The evolving genetic and pathophysiological spectrum of migraine

Vries, B. de

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Genome-wide association study for migraine in a Dutch genetic isolate and meta-analysis with other population-based cohorts

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Abstract

Migraine is a common neurovascular disorder with a genetically complex pattern of inheritance. Linkage and association studies in the common forms of migraine have had limited success as they lacked robust replication of initial findings. Recent availability of high-throughput genotyping methodology makes genome-wide association studies (GWAS) feasible and provided a much better opportunity for identifying gene variants for complex diseases, including migraine. Here we first performed a GWAS on 330 migraine cases and 1,216 controls of the Erasmus Rucphen Family (ERF) study population, a Dutch genetically isolated population. The most significant association was observed with single nucleotide polymorphism (SNP) rs7200027 (*P*-value $1.34x10^{-7}$), an intergenic SNP on chromosome 16. Another 220 SNPs in the ERF GWAS had a *P*-value below 10^{-4}. A subsequent meta-analysis of migraine GWA data from five additional population-based cohorts of the Dutch-Icelandic (DICE) consortium indicated that rs7200027 only showed a significant signal in ERF. In fact, of the SNPs in the ERF GWAS with a *P*-value $<10^{-4}$, only rs11636768 reached that significance level in the meta-analysis. Rs11636768 resides in an intergenic region on chromosome 15 between the ATP/GTP binding protein-like 1 (*AGBL1*) gene and the non-protein coding RNA 52 (*NCRNA00052*) gene. In addition, the meta-analysis itself gave unique opportunities to search for migraine variants that surface in this large set of 10,980 individuals (2,446 cases and 8,534 controls). The strongest association in the meta-analysis was observed with rs9908234 (*P*-value $8.0x10^{-8}$) that is located in the nerve growth factor receptor (*NGFR*) gene. Notably, *NGFR* was previously considered a strong candidate for migraine due to its involvement in the trigeminal pain system. Future studies will have to show the relevance of these findings as GWAS and meta-analysis signals need to be replicated and the functionality of gene variants needs to be investigated with functional studies.

Introduction

Migraine is a common neurovascular brain disorder that is characterized by attacks of severe unilateral, often pulsating headache.[1] Two main types of migraine are distinguished based on the presence of an aura that can precede the headache: migraine with aura (MA) or without aura (MO). Although MA and MO have been considered distinct disease entities[2,3], it is now more and more accepted that they do present different expression forms of the same disease.[4-6]

Gene identification in the common forms of migraine has been notoriously difficult. Except for a genetic association with a single nucleotide polymorphism (SNP) in the 5',10'-methylenetetrahydrofolate reductase (*MTHFR*) gene, no genetic factors have been identified for common migraine; likely because most association studies were underpowered and therefore replicated poorly (for review see De Vries et al. 2009)[7]. Thus far, successes in migraine genetics

come primarily from studies in familial hemiplegic migraine (FHM), a rare monogenic subtype of MA that is considered a suitable model for common migraine.[8] Three genes were identified, all encoding ion transporters.[9-11] Functional research on FHM gene mutations indicated that abnormal neurotransmission of glutamatergic neurons in the cortex plays an important role in FHM and possibly the common forms of migraine.[12]

Here we performed a genome-wide association study (GWAS) which tests for association between a trait and hundreds of thousands of SNPs in the genome. Recently, the first GWAS of migraine was performed using clinic-based cohorts.[13] The present study is the first GWAS in migraine using population-based cohorts. We first performed a GWAS in the Erasmus Rucphen Family (ERF) study, a genetic isolate in the Southwest of the Netherlands, in which we identified 360 migraine cases and 617 non-headache controls.[14] Gene identification is expected to be easier in genetically isolated populations as, i) these populations have limited genetic heterogeneity due to a relatively small number of founders and genetic drift, and ii) environmental factors may be more homogeneous.[15] Subsequently, we performed a meta-analysis by combining migraine GWAS data of, in total, six population-based cohorts (2,446 cases and 8,534 controls) of the Dutch-Icelandic (DICE) consortium.

Materials and Methods

Design

Our study has a two-step design. First, we performed a GWAS on 330 migraine cases from the ERF population. In the second step, we performed a meta-analysis on 2,446 migraine cases from six different population-based cohorts (i.e., ERF, AGES, the Rotterdam study, NESDA, NTR1, and NTR2) of the DICE consortium. Details on the populations and genotyping are described in the following paragraphs.

Populations: Subjects and phenotypes

Six different population-based migraine cohorts (ERF, AGES, the Rotterdam study, NESDA, NTR1, and NTR2) were included in this study (Table 1). Of them, only the ERF population is a genetically isolated population and contains related individuals. In the ERF, AGES, the Rotterdam study, NESDA, NTR1, and NTR2; 330, 357, 349, 756, 378 and 276 migraine cases were included for this study, respectively. Migraine in all populations was diagnosed based on the ICHD-II criteria of the International Headache Society.[1] However, in the NESDA, NTR1 and NTR2 cohorts, migraine diagnoses were determined by means of latent class analysis (LCA) of IHS migraine symptoms.[16] The ERF cohort is a population-based cohort that was not selected based on specific phenotypes. In stead, the NESDA, NTR1 and NTR2 cohorts were initially collected to study major depressive

disorder (MDD). Therefore, MDD is enriched compared to the other cohorts. The AGES cohort and the Rotterdam study were initially collected to study risk factors for disease at older age. A detailed description on the populations and migraine case finding is provided below. All individual GWA studies were approved by local ethics committees.

