Università degli Studi del Piemonte Orientale
"Amedeo Avogadro"

Dipartimento di Scienze del Farmaco

Dottorato di Ricerca in Biotecnologie Farmaceutiche ed Alimentari

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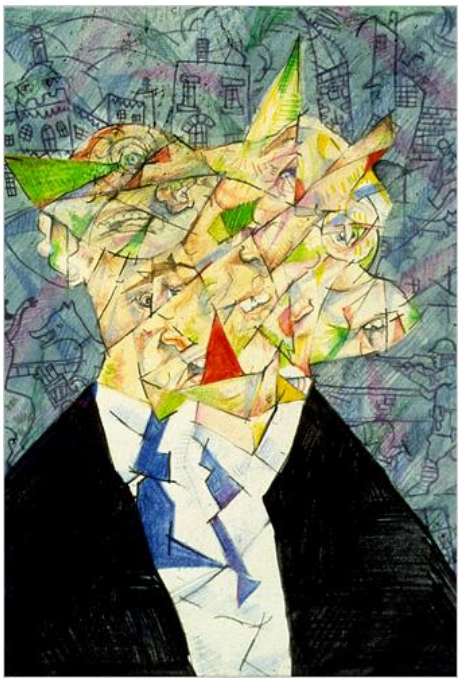

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"Variability is the law of life,
and as no two faces are the same, so no two bodies are alike,
and no two individuals react alike and behave alike under the abnormal conditions
which we know as disease."

Sir William Osler, 1903. *Yale Medical Journal,* Vol. IX, No. 10, p.325

Contents

Chapter 1

Introduction

Headache disorders are ubiquitous, with almost half of the world's adult population having recently experienced one or more headache types [1]. Migraine, tension-type headache and medication overuse headache (MOH) are currently the most common headache disorders worldwide, leading to significantly reduced patients' quality of life and higher costs for society [2]. The Global Burden of Disease Study 2013 (GBD2013) reported migraine as the sixth highest cause of disability in the world and, for the first time, highlighted the relevance of MOH as the 18th most disabling disease [3]. Despite the substantial contribute to public-ill-health of such headache types, their burden is still paradoxically ignored.

Migraine as well as medication overuse headache still remain under-recognized, under-diagnosed and under-treated, with more than 50% of patients worldwide estimated to be primarily self-treated and not consulting a specialist [1].
In the light of the aforementioned remarks, healthcare for headache disorders has the need to be still more improved at multiple levels, with the final aim of successfully diagnose and effectively treat these neurological conditions, primarily including migraine and MOH.

1. Migraine

Migraine is a common primary headache disorder that affects around 10% of the worldwide adult population [4] [5], with a male to female ratio of 1:3 [6].
This neurobiological condition is characterized by altered brain sensitivity and its clinical presentation, in terms of pain intensity, attack frequency and associated

symptoms, broadly varies among migraineurs [7]. Individual variability in migraine phenotype has emerged as presumably resulting from the interaction between multiple genetic and non-genetic (endogenous or exogenous) risk factors [8]. Supporting this, genetic epidemiologic studies reported migraine as a strongly heritable disorder with a substantial risk of familiar occurrence. Twin studies have revealed that almost 50% of migraine susceptibility risk could be attributable to additive genes while the remainder may be due to shared or unshared environmental factors among twins [9]. Exposure to stress, odours, bright lights and sounds, hypoglycemia, consumption of certain foods (for example chocolate, salami, milk, alcohol and excessive caffeine intake) smoking, and sleep disturbances are now listed among potential exogenous triggers of migraine attacks in predisposed subjects [10].

Gender differences in migraine occurrence were also hypothesized on the basis of epidemiological evidences reporting a major prevalence of migraine in females compared to males. Migraine is more common in women than in men during adulthood, with a prevalence peak in women's fertile age. However, this imbalance in terms of prevalence between sexes does not occur in childhood and old age, when the disorder tends to affect the same percentages of males and females [11]. Fluctuation of female sex hormones levels have been proposed as the more plausible endogenous factor responsible for variations of migraine prevalence throughout female life span [12]. Consistent with this, stable and low levels of ovarian hormones, characterizing childhood as well as natural menopause, emerged to be correlated with lower migraine frequency. On the contrary, reduced levels of estradiol and progesterone, typical of menstruation, resulted triggering frequent migraine attacks [13]. In addition to hormonal causes, sex differences in pain perception and stress response have been proposed as additional plausible factors explaining gender differences in migraine [14].

From a physiological viewpoint, a migraine attack could be defined as a complex sequence of brain events that can last from hours to days. For descriptive purposes, several "phases" can be simplistically identified in a migraine attack, each of them corresponding to specific chemical, biological and anatomical mechanisms partially overlapping in the sequence of migraine phases themselves. These phases, chronologically ordered, are named *premonitory*, *aura* (if present), *headache* and *postdrome* phase. It must be highlighted that not all aforementioned phases are manifested by all migraineurs or present in all attacks.

More than 80% of adults and a slightly lower percentage of children experience *premonitory symptoms*, which can occur up to hours before headache. The most commonly reported premonitory symptoms preceding headache are mood changes, fatigue, irritability, difficulty concentrating, stiff neck, phonophobia and nausea. Other symptoms that have been reported in this phase include change in appetite, bloating, piloerection and change in facial expression or body perception. Some symptoms can resolve before the headache phase, whereas others can increase in intensity in headache phase and furthermore persist during the postdrome phase [15].

