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**Identifying common and rare variants in migraine
genetic predisposition**

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Genetics

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ABBREVIATIONS

- 5-HT_{1B} – 5-hydroxytryptamine (serotonin) receptor 1B
- 5-HT_{1D} – 5-hydroxytryptamine (serotonin) receptor 1D
- ATP1A2* – Gene that encodes the catalytic $\alpha 2$ subunit of a glial and neuronal sodium–potassium pump
- CACNA1A* – Gene that encodes P/Q voltage-dependent calcium channel
- Ca_v2.2 – N-type calcium channel α_{1B} subunit
- CI – Confidence interval
- CGRP – Calcitonin gene-related peptide receptor antagonists
- CSD – Cortical spreading depression
- DNA – Deoxyribonucleic acid
- EDTA – Ethylenediaminetetraacetic acid
- EGC – Estonian Genome Center
- ENH1 – Enigma homologue 1
- FHM – Familial hemiplegic migraine
- GABA – Gamma-aminobutyric acid
- GWAS – Genome-wide association study
- Hcy – Homocysteine
- ICHD – The International Classification of Headache Disorders, 3rd edition
- IGV – Integrative Genomics Viewer
- MA – Migraine with aura
- MAF – Minor allele frequency
- MO – Migraine without aura
- MTHFR* – Methylene tetrahydrofolate reductase gene
- Na_v1.7 – Voltage-gated sodium channel
- NSAIDs – Non-steroidal anti-inflammatory drugs
- OR – Odds ratio
- PCR – Polymerase chain reaction
- PCR-RFLP – PCR-restriction fragment length polymorphism
- PEPD – Paroxymal extreme pain disorder
- PKC ϵ – Protein kinase C epsilon
- PRKCE* – Protein kinase C, epsilon gene
- PDLIM5* – PDZ and LIM domain protein 5 gene

RR – Relative risk

SCN1A – Gene that encodes neuronal voltage-gated sodium channel Na_v1.1

SCN9A – Gene that encodes neuronal voltage gated sodium channel Na_v1.7

SNARE – SNAp (Soluble NSF Attachment Protein) REceptor

SNP – Single nucleotide polymorphism,

SNV – Single nucleotide variation

TCA – Tricyclic antidepressants

ABSTRACT

Identifying common and novel variants in migraine genetic predisposition

Migraine is an episodic brain disorder that is characterized by recurrent pain. Etiology of migraine is extremely complex; most likely caused by combination of genetic and environmental risk factors.

The aim of the thesis is to examine the role of *MTHFR* polymorphisms rs1801131 and rs1801133 as risk factors for pediatric migraine; also in migraine subtypes – migraine with aura (MA) and without aura (MO). Second part involved exome sequencing of two family trios to discover novel genetic risk factors for migraine.

Candidate gene study of *MTHFR* did not reveal any statistically significant results. Exome sequencing revealed three novel variants that could precipitate migraine. *PDLIM5*, *PRKCE* and *SCN9A* all affect voltage-gated channels. Mutations in those genes could increase neuronal hyperexcitability and neurotransmitter release, which in turn has been associated with pain and visual aura.

Migraine; MA; MO; exome sequencing. CERCS code: B790 Clinical genetics.

ABSTRAKT

Sagedased ning haruldased variandid migreeni geneetilises põhjuslikkuses

Migreen on episoodiline häire, mida iseloomustavad perioodilised peavalud. Migreeni etioloogia on äärmiselt kompleksne, suurima tõenäosusega põhjustatud geneetiliste ning keskkonna riskifaktorite koosmõjust.

Magistritöö eesmärgiks oli uurida *MTHFR* polümorfismide rs1801131 ja rs1801133 rolli migreeni tekkes; seda ka migreeni alagruppides – auraga migreen (MA) ning aurata migreen (MO). Teiseks eesmärgiks oli leida uusi geneetilisi riskifaktoreid, sekveneerides kahe perekonna-trio eksoomid.

*MTHFR*i kandidaatgeeni uuring ei näidanud statistiliselt olulist seost migreeniga. Eksoomi sekveneermine avaldas kolm varianti, mis võiksid osaleda auraga migreeni tekkes. *PDLIM5*, *PRKCE* kui ka *SCN9A* mõjutavad voltaaz-tundlikke kanaleid. Mutatsioonid nendes geenides võivad põhjustada liigset neuronaalset aktiivsust ja neurotransmitterite vabanemist, mida on varasemalt seostatud valu ja visuaalse auraga.

Migreen; MA; MO; eksoomi sekveneermine. CERCS kood: B790 Kliiniline geneetika.

INTRODUCTION

Migraine is complex disabling primary headache disorder, which affects ~15% of the world's population. The headache attacks vary in frequency, severity and duration, lasting from 4 hours to 72 hours. International Headache Society has classified two main subtypes for migraine, migraine with aura and migraine without aura. Migraine with aura affects 1/3 of migraineurs (migraine sufferers) and is described by visual and sensory disturbances an hour before headache attack. Migraine without aura comprises ~70% of the migraine population. The Global Burden of Disease Survey (2013) declared migraine as 6th cause for disability in the world, 4th in Estonia.

Twin studies have indicated that migraine has a genetic background as 50% of migraineurs have a first-degree relative also suffering from this disorder. A large number of candidate gene, linkage and genome-wide association studies have been done to identify causative gene/genes for migraine, as migraine has been stated to be an inherited disorder. However, only a small part of identified genes has been significantly and reproducibly associated with migraine.

The aims of this master thesis are:

- to investigate the role of two *MTHFR* polymorphisms rs1801131 (A1298C) and rs1801133 (C677T) as migraine risk factors;
- to examine the role of two previously mentioned polymorphisms in migraine subtypes;
- to identify novel mutations for migraine predisposition by sequencing whole-exomes of two family trios, one diagnosed with migraine with aura, another with migraine without aura.

The review of literature in this thesis focuses on migraine's clinical description, pathophysiology and etiology, especially on genetic risk factors. The experimental part concentrates on candidate gene analysis as well as whole-exome sequencing to identify genes that could possibly confer to migraine susceptibility.

1. REVIEW OF LITERATURE

1.1. What is migraine?

Migraine is a common neurological disorder that affects 10–20% of the world's population (Leonardi et al., 2005, Haut et al., 2006). It is mainly characterized by a headache attack that varies in frequency, intensity and duration, but may also be accompanied by nausea and/or vomiting, and photo- and phonophobia (Vries et al., 2009). It has a significant influence on one's quality of life, being a major reason for missing school- or workdays, for decreased social involvement, for increased complexity in performing general cognitive tasks, etc. (Victor et al., 2009, Sarrouilhe et al., 2014). According to the Global Burden of Disease Survey (2013), migraine has become the sixth-highest cause of disability worldwide and is rated as the third most prevalent disorder. In Estonia, migraine is the fourth (same rank in Latvia and Lithuania) cause for years lived with disability after a major depressive disorder, back pain and hearing loss (Global Burden of Disease Survey, 2013).

According to the International Classification of Headache Disorder (ICHD, 2013), migraine is subdivided into two main categories: migraine with aura and migraine without aura. Migraine with aura (MA) is described as a recurring headache preceded by transient focal neurological symptoms (aura). Migraine without aura (MO) is characterized by recurrent headache attacks lasting 4–72 hours (Bhaskar et al., 2013). MA and MO have been identified as two separate disorders (Russell et al., 2002, Ferrari et al., 2015).

The etiology of migraine is extremely complex and it is believed to be a combination of environmental and genetic risk factors (Lin et al., 2015).

1.1.1. Migraine phases

Migraine is divided into 4 phases: prodrome, aura, headache and postdrome. Most migraineurs (migraine sufferers) experience one or more phases, but experiencing any one certain phase is not necessary for the diagnosis of migraine. Aura phase does not occur in patients diagnosed with MO (Anonymous, 1995).

A majority of patients (~60%) with migraine also experience a premonitory phase, also known as the prodrome phase, which may occur in the 24–48 hours prior to the headache. This could be a warning sign for patients for the upcoming migraine episode. These symptoms include fatigue, cognitive change, irritability, depression

etc. (see **Figure 1** for more symptoms) (Waldman, 2011, ICHD, 2013, Burstein et al., 2015).

Approximately 40–60 minutes before the headache attack, patients diagnosed with MA experience transient focal neurological aura symptoms (Waldman, 2011, Ferrari et al., 2015). They are most frequently visual and occur in more than 90% of patients diagnosed with MA (Waldman, 2011, ICHD, 2013). Visual disturbances may include flashing lights (photopsia), zigzag castellations, objects distorted in shape and size, and partial loss of sight (scotoma) (see *Supplement 1*). These symptoms are all caused by the unusual activity in the parietal and occipital cortex (primary visual cortex) or the associated areas (Elkbom, 1993, ICHD, 2013).

The next most common symptoms are sensory disturbances. This aspect of aura may cause the “pins and needles” (tingling) sensation that moves slowly from the point source affecting mainly hands, face and/or tongue, but also may affect the whole body. It may also cause numbness (Waldman, 2011, ICHD, 2013).

Less frequent are speech disturbances. These include difficulty in finding words and/or understanding them, concentration difficulties, or confusion (ICHD, 2013).

Aura lasts less than 60 minutes (typically 20–30 minutes) (ICHD, 2013).

Migraine headache is usually quite severe, throbbing and is commonly, but not always, unilateral (it may fluctuate between sides or become generalized) (Kojić and Stojanović, 2013). It may occur during the day but it is not unusual to wake up with a headache. Pain is often accompanied with nausea or even vomiting, patients are sensitive to light and sound, and feel generally weak (MacGregor, 1999). This makes migraineurs seek out a dark room. Pain might decrease after vomiting and sleep typically terminates it (Elkbom, 1993). The headache lasts from 4 hours to 72 hours (Bhaskar et al., 2013).

Postdrome phase (resolution phase) is like a recovery phase. The headache is gone, but feeling normal could take hours or even days. Patients have illustrated it with the “headache hangover” feeling (Ng-Mak et al., 2011). Sufferers often believe that the symptoms are the result of the medication that was taken to treat migraine, but it could also be due to the migraine attack. Postdromal symptoms occur in the majority of patients and include lower or higher mood levels, physical weakness, fatigue and decreased concentration (Ng-Mak et al., 2011, Charles, 2013).

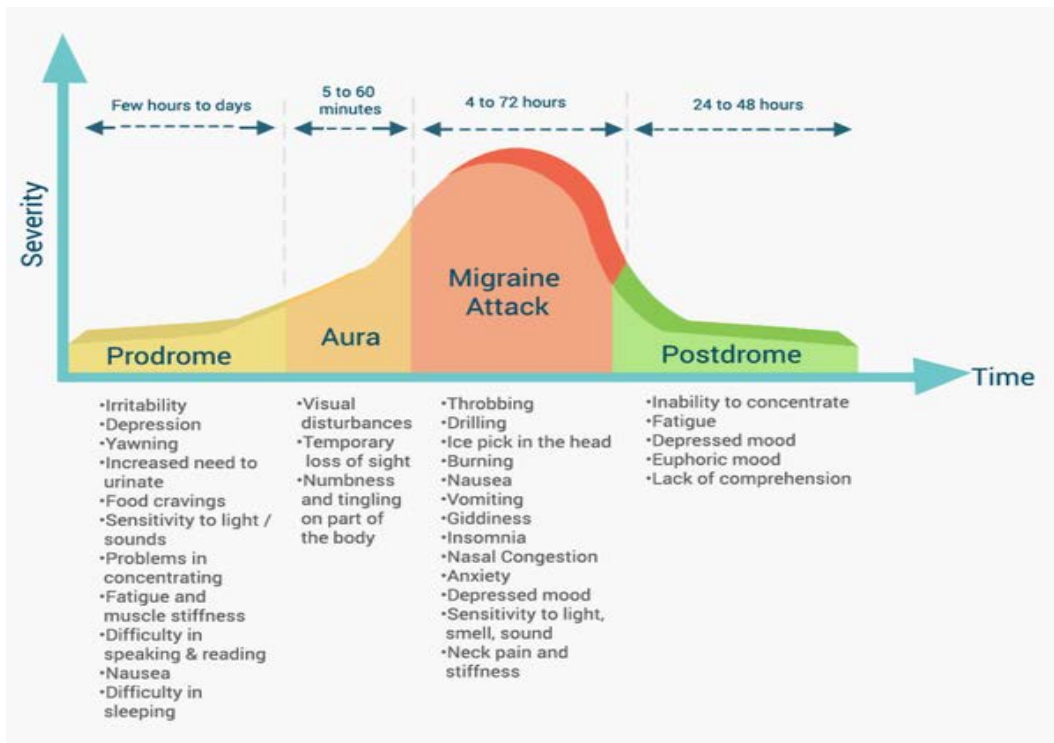


Figure 1. Four phases of migraine and symptoms during each phase (Figure obtained from migrainebuddy.com).

1.1.2. Migraine with aura (MA)

Migraine with aura, also known as classic migraine, is mainly characterized by transient neurological aura symptoms that typically occur before headache, but in rare cases also may not develop until the headache phase. This migraine subtype affects ~30% of all migraine patients (Vries et al., 2009, Goadsby, 2012, ICHD, 2013).

The main symptoms for aura are visual, sensory and speech disturbances. These symptoms last for 20–30 minutes and typically end right before headache (ICHD, 2013).

The duration of the headache episode in patients with MA is typically 6–8 hours (Zupping, 1998).

1.1.3. Migraine without aura (MO)

Migraine without aura, also known as common migraine, is characterized by a throbbing aching headache that may be accompanied by nausea, vomiting etc. Patients without aura do not experience focal neurologic disturbance prior to the headache (Rowland, 1995). This subtype accounts for two thirds of migraine patients (ICHD, 2013).

