non-affected when analysing the risk for MO, and vice-versa (69). Additionally, relatives with MA+MO were considered as affected both when analysing the risk for MA and for MO.

Statistical analysis

Student's *t*-tests were 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 chi-square tests. RR was adjusted for age-at-observation and sex, using the Mantel-Haenszel RR, and its significance analyzed by a Mantel-Haenszel chi-square test (268). The Breslow-Day test for homogeneity between strata was used (283). Comparisons between the estimates of RR were made using the ratio of relative risks (RRR) (270). 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 (116 women and 28 men, mean age $\pm$ SD, 34.6 ± 12.5 years; mean age at onset $\pm$ SD, 17.4 ± 7.7) were selected for this study. 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).

Comparison between probands and population sample

Among 2008 individuals of the population sample, 334 were migraine-sufferers (21, 275) (245 women and 89 men, mean age $\pm$ SD, 38.6 ± 19.9 years; mean age at onset $\pm$ SD, 20.0 ± 12.1). Probands were not different from the migraineurs in the population sample, with regard to gender, age-at-observation and age-at-onset.

Relatives

Interviews were conducted with 143 1st-degree relatives of MA-probands (83 women, 60 men; mean age $\pm$ SD, 41.5 ± 17.5 years), and with 196 1st-degree relatives of MO-probands (121 women, 75 men; mean age $\pm$ SD, 40.6 ± 19.2 years). Only 9% of the first-degree relatives included were below proband's median age at onset. In the group of probands with MA, 36% of the first-relatives observed were parents, 49% were siblings and 15% were offspring, representing 58% of the total first-degree relatives. In case of probands with MO, 35% of the first-relatives interviewed were parents, 41% were siblings and 24% were offspring, representing 43% of the total first-degree relatives. The composition of first-degree relatives by relationship did not differ in both sets of family members ($p>.05$). Co-occurrence of MA and MO was found in 11 first-degree relatives of MA-probands and 14 of MO-probands. A similar proportion of affected relatives were found in both groups (Table 2). The remaining relatives that could not be observed (some were abroad and, in other cases, contact was impossible to obtain) did not differ of those observed, regarding age and gender (data not shown). Of the total 339 first-degree relatives interviewed, 32 were inquired by telephone (9%).

Spouses

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

Familial aggregation

Family history

In the group of MA-probands, 37 (74%) have at least one affected first-degree relative while 13 (26%) do not have affected relatives observed, whereas in the group of MO-probands, 67 (71%) have at least one affected first-degree relative while 27 (29%) do not have affected relatives observed. Regarding multiplex families, there are 24 (48%) in the group of MA-probands and 37 (39%) in the group of MO-probands.

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 2), 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 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 2). In the group of MA-probands, RR of MA in 1st-degree relatives reached a 4-fold increase when compared with RR of MO ($RR_{MA|MA}=12.2$, 95%CI: 7.7-19.5; $RR_{MO|MA}=3.1$, 95%CI: 2.5-3.8), while, in the group of MO-probands, RR of MA was not significantly increased when compared with RR of MO ($RR_{MA|MO}=5.3$, 95%CI: 3.1-9.2; $RR_{MO|MO}=4.0$, 95%CI: 3.5- 4.7).

In a further analysis, we excluded relatives with co-occurrence of MA and MO; RR estimated with and without these relatives did not differ significantly and follow the same pattern (data not shown). Thus, RR estimates were not inflated by the inclusion of first-degree relatives with MA+MO.

Comments

Our study reports a strong evidence for clustering of migraine in 1st-degree relatives of MA and MO probands. Our results clearly show that relatives 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 also shown an increased risk for relatives of migraineurs and our results are, in general, coherent with those findings (66-71, 282).

In our population sample, 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 lower than the one found by Russell et al in Denmark (67, 69, 70). 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 populations (284). Individuals with Portuguese, Italian and Greek ancestry are included in the southern group (284), which could explain the similar MA prevalence in that group.

In our study we had special concern with potential biases, which were considered and reduced. A valid assessment of MA was performed in both clinical and population sample. In what concerns indicators of disease severity such as age at onset, our proband's sample did not differ from the sample of migraineurs ascertained from an earlier population-based study (275). On the other hand, clinical based samples may introduce some selection bias, since individuals with more severe migraine and more complex symptoms (such as MA sufferers) are more likely to look for medical care (68, 285). This fact could lead to higher RR in first-degree relatives, in particular for MA.

Most studies, whether clinical or population-based, point out to MA|MA as the highest risk group (278). RR estimates are in general higher in clinical based-samples, as shown in Kalfakis et al., which reported a high RR for MA in 1st-degree relatives of MA-probands (11.8), which is similar to ours (67).

An interesting feature, however, is that the frequency of MA in first-degree relatives of MA-probands was lower in our study than the one found by Russell et al. in a population-based study (69). Thus, it seems that the high magnitude of our RR for this subgroup is more due to the lower prevalence of MA in our population rather than to the inclusion of the more severe cases.

An attempt was made to contact all first-degree relatives and spouses but we were not able to contact all of them. Although this fact may introduce a bias in our results (68) it is noteworthy that the non-interviewed first-degree relatives did not differ regarding age and gender from the observed ones.

A source of bias could be introduced if relatives, who came to the interview, were mainly the affected ones. To circumvent the possible bias, relatives and spouses who were not able to be inquired in-person, were contacted by telephone (see Methods), a method which has been described as a valid instrument in familial aggregation studies and prevalence studies (13, 53)

Individuals presenting other forms of headaches in addition to migraine were also included, since migraine and tension-type headache are considered as separate entities with different pathophysiology, risk factors and age and sex distribution (19). It has been described that tension-type headache in migraineurs was not significantly more prevalent than in nonmigraineurs and that more than 80% of migraineurs experience also tension-type headache (19). The coexistence of the two conditions must be due to chance and not to a causal relationship (19, 20)

This study has also several strengths. Interviews to 1st-degree relatives and spouses were made by a single neurologist, reducing diagnostic variability (286) 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 aggregation (267). Estimates of RR were adjusted for age and sex, as migraine is an age and sex-dependent trait (69). 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 studies (267).

In this study, migraine prevalence rates from the general population were used, estimated from a previous population-based study (275) performed in the same geographical area where our clinically-based study took place and were similar to other studies (13, 45, 47, 53). 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 RR (271) and to also avoid some problems in the RR estimations due to sporadic cases or incomplete penetrance in controls (277).

Several familial aggregation and twin studies demonstrate that migraine is essentially due to genetic factors (25, 74-76, 78, 80, 276, 278), as it can be also assumed from the increased risk found in our study. Although environmental factors may have an effect, these need to be very high to account entirely for familial clustering of a given disease (287, 288).

We also found that, in our sample, 1st-degree relatives of MA-probands had a higher risk of MA than MO, 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 liability (77). Furthermore, MA and MO can co-occur in the same family and in the same individual (10, 86) as seen in our families. While these may not be distinct entities, sharing common

mechanisms, evidenced by the similar risk of migraine in first-degree relatives of MA and MO probands, MA seems to have a stronger familial aggregation and, thus, a greater genetic predisposition (41), as supported by the data presented here. MA can have a specific genetic component regarding aura and a higher genetic load (88, 89).

Our study is the first on familial aggregation of migraine in a population with this geographical origin. 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.

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Disclosure: The authors report no conflicts of interest

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

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

Probands	1 st -degree relatives	1 st -degree relatives affected (%)	RR age- and sex-adjusted (95% CI)*
MA	Migraine	86 (60)	3.6 (3.0-4.2)
MO		125 (64)	3.7 (3.2-4.3)
MA	MA	32 (22)	12.2 (7.7-19.5)
	MO	65 (45)	3.1 (2.5-3.8)
MO ⁽⁺⁾	MA	19 (9.7)	5.3 (3.1-9.2)
	MO	119 (61)	4.0 (3.5-4.7)

MA and MO co-occur in 11 first-degree relatives of MA-probands and in 14 of MO-probands

⁽⁺⁾ One first-degree relative missing (migraine could not be classified as MA or MO)

* CI, confidence interval.

Article 2. How useful is the probands' description of family history of migraine?

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In Preparation

Abstract

Family history of migraine, as described by the probands, is frequently underreported. Validity of the probands' report was assessed in a group of Portuguese migraine families. From 101 relatives interviewed, 72 were 1st-degree. Sensitivity and specificity of the proband's report were 64% and 96%; Kappa was 0.52 (CI: 0.35-0.69), PPV was 97%, while NPV was only 58%. The Kappa value, sensitivity and NPV were lower for MA (0.33, CI: 0.10-0.55) than MO (0.71, CI: 0.49-0.92) probands. While a clinical interview of relatives is always more reliable than the probands' description, emphasis should be put in interviewing relatives of MA rather than MO probands, since the latest is more reliable. Our results also show that family history method may be appropriate to use in genetic studies (like linkage analysis) where a low rate of false positives is required and is more important than sensitivity.

Keywords: Migraine, clinical interview, Cohen's Kappa, positive predictive value

Introduction

Migraine is a neurological disorder that aggregates in families.(278, 289) Family history of migraine, as described by the probands, is frequently underreported. (271, 272)

We assessed validity of the probands' report in a group of migraine families from the dataset of a previous study (289), by evaluating its agreement with relatives' clinical interview, regarding the use of that information in subsequent genetic studies.

Methods

Relatives (n=101) of 33 migraineurs were contacted, regardless of the information provided by the proband about their affection status, to avoid interviewer bias.(272) Relatives were either personally observed, or interviewed by telephone, a method that has been validated in previous studies.(272) An interview was conducted by the same neurologist (JPM) using a structured questionnaire, based on the 1988's operational diagnostic criteria of the International Headache Society. All participants gave their written informed consent, and the Ethics Committee of HGSA previously approved the project.

Sensitivity (measuring the ability of probands to identify affected relatives) and specificity (evaluating the capacity of probands to report non-migraineur relatives) were calculated

(271, 272), as well as positive predictive value (PPV, proband's identification of a migraineur relative) and negative predictive value (NPV, proband's identification of a healthy relative). Cohen's Kappa statistic was used to assess the extent of agreement between family history reported by probands and their relatives' clinical interview (Table); values of Kappa are given with 95% confidence intervals and P values are one-sided. Statistical analyses used STATA 9.2 software (StataCorp LP, College Station, Texas).

Results

72 relatives interviewed were 1st-degree: in agreement with probands' descriptions, 30 were migraineurs and 24 were healthy; nevertheless, 17 affected relatives had been reported as healthy, while only one non-migraineur was reported as affected. Sensitivity and specificity of the proband's report were 64% and 96%; Kappa was 0.52 (CI: 0.35-0.69), PPV was 97%, while NPV was only 58% (Table).

When classifying data according to migraine with (MA) or without aura (MO) in the proband, the Kappa value was lower for MA (0.33, CI: 0.10-0.55) than MO (0.71, CI: 0.49-0.92); the same happened with sensitivity and NPV (Table). Of the MO relatives, 65% were correctly identified by MO probands while only 27% by MA probands.

When including also 2nd and 3rd-degree relatives (n=29), agreement rate and sensitivity decreased slightly (data not shown).

Discussion

Probands' reports underestimate the occurrence of migraine in 1st-degree relatives, and are more uncertain when 2nd and 3rd-degree relatives are included, probably due to a more indirect knowledge.(272) Despite its low sensitivity, reports were highly specific and had a high PPV.

The overall Kappa's value was moderate (being good for the MO probands group and only fair for MA), according to Landis and Koch criteria.(290)

Unlike Russell et al.(272), we found that MO probands showed a higher sensitivity when identifying their affected relatives; MA probands may undervalue less well-defined migraine episodes in their relatives, when they have *just* MO.

Clinical interviews should ensure accurate diagnosis of migraine, especially in familial aggregation studies, to overcome the low sensitivity of proband's report.(271) In case of scarce resources, these should be put in examining relatives of MA probands, since MO probands reports seem to be more reliable.

In any case, the proband's report will still be useful for deceased or absent relatives.(291) Moreover, when high specificity and PPV are more important than sensitivity, as for

genetic linkage studies (291), it can be a valuable ascertainment method to maximize the number of informative families.

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Table 1. Validity of the affected-by-history method in 72 first-degree relatives

Group/Parameters	Kappa (95% CI)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Migraine Proband	0.52 (0.35-0.69)	64 (30/47)	96 (24/25)	97 (30/31)	58 (24/41)
MA Proband	0.33 (0.10-0.55)	45 (10/22)	100 (9/9)	100 (10/10)	43 (9/21)
MO Proband	0.71 (0.49-0.92)	80 (20/25)	94 (15/16)	95 (20/21)	75 (15/20)

Article 3. Assessing risk factors for migraine: differences in gender transmission

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In Preparation

Abstract

Objective: To assess which specific factors are contributing to an increased risk of migraine in a group of Portuguese families.

Background: The consistent finding of migraine familial aggregation, including in our familial sample, suggests that genetic factors may be implicated in the most common forms of this disorder.

Methods: A multilevel approach was used to account for the dependency among members from the same family. We included in the model relative's gender, the proband's gender and age-at-onset, to evaluate if any of these variables were associated with relative's affection status. We also included in the model proband's migraine subtype. The model was adjusted for relative's age-at-observation. We also assessed maternal and paternal transmissions within the proband nuclear family.

Results: After adjusting for the remaining variables, relatives' gender was found to be a risk factor for migraine (OR= 2.86; 95% CI= 1.75-4.67), with females at a higher risk. When splitting probands according to their migraine subtype, we found that none of the variables studied contributed to relatives of MA-probands affection-status. Our results also show a significant difference between proband's maternal transmission and the gender of the offspring.

Conclusions: Our findings regarding risk factors for migraine subtypes lead us to hypothesize that MA have a higher genetic load than MO. Hormonal events, mtDNA or X-chromosome effects could influence a gender-related risk for migraine, showing a conjunction of environmental and genetic factors. It would be important to identify genes associated with migraine, which could be modulated by genes located in sex chromosomes.

Introduction

Complex diseases have a high impact on human health and a high population incidence (292). Migraine, a highly prevalent disease, is one example among many others (25). Migraine with (MA) and without aura (MO) are the most common forms of this disease. It is a disabling disease leading to a diminished quality of life in both migraineurs and their relatives (8).

The consistent finding of an increased risk for relatives of migraineurs suggests that genetic factors may be implicated in the most common forms of the disease (25). We also found a substantial familial risk of migraine for first-degree relatives in a sample of Portuguese migraineurs, which has led us to conclude that migraine could be essentially due to genetic factors (293).

In a previous study, a lower age at onset in probands was found to be a predictor of migraine familial aggregation (68). Lifetime prevalence of migraine is increased in females compared to males, with a female: male ratio ranging from 2:1 to 4:1 in several populations (50-53). Diverse hypotheses have been raised for this female preponderance such as neurobiological factors, increased sensitivity to environmental stressors or a greater genetic loading for migraine (294, 295).

Our aim was to assess specific factors present in our migraine families are contributing to the increased risk for this disorder, taking into account intrafamilial correlations.

Methods

Data collection

A sample of probands with MA and MO was sequentially enrolled in the study, from 1999 to 2004, from the out-patient Neurology clinic, at HSA, Porto. Probands were interviewed (by JPM or JB), regardless their family history of migraine. First-degree relatives (parents, sibs and offspring) were then inquired about the occurrence of migraine, either personally or, if not feasible, by telephone, by the same neurologist (JPM). In both cases, a diagnostic interview was performed using the same structured questionnaire based on the diagnostic criteria of the “International Headache Society” (IHS). To avoid a selection bias towards affected relatives, family members were contacted regardless of the information provided by the proband about their affection status. The first edition of the IHS criteria (ICHD-I) (279) was used before 2004; when revising the diagnosis using the second

edition (ICHD-II) (6) no differences were found in patients' diagnosis (data not shown). Participants gave their informed consent and the Ethics Committee of HSA approved the project.

Data analysis

Probands were classified according to their migraine subtype. Proband's age at onset was dichotomized (<16, 16+ years). Relatives were divided in to three groups according to their age-at-observation (<30, 30-59, 60+ years) and were also separated by gender, since migraine is an age and gender-dependent trait.

We also assessed maternal and paternal transmissions within the proband nuclear family. We excluded from this analysis bilateral transmissions, i.e. we excluded transmissions where both the mother and the father were affected.

Statistical analysis

A multilevel approach was used to account for the dependency among members from the same family. We included in the model relative's gender, the proband's gender and age-at-onset, to evaluate if any of these variables were associated with relative's affection status in our families. We also included in the model proband's migraine subtype. The model was adjusted for relative's age-at-observation.

Categorical data were compared using a chi-square test, using SPSS version 16.0 for Windows. A 5% significance level was used in all analyses. The models were fit using MLwiN 1.10 software.

Results

Demographic data

Probands

A total of 131 probands were enrolled in this study (104 women and 27 men, mean age $\pm$ SD, 34.4 ± 12.7 years). Regarding age-at-onset, 60 probands had an age-at-onset below 16 years old, while 71 showed migraine after that age. When classifying probands according to their migraine subtype, 85 probands had MO while 46 had MA.

Relatives

Interviews were conducted with 182 first-degree relatives of MO-probands (114 women and 68 men) and 137 first-degree relatives of MA-probands (79 women and 58 men).

In MO-probands group, 81 family members were affected while 56 were not. In MA-probands-group, 116 were affected while 66 were non-migraineurs. Age of relatives was included in the model to adjust for this variable, since migraine is an age-dependent trait. From the total of first-degree relatives, 112 were parents, 139 were siblings whereas 68 were offspring.

Risk Factors

After adjusting for the remaining variables, relatives' gender was found to be a risk factor for migraine (OR= 2.86; 95% CI= 1.75-4.67) (Table 1), with females at higher risk than males. Variables related with proband's, gender and age-at-onset, are not risk factors for migraine ($p>0.05$). When introducing in the model proband's migraine subtype this variable also did not influence relative's affection status ($p>0.05$).

When splitting probands according to their migraine subtype, we found that none of the variables studied contributed to relatives of MA-probands affection-status. Conversely, gender of relatives of MO-probands was associated with their affection status (OR=4.15; 95% CI=2.06-8.34) (Table 1).

We also assessed if risk factors were different according to the first-degree relative group. We found that for parents and offspring, relatives' gender is also a risk factor for migraine (OR=6.47; 95% CI=2.53-16.54, for parents and OR=5.58; 95% CI=1.63-19.12) (Table 2), whereas for siblings no significant differences were found ($p>0.05$)

Maternal and paternal transmissions

Relatives' gender was found to be a risk factor for migraine, with females being at a higher risk than males. Therefore we hypothesized that gender ratio in migraine families could be due to a biased transmission. In order to explore this hypothesis we analysed maternal and paternal transmissions within the proband nuclear family. Our result showed a significant difference between proband's maternal transmission and the gender of the offspring. While for male probands, their sons and daughters are not significantly different regarding their affection status ($p>0.05$), for females probands, we found that daughters are more affected than expected ($\chi^2_{(1)}=4.11$, $p=0.04$). We also found that mothers of probands are more affected than expected when compared to proband's fathers ($\chi^2_{(1)}=22.41$, $p<0.001$). When stratifying for proband's gender, no differences were found for

parents of male probands ($p>0.05$), whereas mothers of female probands are more affected than expected ($X^2_{(1)}=19.61$, $p<0.001$) (Table 3).

We also compared the ratio of affected fathers, mothers and siblings. We found a higher ratio of affected mothers (78%) and siblings (61%) than affected fathers (30%), with mothers being affected twice to three times more than fathers.

Discussion

Familial aggregation is well established for common migraine, nevertheless, several familial factors may be contributing for these increased risk. Our aim was to search for migraine predictors in a sample of Portuguese migraine families. We have assessed proband's age-at-onset and gender as well as relatives age-at-observation and gender as risk factors for migraine in a sample of first-degree relatives.

In a previous study, a lower age at onset in probands was associated with relative's affection status (68) but in contrast, in our study, the proportion of affected relatives was independent of proband's age at onset. After adjusting for the remaining variables, gender was found to be a risk factor for migraine, while proband's age at onset and relative's age at observation were not.

Our findings showed that, as expected, females had a higher risk of migraine than men. Our next aim was then to assess if there was a maternal or paternal effect that could explain this gender ratio. We found that mother-to-daughter transmissions were more frequent than expected by chance alone, although mother-to-son, father-to-daughter and father-to-son transmissions were also observed.

This biased transmission could be explained by a maternally inherited factor such as mitochondrial DNA (mtDNA) (158, 296). Although both sons and daughters inherit mtDNA from their mothers, a gender bias is observed in some mitochondrial disorders, which could be explained by the effect of steroids that influence mitochondrial function (296). As suggested by Boles et al, we assessed if offspring of female and male probands would be differentially affected and in fact we found that daughters of female probands are more affected than expected. Therefore, the offspring of a male proband is at a lower risk for migraine, which favours the mtDNA hypothesis. We also found that the ratio of affected probands' fathers is lower than the ratio of affected mothers and siblings which is another evidence in favour of a maternally inherited factor, according to Boles et al (296). Although mtDNA may not explain by itself the gender differences found, since migraine is a complex disease with several genetic factors involved, variants in mtDNA or in nuclear genes affecting mitochondrial mechanisms could influence migraine susceptibility. Some

authors found that some mtDNA mutations could contribute to the pathogenesis of migraine (158). Also it has been suggested that an impairment of mitochondrial metabolism could lower the threshold for migraine attacks (297). Besides mtDNA, other authors found evidence of a migraine susceptibility locus on chromosome X, although until now no genes in this region have been found (149, 151, 155, 298). Another hypothesis is a sex-conditioned genetic model with sex chromosomes influencing the expression of genes in autosomes, which would lead to an autosomal dominant transmission in female family members and to an autosomal recessive transmission or incomplete penetrance in male relatives (299). This fact could explain our results regarding a preferential transmission mother-to-daughter.

Migraine presents different gender thresholds, with males having a higher threshold (295, 300). The influence of mtDNA and chromosome X could be involved in this process; however, females also differ from males in pain behavior, influenced by differential exposure to gender hormonal effects which can also modulate the expression of migraine in women. Gender hormones are involved in the expression and sustaining of pain states (294). Estrogens exert effects on the pain pathways and other brain structures involved in pain mechanisms and progesterone seems to reduce the occurrence of migraine, by the activation of gamma-aminobutyric acid (GABA) receptor (301). It has been shown that estrogen modifies gene expression in trigeminal neurons which some of these are deeply involved in migraine pathophysiology (302). Additionally, female steroids are involved in other mechanisms related in migraine pathophysiology, such as in neuronal excitability, in the synthesis and release of nitric oxide (NO) and neuropeptides such as calcitonin-gene related peptide (CGRP). Also, the serotonergic, adrenergic and GABA-ergic systems are also modulated by female steroids. Furthermore, some variants have been found in female hormones receptors, such as estrogen and progesterone receptors (229-231). All these evidence show that, in fact, female steroids play an important role in migraine pathophysiology and can also explain the differential gender ratio found for this disorder. Another interesting aspect is that in men, testosterone seem to have a protective role in pain development (301), which could explain the fact that sons of female probands have a lower risk than daughters.

Additionally, a study found for female mice a reduced threshold for cortical spreading depression (CSD), the mechanism that underlies migraine with aura (MA), although it may also be implicated in migraine without aura (MO), suggesting that for females there is an intrinsic difference in cortical excitability, independent of cycling hormonal variation (35, 303).

Our findings regarding risk factors for migraine subtypes lead us to hypothesize that MA have a higher genetic loading while in MO, hormonal events could influence the risk of

having migraine, showing a conjunction of environmental and genetic factors, as suggested previously by Russell et al (69).

It would be important to identify genes associated with migraine that were modulated by genes located in sex chromosomes. Further studies would be important to disentangle the possible effect of mtDNA or X-chromosome and hormonal-related effects.

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Table 1. Risk factors of migraine: results from the logistic regression analysis, adjusted for

Variables	Migraine	MO	MA
Proband's gender	0.79 (0.39-1.62)	0.61 (0.22-1.74)	1.16 (0.41-3.31)
Proband's age at onset	0.75 (0.42-1.33)	0.50 (0.23-1.10)	1.21 (0.48-3.09)
Relative's gender	2.86 (1.75-4.67)	4.15 (2.06-8.34)	1.86 (0.90-3.83)
Proband's migraine subtype	0.78 (0.45-1.36)	-	-
relative's age at observation (Odds ratio, 95% CI)			

Table 2. Risk factors of migraine, according to first-degree relative group: results from the logistic regression analysis, adjusted for relative's age at observation (Odds ratio, 95% CI)

Variables	Parents	Siblings	Offspring
Proband's gender	1.20 (0.39-3.65)	0.64 (0.19-2.22)	0.27 (0.05-1.52)
Proband's age at onset	0.76 (0.27-2.15)	0.57 (0.24-1.36)	1.74 (0.49-6.17)
Relative's gender	6.47 (2.53-16.54)	1.30 (0.60-2.77)	5.58 (1.63-19.12)

Table 3. Distribution of parents and offspring affected and non-affected according to proband's gender

Proband	Mothers		Fathers		Daughters		Sons	
	Affected	Non-affected	Affected	Non-affected	Affected	Non-affected	Affected	Non-affected
Females	43	11	7	18	20	8	12	15
Males	17	6	4	6	7	1	2	3

4.2. Comparison between ICHD-I and ICHD-II

In 1988, the IHS has published the classification and diagnostic criteria of the different subtypes of migraine (ICHD-I). In order to improve the clinical diagnosis, this classification has been revised in 2004 (ICHD-II).

With this work we aimed at comparing the two classifications of the IHS and assessed if the ICHD-II criteria changed the results of the epidemiological studies previously performed. The database which resulted from that study was reviewed and the ICHD-II criteria were applied in order to evaluate the diagnostic distribution. In a second stage, after analysing the results from the epidemiological study, we also compared the diagnosis of our patient's sample, using the two IHS classifications.

We found that this new criteria do not have a major impact in headache epidemiological studies. The high agreement rate found shows that ICHD-II does not change remarkably the epidemiological results in what concerns migraine and tension-type headache, although it presented important clinical implications such as the redefinition of migraine in children. This new classification has some improvements when compared with the first one, although we found that it is still not perfect particularly when there are combinations of headaches subtypes **[article 4]**.

Importantly, we found that in 98% of the patients of our clinical sample, the diagnosis remained the same, showing that, even in clinical samples, ICHD-II does not present significant changes for MO and MA forms **[article 4]**. This is an important finding given that our patients were enrolled in the clinical and genetic study of migraine since 1999 and we can now standardize the diagnosis criteria used in the clinical sample in future studies.

Article 4. Headache epidemiological study: do different criteria make a difference?

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In Preparation

Abstract

The “International Headache Society” classification published in 1988 led to a major improvement in the diagnosis of headaches. Its revision in 2004 aimed to improve clinical diagnosis; however, its consequence on epidemiological data is so far unknown.

Our aim was to apply the new criteria to data obtained from a previous headache epidemiological study performed in 1995 and to evaluate diagnostic distribution.