A consistent proportion of migraineurs also manifests a set of neurological symptoms, called *aura*, which usually occur after premonitory signals and before the headache phase. Sometimes, migraine aura begins after the pain phase has commenced or continues into the headache phase. The most common type of aura is the visual one, characterized by visual symptoms ranging from the most common scintillating scotoma to the rarest visual hallucination. Some subjects can also subsequently experience sensory and language aura, respectively characterized by migrating paresthesias and altered language capacity [16].

The migraine *headache phase* properly named is characterized by headache pain, usually described as of pulsating quality and with unilateral localization. Headache

pain tends to occur in a gradual manner till reaching a stable moderate or severe intensity during the remaining part of headache phase. Photophobia, phonophobia, nausea and vomit can frequently accompany headache pain [17].

After headache resolution, symptoms such as tiredness, weakness, cognitive difficulties, mood changes, residual head pain, dizziness and gastrointestinal symptoms are frequent among migraineurs. These symptoms characterize migraine *postdrome phase*, which can occur from hours to days after resolution of headache. The overlapping between premonitory and postdrome symptoms fostered the hypothesis that postdromal signs could be present throughout the attack, probably disguised by headache, nausea or, if present, aura [18].

1.1 Clinical presentation

The International Classification of Headache Disorders, version III beta (ICHD-IIIb) recognizes two major subtypes of migraine, which are *migraine without aura* and *migraine with aura* [17]. These migraine types are not exclusive but can co-occur in the same migraineur, either alternating or at different phases of patient's life [19].

Migraine without aura (MwoA), previously named "common migraine" or "hemicrania simplex", is defined as a recurrent headache disorder with headache attacks lasting between 4 and 72 hours and characterized by headache pain having at least two of the following characteristics: typical unilateral location, moderate or severe intensity, pulsating quality and aggravation by routinary physical activity, such as walking or climbing stairs. Nausea, vomiting, photophobia and phonophobia may accompany migraine [17].

Table 1: ICHD-IIIb diagnostic criteria for migraine without aura [17].

CRITERIA	DESCRIPTION
A:	At least five attacks fulfilling criteria B–D
B:	Headache attacks lasting 4-72 hours (untreated or unsuccessfully treated)
C:	Headache has at least two of the following four characteristics: 1. unilateral location 2. pulsating quality 3. moderate or severe pain intensity 4. aggravation by or causing avoidance of routine physical activity (e.g. walking or climbing stairs)
D:	During headache at least one of the following: 1. nausea and/or vomiting 2. photophobia and phonophobia
E:	Not better accounted for by another ICHD-3 diagnosis.

In both men and women, migraine without aura is more common than migraine with aura and often seems to correlate with menstrual status in women [11]. On the contrary, the frequency of migraine without aura attacks usually decreases during pregnancy, when the levels of ovarian hormones in serum are stable and high [14]. In children and adolescents aged under 18 years and affected by MwoA, the headache pain is more often bilateral than in adults and unilateral pain usually emerges in late adolescence or in early adult life [17].

Around 20-30% of migraineurs experience an additional complex of transient neurological symptoms, called *aura*, which can precede or accompany headache pain. [19]. *Migraine with aura* (Mwa), previously also named “classic or classical

migraine" "ophthalmic, hemiparaesthetic, hemiplegic or aphasic migraine" or "complicated migraine", is defined by ICHD-IIIb as a migraine characterized by "recurrent attacks, lasting minutes, of unilateral fully reversible visual, sensory or other central nervous system symptoms that usually develop gradually and are usually followed by headache and associated migraine symptoms" [17].

More precisely, migraine aura is defined as a fully reversible neurologic dysfunction, characterized by a gradual onset of specific symptoms, which usually last between 5 and 60 minutes. Visual aura is the most common type of aura, occurring in 99% of migraineurs [20]. Migraineurs describe visual impairments symptoms characterized by "a zigzag figure near the point of fixation that may gradually spread right or left and assume a laterally convex shape with an angulated scintillating edge, leaving absolute or variable degrees of relative scotoma in its wake" [17]. Posphenes, white or coloured dot, curved lines or other geometric forms may also be seen by migraine aura patients.

Sensory aura is the next more frequent aura symptoms, affecting around 54% of MwA patients [21], followed by language aura, which is experienced by 32% of MwA subjects [22]. Sensory aura, is often described as migrating paresthesias, with numbness typically occurring in the hands and then affecting arms, face, lip and tongue (cheiro-oral sensory changes). Language aura may consist of impaired language comprehension and speaking, accompanied or not by decreased ability to read or write. It should be noted that sensory aura may lead to stuttering words and should not be confused with language aura [23]. If co-occurring, aura symptoms types usually follow one other in succession, starting from visual and ending with aphasic symptoms [17].

Table 2: ICHD-IIIb diagnostic criteria for migraine without aura.

CRITERIA	DESCRIPTION
A:	At least two attacks fulfilling criteria B and C
B:	One or more of the following fully reversible aura symptoms: 1. visual 2. sensory 3. speech and/or language 4. motor 5. brainstem 6. retinal
C:	At least two of the following four characteristics: 1. at least one aura symptom spreads gradually over ≥ 5 minutes, and/or two or more symptoms occur in succession 2. each individual aura symptom lasts 5-60 minutes 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.