Table 1 *Descriptives for the samples included in the meta-analysis.*

	ERF	AGES	NESDA	NTR1	NTR2	Rotterdam
Subjects						
Total N	1546	3219	1530	1593	1094	1998
N cases (♂, ♀)	330 (81, 249)	357 (71, 286)	756 (165, 591)	378 (69, 309)	276 (59, 217)	349 (79,270)
N controls (♂, ♀)	1216 (615, 601)	2862 (1281,1581)	774 (322, 452)	1215 (509, 706)	818 (396, 422)	1649 (805,844)
mean age & SD	48.4 (±14.6)	51.22 (±6.33)	42.9 (±12.5)	44.8 (±15.0)	48.6 (±14.4)	55.37 (±4.51)
Genotyping & Imputation						
platform	Illumina HumanHap300 HumanHap370 Affymetrix 250K Nsp array	Illumina 370CNV	Perlegen/ Affymetrix 600K	Perlegen/ Affymetrix 600K	Illumina Human660W-Quad BeadChip	Illumina Infinium II HumanHap550 version 3.0
software used	MACH	MACH 1.0.16	IMPUTE	IMPUTE	IMPUTE	MACH 1.0.15
reference set	HapMap CEU	HapMap CEU	HapMap CEU	HapMap CEU	HapMap CEU	HapMap CEU
NCBI build	36	36	36	36	36	36
hapmap release	22	22	22	22	24	22
# snps analyzed	2,135,034	2,408,991	2,432,125	2,431,993	2,542,087	2,450,030
Software for analysis imputed data	ProbABEL	ProbABEL	SNPTEST	SNPTEST	SNPTEST	ProbABEL

ERF

The ERF study is a family-based study in a genetically isolated population in the Southwest of the Netherlands. This young genetic isolate was founded in the mid-18th century. Minimal immigration and/or marriages occurred between surrounding settlements for social and religious reasons. The ERF population includes 3,465 individuals that are living descendants of 22 couples with at least six children baptized in the community church around 1850–1900. The subjects were unselected with respect to phenotypes. Details about the extensive genealogy and pedigree of the population are described elsewhere.[17]

Migraineurs were identified using a three-stage previously validated screening procedure.[18] The screening procedure in ERF was described by Stam et al.[14] In brief, all participants filled out a concise screening questionnaire on headache and aura symptoms, and those who screened positive also completed a detailed questionnaire. All participants who screened positive were

telephone-interviewed to clarify their clinical symptoms. Final diagnosis was always made after this telephone interview and in consultation with a neurologist (GMT) specialized in headache. The control group consisted of ERF participants negative for migraine based on the written questionnaire.

Data from 1,546 ERF participants; 330 migraineurs and 1,216 (non-migraine) controls were included in this study. Of the migraine cases, 249 (75%) were female and 81 (25%) were male; of the controls, 601 (49%) were female and 615 (51%) were male. The mean age of the study subjects was 48.4 years (SD = 14.6).

AGES

The Reykjavik Study is a population-based cohort study established in 1967 to prospectively study cardiovascular disease in Iceland. The cohort included a random sample of men and women born between 1907 and 1935 originating from Reykjavik. In 2002, the Reykjavik Study continued as the Age, Gene/Environment Susceptibility (AGES)-Reykjavik Study to examine risk factors, genetic susceptibility, and gene-environment interactions in relation to disease and disability in old age. Headache data were collected as part of the Reykjavik study. Details on the Reykjavik and AGES-Reykjavik Studies are described in detail elsewhere.[19-22]

For this study, a modified version of the 1988 International Headache Society (IHS) criteria was used (IHC. 1988). Subjects reporting headache at least once a month were asked whether their headaches were accompanied by any of the following migraine features: nausea/vomiting, unilateral location, photophobia, visual disturbance during or preceding headache, and unilateral numbness preceding headache. Individuals were defined as having migraine with aura if they had visual or sensory aura, or both. Subjects with at least 2 of the non-aura symptoms were classified as having migraine without aura. Details were described elsewhere.[23] In the present study, both migraine with and without aura were included as cases. The remaining individuals were considered controls.

The AGES-Reykjavik study contains 357 migraine cases; 286 were female (80%) and 71 were male (20%). The control group consisted of 1581 females and 281 males. Mean age of all study subjects was 51.03 years (SD = 6.37).

NESDA

The NESDA cohort consisted of 1,530 unrelated individuals from the Netherlands. Most of them had major depressive disorder (MDD) and were genotyped in the context of the Genetic Association Information Network (GAIN) MDD study.[24] For phenotypic assessment, the NESDA participants

underwent a 4-hour baseline assessment at one of seven clinic sites at the beginning of the study. This assessment included an interview on somatic health, functioning and health care use, and the administration of several written questionnaires. Migraine was assessed using a questionnaire that provided information on the symptoms listed in the ICHD-II criteria. Individuals screening positive for a screening question ('do you ever experience headache attacks, for instance migraine?') subsequently answered a set of more detailed questions about their headaches. This information was used to determine the presence of eight of the symptoms present in the ICHD-II criteria: moderate/severe pain intensity, aggravation by physical activity, pulsating quality, nausea/vomiting, photo-/phonophobia. The IHS migraine symptom variables were analyzed with Latent Class Analysis (as in Nyholt et al. 2005)[16] to determine each participant's affection status for migrainous headache. The program Latent Gold 4.0 (Statistical Innovations, Inc., Belmont, MA) was used to perform the LCA. Individuals belonging to LCA classes 2 and 3 (CL2 and CL3) were considered migraine patients; individuals of LCA classes 0 and 1 (CL0 and CL1) were used as controls. Previously it was shown that all individuals that were considered affected in the latent class analysis (i.e., CL2 or CL3) were diagnosed as affected by applying IHS migraine criteria.[5]

In the NESDA sample 1,383 subjects had MDD; 147 had a low risk for MDD. In the sample of the present study, we included 756 migraine cases (713 with MDD and 43 with a low risk for MDD) and 774 controls (670 with MDD and 104 with a low risk for MDD). In the case group, 591 (78%) were female and 165 (22%) were male. In the control group, 452 (58%) females and 322 (42%) males were present. The mean age of the study cohort was 42.9 years (SD = 12.5).