Lifetime prevalence of headaches was 88.6% (1780/2008 individuals). Applying ICHD-II (2004) criteria, 84.7% had primary headaches (80.4%- according to ICHD-I (1988) whereas 6.0% had secondary headaches (6.0%) and 9.3% combined headaches (13.6%). Regarding the two classifications, we found a high significant chance-corrected agreement rate of 78% for primary and combined headaches, of 91% for the frequency of migraine and of 95% for the frequency of tension-type headache.

The ICHD-II criteria have relevant clinical implications, although still needs some improvements but do not change remarkably epidemiological results.

Keywords: Headaches, Clinical Epidemiology, International Headache Society, diagnostic criteria

Introduction

Headache is a common clinical condition and constitutes a major public health problem (4, 304). However, there are several different types of headaches that can vary in incidence, prevalence, duration or severity (1).

The different types of headaches can be classified into primary or secondary headaches. While primary headaches are not associated with an apparent underlying pathology, secondary headaches have an underlying cause, such as an infection, brain tumor or stroke (1).

In headaches' diagnosis, the first task of a neurologist is to discriminate between a primary or secondary headache. However, discrimination between the different types of primary headaches like migraine or tension-type headache is frequently more difficult (30).

Several studies have shown that primary headaches may have a genetic predisposition that can be triggered by physiological and other life events, such as puberty (11). Although the genetics of headaches, especially in migraine's field, has suffered some improvements in the last years (25), it is still difficult to establish genotype–phenotype correlations (11). Consequently, headaches' diagnosis still relies on the clinical diagnosis. In 1988, the International Headache Society has published the classification and diagnostic criteria of the different subtypes of migraine (ICHD-I) (279). This classification has contributed to improve the diagnosis of headaches, introducing more clear definitions which allowed clinical heterogeneity to be reduced in subsequent epidemiological and genetic studies.

However, some specialists' state that this classification has created artificial distinctions since a clinical continuum of headache subtypes can be found, which may introduce some difficulties in clinical diagnosis (11). Some authors showed that this classification has high specificity but low sensitivity (305).

In order to improve the clinical diagnosis, the classification was revised in 2004 (ICHD-II) (306). Some important headache types were included, migraine classification has been adjusted and it has also been modified for pediatric populations with the acceptance of shorter attacks and less associated features (11).

Some studies already showed that this new classification has a higher sensitivity and specificity than ICHD-I, regarding migraine diagnosis in children and adolescents

(305). One study showed that for migraine with aura this new classification has high sensitivity and specificity, allowing the sample of patients classified with migraine with aura to be more homogeneous (307).

Nevertheless, the consequences of the ICHD-II on epidemiological data regarding the different headache subtypes are so far unknown. Our aim was to evaluate the impact of different criteria on (1) our previous population-based sample, obtained from a previous epidemiological study and (2) our clinical sample, on which our association and familial aggregation studies were based.

Methods

Subjects

In 1995 a headache epidemiological study was conducted in the general population of a small-district of Porto. Interviews were made door-to-door to 2008 individuals, using a validated structured headache questionnaire according to the ICHD-I criteria. The validation of the questionnaire was made by a clinical interview and neurological examination by the same neurologist (JPM) of a random sample of 205 individuals (55). The questionnaire had questions regarding demographic data, headache's age at onset, headache's frequency and duration, location, quality and intensity of pain, influence of physical activity, the occurrence of nausea, vomiting, photo and phonophobia, the occurrence of neurological symptoms, head trauma or associated disorders. Children were also included in the study and the questionnaire was fulfilled based on the children's report or on the parent's report to confirm the children's answers (308).

That database was now reviewed and the new criteria (ICHD-II) were applied in order to evaluate the diagnostic distribution and possible changes in diagnosis.

In a second stage, after analysing the results from the epidemiological study, we also compared the diagnosis of our patient's sample, ascertained from the Neurology out-patient clinic, at HSA, Porto, using the two IHS classifications. Comparisons were performed in 150 probands, in which clinical data was available.

Statistical Analysis

Data were summarised using descriptive statistics. The chance-corrected agreement rate (Cohen's Kappa) was estimated in order to compare the ICHD-I and ICHD-II criteria. We estimated Cohen's Kappa to evaluate the agreement between the two classifications regarding 1) primary and combined headaches, and 2) frequency of migraine and tension-type headache in the population. Since our aim was to focus on the possible changes in the two classifications regarding primary and combined headaches, Cohen's Kappa was estimated after excluding secondary headaches from the analyses. A $P < 0.05$ was considered statistically significant. All statistical analyses were performed using SPSS (version 16.0 for Windows).

Results

Demographic data

From the 2008 individuals interviewed, 1780 presented lifetime headaches (88.6%). Mean age (SD) was 39.9 (21.8) with a range between 2-97 years. From the 1780 individuals, 653 were male (36.7%) and 1127 were female (63.3%). Mean age at onset (SD) of headaches was 20.3 (13.4).

Diagnostic distribution according to ICHD-I and ICHD-II criteria

Regarding the ICHD-I classification, 1431 (80.4%) individuals presented primary headaches, 242 (13.6%) presented combined headaches while 107 presented secondary headaches (6.0%) (Table 1).

When comparing the distribution of headaches' type between ICHD-I and ICHD-II (Tables 1 and 2), an increased number of primary headaches was observed, while the number of combined headaches, i.e. individuals that presented more than one headache type, decreased. No changes were found in the diagnostic of individuals with secondary headaches; therefore the frequency of secondary headaches remained the same.

In primary headaches' group, the following distribution was found according to ICHD-I: migraine (including migrainous disorder not fulfilling all criteria) 9.8%, episodic tension-type headache (including headache of the tension-type not fulfilling all criteria) 66.2%, chronic tension-type headache 4.4% and combined headaches 13.6%. According to the ICHD-II diagnostic distribution (Table 2) we found a small

increase in the migraine and episodic tension-type headache groups and a decrease in the combined headache's group. Comparing the two classifications (Table 3), we observed that a small number of individuals previously coded as migraine without aura (ICHD-I) are now classified as having probable migraine without aura (6 individuals), while one case with migrainous disorder not fulfilling all criteria was reclassified as having migraine without aura. Concerning migraine with aura, only one individual was now reclassified as having probable migraine with aura.

With the ICHD-II, it is also clear the subdivision of episodic tension-type headache in frequent and infrequent episodic tension-type headache, being more common cases with frequent episodic tension-type-headache. It is also noteworthy that some cases classified as chronic tension-type headache by the ICHD-I are now classified as having frequent episodic tension-type headache. Also, some cases previously classified as headache of the tension-type not fulfilling all criteria were now categorized as having infrequent episodic tension-type headache. The most striking finding was the decrease of cases with combined headaches, which were now reclassified with only one type of headache (Table3).

Comparing ICHD-I and ICHD-II, we found a Kappa value of 78% ($P<0.001$), between primary and combined headaches (Table 4). We also evaluated the agreement between ICHD-I and ICHD-II regarding the frequency of migraine and tension-type headache in the population. We found a Kappa value of 91% ($P<0.001$) for the frequency of migraine (including migrainous disorder not fulfilling all criteria and migraine associated with other types of headache) and a Kappa value of 95% ($P<0.001$) for the frequency of tension-type headache (including headache of the tension-type not fulfilling all criteria and tension-type associated with other types of headache) (Table 5).

Missing criteria

We also evaluated the missing criteria that lead to a diagnosis of probable headache instead of headache (using ICHD-II). In what concerns probable migraine and probable tension-type headache, the criterion B (attack duration) was the one that was more often missing, followed by criterion D (associated symptoms). Also contributing to a probable tension-type headache diagnosis, a high frequency of missing criterion A (number of attacks) was also observed (Table 6).

Second stage: clinical sample

In a group of 150 unrelated probands of different families, clinical diagnosis was revised using ICHD-II. No differences were found between the two classifications regarding the 42 MA patients and the 19 MAMO patients. In the group of MO patients, 91 individuals were classified with the same diagnosis while only 2 patients were reclassified as having probable MO.

Discussion

Our main aim in this study was to evaluate if the new ICHD criteria introduced remarkable differences in epidemiological studies concerning in particular primary headaches. Our results show that this new criteria do not have a major impact in headache epidemiological studies.

When estimating Cohen's Kappa, we found a an almost perfect agreement (according to Landis and Koch, 1977) (290) regarding the frequency of migraine and tension-type headache in the population (Table 5). This high agreement rate shows that ICHD-II does not change remarkably the epidemiological results in what concerns migraine and tension-type headache.

Furthermore, frequencies of headache types analyzed with both criteria are similar and we found a substantial agreement (according to Landis and Koch, 1977) (290) concerning primary and combined headaches (Table 4). Several individuals presented combined headaches, composed by more than one type of headache as headache types are not mutually exclusive. However, when classified with ICHD-II, the frequency of the combined forms decreased, in particular when there was a combination of headaches not fulfilling all ICHD-I criteria or ICHD-II probable headache types, which may indicate that the new criteria are more adequate to separate the headache's subtypes. Nevertheless, the frequency of combined headaches is still high, which indicates that to establish a final diagnosis, further information is required (309). However, in epidemiological studies, it is difficult to obtain additional information, since data is often obtained by the fulfillment of questionnaires or by telephonic interview (309). Migraine and tension-type headache symptoms frequently overlap and regarding our data, these two primary headaches types are frequently found combined and this symptoms overlap is more pronounced

when individuals have probable forms of headaches. One study suggested that to overcome this question, one solution could be not to combine symptoms like intensity, duration, aggravation by physical activity in one common criterion but to treat each symptom as a separate criterion (310). Probable headache types can not be ignored since some epidemiologic studies showed that, for instance, probable migraine is a common feature and it has been associated with temporary disability and reduction in health-related quality of life (311).

We also found that the basic structure of the classification remains unaltered and the most common subtypes, migraine without aura and episodic tension type headache have essentially the same criteria, also as suggested by Peatfield, 2004 (312).

Additionally, we found that in 98% of the patients of our clinical sample, the diagnosis remained the same, showing that even in clinical samples ICHD-II does not present significant changes for MO and MA forms. This is an important finding given that our patients are being enrolled in the clinical and genetic study of migraine since 1999 and we can now standardize the diagnosis criteria used in the clinical sample in future studies.

Noteworthy, one important difference in the second edition was the classification of tension-type headache into subgroups (7). This subdivision showed to be important also in our data since some cases previously coded as tension-type headache not fulfilling all ICHD-I criteria could now be classified as infrequent episodic tension-type headache by ICHD-II, decreasing the uncertainty of probable headache diagnosis.

Another important aspect was the redefinition of migraine in children in ICHD-II, which allowed also that some cases in our study that were coded as probable migraine in the previous classification were now classified as migraine.

Regarding the methodological approaches used in this study, it has been shown that questionnaire-based approaches are not a satisfactory tool (313). However, a validation study was performed in order to circumvent possible bias (4, 55), although we cannot exclude a recall bias that could explain the missing criteria, which may lead to a probable headache diagnosis, as the case of missing criterion B (attack duration).

This new classification has some improvements when compared with the first one, although it is still not perfect particularly when there are combinations of headaches (312) and still excludes some diagnostic aspects that could be relevant such as family history, age at onset, recurrence patterns of attacks, lifestyle and comorbidity

(314). It will be necessary to revise the classification when new pathophysiological, genetic or therapeutic evidence arise (312). Some prospective studies may be required to determine the evolution of headache disorders, which can result in some improvements in a future classification (315). This may help in further epidemiological studies that are important to observe the evolution of headaches' prevalence and to acquire more detailed information, crucial to obtain better therapeutic strategies. Nevertheless, ICHD-II classification seems to have relevant clinical implications but did not change remarkably the epidemiological results.

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Table 1. Diagnostic distribution of headaches according to the ICHD-I criteria

Group (IHS Code)	Frequency, n (%)
Primary headaches	1431 (80.4)
Migraine without aura (1.1)	120 (6.7)
Migraine with aura (1.2)	29 (1.6)
Migrainous disorder not fulfilling all criteria (1.7)	27 (1.5)
Episodic tension-type headache (2.1)	955 (53.7)
Chronic tension-type headache (2.2)	78 (4.4)
Headache of the tension-type not fulfilling all criteria (2.3)	222 (12.5)
Combined headaches	242 (13.6)
Migraine associated with other headaches	158 (8.9)
Tension-type headache associated with other headaches	81 (4.5)
Secondary headaches associated with primary headaches	3 (0.2)
Secondary headaches	107 (6.0)

Table 2. Diagnostic distribution of headaches according to the ICHD-II criteria

Group (IHS code)	Frequency. n (%)
Primary Headaches	1508 (84.7)
Migraine without aura (1.1)	118 (6.6)
Migraine with aura (1.2)	30 (1.7)
Probable migraine without aura (1.6.1)	44 (2.5)
Probable migraine with aura (1.6.2)	3 (0.1)
Infrequent episodic tension-type headache (2.1)	265 (14.9)
Frequent episodic tension-type headache (2.2)	677 (38.0)
Chronic tension-type headache (2.3)	78 (4.4)
Probable infrequent episodic tension-type headache (2.4.1)	92 (5.2)
Probable frequent episodic tension-type headache (2.4.2)	197 (11.1)
Probable chronic tension-type headache (2.4.3)	4 (0.2)
Combined headaches	165 (9.3)
Migraine associated with other headaches	101 (5.7)
Tension-type headache associated with other headaches	62 (3.5)
Secondary headaches associated with primary headaches	2 (0.1)
Secondary headaches	107 (6.0)

Table 3. Distribution of headaches according to the ICHD-I and ICHD-II classification

		ICHD-II ⁽⁺⁾											Secondary	Total
		MO	MA	PMO	PMA	FETTH	IETTH	CTTH	PFETTH	PIETTH	PCTTH	Combined		
ICHD-I ^(*)	MO	114	-	6	-	-	-	-	-	-	-	-	-	120
	MA	-	28	-	1	-	-	-	-	-	-	-	-	29
	PM	1	-	21	2	-	-	-	2	1	-	-	-	27
	ETTH	-	-	-	-	663	261	3	20	7	-	1	-	955
	CTTH	-	-	-	-	2	-	75	-	-	1	-	-	78
	PTTH	-	-	4	-	6	3	-	130	76	3	-	-	222
	Combined	3	2	13	-	6	1	-	45	8	-	164	-	242
	Secondary	-	-	-	-	-	-	-	-	-	-	-	107	107
Total		118	30	44	3	677	265	78	197	92	4	165	107	1780

(*) MO-migraine without aura; MA-migraine with aura; PM-migrainous disorder not fulfilling all criteria; ETTH-episodic tension-type headache; CTTH-chronic tension-type headache; PTTH-headache of the tension type not fulfilling all criteria

(+) MO-migraine without aura; MA-migraine with aura; PMO-probable migraine without aura; PMA-probable migraine with aura; FETTH-frequent episodic tension-type headache; IETTH-infrequent episodic tension-type headache; CTTH-chronic tension-type headache; PFETTH-probable frequent episodic tension-type headache; PIETTH-probable infrequent episodic tension-type headache; PCTTH-Probable chronic tension-type headache

Table 4. Frequency of primary and combined headaches according to the the ICHD-I and ICHD-II

		ICHD-II		
		Primary headaches	Combined headaches	Total
ICHD-I	Primary headaches	1430	1	1431
	Combined headaches	78	164	242
	Total	1508	165	1712

Table 5. Frequency of migraine and tension-type headache in the population according to the ICHD-I and ICHD-II

		ICHD-II					ICHD-II		
		Migraine	Without migraine	Total			TTH	Without TTH	Total
ICHD-I	Migraine	292	42	334	ICHD-II	TTH	1335	1	1336
	Without migraine	4	1670	1674		Without TTH	40	632	672
	Total	296	1712	2008		Total	1375	633	2008

Table 6. Missing criteria in cases with probable headache types

Missing Criteria	Migraine	Tension-type headache
A	0 (0%)	38 (13%)
B	30 (61%)	158 (53%)
C	4 (8%)	6 (2%)
D	15 (30%)	96 (32%)

4.3. Unravelling susceptibility factors for migraine

Several association studies have been performed for migraine, mostly with contradictory results and replication is therefore needed to confirm the observed association in other populations. One of our aims was to replicate previous positive findings in other populations, in order to assess if those genes were also implicated in migraine susceptibility in our Portuguese sample. We also wanted to study new candidate genes and assess possible interactions between genes.

The first association studied was with *STX1A*, a gene which encodes Syn1A, a protein involved in the SNARE complex, which is crucial for the regulation of presynaptic release of neurotransmitters. We found a significant result regarding two SNPs, rs941298 (which has been previously associated with migraine in a Catalanian sample (316)) and rs6951030 (which has never been associated with migraine before) **[article 5]**. These results strengthen the role of neurotransmitter release in migraine pathophysiology **[article 5]**.

We also studied *EDNRA*, a gene that has been associated with migraine (MO and MA) in other populations (204, 209). We found that rs702757 is a susceptibility factor for MO, as previously reported. Furthermore, we cannot exclude the involvement of this gene in MA susceptibility in our population since we found a trend towards an increased risk of rs5333 **[article 6]**.

We also wanted to further explore a possible role of BDNF and assess for the first time the involvement of CGRP in migraine susceptibility. Due to their connection in migraine pathophysiology, we also assessed a possible interaction between the two genes. No significant results were found for the genes, when analysed independently. However, we found an interaction between *BDNF* and *CGRP*, regarding the SNPs rs2049046 and rs1553005. This interaction should be further explored in larger samples in order to have a broader knowledge of the role of these genes in migraine susceptibility **[article 7]**.

Article 5. Syntaxin 1A in migraine susceptibility- evidence for its involvement in a Portuguese study

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Abstract

Migraine is a common disorder for which several candidate genes have been studied, with contradictory results. Recently, syntaxin 1A was proposed as a risk factor for migraine. The aim of this study was to confirm this, given that syntaxin 1A interacts with several players in migraine pathophysiology.

In a sample of 188 migraineurs (111 without aura (MO) and 77 with aura (MA)) and 287 migraine-free controls, three tagging SNPs (rs3793243, rs941298 and rs6951030) of *STX1A* gene were analyzed. A backward-stepwise multiple-logistic regression was performed. Allelic and haplotypic frequencies were compared between cases and controls.

We found that rs941298 and rs6951030 are risk factors for migraine patients. In particular, the TT-genotype of rs941298 is associated with an increased risk for both migraine in general and MO; the GG and GT-genotypes for rs6951030 are also associated with migraine, while the GT genotype of rs6951030 was found to be significant in the MO group. The SNP rs3793243 did not show any significant association. In the haplotype-based analysis, we found an under-representation of the T-C-T haplotype (rs3793243-rs941298-rs6951030) in the global sample and in MO. We found an enrichment of the G-allele of rs6951030 for female migraineurs only.

We thus confirm the involvement of syntaxin 1A in migraine susceptibility regarding SNP rs941298. In addition, we found rs6951030 to be also associated in Portuguese migraine patients. Gender differences should be further explored to disentangle a possible gender-susceptibility in syntaxin 1A.

Introduction

Migraine is a common neurological disorder, affecting about 15% of the general population (4, 45). Familial aggregation studies have shown that migraine is essentially due to genetic factors, with multiple genes contributing to its liability, in addition to environmental factors (25). We have also confirmed the presence of a genetic component in migraine with aura (MA) and without aura (MO), in a Portuguese population (293).

Although some loci for MA and MO have been proposed, the genes involved have not yet been identified. Several candidate genes have been studied, with contradictory results (25). Some evidence supports that the neurotransmitter system is also involved in migraine pathophysiology (103). Serotonin (5-HT) plays a role in pain modulation and serotonin agonists are an effective therapeutical approach to migraine (317). The serotonin transporter (5-HTT) is involved in 5-HT reuptake and is a target for 5-HT reuptake inhibitors (318).

Syntaxin 1A (Syn1A) is a presynaptic plasma membrane protein of the syntaxin family that, in conjunction with other proteins, compose the SNARE complex, crucial for the regulation of presynaptic release of neurotransmitters. Syn1A, encoded by the *STX1A* gene, is one of the regulatory proteins of the expression and subcellular localization of 5-HTT (318). Furthermore, Syn1A binds to the neuronal Gamma-aminobutyric acid (GABA) transporter (GAT-1) inhibiting its reuptake. GABA is the main inhibitory neurotransmitter in the brain and some GABA receptors agonists are used in migraine prophylaxis (319).

Nitric oxide (NO) is also involved in the formation of the SNARE complex and in the interaction of Syn1A and GAT-1 (320). Also NO has been associated with the nociception mechanism and, consequently, with migraine pathophysiology (321).

The gene encoding the α_{1A} subunit of a voltage-dependent calcium-channel (VDCC) (*CACNA1A*) is involved in a rare form of MA, familial hemiplegic migraine type 1 (FHM1). It has been shown that the association of the SNARE proteins with presynaptic Ca^{2+} channels, including *CACNA1A*, is essential for neurotransmitter release (322).

Recently, Syn1A was proposed as a risk factor for migraine (316). The aim of this study was to confirm the involvement of Syn1A in migraine's susceptibility, using a case-control approach, given that Syn1A interacts with several players in migraine pathophysiology.

Materials and methods

Subjects

A sample of 188 unrelated migraine patients (111 with MO, 77 with MA), from the Neurology out-patient clinic, at HSA, Porto, were sequentially enrolled in this study. Patients with FHM were excluded; migraineurs with co-occurrence of MA and MO were included in the MA group. Control subjects (287), with no history of migraine, were ascertained among healthy blood donors and from the Obstetrics and Gynecology department of HSA. Females with menstrual-related headache were excluded from the control group. Controls were from the same ethnic and geographical origin as the cases (Northern region of the country), and were age-matched to these. A diagnostic interview was performed in cases and controls, based on the operational criteria of the “International Headache Society” (IHS), using the same structured questionnaire. The first edition of the IHS criteria (ICHD-I) (279) was used before 2004; when revising the diagnosis using the second edition (ICHD-II) (6) no differences were found in patients’ diagnosis (data not shown). Participants gave their written informed consent and the project was approved by the Ethics Committee of HSA.

Selection of SNPs and genotyping

Genomic DNA was isolated from peripheral blood, using a standard salting out method(261) or from saliva, using ORAGENE kits according to the manufacturer’s instructions (DNA Genotek Inc.). SNPs were selected based on a data dump from The International HapMap Project (www.hapmap.org); tagging SNPs were selected using Haploview 4.1(263), at an r^2 threshold of 0.80, with a minor allele frequency (MAF) >0.10, by an aggressive tagging approach. Tagging SNPs included rs3793243 (located in chromosome 7: 72,759,283), rs941298 (72,763,199) and rs6951030 (72,771,177, according to the Ensembl database).

In a first stage, allelic discrimination was performed using molecular beacons and Real-Time PCR reactions (iQ5 Real-Time PCR Detection System, Bio-Rad Laboratories). A few discrepancies were found, however, after sequencing (to confirm uncertain genotypes): Real-Time PCR did not show to be a reliable method for allelic discrimination with these SNPs. Therefore, we chose to sequence all samples for rs3793243 and rs941298, while for rs6951030 we used a restriction-enzyme analysis.

All SNPs were PCR-amplified using HotStar Taq Master Mix Kit (Qiagen) according to manufacturer’s instructions (primer sequences are available upon request). Sequencing

was performed using the Big Dye Terminator Cycle Sequencing v1.1 Ready Reaction (Applied Biosystems), according to the manufacturer's instructions and samples were loaded in an ABI-PRISM 3130 XL genetic analyzer (Applied Biosystems). Restriction enzyme analysis was performed with *BsrI*, after PCR amplification, and digestion products were electrophoresed in a 2% agarose gel, stained with ethidium bromide.

Statistical Analysis

Power estimations were performed using the Genetic Power Calculator (<http://pngu.mgh.harvard.edu/~purcell/gpc/>). Analysis of Hardy-Weinberg equilibrium was performed using HWE software (<http://linkage.rockefeller.edu/ott/linkutil.htm>). Demographic data of patients and controls were compared using a chi-square test for categorical variables. To compare SNP allele frequencies between cases and controls, the chi-square test was used and odds ratios (OR) were estimated, with 95% confidence intervals (CI), and the significance level was set to $\alpha=0.05$.

A backward-stepwise multiple-logistic regression was performed (with the most frequent homozygote as the reference category), in order to evaluate the association between the SNPs' genotypes and the occurrence of migraine, by including the three SNPs and gender in the initial model. We also evaluated the role of *STX1A* in the migraine subtypes. All analyses were performed in the total sample, as well as in the MA and MO subsets. Significance was set to $\alpha= 0.016$ (considering three logistic regressions), using a Bonferroni correction, to correct for multiple comparisons.

These analyses were performed using SPSS (version 16.0 for Windows). Haplotype frequencies were compared between cases and controls, using Haploview 4.1(263) with all parameters set at the default values. Haplotypes were estimated, using an accelerated EM algorithm similar to the partition-ligation expectation-maximization algorithm described by Qin et al., and case-control counts were obtained by summing the fractional likelihoods of each individual for each haplotype (263, 273). Frequencies of haplotypes analysed were above >1%, according to the Haploview threshold; to correct for multiple comparisons, regarding estimation of allelic and haplotype frequencies, we used 10,000 permutations.

In the pooled sample, a chi-square and OR were estimated, assuming $\alpha=0.05$, to compare allelic and genotypic frequencies between cases and controls.

Results

We analyzed a sample of 188 unrelated migraineurs and 287 controls age-matched (a case:control ratio of 1:1.5). With this sample, we had a power of 64% to detect association (for a nominal $\alpha=0.05$), assuming a high risk allele frequency of 0.1, a relative risk for AA genotype of 2.25 and 1.5 for Aa genotype. A migraine prevalence of 16% had been previously estimated in a Portuguese population (21, 275).

Demographic and clinical data can be found in Table 1. A family history of migraine was present in 87% of the cases. No significant differences were found regarding gender, between patients and controls ($p>0.05$) (Table1).

Both case and control groups were in Hardy-Weinberg equilibrium for the three SNPs studied. The correlation between the three SNPs was small, denoting the weak LD between them according to the LD plot (Figure 1). In our sample, the three SNPs were also in weak LD (data not shown).

Regarding allele frequencies (Table 2), we found an enrichment of the G-allele of rs6951030 among migraineurs (OR=1.52, 95% CI: 1.12-2.06), as well as in the MO and MA groups (OR=1.48, 95% CI: 1.04-2.12, for MO; OR=1.58, 95% CI: 1.06-2.36, for MA); however, after permutation-based correction, the result for MO and MA did not reach significance. We found an enrichment of the T-allele for rs941298 in MO patients (OR=1.44, 95% CI: 1.04-1.99); however, this was not kept after permutation-based correction. No significant differences were found for this SNP in the other groups. There were no significant differences in the allele frequencies between cases and controls, regarding rs3793243, for any of the groups.

Additionally, we stratified these data by gender and found an enrichment of the G-allele of rs6951030 only for female migraineurs (OR=1.56, 95% CI: 1.11-2.20, but this was not significant after permutation-based correction). In males, no significant results were found (not shown).