Aura symptoms may worsen or appear for the first time during pregnancy, highlighting the role of high estrogen levels in the development of migraine with aura [11].

Some patients may also experience migraine aura followed by a less distinct headache or even without headache. In the first case, they may suffer from *typical aura with headache*, characterized by aura accompanied or followed within 60 minutes by headache with or without migraine characteristics. In the latest case

patients are diagnosed as having had *typical aura without headache*, in which aura is neither accompanied nor followed by headache of any sort within 60 minutes [17].

1.2 Pathophysiology

Migraine pathophysiology has greatly evolved in the last century till currently describing migraine as a "neurovascular" disorder, resulting from the interaction between vascular and neurological events.
The exact cause of migraine is still not completely understood but some key events characterizing migraine pathophysiology have emerged, such as the phenomenon of *cortical spreading depression* and the activation of trigeminovascular systems accompanied by neurogenic inflammation [24].

Historically, two independent theories explaining migraine etiology were proposed. The "vascular theory" was initially introduced by Thomas Willis in 1664, who suggested, for the first time, the involvement of vasodilatation of cerebral and meningeal arteries in migraine onset [25]. In the 20th century Graham and Wolff refined the aforementioned theory, highlighting that the vascular event was mediated by an initial intracranial vasoconstriction subsequently followed by a rebound vasodilatation of the extracranial terminal branches of the external carotid artery. Consistent with this hypothesis was the observed pulsating quality of migraine pain, which they attributed to the amplitude of pulsation of the occipital and superficial temporal branches of the external carotid artery [26].
The alternative theory subsequently proposed was the "neurogenic" one, which reported neuronal networks dysfunctions as key mechanisms underlying migraine pathogenesis. Support for the neurogenic theory came from the fact that typical

neurological symptoms of migraine aura could not be explained by a vascular pathophysiological model alone. In 1944, Aristides Leão, described for the first time the phenomenon of *cortical spreading depression* (CSD), defined as "a self-propagating wave of depolarization that begins in the neuronal/glia cells of local areas of the brain and subsequently spread in all directions at a rate of ≈ 3 mm/min" [27]. Milner in 1958 and then Olsen in the 1980s caught, for the first time, similarities between CSD phenomenon and aura symptoms (previously described in 1941 by the psychologist Karl Spencer Lashley) and fostered pathogenic theories changing from primary vascular to primary neuronal mechanism [28].

However, even if aura is a symptom exclusive for a specific migraine subtype, the observation of the possible co-occurrence of the two subtypes of migraine in the same subject suggested that these two conditions could share at least some mechanisms involved in the initiation and resolution of attacks.

In this context, Moskowitz and collegues, in the 1980s, integrated the vascular theory with the neurogenic one and proposed the "trigeminovascular theory". They hypothesized that probably CSD could depolarize the trigeminocervical nerve terminals innervating meninges which in turn may release pro-inflammatory peptides responsible for "neurogenic inflammation", consisting in meningeal vessels vasodilatation and plasma protein extravasation. Such neurogenic inflammation was hypothesized to lead to migraine pain [29].

Nowadays, migraine is viewed as a complex neurological disorder that affects multiple cortical, subcortical and brainstem areas that regulate several functions, including autonomic, affective, cognitive and sensory ones. The interaction between neurons, glia and blood vessels still appears to be crucial in migraine pathogenesis, with CSD phenomenon and activation of trigeminovascular system accompanied by neurogenic inflammation being recognized as key events implicated in migraine pathogenesis.

In more detail, trigeminovascular system consists of trigeminal nerve and nerve fibers innervating intra- and extra- cranial meningeal blood vessel as well as brainstem [30]. The trigeminovascular system role is to regulate both neurotransmission of pain signals and vascular tone. The transmission pathway originates in trigeminal ganglion neurons whose central axons reach the nociceptive dorsal horn laminae of the spinal trigeminal nucleus. Here, the nociceptors converge on neurons receiving additional inputs from the periorbital skin and precranial muscles. The ascending axons of spinal trigeminal nucleus neurons convey nociceptive signals to brainstem, hypothalamic and basal ganglia nuclei, which overall seem to be crucial in mediating symptoms typical of migraine attacks, such as nausea initiation, vomiting, yawning, loss of appetite, anxiety, irritability and lacrimation [7]. Trigeminal sensory nerves store several vasoactive neuropeptides, including substance P, calcitonin gene-related peptide (CGRP), neurokinin A, nitric oxide (NO) and pituitary adenylate cyclase-activating peptide (PACAP). These neuropeptides, when released, are crucial in provoking an increase in blood flow and vasodilation in meningeal vascular system, where edema and inflammation occur, conceivably causing headache pain. Supporting the hypothesis of trigeminovascular system activation is the evidence of increased levels of CGRP both in external and internal jugular blood of migraineurs during headache attack [31].

Recently Amin and colleagues identified, through a magnetic resonance angiography imaging technique, that the release of neuropeptides and proinflammatory substances (e.g. histamine, bradykinin, serotonin and prostaglandins) from trigeminal afferents in the meningeal vessels, results in an altered molecular environment causing *sensitization* of both peripheral and central neurons of trigeminovascular system. Once sensitized, peripheral trigeminovascular neurons begin to respond to stimuli to which they showed minimal or no response at baseline [32].