NTR1

The Netherlands Twin Registry (NTR) collects phenotype data in Dutch twins, their parents, siblings and partners. The migraine data were collected in the context of a longitudinal study on health, lifestyle and personality. The first NTR (i.e., NTR1) cohort was genotyped as part of the GAIN project, a GWA study originally designed to find genes for major depressive disorder.[24] The majority of the 1,481 subjects were selected for low risk of MDD; 112 subjects were MDD patients. Migraine was assessed with a questionnaire that provided information on the symptoms listed in the ICHD-II criteria. The headache questions were embedded in surveys that were held in the context of a longitudinal study on health, lifestyle and personality. The data used in this study were collected in 2002 and 2004. Both surveys included the same set of headache items. Data collection procedures are described in detail elsewhere.[25,26] When a participant answered the headache section in both surveys, the survey of 2004 was used. Final migraine diagnosis was based on the LCA method as described above for the NESDA cohort.

Migraine data were available for 1,593 individuals: 378 cases (56 with MDD and 322 with a low risk for MDD), and 1,215 controls (56 with MDD and 1,159 with a low risk for MDD). In the case group, 309 (82%) were female and 69 (18%) were male. In the control group, 706 (58%) females and 509 (42%) males present. The mean age of the study population was 44.8 years (SD = 15.0).

NTR2

The second cohort from the Netherlands Twin Registry (i.e., NTR2) was an unselected sample. For 1,094 individuals, migraine data were available. Migraine case finding in NTR2 was similar as for NTR1 and is described in the section above.

NTR2 contained 276 migraine cases, including 217 (79%) females and 59 (21%) males. The control group consisted of 818 controls, consisted of 422 (52%) females and 396 (48%) males. The mean age in this cohort was 48.6 years (SD = 14.4).

Rotterdam Study

This sample included participants of the Dutch Rotterdam Study, a prospective population-based cohort study among persons 55 years or older who were living in Ommoord, a well-defined district of Rotterdam.[27] The aim of the study was to investigate causes of frequent chronic diseases, with a focus on cardiovascular, neurologic, psychiatric, and ophthalmic diseases. The original cohort consisted of 7,983 participants and was expanded in 2000 with 3,011 participants and again in 2006 with another 3,919 persons who were 45 years of age or older. At study entry, all participants underwent a structural interview and a physical examination, which was repeated every 3-4 years. The migraine questionnaire was introduced into the core study protocol in 2006 (response rate of 64.8%). The migraine questionnaire was based on the ICHD-II criteria and was a modified questionnaire according to the questionnaire used in the GEM study.[18] The first question was “Have you ever experienced a severe headache that affected your daily activities?” If the answer was negative or if it was clearly indicated that the participants experienced a severe headache due to other causes, such as a tumor, sinusitis, stroke, trauma or meningitis, no further questions on headaches were asked. If the answer to the first question was positive, headache duration and headache frequency were asked. Next, if a person experienced headaches of which, 1) the duration was between 4 and 72 hours (untreated) or the participant did not know the answer to this question, because they always treated their headache attacks, and 2) the attack frequency was two or more attacks in a lifetime, details on the characteristics and symptoms of the headaches were asked. These included age of onset, unilateral location, pulsating quality, aggravation by daily activities, sensitivity to light and sound, nausea or vomiting. The frequency of the symptoms accompanying the headaches was assessed and defined as never, sometimes, half of the time and more than half of the time. In this group of participants, questions on

medication use were assessed. Furthermore, every participant was asked about aura symptoms and physician diagnosis, if they ever had a severe headache. If the participant experienced an aura or the physician had diagnosed migraine, questions on medication use were assessed. Participants whose duration of headache was unknown, because they always used medication to prevent or treat the attack, were considered migraineurs if they fulfilled the remaining ICHD-II criteria. Individuals who were not classified as migraineurs were regarded as controls.

For the present study, we used data from persons from the second cohort expansion (2006 to 2008) who completed the migraine questionnaire. Migraine data were available for 1,998 unrelated individuals, including 349 cases (270 females (77%) and 79 males (23 %)) and 1,649 controls (844 females (51%) and 805 males (49%)). The mean age of the study sample was 55.37 years (SD = 4.51).

Genotyping and imputation

Genotypes were already available for all cohorts, and were not generated for this meta-analysis which explains why different genotyping platforms were used. After imputation, for all populations approximately 2.5 million genotypes were available for GWA. All SNPs were located in autosomes. The meta-analysis was performed on 2,394,913 SNPs. Detailed information for the genotyping of the individual cohorts is provided below.

ERF

Genotyping was performed on several different platforms (Illumina HumanHap300, HumanHap370, Affymetrix 250K Nsp array). These sets were merged and genotypes for 2,585,854 SNPs were imputed to HapMap CEU, release 22, NCBI build 36 using the MACH program. Data were filtered for rare variants and linkage disequilibrium (LD). SNPs with a minor allele frequency (MAF) below 5% were excluded, and SNPs with an r^2 below 0.3 were excluded.

AGES

Genotyping was performed using the Illumina 370CNV platform. Genotypes for approximately 2.5 million SNPs were imputed using the MACH 1.0.16 program, using HapMap CEU as the reference set, based on NCBI build 36, HapMap release 22.

NTR1 and NESDA

Genotyping for the GAIN sample was conducted by Perlegen Sciences (Mountain View, CA, USA). The unfiltered dataset contained 599,156 unique SNPs. For the final analysis dataset, SNPs were required not to have gross mapping problems, ≥2 genotype disagreements in 40 duplicated samples, ≥2 Mendelian inheritance errors in 38 complete trio samples, MAF below 1%, or over 5% missing genotypes in either cases or controls. A total of 427,049 autosomal SNPs met these

criteria and were included in the analyses. Genotypes for approximately 2.5 million SNPs were imputed using the IMPUTE software, using the HapMap CEU data (release 22, NCBI build 36) (https://mathgen.stats.ox.ac.uk/impute/impute.html), as reference. For each SNP, an r^2 value was calculated using the QUICKTEST program (http://toby.freeshell.org/software/quicktest.shtml). SNPs were excluded if the Hardy-Weinberg equilibrium (HWE) test in controls produced a *P*-value $<10^{-6}$, the MAF was smaller than 1%, and the r^2 was smaller than 0.3, leaving 2,432,125 SNPs for analysis in the NESDA sample and 2,431,994 in the NTR1 sample.