Genotypic frequencies of the SNPs are shown in Table 3. Results from the backward-stepwise multiple-logistic regression, with the three SNPs and gender included in the initial model, are shown in Table 4. Values for each SNP were adjusted for the remaining significant variables in the model. For the migraine sample, the TT genotype of rs941298 showed an increased risk for migraine patients (OR=2.22, 95% CI: 1.19-4.12), significant

after Bonferroni correction; the GT and GG genotypes of rs6951030 were also associated with an increased risk for migraine (OR=1.68, 95% CI: 1.13-2.51 for GT, OR=3.27, 95% CI: 1.35-7.88 for GG), also significant after Bonferroni correction. Interestingly, the TT genotype of rs941298 showed an increased risk also for MO patients (OR=3.11, 95% CI: 1.52-6.33); and heterozygosity for rs6951030 was associated with an increased risk for MO (OR=1.85, 95% CI: 1.14-2.99), withstanding Bonferroni correction. An OR higher than 2 (OR=3.01, 95% CI: 1.05-8.64) was found for the GG-genotype, which may indicate that this genotype is also a risk factor for MO (although not statistically significant, after Bonferroni correction) similar to what was found in the MA sample (OR=3.20, 95% CI: 1.14-8.92). The SNP rs3793243 did not show any significant association.

In a haplotype-based analysis (Table 5), C-C-G was more frequent in migraineurs than in controls (OR=1.40, but did not withstand after permutation-based correction). Also, T-C-T was less frequent in migraineurs and in MO patients than in controls, suggesting a possible protective role of the T-allele of rs6951030 (OR=0.54, $p=0.03$, for migraine sample, OR=0.43, $p=0.02$ for the MO subgroup, after a permutation-based correction). Interestingly, we found an increased risk for the T-T-T haplotype in the MO sample, showing that the T-allele of rs941298 might confer an increased risk for MO (OR=1.44), but this was not significant after permutation-based correction.

We also stratified the haplotype-based analysis regarding gender and found a protective role for T-C-T in females (non-significant after multiple-testing correction). In males, we did not find any differences in haplotype frequencies between cases and controls (data not shown).

Patients with co-occurrence of MA and MO were first included in the MA sample. In a further analysis, we decided to exclude these patients from the analysis and the results found were similar regarding the involvement of the three SNPs in MA (data not shown).

Additionally, we performed a pooled analysis of our data and those published by Corominas et al. (316), excluding the Catalanian hemiplegic migraine patients. We found a significant association between rs941298 and the total and MO samples (not shown). With allele frequencies of rs941298 pooled together, risk was significantly increased for migraine sample and MO in patients with the T-allele (not shown). For rs6951030, we did not find any significant results after pooling.

We performed a PupaSuite database search (266), to assess a putative functional role of these SNPs: (1) rs6951030 is located in a conserved region, what may indicate it is important for gene regulation; (2) rs941298 is located in a triplex sequence, and may affect its structure formation, altering regulation. Additionally, we assessed if there were

any regulatory SNPs in LD with rs6951030 and rs941298. The SNP rs6951030 is not in LD with any other SNP genotyped in the HapMap project. We found that rs867500 (in LD with rs941298) is located in an exonic-splicing enhancer-motif.

Comments

We analyzed the role of *STX1A* in migraine susceptibility and, more specifically, in migraine subtypes MO and MA. Our findings confirm the involvement of *STX1A* as a risk factor for migraine. The intronic variants rs941298 and rs6951030 may play a role on migraine and, particularly, in MO. Additionally, we cannot exclude the effect of rs6951030 in MA, since homozygous state GG results in an OR above 2, however the result was not significant after Bonferroni correction.

The haplotype analysis also supports the involvement of the rs6951030 G-allele in migraine susceptibility and a protective role for the T-allele both for migraine and MO. With this we also confirmed the increased risk conferred by rs941298 T-allele. Comparing allele frequencies, we found an association of rs6951030 with migraine and migraine subtypes, while for rs941298 only an association with MO was found; results for rs3793243 were not significant.

We found a gender-related effect in rs6951030, since only the female group showed enrichment of the G-allele. This may be due to hormonal effects mediated by epigenetic modifications (323). We cannot exclude, however, that the non-increased risk in males is due to its small size in our sample. It would be important to explore this in larger samples, to disentangle a possible gender-susceptibility regarding *Syn1A*.

In the recent study from Catalonia (316) significant differences were found between cases and controls, both in allele and genotype frequencies of rs941298. The T-allele was overrepresented in cases, either with MO or MA. In our study, we also found an effect of the TT-genotype in the global sample and in the MO subset. In contrast to our results, no association with rs6951030 was found in the Catalanian study.

Although our study replicates the involvement of *STX1A* gene in migraine susceptibility, different variants were associated. Our data suggests a stronger effect of this gene in MO, regarding rs6951030 and rs941298.

The difference between our results and those from Catalonia (316) regarding rs6951030 may be due to allele-frequency variation across populations; it may be more marked due

to gene-gene and gene-environment interactions (324). This emphasizes the importance of replicating association studies in several populations.

Additionally, when pooling both samples, we found a significant effect of the T-allele of rs941298 in migraineurs; this was expected, as the Catalanian study found significant results for this SNP and we saw an increased risk for the TT-genotype in our sample. These results show that this SNP may be involved in susceptibility to migraine in both populations. More importantly, we found an increased risk of the G-allele of rs6951030 for Portuguese migraineurs, not present in the pooled sample: we can postulate that the association with rs6951030 is specific of our population.

In FHM, glutamate release, facilitated by the mutated VDCC α_{1A} subunit, results in cortical spreading depression (CSD), a slowly propagating wave of neuronal and glial depolarization, spreading across the cortex (28). CSD is the underlying mechanism implicated in the visual, sensory or motor aura observed in MA, during which several neurotransmitters are released (28). In MO, imaging studies suggest that CSD may occur in silent brain areas, leading to pain but not other symptoms (35, 36). The association of the SNARE proteins (including Syn1A) with presynaptic Ca^{2+} channels, namely the VDCC α_{1A} subunit, is essential for neurotransmitter release (322). Therefore, polymorphisms in *STX1A* gene may alter the interaction between Syn1A and calcium channels, modifying the functional state of the VDCC α_{1A} , altering neurotransmitter release and, ultimately, contributing to CSD and the migraine phenotype (316, 325).

Although no functional role has been described yet for any of these intronic SNPs, it would be important to evaluate further that possibility. We found that both rs941298 and rs6951030, but also rs867500 (which is LD with rs941298), are located in regions potentially important for regulation. Further analyses are needed to evaluate a potential functional role of these SNPs and understand their effect on Syn1A.

Although our sample is not very large, we had special concern in obtaining a high case:control ratio, to increase power. Also, cases and controls were matched for age at observation and gender, and were from the same geographic region (several studies, using markers sensitive to population stratification, as lineage markers, have showed there is no population substructure among the Portuguese population (326-328)). Also, we used corrections for multiple testing to prevent type-I errors (Bonferroni and permutation-based corrections) and finally, we used logistic regression analyses in order to analyze the SNPs effects altogether.

In conclusion, our study favours and brings new insight into the involvement of syntaxin 1A in susceptibility to migraine, and strengthens the role of neurotransmitter release in its pathophysiology. We can thus confirm the association between Syn1A and migraine found in Catalanian patients. Further studies with larger samples in other populations will be important to disentangle the role of this gene in migraine subtypes.

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Table 1. Demographic and clinical data of migraine patients and controls

Migraine patients (<i>n</i>)	188
MA	77
MO	111
Females	153
Males	35
mean age at observation (SD)	36.14 yrs (12.84)
mean age at onset (SD)	17.67 yrs (8.15)
family history of migraine	87%
Migraine-free controls (<i>n</i>)	287
females	217
males	70
mean age at observation (SD)	36.42 yrs (12.35)

Table 2. Allele frequencies of SNPs studied in migraine patients and controls

SNP	Alleles (%)		χ^2	OR (95% CI)	<i>P</i>	<i>P</i> (after permutation)
rs3793243	T	C				
all patients	159 (42.3)	217 (57.7)	0.00	1.00 (0.77-1.30)	0.99	1.00
MO	100 (45.0)	122 (55.0)	0.48	1.12 (0.82-1.52)	0.48	0.97
MA	59 (38.3)	95 (61.7)	0.81	0.85 (0.59-1.22)	0.37	0.89
controls	243 (42.3)	331 (57.7)	-	-	-	
rs941298	T	C				
all patients	130 (34.6)	246 (65.4)	2.05	1.22 (0.93-1.62)	0.15	0.56
MO	85 (38.3)	137 (61.7)	4.85	1.44 (1.04-1.99)	0.03	0.13
MA	45 (29.2)	109 (70.8)	0.05	0.96 (0.65-1.41)	0.82	1.00
controls	173 (30.1)	401 (69.9)	-	-	-	
rs6951030	G	T				
all patients	107 (28.5)	269 (71.5)	7.48	1.52 (1.12-2.06)	0.006	0.03
MO	62 (27.9)	160 (72.1)	4.72	1.48 (1.04-2.12)	0.03	0.14
MA	45 (29.2)	109 (70.8)	5.01	1.58 (1.06-2.36)	0.02	0.12
controls	119 (20.7)	455 (79.3)	-	-	-	

Abbreviations:OR: odds ratio; CI: confidence interval. Nominal and corrected- significant values are shown in bold

Table 3. Genotype frequencies in patients and controls

SNPs	Genotypes n (%)			
	CC	TC	TT	Total
rs3793243				
all patients	67 (35.6)	83 (44.2)	38 (20.2)	188
MO	37 (33.3)	48 (43.2)	26 (23.4)	111
MA	30 (39.0)	35 (45.5)	12 (15.6)	77
controls	89 (31.0)	153 (53.3)	45 (15.7)	287
rs941298	CC	CT	TT	Total
all patients	28 (14.9)	74 (39.4)	86 (45.7)	188
MO	46 (41.4)	45 (40.5)	20 (18.0)	111
MA	40 (51.9)	29 (37.7)	8 (10.4)	77
controls	28 (9.8)	117 (40.8)	142 (49.5)	287
rs6951030	TT	TG	GG	Total
all patients	95 (50.5)	79 (42.0)	14 (7.5)	188
MO	56 (50.5)	48 (43.2)	7 (6.3)	111
MA	39 (50.6)	31 (40.3)	7 (9.1)	77
controls	178 (62.0)	99 (34.5)	10 (3.5)	287

Table 4. Results from the backward-stepwise multiple-logistic regression

Genotypes	Migraine			MO			MA		
	OR	95% CI	<i>P</i>	OR	95% CI	<i>P</i>	OR	95% CI	<i>P</i>
rs3793243			<i>0.25</i>			<i>0.30</i>			<i>0.67</i>
CC (ref ^a)	1.00	-	-	1.00	-	-	1.00	-	-
CT	0.66	0.39-1.11	<i>0.12</i>	0.62	0.32-1.19	<i>0.15</i>	0.80	0.44-1.42	<i>0.44</i>
TT	0.86	0.38-1.94	<i>0.71</i>	0.88	0.33-2.34	<i>0.80</i>	1.02	0.46-2.29	<i>0.96</i>
rs941298			<i>0.04</i>			<i>0.008</i>			<i>0.85</i>
CC (ref ^a)	1.00	-	-	1.00	-	-	1.00	-	-
CT	1.16	0.77-1.75	<i>0.48</i>	1.32	0.80-2.18	<i>0.27</i>	1.16	0.59-2.26	<i>0.67</i>
TT	2.22	1.19-4.12	<i>0.012</i>	3.11	1.52-6.33	<i>0.002</i>	1.36	0.42-4.45	<i>0.61</i>
rs6951030			<i>0.004</i>			<i>0.015</i>			<i>0.06</i>
TT (ref ^a)	1.00	-	-	1.00	-	-	1.00	-	-
GT	1.68	1.13-2.51	<i>0.011</i>	1.85	1.14-2.99	<i>0.013</i>	1.43	0.84-2.43	<i>0.19</i>
GG	3.27	1.35-7.88	<i>0.008</i>	3.01	1.05-8.64	<i>0.04</i>	3.20	1.14-8.92	<i>0.03</i>

Abbreviations: OR: odds ratio; CI: confidence interval, ^aindicates the reference category; after Bonferroni correction, significance level was set to $\alpha=0.016$. Significant values are shown in bold.

Table 5. Results from the haplotype analysis

Haplotypes (rs3793243-rs941298- rs6951030)	Cases		Controls		OR	95% CI	X ²	P ^a	P (after permutation)
	carriers	non-carriers	carriers	non-carriers					
All patients									
C-C-G	89.9	286.1	105.4	468.6	1.40	1.02-1.92	4.28	0.04	0.17
T-C-T	30.8	345.2	81.7	492.3	0.54	0.35-0.83	7.94	0.005	0.03
MO									
T-C-T	14.9	207.1	81.7	492.3	0.43	0.24-0.77	8.49	0.004	0.02
T-T-T	74.1	147.9	147.9	426.1	1.44	1.03-2.02	4.61	0.03	0.15

^aOnly haplotypes with any significant result are shown. Nominal and corrected- significant values are shown in bold.

Article 6. A variant in endothelin receptor type A is associated with migraine without aura

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Submitted

Abstract

Objective: We aim to assess the possible involvement of *EDNRA* in migraine in a large sample of Portuguese migraineurs (with and without aura).

Background: Migraine is a common disabling disorder. Anomalies of vascular function have been implied in its pathophysiology. Several findings point to a possible role of endothelin receptor type A (*EDNRA*) in migraine.

Methods: Using a case-control approach, three tagging SNPs (rs702757, rs5333 and rs5335) of *EDNRA* were analyzed in 188 cases – 111 without (MO) and 77 with aura (MA) – and 287 controls. A multivariable logistic regression included the three SNPs in a model adjusted for gender. Allelic and haplotypic frequencies were compared between cases and controls.

Results: We found an increased risk for the rs702757 A-allele (OR=1.44, 95% CI: 1.05-1.99), in MO patients. The logistic regression showed a borderline significant increased risk of AA genotype (OR=2.34, 95% CI: 1.12-4.90) for MO after correction for multiple testing. A trend towards an increased risk for MA patients regarding the C-allele of rs5333 was also found. The A-C-G haplotype was overrepresented in the general migraine sample (OR=1.75, 95% CI: 1.06-2.90) and the MO subgroup (OR=2.06, 95% CI: 1.18-3.59); after permutation-based correction, this was significant only for MO patients.

Conclusions: Our results reinforce the role of *EDNRA* (rs702757) as a susceptibility factor for MO, and, thus, of the vascular component in migraine pathophysiology. We cannot exclude the involvement of this gene in MA susceptibility in our population. Our study also emphasizes the need for replication of association findings in different populations.

Introduction

Migraine is a common disabling primary headache, leading to a diminished quality of life in migraineurs and their relatives. (8) Anomalies of vascular function, with dilatation of cerebral blood vessels and release of vasoactive neuropeptides have been implied its pathophysiology. (28, 193)

Endothelin-1 (ET-1) is one of the most potent vasoconstrictors, synthesized by endothelial and vascular smooth muscle cells. (329) Plasma ET-1 levels may increase during migraine attacks and remain so hours after. (200, 201) Also, ET-1 is an inducer of cortical spreading depression (CSD) that underlies migraine with aura (MA), although it may also be implicated in migraine without aura (MO). This may be one of the links between the vascular theory of Woolf and the neuronal theory of Leão and Morrison. (34, 35, 199)

Endothelin type A receptor (EDNRA) mediates the biological effects of ET-1. It is expressed in vascular smooth muscle cells, and its activation by ET-1 leads to vasoconstriction. (203) EDNRA also mediates the inhibitory effect of ET-1 on nitric oxide (NO) synthesis, which, on its turn, has been associated with nociception and, consequently, with migraine pathophysiology. (201, 321) One study in rats showed that the effect of ET-1 on CSD may be mediated by EDNRA and lead to the formation of a microarea of selective neuronal necrosis. (199) Some described an association between a polymorphism of the *ENDRA* gene and migraine, although this was not replicated in another study in a paediatric sample. (204, 210) A recent study found an association between an *EDNRA* polymorphism and MA and with age at onset below 20 years. (209) All these findings point to a possible role of EDNRA in migraine. Our aim was to assess its possible involvement in a Portuguese population.

Materials and methods

Subjects

A total of 188 unrelated migraine patients (111 with MO, 77 with MA) from the Neurology out-patient clinic, at HSA, Porto, were sequentially enrolled. Patients with familial hemiplegic migraine were excluded; those with co-occurrence of MA and MO were included in the MA group. Controls (287), with no history of migraine, were ascertained among healthy blood donors and from the Obstetrics and Gynecology department of HSA. Controls were from the same ethnic and geographical origin as cases, and were age-matched to these. A diagnostic interview was performed in all, based on the operational criteria of the International Headache Society (IHS), using a structured questionnaire. ICHD-I, the first edition of the IHS criteria (279), was used before 2004; when reviewing the diagnoses with ICHD-II (6) no differences were found (not shown). Participants gave written informed consent; the project was approved by the Ethics Committee of HSA.

Selection of SNPs and genotyping

Genomic DNA was isolated from peripheral blood, using a standard salting-out method (261); or from saliva, using ORAGENE kits according to the manufacturer's instructions (DNA Genotek Inc.). SNPs were selected based on a data dump from the HapMap Project (www.hapmap.org), with a minor allele frequency (MAF) of 0.10. Using Haploview 4.1 (263), one tagging SNP was selected from three haplotype blocks of the *EDNRA* gene. These tagging SNPs included rs702757 (located in chromosome 4: 148,409,772; on intron 2), rs5333 (148,461,537; on exon 6, p.H323H) and rs5335 (148,464,340 according to the Ensembl database; on 3'UTR). Allelic discrimination was performed using Real-Time PCR (iQ5 RT PCR Detection System, Bio-Rad). Primers and molecular beacons were designed using Beacon Designer 6.0. Reactions were performed using 20 ng/ μ L DNA, 10 μ L iQTM Supermix or iQTM Multiplex Powermix, 1.5-8.0 mM MgCl₂ and 0.5 μ M of each primer, in 20 μ L final reaction volume. The concentration of the probes used was: for rs702757, 0.05 μ M of probe 1 (labelled with FAM) and 0.15 μ M of probe 2 (labelled with Cy3) and for rs5335, 0.05 μ M of probe 1 (labelled with FAM) and 0.05 μ M of probe 2 (labelled with Cy3). Primer and probe sequences are available upon request.

Cycling conditions used were: an initial denaturing at 95°C for 3m and 30s, followed by 35 cycles of 30s at 95°C, 60s at 60°C (for the annealing; at this point fluorescence was measured) and 30s at 72°C. Genotypes were determined automatically by the signal processing algorithms of the iQ5 Optical System Software, version 2.0 (Bio-Rad) and

results compared with the genotypes visually scored with the fluorescent emission data depicted (RFUs).

To validate RT PCR, we included three controls genotyped by sequencing (one heterozygote and homozygotes for variants 1 and 2). Uncertain genotypes were sequenced. A few discrepancies were found, however, after sequencing only for rs5333: Real-Time PCR did not show to be a reliable method for allelic discrimination of this SNP. Therefore, for this SNP, all cases and controls were sequenced.

The Big Dye Terminator Cycle Sequencing v1.1 Ready Reaction and an ABI-PRISM 3130 XL (Applied Biosystems) were used according to manufacturer's instructions.

Statistical Analysis

Power to detect association was estimated with the Genetic Power Calculator (<http://pngu.mgh.harvard.edu/~purcell/gpc/>). Hardy-Weinberg equilibrium was tested, using HWE software (<http://linkage.rockefeller.edu/ott/linkutil.htm>). Demographic data of patients and controls were compared using a chi-square test for categorical variables. SNP allele frequencies were compared between cases and controls, a chi-square test was used and odds ratios (OR) were estimated with 95% confidence intervals (CI) and the level of significance was set at $\alpha=0.05$. A multivariable logistic regression was performed (with the most frequent homozygote as the reference), including the three SNPs and adjusting for gender in the model. All analyses were done in the total sample, as well as in MA and MO subsets. Significance was set to $\alpha=0.016$ (considering three logistic regressions), using a Bonferroni correction for multiple comparisons.

These analyses were performed with SPSS (version 16.0 for Windows). Haplotype frequencies were compared between cases and controls, using Haploview 4.1 (263), with all parameters set at the default. Haplotypes were estimated using an accelerated EM algorithm similar to the partition-ligation expectation-maximization one; cases-controls were counted by summing the fractional likelihoods of each individual for each haplotype. (263, 273) Haplotypes analysed were above 1%, according to the Haploview threshold; to correct for multiple comparisons, when estimating allelic and haplotype frequencies, 10,000 permutations were used.

Results

Demographic data from patients and controls are shown in Table 1. There were no differences between patients and controls regarding gender. Case-control ratio was 1:1.5; power to detect association (significance level of 0.05) was 64%, assuming a high risk allele frequency of 0.1, a relative risk for homozygotes of 2.25 and 1.5 for heterozygotes. A prevalence of 16% had been estimated before for migraine in this population. (275)

All SNPs analyzed were in Hardy-Weinberg equilibrium, in case and control groups. The three SNPs were in weak LD ($r^2 < 0.80$) (Fig. 1). Allele frequencies are shown in Table 2. We found an increased risk of the rs702757A-allele (OR=1.44, 95% CI: 1.05-1.99) in the group of MO patients; however, this did not reach significance after correction for multiple testing. No other differences were found.

Genotype frequencies are shown in Table 3, and results from the multivariable logistic regression are in Table 4. When incorporating all SNPs and adjusting for gender in the model, there was an increased risk of the AA genotype of rs702757 (OR=2.34, 95% CI: 1.12-4.90) for MO. Interestingly, we found a trend towards an increased risk for MA patients regarding the C-allele of rs5333 (OR=2.21, 95% CI: 0.96-5.09). No other significant results were found, either in the multivariable or in the univariable logistic analysis.

The haplotype A-C-G (Table 5) was overrepresented in the migraine sample (OR=1.75, 95% CI: 1.06-2.90) and in MO patients (OR=2.06, 95% CI: 1.18-3.59). After permutations, the result is only significant for MO patients. No other differences were found.

Allele and haplotype frequencies did not differ significantly with gender (data not shown).

Discussion

Vascular events have been associated with pathophysiology of migraine, in particular the vasodilatation, followed by constriction, during CSD. (330) Functional arterial properties may also be altered in migraine. (331)

EDNRA is involved in vasoconstriction and has been suggested as a susceptibility factor. (204, 209) It was associated with MA in Finnish patients and a pooled Finnish-German sample. (209) It was also associated with migraine in French patients, in particular with MO. (204)

In our sample of Portuguese patients, we now found significant differences between cases and controls, for rs702757, in the MO subgroup. The frequency of the A-allele and the AA-genotype were enriched in MO, both borderline significant after multiple testing corrections. Also, we found an enrichment of the A-C-G haplotype in MO patients, significant after permutation-based correction.

Cases and controls were matched for age at observation and gender, and were from the same geographic region; lineage-markers have shown there was no stratification in the Portuguese population. (326, 328) Also, we corrected for multiple testing (to prevent type-I errors) and used logistic regression to analyze the effects of all SNPs together. We paid special attention in obtaining a high case:control ratio, to increase power.

Though CSD is more often associated with MA, imaging studies suggested CSD may occur in silent brain areas, leading to pain but not other symptoms. (28, 35)

Our results suggest *EDNRA* is involved in susceptibility to migraine in our population, particularly in MO. Linkage to a region near the *EDNRA* locus was reported before. (151) Moreover, rs702757 is located in a conserved region and may be involved in gene regulation. (266) Even if its A-allele is not the effective risk allele, it may be in linkage disequilibrium (LD) with a true one; e.g., it was shown to be in strong LD ($r^2 > 0.92$) with allele G of rs1801708. (204) Though a protective role for the G-allele has been postulated in French patients (204), risk alleles and their frequency vary across populations, and may be enhanced by other gene and environment interactions. (324) This emphasizes the need for replication of association findings in different populations. Importantly, Tikka-Kleemola et al. did not find a significant result for rs702757 in their MA patients while our results show involvement of this SNP in MO. (209) We cannot exclude a role of this gene in MA for our population, since we found a trend towards an increased risk of CC-genotype of rs5333 in MA group. The C-allele of this SNP is in LD with T-allele of rs2048894, which has been found to be associated with MA in the study of Tikka-Kleemola et al. (209) These data show that this gene may be associated with MO and MA susceptibility.

An antagonist of both *EDNRA* and *EDNRB* proved not to be effective in migraine treatment. (332, 333) These results could be explained by the population in study and/or opposite effects of *EDNRA* and *EDNRB*. Pharmacological studies with *EDNRA* only would clarify its importance as a potential target. (334)

Our results thus reinforce the role of *EDNRA* as a susceptibility factor and of the vascular component in migraine pathophysiology. Dissecting these genetic susceptibilities will be crucial to develop better therapeutic strategies, as the risk variants implied and their effects may vary in different populations.

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We would like to thank all patients and controls for participating in this study. We thank also Dr. Serafim Guimarães, nurses Teresa Gomes and Palmira Gouveia for their assistance with sample collection, Paula Magalhães for technical assistance and Zoltán Bochnanovits for his helpful suggestions. The authors report no conflicts of interest.

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Table 1. Demographic and clinical data of migraine patients and controls

Migraine patients (<i>n</i>)	188
MA	77
MO	111
females	153
males	35
mean age at observation ($\pm$ SD)	36.14 yrs ($\pm$ 12.84)
mean age at onset ($\pm$ SD)	17.67 yrs ($\pm$ 8.15)
family history of migraine	87%
Migraine-free controls (<i>n</i>)	287
females	217
males	70
mean age at observation ($\pm$ SD)	36.42 yrs ($\pm$ 12.35)

Table 2. Allele frequencies of SNPs studied in migraine patients and controls.