Endogenous events that may trigger trigeminovascular system, and so neuropeptides release, are still largely unknown. Several experimental models report CSD as an important upstream trigger of trigeminovascular system activation. Cortical SD seems to activate trigeminovascular pathway, leading to a prolonged neurogenic inflammation around meningeal vessels [33]. Even if it is not clear how CSD begins in human brain, genetic factors are likely to play a substantial role in modulating individual CSD susceptibility by regulating brain excitability.

1.3 Genetic basis of migraine

The current knowledge of genetic basis of migraine has come from different scientific approaches, encompassing linkage studies in family pedigrees and candidate genes or genome-wide association studies.

Much interest of geneticists has been initially focused on the study of genetic basis of familial hemiplegic migraine (FHM), a rare subtype of migraine with aura, characterized by a prolonged visual aura typically accompanied by hemiparesis [24]. FHM is inherited in an autosomal dominant fashion and was identified as the first primary headache disorder with a genetic basis. The substantial overlapping between phenotypes of FHM and migraine with aura fostered FHM to be considered a good model to study the genetic architecture of migraine. In this context, CACNA1A, ATP1A2 and SCNA1A have been identified as causative genes in FHM. More precisely, the proteins encoded by these three genes form channels involved in the regulation of ions flow across neuronal and glial cell membranes. Collectively, these genes regulate glutamate availability in the synaptic cleft by means of a fine tuning of glutamate release and re-uptake as well as the generation of action potentials. It has been hypothesized that mutations in all

three genes may result in an increased efflux of glutamate and potassium in the synaptic cleft, in turn leading to an increased susceptibility to cortical spreading depression. Mutations in these genes specifically identify three different forms of FHM, named, respectively, FHM1, FHM2 and FHM3 [7]. However, these identified genetic variants do not account for the totality of FHM cases and it has been suggested that additional genetic variants resulting in altered ions flow may lead individuals to be more susceptible to FHM [34] [35].
Strongly supporting the hypothesis of common genetic basis between FHM and other migraine subtypes is the subsequent evidence provided by Cuenca-Leon and colleagues which, in 2009, reported 14q32 locus as a shared susceptibility locus in a Spanish family affected by FHM, MwoA and MwA [36].

Numerous linkage studies were also performed on families of different ethnic origin. Several migraine susceptibility loci have been identified in a wide range of chromosomes, suggesting how migraine could be substantially considered as a polygenic disease [37]. However, many of the findings in the field have not been replicated in populations of different origin. Heterogeneity of analyzed cohorts, both in terms of phenotype and diagnosis, or the existence of rare high-impact family specific markers may explain, almost in part, inconsistency of results between studies.

Another commonly used approach to identify genetic variants potentially influencing migraine susceptibility is represented by the conduction of candidate-genes association studies performed in a case-control setting. These studies generally attempted to investigate genetic basis of migraine through the analysis of tagging SNPs or selected functional variants located on specific genes plausibly involved in migraine susceptibility. On the basis of available evidences regarding migraine pathophysiological mechanisms, the majority of studied SNPs were located in genes primarily involved in neurological, hormonal and vascular

functions. Among neurological candidate genes, genes involved in serotonin neurotransmission have been extensively studied. Even if the exact mechanisms of the serotoninergic system in migraine are still unknown, a deficit on 5-HT descending pain inhibitory system is still probably today the most implicated in migraine pathophysiology [38]. These genes included 5-HT1B, 1D and 2C receptors, SLC6A4 (encoding for SERT, a serotonin transporter), TPH2 (encoding for tryptophan hydroxylase, a rate-limiting enzyme in the synthesis of serotonin), and MAOA (encoding for monoamine oxidase A enzyme which degrade serotonin). Similarly, genes regulating dopaminergic neurotransmission were studied, such as DRD2 (dopamine receptor D2), DRD3 (dopamine receptor D3), SLC6A3 (encoding for the dopamine active transporter), DBH (encoding for dopamine beta hydroxylase, an enzyme converting dopamine into noradrenaline), TH (tyrosine hydroxylase) and COMT (catechol-O-methyltansferase). In the same way, genes implicated in glutamate transmission, such as GRIA1 (glutamate receptor 1) and GRIA3 (glutamate receptor 3) were considered as well good candidate genes [37].

As previously mentioned, fluctuations of hormonal levels, in particular estrogen, seem to trigger migraine attacks. In this context, genetic variations on ESR1 (encoding for estrogen receptor 1) and PGR genes (progesterone receptor gene) emerged as potential predictors of migraine susceptibility [39] [40]. Being vascular events also crucial in migraine pathogenesis, genetic variations in CGRP (calcitonin gene related peptide), ACE (angiotensin I-converting enzyme), MTHFR (methylenetetrahydrofolate reductase) and NOTCH3 were analyzed in correlation with migraine risk [37].

However, even if several replication studies were performed to validate significant associations between SNPs in all aforementioned genes and migraine risk, a consistent part of these results still remains controversial or inconclusive. The small effect size of these genetic variants, the generally small sample size of studies

as well as the strong heterogeneity among migraineurs included in the patient sets may explain, almost in part, the difficulty in replicating such results.