Headache normally starts during the day or right after waking up. During the following several hours, the pain increases. Pain is unilateral with a pulsating quality. Any head movement, noises and smells can increase headache (Zupping, 1988). Severe headache, together with nausea or insensitivity to external stimuli, make patients look for a dark space (Zupping, 1998). Pain lasts longer than in patients with MA, lasting from 4-72 hours (MacGregor, 1999).

1.1.3.1. ICHD-III diagnostic criteria for migraine

The first edition of the International Classification of Headache Disorders (ICHD) was published already in 1988 and consisted of 165 diagnoses (Levin, 2008). Currently, the 3rd edition of the ICHD (2013) is in use.

The diagnosis of migraine is based on the patient's recollection of their previous medical history, a review of symptoms, family history, and a neurological and physical examination by a physician. If all other disorders and diseases are ruled out, the doctor makes a diagnosis according to the ICHD (see **Table 1** for diagnosis criteria for MA and MO) (Levin, 2008).

Table 1. ICHD-III diagnostic criteria for both migraine subtypes (ICHD, 2013).

Diagnostic criteria for MA	Diagnostic criteria for MO
A. At least two attacks fulfilling criteria B and C	A. At least five attacks fulfilling criteria B–D
B. One or more of the fully reversible aura symptoms:	B. Headache attacks lasting 4–72 hours (untreated or unsuccessfully treated)
1. visual	C. Headache has at least two of the following four characteristics:
2. sensory	1. unilateral location
3. speech and/or language	2. pulsating quality
4. motor	3. moderate or severe pain intensity
5. brainstem	4. aggravation by or causing avoidance of routine physical activity (e.g. walking or climbing stairs)
6. retinal	D. During headache at least one of the following:
C. At least two of the following four characteristics:	1. nausea and/or vomiting
1. at least one aura symptom spreads gradually over 5 min and/or two or more symptoms occur in succession	2. photophobia and phonophobia
2. each individual aura symptom lasts 5–60 minutes	E. Not better accounted for by another ICHD-3 diagnosis
3. at least one aura symptom is unilateral	
4. the aura is accompanied, or followed within 60 minutes, by headache	
D. Not better accounted for by another ICHD-3 diagnosis, and transient ischaemic attack has been excluded	

1.2. Migraine triggers

A trigger is any factor that on exposure or withdrawal causes the development of migraine attack. Migraine can be triggered by several environmental factors. Activators can be certain types of food or beverages, weather, visual, olfactory or acoustic stimuli, but stress, hormones and sleep are the most common (Zupping 1988, Kojić and Stojanović, 2013).

Stress is the most common migraine-provoking factor. In addition to triggering migraine, stress can also make migraine attacks worse, make them longer in duration or make them more frequent. Some people have reported that they get a headache when stress is decreasing. This is called the “weekend headache”, as people have ended their stressful workweek and are relaxing. A definite reason explaining why stress causes migraine has not yet been confirmed, but it is believed that the release of peptides that make blood vessels expand and become inflamed might be the source (Nattero et al., 1989).

It has been stated that some patients have a migraine attack after eating or drinking certain things. For example, the most common dietary cause is red wine. Tannins and the phenolic flavonoid components of red wine have been linked to the trigger of migraine attacks through their interaction with the metabolism of certain monoamines, as well as their capacity to mobilize serotonin (Ekbom, 1993, Krymchantowski and da Cunha Jevoux, 2014). Another compound suggested as a possible migraine trigger is phenylethylamine, which is found in chocolate (Diamond and Marcus, 2008).

A handful of migraine patients have said their migraine is provoked by flickering lights, loud and persistent noise, or inhalation of specific fumes or odours (Ekbom, 1993).

In prepubescent children, the frequency of migraine is higher in boys than in girls. During and after puberty, more and more women start having migraine (Genzini et al., 2015). It has been reported that migraine is two to three times more common in women, during their reproductive years, than in men of a similar age, which could indicate that female sex hormones play a role in migraine. Hormones have an effect on frequency, severity and type of migraine. The fundamental mechanisms for sex hormones in migraine have so far eluded researchers (Bhaskar et al., 2013, Gasparini et al., 2013, Faria et al., 2015).

1.3. Heritability

Heritability has shown to play a big role in migraine: twin and family studies have demonstrated that ~50% of patients with migraine have a first-degree relative who also suffers from migraine (Ashina et al., 2012, Persico et al., 2015). Among patients with MA, the relative risk (RR) of a recurrence of MA in the family is 3.8, and among patients with MO, the RR is 1.9 (Russell et al., 1996).

Previous large population-based studies have concluded that both migraine subtypes are multifactorial inherited disorders, that are most probably caused by a combination of genetic and environmental risk factors (Ekbom, 1993, Russell et al., 2002, An et al., 2013). Svensson et al. (2002) reported that during childhood and adolescence, the occurrence of migraine is mainly caused by genetic factors, whereas in adulthood, environmental factors seem to have an increased effect. Heritability studies in population-based twin cohorts have indicated that the heritability varies between 0.34 and 0.57 (in the overall migraine population; heritability value of 1 implies complete regulation by genetic factors) (Mulder et al., 2003). The heritability in migraine subtypes is 0.65 for MA (Ulrich et al., 1999) and 0.61 for MO (Gervil et al., 1999).

1.4. Migraine in childhood

Migraine has a great negative influence on the physical and mental health of children, which also influences their school performance, family and social life, as well as their quality of life. Headaches, including migraine, have a much bigger destructive consequence on children than for example, back pain, abdominal pain, etc. (Rocha-Filho and Santos, 2014, Casucci et al., 2015).

The incidence of migraine has increased notably over the last decade, as the estimated prevalence of migraine in the pediatric population is approximately 8% (Rocha-Filho and Santos, 2014, Casucci et al., 2015). The incidence for this disorder accounts for 3% in preschool years, increasing to 4–11% in elementary school and reaching up to 23% during high school years. The mean age of onset is 7 years for boys and 11 years for girls (Genzini et al., 2015). Genzini et al. (2015) found in their study that prepubescent children were three times less likely to experience aura than adolescents, and the average age of reporting aura was 13.1 years.

Migraine headache in children and adolescents is often bilateral and frontotemporal. If pain is occipital, it requires immediate attention, as it is extremely

rare and could be due to something else (Winner et al., 2008, ICHD, 2013). Their headache attacks are less frequent and shorter in duration. Migraine is often underdiagnosed in children and adolescents (Winner et al., 2008).

1.5. Migraine pathophysiology

Migraine is considered to have a multifactorial basis. Although the pathogenesis has not yet been completely explained, due to the extensive number of trigger factors, and functional and biological abnormalities of migraine, countless concepts have been presented that could elucidate migraine pathophysiology to some extent (Mulder et al., 2003, Kojić and Stojanović, 2013).

The main mechanisms underlying migraine pathophysiology are hyperexcitability of the cerebral cortex, cortical spreading depression and neurovascular inflammation of meningeal blood vessels (Kojić and Stojanović, 2013).

1.5.1. Neuronal hyperexcitability

In a normally functioning neuron, neuronal excitability is caused by the movement of sodium (Na^+) and potassium (K^+) ions from one side of the membrane to the other. At resting membrane potential (-70 mV), voltage-gated channels for Na^+ and K^+ are closed. During the depolarization caused by the action potential (Figure 2), when the threshold has been reached (-55mV), the sodium channels are opened, but potassium channels are yet not completely opened, as they have not responded to the polarization. During the repolarization phase, the potassium channels are

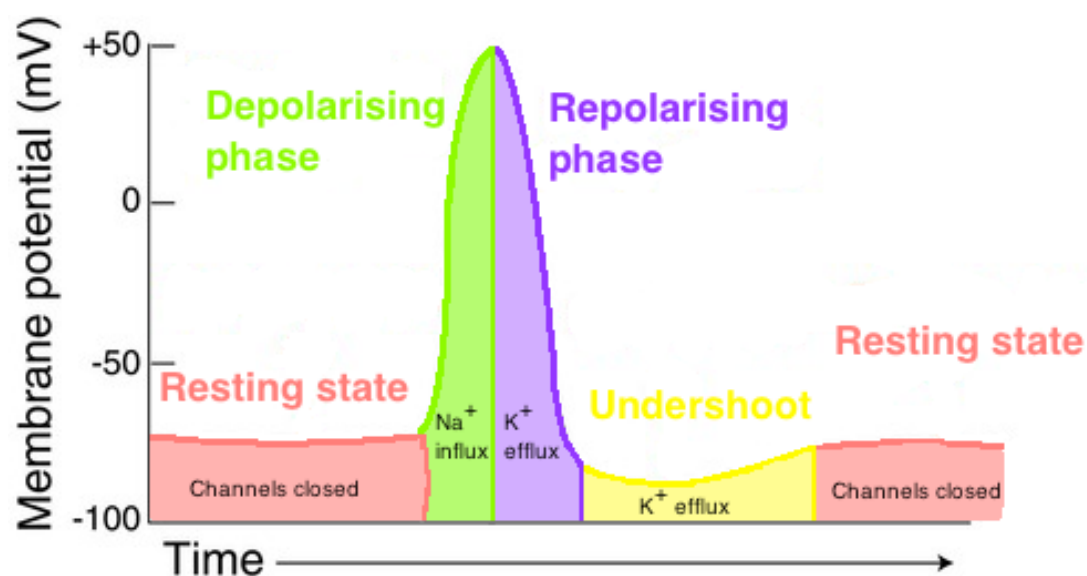


Figure 2. Action potential phases. The opening and closing of voltage-gated channels.

opened, whereas sodium channels are closed. In the refractory period, the sodium channels are closed and inactivated, while the potassium channels continue their work. Eventually the potassium channels also close and sodium channel inactivation is stopped. The membrane potential returns to its resting state (-70 mV) (Rhoades and Bell, 2009). This depolarization moves along the axon of the neuron until it reaches the axon terminal. At the terminal, there are voltage-gated Ca^{2+} channels that are important for Ca^{2+} ions to enter the neuron. As soon as the nerve impulse arrives at the presynaptic terminal, the charge across the membrane changes, which in turn opens the voltage-gated calcium channels for Ca^{2+} ions to enter the neuron. This forces vesicles full of neurotransmitters to bind with the presynaptic membrane, to be emptied into the synaptic cleft. Presynaptic Ca^{2+} channels are mainly specialized in neurotransmitter release, due to their attachment to synaptic vesicles (Yokoyama et al., 2004).

According to one possible theory of migraine pathophysiology, migraine and its symptoms may be caused by a hyperexcitable brain state (Borsook et al, 2012, Diamond et al., 2015). Excessive neuronal activity (hyperexcitability) is mainly caused by changes in the ion channels, receptors and signaling molecules (Diamond et al., 2015). Voltage-gated calcium and sodium channels, due to their function and synaptic transmission properties, are targets for several mutations that cause abnormal excitability in neurons. Genetic forms of chronic pain, epilepsy, cardiac arrhythmia, etc. are disorders/diseases that are caused by alterations in the genes coding ion channels (these disorders/diseases are collectively called channelopathies) (Catterall et al., 2008, Rhoades and Bell, 2009). Several studies in electrophysiology and magnetoencephalography have demonstrated that increased excitability of the brain cortex may precipitate migraine (Welch, 2005, Borsook et al., 2012).

For several decades it has been implied, that neuronal hyperexcitability is caused by genetic factors, as 50% of migraineurs also have a first-degree relative with this disorder. Unfortunately, investigations into those factors causing abnormal excitability have been quite unsuccessful (excluding familial hemiplegic migraine) (Welch, 2005, Rhoades and Bell, 2009).

1.5.2. Cortical spreading depression (CSD)

Cortical spreading depression (CSD), which is considered the neurological basis of visual aura, was first described by Aristides Leão in 1944 (Gasparini et al., 2013, Persico et al., 2015). CSD is characterized by slow self-propagating depolarization waves across the cerebral cortex that proceed at the velocity of 3–5 mm/min. It starts from the occipital cortex and propagates to the frontal cortex, after which pain is experienced (Ashina et al., 2012). CSD is acknowledged as the basis for visual aura (Gasparini et al., 2013).

CSD has been shown to activate and sensitize the trigeminovascular system, that in turn starts the neural, vascular and inflammatory events that cause pain. However, mechanisms by which CSD activates are not well known (Kojić and Stojanović, 2013), but it might be set in motion by migraine triggers that enhance cortical neuronal network excitability (Striessnig, 2005). Experiments have shown that CSD, in turn, may cause the disruption of ionic gradients – triggers the outflow of potassium ions, hydrogen ions and neurotransmitters such as glutamate into the extracellular space, and sodium and calcium ion inflow into the intracellular space (Gasparini et al., 2013, Yan and Dussor, 2014, Burstein et al., 2015). The flow of ions across the plasma membrane causes depolarization, followed by a prolonged inhibition of neuronal activity (Bhaskar et al., 2013, Kojić and Stojanović, 2013).

1.5.3. Genetics in migraine pathophysiology

According to countless family and twin studies, migraine is evidently identified as a genetic disorder. It is a complex, inherited disorder that comprises gene–gene, gene–environment, as well as epigenetic factors. Due to the heterogeneity of migraine expression and comorbid disorders, the investigation of potential genes/genomic areas is particularly complex as many gene variants, each with a certain amount of effect, together might have an impact on migraine susceptibility (Shyti et al., 2011, Bhaskar et al., 2013, Gasparini et al., 2013). Also, across different populations, the same genes do not account for the susceptibility of migraine (Gasparini et al., 2013) and it has been suggested that both genetic and environmental factors are determinants of MO, whereas MA is mainly determined by genetic factors (Ashina et al., 2012). Additionally, Russell and colleagues (1995) and Stewart and colleagues (1997) stated that migraine with aura has stronger genetic influences than migraine without aura.