NTR2

Genotyping for 657,366 was performed SNPs on the Human660W-Quad BeadChip. SNPs were excluded based on MAF below 1%, missing genotype rate above 5% or HWE *P*-value $<10^{-5}$. After quality control, 515,781 SNPs remained for further analysis. Genotypes of approximately 3.8 million SNPS were imputed with the IMPUTE program[28], using the HapMap CEU data (release 24, NCBI build 36), available from the IMPUTE website, as reference. Imputed SNPs were excluded if they had a MAF below 1% or an r^2 below 0.3, leaving 2,506,433 SNPs for analysis.

Rotterdam Study

Genotyping was performed using the Illumina Infinium II HumanHap550 chip, version 3.0. A total of 572,129 SNPs were genotyped. SNPs were excluded based on the following criteria: HWE *P*-value $<10^{-6}$, call rate <98% and a MAF <1%. The number of SNPs that survived quality control was 514,139. Genotypes were imputed for 2,543,888 SNPs, using the Hapmap CEU (build 36, rel. 22) as reference. Imputations were performed in MACH 1.0.15. SNPs were excluded if they had a MAF <0.01 or an $r^2 < 0.3$, leaving a total of 2,450,030 SNPs for analysis.

GWA analysis in ERF

For each of the 2,135,034 SNPs, logistic regression was performed, using an additive genetic model, while adjusting for age and sex. Uncertainty in the inferred genotype from the imputation was accounted for by utilizing the estimated genotype probabilities (implemented in ProbABEL). Data were filtered for rare variants and LD (MAF <0.05 were excluded; SNPs with r^2 below 0.3 were excluded). We accounted for relatedness between study participants; genomic control was applied with a study-specific λ factor being 1.17.

GWAS for meta-analysis

In each sample, a logistic regression association test was performed, with sex, age, and age^2 included as covariates, under an additive model. Age^2 was included to account for potential nonlinearity of the age effect, because the prevalence of migraine is lower in both younger and older individuals.[29] Uncertainty of imputation was taken into account in the analyses. The data of

AGES, ERF and the Rotterdam Study were analyzed with ProbABEL[30], NESDA, NTR1 and NTR2 were analyzed using SNPTEST.[28] The study specific genomic inflation factors (λ) were 1.002, 1.000, 1.006, 1.013, 1.000 and 1.021 for AGES, ERF, NESDA, NTR1, NTR2 and Rotterdam, respectively.

Next, a meta-analysis was performed on 10,890 individuals of the six population-based migraine cohorts using the METAL program (http://www.sph.umich.edu/csg/abecasis/metal/). Since different phenotype definitions were used in the different samples, the effect sizes are not directly comparable between studies. Therefore, a pooled Z-score approach was used. With the pooled Z-score method, an overall Z-score is calculated based on the summed Z-scores from the individual studies, weighted by each study's sample size. The weights are calculated as the square root of (Nstudy/Ntotal). The squared weights sum to one. The sign of the Z-score indicates the direction of effect. To ensure that meta-analysis results were indeed based on a substantial number of samples, hence SNPs (N=184,350 present for less than 70% of all participants) were excluded from the meta-analysis. This left a total of 2,394,913 SNPs for analysis. Annotation of GWAS results was performed with WGA viewer, version 1.26E.

Literature-based relationships

Literature-based relationships between genes in the specific gene sets and migraine were studied using the Anni text-mining program (Anni version 2.1)[31]. For each gene or disorder a concept profile was generated by the program. A concept profile is a summary of all concepts directly co-mentioned with the disease or gene concept (i.e. the main concept) in PubMed abstracts. The strength of association for each concept with the main concept is calculated using 2x2 contingency tables and the uncertainty coefficient. The association between two concept profiles is calculated using vector based matching (e.g. inner product score) over the concepts that the two profiles have in common.

Results

GWAS in the ERF population

Using the ProbABEL package, which is suitable for imputed genotypes, we performed a GWAS with 2,585,854 SNPs in ERF. The Q-Q plot for the GWA-analysis is shown in figure 1A. We corrected for residual inflation using genomic control using the genomic inflation factor λ, which is calculated as the median observed χ^2 divided by the median expected χ^2 based on 1 df, and was 1.17 for ERF. The genome-wide plot of probability values for individual SNPs against their genomic position shows that none of the SNPs reached the threshold for genome-wide significance (set to a *P*-value of 5.0×10^{-8}) (figure 1B). However, 22 SNPs showed suggestive associations with *P*-values below 10^{-5} (Table 2); 221 SNPs had *P*-values below 10^{-4}.

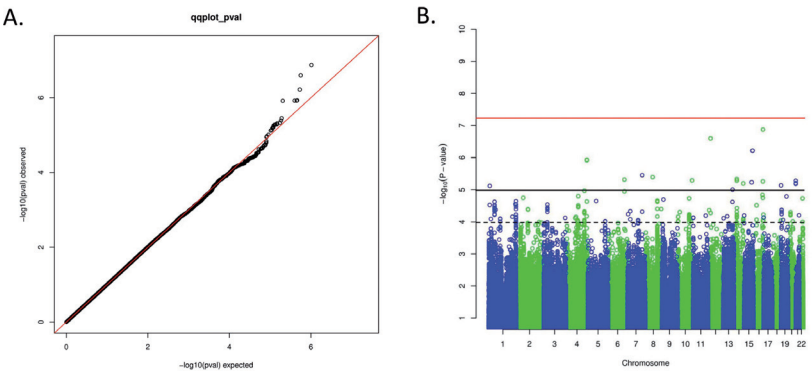

Figure 1 A *Quantile-Quantile (Q-Q) plot showing the expected (x-axis) and observed distribution of –log10 (P-value) in our GWAS analysis in the ERF population. The genomic factor (λ) was 1.17.* **B** *Genome-wide signal intensity (Manhattan) plot showing individual probability values per chromosome for the GWAS in ERF. Solid red line indicates the threshold for genome-wide significance (P-value = 5.0×10^{-8}), solid black line indicates the threshold for suggestive association (P-value = 1.0×10^{-5}), and the dotted black lines indicates a P-value threshold of 1.0×10^{-4}.*