A recent advance in the knowledge of genetic basis of migraine has been provided by genome-wide association studies (GWAS). More precisely, two GWAS were performed on clinic based collections of patients affected by MwA [41] or MwoA [42] whilst two other were conducted on population-based collections of migraineurs [43] [44]. A meta-analysis performed by Anttila and colleagues in 2013 quantitatively summarized previously reported results in the four GWAS added together with unreported GWAS findings for migraine susceptibility obtained in additional 9 population-based collections [45]. A total of 12 loci emerged as significantly associated with migraine susceptibility. Among them, 5 loci were new (near AJAP1, near TSPAN2, FHL5, c7orf10, and near MMP16) while 7 confirmed previously found migraine loci (PRDM16 [43], MEF2D [42], TRPM8 [41] [43], near TGFBR2 [42], PHACTR1 [42], ASTN2 [42], and LRP1 [43]). More in detail, LRP1 modulates synaptic transmission through the NMDA receptor while MEF2D is involved in glutamatergic excitatory synapse. AJAP1, TSPAN2 and MMP16 (whose encoded protein cleaves LPR1) seem to regulate activity of metalloproteinase, which are responsible for the breakdown of extracellular matrix in normal physiological processes. ASTN2 and FHL5 are involved in neuronal neurodevelopment and synaptic plasticity.

Overall, these results support the importance of glutamatergic neurotransmission and neuronal development/plasticity in migraine pathogenesis and, in conjunction with all evidences retrieved from other genetic studies, make conceivable the hypothesis of a “generalized” neuronal hyperexcitability in migraine brain.

1.4 Pharmacological treatment

The pharmacological treatment of migraine traditionally includes:
i) the *acute/symptomatic treatment*, able to relieve headache pain and corollary symptoms accompanying migraine;
ii) the *prophylactic therapy*, aimed to reduce intensity and frequency of headache attacks.

The Italian guidelines for primary headache (revised version 2012) recommend the symptomatic treatment alone when migraine attacks are not-disabling or if they occur < 4 days per month. On the contrary, preventive therapy is suggested when disabling migraine attacks are present for ≥ 4 days per month or in case of poor response to symptomatic drugs [46].

Acute treatment

Nowadays, several drugs are available for symptomatic pharmacological treatment of migraine. Antimigraine drugs can be divided in *specific antimigraine drugs*, characterized by being effective only for headache pain (i.e. triptans and ergot derivates), and *non-specific antimigraine drugs*, resulting effective in relieving pain of several origins, including headache (e.g. non-steroidal anti-inflammatory drugs, simple analgesics and antiemetics) [47].
The Italian guidelines for primary headache (revised version 2012) propose a "stratified approach" when the initial pharmacological acute treatment of migraine attacks must be outlined. More precisely, *specific drugs* are recommended for the treatment of moderate or severe attacks while *non-specific drugs* should be administered for the therapy of migraine attacks of mild/moderate intensity. Moreover, preparations with only one active principle should be preferred and the

most appropriate drug should be taken at the lowest effective dosage and as early as possible from migraine attack onset [46].

As mentioned before, *specific antimigraine drugs* include triptans and ergot derivates.

Triptans are considered by many specialists as the gold-standard therapy of migraine. However, there's a lack of knowledge regarding the complexity of their hypothesized multiple mechanisms of action. Their action is primarily attributed to their agonist effect on serotonin $5HT1_{B/D/F}$ receptors, resulting in the vasoconstriction of meningeal vessels and in the inhibition of the neurogenic inflammation. In addition to this well-known mechanism, recent evidences support the potential action of triptans on modulating calcium and potassium currents in dural-projecting trigeminal neurons in vitro [48].

Nowadays, six triptans are on Italian market: sumatriptan, frovatriptan, eletriptan, rizatriptan, almotriptan, zolmitriptan. All triptans are available in conventional oral formulations. Sumatriptan is also marketed in other several formulations, such as nasal spray, suppository and subcutaneous, the latest one being considered the most effective one [49]. Zolmitriptan has also the nasal spray formulation and, as rizatriptan, is available in rapid-dissolving formulations (RPD), which have an effectiveness similar to that of tablet formulations of the same drugs at the same dosages [50], [51].

Triptans differ from each other in terms of specific pharmacokinetic and lipophilic properties [52].

Table 3: Pharmacokinetic parameters and lipophilicity of oral triptans available on Italian market [52].

Drug	Tmax (h)	$T_{1/2}$ (h)	Bioavailability (%)	Lipophilicity Log $D_{ph=7.4}$	Relative brain penetration
sumatriptan	2.0-3.0	2	14	-1.5	low
almotriptan	1.5-2.0	3	70	-2.1	low
frovatriptan	2.0-3.0	26	24-30	-1.0	high
rizatriptan	1.0-1.5	2	40-45	-0.7	high
zolmitriptan	1.5-2.0	3	41-48	-1.0	high
eletriptan	1.0-1.5	4	50	+0.5	high

T_{max}: time to maximum concentration; $t_{1/2}$: biological half-life; Log $D_{ph=7.4}$ quantifies triptan lipophilicity (increasing numbers indicate greater lipid solubility).