So far, numerous linkage, candidate gene and genome-wide association studies (GWASs) have been used in family and case-control cohorts to identify a possible genetic component for migraine. Via linkage studies, three genes for familial hemiplegic migraine, that are all associated with ion transport or ion channel formation, have been isolated – *CACNA1A*, *SCN1A* and *ATP1A2* (Harrington et al., 2009). Mainly, genes involved in neurological, vascular, hormonal and mitochondrial functions have been examined to identify candidate genes involved in migraine pathological pathways. In the 1930s, Graham and Wolff suggested that the pain in migraine is triggered by dilated blood vessels (Ahn, 2012). One of the most investigated and cited genes is *MTHFR*, that causes accumulation of homocysteine, that in turn dilates cerebral vessels (Stuart et al., 2010).

Since the first migraine GWAS was done by Anttila et al. (2010), many more genes have been identified (see the list of several genes identified by GWAS in **Supplement 2**) for both migraine subtypes; for migraine with aura as well as for migraine without aura (Ashina et al., 2012). Unfortunately, only a handful of them have been significantly and reproducibly associated. In 2013, Gasparini et al. stated that GWAS is an excellent way to identify novel genes or genomic areas linked to disease phenotype; however it neither detects causal variants involved at the detected locus, nor addresses gene function.

1.5.3.1. Familial hemiplegic migraine (FHM)

Familial hemiplegic migraine (FHM) is a rare subtype of migraine with aura. In 1910, J.K. Clarke described this disorder in a family of 4 generations in which hemicranial pain and associated hemiparesis was seen. FHM attacks are characterized by the presence of hemiparesis or hemiplegia that may or may not be accompanied by other aura symptoms. These symptoms, as for classic migraine, last for a maximum of 60 minutes and are followed by a severe pulsatile headache (Joutel et al. 1994, Gasparini et al., 2013).

To date, three causative genes have been connected with FHM: *CACNA1A*, *ATP1A2*, *SCN1A*. All these genes encode proteins that are involved in ion transport or forming channels to organize the flow of ions from one side of the plasma membrane to the other (Gasparini et al., 2013).

The *CACNA1A* gene, located on the chromosome 19p13, encodes a protein that is a pore-forming $\alpha 1$ subunit of neuronal Ca_v2.1 (P/Q type) voltage-gated calcium

channels. This channel regulates Ca^{2+} ion inflow into excitable cells and is expressed in neuronal tissue. A defect in this gene causes a gain of slow calcium channel function. This also contributes to the cause of cerebellar ataxia and epilepsy (Harrington et al., 2009, Gasparini et al., 2013).

Another gene associated with FHM is *ATPIA2*, located on chromosome 1q23. This gene encodes a transporter protein that is a catalytic $\alpha 2$ subunit of a glial and neuronal sodium-potassium pump. Astrocytes are the main cells expressing this kind of channel. A mutation in the *ATPIA2* gene causes a loss of function in the transporter protein (Harrington et al., 2009, Russell and Ducros, 2011).

The third gene, located on chromosome 2q24, is *SCN1A*, which encodes a neuronal voltage-gated sodium channel. This channel is essential in the generation of action potential in neurons. A defective *SCN1A* gene causes sodium channel function gain. This defect has also been seen in patients with epilepsy syndrome and severe myoclonic epilepsy in infancy (Harrington et al., 2009, Russell and Ducros, 2011).

Although as scientific association has been found between these genes and FHM, they do not account for 100% of all FHM cases. This could indicate that there may be an additional mutation at another location, which could cause FHM (Harrington et al., 2009, Gasparini et al., 2013).

1.5.3.2. Methylene tetrahydrofolate reductase (*MTHFR*)

The methylene tetrahydrofolate reductase (*MTHFR*) gene, located on chromosome 1p36, encodes a protein of the same name, which converts 5,10-methylene tetrahydrofolate to 5-methyl tetrahydrofolate, which is a fundamental enzyme in the metabolism of folate (Liu et al., 2014). Folate, in turn, is needed for the conversion of homocysteine to methionine (**Figure 3**). The lack of dietary folate or decreased activity of methylene tetrahydrofolate reductase (*MTHFR*), which is caused by the common polymorphisms C677T and A1298C, is thought to cause accumulation of the sulfur-containing amino acid homocysteine ($\text{HSCH}_2\text{CH}_2\text{CH}[\text{NH}_2]\text{CO}_2\text{H}$) in blood plasma (Stuart et al., 2010, Liu et al., 2014). Patients carrying the homozygous variant of C677T, express only about 30% of the mean activity of the *MTHFR* enzyme, while the defective A1298C variant decreases

40% of the enzyme activity ¹. The C677T polymorphism has also been stated as a risk factor for various cancers, coronary heart disease, depression and ischemic stroke (Liu et al., 2014), as well as for migraine. *MTHFR* has been shown as a part of the genetic basis for abnormally increased homocysteine levels (homocysteinemia) (Lippi et al., 2014).

Elevated homocysteine plasma levels are a source of endothelial cell injury, spontaneous trigeminal cell firing, and alteration in the coagulant properties of blood. Vascular theory indicates that the pain in migraine is caused by the dilation of cerebral vessels together with inflammation in meninges that due to trigeminal cell firing (Berstein and Burstein, 2012).

Homocysteine has been implicated as part of the pathophysiology of several neurological disorders/diseases, such as stroke, Parkinson's disease, epilepsy, etc. (Obeid et al., 2008, Liu et al., 2014).

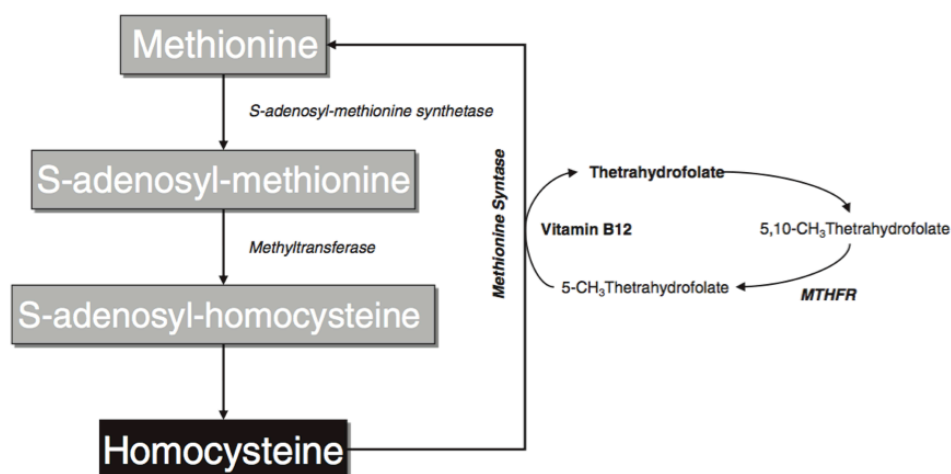


Figure 3. Metabolism of homocysteine. *MTHFR* – methylenetetrahydrofolate reductase. Figure adjusted from Lippi et al., 2014.

1.6. Treatment

Treatment for migraine is divided into acute (also known as abortive) and preventive (also known as prophylactic), stopping the evolving attack or stopping the onset of attack respectively (Sarrouilhe et al., 2014).

Acute treatments (**Table 2**) mainly include triptans, which specifically target serotonin, and ergotamine, which is vasoconstrictor of painfully dilated cranial arteries (Ekbom, 1993). Triptans work as 5-HT_{1B} and 5-HT_{1D} receptor agonists by

¹<http://www.kliinikum.ee/yhendlabor/images/stories/kasiraamat/HIJ/homotssteiin%20.pdf>

causing vasoconstriction of cerebral vessels and prohibiting the release of neuropeptides. The first triptan that was specifically developed for acute migraine therapy was sumatriptan (in tablets, injection kits and nasal spray), although an additional six triptans have been developed to this day (Sheikh and Mathew, 2012, Diener et al., 2015). In the presence of cardiovascular diseases, triptans are contraindicated (Ferrari et al., 2001).

In the last decade, another quite promising abortive drug group (calcitonin gene-related peptide (CGRP) receptor antagonists) has been developed. These drugs function by inhibiting pain and inflammatory neurotransmitter release in the brain and brainstem. CGRP receptor antagonism has a major benefit in its lack of vasoconstrictive effect, which means it could also be used in patients with cardiovascular diseases (Sherwood and Jones, 2011).

Migraineurs also use non-steroidal anti-inflammatory drugs, e.g. aspirin (Sarrouilhe et al., 2014).

Table 2. The diverse drug groups for the acute treatment of migraine.

<u>ABORTIVE DRUGS</u>	
Triptans - acts on serotonin 5-HT _{1B} and 5-HT _{1D} receptors on cranial blood vessels and inhibits inflammatory neuropeptide release	
sumatriptan	eletriptan
rizatriptan	almotriptan
zolmitriptan	frovatriptan
naratriptan	
Ergotamines - vasoconstrictor via 5-HT _{1B} receptor, inhibiting neurotransmission by 5-HT _{1D} receptors	
dihydroergotamine	ergotamine tartrate
Calcitonin gene-related peptide (CGRP) receptor antagonists - works on CGRP receptor	
olcegepant	telcagepant
Non-steroidal anti-inflammatory drugs (NSAIDs) - block the enzyme cyclooxygenase from synthesizing pain and inflammation-causing lipids and prostaglandins.	
aspirin	ibuprofen
naproxen	

Preventive treatments for migraine are used considerably less than they should be. Lipton and colleagues (2007) stated that more than one in four migraineurs are suitable for preventative therapy but a substantial portion of them have not received it. The purpose of this kind of treatment is to decrease the frequency and intensity of migraine (Sheikh and Mathew, 2012). Different types of preventive drugs are antiepileptic drugs, tricyclic antidepressants (TCAs), excitatory mechanism (glutamatergic neurotransmission, ion channel) inhibitors, etc. (See **Table 3** for drugs used for migraine prevention) (Rizzoli, 2013, Sarrouilhe et al., 2014). Valproate and

topiramate are the most used antiepileptic drugs for migraine prevention, as they influence the activity of Na⁺ and Ca²⁺ channels, GABA-A and glutamate receptors (Pelzer et al., 2013). The dosing can reach the amount used in epilepsy treatment but is usually lower for migraine. These drugs have serious side effects (e.g. depression, seizures, concentration difficulties etc.) (Rizzoli, 2013). Verapamil blocks L-type Ca²⁺-channels, but on higher doses can also work on P/Q-type channels. Effectiveness is often reported, but might also have quite severe side effects (hypotension, increased risk of heart failure, bradycardia etc). Another medication, which works on voltage-gated channels, is Lamotrigine. This medication inhibits glutamate release by blocking Na⁺ and N- and P/Q-type Ca²⁺ channels. This medication does not work on headaches, however has shown somewhat an improvement on frequency and duration of aura (Pelzer et al., 2013).

Table 3. The diverse drug groups used for the preventative treatment of migraine.

<u>PREVENTIVE DRUGS</u>	
Beta-Blockers - used for relaxing blood vessels, not clear how they prevent migraine	
propranolol timolol nadolol	atenolol metoprolol
Calcium channel agonists - calcium channel blockers reduce the constriction of blood vessels	
diltiazem nifedipine lamotrigine	nimodipine verapamil
Antiepileptics - not known how they work but thought to have an impact on neurotransmitters	
topiramate	valproate
Tricyclic antidepressants - have pain-relieving characteristics and may reduce duration and frequency	
amitriptyline	nortriptyline

2. EXPERIMENTAL PART

1.7. Aims of the study

The aims of the study are:

- to examine the role of two *MTHFR* polymorphisms (rs1801131 – A1298C and rs1801133 – C677T) as the risk factors for Estonian pediatric migraine population;
- to investigate the allele and genotype frequencies of both polymorphisms in migraine subtypes;
- to discover possible genetic risk factors for both migraine subtypes by sequencing the exomes of two family-based trios, one diagnosed with migraine with aura, another with migraine without aura.

1.8. Materials and methods

1.8.1. Genetic analysis of the *MTHFR* gene in migraine

1.8.1.1. Description of patient and control groups

In the study, 110 pediatric migraine patients (F=62, M=48) diagnosed according to the International Classification of Headache Disorders were enrolled. They were treated in the Tartu University Hospital Children's Clinic during the period 2011–2016. Of these patients, 46 were diagnosed with migraine with aura and 64 with migraine without aura. The age of the patients varied between 5–18 years, with a mean age of 12.5 ± 3.1 years. Migraine patients who experience or have experienced comorbid conditions like cardiovascular disorders (e.g. stroke) and non-migrainous headaches (e.g. tension headaches) were excluded from the study. Also, patients with secondary causes for migraine (e.g. post-head injury headache) were excluded.

The control samples were obtained from the Biobank of the Estonian Genome Center. The control group consisted of 220 randomly selected healthy individuals (F=124, M=96), without any kind of cerebrovascular diseases (e.g. stroke, hypertension) and migraine. The control group was sex-matched to the patient group (2:1). Also, the control groups for migraine with aura and without aura subgroups were randomly selected. The age of controls ranged from 18 to 45 years with an average of 30.1 ± 3.4 years. Genotyping for rs1801131 as well as for rs1801133 in the control sample was done with Illumina Human Omni 770K BeadChip in the Core

Laboratory of the Estonian Genome Center, University of Tartu, Estonia. Quality control and filtration was accomplished using IlluminaGenomeStudio 3.1 and PLINK 1.07 software (Purcell et al., 2007).

Migraine patients and gene donors gave informed consent to participate in this study, which was approved by the Research Ethics Committee of Tartu University (protocol nr: 233/M-19).

1.8.1.2. DNA extraction and genotyping of the sample group

Genomic DNA was extracted from EDTA-collected peripheral blood using the standard high-salt extraction method.