The strongest associated SNP in ERF, rs7200027 (*P*-value 1.34×10^{-7}, OR 0.63 (CI 0.53-0.75), resides on chromosome 16. The minor allele is overrepresented in the controls compared to the cases, indicating a protective effect of the minor allele. Rs7200027 is an intergenic SNP located 50 Kb upstream of the *TMEM148* gene, which encodes a transmembrane protein with unknown function. The regional association plot shows that the SNPs surrounding rs7200027 are in relatively low LD and therefore show only limited to no association (figure 2). The second best SNP on the list is rs17379695 (*P*-value 2.52×10^{-7}) which is an intronic SNP located in the solute carrier organic anion transporter *SLCO1C1* gene on chromosome 12.

Table 2 *Overview of most significant SNPs in the ERF GWA study (with P-value of 1.0x10^{-5} or smaller)*

SNP	Chr	Position	Nearest gene	Gene description	Alleles*	MAF	Beta	SE	*P*-value	OR (CI)	SNPs in region **	Located in migraine linkage peaks
rs7200027	16	83957068	*TMEM148*	Transmembrane protein 148	A/G	0.36	-0.47	0,09	1.34x10^{-7}	0.63 (0.53-0.75)	1	No
rs17379695	12	20794062	*SLCO1C1*	Solute carrier organic anion transporter family, member 1C1	T/C	0.06	0.97	0.19	2.52x10^{-7}	2.64 (1.81-3.83)		No
rs8029074	15	92528658	*MCTP2*	Multiple C2 domains, transmembrane 2	T/C	0.06	-2.02	0.41	6.08x10^{-7}	0.13 (0.06-0.30)		No
rs11735224	4	182548055	AC093840.1	Hypothetical protein LOC100288373	T/C	0.28	-0.41	0.08	1.18x10^{-6}	0.66 (0.57-0.78)	3	No
rs6963861	7	129380575	*UBE2H*	Ubiquitin-conjugating enzyme E2H (UBC8 homolog, yeast)	C/T	0.34	0.38	0.08	3.55x10^{-6}	1.46 (1.25-1.71)		No
rs17280878	8	64210464	AC120042.1	Hypothetical LOC643763	G/A	0.08	0.63	0.14	4.08x10^{-6}	1.88 (1.42-2.47)		No
rs17212806	14	43521554	LOC390472	Similar to keratin 8	T/C	0.08	0.58	0.13	4.66x10^{-6}	1.79 (1.38-2.30)		Yes (Sorogna et al. 2003)
rs2184359	6	143280911	*HIVEP2*	Human immunodeficiency virus type I enhancer binding protein 2	C/T	0.09	0.58	0.13	4.86x10^{-6}	1.79 (1.38-2.30)		No
rs1040150	10	126062815	*OAT*	Ornithine aminotransferase	T/C	0.07	0.71	0.15	5.16x10^{-6}	2.03 (1.52-2.73)		No
rs17212778	14	44439714	LOC390472	Similar to keratin 8	A/G	0.08	0.57	0.13	5.25x10^{-6}	1.77 (1.37-2.28)		No
rs2207843	21	15154158	LOC654338	Brain cytoplasmic RNA 1, pseudogene	C/A	0.09	0.91	0.20	5.29x10^{-6}	2.48 (1.68-3.68)	2	No
rs11636768	15	87695511	NCRNA00052	Non-protein coding RNA 52	G/A	0.19	0.55	0.12	5.90x10^{-6}	1.73 (1.37-2.19)		No
rs4906086	14	101707363	LOC100128373	Similar to AKT interacting protein	T/A	0.45	-0.33	0.07	6.38x10^{-6}	0.72 (0.63-0.82)		No
rs3760877	19	542672	*CDC34*	Cell division cycle 34 homolog (S. cerevisiae)	C/T	0.45	0.46	0.10	7.29x10^{-6}	1.58 (1.20-1.93)		Yes (Nyholt et al. 1998; Jones et al. 2001)
rs3010223	1	11372638	*UBIAD1*	UbiA prenyltransferase domain containing 1	A/G	0.28	-0.36	0.08	7.57x10^{-6}	0.70 (0.60-0.82)		No
rs9300671	13	102155537	*ITGBL1*	Integrin, beta-like 1 (with EGF-like repeat domains)	G/A	0.30	-0.36	0.08	9.81x10^{-6}	0.70 (0.60-0.82)		No

**Alleles: major allele/minor allele. MAF = minor allele frequency, SE = standard error, OR = odds ratio, CI = 95% confidence interval*
***Table shows an overview of the meta-analysis results, for all SNPs with a P-value of 10^{-5} or smaller, but when multiple SNPs within a 0.5 Mb region had P-values < 10^{-5}, the SNP with the best P-value is reported, and the number of SNPs with small p-values surrounding the best SNP is given.*

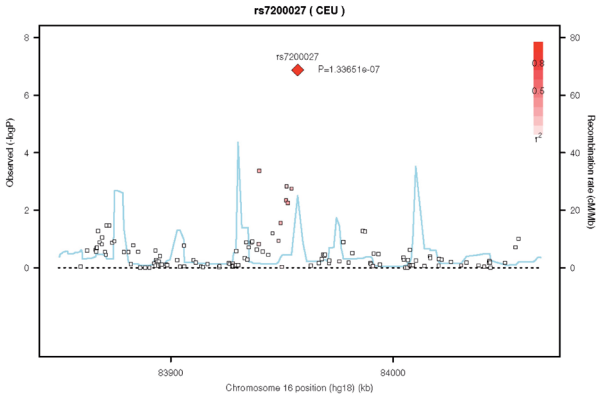

Figure 2 *Regional plot for associations in the region surrounding the top hit (rs720027). All SNPs (indicated as squares) are plotted with their –log (P-value) against their genomic position. Colour of the squares represents the degree of LD between the SNPs, with white representing no LD till dark red representing complete LD. Light blue line represents estimated recombination rates.*