They are all effective on relieving migraine pain, associated symptoms and functional disability, without provoking common relevant side effects in patients. Only the 4-5% of migraineurs treated with subcutaneous sumatriptan experience the "triptan syndrome", characterized by chest pain and chest and neck tightness, while the 2-4% of subject also complaint symptoms such as somnolence, dizziness, paresthesia, asthenia, nausea and facial flush [46]. Myocardial infarction and ictus related to triptan use were rarely reported. Contraindications to triptans are uncontrolled blood hypertension, coronary artery disease, history of ischemic stroke, peripheral artery disease, pregnancy, lactation and age <18 years and > 65years.

Despite the well documented efficacy of triptans, about one third of migraineurs do not respond to a specific triptan [53]: in this case, other triptans can be administered and, if ineffective, non-steroidal anti-inflammatory drugs can be used [54].

Ergot derivates have been used for many years in migraine treatment before the advent of triptans. These drugs are partial agonists of α-adrenergic and dopaminergic receptors and interact with $5HT1_{B,D,F}$ receptors, so resulting in vasoconstriction of meningeal vessels and inhibition of pro-inflammatory neuropeptides release.

Ergotamine (not available in Italy) and dihydroergotamine are the most common used ergot derivates and have emerged to be more effective vs placebo in migraine treatment, but less effective compared to triptans [55]. Their use should be restricted to migraineurs which fail to be responsive to other drugs. Ergot derivates may worsen nausea and vomiting in migraineurs, so contemporary administration of antiemetic drugs is generally indicated. Major side effects include nausea, vomiting, diarrhea and ergotism. Cardiovascular and cerebrovascular diseases, uncontrolled blood hypertension, Raynaud disease, renal failure, pregnancy and lactation represent contraindications for ergot derivates treatment.

Non-specific antimigraine drugs include non-steroidal anti-inflammatory drugs, simple and combination analgesics, antiemetics, barbiturates, lidocaine and steroids.

Non-steroidal anti-inflammatory drugs (NSAIDs) are indicated for the treatment of migraine attacks of mild or moderate intensity or when triptans resulted ineffective or contraindicated [46]. Acetaminophen, acetylsalicylic acid, ibuprofen, naproxen sodium, and diclofenac potassium have good evidence supporting their use for migraine. Among the latest, acetaminophen is considered less effective than other NSAIDs and is generally administered for the treatment of relatively mild migraine attacks [56]. Acetylsalicylic acid is recommended in subjects with cardio- and cerebrovascular comorbidities. Only ibuprofen (the most commonly used one), ketoprofen and morniflumate can be used in patients under 14 years of age. Due to

their well-known mechanisms of action, adverse events mainly consist in gastrointestinal toxicities (e.g. gastric pain, gastric or duodenal ulcer).

Concerning simple analgesics, paracetamol has a good efficacy on mild-moderate migraine pain and corollary symptoms and it is the first-choice drug for migraine treatment during pregnancy.

Analgesics in combination have the same indication of NSAIDs and simple analgesics. Analgesic combinations available in Italy include: acetylsalicylic acid + acethaminophen + propyhenazone, acetylsalicylic acid + acethaminophen + indomethacin (with/without caffeine), and acetaminophen + propyphenazone and acetaminophen + codeine. Side effects and contraindications of combinations are the same as those for each component [46].

Antiemetics are considered adjuvant drugs in migraine therapy, especially during attacks characterized by prominent nausea and vomiting. The association between antiemetics and other classes of antimigraine drugs, such as NSAIDs, ergotamine or triptans, may result in a major absorption of antimigraine drugs and in the effective treatment of nausea and vomiting. Among common used antiemetics, metoclopramide emerged effective in reducing headache pain if intravenously administered alone in migraineurs [57].

Barbiturates are usually prescribed in addition to other antimigraine drugs. However, this class of drugs should in principle be avoided because barbiturates may induce intoxication, addiction and dependence.

Steroids, such as dexamethasone and prednisone, seem also to be effective in migraine treatment but available findings are conflicting. Only one study demonstrated the superiority of the association dexamethasone-rizatriptan compared with rizatriptan alone in women affected by menstrual migraine.

New specific drugs for the abortive treatment of migraine: a focus on CGRP as a therapeutic target

In the light of the substantial abundance of non-responders among migraineurs treated with triptans, a digression concerning new developed drugs representing a valid alternative to triptans is mandatory.

The evidence of CGRP involvement in the transmission of pain [58] as well as its release during migraine attack [31], fostered the hypothesis that CGRP could be a potential good target for new drugs. In this context, CGRP receptor antagonists emerged as effective molecules compared to placebo. CGRP receptor antagonists include, telcagepant, olcegepant, rimegepant, MK-3207, BI 44370 TA and MK-1602. However, despite the promising results in term of efficacy, the drug development programmes of all these new drugs terminated because of different reasons, including toxicity evidences and commercial decision. Only MK-1602 is in phase 2 study [59].

In addition to CGRP receptor antagonist, four CGRP monoclonal antibodies (mAbs) were developed, of which three targeting CGRP ligand (LY2951742, ALD-403 and TEV-48125) and one targeting CGRP receptor (AMG 334). The mAbs against the ligand are thought to remove CGRP that is released at perivascular trigeminal sensory nerve fibers, while the receptor mAbs block CGRP signalling. Preliminary data showed positive results in terms of efficacy and safety for all four mAbs. However, subcutaneous and intravenous administration routes, that are mandatory for mAbs, represent a significant limitation for their everyday use in clinical practice [60].