Genotyping of *MTHFR* polymorphisms rs1801131 and rs1801133 was done via PCR-RFLP analysis using *Hin*I and *Mbo*II restriction enzymes respectively. To identify the rs1801133 mutation in the *MTHFR* gene, the amplification of a 198 bp PCR fragment was done using primers: forward – 5'TGAAGGAGAAGGTGTCTGCGGGA3' and reverse – 5'AGGACGGTGCGGTGAGAGAGT3'. The *Hin*I restriction site causes the 198 bp amplicon to divide into 175 bp and 23 bp fragments. The modified protocol (Hanson et al., 2001) for genotyping the rs1801131 mutation was applied. For the detection of this *MTHFR* polymorphism, a 256 bp fragment was amplified using following primers: forward – 5'CTTCTACCTGAAGAGCAAGTC3' and reverse – 5'CATGTCCACAGCATGGAG3'. In carriers having 1298AA genotype, the digestion of the 256 bp fragment results in four fragments of 176 bp, 30 bp, 28 bp and 22 bp, whereas the 1298CC genotype gives three fragments of 204 bp, 30 bp and 22 bp.

Fragments were visualized on the 2.5% agarose gel in 1x TBE buffer (SeaKem® LE, Lonza) electrophoresis using ethidium bromide.

DNA extraction and genotyping was done at Tartu University Hospital, in the Genetics Center.

1.8.1.3. Statistical analysis

The genotype frequencies of both single nucleotide polymorphisms (SNPs) were examined to determine whether adherence to Hardy–Weinberg equilibrium was present. Statistical analysis for the allele association study was carried out using the software PLINK 1.07 (Purcell et al., 2007), and for genotypes using software R. The

odds ratios with 95% confidence intervals, Fisher exact test for p-values and chi-squared analysis were calculated using R and PLINK 1.07.

1.8.1.4. Results

A total of 110 migraine patients and 220 healthy controls were enrolled in the study. The aim of genotyping was to investigate the possibility of two *MTHFR* polymorphisms as causative mutations of migraine.

Both genotyped SNPs – rs1801131 and rs1801133 – were in Hardy–Weinberg equilibrium, in the patient group, in the control group, as well as in the combined group.

The allele and genotype frequencies of the studied SNPs for patients and controls are seen in **Table 4**, together with the corresponding p-values and odds ratios (OR) for minor allele (MAF) and genotype frequencies.

Table 4. The frequencies of alleles and genotypes for both *MTHFR* rs1801131 and rs1801133 SNPs in patient and control groups. MAF – minor allele frequency; CI – confidence interval.

<i>MTHFR</i> allele	Cases (n=110) MAF	Control group (n=220) MAF	Odds ratio (95% CI)	p-value
677T	0.300	0.296	1.02 (0.72–1.46)	0.90
1298C	0.341	0.277	1.35 (0.95–1.91)	0.09

<i>MTHFR</i> genotype	Cases (n=110) frequency	Control group (n=220) frequency	Odds ratio (95% CI)	p-value
677CC	0.472	0.495	0.95 (0.64-1.43)	0.839
677CT	0.454	0.427	1.06 (0.70-1.61)	0.832
677TT	0.073	0.077	0.94 (0.39-2.25)	1
1298AA	0.418	0.486	0.86 (0.57-1.30)	0.531
1298AC	0.481	0.450	1.07 (0.71-1.60)	0.757
1298CC	0.100	0.063	1.57 (0.69-3.58)	0.282

For the *MTHFR* rs1801131 and rs1801133 polymorphisms, there was no statistically significant (threshold for significance $p < 0.05$) difference between cases and controls, either among genotype nor allele frequencies. The *MTHFR* 1298C allele and 677T allele were not associated with increased risk for migraine. In addition, no individual genotypes of both *MTHFR* polymorphisms showed statistically significant results in distribution between cases and controls ($\chi^2 = 2.18$, $p = 0.34$ for A1298C and $\chi^2 = 0.22$, $p = 0.89$ for C677T). Both *MTHFR* mutations are common in patients, with frequencies of 0.300 for the 677T allele and 0.341 for the 1298C allele.

Similarly, the allele frequencies in the migraine with aura (MA) subtype did not reveal any statistically relevant associations (**Table 5**). The observed allele frequency of 1298C was more prevalent in the patient group (MAF = 0.304) compared to controls (MAF = 0.266). Similarly to the MA subtype, no significant discrepancy between either variant allele was found between the migraine without aura (MO) group and control group. The frequency of A1298C polymorphism in MO group was 0.367 and 0.285, respectively for patients and controls. The 1298C and 677T alleles did not suggest significant overall risk for migraine subtypes.

Table 5. The allele frequency among migraine subtypes – migraine with aura (MA) and migraine without aura (MO). MAF – minor allele frequency; CI – confidence interval.

<i>MTHFR</i> allele	MA group (n=46) MAF	Control group (n=92) MAF	Odds ratio (95% CI)	p-value
677T	0.304	0.337	0.86 (0.50–1.48)	0.586
1298C	0.304	0.266	1.21 (0.69–2.09)	0.506
<i>MTHFR</i> allele	MO group (n=64) MAF	Control group (n=128) MAF	Odds ratio (95% CI)	p-value
677T	0.297	0.266	1.17 (0.73–1.87)	0.518
1298C	0.367	0.285	1.46 (0.93–2.28)	0.102

The genotypic distribution analysis revealed an interesting observation within the migraine with aura subtype. The control group had the defective genotypes 1298CC and 677CT with a higher frequency (**Table 6**) than patients with aura. The same thing was detected for the 1298AC and 677TT genotypes in the migraine without aura subtype. The analysis of genotype distribution of both *MTHFR* polymorphisms in migraine subtypes did not reveal their significant role (**Supplement 3**) in susceptibility to migraine with aura nor migraine without aura.

Table 6. The genetic distribution of *MTHFR* C677T and A1298C genotypes in migraine with aura (MA) and migraine without aura. CI – confidence interval.

<i>MTHFR</i> genotype	MA group (n=46) frequency	Control group (n=92) frequency	Odds ratio (95% CI)	p-value
677CC	0.478	0.413	1.16 (0.61-2.18)	0.745
677CT	0.434	0.511	0.85 (0.45-1.60)	0.637
677TT	0.09	0.08	1.14 (0.32-4.10)	1
1298AA	0.435	0.478	0.91 (0.48-1.72)	0.872
1298AC	0.523	0.467	1.12 (0.61-2.06)	0.755
1298CC	0.043	0.054	0.8 (0.15-4.28)	1

<i>MTHFR</i> genotype	MO group (n=64) frequency	Control group (n=128) frequency	Odds ratio (95% CI)	p-value
677CC	0.156	0.370	0.85 (0.50–1.42)	0.598
677CT	0.156	0.245	1.28 (0.74–2.20)	0.398
677TT	0.021	0.052	0.80 (0.24–2.65)	1
1298AA	0.135	0.328	0.82 (0.48–1.43)	0.583
1298AC	0.151	0.292	1.04 (0.60–1.78)	0.891
1298CC	0.047	0.047	2.00 (0.75–5.28)	0.196

Analyzing the homocysteine (Hcy) level association for either *MTHFR* polymorphism, a significant effect of the 677T allele ($p = 0.03$, $\beta = 1.16$, standard deviation = 0.54) on plasma homocysteine concentrations was seen. It appears that the 677T defective allele affects homocysteine levels. According to the Tartu University Hospital United Laboratory Handbook², Hcy levels increase with age (1–7 yrs < 7.6 $\mu\text{mol/L}$; 7–12 yrs < 8.4 $\mu\text{mol/L}$; 12–19 yrs < 11.9 $\mu\text{mol/L}$), so the patients were divided into age groups corresponding to the Handbook. Following investigation into the Hcy levels in patient age groups, no significantly higher levels were seen in any of the groups compared to the reference levels described.

²<http://www.kliinikum.ee/yhendlabor/images/stories/kasiraamat/HIJ/homotssteiin%20.pdf>

1.8.2. Exome sequencing to identify novel genes for migraine

1.8.2.1. Family with migraine with aura

The first examined family trio consists of three members – a child and mother affected with migraine with aura, and a clinically healthy father. The proband also has aunt and grandmother who have been diagnosed with migraine (Figure 4). Both of her brothers have died – one due to diabetes and another due to cancer.

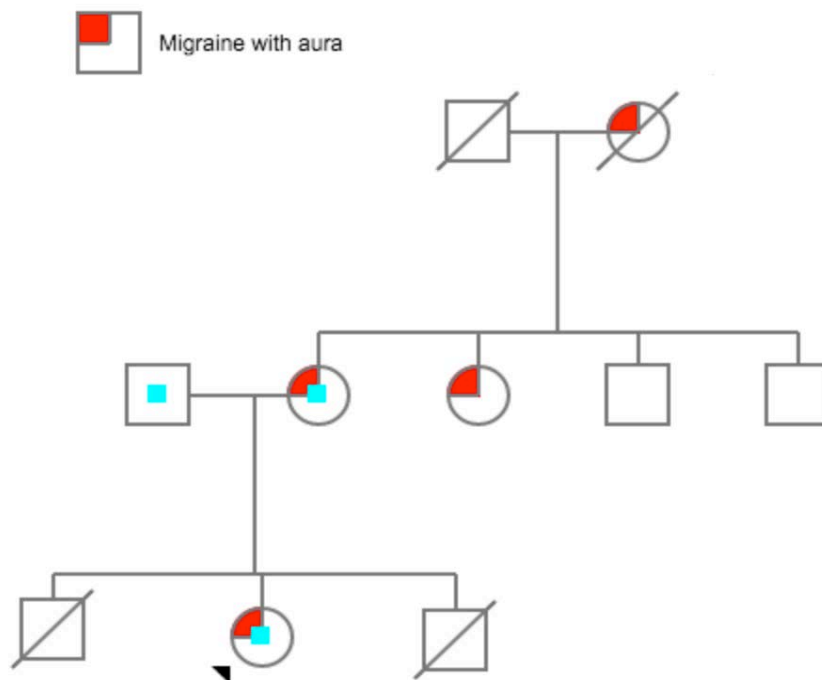


Figure 4. Family tree of patient with migraine with aura. Individuals whose exomes were sequenced are marked with a light blue square. Proband is marked with an arrow. (Figure created with <https://pedigree.progenygenetics.com/>)

The proband is a 36-year-old woman, who has received higher education and is a college lecturer. She is married and is mother to 4 children. She experienced her first migraine attack at the age of 15. Since age 20, headaches have been regular. In the first several years, the headache attacks appeared once a month, but for approximately 15 years, attacks are weekly (in some weeks even for 4 to 5 days). Headaches have become much worse in the last 10 of these years. During her first two pregnancies and breastfeeding (first 3–4 months), the headaches were reduced or even disappeared but during the last two pregnancies, the headaches returned with the same frequency and severity. She describes her headaches as unilateral (sides can change) and drilling, sharp and cutting. She also experiences prodrome symptoms (lethargy,

photophobia) as well as visual aura and nausea accompanying the pain. An MRI was done in 2005 and it did not show any abnormalities.

She has tried number of medications, acute as well as preventative, however most of them have not worked as migraine relievers. She went to the emergency room in 2010 and in 2015 due to being unable to reduce headache with prescription drugs.

Chocolate and wine are definite migraine triggers for her.

The proband's mother is a 62-year-old woman, who is currently working as a laboratory technician. She has been experiencing headaches since her twenties. The headaches were extremely severe in her youth; it took several days to recover from the migraine attack. Several times she could not even attend work due to the severity of headaches. She has also tried several medications, preventative as well as acute. After menopause, her attacks have reduced in severity and frequency but still occur once a month.

The proband's father is also a 65-year-old healthy man. He has retired, but still contributes a lot to the community of his small residential town. He takes care of his grandchildren and lives his life to the fullest.

The second family trio is diagnosed with migraine without aura but as no significant findings were discovered in this family, the family's medical history will not here be described in depth.

1.8.2.2. The collection of blood samples and the extraction of DNA

Two families gave their informative consent for blood extraction in order to discover possible risk factors for migraine. The extraction of whole venous blood was done in Tartu University Hospital. From each patient, 8 ml of blood was transported to Tartu Estonian Genome Center, where the author of the thesis extracted DNA following a specific high-salt extraction protocol (*Supplement 4*). After the extraction, the DNA was sent to the Core Facility of the Estonian Genome Center for exome sequencing (approved by the Research Ethics Committee of Tartu University, protocol nr: 233/M-19).

1.8.2.3. Exome sequencing and data annotation

The preparation of samples and exome sequencing of the two trios was carried out in the Estonian Genome Center (EGC), Core Facility using a HiSeq2500 Ultra-High-Throughput Sequencing System.

The purified genomic DNA's concentration was measured with a Qubit 2.0 Fluorometer (Life Technologies, Grand Island, California, USA). For the preparation of next-generation sequencing libraries, 50 ng of DNA was used for the input. The preparation was done according to the Illumina Nextera Rapid Capture Exome protocol (Illumina, San Diego, California, USA). Two family trios (6 samples) were indexed, pooled together and then hybridized to Nextera Coding Exome Oligos according to the protocol of the manufacturer. Next the library mixture was quantified by a Qubit 2.0 Fluorometer and validated via an Agilent 2200 TapeStation analysis (Agilent Technologies, Santa Clara, California, USA). The library was quantified by qPCR with a Kapa Library Quantification Kit (Kapa Biosystems, Woburn, USA) in order to optimize cluster generation. Thereafter sequencing on HiSeq2500 platform (Illumina, San Diego, USA) with 2 x 98 bp paired-end reads was done. Of the bases sequenced, 93% were above a quality of Q30. Demultiplexing was done with bcl2fastq2 Conversion Software v2.17 (Illumina, San Diego, California, USA) allowing one mismatch per 8 bp index read. The number of reads was between 45 M and 55 M, varying between samples.