<i>EDNRA</i> SNPs	Alleles (%)		χ^2	OR (95% CI)	<i>P</i>	<i>P</i> (after permutation)
rs702757	A	T				
all patients	139 (37.0)	237 (63.0)	2.81	1.26 (0.96-1.66)	0.09	0.37
MO	89 (40.1)	133 (59.9)	5.01	1.44 (1.05-1.99)	0.02	0.10
MA	50 (32.5)	104 (67.5)	0.03	1.04 (0.71-1.52)	0.86	1.00
controls	182 (31.7)	392 (68.3)	-	-	-	
rs5333	C	T				
all patients	111 (29.5)	265 (70.5)	2.46	1.26 (0.94-1.69)	0.12	0.45
MO	63 (28.4)	159 (71.6)	1.00	1.19 (0.84-1.69)	0.32	0.88
MA	47 (30.5)	107 (69.5)	1.98	1.32 (0.90-1.96)	0.16	0.58
controls	143 (24.9)	431 (75.1)	-	-	-	
rs5335	C	G				
all patients	155 (41.2)	221 (58.8)	0.25	0.94 (0.72-1.22)	0.62	1.00
MO	90 (40.5)	132 (59.5)	0.35	0.91 (0.66-1.24)	0.55	0.99
MA	65 (42.2)	89 (57.8)	0.02	0.97 (0.68-1.4)	0.88	1.00
controls	246 (42.9)	328 (57.1)	-	-	-	

OR: odds ratio; CI: confidence interval; Nominal-significant values are shown in bold

Table 3. Genotype frequencies in patients and controls

EDNRA SNPs		Genotypes n (%)		
rs702757	TT	TA	AA	Total
all patients	71 (37.8)	95 (50.5)	22 (11.7)	188
MO	39 (35.1)	55 (49.5)	17 (15.3)	111
MA	32 (41.6)	40 (51.9)	5 (6.5)	77
controls	128 (44.6)	136 (47.4)	23 (8.0)	287
rs5333	TT	TC	CC	Total
all patients	98 (52.1)	70 (37.2)	20 (10.6)	188
MO	57 (51.4)	45 (40.5)	9 (8.1)	111
MA	41 (53.2)	25 (32.5)	11 (14.3)	77
controls	164 (57.1)	103 (35.9)	20 (7.0)	287
rs5335	GG	GC	CC	Total
all patients	67 (35.6)	87 (46.3)	34 (18.1)	188
MO	40 (36.0)	52 (46.8)	19 (17.1)	111
MA	27 (35.1)	35 (45.5)	15 (19.5)	77
controls	87 (30.3)	154 (53.7)	46 (16.0)	287

Table 4. Results from a multivariable logistic regression, gender-adjusted

Genotypes	Migraine			MO			MA		
	OR	CI 95%	P	OR	CI 95%	P	OR	CI 95%	P
rs702757			<i>0.34</i>			<i>0.08</i>			<i>0.78</i>
TT (ref)	1.00	-	-	1.00	-	-	1.00	-	-
TA	1.23	0.82-1.85	<i>0.31</i>	1.31	0.80-2.14	<i>0.28</i>	1.13	0.66-1.94	<i>0.67</i>
AA	1.59	0.81-3.10	<i>0.18</i>	2.34	1.12-4.90	0.02	0.80	0.27-2.32	<i>0.67</i>
rs5333			<i>0.50</i>			<i>0.78</i>			<i>0.14</i>
TT (ref)	1.00	-	-	1.00	-	-	1.00	-	-
TC	1.09	0.73-1.63	<i>0.67</i>	1.18	0.73-1.90	<i>0.49</i>	0.97	0.55-1.70	<i>0.92</i>
CC	1.50	0.76-2.99	<i>0.24</i>	1.14	0.48-2.71	<i>0.77</i>	2.21	0.96-5.09	<i>0.06</i>
rs5335			<i>0.24</i>			<i>0.38</i>			<i>0.36</i>
GG (ref)	1.00	-	-	1.00	-	-	1.00	-	-
GC	0.71	0.46-1.07	<i>0.10</i>	0.70	0.43-1.16	<i>0.16</i>	0.71	0.40-1.26	<i>0.24</i>
CC	0.91	0.52-1.59	<i>0.74</i>	0.83	0.42-1.61	<i>0.58</i>	1.08	0.52-2.26	<i>0.84</i>

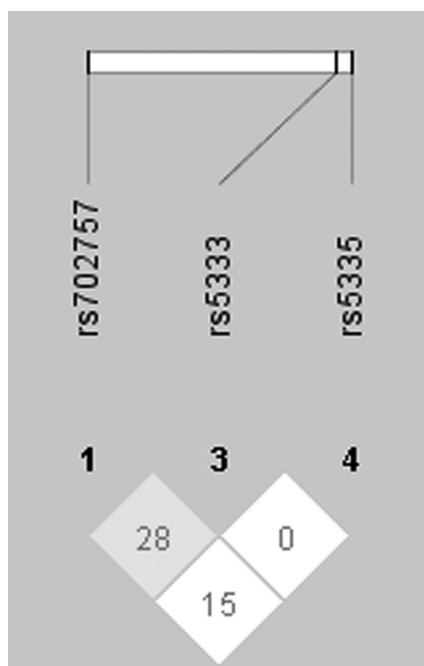
(ref) indicates the reference category; OR: odds ratio; CI: confidence interval; after Bonferroni correction, significance level was set to $p < 0.016$.

Table 5. Haplotype analysis for the *EDNRA* gene (rs702757- -rs5333-rs5335)

Haplotypes	Cases		Controls		OR	CI 95%	X ²	P	P (after permutation)
	carriers	non-carriers	carriers	non-carriers					
All patients									
A-C-G	34.3	341.7	31.1	542.9	1.75	1.06-2.90	4.86	0.03	0.11
MO									
A-C-G	23.7	198.3	31.5	542.5	2.06	1.18-3.59	6.72	0.009	0.03
MA									
A-C-G	9.2	144.8	26.3	547.7	1.32	0.61-2.87	0.52	0.47	0.98

Only haplotypes with a significant result in any of the patients sample are shown. Nominal and corrected significant values are shown in bold

Figure 1. LD plot of SNPs analysed within *EDNRA* gene; r^2 values are given within each square.



Article 7. BDNF and CGRP interaction: implications in migraine susceptibility

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In Preparation

Abstract

Migraine pathophysiology involves several pathways. Our aims were to explore a possible role of *BDNF* in migraine susceptibility, to study, for the first time, *CGRP* and a possible interaction between the two genes.

Using a case-control approach, four tagging SNPs (rs7124442, rs6265, rs11030107, and rs2049046) of *BDNF* and one tagging SNP – rs1553005 of *CGRP* were analyzed in 188 cases – 111 without (MO) and 77 with aura (MA) – and 287 controls. A multivariable-logistic regression was performed, adjusting for gender. Allelic and haplotypic frequencies were estimated. Interaction was assessed by a stepwise multivariable-logistic regression.

No significant main effects were found; however, a significant interaction was found between *BDNF* and *CGRP*, showing an increased risk for the AT-genotype of rs2049046 and the GC-genotype of rs1553005 (OR= 1.88, 95% CI: 1.20-2.93) for migraineurs.

Our data support the hypothesis of an interaction between *BDNF* and *CGRP* in migraine susceptibility that should be further explored.

Keywords: Migraine, association, *BDNF*, *CGRP*, pathophysiology

Introduction

Migraine is a common multifactorial disease (67, 83). Several different processes are involved in its pathophysiology, such as alteration of pain and sensory input, increased sensitivity of the cortex leading to aura, central pain facilitation, neurogenic inflammation and brain stem nociceptor sensitization (335, 336).

BDNF (brain-derived neurotrophic factor) is important for neuronal growth, development and survival in the central nervous system, and is the most abundant neurotrophin in the brain (337, 338). BDNF is expressed in the nociceptive sensory neurons, acting as central pain modulator at both the spinal and supra-spinal level, contributing to central sensitization and modulating the activation of glutamatergic, N-methyl-d-aspartate (NMDA) receptors (339-341). A significant decrease in the levels of BDNF in platelets was found in migraineurs, showing that it may also have a role in migraine pathophysiology (340). One of the mechanisms involved in migraine is central sensitization, characterized by activity-dependent plastic changes in second-order trigeminal neurons (33). BDNF has been pointed as a mediator of the trigeminal nociceptive plasticity (341, 342). A previous study evaluated several genes that can be expressed after cortical spreading depression (CSD), the mechanism hypothesized to underlie migraine pathophysiology, especially migraine with aura (MA) (33, 343). *BDNF* was one of the genes found to be differentially expressed after CSD (343).

Several studies showed that neurogenic inflammation is associated with pathogenesis of migraine (344). When neuronal excitability occurs, leading to primary brain dysfunction, the perivascular trigeminal sensory nerve fibers are activated, resulting in the release of vasoactive peptides, such as substance P and calcitonin-gene related peptide (CGRP), from trigeminal fibers (344). CGRP is the most abundant neuropeptide in perivascular sensory trigeminal nerve fibres and one of the mediators of neurogenic inflammation; it is a potent vasodilator (345, 346), enhancing the activation of second-order neurons, thus contributing to pain transmission (28, 344). CGRP has an active role in the activation and sensitisation of nociceptors at the peripheral and central level (347), modulating nociceptive transmission in the trigeminovascular system (346).

The involvement of CGRP in migraine pathophysiology has been supported by the finding that the serum levels of CGRP are increased during migraine attacks and there is a decrease after treatment with triptans, although this was not observed in a more recent study (348-351).

CGRP is coexpressed with BDNF in trigeminal ganglion neurons in rats. In addition, CGRP enhances BDNF release from trigeminal neurons *in vitro*; this is independent of extracellular calcium and is abolished by a CGRP-receptor antagonist (342).

A case-control study found no evidence of a *BDNF* SNP (rs6265) being associated with migraine susceptibility (352). The aims of this study were (1) to further explore a possible role of *BDNF* in migraine susceptibility and (2) to study, for the first time, *CGRP* as a candidate gene. Furthermore, since both genes are coexpressed and involved in migraine pathophysiology, (3) we also assessed their possible interaction in migraine's susceptibility.

Subjects and methods

Subjects

A sample of 188 unrelated migraine patients (111 with MO, 77 with MA), from the Neurology clinic, at HSA, Porto, were sequentially enrolled in this study. Patients with familial hemiplegic migraine were excluded; migraineurs with co-occurrence of MA and MO were included in the MA group. Control subjects (287), with no personal history of migraine, were ascertained among healthy blood donors and from the Obstetrics and Gynecology department of HSA. Controls were from the same ethnic and geographical origin (north of Portugal) as cases, and were age-matched to these. A diagnostic interview was performed both in cases and controls, based on the operational criteria of the “International Headache Society” (IHS), using the same structured questionnaire. The first edition of these criteria (ICHD-I) (279) was used before 2004; revising the diagnosis using the second edition (ICHD-II) (6) showed no differences in patients' diagnosis (data not shown). Participants gave their written informed consent and the project was approved by the Ethics Committee of HSA.

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes, using a standard salting-out method (261); or from saliva using ORAGENE kits and DNA extraction according to the manufacturer's instructions (DNA Genotek Inc.).

SNPs were selected based on a data dump from The International HapMap Project (www.hapmap.org) and tagging SNPs were selected using Haploview 4.1 (263), at an r^2 threshold of 0.80 and with a minor allele frequency (MAF) of 0.10, by an aggressive tagging approach. For *BDNF*, these included rs712442 (located on chromosome 11: 27,634,117; on 3' UTR), rs6265 (27,636,992; a functional SNP: p.V66M), rs11030107 (27,650,911; on intron

1) and rs2049046 (27,679,851, according to the Ensembl database; on 3' gene region). Two of the SNPs were combined as a 2-marker haplotype, allowing coverage of a broader region of SNPs tagged. For *CGRP*, rs1553005 (located in chromosome 11: 14,950,566; on 3' gene region) was selected.

Allelic discrimination was performed using Real-Time PCR (iQ5 Real-Time PCR Detection System, Bio-Rad Laboratories). Primers and molecular beacons were designed using Beacon Designer 6.0.

For *BDNF*, Real-Time PCR reactions were performed using 20 ng/μL DNA, 10 μL of iQTM *Supermix* or iQTM Multiplex Powermix, 1.2-8.0 mM MgCl₂ and 0.5 μM of each primer, in 20 μL final reaction volume. Concentration of the probes used was 0.06 μM of probe 1 (labelled with FAM) and 0.25 μM of probe 2 (labelled with Cy3), for rs6265; 0.04 μM of probe 1 (labelled with FAM) and 0.16 μM of probe 2 (labelled with Cy3), for rs2049046; 0.03 μM of probe 1 (labelled with FAM) and 0.15 μM of probe 2 (labelled with Cy3), for rs7124442; 0.03 μM of probe 1 (labelled with FAM) and 0.30 μM of probe 2 (labelled with Cy3), for rs11030107. For the rs1553005 SNP of *CGRP*, Real-Time PCR reactions were performed using 20 ng/μL DNA, 10 μL of iQTM *Supermix*, 8 mM MgCl₂, 0.5 μM of each primer, 0.03 μM of probe 1 (labelled with FAM) and 0.15 μM of probe 2 (labelled with Texas Red), in 20 μL final volume.

Primer and probe sequences are available upon request. Cycling conditions used were: an initial denaturing at 95°C, for 3 min and 30 sec, followed by 35 cycles of 30 sec, at 95°C, 60 sec at 60°C (for annealing, and at this point fluorescence was measured), and 30 sec at 72°C. Genotypes were determined automatically by the signal processing algorithms of the iQ5 Optical System Software, version 2.0 (Bio-Rad Laboratories); results were compared with the genotypes visually scored, based on the fluorescent emission data depicted (RFUs). To validate the Real-Time PCR reaction, we included three individuals previously genotyped by sequencing (a heterozygote, a homozygote for variant 1 and a homozygote for variant 2). If genotypes were uncertain, they were additionally sequenced, using the Big Dye Terminator Cycle Sequencing v1.1 Ready Reaction (Applied Biosystems), according to the manufacturer's instructions, and an ABI-PRISM 3130 XL genetic analyzer (Applied Biosystems).

Statistical Analysis

The Genetic Power Calculator (<http://pengu.mgh.harvard.edu/~purcell/gpc/>) was used, assuming a high-risk allele frequency of 0.1, a relative risk for a homozygous genotype of 2.25 and 1.5 in heterozygosity. Hardy-Weinberg equilibrium was tested, using HWE software (<http://linkage.rockefeller.edu/ott/linkutil.htm>). Demographic data of patients and controls

were compared using a chi-square test for qualitative variables. To compare allele frequencies between cases and controls, a chi-square test was used and odds ratios (OR) were estimated, with 95% confidence intervals (CI). The significance level was set at $\alpha=0.05$.

A multivariable-logistic regression was performed (with the most frequent homozygote as the reference), to evaluate association between SNPs and migraine, including the four SNPs of *BDNF* and the *CGRP* SNP in the model, and adjusting for gender. All analyses were performed in the global sample, as well as in the MA and MO subsets. We assessed interaction between *BDNF* and *CGRP* by a stepwise multivariable-logistic regression. For the logistic regression analyses, significance was set to $\alpha=0.012$, using a Bonferroni correction for multiple comparisons.

These analyses were performed using SPSS (version 16.0 for Windows). Haplotype frequencies were compared between cases and controls, for the *BDNF* gene, using Haploview 4.1 (263) with all parameters set at the default values. Frequencies of haplotypes analysed were above 1%, according to the Haploview threshold; to correct for multiple comparisons, when estimating allelic and haplotype frequencies, 10,000 permutations were used.

Results

Demographic and clinical data of 188 unrelated migraineurs and 287 age-matched controls can be found in Table 1. The cases-control ratio was 1:1.5 and no significant differences were found between cases and controls regarding gender.

A migraine prevalence of 16% has been estimated in a Portuguese population (21, 275). We found that we had a power of 64% to detect an association with our sample (for a nominal significance level of 0.05).

Both case and control groups were in Hardy-Weinberg (H-W) equilibrium for the four SNPs of the *BDNF* gene (rs712442, rs6265, rs11030107 and rs2049046). For rs1553005 only the control group was in H-W equilibrium. The correlation between the *BDNF* SNPs was small, denoting the weak LD between them according to the LD plot (Figure 1); in our sample, these SNPs were also in weak LD (data not shown).

No significant differences were found between cases and controls regarding allele frequencies in all the sample sets analyzed (Table 2). Genotypic frequencies can be found in Table 3. Results from the multivariable logistic models showed that in the global sample, as in the MO and MA subgroups, no significant independent effects of *BDNF* or *CGRP* were found (Table 4). A significant interaction was found, however, between *BDNF* and *CGRP*, showing an increased risk for the AT-genotype of rs2049046 and the GC-genotype of rs1553005 (OR= 1.88, 95% CI: 1.20-2.93, p=0.005) in the global migraine sample. This result was still significant after multiple testing correction. Other possible two-way interactions were non-significant (data not shown).

For *BDNF*, we also performed a haplotype-based analysis. No differences were found between cases and controls for any of the haplotypes evaluated (data not shown).

Discussion

The aim of this study was to assess the possible involvement of *BDNF* and *CGRP* in migraine susceptibility and their possible interaction. A previous study found no evidences of *BDNF* as a risk factor for migraine, regarding the functional SNP rs6265 (352). We now used a haplotype-block tagging approach, selecting SNPs that cover common variation within this gene. No significant differences were found between cases and controls regarding the SNPs analysed.

For *CGRP*, only one tagging SNP (rs1553005) was selected with the software used. Also, no differences were found between cases and controls. Several polymorphisms of *CGRP* are described as non-informative in dbSNP (<http://www.ncbi.nlm.nih.gov/SNP>) in European populations, although they may be informative for other ones. This may be a reason why these SNPs are not captured when searching for tagging SNPs. Although we were interested in studying the role of common variants (MAF>0.10), rare alleles may also be involved in the susceptibility of complex disorders (122).

No differences were found regarding gender for *BDNF* and *CGRP* (data not shown).

We have also assessed a possible interaction between *BDNF* and *CGRP*. The Hierarchically Well-Formulated Rule (HWFR) is commonly used when a logistic regression is performed and assumes that lower order terms of the highest order interaction term are included in the model (353). With limited number of SNPs (as in our study), a stepwise logistic regression without HWFR may be a better approach to test for SNP-SNP interactions (353). Therefore, we performed a stepwise logistic regression, allowing that the final model included only the interaction terms between the *BDNF* and the *CGRP* SNPs, in an exploratory analysis of a putative interaction between the two genes. Further studies are now needed to confirm these results.

A significant interaction between rs2049046 and rs1553005 was found, showing an increased risk for the double heterozygotes AT and GC (OR=1.88, 95% CI: 1.20-2.93) when compared with the most frequent double homozygotes (AA of rs2049046 and GG of rs1553005). The less frequent double homozygotes (TT of rs2049046 and CC of rs1553005) showed a non-significant decreased risk (data not shown).

Therefore, one protective allele may not be sufficient to decrease risk for migraine. It would be interesting to assess if this trend remains in larger samples, and if the decreased risk between the less frequent homozygotes reaches significance. That would indicate that an

interaction between these variants, or between variants in LD with them, may protect from migraine.

The high case:control ratio increases the power to detect any effects in our sample. In addition, cases and controls were matched for age-at-observation and gender, and were from the same population and geographic region; several studies using lineage markers, sensitive to population stratification, have showed there is no substructure within the Portuguese population (326-328). Also, we corrected for multiple testing, to prevent type-I errors, and used logistic regression analyses to incorporate all SNPs in the model and analyze their effects together.

Several findings support a role of CGRP in initiating and sustaining migraine episodes (354). CGRP has been extensively studied regarding its possible role in migraine treatment. Antagonists for CGRP receptors may act at the central level and modulate nociceptive trigeminovascular transmission in the cat, and are arising as a new generation of migraine drugs (334, 354, 355). Furthermore, studies in transgenic mice showed that modulation of one of the *CGRP* receptors (*RAMP1*) levels may contribute to migraine susceptibility and, in particular, to photophobia (356). More interestingly, a recent study showed that adenosine triphosphate (ATP)-gated *P2X3* receptors may be involved in chronic pain (including migraine) and CGRP seems to enhance its transcription, involving also mediation by *BDNF* (357). *BDNF* is also a mediator of neuronal survival and plasticity of dopaminergic, cholinergic and serotonergic neurons, and its involvement in migraine seems clear as a mediator of the trigeminal nociceptive plasticity (341, 342, 358).

Our data support the hypothesis of an interaction between *BDNF* and *CGRP* in migraine susceptibility. The results obtained also reinforce the importance of further association studies with these genes in larger samples and in other populations. Interaction between candidate genes for migraine pathophysiology should be further explored, in order to have a broader knowledge of genes interplay in susceptibility for this common disease.

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Table 1. Demographic and clinical data of migraine patients and controls

Migraine patients (<i>n</i>)	188
MA	77
MO	111
females	153
males	35
mean age at observation ($\pm$ SD)	36.14 yrs ($\pm$ 12.84)
mean age at onset ($\pm$ SD)	17.67 yrs ($\pm$ 8.15)
family history of migraine	87%
Migraine-free controls (<i>n</i>)	287
females	217
males	70
mean age at observation ($\pm$ SD)	36.42 yrs ($\pm$ 12.35)

Table 2. Allele frequencies of SNPs studied in migraine patients and controls

<i>BDNF</i> SNPs	Alleles (%)		χ^2	OR (95% CI)	<i>P</i>
rs7124442	C	T			
all patients	112 (29.8)	264 (70.2)	0.053	1.03 (0.78-1.38)	0.82
MO	65 (29.3)	157 (70.7)	0.003	1.01 (0.72-1.42)	0.96
MA	47 (30.5)	107 (69.5)	0.12	1.07 (0.73-1.58)	0.73
controls	167 (29.1)	407 (70.9)	-	-	-
rs6265	A	G			
all patients	76 (20.2)	300 (79.8)	0.04	1.03 (0.75-1.43)	0.84
MO	44 (19.8)	178 (80.2)	0.002	1.01 (0.68-1.49)	0.97
MA	32 (20.8)	122 (79.2)	0.09	1.07 (0.69-1.66)	0.76
controls	113 (19.7)	461 (80.3)	-	-	-
rs11030107	G	A			
all patients	85 (22.6)	291 (77.4)	0.13	1.06 (0.78-1.45)	0.72
MO	53 (23.9)	169 (76.1)	0.48	1.14 (0.79-1.64)	0.49
MA	32 (20.8)	122 (79.2)	0.05	0.95 (0.62-1.47)	0.82
controls	124 (21.6)	450 (78.4)	-	-	-
rs2049046	T	A			
all patients	175 (46.5)	201 (53.5)	0.095	0.96 (0.74-1.25)	0.76
MO	105 (47.3)	117 (52.7)	0.004	0.99 (0.73-1.35)	0.95
MA	70 (45.5)	84 (54.5)	0.22	0.92 (0.64-1.1)	0.64
controls	273 (47.6)	301 (52.4)	-	-	-
rs7124442/ rs2049046	TA	Others			
all patients	92 (24.5)	284 (75.5)	0.003	0.99 (0.73-1.34)	0.95
MO	52.5 (23.6)	169.5 (76.4)	0.08	0.95 (0.66-1.36)	0.78
MA	39.5 (25.6)	114.5 (74.4)	0.06	1.05 (0.70-1.58)	0.81
controls	141.4 (24.6)	432.6 (75.4)	-	-	-
<i>CGRP</i> SNP	Alleles (%)		χ^2	OR (95% CI)	<i>P</i>
rs1553005	C	G			
all patients	124 (33.0)	252 (67.0)	0.75	1.13 (0.86-1.50)	0.39
MO	77 (34.7)	145 (65.3)	1.42	1.22 (0.88-1.70)	0.23
MA	47 (30.5)	107 (69.5)	0.002	1.01 (0.69-1.49)	0.96
controls	174 (30.3)	400 (69.7)	-	-	-

Table 3. Genotype frequencies in patients and controls

BDNF SNPs		Genotypes, n (%)		
rs7124442	TT	TC	CC	Total
all patients	96 (51.1)	72 (38.3)	20 (10.6)	188
MO	58 (52.3)	41 (36.9)	12 (10.8)	111
MA	38 (49.4)	31 (40.3)	8 (10.4)	77
controls	149 (51.9)	109 (38.0)	29 (10.1)	287
rs6265	GG	GA	AA	Total
all patients	118 (62.8)	64 (34.0)	6 (3.2)	188
MO	69 (62.2)	40 (36.0)	2 (1.8)	111
MA	49 (63.6)	24 (31.2)	4 (5.2)	77
controls	183 (63.8)	95 (33.1)	9 (3.1)	287
rs11030107	AA	AG	GG	Total
all patients	114 (60.6)	63 (33.5)	11 (5.9)	188
MO	66 (59.5)	37 (33.3)	8 (7.2)	111
MA	48 (62.3)	26 (33.8)	3 (3.9)	77
controls	174 (60.6)	102 (35.5)	11 (3.8)	287
rs2049046	AA	AT	TT	Total
all patients	51 (27.1)	99 (52.7)	38 (20.2)	188
MO	28 (25.2)	61 (55.0)	22 (19.8)	111
MA	23 (29.9)	38 (49.4)	16 (20.8)	77
controls	79 (27.5)	143 (49.8)	65 (22.6)	287
CGRP SNPs		Genotypes n (%)		
rs1553005	GG	GC	CC	Total
all patients	78 (41.5)	96 (51.1)	14 (7.4)	188
MO	44 (39.6)	57 (51.4)	10 (9.0)	111
MA	34 (44.2)	39 (50.6)	4 (5.2)	77
controls	140 (48.8)	120 (41.8)	27 (9.4)	287

Table 4. Results from a multivariable logistic regression, gender-adjusted

Genotypes	Migraine			MO			MA		
	OR	CI 95%	P	OR	95% CI	P	OR	95% CI	P
rs7124442			<i>0.91</i>			<i>0.90</i>			<i>0.78</i>
TT (ref)	1.00	-	-	1.00	-	-	1.00	-	-
TC	1.13	0.58-2.22	<i>0.72</i>	0.96	0.42-2.20	<i>0.93</i>	1.38	0.56-3.43	<i>0.48</i>
CC	1.03	0.33-3.22	<i>0.96</i>	0.74	0.17-3.12	<i>0.40</i>	1.39	0.30-6.40	<i>0.67</i>
rs6265			<i>0.94</i>			<i>0.74</i>			<i>0.65</i>
GG (ref)	1.00	-	-	1.00	-	-	1.00	-	-
GA	1.09	0.60-2.00	<i>0.51</i>	1.25	0.59-2.66	<i>0.56</i>	0.90	0.40-2.05	<i>0.81</i>
AA	1.26	0.30-5.35	<i>0.55</i>	0.86	0.12-6.35	<i>0.88</i>	1.70	0.29-10.10	<i>0.56</i>
rs11030107			<i>0.55</i>			<i>0.29</i>			<i>0.58</i>
AA (ref)	1.00	-	-	1.00	-	-	1.00	-	-
AG	0.87	0.49-1.54	<i>0.63</i>	1.07	0.53-2.15	<i>0.84</i>	0.68	0.32-1.43	<i>0.31</i>
GG	1.55	0.46-5.24	<i>0.48</i>	3.15	0.70-14.29	<i>0.14</i>	0.59	0.10-3.40	<i>0.56</i>
rs2049046			<i>0.69</i>			<i>0.47</i>			<i>0.97</i>
AA (ref)	1.00	-	-	1.00	-	-	1.00	-	-
AT	1.20	0.63-2.28	<i>0.58</i>	1.44	0.65-3.20	<i>0.37</i>	0.94	0.40-2.22	<i>0.90</i>
TT	1.01	0.35-2.89	<i>0.99</i>	1.14	0.30-4.28	<i>0.85</i>	0.85	0.20-3.47	<i>0.82</i>
rs1553005			<i>0.16</i>			<i>0.26</i>			<i>0.20</i>
GG (ref)	1.00	-	-	1.00	-	-	1.00	-	-
GC	1.40	0.94-2.08	<i>0.09</i>	1.48	0.92-2.40	<i>0.11</i>	1.37	0.80-2.34	<i>0.25</i>
CC	0.86	0.42-1.77	<i>0.69</i>	1.11	0.49-2.52	<i>0.80</i>	0.55	0.18-1.71	<i>0.30</i>

(ref) indicates the reference category; OR: odds ratio; CI: confidence interval;

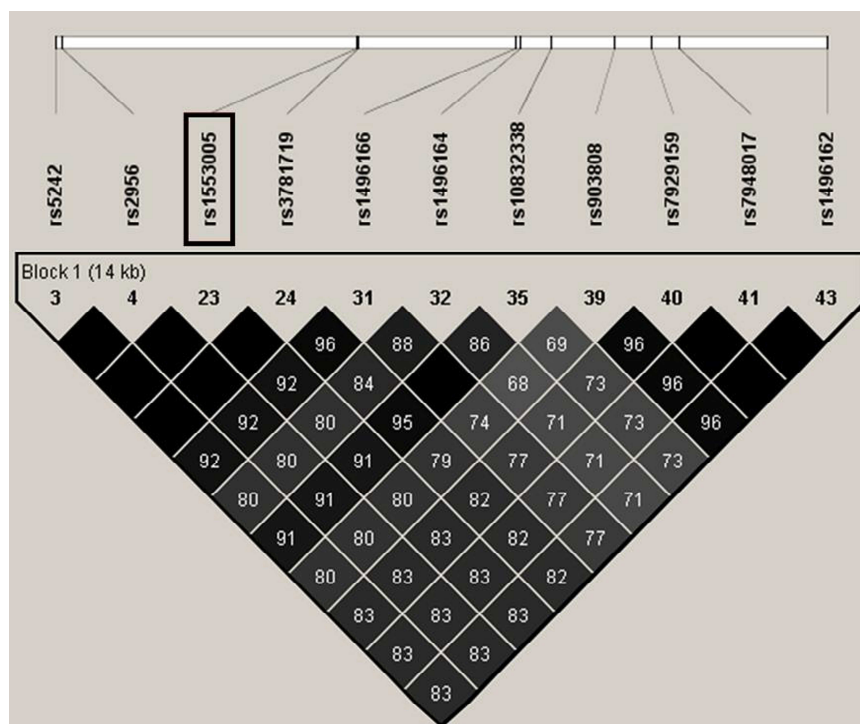


Figure 2. LD plot of SNPs within *CGRP* gene (HaploView 4.1.); r^2 values are given within each square.