Prophylactic therapy

As previously mentioned, preventive therapy is recommended when disabling migraine attacks are present for ≥ 4 days per month or in case of poor response to symptomatic drugs. A wide range of drugs have been studied for the purpose of

reducing both frequency and intensity of migraine attacks. However, the majority of preventive medications currently available were not specifically design for migraine treatment and their use is often limited by their toxicities or eventual inefficacy.

Preventive drugs must be chosen on the basis of patient's comorbidities, with a particular attention to drug-drug and food-drug interactions. To minimize side effects, the most appropriate drug should be administered at the lower dose and preferentially as a monotherapy. The preventive therapy will be considered effective if the frequency of migraine attacks will be reduced by at least half and if a significant improvement in quality of life will be reached. Clinical benefit may take some time to be obtained, so preventive treatment should be maintained for at least 3 months. Due to the teratogenic effect of the majority of prophylactic drugs, preventive treatment during pregnancy should be limited to special situations for which the use of drugs with the lowest teratogenic effect should be preferred [46].

Preventive drugs include beta-blockers, calcium channel blockers, antidepressants, antiepileptic drugs, angiotensin inhibitors, botulinum toxin A and other supplements. However, few evidences clearly stated their efficacy and no universally accepted guideline unequivocally suggest the preventive treatment of choice for migraine. Depending on the methodological assessment underlying guidelines development, the levels of recommendation reported for every single active principle broadly vary among national and international guidelines.

Table 4 simplistically summarized the most commonly recommended preventive drugs on the basis of two Italian [46] [61] and five international guidelines [62] [63] [64] [65] [66] for preventive migraine treatment. No emphasis was herein put on the levels of recommendation for single drugs reported by each guideline. Specific indications for each class of drugs are also reported.

Table 4: Prophylactic drugs for migraine.

Prophylactic medications		
Pharmacological class	*Molecule*	*Indications* [46]
Beta-blockers	metoprolol [46] [61] [62] [63] [64] [65] [66] propranolol [46] [61] [62] [63] [64] [65] [66] atenolol [46] [62] [63] [65] [66] nadolol [46] [61] [63] [65] [66] bisoprolol [46] [62] [64] [65] timolol [63] [65] [66] nebivolol [65] [66]	First-choice treatment for patients with hypertension or tachycardia
Angiotensin inhibitors	candesartan [46] [64] [65] [66] lisinopril [46] [64] [65]	Second-choice treatment for patients with concomitant hypertension
Calcium channel antagonists	flunarizine [46] [61] [63] [66]	Particularly indicated for patients with concomitant anxiety and insomnia
Antiepileptic drugs	topiramate [46] [61] [62] [63] [64] [65] [66] sodium valproate [46] [61] [62] [63] [64] [65] [66] gabapentin [46] [63] [64] [65] [66] lamotrigine [46][65]	First choice treatment of high-frequency migraine attacks, chronic migraine, MOH and in migraineurs with concomitant epilepsy
Antidepressants	amitriptyline [46] [61] [62] [63] [64] [65] [66] venlafaxine [46] [61] [63] [64] [65] [66] fluoxetine [46] [61]	Indicated for patients with anxiety and depression or concomitant tension-type headache
Analgesics	pizotifen [46] [61] [63][66]	NS
Other drugs	onabutlinum toxin A [46] [61] [64] [65] riboflavin [46] [64]	NS

NS: not specified

1.5 Pharmacogenetics of migraine

Pharmacogenetics is the study of the influence of individual genetic background on drug response, in terms of both therapeutic effect and drug safety. The identification of subpopulations inadequately responding to a specific drug may lead to a personalized pharmacological therapy, resulting in improved patients' quality of life and optimization of healthcare resources.

Despite rigorous pharmacogenetic studies have been carried out for a multitude of diseases with interesting and clinical-relevant results, surprisingly a very few pharmacogenetic evidences have been reported in migraine. Nevertheless, strong clinical evidences have highlighted a significant individual variability in response to all drugs routinely used for the acute treatment of migraine. A recent network meta-analysis, aimed to compare relative efficacy of triptans with respect to other acute antimigraine drugs, reported that the proportions of responders to abortive antimigraine drugs were 42-76% for triptans, 38% for ergots, 46-52% for NSAIs and paracetamol, and 62-80% for combination therapies [67]. In this context, non-response to acute medications can be hypothesized not to be only influenced by factors such as altered drug absorption, inadequate dosing or incorrect time of drug administration, but also by individual genetic background, plausibly impacting on both pharmacokinetic and pharmacodynamic drug variability. In addition to this, the observation that migraineurs may experience adverse reactions more frequently than other groups of patients [68] further supports the need of improving pharmacogenetic knowledge in migraine, which in turn may allow to unravel subpopulations of migraineurs that, at present, are inadequately treated by antimigraine drugs on the market.