The annotation of exome sequencing data was done by Estonian Genome Center specialist M. Kals using special in-house scripts. Variant annotation is based on Ensembl Variation API (http://www.ensembl.org/info/docs/api/variation/variation_tutorial.html), determining the gene and transcript on which the variant is located, also the importance and location of variant on the protein, along with SIFT and PolyPhen-2 scores, minor allele frequency from 1000 Genomes Project, etc. Annotation includes information from other databases such as CADD-score and phyloP-score, ESP6500 and GenomeTrax (includes several disease databases – OMIM, HGMD, ClinVar, Cosmic). Also the data from whole-exome sequencing and whole-genome sequencing from Estonian Genome Center was added to the annotation.

1.8.2.4. Variant prioritization

The interpretation of data was done by the author, using the scheme shown below (**Figure 5**). The first step in cleaning up the huge data file was to discard any mutations of insufficient quality (e.g. HARD_TO_VALIDATE, LowQD, LowCoverage etc). As mother and proband were diagnosed with the disorder, all variants that were in the homozygous-reference state for them, were discarded. The

father is healthy individual without migraine, so he was the homozygous-reference. Since rare novel variants were being looked for, anything which had an occurrence in the population higher than 0.05 (according to 1000 Genome Project data – 1000G_p3_EUR) were overlooked (mutations with no available data were included). The CADD-score threshold was set higher than 20. Probably/possibly damaging variants (according to Polyphen-2 <http://genetics.bwh.harvard.edu/pph2/>, SIFT scores <http://sift.jcvi.org/>, and Mutation Taster <http://www.mutationtaster.org/>) were all included. The highest SIFT score of a mutation that was included was 0.05, for Polyphen-2, the lowest was 0.80. Next tissue expression was studied using UniProt (<http://www.uniprot.org/>) and GeneCards (<http://www.genecards.org/>) databases. Any proteins not expressed in any part of the brain were discarded. After all these steps, only 37 mutations out of 92 994 remained.

The following steps taken to reduce the number of mutations that possibly cause migraine included the study of protein function and the selection of possible variations causing migraine. Finally, the three most likely mutations were selected, which were then reviewed together with T. Nikopensius, Estonian Genome Center researcher. The alignments of three genes were visualized using Integrative Genomics Viewer Version 2.3.72 (IGV).

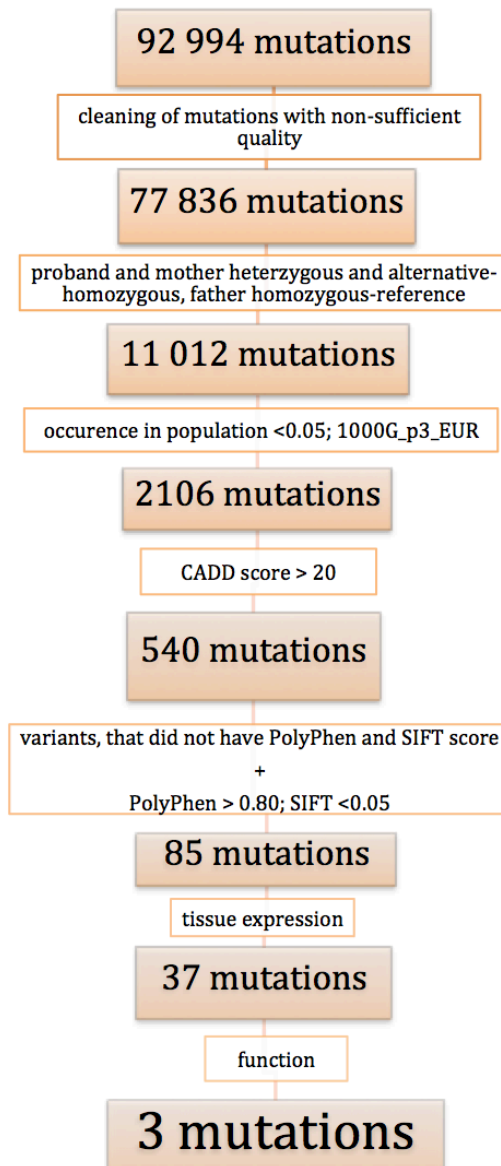


Figure 5. Scheme showing how the novel genes/mutations for migraine in the migraine with aura trio were prioritized.

1.8.2.5. Sanger sequencing

The IGV image interpretation was not definitive for the *SCN9A* mutation, therefore validation of this mutation was done using Sanger sequencing. The confirmation of the mutation in the *SCN9A* was carried out using self-designed primers (used program <http://primer3.ut.ee/> developed by University of Tartu scientists): 5'-GGCTGGGATTGTGAATAAATG-3' and 3'-AGAAGACCCTGATGCAAACAA-5'. First, the interesting part of DNA was amplified via a PCR reaction. The total volume of PCR mixture was 20 μ l, which contained:

- 10x Hot FIREPol® PCR buffer (Solis Biodyne, Estonia) 2 µl
- MgCl₂ (25 mM) 1.2 µl
- dNTP (2 mM) 3 µl
- Hot FIREPol® DNA polymerase (Solis Biodyne, Estonia) 0.3 µl
- Forward primer (5 µM) 1 µl
- Reverse primer (5 µM) 1 µl
- Deionized water (MQ) 9.5 µl

To the PCR reaction mixture, 2 µl of DNA was added. The reaction was done in the *Applied Biosystems Veriti 96-Well Thermal Cycle* according to the program shown in **Supplement 5**. The PCR product was visualized using ethidium bromide (**Supplement 6**). The product size was 304 bp. The products of PCR were sent to the Estonian Biocenter, Core Laboratory for Sanger sequencing.

1.8.2.6. Results

Exome sequencing in two family trios diagnosed with migraine were performed. Variant calling resulted in 84 150 – 84 282 single-nucleotide variants (SNVs) and 5 214 – 5 318 small insertion and deletion variants (indels) in both family trios.

As a result of exome sequencing in the migraine without aura trio, no genetic risk factors likely attributable to the development of migraine were discovered. However in the family with aura, three possible mutations that might be involved in migraine pathophysiology were identified – in the *PDLIM5*, *PRKCE* and *SCN9A* genes. As the sequencing quality of *SCN9A* was not definitive for decision-making, Sanger sequencing was used for validation of this variant.

The proband is heterozygous for the *PDLIM5* gene missense mutation rs76352571, c.1448A>G (p.His483Arg, RefSeq NM_006457), located in the 13th exon (**Figure 6**). She was also heterozygous for the missense mutation in *PRKCE* gene rs34077350, c.1688C>T (p.Thr563Met, RefSeq NM_005400), located in 12th exon (**Figure 7**). The proband's mother was heterozygous for the same missense mutations rs76352571 and rs34077350. The father was reference-homozygous for both variations. Mutations rs76352571 in the *PDLIM5* gene and rs34077350 in the *PRKCE* gene are considered as probably damaging according to the PolyPhen-2 database; damaging by SIFT (**Table 7**) and disease-causing by Mutation Taster. Both variants rs76352571 and rs34077350 are with low frequencies. In EGC whole-exome

(334) database, there were only 6 and 5 additional people respectively carrying heterozygous genotypes. The 1000 Genome Project p3 has stated that the European allele frequencies for these previously mentioned variants are 0.015 and 0.008, respectively (**Table 8**).



Figure 6. The results of exome sequencing. IGV picture of the *PDLIM5* mutation c.1448A>G, for the proband, mother (both diagnosed with migraine) and father (healthy individual).

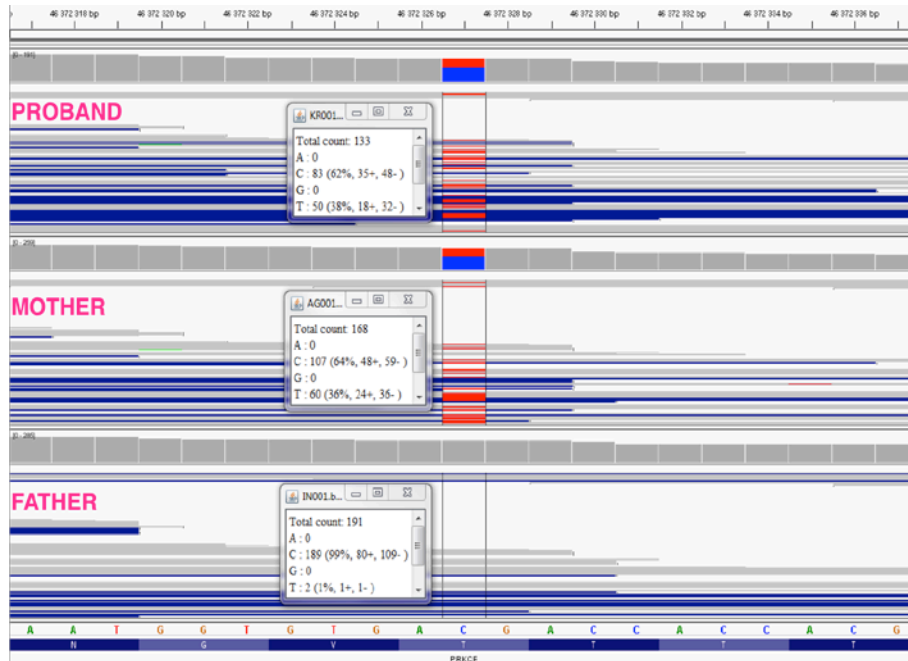


Figure 7. The results of exome sequencing. IGV picture of the *PRKCE* mutation c.1688C>T for the proband, mother (both diagnosed with migraine) and father (healthy individual).

The human *PDLIM5* gene (Ensembl: ENSG00000163110) encodes PDZ and LIM domain protein 5 (UniProt: Q96HC4), which contains a PDZ domain (100 amino acids) at the N-terminus and three LIM domains at the C-terminus. The SNP (rs76352571) is located in the second LIM domain (**Figure 8**) (Maturana et al., 2011).



Figure 8. The structure of *PDLIM5*. PDZ domain in grey, domain with unknown function in yellow and LIM domains in blue. Mutation rs76352571 is located in the second LIM domain. Figure adapted from the InterPro webpage.

The human *PRKCE* gene (Ensembl: ENSG00000171132) encodes a protein kinase C epsilon ($PKC\epsilon$) type enzyme (UniProt: Q02156), which is a novel isoform from the large PKC protein kinase family. $PKC\epsilon$ has many similar structural features to as other members of the PKC family, including a C1 domain (two cysteine-rich motifs binding diacylglycerols), a C2-like (phospholipid-binding) domain and catalytic domains (C3 and C4). The SNP rs34077350 is located in the kinase/catalytic domain (**Figure 9**) (Shirai et al., 2008, Newton and Messing, 2010).

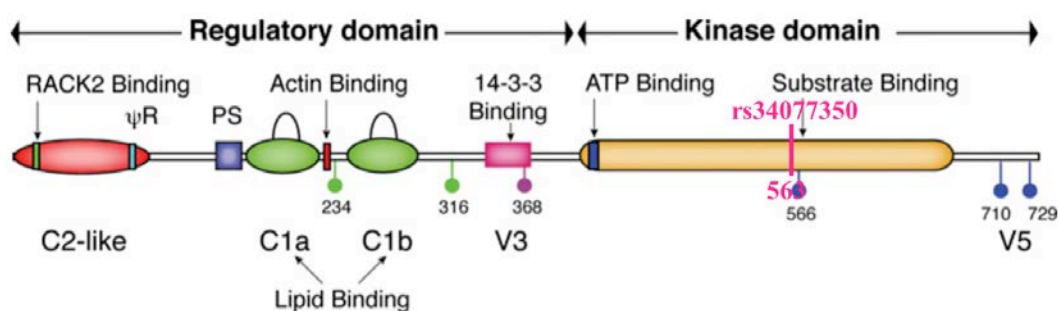


Figure 9. The structure of $PKC\epsilon$. C1a and C1b (green) – diacylglycerol binding domain; C2-like (red) – phospholipid-binding domain; catalytic domain (yellow) – kinase domain. Mutation rs34077350 is located in the kinase domain. Figure adapted from Newton and Messing (2010).

After Sanger sequencing (see previous Chapter), the missense mutation rs199692186, c.2969A>G (p.Tyr990Cys, NM_002977) of the *SCN9A* gene was confirmed (**Figure 10**). The proband and her mother were heterozygous for this variation, whereas her father was reference-homozygous. According to Polyphen-2, rs199692186 is also considered to be probably damaging, by SIFT damaging (**Table 7**) and by Mutation Taster disease-causing. According to Estonian whole-exome (334) sequencing data, only 2 additional people had a heterozygous genotype.

The allele frequency stated by the 1000 Genome Project p3 is not reported, so in EUR as well as global (**Table 8**).

The human *SCN9A* gene (Ensembl: ENSG00000169432) encoding the major voltage-gated sodium channel (Na_v1.7) alpha subunit is located in the 17th exon (Harrington et al., 2009).

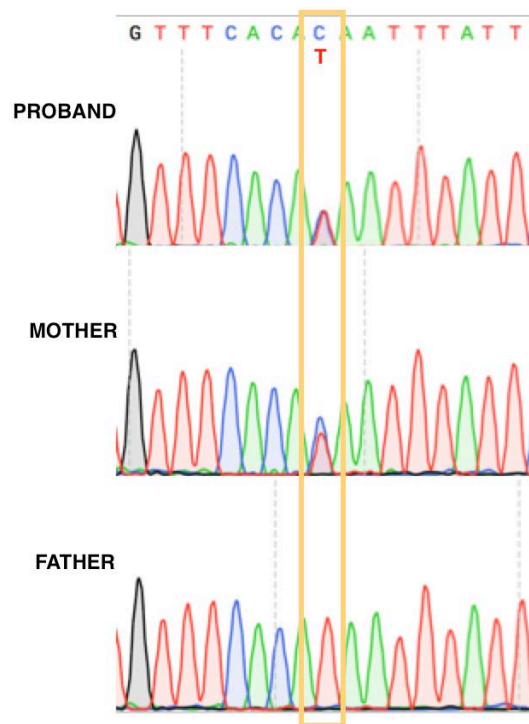


Figure 10. The verification of the c.2969T>C mutation in the *SCN9A* gene by Sanger sequencing. Sequencing showed the proband and her mother to be heterozygous for the loci, and father to be wild-type homozygous.