5. DISCUSSION

5. Discussion

5.1. Genetic component in migraine

Migraine is a chronic neurological disorder with recurrent headache episodes and associated symptoms (6, 39). This common disorder is, among primary headaches, the one with the highest burden to patients, their relatives and to public health (1, 4, 8, 46). It has been shown that migraineurs may have more physical, mental or social problems than non-migraineurs (8).

By studying migraine epidemiology and genetic factors involved in this pathology, our aim was to study risk factors in migraine patients and their families that could contribute to a deeper understanding of migraine pathophysiology and, consequently, to the future development of better and more specific treatment strategies.

5.1.1. Migraine prevalence

Migraine prevalence has been widely studied, with high prevalence values reported (3, 4). Studies have been mainly performed in Western Europe and North America, but migraine prevalence has also been estimated in Asia and Africa (3). In Portugal, the major prevalence study performed until now was conducted in 1995, in a small urban district of northern Portugal (21, 48, 55). The results from this survey are similar to the ones found worldwide and have been used in our study as a reference of migraine prevalence in general population, since most of our patients and their families are from the northern region of the country.

5.1.2. Familial aggregation studies

Several studies in other populations have described an increased risk for migraine in relatives of migraineurs (66-71). With the vast collection of migraine patients ascertained in the Neurology out-patient clinic, at HGSA, it seemed crucial to study the occurrence of migraine in their relatives and to assess if a genetic component of migraine was also present in our families, since, due to the high prevalence of this disorder, migraine could occur in families simply by chance (359).

In a sample of 144 Portuguese families, we assessed familial aggregation of migraine by estimating the RR, in order to compare the frequency of the disease in relatives with the prevalence in the population. We found that first-degree relatives of migraineurs have a 3 to 4-fold increased risk, when compared to the general population. One important finding was that probands' spouses did not present a significantly higher risk of migraine, showing that the results of RR found are not due to shared environmental factors. Although environmental factors may have an effect, these would need to be very high to account entirely for familial clustering of a given disease (287, 288) **[article 1]**.

Additionally, it would be interesting to have analysed if RR would change with the degrees of kinship. We would expect that RR increased with degree of kinship, which would be another indication of a genetic contribution to migraine etiology (65, 277). In a study of familial aggregation of Parkinson disease (PD), the authors found a significant, though lower RR for second-degree relatives, indicating that familial aggregation extends beyond the nuclear family and demonstrating evidence of a genetic component in PD (360). In our families, however, the group of second and third-degree relatives ascertained was small and did not achieve enough statistical power to be incorporated in the analyses.

Our results are in line with the studies performed in other populations (66-71), showing that in Portuguese families, there is also a genetic contribution in migraine pathogenesis. Small sample sizes may lead to a decrease in the precision of estimates and do not allow subgroup analysis, which is important to understand how familial aggregation varies in the different subgroups (271). Our sample size was large enough to enable the analysis of MO and MA subgroups.

In most studies, whether clinical or population-based, MA was found to be the migraine subtype with a higher risk for first-degree relatives of migraineurs (278). In our sample, we found that first-degree relatives of MA-probands had a higher risk of MA than MO ($RR_{MA|MA}=12.2$; $RR_{MO|MA}=3.1$). These results suggest that MA may have a stronger familial aggregation and, thus, a greater genetic predisposition **[article 1]**.

Another interesting finding was that in our population sample, prevalence of MA was very similar to the frequency of MA in the control group of a study performed in Greece (67) and also the study in a control population from Italy (70), but lower than the one found in Denmark (67, 69, 70). These findings made us hypothesize that prevalence of MA in South of Europe is lower than in the North, supported by studies showing a population

substructure in northern and southern Europe (284). Individuals with Portuguese, Italian and Greek ancestry are included in the southern group (284), which could explain their similar MA prevalence. It would be interesting to have more data on familial aggregation for other northern and southern countries that could help us elucidating if, in fact, there is a different prevalence for MA, which could have some influence in the search for susceptibility genes in the different populations.

These findings encouraged us to further explore genetic factors possibly involved in migraine susceptibility.

5.1.3. Validation of familial history

In addition to the relatives observed and included in the familial aggregation study, probands also described the affection status of relatives which were never clinically observed. From the 190 families enrolled in this study, 267 first-degree relatives and 149 second- and third-degree relatives were directly observed by a neurologist; additionally, probands described the affection status of more 263 first-degree relatives and 80 second and third-degree relatives. Therefore, it seemed important to assess if the probands' description about their relatives was reliable enough to be used in subsequent studies.

Ottman et al. have found that the occurrence of migraine is underestimated by the family history method and suggested that direct interviews with relatives are required for an accurate perspective on migraine occurrence within families (271). Russell et al. also concluded that the probands report is not valid, given the misclassification of relatives and underestimation of migraine occurrence (272).

By estimating the chance-corrected agreement, we found that the overall Kappa's value was moderate (being good for the MO probands group and only fair for MA), according to Landis and Koch criteria (290).

Unlike Russell et al. (272), we found that our MO probands showed a higher sensitivity when identifying their affected relatives; but MA probands might have undervalued less well-defined migraine episodes in their relatives, when they have just MO. We concluded that probands' reports underestimate the occurrence of migraine in 1st-degree relatives, and are more uncertain when 2nd and 3rd-degree relatives are included, probably due to a

more indirect knowledge, as suggested by Russell et al. (272). Noteworthy, we found that reports were highly specific and had a high PPV, in spite of their low sensitivity.

To overcome the low sensitivity of proband's report, clinical interviews should be preferred to ensure an accurate diagnosis of migraine, especially in familial aggregation studies, as we did in our study (271). However, in case of scarce resources, these should be put in examining relatives of MA probands, since MO probands reports seem to be more reliable.

In any case, the proband's report will still be useful for deceased or absent relatives (291) and can be improved if information from multiple informants is used, increasing sensitivity of family history method without decreasing specificity (271, 361). Also, it can be useful in a pilot phase of a genetic investigation or to identify relatives that can be useful and later directly interviewed (291, 362).

Moreover, when high specificity and PPV are more important than sensitivity, as for genetic linkage studies (291), this can be a valuable ascertainment method to maximize the number of informative families.

5.1.4. Risk factors

After assessing familial aggregation of migraine in our families, our aim was to assess specific factors contributing to the increased risk for migraine. We put special care to take into account intrafamilial correlations (363). Several studies showed that variation in age at onset is associated with the disease liability and genetic heterogeneity in some complex disorders, such as Alzheimer disease, bipolar disorder and major depression (363-366). Also for some forms of epilepsy, a study found that offspring had a higher risk when the probands had an earlier age at onset, while no differences were found in parents and siblings (367). On the contrary, a study in schizophrenia showed no differences between age at onset variation and risk for relatives (365). In a previous study, a lower age at onset in probands was also associated with relative's affection status for migraine (68).

In contrast, in our study, we found that the proportion of affected relatives was independent of the proband's age at onset. After adjusting for the age at observation with relatives, gender was found to be the only risk factor for migraine, while the proband's age at onset and gender were not. Our findings showed that, as expected, females had a higher risk of migraine than men.

Our next aim was to assess if there was a maternal or paternal effect that could explain this gender ratio. We found that mother-to-daughter transmissions were more frequent than expected by chance alone, although mother-to-son, father-to-daughter and father-to-son transmissions have also been observed.

This biased transmission could be explained by a maternally inherited factor, such as mitochondrial DNA (mtDNA) (158, 296). Besides mtDNA, other authors found evidence of a migraine susceptibility locus on chromosome X, although until now no genes have been found in this region (149, 151, 155, 298).

Another hypothesis is a sex-conditioned genetic model, with sex chromosomes influencing the expression of genes in autosomes, which would lead to an autosomal dominant transmission in female family members and an autosomal recessive-like transmission or incomplete penetrance in male relatives (299). This could explain our results regarding a preferential mother-to-daughter transmission. Females also differ from males in pain behavior, influenced by differential exposure to sex hormones effects, which can also modulate the expression of migraine in women, since these are involved in the expression and sustaining of pain states (294).

Other complex diseases, such as multiple sclerosis, rheumatoid arthritis or autism, have also a differential gender ratio, with one of the genders being more susceptible to the disease (323). The gender-specific effects found in some association or linkage studies can be explained by the effect of sex hormones, which can control gene expression through epigenetic modifications (323). Furthermore, epigenetic factors may be also influencing the multifactorial threshold observed in complex diseases, determining different liabilities for males and females (368).

In multiple sclerosis (MS), a maternal effect was suggested, by half-siblings and twin studies. For MS, it has been suggested that this parent-of-origin effect can be due to mitochondrial inheritance, epigenetic factors or gene-environment mechanisms (369). However, it was also found that, contrarily to what was expected, affected mother-child pairs are less frequent than affected father-child pairs (370). This may be due to the inclusion of small families or small sample size introducing some bias leading to the enrichment of maternal or paternal effects (368).

It would also be important to study this parent-of-origin effect in larger samples in migraine. As already performed in MS (369), it would be also interesting to study

avuncular pairs, in order to confirm the maternal effect observed and the increased risk of migraine for females.

5.2. Comparison between ICHD-I and ICHD-II

We felt the need to evaluate if the new ICHD criteria introduced remarkable differences in epidemiological studies, in particular of primary headaches. Our results suggested that these new criteria do not have a major impact in headache epidemiological studies. The high agreement rate found showed that ICHD-II does not significantly alter the results of epidemiological, concerning migraine and tension-type headache.

Furthermore, the frequency of headache types analyzed were similar with both criteria and we found a substantial agreement (according to Landis and Koch, 1977) (290) concerning primary and combined headaches. Several individuals presented combined headaches, composed by more than one type of headache, as these are not mutually exclusive. However, when classified with ICHD-II, the frequency of the combined forms decreased, in particular when there was a combination of headaches not fulfilling all ICHD-I criteria or ICHD-II probable headache type, which may indicate that the new criteria are more adequate to separate headache types.

This is an important finding, since 10-45% of the patients are diagnosed as having *probable* migraine, due to missing diagnosis criteria (7). Probable headache type can not be ignored, since some epidemiologic studies showed that, for instance, probable migraine is common and has been associated with temporary disability and reduction in health-related quality of life (311).

Nevertheless, the frequency of combined headaches is still high, which indicates that to establish a final diagnosis, further information is required (309). However, it is difficult to obtain additional information in epidemiological studies, since data is often obtained by the fulfillment of questionnaires or by telephonic interview (309). Importantly, in our study, we found that the diagnosis remained the same in 98% of the patients of our clinical sample, showing that ICHD-II does not present significant changes in MO and MA forms. This is an important finding given that our patients are being enrolled in the clinical and genetic study of migraine since 1999, and we can now standardize the diagnosis criteria used in the clinical sample in future studies.

The basic structure of the classification remains unaltered and the most common subtypes, MO and episodic tension type headache have essentially the same criteria, also as suggested by Peatfield, 2004 (312). This is the first study comparing the two classifications regarding primary and combined headaches. Eriksen et al. found a high sensitivity and specificity of ICHD-II for MA only (307).

Lima et al. performed a critical analysis of the two classifications regarding migraine in children and adolescents, and found a higher sensitivity and specificity, which enabled an improved migraine diagnosis in this group of patients (305).

Bigal et al. compared ICHD-I and ICHD-II regarding chronic daily headache, which has been readjusted in ICHD-II in four categories. This study found that some features need to be revised in a third edition of the classification (371).

We conclude that ICHD-II classification seems to have relevant clinical implications and suffered important improvements. However, our study showed that ICHD-II did not change remarkably previous epidemiological results, in what concerns primary and combined headaches.

5.3. Unravelling susceptibility factors for migraine

The susceptibility factors involved in migraine pathophysiology are still not well understood (25). Several studies have described some variants in pathways involved in migraine pathophysiology but results are still scarce or lacking confirmation (25).

5.3.1. FHM genes and common forms of migraine

Special attention has been given to the genes involved in FHM, the Mendelian form of migraine. The possible involvement of *CACNA1A* and *ATP1A2* in the common forms of migraine have been studied; however, the results were not conclusive (25, 157). As for the third gene, responsible for FHM3, *SCN1A*, no studies in the common forms of migraine have been performed until now. It would be interesting to examine if this gene is involved in the common forms of migraine, since epilepsy and migraine have high comorbidity and may be part of a clinical continuum, with some common and some different features underlying both conditions (274). Also, several mutations in this gene

have been implicated in epilepsy and recently some mutations have been described in patients with FHM (96, 101).

With the contradictory results found in the several studies performed, the question remains if it is valuable to study, for common traits, genes involved in the monogenic forms (359). For other common diseases, as hypertension and epilepsy, this strategy has also not been very successful yet (25).

5.3.2. Candidate gene approach

In this study, we devoted special attention to genes involved in the pathophysiology of migraine. We chose not to use linkage analysis to search for susceptibility genes, since linkage is more powerful to find rare variants with major effects, than common variants with small effects, as is hypothesized in complex diseases (111, 117, 119-121). In the linkage studies performed so far, no genes responsible for migraine have been found, what could be due to the small power of this approach, to the families ascertainment methods, since migraine is a highly prevalent disorder and to the fact that spouses can introduce other susceptibility factors in the families, or to incorrect diagnosis (157).

Therefore, we chose to perform association studies, in a case-control sample, in order to look for the possible involvement of susceptibility genes in migraine. Our aim was also to perform family-based studies, in order to confirm the results found in the case-control sample. This method has been described as a useful complementary strategy, avoiding population stratification since the genetic background of the alleles transmitted to the offspring is the same of the alleles untransmitted (112, 114). However, with the number of trios (parent-offspring) and sibpairs ascertained, the power of our sample was very small (only 4%). Consequently, we decided not to analyze this, since results would be inconclusive.

Population stratification can decrease the power of an association study and introduce some biases in the results due to the presence of several subgroups with different allele frequencies in a population (114). However, in our Portuguese case-control samples population stratification this is not expected, since: 1) cases and controls are matched by geographical origin; and 2) several studies, using markers sensitive to population stratification, as lineage markers, have shown there is no population substructure among the Portuguese population (326-328).

In the era of GWAS, performing a candidate-gene approach seems less attractive, since GWAS have been proven to be fruitful for some common disorders (118, 372, 373). However, GWAS requires larger samples, is a more expensive approach and can lead to some false positives due to the high density of markers (117, 157, 374).

The candidate gene approach relies on the premise that the disease pathways are well characterized and the biological function of the genes selected is known. This can be an advantageous and cost-effective approach, in particular if GWAS in the disease of interest have not yet been performed (117). Also, with the increasing availability of information regarding gene function, the number of known SNPs and better technologies to study functional effects, this kind of approach is very useful (375).

5.3.3. Selection of candidate genes

Several candidate genes have been studied regarding their possible involvement in migraine susceptibility. Genes involved in neurotransmitter, vascular and hormonal functions have been searched, although several other genes, such as *TNF- α* , *INSR*, *LDLR* and others, have also been studied (103).

Since, so far, no previous association studies have been performed on migraine in our population (except for *MTHFR*, with negative results (217)), one of our aims was to try to replicate previous findings in other populations, in order to assess if those genes were also implicated in migraine susceptibility in our Portuguese sample. We also wanted to study new candidate genes and assess possible gene-gene interactions.

We thus chose to select genes involved in several pathways, rather than focus in one particular pathway, in order to gain a broader insight into migraine pathophysiology. We assessed a possible association between migraine and *STX1A* (involved in neurotransmitter release) [article 5], *EDNRA* (a potent vasoconstrictor, which may reflect the role of a vascular component) [article 6], *BDNF* (involved in mediation of trigeminal nociceptive plasticity) and *CGRP* (neuropeptide involved in the modulation of nociceptive transmission) [article 7].

5.3.4. Comparison of our findings with other populations

5.3.4.1. Syntaxin 1A

Syntaxin 1A (Syn1A) has also been studied as a candidate gene for other neurological complex diseases such as autism (with nominal association with two *STX1A* SNPs) and schizophrenia (with contradictory results) (318, 376, 377).

STX1A seemed to be a good candidate gene to assess, since it interacts with some players in migraine pathophysiology, such as NO and GAT-1 and is involved in neurotransmitter release (318, 320). For *STX1A*, we confirmed the association of rs941298, previously found by Corominas et al. (316). In addition, we found rs6951030 to be also associated with migraine in Portuguese patients. These are important findings since some susceptibility variants may be shared among populations, while others will be more population specific.

5.3.4.2. Endothelin receptor type-A (EDNRA)

Previous studies found association of this gene and migraine in other populations (204, 209). *EDNRA* has been associated with MA in a group of Finnish patients and in the pooled sample of Finnish and German patients (209). Also, it has been previously associated with a migraine sample of French patients and, in particular, with the MO subgroup (204).

In our sample of Portuguese patients, we found significant differences between the case and control groups regarding the SNP rs702757 in the MO subgroup, with an enrichment of the A-allele in this subgroup. Although this SNP may not be the effective risk variant, it may be in LD with the true variant, since it is in strong LD ($r^2 > 0.92$) with allele G of rs1801708 (204), which is another argument in favour of our findings. However, while Tzourio et al. (204) found a protective role for the variant associated with migraine, in our study, the A-allele of rs702757 showed an increased risk for MO **[article 6]**.

Furthermore, we cannot exclude a role of this gene in MA for our population, since we found a trend towards an increased risk for rs5333. The C-allele of this SNP is in LD with T-allele of rs2048894, which has been found to be associated with MA in the study of Tikka-Kleemola et al. (209). The findings of this study show that, besides the importance of replicating the association studies in different populations, different variants in the same gene may be associated with MA or MO, which can add some light into the debate if MA and MO are the same or different entities.

5.3.4.3. BDNF and CGRP

A previous study found no evidence of *BDNF* being a risk factor for migraine, regarding the functional rs6265 (352). This SNP has been widely used in association studies for several disorders, such as schizophrenia, bipolar disorder and depression, among other diseases, with conflicting results (378-383).

In our study, we used a haplotype-block tagging approach, selecting SNPs that covered common variation within this gene, but no significant differences were found between cases and controls, regarding the SNPs analysed [article 7].

Also for *CGRP*, no significant results were found, although this gene has been extensively studied regarding its possible therapeutical role in migraine. Interestingly, a recent study showed that adenosine triphosphate (ATP)-gated P2X3 receptors may be involved in chronic pain (including migraine) and *CGRP* seems to enhance its transcription, involving also mediation by *BDNF* (357). These data supports a connection between the two genes in migraine susceptibility, which was also seen in our results, since we found a significant interaction between *BDNF* and *CGRP*, modifying migraine susceptibility, despite these being only preliminary findings, requiring confirmation.

These results reinforce the importance of further association studies with these genes in larger samples and in other populations. Other gene interactions have been assessed for migraine, such as the interaction between a *5-HTTLPR* variant and a polymorphism in the *5-HT2a* receptor gene; between *AGTR1* gene and *AGT* gene; and between *HTR2B* and *MAOA*; all with negative results (173, 198, 384). However, a strong interaction was found between thymidylate synthase (*TS*) and *MTHFR* polymorphisms (385).

Interaction between candidate genes involved in migraine should be further explored, in order to have a broader knowledge of genes interplaying in its susceptibility.

5.3.4.4. General considerations

Allele and haplotype diversity of *BDNF* were observed among global populations, with evidence for a positive selection at the *BDNF* locus. Therefore, the differences regarding association of *BDNF* and other genes in the several studies may be due to allele frequency variation across populations and be more marked due to gene-gene and gene-environment interactions (324, 386).

Furthermore, environmental components and gene-environment interactions may play a role in the susceptibility to common diseases. Some examples of gene-environment interactions have been described, such as different cholesterol levels in APOE4 carriers, due to cholesterol intake, or a different risk of colorectal cancer associated with the Ala222Val variant of *MTHFR* and folic acid intake (387). The negative results found for the ENDRA/EDNRB antagonist may also be due to the population in study and not only to the opposite effects of EDNRA and EDNRB (334). We can hypothesize that pharmacological studies should be performed in different populations and that gene-environment interactions should be explored in order to develop more efficient treatments (387).

Finding susceptibility genes will depend on allelic heterogeneity, allele frequency and inter-ethnic differences. Therefore, positive association findings are more easily replicated in similar populations with the same alleles. The number and frequency of susceptibility alleles will also depend on the effects of mutation, genetic drift and selection (388).

Part of the selective pressures acting on susceptibility alleles for complex diseases might have changed in the course of time, as a result of cultural and environmental modifications, and, therefore, alleles that were deleterious in ancestral populations may now be protective or neutral. No single model can explain the evolution of susceptibility alleles for the same disease or the same susceptibility gene, and both common and rare variants in the same gene may contribute to disease susceptibility (388).

For *CGRP*, this was the first study assessing a possible association of this gene with migraine, although no differences were found [article 7]. However, we cannot exclude that other variants are associated with migraine in other populations. Several polymorphisms of *CGRP* are described as non-informative in the SNP database for Europeans, although they may be more informative in African and Asian populations. Therefore, these SNPs are not captured when searching for tagging SNPs and their effect has not been evaluated.

In our study, for the four candidate genes studied, we used both a multimarker and a haplotype-based approach, in order to evaluate a possible association between haplotypes and disease status and to prevent loss of information if only a tagging method was used [articles 5, 6 and 7] (262). Combination of SNPs can be helpful in the detection of epistatic effects and haplotypes may provide useful information about the several SNPs, which may be in LD with the causal variant (389).

The International HapMap project has produced information on common variation in four population groups from Africa, Asia and Europe, making the selection of tagging SNPs more

accurate (390). It has proved to be a powerful resource for selecting tagging SNPs for GWAs and candidate gene studies (391). Nevertheless, the HapMap does not have information on all SNPs in the genome, which may result in loss of information, since some genes may have few tagging SNPs genotyped in its database (391). One way to overcome this problem in the future is to supplement the information of HapMap with additional SNPs from gene resequencing data (391). Furthermore, the HapMap project has scarce information on less common variants, which are thought to be involved also in complex diseases, with a substantial contribution to the multifactorial inheritance underlying these disorders due to the higher genotypic RR of these rare variants, justifying their screening (392, 393). It is also hypothesized that common variants could act as modifiers of the rare variants effects and that these rare variants will be more population specific due to founder effects (392). Some authors state that the selection of tagging SNPs based on MAF will lead to loss of information and, therefore, unrestricted tagging SNPs selection should be preferred (394). If the sample sizes increases, in large case-control association studies, using SNPs with MAF <5% will allow the identification of causal SNPs underlying disease susceptibility (395). Also, sequencing the entire candidate genes will allow unraveling the rare variants present in those genes (393).

5.3.5. Gender-related susceptibility

Another fact to take into account is a differential gene susceptibility regarding gender. In this study, we used a regression logistic model adjusted for gender, since migraine is a gender-dependent trait, and no significant results were found regarding gender in this analysis.

However, for *STX1A*, we found that the increased risk for rs6951030 was only significant in the female group [article 5], which can be due to hormonal effects mediated by epigenetic modifications and may explain the gender differences found in some association studies (323). Female steroids seem to modulate mechanisms involved in migraine pathophysiology, such as the serotonergic, adrenergic and gamma-aminobutyric acid (GABA)-ergic systems, the synthesis and release of NO and neuropeptides, such as CGRP and other receptors involved in vasodilatation and vasoconstriction (226).

Also, this gender-related susceptibility is line with our previous findings, regarding a preferential mother-daughter transmission, showing that females have an increased risk for migraine [article 3].

We cannot exclude, however, that in our sample, the non-increased risk found in males is due to the small sample size of this subgroup. It would be interesting to explore this finding in larger samples, in order to disentangle a possible gender-susceptibility regarding *STX1A* and other genes.

5.3.6. An integrated perspective of migraine pathophysiology

Migraineurs seem to have an enhanced neuronal excitability, which facilitates the occurrence of CSD, the mechanism that is hypothesized as underlying MA and possibly also MO (28). In the pathophysiology of migraine, both central and peripheral components of the trigeminal pain pathway are probably involved (35), as well as structures involved in nociception control and regulation (30).

Alterations in the synthesis of neurotransmitters, receptors and ion channels may lead to an increased functional neuronal activity in nociceptive pathways or, on the contrary, to a decrease in pain inhibitory systems (396, 397). Additionally, a neurogenic inflammatory reaction occurs with the release of neuropeptides at the trigeminovascular nerve endings near the meningeal vessels, leading to vasodilatation, plasma extravasation and release of pro-inflammatory substances (28, 30, 31). We studied candidate genes involved in some of these pathways, all possibly contributing to migraine pathophysiology.

Regarding *Syn1A*, the association of the SNARE proteins (including *Syn1A*) with presynaptic Ca^{2+} channels, namely the VDCC α_{1A} subunit, is essential for neurotransmitter release (322). Therefore, the polymorphisms found in our patients in *STX1A* **[article 5]** may alter the interaction between *Syn1A* and calcium (Ca^{2+}) channels, modifying the functional state of the VDCC α_{1A} , thus altering neurotransmitter release and, ultimately, contributing to CSD and the migraine phenotype (316, 325).