Furthermore, we explored the 221 top SNPs (with *P*-values $<10^{-4}$) from the ERF GWAS using a text-mining program and by comparing the SNP locations to previously reported migraine linkage regions. The 221 top SNPs are located within or close to 84 different genes. Literature-based relationships between these genes and migraine were studied using the Anni text-mining program (Anni version 2.1)[31]. Neuronal cell adhesion molecule 1 (*NCAM1*), located on chromosome 11, was identified as the best migraine candidate gene according to literature-based connections with the concept ´migraine´. In the top list, two SNPs located upstream of *NCAM1* had a *P*-value $<10^{-4}$ (rs4937776 ($6.37x10^{-5}$) and rs4937786 ($6.39x10^{-5}$)). The text-mining program did not show any meaningful results for the 16 genes (for 7 a concept profile was present) in the 22 top SNPs with *P*-value $<10^{-5}$. None of the 22 top SNPs (*P*-value $<10^{-5}$) were located in known migraine linkage regions, although rs17212806 is located in relative proximity to the linkage region on chromosome 14q21.2-q22.3.[32] Of the 221 top SNPs, 46 SNPs were located in 8 known migraine linkage loci.

Most significant SNPs from the ERF GWAS in other population-based cohorts

As described above, the most significant association in the ERF GWAS was obtained with rs7200027. When performing the meta-analysis for all six population-based migraine cohorts the *P*-value of this SNP increased to $>10^{-4}$, indicating that the association was less clear in other populations. In fact, the association with rs7200027 was only observed in ERF. When comparing the top SNPs (*P*-value $<10^{-4}$) from the ERF GWAS and the meta-analysis, only rs11636768 surfaced in both studies ($P^{\text{ERF-GWAS}}$ $5.9x10^{-6}$ and $P^{\text{Meta-analysis}}$ $3.2x10^{-7}$). Importantly, the direction of the effect for this SNP was identical for all six populations, which adds weight to the association finding. Moreover, it was the second best SNP in the meta-analysis (Table 4). SNP rs11636768 is located on chromosome 15q25 between the *NCRNA00052* gene encoding non-protein coding RNA 52 and the

AGBL1 gene encoding ATP/GTP binding protein-like 1, but the SNP is located outside the known migraine linkage regions on chromosome 15.[33,34] Interestingly, approximately 0.5 Mb downstream of the rs11636768 SNP the *NTRK3* gene is located, which encodes a member of the neurotrophic tyrosine receptor kinase (NTRK) family.

FHM genes in ERF GWA and in the meta-analysis

This study also provides an excellent opportunity to investigate the role of FHM genes in large population-based migraine cohorts. We investigated whether SNPs in the three known FHM genes showed any signal in the ERF GWAS and meta-analysis data sets. Genotypic information was available for *CACNA1A* (202 SNPs in ERF, 241 SNPs in the meta-analysis), *ATP1A2* (19 SNPs in ERF, 20 SNPs in the meta-analysis), and *SCN1A* (97 SNPs in ERF, 99 SNPs in the meta-analysis) (Table 3). For both the ERF GWAS and the meta-analysis the most significant association was obtained with SNPs in the *ATP1A2* gene. In the ERF GWAS, 5 SNPs had a *P*-value <0.01 (all were intronic SNPs). Three of them were in close LD (r^2<0.8). Highest association in *ATP1A2* was obtained for SNP rs4656883 (*P*-value $1.4x10^{-3}$). In the meta-analysis, 5 SNPs had a *P*-value <0.001; highest association was found for rs2854248 (*P*-value $3.62x10^{-4}$). This SNP had a *P*-value of 0.009 in ERF. For the *CACNA1A* gene, only weak association signals were observed: in both data sets less than 10% of the *CACNA1A* SNPs showed *P*-values below 0.01; none were below 0.003. For the *SCN1A* gene, none of the SNPs showed any sign of association, neither in the GWA study nor in the meta-analysis.

Table 3A *Results in ERF GWAS for monogenic migraine genes*

FHM genes									
Gene Symbol	**Location**	**Most significant SNP in ERF GWAS**	**P-value**	**Beta**	**SNPs *P* < 0.001**	**SNPs *P* < 0.01**	**SNPs *P* < 0.05**	**SNPs *P* < 0.1**	**Total nr's of SNPs**
CACNA1A	19p13	rs7248281	0.0030	0.29	0	16	44	56	202
ATP1A2	1q21-q23	rs4656883	0.0014	0.46	0	5	7	7	19
SCN1A	2q24.3	rs13397210	0.1434	0.26	0	0	0	0	97

Table 3B *Results in meta-analysis for monogenic migraine genes*

FHM genes										
Gene Symbol	**Location**	**Most significant SNP in meta-analysis**	**Pooled Z-score**	**Pooled P-value**	**Direction of effect**	**SNPs *P*<0.001**	**SNPs *P*<0.01**	**SNPs *P*<0.05**	**SNPs *P*<0.1**	**Total number of SNPs**
CACNA1A	19p13	rs3764615	2.903	0.003695	-+++-+	0	9	17	37	241
ATP1A2	1q21-q23	rs2854248	3.566	0.0003618	++++++	3	4	5	8	20
SCN1A	2q24.3	rs12151636	2.142	0.03218	+?+-++	0	0	1	1	99

Previously identified common migraine genes; MTHFR and AEG-1

Next, we investigated whether previously identified common migraine gene variants showed a signal for association in our population-based cohorts. The first gene variant that we studied was the C677T SNP (rs1801133) in the *MTHFR* gene that surfaced in several candidate-gene-based association studies performed for migraine (for review see De Vries et al. 2009)[7]. However, in both the ERF GWAS and the meta-analysis, no significant association was found for this SNP. In addition, we testes the top SNP (rs1835740) that was identified in a GWAS of large clinic-based cohorts (testing in total 2,748 MA patients and 10,747 controls).[13] SNP rs1835740 resides close to the astrocyte elevated gene-1 (*AEG-1*) gene, which has relevance to the pathway identified in FHM. Neither the ERF GWAS nor the meta-analysis showed any sign of association for this specific SNP.