Table 7. Potential candidate genes for migraine with aura.

ID	Gene	Proband	Mother	Father	Chromosome	Exon	Ref/Alt	Mutation type	Change	SIFT	Polyphe n	CAD D score	phyloP
rs76352571	<i>PDLIM5</i>	het	het	hom_ref	4	13	A/G	missense	p.His483Arg	0.02	1.000	27.4	5.142
rs34077350	<i>PRKCE</i>	het	het	hom_ref	2	12	C/T	missense	p.Thr563Met	0.02	0.984	32	4.176
rs199692186	<i>SCN9A</i>	het	het	hom_ref	2	17	T/C	missense	p.Tyr990Cys	0.01	0.988	24.1	0.608

Table 8. The frequency of potential candidate genes in different populations and in Estonian exome and whole genome sequencing data. AF – 1000 Genome Project, global allele frequency (phase 3); EUR_AF – 1000 Genome Project, European allele frequency (phase 3); het/hom.alt.est.334.ex – count of population specific heterozygous/alternative homozygous genotypes based on whole-exome samples; het/hom.alt.est.2244.wg – count of population specific heterozygous/alternative homozygous genotypes based on whole-genome samples; ExAC_NFE_AC_het_hom – non-Finnish European population allele counts, heterozygous and homozygous counts; ESP_EA_GTC – European American genotype count.

ID	Gene	AF	EUR_AF	het.est. 334.ex	hom.alt. est.334.ex	het.est. 2244.wg	hom.alt. est.2244.wg	ExAC_NFE_A C het_hom	ESP_EA_GTC
rs76352571	<i>PDLIM5</i>	0.0039	0.015	8	0	79	0	670;666;2	GG=1/GA=85/AA=4214
rs34077350	<i>PRKCE</i>	0.0015	0.008	7	0	127	1	263;263;0	TT=0/TC=16/CC=4116
rs199692186	<i>SCN9A</i>	.	.	4	0	.	.	59;59;0	CC=0/CT=9/TT=4060

1.9. Discussion

Migraine is a common neurological disorder that causes millions of people serious health and disability problems daily. It is mainly characterized by recurrent headaches that in 30% of migraineurs are accompanied by aura symptoms (visual, speech and sensory disturbances). Twin and family studies have firmly indicated that migraine is a genetic disorder. Several genes using genome-wide association, candidate gene and linkage studies have been identified for migraine, but unfortunately only a small portion of them have been significantly and reproducibly associated (Waldman, 2011, Gasparini et al., 2013).

The purpose of this candidate gene association study was to determine the role of both polymorphisms rs1801131 and rs1801133 of methylenetetrahydrofolate reductase (*MTHFR*) in the pediatric migraine population, to assess their allelic and genotypic effect. Also to examine whether any allele/genotype could contribute to the disorder's predisposition in either migraine subtypes (migraine with aura – MA, migraine without aura – MO). In addition, investigate the role of homocysteine in migraine susceptibility. *MTHFR* 677T and 1298C minor alleles, as migraine risk factors in Estonia, have been studied once before (Lorenz et al., 2014); however the genotypic distribution and homocysteine levels in migraineurs have not been reported.

MTHFR encodes 5-methylenetetrahydrofolate, which is the major carbon donor needed for the efficient remethylation of homocysteine to methionine. In the presence of A1298C or/and C677T polymorphisms, *MTHFR* enzyme activity is reduced. This in turn causes moderate hyperhomocysteinemia. It is suggested that the dysfunction of vascular endothelium produced by hyperhomocysteinemia influences migraine susceptibility, especially MA, through activating trigeminal fibers, which causes dilation of cerebral vessels and inflammation in meninges (Liu et al., 2010, Stuart et al., 2010, Liu et al., 2014).

A number of epidemiological studies have been performed in recent years to investigate the association of *MTHFR* polymorphisms as migraine risk factors in different populations. Unfortunately, the results have been controversial and have not provided any conclusive finding (Liu et al., 2014).

An investigation into the *MTHFR* A1298C and C677T minor allele frequencies (MAF) in pediatric migraine patients, we found that 1298C and 677T alleles are common in Estonian population (**Table 4**). This is concordant with the European

allele frequency stated by HapMap Project (MAF=0.358 and MAF=0.309, respectively). Analyzing the MAF between controls and patients showed none of the findings to be statistically significant. The 1298C allele was seen more frequently in patients than controls, however non-significantly ($p = 0.09$, OR = 1.35), whereas the 677T allele almost had the same frequency in patients and controls (**Table 4**). These findings are in conflict with several studies, where the 677T variant allele has been found to be associated with migraine susceptibility (Samaan et al., 2011, Liu et al., 2014, Saeedi et al, 2015) rather than the 1298C allele. Furthermore, no significant difference resulted when analyzing migraine subtype (MA and MO) association with *MTHFR* 1298C and 677T alleles (**Table 5**).

The statistical analysis of genotypic distribution of *MTHFR* polymorphisms A1298C and C677T also revealed no significant differences between migraineurs and the control group. In migraine subtypes, the 1298CC and 677CT genotypes were more frequent in control groups than in patients with MA (**Table 6**). Analogous observation was done in the MO group, where the 1298AC and 677TT genotypes were highly frequent in controls (**Table 6**). The findings in the MA group are supported by the study done in the Finnish population by Kaunisto et al. (2006), who also found no association between migraine with aura and C677T related genotypes.

A common polymorphism – C677T in *MTHFR* gene – is known to be contributing to increased homocysteine (Hcy) levels. In the current study, the *MTHFR* 677T variant allele was found to be nominally associated with circulating homocysteine levels, whereas no link between the 1298C variant allele and increased Hcy levels was seen. These findings are in accordance with Vohnout et al. (2011), who also indicated that the *MTHFR* 677T allele increased Hcy levels. However, analyses of the homocysteine levels in our patients showed no significantly higher levels of homocysteine in those carrying defective 677T allele was observed. This could indicate that 677T allele contributes to increasing Hcy level, however not enough to be causing hyperhomocysteinemia.

In conclusion, neither the *MTHFR* C677T nor A1298C polymorphisms, responsible for reduction of the *MTHFR* activity in homocysteine metabolism, do act as a genetic susceptibility factors for migraine in Estonian migraine population. These controversial results could be due to the small sample size or due to ethnic differences (An et al., 2013).

Since the candidate gene association studies for *MTHFR* did not reveal any significant results, and GWAS is not considered to be the holy grail for migraine genetics³, since common gene variants could only explain little of the genetic variance, next generation sequencing was decided to use, in order to reveal novel variants with the population frequency < 1%. Exome sequencing was selected over whole genome sequencing due to the cost and fact that coding regions could be a potential area for identifying rare variations in complex diseases, like migraine (Rudkjobing et al., 2012). To our knowledge, there have been only two previous investigations into migraine genetic susceptibility using whole-exome and whole-genome sequencing (Calafato, 2011, Jiang et al., 2015). The aim of whole-exome sequencing was to identify possible novel variants that could, to some extent, describe migraine pathophysiology.

Exome sequencing in family trio diagnosed with migraine without aura did not reveal any clinically significant variants. MO is said to be determined by both genetic and environmental factors, which could indicate that its genetic influences are less powerful than in MA (Russell et al., 1995, Stewart et al., 1997, Ashina et al., 2012).

However, exome sequencing in migraine with aura trio revealed three rare mutations that could possibly be associated with migraine susceptibility. The proband and mother, diagnosed with MA, were heterozygous for all mutations (rs34077350 in *PRKCE*, rs76352571 in *PDLIM5* and rs199692186 in *SCN9A*), whereas the father was reference-homozygous (a healthy individual). Since no previous studies have been done with the mutations found by the thesis author, only assumptions about the expression and function of the mutated genes could be made.

The first mutation, rs76352571 c.1448A>G, is located in the *PDLIM5* gene on chromosome 4q22. *PDLIM5* encodes PDZ and LIM domain protein 5 (also known as ENH1, forwardly referred as PDLIM5), which is 595 amino acids in length with a molecular weight of 63.9 kDa. LIM domains at the C-terminus are 50–60 amino acid long double zinc finger motifs that serve as protein–protein interaction sites in numerous proteins (Horiuchi et al., 2006, Zhao et al., 2009). Chen and colleagues (2006) reported that the second LIM domain is vital for forming protein–protein interactions. The mutation found by the thesis author is also localized in the second LIM domain of the protein. The cytoplasmic PDLIM5 is expressed in various brain

³ <http://iasppain.org/files/Content/ContentFolders/GlobalYearAgainstPain2/HeadacheFactSheets/14-Genetics.pdf>

regions, particularly in the hippocampus, cortex, thalamus, hypothalamus, amygdala and cerebellum (Zhao et al., 2009, Newton and Messing, 2010, Maturana et al., 2011).

The second mutation, rs34077350 c.1688C>T, is located in the *PRKCE* gene on chromosome 2p21. The *PRKCE* gene encodes a protein kinase C epsilon (PKC ϵ) type enzyme with a molecular weight of 83.6 kDa and a length of 737 amino acids. PKC ϵ is highly expressed in the brain and has been found to have an important role in neurotransmitter release, membrane excitability and ion channel regulation. The protein is mainly found in the cerebral cortex, cerebellum and hippocampus. The mutation is located in the Ser/Thr kinase domain (Maeno-Hikichi et al., 2003, Shirai et al., 2008, Newton and Messing, 2010).

PDZ and LIM domain protein 5 is known to interact specifically with PKC ϵ (**Figure 11**) to recruit the kinase directly to its substrate (voltage-gated calcium channel). The formation of kinase–substrate aggregation is the molecular basis for the specificity and efficiency of cellular signaling. Direct protein–protein interaction between the LIM domain protein and the enzyme modulates the activity of the N-type

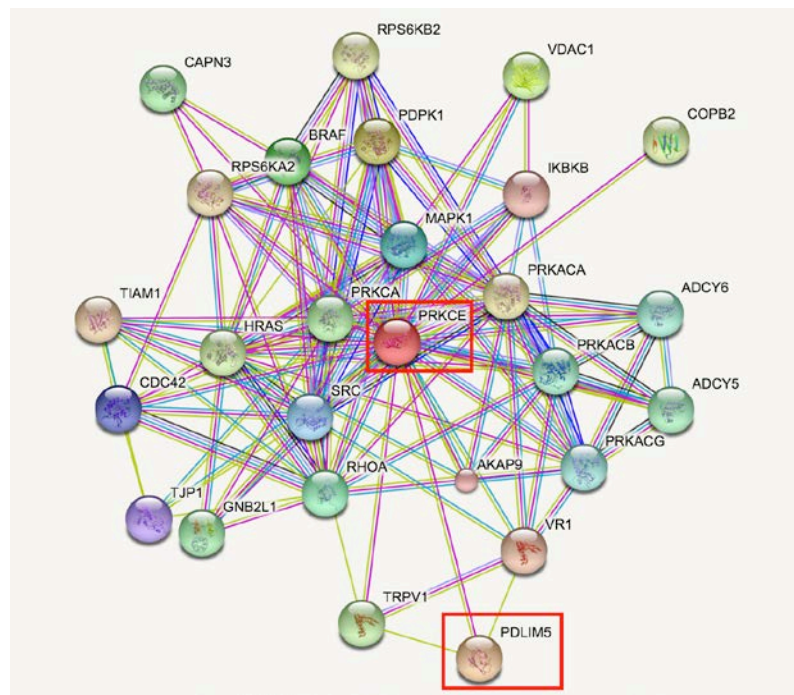


Figure 11. The gene–gene interaction between *PRKCE* and *PDLIM5* (Figure obtained from *string-db.org*). The pink connecting line indicates that the interaction is determined by experiments.

voltage-gated calcium channel (Ca $_v$ 2.2), which is induced via the phosphorylation of the channel's α_1 subunit by PKC ϵ . The forming of the PKC ϵ -ENH1-N-type Ca $^{2+}$ channel complex is essential for the potentiation of high voltage-activated calcium channels in neurons (Chen et al., 2006, Zain et al., 2012, Liu et al., 2013).

Studies done on protein–protein interactions have shown that the LIM domains have high specificity to particular sequences of other proteins, which ensures the effective forming of the complex (Pawson, 1998). In the presence of mutation rs76352571, located in the second LIM domain, the binding ability of PDLIM5 to PKC ϵ could be affected, which could cause an increase in affinity towards protein kinase C. Arimura et al. (2004) declared that a mutation in the third LIM domain (has four all together) of Cypher/ZASP protein (also belonging to the Enigma subfamily like *PDLIM5*) alters the binding affinity to protein kinase C, by increasing it. Consequently, due to this gain-of-function, the specificity and efficiency, as well as the probability of binding between the kinase and Ca $_v$ 2.2 could also be increased to some extent.

The expression of the *PDLIM5* have been previously reported to be significantly and commonly increased in the patients with bipolar disorder, schizophrenia and major depressive disorder (Iwamoto et al., 2004). Several mutations (rs2433320, rs10008257, rs2433322) in the *PDLIM5* gene have been found to be associated with previously mentioned major mental disorders (Horiuchi et al., 2006, Liu et al., 2008, Zhao et al., 2009). For example, the increase in protein level in schizophrenia patients has been attributed to SNP rs2433320, where the A allele is associated with higher expression contrary to the G allele (Horiuchi et al., 2006). Even though, all these previously mentioned mutations seem to be associated with transcriptional activities, we can still hypothesize that our variation has totally distinct effect on the protein. It could be due to the fact that all these SNPs are located in the upstream region of the gene (Zain et al., 2012), whereas our variation is located towards the 3' end of the protein. This could also exclude the possibility of the family trio suffering from one of these disorders/diseases. Unfortunately, relatively little is known about the biological function of the cytoplasmic LIM domains; however important neuronal roles (receptor trafficking, signal transduction etc.) have been suggested (Pawson, 1998).