Glutamate is the major excitatory neurotransmitter and its release is facilitated by the mutated VDCC α_{1A} subunit, also leading to CSD (28, 398). Therefore, glutamate could be one of the neurotransmitters, the release of which is modified due to alterations in the interaction between *Syn1A* and VDCC α_{1A} subunit, inducing neuronal hyperexcitability due to an increase of the extracellular glutamate levels (398).

Interestingly, we found an association with *EDNRA* in the same group of patients **[article 6]**. This gene mediates the biological effects of ET-1 and the inhibitory effect of ET-1 on NO synthesis, which, on its turn, has been associated with nociception and, consequently, with

migraine pathophysiology (201, 321). This gene also has a role in the development of CSD, since the effect described of ET-1 on CSD may be mediated by EDNRA (199). Interestingly, when ET-1 binds with EDNRA it activates phospholipase C to hydrolyse phosphatidylinositol- 4, 5-bisphosphate to form inositol-1, 4, 5-trisphosphate (IP3) and diacylglycerol; IP3 promotes the downstream release of Ca^{2+} from the endoplasmic reticulum (329). This can be another evidence favouring the role of Ca^{2+} in the pathophysiology of migraine, since an increase in intracellular Ca^{2+} will lead to neurotransmitter release, but can also activate a number of enzymes, including PLA2, CaM kinases and PKCs which phosphorylate ion channels and synaptic proteins, leading to an increase in neurotransmitter release (399).

This cascade of events will lead to an increase of intracellular Ca^{2+} and neuropeptide release, such as CGRP. The influx of Ca^{2+} can activate protein kinase C (PKC), which will also participate in the release of neuropeptides (399). We were also interested in studying the involvement of CGRP in migraine susceptibility **[article 7]**, since CGRP release is crucial for the development of neurogenic inflammation, promoting the release of inflammatory factors, such as cytokines, which may also be involved in pain generation and affect glutamate release (397, 398). CGRP has an active role in the activation and sensitisation of nociceptors, both at peripheral and central level (347), by promoting the release of inflammatory mediators resulting in sensitization of the trigeminal fibers. Sensitisation is associated with a lower threshold of pain response to apparently innocuous stimuli, e.g., head movements (397).

We focused also on the possible association of BDNF and migraine **[article 7]**, as BDNF is expressed in the nociceptive sensory neurons, acting as central pain modulator both at the spinal and supra-spinal level, contributing to central sensitization and modulating the activation of glutamatergic and N-methyl-d-aspartate (NMDA) receptors (339-341).

Furthermore, BDNF is also a mediator of neuronal survival and plasticity of dopaminergic, cholinergic and serotonergic neurons, and its involvement in migraine seems clear as a mediator of the trigeminal nociceptive plasticity (341, 342, 358). This neuronal plasticity is also responsible for alterations in pain threshold, modulating the response to stimuli. Plasticity can result in short-term changes that last minutes to hours, or long-term changes that may be permanent (396, 397). Interestingly, CGRP is coexpressed with BDNF in trigeminal ganglion neurons in rats and, in addition, CGRP enhances BDNF release from trigeminal neurons in vitro (Buldyrev, Tanner et al. 2006), which reinforces the possible interaction we have found between CGRP and BDNF **[article 7]**.

All these data support a role of the candidate genes studied in this work in the pathophysiology of migraine. The different mechanisms may be interconnected, and variants in each gene may contribute to susceptibility to migraine, through the different pathways in which they act.

6. CONCLUSIONS

6. Conclusions

1. We performed for the first time a familial aggregation study of migraine in a Portuguese population and we found that first-degree relatives of migraineurs had a 3 to 4-fold increased risk when compared to the general population, while spouses did not present a significantly higher risk, showing that 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.
2. We also found that, in our sample, 1st-degree relatives of MA-probands had a higher risk of MA than MO. In spite of these not being distinct entities, MA seems to have a stronger familial aggregation and, thus, a greater genetic predisposition, as supported by our results.
3. We found that MO probands showed a higher sensitivity when identifying their affected relatives, whereas MA probands may undervalue less well-defined migraine episodes in their relatives, when they have *just* MO. When high specificity and PPV are more important than sensitivity, probands reports can be a valuable ascertainment method to maximize the number of informative families.
4. Regarding risk factors for relatives of migraineurs, our findings showed that, as expected, females had a higher risk of migraine than men. We also found that mother-to-daughter transmissions were more frequent than expected, what may be due to the possible effect of mtDNA or X-chromosome genes and hormonal-related effects. Importantly, we also concluded that MA has a higher genetic load than MO.
5. We found a high significant chance-corrected agreement rate for primary and combined headaches and also for the frequency of migraine and tension-type headache. The ICHD-II criteria have relevant clinical implications; although they still need some improvement, these criteria do not change remarkably results from epidemiological studies. Given that our patients had been enrolling in the clinical and genetic study of migraine since 1999, we concluded that we could use the new classification in our patients and standardize the diagnosis criteria used in the clinical sample in future studies.

6. We confirmed the involvement of *STX1A* as a risk factor for migraine; also, new insight was provided by this study, since we found, in our population, an association between migraine and variants that were different from those described in previous studies. Our results also strengthen the role of neurotransmitter release in migraine pathophysiology.
7. We also confirmed *EDNRA* as a susceptibility factor for MO, although with different risk patterns regarding others populations studied. We also concluded that we could not exclude a role of this gene in MA susceptibility, showing that variants of this gene may affect the two migraine subtypes. This study reinforces also the role of the vascular component in the pathophysiology of migraine, and stresses the need to replicate findings from association studies in several populations.
8. We found an interaction between *BDNF* and *CGRP* in migraine susceptibility, but, due to small sample size, this interaction needs to be further investigated with larger samples. Nevertheless, this shows that genes interaction might be important in susceptibility to migraine.
9. Regarding the controversy if MA and MO are one or two different entities, our results show some evidence that MA seems to have a higher genetic predisposition than MO. Although sharing some mechanisms and susceptibility genes, MA can have a specific genetic component regarding aura.

7. FUTURE PERSPECTIVES

7. Future Perspectives

1. To enlarge the number of probands and controls ascertained in order to increase the power for subsequent studies.
2. To increase the number of trios (parents-child) and discordant sib-pairs ascertained, in order to perform family-based association studies that could be used to confirm the results found in the case-control sample.
3. To ascertain avuncular pairs in order to study the observed maternal effect.
4. To disentangle the possible role of mtDNA, X-linked genes and hormonal-related effects on female's liability for migraine.
5. To study the involvement of epigenetic modifications in susceptibility for migraine, in its possible parent-of-origin effects and in gender differences, as well as in differential response to treatment observed among patients.
6. To study new candidate genes underlying migraine susceptibility, such as glutamate receptors or genes related with synaptic vesicles and consequently with neurotransmitter release.
7. To assess possible regulatory functions of the polymorphisms found to be associated with migraine, by *in silico* or *in vivo* analysis, and try to determine the causative variant that might be in LD with them.

8. REFERENCES

8. References

1. Bigal ME, Lipton RB. Overview on the prevalence and impact of migraine. *J Headache Pain* 2004;5:S88-S91.
2. Carson AJ, Ringbauer B, MacKenzie L, Warlow C, Sharpe M. Neurological disease, emotional disorder, and disability: they are related: a study of 300 consecutive new referrals to a neurology outpatient department. *J Neurol Neurosurg Psychiatry* 2000;68:202-206.
3. Stovner L, Hagen K, Jensen R, et al. The global burden of headache: a documentation of headache prevalence and disability worldwide. *Cephalalgia* 2007;27:193-210.
4. Stovner LJ, Zwart JA, Hagen K, Terwindt GM, Pascual J. Epidemiology of headache in Europe. *Eur J Neurol* 2006;13:333-345.
5. Classification and diagnostic criteria for headache disorders, cranial neuralgias and facial pain. Headache Classification Committee of the International Headache Society. *Cephalalgia* 1988;8 Suppl 7:1-96.
6. Headache Classification Subcommittee of the International Headache Society. The International Classification of Headache Disorders, 2nd edition. . *Cephalalgia* 2004;24 (suppl 1):1–160
7. Lipton RB, Bigal ME, Steiner TJ, Silberstein SD, Olesen J. Classification of primary headaches. *Neurology* 2004;63:427-435.
8. Terwindt GM, Ferrari MD, Tijhuis M, Groenen SM, Picavet HS, Launer LJ. The impact of migraine on quality of life in the general population: the GEM study. *Neurology* 2000;55:624-629.
9. World Health Organization WHO. The world health report 2001,Chapter 2 [online]. Available at: <http://www.who.int/whr/2001/chapter2/en/index3.html>. Accessed 2009.06.24.
10. Ferrari MD. Migraine. *Lancet* 1998;351:1043-1051.
11. Goadsby PJ. Advances in the understanding of headache. *British Medical Bulletin* 2005;73-74
12. De Vries B, Haan J, Frants RR, Van den Maagdenberg AM, Ferrari MD. Genetic biomarkers for migraine. *Headache* 2006;46:1059-1068.
13. Launer LJ, Terwindt GM, Ferrari MD. The prevalence and characteristics of migraine in a population-based cohort: the GEM study. *Neurology* 1999;53:537-542.
14. Rasmussen BK, Olesen J. Migraine with aura and migraine without aura: an epidemiological study. *Cephalalgia* 1992;12:221-228; discussion 186.
15. Cady R, Schreiber C, Farmer K, Sheftell F. Primary headaches: a convergence hypothesis. *Headache* 2002;42:204-216.
16. Pietrobon D. Familial hemiplegic migraine. *Neurotherapeutics* 2007;4:274-284.
17. Thomsen LL, Eriksen MK, Roemer SF, Andersen I, Olesen J, Russell MB. A population-based study of familial hemiplegic migraine suggests revised diagnostic criteria. *Brain* 2002;125:1379-1391.

18. Kaniecki RG. Migraine and tension-type headache: an assessment of challenges in diagnosis. *Neurology* 2002;58:S15-20.
19. Rasmussen BK. Migraine and tension-type headache are separate disorders. *Cephalalgia* 1996;16:217-220; discussion 223.
20. Rasmussen BK, Jensen R, Schroll M, Olesen J. Interrelations between migraine and tension-type headache in the general population. *Arch Neurol* 1992;49:914-918.
21. Pereira Monteiro JM, R.Maio and J.M.Calheiros. Overlap of Migraine and Tension-Type Headaches in a Population-Based Study. In: Olesen J, ed. *Headache Classification and Epidemiology*. New York: Raven Press, 1994: 103-106.
22. Silberstein SD. Migraine. *Lancet* 2004;363:381-391.
23. Blau JN. Migraine pathogenesis: the neural hypothesis reexamined. *J Neurol Neurosurg Psychiatry* 1984;47:437-442.
24. Blau JN. Feeling and seeing headaches. *J Headache Pain* 2005;6:10-19.
25. Wessman M, Terwindt GM, Kaunisto MA, Palotie A, Ophoff RA. Migraine: a complex genetic disorder. *Lancet Neurol* 2007;6:521-532.
26. Aguggia M, D'Andrea G, Bussone G. Neurophysiology and neuromodulators. *Neurol Sci* 2007;28 Suppl 2:S97-S100.
27. Goadsby PJ. Pathophysiology of migraine. *Neurol Clin* 2009;27:335-360.
28. Striessnig J. Pathophysiology of migraine headache: Insight from pharmacology and genetics. *Drug Discovery Today:Disease Mechanisms* 2005. DOI: 10.1016/j.ddmec.2005.11.002.
29. Goadsby PJ, Charbit AR, Andreou AP, Akerman S, Holland PR. Neurobiology of migraine. *Neuroscience* 2009.
30. Bonavita V, Sorrentino G. Headache and neurology. *Neurol Sci* 2004;25 Suppl 3:S61-65.
31. Waeber C, Moskowitz MA. Migraine as an inflammatory disorder. *Neurology* 2005;64:S9-15.
32. Somjen GG. Mechanisms of spreading depression and hypoxic spreading depression-like depolarization. *Physiol Rev* 2001;81:1065-1096.
33. Pietrobon D. Migraine: new molecular mechanisms. *Neuroscientist* 2005;11:373-386.
34. Lauritzen M. Pathophysiology of the migraine aura. The spreading depression theory. *Brain* 1994;117 (Pt 1):199-210.
35. Buzzi MG, Moskowitz MA. The pathophysiology of migraine: year 2005. *J Headache Pain* 2005;6:105-111.
36. Sanchez-Del-Rio M, Reuter U, Moskowitz MA. New insights into migraine pathophysiology. *Curr Opin Neurol* 2006;19:294-298.
37. Wassertheil-Smoller S. *Biostatistics and Epidemiology- A primer for health and biomedical professionals*, Third Edition ed. New York: Springer-Verlag New York, 2003.
38. Lucas S. Epidemiology of Primary Headache in Women. In: Loder E, Marcus DA, eds. *Migraine in Women: PMPH-USA*, 2004: 3-11.
39. Lipton RB, Bigal ME. Migraine: epidemiology, impact, and risk factors for progression. *Headache* 2005;45 Suppl 1:S3-S13.

40. Lyngberg AC, Rasmussen BK, Jorgensen T, Jensen R. Incidence of primary headache: a Danish epidemiologic follow-up study. *Am J Epidemiol* 2005;161:1066-1073.
41. Stewart WF, Linet MS, Celentano DD, Van Natta M, Ziegler D. Age- and sex-specific incidence rates of migraine with and without visual aura. *Am J Epidemiol* 1991;134:1111-1120.
42. Rasmussen BK. Epidemiology of headache. *Cephalalgia* 1995;15:45-68.
43. Breslau N, Davis GC, Schultz LR, Peterson EL. Joint 1994 Wolff Award Presentation. Migraine and major depression: a longitudinal study. *Headache* 1994;34:387-393.
44. Stang PE, Yanagihara PA, Swanson JW, et al. Incidence of migraine headache: a population-based study in Olmsted County, Minnesota. *Neurology* 1992;42:1657-1662.
45. Bigal ME, Liberman JN, Lipton RB. Age-dependent prevalence and clinical features of migraine. *Neurology* 2006;67:246-251.
46. Lipton RB, Bigal ME. The epidemiology of migraine. *Am J Med* 2005;118 Suppl 1:3S-10S.
47. Henry P, Auray JP, Gaudin AF, et al. Prevalence and clinical characteristics of migraine in France. *Neurology* 2002;59:232-237.
48. Pereira Monteiro J. Cefaleias: estudo epidemiológico e clínico de uma população urbana. Porto: Universidade do Porto - ICBAS, 1995.
49. Stewart WF, Simon D, Shechter A, Lipton RB. Population variation in migraine prevalence: a meta-analysis. *J Clin Epidemiol* 1995;48:269-280.
50. Cucurachi L, Devetak M, Torelli P, Lambru G, Manzoni GC. Gender ratio of migraine without aura: observations over time. *Neurol Sci* 2006;27:47-50.
51. Tellez-Zenteno JF, Garcia-Ramos G, Zermeno-Pohls F, Velazquez A. Demographic, clinical and comorbidity data in a large sample of 1,147 patients with migraine in Mexico City. *J Headache Pain* 2005;6:128-134.
52. Radtke A, Neuhauser H. Prevalence and burden of headache and migraine in Germany. *Headache* 2009;49:79-89.
53. Russell MB, Rasmussen BK, Thorvaldsen P, Olesen J. Prevalence and sex-ratio of the subtypes of migraine. *Int J Epidemiol* 1995;24:612-618.
54. Monteiro JM, Matos E, Calheiros JM. Headaches in medical school students. *Neuroepidemiology* 1994;13:103-107.
55. Pereira Monteiro JM, R.Maio and J.M.Calheiros. Comparison of Questionnaire with Clinical Interview. In: Olesen J, ed. *Headache Classification and Epidemiology*. New York: Raven Press, 1994: 217-220.
56. Feinstein AR. The pre-therapeutic classification of co-morbidity in chronic disease. *J Chronic Dis* 1970;23:455-468.
57. Sacco S, Olivieri L, Bastianello S, Carolei A. Comorbid neuropathologies in migraine. *J Headache Pain* 2006;7:222-230.
58. Gijzen R, Hoeymans N, Schellevis FG, Ruwaard D, Satariano WA, van den Bos GA. Causes and consequences of comorbidity: a review. *J Clin Epidemiol* 2001;54:661-674.

59. Nuyen J, Schellevis FG, Satariano WA, et al. Comorbidity was associated with neurologic and psychiatric diseases: a general practice-based controlled study. *J Clin Epidemiol* 2006;59:1274-1284.
60. Artto V, Wessman M, Nissila M, et al. Comorbidity in Finnish migraine families. *J Headache Pain* 2006;7:324-330.
61. Hottenga JJ, Vanmolkot KR, Kors EE, et al. The 3p21.1-p21.3 hereditary vascular retinopathy locus increases the risk for Raynaud's phenomenon and migraine. *Cephalalgia* 2005;25:1168-1172.
62. Terwindt GM, Haan J, Ophoff RA, et al. Clinical and genetic analysis of a large Dutch family with autosomal dominant vascular retinopathy, migraine and Raynaud's phenomenon. *Brain* 1998;121 (Pt 2):303-316.
63. Goldgar DE. Sib Pair Analysis. In: Haines JL, Pericak-Vance MA, eds. *Approaches to Gene Mapping in complex Human Diseases*. New York: Wiley-Liss, Inc, 1998: 277-278.
64. Gardner KL. Genetics of migraine: an update. *Headache* 2006;46 Suppl 1:S19-24.
65. Cantor RM, and J.I. Rotter. Analysis of Genetic Data: Methods and Interpretation. In: King RA, Rotter J.I., Motulsky A.G., ed. *The Genetic Basis of Common Diseases*. New York: Oxford University Press, 1992: 49-70.
66. Hemminki K, Li X, Johansson SE, Sundquist K, Sundquist J. Familial risks for migraine and other headaches among siblings based on hospitalizations in Sweden. *Neurogenetics* 2005;6:217-224.
67. Kalfakis N, Panas M, Vassilopoulos D, Malliara-Loulakaki S. Migraine with aura: segregation analysis and heritability estimation. *Headache* 1996;36:320-322.
68. Stewart WF, Bigal ME, Kolodner K, Dowson A, Liberman JN, Lipton RB. Familial risk of migraine: variation by proband age at onset and headache severity. *Neurology* 2006;66:344-348.
69. Russell MB, Iselius L, Olesen J. Migraine without aura and migraine with aura are inherited disorders. *Cephalalgia* 1996;16:305-309.
70. Cologno D, De Pascale A, Manzoni GC. Familial occurrence of migraine with aura in a population-based study. *Headache* 2003;43:231-234.
71. Gervil M, Ulrich V, Kaprio J, Russell MB. Is the genetic liability in multifactorial disorders higher in concordant than discordant monozygotic twin pairs? A population-based family twin study of migraine without aura. *Eur J Neurol* 2001;8:231-235.
72. Farrer LA, Cupples LA. Determining the genetic component of a disease. In: Haines JL, Pericak-Vance MA, eds. *Approaches to Gene Mapping in Complex Human Diseases*. New York: Wiley-Liss, Inc., 1988: 93-129.
73. Gervil M, Ulrich V, Kaprio J, Olesen J, Russell MB. The relative role of genetic and environmental factors in migraine without aura. *Neurology* 1999;53:995-999.
74. Larsson B, Bille B, Pedersen NL. Genetic influence in headaches: a Swedish twin study. *Headache* 1995;35:513-519.
75. Mulder EJ, Van Baal C, Gaist D, et al. Genetic and environmental influences on migraine: a twin study across six countries. *Twin Res* 2003;6:422-431.

76. Svensson DA, Larsson B, Waldenlind E, Pedersen NL. Shared rearing environment in migraine: results from twins reared apart and twins reared together. *Headache* 2003;43:235-244.
77. Nyholt DR, Gillespie NG, Heath AC, Merikangas KR, Duffy DL, Martin NG. Latent class and genetic analysis does not support migraine with aura and migraine without aura as separate entities. *Genet Epidemiol* 2004;26:231-244.
78. Honkasalo ML, Kaprio J, Winter T, Heikkila K, Sillanpaa M, Koskenvuo M. Migraine and concomitant symptoms among 8167 adult twin pairs. *Headache* 1995;35:70-78.
79. Ligthart L, Boomsma DI, Martin NG, Stubbe JH, Nyholt DR. Migraine with aura and migraine without aura are not distinct entities: further evidence from a large Dutch population study. *Twin Res Hum Genet* 2006;9:54-63.
80. Ulrich V, Gervil M, Kyvik KO, Olesen J, Russell MB. The inheritance of migraine with aura estimated by means of structural equation modelling. *J Med Genet* 1999;36:225-227.
81. Ulrich V, Gervil M, Kyvik KO, Olesen J, Russell MB. Evidence of a genetic factor in migraine with aura: a population-based Danish twin study. *Ann Neurol* 1999;45:242-246.
82. Khoury MJ, Beaty TH, Cohen BH. *Fundamentals of Genetic Epidemiology*: Oxford University Press, 1993.
83. Devoto M, Lozito A, Staffa G, D'Alessandro R, Sacquegna T, Romeo G. Segregation analysis of migraine in 128 families. *Cephalalgia* 1986;6:101-105.
84. Russell MB, Iselius L, Olesen J. Inheritance of migraine investigated by complex segregation analysis. *Hum Genet* 1995;96:726-730.
85. Falconer DS. The inheritance of liability to certain diseases, estimated from the incidence among relatives. *Ann Hum Genet* 1965;29:51-76.
86. Kallela M, Wessman M, Havanka H, Palotie A, Farkkila M. Familial migraine with and without aura: clinical characteristics and co-occurrence. *Eur J Neurol* 2001;8:441-449.
87. Ophoff RA, van Eijk R, Sandkuijl LA, et al. Genetic heterogeneity of familial hemiplegic migraine. *Genomics* 1994;22:21-26.
88. Noble-Topham SE, Cader MZ, Dymont DA, Rice GP, Brown JD, Ebers GC. Genetic loading in familial migraine with aura. *J Neurol Neurosurg Psychiatry* 2003;74:1128-1130.
89. Thomsen LL, Olesen J, Russell MB. Increased risk of migraine with typical aura in probands with familial hemiplegic migraine and their relatives. *Eur J Neurol* 2003;10:421-427.
90. Manzoni GC, Torelli P. Migraine with and without aura: a single entity? *Neurol Sci* 2008;29 Suppl 1:S40-43.
91. Ophoff RA, Terwindt GM, Vergouwe MN, et al. Familial hemiplegic migraine and episodic ataxia type-2 are caused by mutations in the Ca²⁺ channel gene CACNL1A4. *Cell* 1996;87:543-552.
92. De Fusco M, Marconi R, Silvestri L, et al. Haploinsufficiency of ATP1A2 encoding the Na⁺/K⁺ pump alpha2 subunit associated with familial hemiplegic migraine type 2. *Nat Genet* 2003;33:192-196.

93. Vanmolkot KR, Kors EE, Hottenga JJ, et al. Novel mutations in the Na⁺, K⁺-ATPase pump gene ATP1A2 associated with familial hemiplegic migraine and benign familial infantile convulsions. *Ann Neurol* 2003;54:360-366.
94. Kors EE, Vanmolkot KR, Haan J, et al. Alternating hemiplegia of childhood: no mutations in the second familial hemiplegic migraine gene ATP1A2. *Neuropediatrics* 2004;35:293-296.
95. Spadaro M, Ursu S, Lehmann-Horn F, et al. A G301R Na⁺/K⁺-ATPase mutation causes familial hemiplegic migraine type 2 with cerebellar signs. *Neurogenetics* 2004;5:177-185.
96. Dichgans M, Freilinger T, Eckstein G, et al. Mutation in the neuronal voltage-gated sodium channel SCN1A in familial hemiplegic migraine. *Lancet* 2005;366:371-377.
97. Alonso I, Barros J, Tuna A, et al. Phenotypes of spinocerebellar ataxia type 6 and familial hemiplegic migraine caused by a unique CACNA1A missense mutation in patients from a large family. *Arch Neurol* 2003;60:610-614.
98. Alonso I, Barros J, Tuna A, et al. A novel R1347Q mutation in the predicted voltage sensor segment of the P/Q-type calcium-channel alpha-subunit in a family with progressive cerebellar ataxia and hemiplegic migraine. *Clin Genet* 2004;65:70-72.
99. Castro MJ, Nunes B, de Vries B, et al. Two novel functional mutations in the Na⁺,K⁺-ATPase alpha2-subunit ATP1A2 gene in patients with familial hemiplegic migraine and associated neurological phenotypes. *Clin Genet* 2008;73:37-43.
100. Castro MJ, Stam AH, Lemos C, et al. Recurrent ATP1A2 mutations in Portuguese families with familial hemiplegic migraine. *J Hum Genet* 2007;52:990-998.
101. Castro MJ, Stam AH, Lemos C, et al. First mutation in the voltage-gated Nav1.1 subunit gene SCN1A with co-occurring familial hemiplegic migraine and epilepsy. *Cephalalgia* 2009;29:308-313.
102. Cuenca-Leon E, Corominas R, Montfort M, et al. Familial hemiplegic migraine: linkage to chromosome 14q32 in a Spanish kindred. *Neurogenetics* 2009;10:191-198.
103. Colson NJ, Fernandez F, Lea RA, Griffiths LR. The search for migraine genes: an overview of current knowledge. *Cell Mol Life Sci* 2007;64:331-344.
104. Khoury MJ, Yang Q. The future of genetic studies of complex human diseases: an epidemiologic perspective. *Epidemiology* 1998;9:350-354.
105. McCarroll SA, Altshuler DM. Copy-number variation and association studies of human disease. *Nat Genet* 2007;39:S37-42.
106. Dawn Teare M, Barrett JH. Genetic linkage studies. *Lancet* 2005;366:1036-1044.
107. Schulze TG, McMahon FJ. Genetic linkage and association studies in bipolar affective disorder: a time for optimism. *Am J Med Genet C Semin Med Genet* 2003;123:36-47.
108. Pericak-Vance MA. Basic Concepts in Linkage Analysis. In: Haines JL, Pericak-Vance MA, eds. *Approaches to Gene Mapping in Complex Human Diseases*. New York: Wiley-Liss, Inc, 1998: 77-92.
109. Lander E, Kruglyak L. Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. *Nat Genet* 1995;11:241-247.