Meta-analysis-specific migraine variants

The meta-analysis comprising 2,446 migraine cases and 8,534 control individuals revealed a unique large data set with potential for identification of novel genetic migraine factors itself. The Q-Q plot for this meta-analysis is shown in figure 3A. The genomic inflation factor λ was 1.022. None of the SNPs reached a *P*-value $<5x10^{-8}$; the threshold for genome-wide significance (figure 3B). However, the threshold for suggestive association (*P*-value $<10^{-5}$) was observed for 32 SNPs (Table 4). The most significant result was obtained for SNP rs9908234 (*P*-value $8.00x10^{-8}$), which is located in the nerve growth factor receptor gene *NGFR*. Genotypic information was available for 17 SNPs in this gene, but none of the other SNPs were found associated with migraine, likely because none were in high LD with rs9908234 (figure 4). Notably, this SNP was genotyped only in the NTR1 and NESDA samples, but was imputed in the other samples.

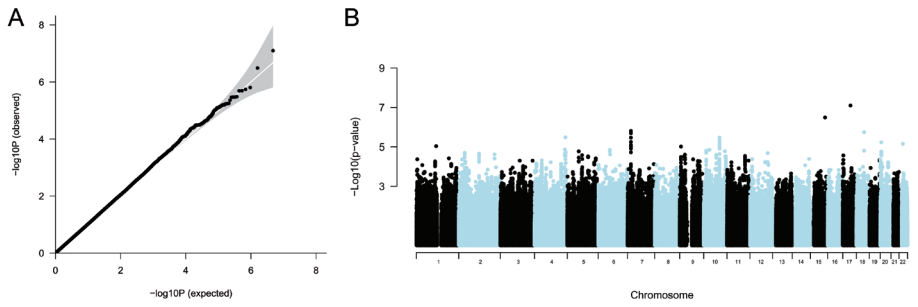

Figure 3 A. *Quantile-Quantile (Q-Q) plot showing the expected (x-axis) and observed distribution of –log10 (P-value) in our meta-analysis. The genomic factor (λ) for all six samples was 1.022. B. Genome-wide signal intensity (Manhattan) plot showing individual probability values per chromosome for the meta-analysis. Solid red line indicates the threshold for genome-wide significance (P-value = $5.0x10^{-8}$), solid black line indicates the threshold for highly suggestive association (P-value = $1.0x10^{-5}$), and the dotted black lines indicates the P-value threshold of $1.0x10^{-4}$.*

Table 4 *Most significant SNPs per region in the meta-analysis.*

SNP	Chr	Position (bp)	Type	Closest gene	Distance to gene (bp)	A1	A2	Freq A1	*P*-value	Direction of effect	N SNPs in region ($P<10^{-5}$)
rs9908234	17	44932347	intronic	*NGFR*	0	A	G	0.93	$8.0x10^{-08}$	------	1
rs11636768	15	85496515	intergenic	AC020687	321903	A	G	0.15	$3.23x10^{-07}$	++++?+	1
rs10275320	7	20148579	intronic	*MACC1*	0	A	G	0.15	$1.56x10^{-06}$	------	8
rs4939879	18	45399981	intergenic	*LIPG*	26705	A	G	0.47	$1.82x10^{-06}$	++++++	1
rs4861775	4	180553645	intergenic	AC017087.1	-709541	A	C	0.81	$3.28x10^{-06}$	------	1
rs986222	10	91920867	intergenic	AL139340.2	-7170	A	G	0.46	$3.37x10^{-06}$	++++++	16
rs6107848	20	6539116	intergenic	AL121911	82010	A	G	0.37	$5.90x10^{-06}$	+++++-	1
rs140174	22	22252983	intronic	*IGLL1*	0	A	G	0.75	$6.98x10^{-06}$	------	1
rs1146161	1	115460299	intergenic	AL109660.1	13497	A	C	0.18	$9.27x10^{-06}$	++++++	1
rs4742323	9	7276743	intergenic	*KDM4C*	111095	C	G	0.61	$9.70x10^{-06}$	------	1

Note. The best SNP per region is shown, as well as the number of SNPs in the region with a P-value <10^{-5}. The "Direction" column shows the direction of effect of the best SNP in the region, for each of the six samples, in the following order: AGES, ERF, NESDA, NTR1, NTR2, Rotterdam. A question mark indicates the SNP has not been tested for a particular sample, because it was removed during quality control. A1 is the effect allele in the meta-analysis, A2 is the non-effect allele. Positions are based on NCBI Build 36. The frequency of A1 was calculated as a weighted average across all samples.

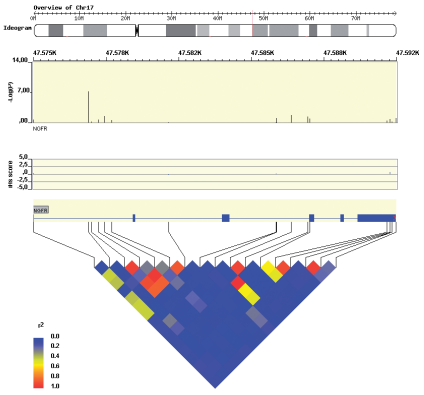

Figure 4 *Plot showing the LD structure between the SNPs tested in the NGFR gene. Rs9908234 was not in LD with any of the other SNPs tested.*

No less than 340 SNPs had *P*-values <10^{-4}, and were analyzed with text-mining program Anni that can prioritize genes for follow up studies. Of all 86 genes associated with these SNPs, *NGFR* was identified as the best candidate based on literature connections with the concept 'migraine'.