The modulation of calcium release in the presynaptic terminal is an excellent regulation mechanism for releasing neurotransmitters into the synaptic cleft (Catterall, 2011). The second identified rare mutation, rs34077350, is located in the catalytic domain of the PKC ϵ enzyme. The variation in the kinase could stimulate the phosphorylation of the N-type calcium channel α_1 subunit (**Figure 12**) as well as G-

protein coupled receptors (GPCRs), as these phosphorylations are the foundation of considerable up-regulation of the calcium channel activity. In neurons, the inhibition of voltage-gated N-type calcium channels ($Ca_v2.2$) are carried out by G-protein coupled receptors, by decreasing the N-type channel activity by 60%. This kind of inhibition could be overturned by the PKC-dependent phosphorylation of GPCRs, G-protein interaction sites on the channel, or the G-protein itself. It has been found that the phosphorylation of G-protein can also result in enhancement of the calcium current (the channel activity is increased by approximately 50%), while G-protein inhibition is fully antagonized (Hamid et al., 1999, Bourinet and Zamponi, 2005, Zamponi and Currie., 2013).

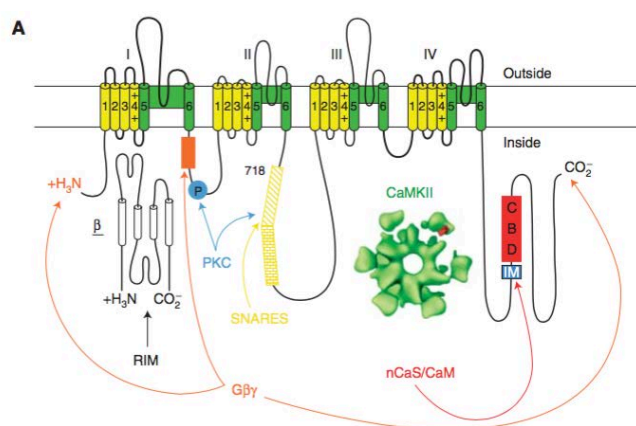


Figure 12. The phosphorylation of the $Ca_v2.2$ α_1 subunit by PKC ϵ . Protein kinase C is marked in light blue (Catterall, 2011).

The $Ca_v2.2$ channels encoded by the single α_1 -subunit gene are located in the presynaptic nerve terminals. The N-type channels have an important responsibility to regulate neurotransmitter release in presynaptic neurons, as the channels are linked to synaptic vesicles by SNARE proteins. Chronic pain has been characterized by the change in the performance of the ion channels, as well as by extensive neurotransmitters release, which leads to a state of hyperexcitability. The up-regulation of $Ca_v2.2$ expression has been associated with pathophysiology of pain, which is supported by the fact that the absence of N-type channels in mice has shown a decreased response to pain (Yokoyama et al., 2004, Bourinet and Zamponi, 2005, Chen, 2011).

Formation of the PKC ϵ -ENH1-N-type calcium channel complex allows quick and selective modulation of calcium channel activity (Maeno-Hikichi et al., 2003). In the presence of the mutations rs34077350 in *PRKCE* and rs76352571 in *PDLIM5*, the inflow of calcium into neurons could significantly increase. This in turn could cause

extensive release of neurotransmitters, which has previously been associated with pain (Bourinet and Zamponi, 2005). Also, excessive calcium influx causes hyperexcitability, which has been related to cortical spreading depression (CSD), known to be the basis for visual aura (Gasparini et al., 2013).

The third mutation of interest, rs199692186 c.2969A>G, is located in the *SCN9A* gene on chromosome 2q24. *SCN9A* encodes the α subunit of the Na_v1.7 sodium channel protein that is expressed highly in pain-sensing neurons. Voltage-gated sodium channels consist of a 1988 amino acid long α subunit (~260 kDa) and two auxiliary β subunits (~35 kDa) (Waszkielewicz et al., 2013). This channel is an essential component in pain perception in humans, as mutations in them cause functional abnormality in neurons. Changes caused by the heterozygous mutations in amino acids of Na_v1.7 are the source of different pain-related phenotypes. Loss-of-function mutations in Na_v1.7 cause a complete incapability to perceive pain (disorder: congenital insensitivity to pain). Patients with these kind of mutations are unable to sense any form of pain, often experiencing painless fractures, burns and injuries. Gain-of-function mutations, on the other hand, cause attacks of severe pain, often with a burning quality (disorders: erythromelalgia, paroxysmal extreme pain disorder, small fiber neuropathy etc.). This is due to the increase of Na_v1.7 channel activity (Raouf et al., 2010, Reimann et al., 2010). The mutation rs199692186 is located near the missense variation R996C that contributes to paroxysmal extreme pain disorder (**Figure 13**). Paroxysmal extreme pain disorder (PEPD) is caused by a gain-of-function mutation and is characterized by episodes of severe perineal, periocular and perimandibular pain (Zorina-Lichtenwalter et al., 2016). The mutation, found by the thesis author, could also cause gain of function of the Na_v1.7 channel in migraine.

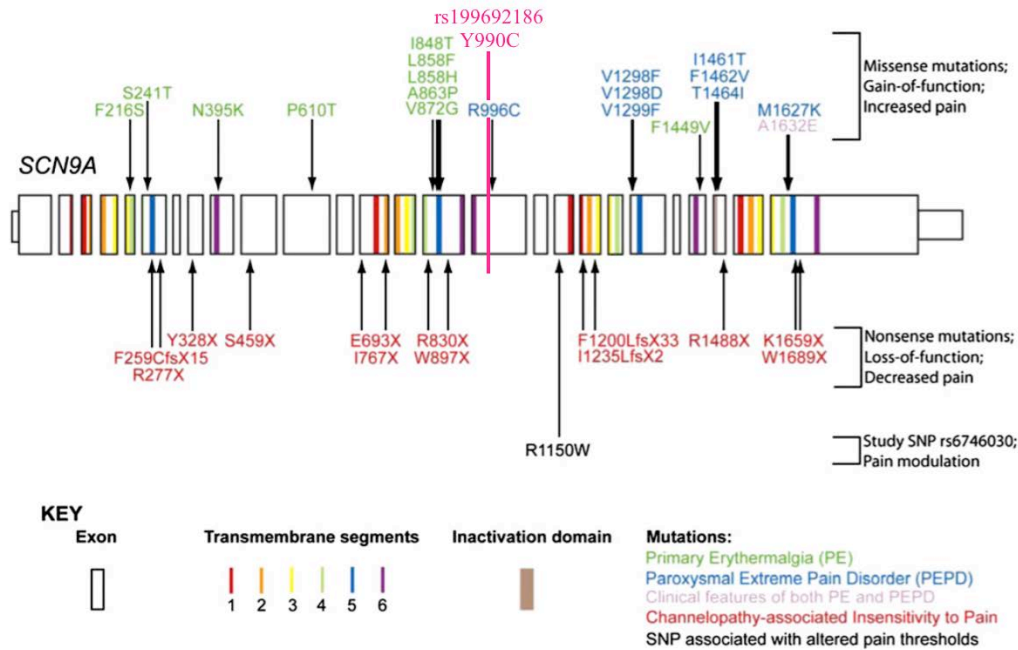


Figure 13. The location of the rs199692186 mutation found by the thesis autor (marked in pink) in the *SCN9A* gene. All labelled mutations are contributing to different pain disorders. Adapted from Reimann et al., (2010)

The rs199692186 variation in *SCN9A* could have a modifier effect regarding the enhancement of pain perception in migraine pathophysiology. To date, the role of *SCN9A* mutations in central nervous system abnormalities is unclear; however, mutations causing a spectrum of syndromes (PEPD, congenital insensitivity to pain etc.) show that $Na_v1.7$ has a critical role in altering neuronal excitability, which in turn is linked to human pathologies (Raouf et al., 2010).

All these previously discussed mutations affect, either directly or indirectly, voltage-gated ion channels that are known to alter neuronal excitability. Higher neuronal excitability has been associated with cortical spreading depression, which is the foundation of visual aura (Gasparini et al., 2013). Hyperexcitability in turn causes the excessive release of neurotransmitters that is related to pain pathophysiology (Bourinet and Zamponi, 2005). These findings suggest that alterations in the *PDLIM5* and *PRKCE* could contribute synergistically for migraine with aura pathophysiology. *SCN9A* variant in the other hand could act as a modifier effect that could likely increase the pain perception in migraine. These candidate genes however require further investigation in a larger cohort to confirm their involvement in migraine.

In summary, whole-exome sequencing did not reveal significant variations for migraine predisposition in migraine without aura family trio. However, in a family diagnosed with migraine with aura three rare variants that could cause migraine were

identified. These rare probably pathogenic variants were located in the *PDLIM5* (rs76352571), *PRKCE* (rs34077350) and *SCN9A* (rs199692186) genes. Exome sequencing seems to be a potentially effective tool for identifying rare genes for migraine with aura susceptibility.

SUMMARY

Migraine is a complex central nervous system disorder with approximately 1 billion people all over the world suffering from it. Migraine is subdivided into two groups: migraine with aura (MA) and migraine without aura (MO). The inherited nature of migraine is considered as an important aspect of the disorder's pathophysiology. To date only a small number of genes have been associated with migraine, all being common variants. However, the possibility of using next-generation sequencing could establish genetic background for migraine with aura and without aura.

Candidate gene association study done in this thesis analyzed the possibility of *MTHFR* polymorphisms rs1801131 (A1298C) and rs1801133 (C677T) as migraine risk factors. The study revealed no significant association between migraine and both *MTHFR* polymorphisms, so in overall migraine group as well as in migraine subtypes. This finding is controversial with several meta-analyses, which have found that 677TT genotype has significant association with migraine with aura. This controversy could be due to ethnic-specific effect or small sample size.

Sequencing the exomes of a family trio (proband and mother diagnosed with migraine without aura, father healthy individual) revealed no clinically significant findings. However, in another family trio experiencing severe migraine phenotype (MA), three potential rare deleterious variants for migraine predisposition were discovered. Previous studies have not associated these genes with migraine susceptibility.

The variants rs34077350 in the *PRKCE* gene and rs76352571 in the *PDLIM5* gene could contribute to a digenic causality for familial MA. Due to the mutation in second LIM domain, which has been reported to be important in the protein-protein interaction, the affinity towards the Ser/Thr kinase (*PDLIM5* interacts with PKC ϵ , which is encoded by *PRKCE*) could increase, and transport of the kinase to the voltage-gated N-type calcium channels (Ca $_v$ 2.2) could be with increased specificity and efficiency. The mutation in the *PRKCE* gene could in turn stimulate the phosphorylation of the Ca $_v$ 2.2 channel α_1 subunit or/and G-protein coupled receptor. Both these previously mentioned phosphorylations increase calcium channel activity, which in turn leads to neuronal hyperexcitability and increase in neurotransmitter

release. Chronic pain has been associated with extensive neurotransmitter release whereas visual aura (visual disturbances) with hyperexcitability.

Third mutation rs199692186, in the *SCN9A* gene could have a modifier effect on migraine with aura predisposition due to the gain-of function effect, which causes the increase in activity of Na_v1.7 channel. It has been previously reported to cause severe pain disorders (paroxymal extreme pain disorder and primary erythermalgia), which are caused by gain-of-function mutations in *SCN9A*.

The variations in three genes – *PRKCE*, *PDLIM5* and *SCN9A* – could contribute to the pain and/or visual aura of MA subtype. These findings although are preliminary and need further investigations in bigger cohorts.

KOKKUVÕTE

Sagedased ning haruldased variandid migreeni geneetilises põhjuslikkuses

Anna-Liisa Lorenz

Migreen on kompleksne kesknärvisüsteemi häire, mis mõjutab umbes üht miljardit inimest üle maailma. Migreen on jaotatud kaheks alatüübiks: migreen auraga ja migreen aurata. Migreeni patofüsioloogia oluline osa on selle pärilikkus. Tänapäevani on migreeni tekkega seostatud vaid väikest hulka genee ning siiani on uuritud peamiselt sageli esinevaid variante. Järgmise põlvkonna sekveneerimistehnoloogia võimaldab põhjalikumalt uurida migreeni alatüüpide geneetilist olemust ka haruldaste variantide tasandil.

Magistritöös läbiviidud assotsiatsiooniuuringu eesmärgiks oli esmalt kindlaks teha *MTHFR* geeni kahe polümorfismi rs1801131 (A1298C) ja rs1801133 (C677T) osa migreeni kujunemises. Kumbki *MTHFR*i polümorfism ei näidanud statistiliselt olulist seost migreeniga, seda nii migreeni üleüldises grupis kui ka migreeni alatüüpides. Saadud tulemus on aga vastuolus mitmete meta-analüüsidega, kus on leitud, et 677TT genotüübil on statistiliselt oluline seos auraga migreeni tekkes. Nimetatud vastuoluline leid võib olla tingitud nii etniliste taustade erinevustest kui ka meiepoolsest väikesest uuringurühmast.