110. Risch N. Searching for genes in complex diseases: lessons from systemic lupus erythematosus. *J Clin Invest* 2000;105:1503-1506.
111. Strachan T, Read AP. *Human Molecular Genetics* 3. London & New York: Garland Science, 2004.
112. Cordell HJ, Clayton DG. Genetic association studies. *Lancet* 2005;366:1121-1131.
113. Pericak-Vance MA. Linkage Disequilibrium and Allelic Association. In: Haines JL, Pericak-Vance MA, eds. *Approaches to Gene Mapping in Complex Human Diseases*. New York: Wiley-Liss, Inc., 1998: 323-333.
114. Cardon LR, Bell JI. Association study designs for complex diseases. *Nat Rev Genet* 2001;2:91-99.
115. Spielman RS, McGinnis RE, Ewens WJ. Transmission test for linkage disequilibrium: the insulin gene region and insulin-dependent diabetes mellitus (IDDM). *Am J Hum Genet* 1993;52:506-516.
116. Spielman RS, Ewens WJ. A sibship test for linkage in the presence of association: the sib transmission/disequilibrium test. *Am J Hum Genet* 1998;62:450-458.
117. Wilkening S, Chen B, Bermejo JL, Canzian F. Is there still a need for candidate gene approaches in the era of genome-wide association studies? *Genomics* 2009;93:415-419.
118. Manolio TA, Brooks LD, Collins FS. A HapMap harvest of insights into the genetics of common disease. *J Clin Invest* 2008;118:1590-1605.
119. Lohmueller KE, Pearce CL, Pike M, Lander ES, Hirschhorn JN. Meta-analysis of genetic association studies supports a contribution of common variants to susceptibility to common disease. *Nat Genet* 2003;33:177-182.
120. Risch NJ. Searching for genetic determinants in the new millennium. *Nature* 2000;405:847-856.
121. Risch N, Merikangas K. The future of genetic studies of complex human diseases. *Science* 1996;273:1516-1517.
122. Hemminki K, Forsti A, Bermejo JL. The 'common disease-common variant' hypothesis and familial risks. *PLoS ONE* 2008;3:e2504.
123. Terwindt GM, Ophoff RA, Haan J, et al. Variable clinical expression of mutations in the P/Q-type calcium channel gene in familial hemiplegic migraine. Dutch Migraine Genetics Research Group. *Neurology* 1998;50:1105-1110.
124. Ducros A, Denier C, Joutel A, et al. The clinical spectrum of familial hemiplegic migraine associated with mutations in a neuronal calcium channel. *N Engl J Med* 2001;345:17-24.
125. Wieser T, Mueller C, Evers S, Zierz S, Deufel T. Absence of known familial hemiplegic migraine (FHM) mutations in the CACNA1A gene in patients with common migraine: implications for genetic testing. *Clin Chem Lab Med* 2003;41:272-275.
126. Hovatta I, Kallela M, Farkkila M, Peltonen L. Familial migraine: exclusion of the susceptibility gene from the reported locus of familial hemiplegic migraine on 19p. *Genomics* 1994;23:707-709.

127. Nyholt DR, Lea RA, Goadsby PJ, Brimage PJ, Griffiths LR. Familial typical migraine: linkage to chromosome 19p13 and evidence for genetic heterogeneity. *Neurology* 1998;50:1428-1432.
128. May A, Ophoff RA, Terwindt GM, et al. Familial hemiplegic migraine locus on 19p13 is involved in the common forms of migraine with and without aura. *Hum Genet* 1995;96:604-608.
129. Terwindt GM, Ophoff RA, van Eijk R, et al. Involvement of the CACNA1A gene containing region on 19p13 in migraine with and without aura. *Neurology* 2001;56:1028-1032.
130. Noble-Topham SE, Dymment DA, Cader MZ, et al. Migraine with aura is not linked to the FHM gene CACNA1A or the chromosomal region, 19p13. *Neurology* 2002;59:1099-1101.
131. Lea RA, Curtain RP, Hutchins C, Brimage PJ, Griffiths LR. Investigation of the CACNA1A gene as a candidate for typical migraine susceptibility. *Am J Med Genet* 2001;105:707-712.
132. Jones KW, Ehm MG, Pericak-Vance MA, Haines JL, Boyd PR, Peroutka SJ. Migraine with aura susceptibility locus on chromosome 19p13 is distinct from the familial hemiplegic migraine locus. *Genomics* 2001;78:150-154.
133. Kaunisto MA, Tikka PJ, Kallela M, et al. Chromosome 19p13 loci in Finnish migraine with aura families. *Am J Med Genet B Neuropsychiatr Genet* 2005;132:85-89.
134. Brugnioni R, Leone M, Rigamonti A, et al. Is the CACNA1A gene involved in familial migraine with aura? *Neurol Sci* 2002;23:1-5.
135. Kirchmann M, Thomsen LL, Olesen J. The CACNA1A and ATP1A2 genes are not involved in dominantly inherited migraine with aura. *Am J Med Genet B Neuropsychiatr Genet* 2006;141B:250-256.
136. Curtain RP, Lea RA, Tajouri L, et al. Analysis of chromosome 1 microsatellite markers and the FHM2-ATP1A2 gene mutations in migraine pedigrees. *Neurol Res* 2005;27:647-652.
137. Ambrosini A, D'Onofrio M, Grieco GS, et al. Familial basilar migraine associated with a new mutation in the ATP1A2 gene. *Neurology* 2005;65:1826-1828.
138. Todt U, Dichgans M, Jurkat-Rott K, et al. Rare missense variants in ATP1A2 in families with clustering of common forms of migraine. *Hum Mutat* 2005;26:315-321.
139. Jen JC, Kim GW, Dudding KA, Baloh RW. No mutations in CACNA1A and ATP1A2 in probands with common types of migraine. *Arch Neurol* 2004;61:926-928.
140. Netzer C, Todt U, Heinze A, et al. Haplotype-based systematic association studies of ATP1A2 in migraine with aura. *Am J Med Genet B Neuropsychiatr Genet* 2006;141:257-260.
141. Deprez L, Peeters K, Van Paesschen W, et al. Familial occipitotemporal lobe epilepsy and migraine with visual aura: linkage to chromosome 9q. *Neurology* 2007;68:1995-2002.
142. Wessman M, Kallela M, Kaunisto MA, et al. A susceptibility locus for migraine with aura, on chromosome 4q24. *Am J Hum Genet* 2002;70:652-662.
143. Cader ZM, Noble-Topham S, Dymment DA, et al. Significant linkage to migraine with aura on chromosome 11q24. *Hum Mol Genet* 2003;12:2511-2517.
144. Bjornsson A, Gudmundsson G, Gudfinnsson E, et al. Localization of a gene for migraine without aura to chromosome 4q21. *Am J Hum Genet* 2003;73:986-993.

145. Soragna D, Vettori A, Carraro G, et al. A locus for migraine without aura maps on chromosome 14q21.2-q22.3. *Am J Hum Genet* 2003;72:161-167.
146. Carlsson A, Forsgren L, Nylander PO, et al. Identification of a susceptibility locus for migraine with and without aura on 6p12.2-p21.1. *Neurology* 2002;59:1804-1807.
147. Russo L, Mariotti P, Sangiorgi E, et al. A new susceptibility locus for migraine with aura in the 15q11-q13 genomic region containing three GABA-A receptor genes. *Am J Hum Genet* 2005;76:327-333.
148. Lea RA, Shepherd AG, Curtain RP, et al. A typical migraine susceptibility region localizes to chromosome 1q31. *Neurogenetics* 2002;4:17-22.
149. Nyholt DR, Curtain RP, Griffiths LR. Familial typical migraine: significant linkage and localization of a gene to Xq24-28. *Hum Genet* 2000;107:18-23.
150. Merikangas KR, Dartigues JF, Whitaker A, Angst J. Diagnostic criteria for migraine. A validity study. *Neurology* 1994;44:S11-16.
151. Anttila V, Kallela M, Oswell G, et al. Trait components provide tools to dissect the genetic susceptibility of migraine. *Am J Hum Genet* 2006;79:85-99.
152. Ligthart L, Nyholt DR, Hottenga JJ, Distel MA, Willemsen G, Boomsma DI. A genome-wide linkage scan provides evidence for both new and previously reported loci influencing common migraine. *Am J Med Genet B Neuropsychiatr Genet* 2008;147B:1186-1195.
153. Lea RA, Nyholt DR, Curtain RP, et al. A genome-wide scan provides evidence for loci influencing a severe heritable form of common migraine. *Neurogenetics* 2005;6:67-72.
154. Nyholt DR, Morley KI, Ferreira MA, et al. Genomewide significant linkage to migrainous headache on chromosome 5q21. *Am J Hum Genet* 2005;77:500-512.
155. Anttila V, Nyholt DR, Kallela M, et al. Consistently replicating locus linked to migraine on 10q22-q23. *Am J Hum Genet* 2008;82:1051-1063.
156. Chen CC, Keith JM, Nyholt DR, Martin NG, Mengersen KL. Bayesian latent trait modeling of migraine symptom data. *Hum Genet* 2009. DOI 10.1007/s00439-009-0671-4
157. de Vries B, Frants RR, Ferrari MD, van den Maagdenberg AM. Molecular genetics of migraine. *Hum Genet* 2009. DOI 10.1007/s00439-009-0684-z
158. Montagna P. Molecular genetics of migraine headaches: a review. *Cephalalgia* 2000;20:3-14.
159. Anthony M, Hinterberger H, Lance JW. Plasma serotonin in migraine and stress. *Arch Neurol* 1967;16:544-552.
160. Panconesi A. Serotonin and migraine: a reconsideration of the central theory. *J Headache Pain* 2008;9:267-276.
161. Ferrari MD, Odink J, Tapparelli C, Van Kempen GM, Pennings EJ, Bruyn GW. Serotonin metabolism in migraine. *Neurology* 1989;39:1239-1242.
162. Goadsby PJ. Serotonin receptor ligands: treatments of acute migraine and cluster headache. *Handb Exp Pharmacol* 2007:129-143.
163. Erdal N, Herken H, Yilmaz M, Erdal E, Bayazit YA. The A218C polymorphism of tryptophan hydroxylase gene and migraine. *J Clin Neurosci* 2007;14:249-251.

164. Johnson MP, Griffiths LR. A genetic analysis of serotonergic biosynthetic and metabolic enzymes in migraine using a DNA pooling approach. *J Hum Genet* 2005;50:607-610.
165. Marziniak M, Mossner R, Benninghoff J, Syagailo YV, Lesch KP, Sommer C. Association analysis of the functional monoamine oxidase A gene promoter polymorphism in migraine. *J Neural Transm* 2004;111:603-609.
166. Filic V, Vladoic A, Stefulj J, et al. Monoamine oxidases A and B gene polymorphisms in migraine patients. *J Neurol Sci* 2005;228:149-153.
167. Marziniak M, Mossner R, Kienzler C, Riederer P, Lesch KP, Sommer C. Functional polymorphisms of the 5-HT1A and 5-HT1B receptor are associated with clinical symptoms in migraineurs. *J Neural Transm* 2007;114:1227-1232.
168. Oterino A, Castillo J, Pascual J, et al. Genetic association study and meta-analysis of the HTR2C Cys23Ser polymorphism and migraine. *J Headache Pain* 2007;8:231-235.
169. Yang XS, Xu XP, Yang QD. No association of C-1019G promoter polymorphism of 5-HT1A receptor gene with migraine. *Eur J Neurol* 2006;13:533-535.
170. Racchi M, Leone M, Porrello E, et al. Familial migraine with aura: association study with 5-HT1B/1D, 5-HT2C, and hSERT polymorphisms. *Headache* 2004;44:311-317.
171. Kusumi M, Araki H, Ijiri T, et al. Serotonin 2C receptor gene Cys23Ser polymorphism: a candidate genetic risk factor of migraine with aura in Japanese population. *Acta Neurol Scand* 2004;109:407-409.
172. Johnson MP, Lea RA, Curtain RP, MacMillan JC, Griffiths LR. An investigation of the 5-HT2C receptor gene as a migraine candidate gene. *Am J Med Genet B Neuropsychiatr Genet* 2003;117:86-89.
173. Juhasz G, Zsombok T, Laszik A, et al. Association analysis of 5-HTTLPR variants, 5-HT2a receptor gene 102T/C polymorphism and migraine. *J Neurogenet* 2003;17:231-240.
174. Erdal ME, Herken H, Yilmaz M, Bayazit YA. Association of the T102C polymorphism of 5-HT2A receptor gene with aura in migraine. *J Neurol Sci* 2001;188:99-101.
175. Burnet PW, Harrison PJ, Goodwin GM, et al. Allelic variation in the serotonin 5-HT2C receptor gene and migraine. *Neuroreport* 1997;8:2651-2653.
176. Nyholt DR, Curtain RP, Gaffney PT, Brimage P, Goadsby PJ, Griffiths LR. Migraine association and linkage analyses of the human 5-hydroxytryptamine (5HT2A) receptor gene. *Cephalalgia* 1996;16:463-467.
177. Todt U, Freudenberger J, Goebel I, et al. Variation of the serotonin transporter gene SLC6A4 in the susceptibility to migraine with aura. *Neurology* 2006;67:1707-1709.
178. Marziniak M, Mossner R, Schmitt A, Lesch KP, Sommer C. A functional serotonin transporter gene polymorphism is associated with migraine with aura. *Neurology* 2005;64:157-159.
179. Yilmaz M, Erdal ME, Herken H, Cataloluk O, Barlas O, Bayazit YA. Significance of serotonin transporter gene polymorphism in migraine. *J Neurol Sci* 2001;186:27-30.
180. Ogilvie AD, Russell MB, Dhall P, et al. Altered allelic distributions of the serotonin transporter gene in migraine without aura and migraine with aura. *Cephalalgia* 1998;18:23-26.

181. Fernandez F, Colson NJ, Griffiths LR. Pharmacogenetics of migraine: genetic variants and their potential role in migraine therapy. *Pharmacogenomics* 2007;8:609-622.
182. Todt U, Netzer C, Toliat M, et al. New genetic evidence for involvement of the dopamine system in migraine with aura. *Hum Genet* 2009;125:265-279.
183. Mochi M, Cevoli S, Cortelli P, et al. A genetic association study of migraine with dopamine receptor 4, dopamine transporter and dopamine-beta-hydroxylase genes. *Neurol Sci* 2003;23:301-305.
184. Shepherd AG, Lea RA, Hutchins C, Jordan KL, Brimage PJ, Griffiths LR. Dopamine receptor genes and migraine with and without aura: an association study. *Headache* 2002;42:346-351.
185. Maude S, Curtin J, Breen G, et al. The -141C Ins/Del polymorphism of the dopamine D2 receptor gene is not associated with either migraine or Parkinson's disease. *Psychiatr Genet* 2001;11:49-52.
186. Dichgans M, Forderreuther S, Deiterich M, Pfaffenrath V, Gasser T. The D2 receptor NcoI allele: absence of allelic association with migraine with aura. *Neurology* 1998;51:928.
187. Del Zompo M, Cherchi A, Palmas MA, et al. Association between dopamine receptor genes and migraine without aura in a Sardinian sample. *Neurology* 1998;51:781-786.
188. Peroutka SJ, Wilhoit T, Jones K. Clinical susceptibility to migraine with aura is modified by dopamine D2 receptor (DRD2) NcoI alleles. *Neurology* 1997;49:201-206.
189. Fernandez F, Colson N, Quinlan S, MacMillan J, Lea RA, Griffiths LR. Association between migraine and a functional polymorphism at the dopamine beta-hydroxylase locus. *Neurogenetics* 2009;10:199-208.
190. Lea RA, Dohy A, Jordan K, Quinlan S, Brimage PJ, Griffiths LR. Evidence for allelic association of the dopamine beta-hydroxylase gene (DBH) with susceptibility to typical migraine. *Neurogenetics* 2000;3:35-40.
191. Mossner R, Freitag CM, Marziniak M, Moser D, Sommer C, Meyer J. The functional Val158Met variant of the COMT gene is not associated with migraine with or without aura. *J Headache Pain* 2006;7:165-166.
192. Hagen K, Pettersen E, Stovner LJ, Skorpen F, Zwart JA. The association between headache and Val158Met polymorphism in the catechol-O-methyltransferase gene: the HUNT Study. *J Headache Pain* 2006;7:70-74.
193. Silvestrini M, Cupini LM, Troisi E, Matteis M, Bernardi G. Estimation of cerebrovascular reactivity in migraine without aura. *Stroke* 1995;26:81-83.
194. Kowa H, Yasui K, Takeshima T, Urakami K, Sakai F, Nakashima K. The homozygous C677T mutation in the methylenetetrahydrofolate reductase gene is a genetic risk factor for migraine. *Am J Med Genet* 2000;96:762-764.
195. Harker LA, Harlan JM, Ross R. Effect of sulfinpyrazone on homocysteine-induced endothelial injury and arteriosclerosis in baboons. *Circ Res* 1983;53:731-739.
196. Johnson MP, Fernandez F, Colson NJ, Griffiths LR. A pharmacogenomic evaluation of migraine therapy. *Expert Opin Pharmacother* 2007;8:1821-1835.

197. Lea RA, Ovcaric M, Sundholm J, Solyom L, Macmillan J, Griffiths LR. Genetic variants of angiotensin converting enzyme and methylenetetrahydrofolate reductase may act in combination to increase migraine susceptibility. *Brain Res Mol Brain Res* 2005;136:112-117.
198. Schurks M, Zee RY, Buring JE, Kurth T. Polymorphisms in the renin-angiotensin system and migraine in women. *Headache* 2009;49:292-299.
199. Dreier JP, Kleeberg J, Alam M, et al. Endothelin-1-induced spreading depression in rats is associated with a microarea of selective neuronal necrosis. *Exp Biol Med (Maywood)* 2007;232:204-213.
200. Gallai V, Sarchielli P, Firenze C, et al. Endothelin 1 in migraine and tension-type headache. *Acta Neurol Scand* 1994;89:47-55.
201. Ikeda U, Yamamoto K, Maeda Y, Shimpo M, Kanbe T, Shimada K. Endothelin-1 inhibits nitric oxide synthesis in vascular smooth muscle cells. *Hypertension* 1997;29:65-69.
202. Kallela M, Farkkila M, Saijonmaa O, Fyhrquist F. Endothelin in migraine patients. *Cephalalgia* 1998;18:329-332.
203. Arai H, Hori S, Aramori I, Ohkubo H, Nakanishi S. Cloning and expression of a cDNA encoding an endothelin receptor. *Nature* 1990;348:730-732.
204. Tzourio C, El Amrani M, Poirier O, Nicaud V, Bousser MG, Alperovitch A. Association between migraine and endothelin type A receptor (ETA -231 A/G) gene polymorphism. *Neurology* 2001;56:1273-1277.
205. Bruhwylter J, Chleide E, Liegeois JF, Carreer F. Nitric oxide: a new messenger in the brain. *Neurosci Biobehav Rev* 1993;17:373-384.
206. Toriello M, Oterino A, Pascual J, et al. Lack of association of endothelial nitric oxide synthase polymorphisms and migraine. *Headache* 2008;48:1115-1119.
207. Borroni B, Rao R, Liberini P, et al. Endothelial nitric oxide synthase (Glu298Asp) polymorphism is an independent risk factor for migraine with aura. *Headache* 2006;46:1575-1579.
208. Griffiths LR, Nyholt DR, Curtain RP, Goadsby PJ, Brimage PJ. Migraine association and linkage studies of an endothelial nitric oxide synthase (NOS3) gene polymorphism. *Neurology* 1997;49:614-617.
209. Tikka-Kleemola P, Kaunisto MA, Hamalainen E, et al. Genetic association study of Endothelin-1 and its receptors EDNRA and EDNRB in migraine with aura. *Cephalalgia* 2009.
210. Lisi V, Garbo G, Battistella P, et al. Endothelin receptor A -231 G>A polymorphism: no linkage to primary pediatric headache. *Headache* 2006;46:486-491.
211. Schurks M, Zee RY, Buring JE, Kurth T. ACE D/I polymorphism, migraine, and cardiovascular disease in women. *Neurology* 2009;72:650-656.
212. Tronvik E, Stovner LJ, Bovim G, et al. Angiotensin-converting enzyme gene insertion/deletion polymorphism in migraine patients. *BMC Neurol* 2008;8:4.
213. Kowa H, Fusayasu E, Ijiri T, et al. Association of the insertion/deletion polymorphism of the angiotensin I-converting enzyme gene in patients of migraine with aura. *Neurosci Lett* 2005;374:129-131.

214. Paterna S, Di Pasquale P, Cottone C, et al. Migraine without aura and ACE-gene deletion polymorphism: is there a correlation? Preliminary findings. *Cardiovasc Drugs Ther* 1997;11:603-604.
215. Paterna S, Di Pasquale P, D'Angelo A, et al. Angiotensin-converting enzyme gene deletion polymorphism determines an increase in frequency of migraine attacks in patients suffering from migraine without aura. *Eur Neurol* 2000;43:133-136.
216. Schurks M, Zee RY, Buring JE, Kurth T. Interrelationships among the MTHFR 677C>T polymorphism, migraine, and cardiovascular disease. *Neurology* 2008;71:505-513.
217. Ferro A, Castro MJ, Lemos C, et al. The C677T polymorphism in MTHFR is not associated with migraine in Portugal. *Dis Markers* 2008;25:107-113.
218. Rubino E, Ferrero M, Rainero I, Binello E, Vaula G, Pinessi L. Association of the C677T polymorphism in the MTHFR gene with migraine: a meta-analysis. *Cephalalgia* 2007; 29: 818-825.
219. Kaunisto MA, Kallela M, Hamalainen E, et al. Testing of variants of the MTHFR and ESR1 genes in 1798 Finnish individuals fails to confirm the association with migraine with aura. *Cephalalgia* 2006;26:1462-1472.
220. Scher AI, Terwindt GM, Verschuren WM, et al. Migraine and MTHFR C677T genotype in a population-based sample. *Ann Neurol* 2006;59:372-375.
221. Todt U, Freudenberg J, Goebel I, et al. MTHFR C677T polymorphism and migraine with aura. *Ann Neurol* 2006;60:621-622; author reply 622-623.
222. Lea RA, Ovcarić M, Sundholm J, MacMillan J, Griffiths LR. The methylenetetrahydrofolate reductase gene variant C677T influences susceptibility to migraine with aura. *BMC Med* 2004;2:3.
223. Oterino A, Valle N, Bravo Y, et al. MTHFR T677 homozygosis influences the presence of aura in migraineurs. *Cephalalgia* 2004;24:491-494.
224. Kara I, Sazci A, Ergul E, Kaya G, Kilic G. Association of the C677T and A1298C polymorphisms in the 5,10 methylenetetrahydrofolate reductase gene in patients with migraine risk. *Brain Res Mol Brain Res* 2003;111:84-90.
225. Granella F, Sances G, Zanferrari C, Costa A, Martignoni E, Manzoni GC. Migraine without aura and reproductive life events: a clinical epidemiological study in 1300 women. *Headache* 1993;33:385-389.
226. Gupta S, Mehrotra S, Villalon CM, Perusquia M, Saxena PR, MaassenVanDenBrink A. Potential role of female sex hormones in the pathophysiology of migraine. *Pharmacol Ther* 2007;113:321-340.
227. Mattsson P. Hormonal factors in migraine: a population-based study of women aged 40 to 74 years. *Headache* 2003;43:27-35.
228. Corominas R, Ribases M, Cuenca-Leon E, Cormand B, Macaya A. Lack of association of hormone receptor polymorphisms with migraine. *Eur J Neurol* 2009;16:413-415.
229. Oterino A, Toriello M, Cayon A, et al. Multilocus analyses reveal involvement of the ESR1, ESR2, and FSHR genes in migraine. *Headache* 2008;48:1438-1450.
230. Colson NJ, Lea RA, Quinlan S, MacMillan J, Griffiths LR. Investigation of hormone receptor genes in migraine. *Neurogenetics* 2005;6:17-23.

-
231. Colson NJ, Lea RA, Quinlan S, MacMillan J, Griffiths LR. The estrogen receptor 1 G594A polymorphism is associated with migraine susceptibility in two independent case/control groups. *Neurogenetics* 2004;5:129-133.
232. Netzer C, Freudenberg J, Tolia MR, et al. Genetic association studies of the chromosome 15 GABA-A receptor cluster in migraine with aura. *Am J Med Genet B Neuropsychiatr Genet* 2008;147B:37-41.
233. Fernandez F, Esposito T, Lea RA, et al. Investigation of gamma-aminobutyric acid (GABA) A receptors genes and migraine susceptibility. *BMC Med Genet* 2008;9:109.
234. Oswell G, Kaunisto MA, Kallela M, et al. No association of migraine to the GABA-A receptor complex on chromosome 15. *Am J Med Genet B Neuropsychiatr Genet* 2008; 147B:33-36
235. Joutel A, Corpechot C, Ducros A, et al. Notch3 mutations in CADASIL, a hereditary adult-onset condition causing stroke and dementia. *Nature* 1996;383:707-710.
236. Wang W, Prince CZ, Mou Y, Pollman MJ. Notch3 signaling in vascular smooth muscle cells induces c-FLIP expression via ERK/MAPK activation. Resistance to Fas ligand-induced apoptosis. *J Biol Chem* 2002;277:21723-21729.
237. Smith RA, Curtain R, Ovcacic M, Tajouri L, Macmillan J, Griffiths L. Investigation of the NOTCH3 and TNFSF7 genes on C19p13 as candidates for migraine. *Open Neurol J* 2008;2:1-7.
238. Scher AI, Terwindt GM, Picavet HS, Verschuren WM, Ferrari MD, Launer LJ. Cardiovascular risk factors and migraine: the GEM population-based study. *Neurology* 2005;64:614-620.
239. Netzer C, Freudenberg J, Heinze A, et al. Replication study of the insulin receptor gene in migraine with aura. *Genomics* 2008;91:503-507.
240. Nyholt DR, LaForge KS, Kallela M, et al. A high-density association screen of 155 ion transport genes for involvement with common migraine. *Hum Mol Genet* 2008;17:3318-3331.
241. Asuni C, Stochino ME, Cherchi A, et al. Migraine and tumour necrosis factor gene polymorphism. An association study in a Sardinian sample. *J Neurol* 2009;256:194-197.
242. Lee KA, Jang SY, Sohn KM, et al. Association between a polymorphism in the lymphotoxin-a promoter region and migraine. *Headache* 2007;47:1056-1062.
243. Rainero I, Grimaldi LM, Salani G, et al. Association between the tumor necrosis factor-alpha -308 G/A gene polymorphism and migraine. *Neurology* 2004;62:141-143.
244. Trabace S, Brioli G, Lulli P, et al. Tumor necrosis factor gene polymorphism in migraine. *Headache* 2002;42:341-345.
245. Rainero I, Salani G, Valfre W, et al. Absence of linkage between the interleukin-6 gene (-174 G/C) polymorphism and migraine. *Neurosci Lett* 2003;343:155-158.
246. Rainero I, Pinessi L, Salani G, et al. A polymorphism in the interleukin-1alpha gene influences the clinical features of migraine. *Headache* 2002;42:337-340.
247. Oswell G, Kaunisto MA, Kallela M, et al. No association of migraine to the GABA-A receptor complex on chromosome 15. *Am J Med Genet B Neuropsychiatr Genet* 2008;147B:33-36.
248. Borroni B, Brambilla C, Liberini P, et al. Investigating the association between Notch3 polymorphism and migraine. *Headache* 2006;46:317-321.