Discussion

ERF GWAS

Here we performed the first GWA study for migraine in population-based cohorts. Initially, we performed a GWAS in the ERF population, a genetically isolated population in the Southwest of the Netherlands, in which several loci and/or variants for complex disorders, such as metabolic syndrome[35] and type 2 Diabetes[36] were identified. The present study was designed to identify genetic variants for migraine using available genotypic data from 1,546 individuals of the ERF population. The most significant association with migraine was obtained for SNP rs7200027 located on chromosome 16q24 (*P*-value $1.34x10^{-7}$, OR 0.63 (CI 0.53-0.75)), indicating a protective effect for the minor allele of this SNP. The SNP is located 50 Kb upstream region of *TMEM148*, a gene with a thus far unknown function. The second-best SNP in the top list, rs17379695 (*P*-value $2.52x10^{-7}$) is an intronic SNP located in the *SLCO1C1* gene located on chromosome 12p12, which encodes the solute carrier member 1C1 from the organic anion transporter family. At present, it is not clear how this organic anion transporter that is predominantly expressed in the microvessels of the brain and the choroid plexus that transports brain-specific thyroid hormone into the brain, may cause migraine. Also, no association with a brain disorder is at present known with genetic variations in this gene. Notably, other solute carriers have previously been implicated in several brain disorders, such as episodic ataxia, epilepsy and mental retardation.[37-39] Of note, no migraine linkage peaks have been reported on chromosomes 12p12 and 16q24. Clearly, without robust replications and/or functional analysis of the identified variants, the relevance of these findings remains uncertain.

GWA meta-analysis

The availability of five additional population-based cohorts with GWA data gave unique opportunities for performing the first meta-analysis for migraine in population-based cohorts. This analysis was based on GWAS data from 10,890 individuals (2,446 cases, 8,534 controls) of European ancestry. The highest association in the meta-analysis was observed for SNP rs9908234 (*P*-value $8.00x10^{-8}$) in the nerve growth factor receptor (*NGFR*) gene on chromosome 17. NGFR is part of a large superfamily of tumor necrosis factor receptors.[40] Together with tyrosine kinase receptor A (TrkA), NGFR belongs to the two receptors that bind neural growth factor (NGF), which acts as a peripheral pain mediator, and is upregulated in many chronic pain conditions, particularly in inflamed tissues.[40] NGF can activate and sensitize primary afferent neurons that express TrkA, thereby producing hyperalgesia[41], which has some relevance to allodynia that is reported for many migraine patients.[42] NGFR is not directly linked to migraine in the literature, however there is evidence for the involvement of NGF in chronic headache disorders[43]; increased levels of NGF were observed in the cerebrospinal fluid (CSF) of patients with chronic daily

headache. In contrast, Blandini et al.[44] found reduced peripheral levels of NGF in migraineurs. Based on current knowledge, the hypothesis that NGFR mediates NGF-induced sensitization of trigeminal neurons is proposed as an explanation for migraine headache.

When we compared the top SNPs from the ERF GWAS data with those of the meta-analysis data, SNP rs11636768 is the only SNP that obtained a *P*-value $<10^{-4}$ in both studies. This SNP is located between the *NCRNA00052* gene encoding non-protein coding RNA 52 and the *AGBL1* gene that encodes the ATP/GTP binding protein-like 1. No other SNPS in this region showed a *P*-value $<10^{-4}$. The *NTRK3* gene, encoding a member of the neurotrophic tyrosine receptor kinase (NTRK) family, is located ~0.5 Mb upstream to this SNP. This gene is located in the same pathway as the *NGFR* gene and can be linked to migraine and/or pain pathophysiology. Future studies need to show whether this association can be replicated in other populations, and whether the affect allele of the rs11636768 SNP has an effect on expression of the *NTRK3* gene.

Migraine linkage studies; loci previously implicated in migraine

Except for the hemiparesis, migraine symptoms largely overlap between FHM and common migraine patients. Consequently, FHM genes are considered good candidate genes for common migraine. However, there is debate whether the same genes (i.e., ion transporters) play a role in common migraine. Recently, Nyholt et al studied 155 ion transport genes in the human genome in Finnish MA patients to investigate their involvement in common migraine.[45] There were no indications that the FHM genes played an important role in common migraine. Except for a few SNPs in the *CACNA1A* gene, none had nominal significant *P*-values. It was suggested that there still may be SNPs in these genes, or in other ion transporters, that are associated with migraine but with very low effect sizes and could therefore not be detected in this study due to the power. However, because of the much larger sample size of our present study, we had the opportunity to test SNPs in FHM genes. A possible role of the *ATP1A2* gene is most likely for the *ATP1A2* gene, similar to what was described in a genetic study by Todt et al.[46] Also several linkage studies supported a possible role of *ATP1A2* in common migraine.[47,16]

Migraine association studies; genes previously implicated in migraine

Many other candidate genes are tested and reported for migraine, however the majority of these genes were tested in small samples sizes with limited power and replication was lacking.[7] Of them the *MTHFR* gene, and more specifically the functional SNP (rs1801133), is the most promising genetic finding. This SNP is associated with MA in several studies[48,49], but did not replicate neither in the ERF GWA study nor in the meta-analysis. Also a very recent finding from a clinic-

based GWAS in MA, the SNP (rs1835740) that is located in the vicinity of the *AEG-1* gene[13], did not replicate in the present study. For rs1835740 this could be explained by the fact that we studied a population-based cohort of common migraine patients, not specified for MO or MA. Perhaps this *AEG-1* SNP is only associated with clinic-based migraine with aura.

Future studies will show whether our results can be replicated in other migraine populations, and what the true value of these GWA studies will be for improvement of our knowledge on migraine pathophysiology and in the end treatment of migraine patients.

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