Perekonna trio (emal ja lapsel diagnoositud aurata migreen, isa on terve indiviid) eksoomide sekveneerimine ei leidnud olulist kliinilist tähendust omavaid geenivariante aurata migreeniga perekonnas. Seevastu auraga migreeni (MA) perekonnas leiti kolm potentsiaalset haruldast deleterioosset mutatsiooni, mis võiksid põhjustada migreeni. Varasemalt ei ole nende geenide variante migreeniga seoses kirjeldatud. Mutatsioonid rs34077350 *PRKCE* geenis ning rs76352571 *PDLIM5* geenis võivad omada sünergistlikku mõju auraga migreeni kujunemises. *PDLIM5* teises LIM domäänis, mida on varasemalt kirjeldatud kui tähtsat domääni valk-valk interaktsioonis, asuva mutatsiooni tulemusena võib afiinsus Ser/Thr kinaasi (*PDLIM5* seondub PKC ϵ kinaasiga, mida kodeerib *PRKCE*) suhtes suurenedada ning samuti tõusta efektiivsus ja spetsiifilisus kinaasi transpordil voltaaz-tundliku N-tüüpi kaltsium kanalile. Mutatsioon rs34077350 aga *PRKCE* geenis võib stimuleerida kaltsiumkanali α_1 -subühiku või G-proteiini retseptori fosforüleerimist, mis põhjustab kaltsiumkanali aktiivsuse tõusu ja neuronite erutuvuse suurenemist. Protsessi

tagajärjel toimub neurotransmitterite suurenenud vabanemine. Kroonilist valu on varem seostatud rohkete neurotransmitterite vabanemisega ning visuaalset aurat (nägemishäireid) kõrgema neuronaalse aktiivsusega.

Kolmas mutatsioon rs199692186, mis asub *SCN9A* geenis võib omada modifitseerivat efekti auraga migreeni kujunemises. Mutatsioon võib olla *gain-of-function*, mis põhjustab naatriumkanali aktiivsuse tõusu ning varasemalt on seostatud erinevate tugevat valu põhjustavate häiretega (nt. episoodiline kobarpeavalu).

Variandid kolmes geenis – *PDLIM5*, *PRKCE*, *SCN9A* – võivad põhjustada migreeni eelsoodumust. Need leiud on esmased ning vajavad laialdasemaid uuringuid suuremates kohortides.

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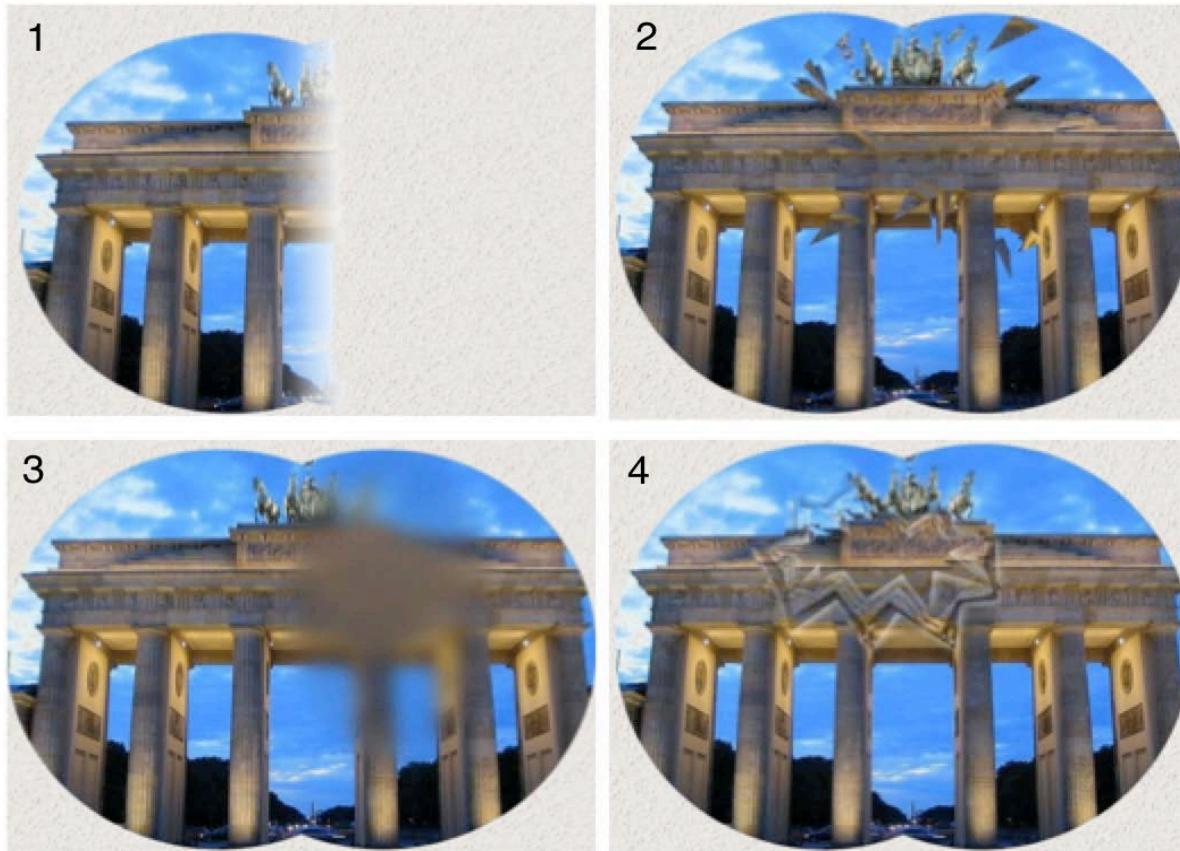
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SUPPLEMENTS



Supplement 1. Four types of visual aura. 1 – Mostly one-sided loss of perception; 2 – positive scotoma, local perception of additional structures; 3 – Negative scotoma, loss of awareness of local structures; 4 – Enhancements reminiscent of a zigzag fort structure. (<http://www.halinaking.co.uk/Location/Yorkshire/Frames/Health/Migraine/Migraine%20Auras.htm>)

SNP	Gene	Chromosome	Position	Minor allele	MAF	OR (95% CI)	Migraine subtype	Reference
rs2651899	<i>PRDM16</i>	1	3,070,572	C	0.43	1.13 (1.08-1.18)	Both subtypes	Chasman et al., 2011; Ran et al., 2014.
rs10915437	near <i>AJAP1</i>	1	4,082,866	G	0.36	0.86 (0.82-0.91)	Both subtypes	Anttila et al., 2013
rs12134493	near <i>TSPAN2</i>	1	115,479,469	A	0.46	1.14 (1.10-1.18)	Both subtypes	Anttila et al. 2013; Sintas et al., 2014.
rs2274316	<i>MEF2D</i>	1	154,712,866	C	0.37	1.07 (1.04-1.09)	Both subtypes, MO	Anttila et al., 2013; Freilinger et al., 2012.
rs11172113	<i>LRP1</i>	12	55,813,550	C	0.41	0.9 (0.86-0.94)	MO	Chasman et al., 2011
rs13208321	<i>FHL5</i>	6	96,967,075	A	0.22	1.18 (1.13-1.24)	MO	Anttila et al., 2013.
rs10504861	near <i>MMP16</i>	8	89,617,048	T	0.16	0.86 (0.81-0.90)	MO	Anttila, et al., 2013; Sintas et al., 2014
rs6478241	<i>ASTN2</i>	9	118,292,450	A	0.38	1.16 (1.11-1.22)	Both subtypes	Anttila et al., 2013
rs10166942	<i>TRPM8</i>	2	234,489,832	C	0.19	0.86 (0.81-0.91)	Both subtypes	Chasman et al., 2011

Supplement 2. Different genes identified through GWAS. MAF – minor allele frequency, OR – odds ratio; all migraine – no specification for migraine type; MO – migraine without aura; both subtypes – includes migraine with aura as well as migraine without aura.

	<i>MTHFR</i> rs1801133 (C677T)				<i>MTHFR</i> rs1801131 (A1298C)			
	CT vs CC		TT vs CC		AC vs AA		CC vs AA	
	OR (95% CI)	p-value	OR (95% CI)	p-value	OR (95% CI)	p-value	OR (95% CI)	p-value
All migraines	1.07 (0.73-1.58)	0.767	0.8 (0.39-1.79)	0.704	1.24 (0.77-2.01)	0.394	1.83 (0.77-4.33)	0.173
Migraine with aura	0.73 (0.35-1.54)	0.454	0.99 (0.25-3.75)	1	1.23 (0.59-2.54)	0.712	0.88 (0.16-4.92)	1
Migraine without aura	1.51 (0.81-2.83)	0.205	0.94 (0.27-3.26)	1	1.25 (0.66-2.38)	0.517	2.42 (0.86-6.79)	0.103

Supplement 3. The association between C677T and A1298C polymorphism and migraine. OR – odds ratio, CI – confidence interval.

THE PROTOCOL FOR DNA EXTRACTION

First day:

1. 4 ml of blood placed in 15 ml centrifuge tube. Add 8 ml of A-lysis buffer (RBC), shake and place it on ice for 30 minutes
2. Centrifuge at 3100 rpm for 15 minutes (4°C)
3. Discard supernatant, add 8 ml of A-lysis buffer (RBC) again
4. Centrifuge at 3100 rpm for 15 minutes (4°C)
5. Discard supernatant and add 1,6 ml of TKM1, shake briefly and add straight away 100 ml of 25% NP-40, mix thoroughly
6. Place the tube in -20°C overnight

Second day:

1. Slowly melt the tube and centrifuge at 3100 rpm for 15 minutes
2. Discard supernatant and add 1,6 ml of TKM2, pipette slowly to form a homogeneous mixture. Add 80 ml of 10% SDS and finally 0.8 ml of TKM2. Pipette to form homogeneous mixture
3. Place tube in thermostat at 56°C for 20 minutes
4. Add quickly 800 ml 5M NaCl, pipette to form homogenous mixture
5. Centrifuge at 3100 rpm for 15 minutes
6. Pour supernatant to previously prepared 50 ml tube, where is 2,5 volumes of cold (-20°C) 96% ETOH
7. Formed DNA clot spin around stick (e.g Pasteur pipette) and place it in ~1,5 ml of cold 70% ETOH
8. Place the stick upwards for air-drying
9. Dissolve the DNA in TE (~300 , ml), let it dissolve at end-over at least for 24 hours

REAGENTS

TKM1:

- 10 ml of 1M TrisHCl
- 10 ml of 1M KCl
- 5 ml of MgCl₂
- 4 ml of 0.5M EDTA
- MQ

TKM2:

- 25 ml 5M NaCl
- TKM1 till 200 ml

25% NP-40 (nonident):

- 25 g of NP-40
- MQ till 100 g

10% SDS

5M NaCl

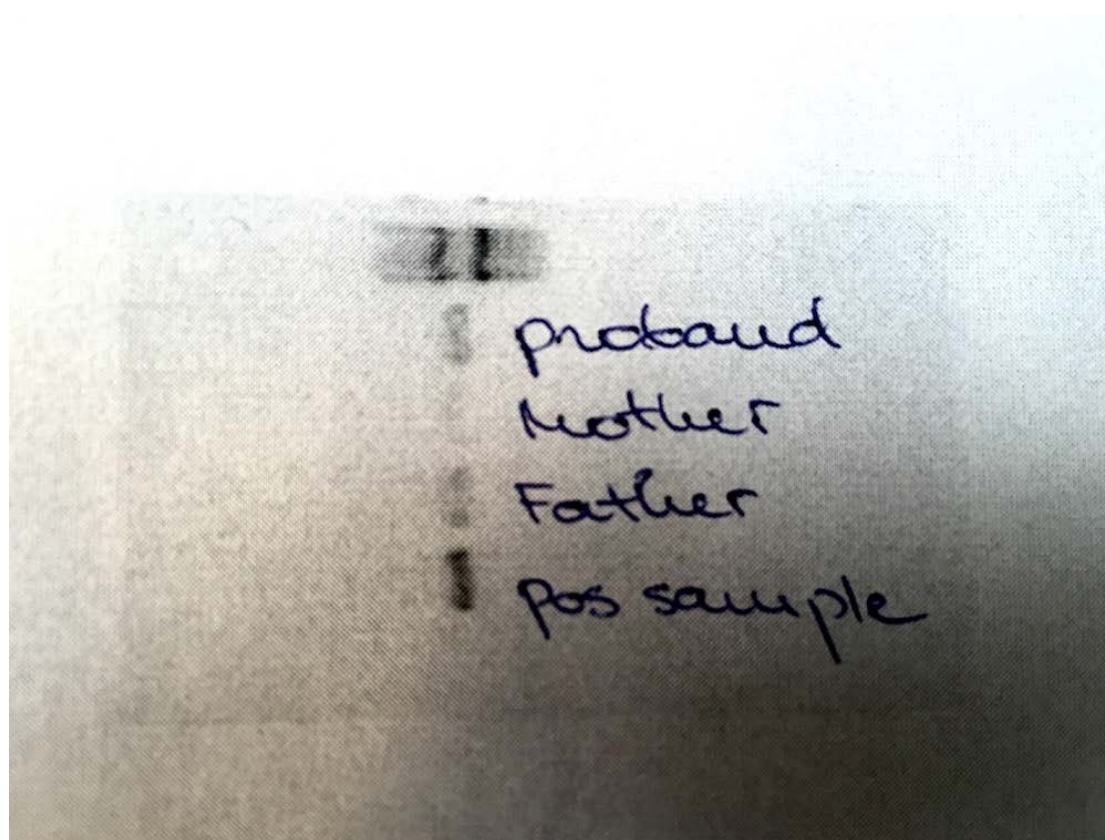
Supplement 4. The extraction protocol for DNA.

Primary denaturation	95°C	15 min
denaturation	95°C	30 sec
binding of primers	62°C	30 sec
DNA synthesis	72°C	40 sec

5 cycles using touchdown method		
denaturation	95°C	30 sec
binding of primers	59°C	30 sec
DNA synthesis	72°C	40 sec

35 cycles		
Final elongation	72°C	10 min

Supplement 5. The PCR program for Sanger sequencing.



Supplement 6. Agarose gel electrophoresis of genomic DNA.

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