249. Schwaag S, Evers S, Schirmacher A, Stogbauer F, Ringelstein EB, Kuhlenbaumer G. Genetic variants of the NOTCH3 gene in migraine--a mutation analysis and association study. *Cephalalgia* 2006;26:158-161.
250. McCarthy LC, Hosford DA, Riley JH, et al. Single-nucleotide polymorphism alleles in the insulin receptor gene are associated with typical migraine. *Genomics* 2001;78:135-149.
251. Curtain R, Lea RA, Quinlan S, et al. Investigation of the low-density lipoprotein receptor gene and cholesterol as a risk factor for migraine. *J Neurol Sci* 2004;227:95-100.
252. Mochi M, Cevoli S, Cortelli P, et al. Investigation of an LDLR gene polymorphism (19p13.2) in susceptibility to migraine without aura. *J Neurol Sci* 2003;213:7-10.
253. Henley SM, Bates GP, Tabrizi SJ. Biomarkers for neurodegenerative diseases. *Curr Opin Neurol* 2005;18:698-705.
254. Loder E, Rizzoli P. Biomarkers in migraine: their promise, problems, and practical applications. *Headache* 2006;46:1046-1058.
255. Nagata E, Hattori H, Kato M, et al. Identification of biomarkers associated with migraine with aura. *Neurosci Res* 2009;64:104-110.
256. Loder E, Harrington MG, Cutrer M, Sandor P, De Vries B. Selected confirmed, probable, and exploratory migraine biomarkers. *Headache* 2006;46:1108-1127.
257. Imamura K, Takeshima T, Fusayasu E, Nakashima K. Increased plasma matrix metalloproteinase-9 levels in migraineurs. *Headache* 2008;48:135-139.
258. Fusayasu E, Kowa H, Takeshima T, Nakaso K, Nakashima K. Increased plasma substance P and CGRP levels, and high ACE activity in migraineurs during headache-free periods. *Pain* 2007;128:209-214.
259. Piane M, Lulli P, Farinelli I, et al. Genetics of migraine and pharmacogenomics: some considerations. *J Headache Pain* 2007;8:334-339.
260. Tfelt-Hansen P, Broesen K. Pharmacogenomics and migraine: possible implications. *J Headache Pain* 2008;9:13-18.
261. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988;16:1215.
262. Goode EL, Fridley BL, Sun Z, et al. Comparison of tagging single-nucleotide polymorphism methods in association analyses. *BMC Proc* 2007;1 Suppl 1:S6.
263. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005;21:263-265.
264. Mhlanga MM, Malmberg L. Using molecular beacons to detect single-nucleotide polymorphisms with real-time PCR. *Methods* 2001;25:463-471.
265. Tan W, Fang X, Li J, Liu X. Molecular beacons: a novel DNA probe for nucleic acid and protein studies. *Chemistry* 2000;6:1107-1111.
266. Conde L, Vaquerizas JM, Dopazo H, et al. PupaSuite: finding functional single nucleotide polymorphisms for large-scale genotyping purposes. *Nucleic Acids Res* 2006;34:W621-625.
267. Zhang J, Yu KF. What's the relative risk? A method of correcting the odds ratio in cohort studies of common outcomes. *Jama* 1998;280:1690-1691.

-
268. Mantel N, Haenszel W. Statistical aspects of the analysis of data from retrospective studies of disease. *J Natl Cancer Inst* 1959;22:719-748.
269. Breslow NE, Day NE. Statistical methods in cancer research. Volume I - The analysis of case-control studies. IARC Sci Publ 1980;5:338.
270. Altman DG, Bland JM. Interaction revisited: the difference between two estimates. *Bmj* 2003;326:219.
271. Ottman R, Hong S, Lipton RB. Validity of family history data on severe headache and migraine. *Neurology* 1993;43:1954-1960.
272. Russell MB, Fenger K, Olesen J. The family history of migraine. Direct versus indirect information. *Cephalalgia* 1996;16:156-160.
273. Qin ZS, Niu T, Liu JS. Partition-ligation-expectation-maximization algorithm for haplotype inference with single-nucleotide polymorphisms. *Am J Hum Genet* 2002;71:1242-1247.
274. Haut SR, Bigal ME, Lipton RB. Chronic disorders with episodic manifestations: focus on epilepsy and migraine. *Lancet Neurol* 2006;5:148-157.
275. Pereira Monteiro JM, and J.R.Barros. Familial Incidence of Primary Headaches in a General Population. In: Olesen J, and M-G. Bousser, ed. *Genetics of Headache Disorders*. Philadelphia: Lippincott-Williams & Wilkins, 2000: 37-40.
276. Wessman M, Kaunisto MA, Kallela M, Palotie A. The molecular genetics of migraine. *Ann Med* 2004;36:462-473.
277. Weiss KM, Chakraborty R, Majumder PP, Smouse PE. Problems in the assessment of relative risk of chronic disease among biological relatives of affected individuals. *J Chronic Dis* 1982;35:539-551.
278. Russell MB. Genetics in primary headaches. *J Headache Pain* 2007;8:190-195.
279. Headache Classification Committee of the International Headache Society. Classification and diagnostic criteria for headache disorders, cranial neuralgias and facial pain. *Cephalalgia* 1988;8:1-96.
280. Russell MB, Rasmussen BK, Fenger K, Olesen J. Migraine without aura and migraine with aura are distinct clinical entities: a study of four hundred and eighty-four male and female migraineurs from the general population. *Cephalalgia* 1996;16:239-245.
281. Hemminki K, Sundquist K, Li X. Familial risks for main neurological diseases in siblings based on hospitalizations in Sweden. *Twin Res Hum Genet* 2006;9:580-586.
282. Mochi M, Sangiorgi S, Cortelli P, et al. Testing models for genetic determination in migraine. *Cephalalgia* 1993;13:389-394.
283. Breslow N, Day N. Statistical methods in cancer research. In: Breslow N, Day N, eds. *Analysis of case-control studies*. Lyon, France: International Agency for Research on Cancer, 1980: 142-146.
284. Seldin MF, Shigeta R, Villoslada P, et al. European population substructure: clustering of northern and southern populations. *PLoS Genet* 2006;2:e143.
285. Stewart WF, Staffa J, Lipton RB, Ottman R. Familial risk of migraine: a population-based study. *Ann Neurol* 1997;41:166-172.

286. Russell MB, Olesen J. Migrainous disorder and its relation to migraine without aura and migraine with aura. A genetic epidemiological study. *Cephalalgia* 1996;16:431-435.
287. Khoury MJ, Beaty TH, Liang KY. Can familial aggregation of disease be explained by familial aggregation of environmental risk factors? *Am J Epidemiol* 1988;127:674-683.
288. Lorenzo Bermejo J, Hemminki K. Familial lung cancer and aggregation of smoking habits: a simulation of the effect of shared environmental factors on the familial risk of cancer. *Cancer Epidemiol Biomarkers Prev* 2005;14:1738-1740.
289. Lemos C, Castro MJ, Barros J, et al. Familial Clustering of Migraine: Further Evidence From a Portuguese Study. *Headache* 2009;49:404-411.
290. Landis JR, Koch GG. The measurement of observer agreement for categorical data. *Biometrics* 1977;33:159-174.
291. Fogelson DL, Nuechterlein KH, Asarnow RF, Payne DL, Subotnik KL. Validity of the family history method for diagnosing schizophrenia, schizophrenia-related psychoses, and schizophrenia-spectrum personality disorders in first-degree relatives of schizophrenia probands. *Schizophr Res* 2004;68:309-317.
292. Rannala B. Finding genes influencing susceptibility to complex diseases in the post-genome era. *Am J Pharmacogenomics* 2001;1:203-221.
293. Lemos C, Castro MJ, Barros J, et al. Familial clustering of migraine: further evidence from a Portuguese study. *Headache* 2009;49:404-411.
294. Aloisi AM. Gonadal hormones and sex differences in pain reactivity. *Clin J Pain* 2003;19:168-174.
295. Low NC, Cui L, Merikangas KR. Sex differences in the transmission of migraine. *Cephalalgia* 2007;27:935-942.
296. Boles RG, Gardner A. Sex ratios and mitochondrial genetics in migraine. *Cephalalgia* 2008;28:1001-1002; author reply 1002.
297. Sparaco M, Feleppa M, Lipton RB, Rapoport AM, Bigal ME. Mitochondrial dysfunction and migraine: evidence and hypotheses. *Cephalalgia* 2006;26:361-372.
298. Nyholt DR, Dawkins JL, Brimage PJ, Goadsby PJ, Nicholson GA, Griffiths LR. Evidence for an X-linked genetic component in familial typical migraine. *Hum Mol Genet* 1998;7:459-463.
299. Wang XP, Liu JM, Zhao YB. Migraine: sex-influenced trait model? *Med Hypotheses* 2008;71:14-21.
300. Kidd KK, Spence MA. Genetic analyses of pyloric stenosis suggesting a specific maternal effect. *J Med Genet* 1976;13:290-294.
301. Cairns BE, Gazerani P. Sex-related differences in pain. *Maturitas* 2009;63:292-6
302. Puri V, Puri S, Svojanovsky SR, et al. Effects of oestrogen on trigeminal ganglia in culture: implications for hormonal effects on migraine. *Cephalalgia* 2006;26:33-42.
303. Brennan KC, Romero Reyes M, Lopez Valdes HE, Arnold AP, Charles AC. Reduced threshold for cortical spreading depression in female mice. *Ann Neurol* 2007;61:603-606.
304. Beghi E. Methodology of studies on the epidemiology of headache. *Neurol Sci* 2004;25 Suppl 3:S70-73.

-
305. Lima MM, Padula NA, Santos LC, Oliveira LD, Agapejev S, Padovani C. Critical analysis of the international classification of headache disorders diagnostic criteria (ICHD I-1988) and (ICHD II-2004), for migraine in children and adolescents. *Cephalalgia* 2005;25:1042-1047.
306. The International Classification of Headache Disorders: 2nd edition. *Cephalalgia* 2004;24 Suppl 1:9-160.
307. Eriksen MK, Thomsen LL, Olesen J. Sensitivity and specificity of the new international diagnostic criteria for migraine with aura. *J Neurol Neurosurg Psychiatry* 2005;76:212-217.
308. Zebenholzer K, Wober C, Kienbacher C, Wober-Bingol C. Migrainous disorder and headache of the tension-type not fulfilling the criteria: a follow-up study in children and adolescents. *Cephalalgia* 2000;20:611-616.
309. Pfaffenrath V, Fendrich K, Vennemann M, et al. Regional variations in the prevalence of migraine and tension-type headache applying the new IHS criteria: the German DMKG Headache Study. *Cephalalgia* 2008; 29:48-57.
310. Messinger HB, Spierings EL, Vincent AJ. Overlap of migraine and tension-type headache in the International Headache Society classification. *Cephalalgia* 1991;11:233-237.
311. Patel NV, Bigal ME, Kolodner KB, Leotta C, Lafata JE, Lipton RB. Prevalence and impact of migraine and probable migraine in a health plan. *Neurology* 2004;63:1432-1438.
312. Peatfield R. A revised classification of headache disorders. *BMJ* 2004;328:119-120.
313. Rasmussen BK, Jensen R, Olesen J. Questionnaire versus clinical interview in the diagnosis of headache. *Headache* 1991;31:290-295.
314. Manzoni GC, Torelli P. Headache classification: criticism and suggestions. *Neurol Sci* 2004;25 Suppl 3:S67-69.
315. Sancisi E, Cevoli S, Pierangeli G, et al. Application of ICHD-II and revised diagnostic criteria to patients with chronic daily headache. *Neurol Sci* 2007;28:2-8.
316. Corominas R, Ribases M, Cuenca-Leon E, et al. Contribution of syntaxin 1A to the genetic susceptibility to migraine: a case-control association study in the Spanish population. *Neurosci Lett* 2009;455:105-109.
317. Ferrari MD, Roon KI, Lipton RB, Goadsby PJ. Oral triptans (serotonin 5-HT(1B/1D) agonists) in acute migraine treatment: a meta-analysis of 53 trials. *Lancet* 2001;358:1668-1675.
318. Nakamura K, Anitha A, Yamada K, et al. Genetic and expression analyses reveal elevated expression of syntaxin 1A (STX1A) in high functioning autism. *Int J Neuropsychopharmacol* 2008;11:1073-1084.
319. Fan HP, Fan FJ, Bao L, Pei G. SNAP-25/syntaxin 1A complex functionally modulates neurotransmitter gamma-aminobutyric acid reuptake. *J Biol Chem* 2006;281:28174-28184.
320. Meffert MK, Calakos NC, Scheller RH, Schulman H. Nitric oxide modulates synaptic vesicle docking fusion reactions. *Neuron* 1996;16:1229-1236.
321. Olesen J. The role of nitric oxide (NO) in migraine, tension-type headache and cluster headache. *Pharmacol Ther* 2008;120:157-171.

322. Jarvis SE, Zamponi GW. Interactions between presynaptic Ca²⁺ channels, cytoplasmic messengers and proteins of the synaptic vesicle release complex. *Trends Pharmacol Sci* 2001;22:519-525.
323. Kaminsky Z, Wang SC, Petronis A. Complex disease, gender and epigenetics. *Ann Med* 2006;38:530-544.
324. Adeyemo A, Rotimi C. Genetic Variants Associated with Complex Human Diseases Show Wide Variation across Multiple Populations. *Public Health Genomics* 2009.
325. Keith RK, Poage RE, Yokoyama CT, Catterall WA, Meriney SD. Bidirectional modulation of transmitter release by calcium channel/syntaxin interactions in vivo. *J Neurosci* 2007;27:265-269.
326. Pereira L, Prata MJ, Amorim A. Diversity of mtDNA lineages in Portugal: not a genetic edge of European variation. *Ann Hum Genet* 2000;64:491-506.
327. Pereira R, Fondevila M, Philips C, Amorim A, Carracedo A, Gusmao L. Genetic characterization of 52 autosomal SNPs in the Portuguese population. *Forensic Science International* 2008;Genetics Supplement Series 1:358-360.
328. Beleza S, Gusmao L, Lopes A, et al. Micro-phylogeographic and demographic history of Portuguese male lineages. *Ann Hum Genet* 2006;70:181-194.
329. Rahman T, Baker M, Hall DH, Avery PJ, Keavney B. Common genetic variation in the type A endothelin-1 receptor is associated with ambulatory blood pressure: a family study. *J Hum Hypertens* 2008;22:282-288.
330. Moskowitz MA. Pathophysiology of headache--past and present. *Headache* 2007;47 Suppl 1:S58-63.
331. Vanmolkot FH, Van Bortel LM, de Hoon JN. Altered arterial function in migraine of recent onset. *Neurology* 2007;68:1563-1570.
332. May A, Gijsman HJ, Wallnofer A, Jones R, Diener HC, Ferrari MD. Endothelin antagonist bosentan blocks neurogenic inflammation, but is not effective in aborting migraine attacks. *Pain* 1996;67:375-378.
333. Edvinsson L. Neuronal signal substances as biomarkers of migraine. *Headache* 2006;46:1088-1094.
334. Mehrotra S, Gupta S, Chan KY, et al. Current and prospective pharmacological targets in relation to antimigraine action. *Naunyn Schmiedebergs Arch Pharmacol* 2008;378:371-394.
335. Menken M, Munsat TL, Toole JF. The global burden of disease study: implications for neurology. *Arch Neurol* 2000;57:418-420.
336. Mazaheri S, Hajilooi M, Rafiei A. The G-308A promoter variant of the tumor necrosis factor-alpha gene is associated with migraine without aura. *J Neurol* 2006;253:1589-1593.
337. Xu MQ, St Clair D, Ott J, Feng GY, He L. Brain-derived neurotrophic factor gene C-270T and Val66Met functional polymorphisms and risk of schizophrenia: a moderate-scale population-based study and meta-analysis. *Schizophr Res* 2007;91:6-13.
338. Lipsky RH, Marini AM. Brain-derived neurotrophic factor in neuronal survival and behavior-related plasticity. *Ann N Y Acad Sci* 2007;1122:130-143.

-
339. Thompson SW, Bennett DL, Kerr BJ, Bradbury EJ, McMahon SB. Brain-derived neurotrophic factor is an endogenous modulator of nociceptive responses in the spinal cord. *Proc Natl Acad Sci U S A* 1999;96:7714-7718.
340. Blandini F, Rinaldi L, Tassorelli C, et al. Peripheral levels of BDNF and NGF in primary headaches. *Cephalalgia* 2006;26:136-142.
341. Mannion RJ, Costigan M, Decosterd I, et al. Neurotrophins: peripherally and centrally acting modulators of tactile stimulus-induced inflammatory pain hypersensitivity. *Proc Natl Acad Sci U S A* 1999;96:9385-9390.
342. Buldyrev I, Tanner NM, Hsieh HY, Dodd EG, Nguyen LT, Balkowiec A. Calcitonin gene-related peptide enhances release of native brain-derived neurotrophic factor from trigeminal ganglion neurons. *J Neurochem* 2006;99:1338-1350.
343. Urbach A, Bruehl C, Witte OW. Microarray-based long-term detection of genes differentially expressed after cortical spreading depression. *Eur J Neurosci* 2006;24:841-856.
344. Durham PL. Calcitonin gene-related peptide (CGRP) and migraine. *Headache* 2006;46 Suppl 1:S3-8.
345. Lassen LH, Haderslev PA, Jacobsen VB, Iversen HK, Sperling B, Olesen J. CGRP may play a causative role in migraine. *Cephalalgia* 2002;22:54-61.
346. Greco R, Tassorelli C, Sandrini G, Di Bella P, Buscone S, Nappi G. Role of calcitonin gene-related peptide and substance P in different models of pain. *Cephalalgia* 2008;28:114-126.
347. Benemei S, Nicoletti P, Capone JA, Geppetti P. Pain pharmacology in migraine: focus on CGRP and CGRP receptors. *Neurol Sci* 2007;28 Suppl 2:S89-93.
348. Goadsby PJ, Edvinsson L. The trigeminovascular system and migraine: studies characterizing cerebrovascular and neuropeptide changes seen in humans and cats. *Ann Neurol* 1993;33:48-56.
349. Goadsby PJ, Edvinsson L, Ekman R. Vasoactive peptide release in the extracerebral circulation of humans during migraine headache. *Ann Neurol* 1990;28:183-187.
350. Tvedskov JF, Lipka K, Ashina M, Iversen HK, Schifter S, Olesen J. No increase of calcitonin gene-related peptide in jugular blood during migraine. *Ann Neurol* 2005;58:561-568.
351. Edvinsson L. Blockade of CGRP receptors in the intracranial vasculature: a new target in the treatment of headache. *Cephalalgia* 2004;24:611-622.
352. Marziniak M, Herzog AL, Mossner R, Sommer C. Investigation of the functional brain-derived neurotrophic factor gene variant Val66MET in migraine. *J Neural Transm* 2009.
353. Lin HY, Desmond R, Bridges SL, Jr., Soong SJ. Variable selection in logistic regression for detecting SNP-SNP interactions: the rheumatoid arthritis example. *Eur J Hum Genet* 2008;16:735-741.
354. Recober A, Russo AF. Calcitonin gene-related peptide: an update on the biology. *Curr Opin Neurol* 2009;22:241-246.
355. Storer RJ, Akerman S, Goadsby PJ. Calcitonin gene-related peptide (CGRP) modulates nociceptive trigeminovascular transmission in the cat. *Br J Pharmacol* 2004;142:1171-1181.

356. Recober A, Kuburas A, Zhang Z, Wemmie JA, Anderson MG, Russo AF. Role of calcitonin gene-related peptide in light-aversive behavior: implications for migraine. *J Neurosci* 2009;29:8798-8804.
357. Simonetti M, Giniatullin R, Fabbretti E. Mechanisms mediating the enhanced gene transcription of P2X3 receptor by calcitonin gene-related peptide in trigeminal sensory neurons. *J Biol Chem* 2008;283:18743-18752.
358. Angelucci F, Brene S, Mathe AA. BDNF in schizophrenia, depression and corresponding animal models. *Mol Psychiatry* 2005;10:345-352.
359. Montagna P. Migraine: a genetic disease? *Neurol Sci* 2008;29 Suppl 1:S47-51.
360. Sveinbjornsdottir S, Hicks AA, Jonsson T, et al. Familial aggregation of Parkinson's disease in Iceland. *N Engl J Med* 2000;343:1765-1770.
361. Roy MA, Walsh D, Kendler KS. Accuracies and inaccuracies of the family history method: a multivariate approach. *Acta Psychiatr Scand* 1996;93:224-234.
362. Farcone SV, Tsuang MT, Tsuang DW. *Genetics of Mental Disorders-A Guide for Students, Clinicians and Researchers*. New York: Guilford Publications, 1999.
363. Lin PI, McInnis MG, Potash JB, et al. Clinical correlates and familial aggregation of age at onset in bipolar disorder. *Am J Psychiatry* 2006;163:240-246.
364. Brooks JO, 3rd. A comment on age at onset as a subtype of Alzheimer disease. *Alzheimer Dis Assoc Disord* 1995;9 Suppl 1:S28-29.
365. Kendler KS, Karkowski-Shuman L, Walsh D. Age at onset in schizophrenia and risk of illness in relatives. Results from the Roscommon Family Study. *Br J Psychiatry* 1996;169:213-218.
366. Weissman MM, Wickramaratne P, Merikangas KR, et al. Onset of major depression in early adulthood. Increased familial loading and specificity. *Arch Gen Psychiatry* 1984;41:1136-1143.
367. Ottman R, Lee JH, Risch N, Hauser WA, Susser M. Clinical indicators of genetic susceptibility to epilepsy. *Epilepsia* 1996;37:353-361.
368. Kantarci OH, Spurkland A. Parent of origin in multiple sclerosis: understanding inheritance in complex neurologic diseases. *Neurology* 2008;71:786-787.
369. Herrera BM, Ramagopalan SV, Lincoln MR, et al. Parent-of-origin effects in MS: observations from avuncular pairs. *Neurology* 2008;71:799-803.
370. Kantarci OH, Barcellos LF, Atkinson EJ, et al. Men transmit MS more often to their children vs women: the Carter effect. *Neurology* 2006;67:305-310.
371. Bigal ME, Tepper SJ, Sheftell FD, Rapoport AM, Lipton RB. Chronic daily headache: correlation between the 2004 and the 1988 International Headache Society diagnostic criteria. *Headache* 2004;44:684-691.
372. Aulchenko YS, Hoppenbrouwers IA, Ramagopalan SV, et al. Genetic variation in the KIF1B locus influences susceptibility to multiple sclerosis. *Nat Genet* 2008;40:1402-1403.
373. Hakonarson H, Grant SF. Genome-wide association studies in type 1 diabetes, inflammatory bowel disease and other immune-mediated disorders. *Semin Immunol* 2009.
374. Thomson G. Significance levels in genome scans. *Adv Genet* 2001;42:475-486.

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375. Daly AK, Day CP. Candidate gene case-control association studies: advantages and potential pitfalls. *Br J Clin Pharmacol* 2001;52:489-499.
376. Kawashima K, Kishi T, Ikeda M, et al. No association between tagging SNPs of SNARE complex genes (STX1A, VAMP2 and SNAP25) and schizophrenia in a Japanese population. *Am J Med Genet B Neuropsychiatr Genet* 2008;147B:1327-1331.
377. Wong AH, Trakalo J, Likhodi O, et al. Association between schizophrenia and the syntaxin 1A gene. *Biol Psychiatry* 2004;56:24-29.
378. Brunoni AR, Lopes M, Fregni F. A systematic review and meta-analysis of clinical studies on major depression and BDNF levels: implications for the role of neuroplasticity in depression. *Int J Neuropsychopharmacol* 2008;11:1169-1180.
379. Chen L, Lawlor DA, Lewis SJ, et al. Genetic association study of BDNF in depression: finding from two cohort studies and a meta-analysis. *Am J Med Genet B Neuropsychiatr Genet* 2008;147B:814-821.
380. Frustaci A, Pozzi G, Gianfagna F, Manzoli L, Boccia S. Meta-analysis of the brain-derived neurotrophic factor gene (BDNF) Val66Met polymorphism in anxiety disorders and anxiety-related personality traits. *Neuropsychobiology* 2008;58:163-170.
381. Kawashima K, Ikeda M, Kishi T, et al. BDNF is not associated with schizophrenia: data from a Japanese population study and meta-analysis. *Schizophr Res* 2009;112:72-79.
382. Naoe Y, Shinkai T, Hori H, et al. No association between the brain-derived neurotrophic factor (BDNF) Val66Met polymorphism and schizophrenia in Asian populations: Evidence from a case-control study and meta-analysis. *Neurosci Lett* 2007;415:108-112.
383. Verhagen M, van der Meij A, van Deurzen PA, et al. Meta-analysis of the BDNF Val66Met polymorphism in major depressive disorder: effects of gender and ethnicity. *Mol Psychiatry* 2008; DOI: 10.1038/mp.2008.109
384. Corominas R, Sobrido MJ, Ribases M, et al. Association study of the serotonergic system in migraine in the spanish population. *Am J Med Genet B Neuropsychiatr Genet* 2009. DOI: 10.1002/ajmg.b.30972
385. Oterino A, Valle N, Pascual J, et al. Thymidylate synthase promoter tandem repeat and MTHFD1 R653Q polymorphisms modulate the risk for migraine conferred by the MTHFR T677 allele. *Brain Res Mol Brain Res* 2005;139:163-168.
386. Petryshen TL, Sabeti PC, Aldinger KA, et al. Population genetic study of the brain-derived neurotrophic factor (BDNF) gene. *Mol Psychiatry* 2009. DOI: 10.1038/mp.2009.24
387. Hunter DJ. Gene-environment interactions in human diseases. *Nat Rev Genet* 2005;6:287-298.
388. Di Rienzo A. Population genetics models of common diseases. *Curr Opin Genet Dev* 2006;16:630-636.
389. Havill LM, Dyer TD. Association mapping: methodologies, strategies, and issues. *Genet Epidemiol* 2005;29 Suppl 1:S77-85.
390. The International HapMap Project. *Nature* 2003;426:789-796.

391. Xu Z, Kaplan NL, Taylor JA. Tag SNP selection for candidate gene association studies using HapMap and gene resequencing data. *Eur J Hum Genet* 2007;15:1063-1070.
392. Bodmer W, Bonilla C. Common and rare variants in multifactorial susceptibility to common diseases. *Nat Genet* 2008;40:695-701.
393. Li B, Leal SM. Discovery of rare variants via sequencing: implications for the design of complex trait association studies. *PLoS Genet* 2009;5:e1000481.
394. Curtin K, Iles MM, Camp NJ. Identifying rarer genetic variants for common complex diseases: diseased versus neutral discovery panels. *Ann Hum Genet* 2009;73:54-60.
395. Gorlov IP, Gorlova OY, Sunyaev SR, Spitz MR, Amos CI. Shifting paradigm of association studies: value of rare single-nucleotide polymorphisms. *Am J Hum Genet* 2008;82:100-112.
396. Stucky CL, Gold MS, Zhang X. Mechanisms of pain. *Proc Natl Acad Sci U S A* 2001;98:11845-11846.
397. Fusco M, D'Andrea G, Micciche F, Stecca A, Bernardini D, Cananzi AL. Neurogenic inflammation in primary headaches. *Neurol Sci* 2003;24 Suppl 2:S61-64.
398. Longoni M, Ferrarese C. Inflammation and excitotoxicity: role in migraine pathogenesis. *Neurol Sci* 2006;27 Suppl 2:S107-110.
399. Richardson JD, Vasko MR. Cellular mechanisms of neurogenic inflammation. *J Pharmacol Exp Ther* 2002;302:839-845.

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