With the aim of collecting all available pharmacogenetic evidences in episodic migraine, we performed in 2012 a systematic review of literature concerning

genetic predictors of response to acute or preventive drugs commonly used in migraine treatment [1]. We identified only 7 candidate-gene association studies investigating the correlation between genetic polymorphisms and clinical response to acute antimigraine drugs [69] [70] [71] [72] [73] [74] [75]. Despite the wide range of symptomatic drugs available for migraine therapy, all 7 pharmacogenetic studies were performed on patients exclusively treated with triptans. Triptan efficacy was always included as the primary outcome while four out of seven studies also investigated the potential correlation between gene polymorphisms and triptan-induced side effects as secondary endpoint [70] [71] [72] [75]. The polymorphisms studied were related to 5HT1BR (serotonin receptor 5HT1BR) [69] [71] [72] [73] [75], SLC6A4 (serotonin transporter SERT) [69] [73] [74], DRD2 (D_2 subtype of dopamine receptor) [69] [73] [74], MAOA (monoamine oxidase A) [69] [73], GNB3 (guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-3) [69] [74], 5HT2A ($5HT_{2A}$ receptor) [69], 5HT1F ($5HT_{1F}$ receptor) [70] , MTHFR (methylene tetrahydrofolate reductase) [69], ACE (angiotensin-converting enzyme) [69], ESR1 (estrogen receptor alpha) and TNF-B (tumor necrosis factor-beta) genes [69]. Among them, only DRD2 rs6275 and SLC6A4 Stin2 VNTR genetic variations have been reported to be potentially associated with triptan response, evaluated in terms of drug efficacy. More precisely, Asuni and colleagues reported that carriers of DRD2 rs6275 C/C genotype showed a better response to triptans compared to other DRD2 rs6275 genotypes [73]. Conversely, Ishii et al found an association between rs6275 C/C genotype and a lack of response to triptan class [69] whereas our group did not confirm any correlation between the aforementioned genetic variant and triptan response [74]. Moreover, we found a significant association between SLC6A4 Stin2 VNTR and response to triptans [74] but Ishii and collegues subsequently fail to replicate our results [69].

[1] Complete results of our systematic review are reported in Chapter 3.

Four additional pharmacogenetic studies investigating potential genetic predictors of triptan response were published after our database search for the systematic review of literature was performed. More precisely, three out of four studies were performed by our group [2], whereas the remainder was conducted by Christensen and colleagues in 2015 [76]. Christensen et al aimed to test the role of 12 SNPs, previously emerged to be correlated with migraine susceptibility [45], as genetic predictors of response to both acute antimigraine drugs (triptans and ergotamine) and prophylactic medications (beta-blockers, calcium antagonists, angiotensin II receptor antagonists and anti-epileptics) in two large cohorts of migraineurs. A number of tested SNPs showed a trend of association with the efficacy of triptans or some of the prophylactic drugs. Among them, only the genetic variant PRDM16 rs2651899 resulted significantly associated with the efficacy of triptans in the exploratory cohort. However, this correlation failed to be replicated in the validation cohort.

As previously mentioned, pharmacological treatment of migraine includes also preventive therapy. Through our systematic review of the literature, we identified only two pharmacogenetic studies investigating the role of genetic variants as predictors of preventive therapy response [77] [78]. One study did not report any correlation between ACE I/D polymorphism and the response to ACE inhibitors (i.e. lisinopril and candesartan) [77]. The other one found a potential association between non-H mitochondrial DNA haplotypes and better response to riboflavin [78]. No replication cohorts were present in these two studies.

After the publication of our systematic review, only one pharmacogenetic study investigating the role of genetic background in modulating the response to preventive drugs was reported in the literature [79]. This study included 80 migraine patients treated with tricyclic antidepressants (the molecules were not

[2] Complete results of the three candidate-gene association studies performed by our group are reported in Chapter 4, 5 and 6.

specified). Being nitric oxide involved in migraine pathogenesis, the genetic variant Glu298Asp of NOS3 gene was investigated as genetic predictor of tricyclic antidepressants response. As results, they found that homozygous carries of T allele of the Glu298Asp SNP showed a better response to tricyclic antidepressants compared to other genotypes. However, the absence of a replication cohort as well as the limited sample size of the study do not allow to consider these results as conclusive.

The data reported herein highlight that only little information is still available in the field of migraine pharmacogenetics. Given the high burden of migraine, in terms of high prevalence, disability and healthcare costs, it is surprising that a tool aimed to tailor and optimize antimigraine therapies has not received enough attention. Moreover, the absence of genome-wide association studies in the field emphasizes the immense distance between research in migraine genetics and migraine pharmacogenomics.

2. Medication overuse headache

Epidemiological evidences report that each year about 2.5% of people with migraine show a progressive worsening of headache clinical presentation, in terms of both increased frequency and severity of migraine attacks [80]. This "migraine transformation" usually takes place gradually over months to years [81] and leads to the development of *chronic migraine*, defined by the International Classification of Headache Disorders, version III- beta (ICHD-IIIb) as a "headache occurring on 15 or more days per month for more than 3 months, which has the features of migraine headache on at least 8 days per month" [17].

Chronic migraine has an estimated worldwide prevalence ranging from 1.2% to 2.2%, with a prevalence peak in women aged 18-49 years [82]. It mainly affects people during their most productive years of life, resulting in greater disability and higher direct/indirect costs than episodic migraine [83]. It is often comorbid with fatigue, sleep disorders, cerebrovascular disease, cardiovascular disease, and gastrointestinal problems. Compared to subjects affected by episodic migraine, patients with chronic migraine are twice as likely to have depression, anxiety, other chronic pain, bipolar disorders and respiratory illness, such as asthma and chronic obstructive pulmonary disease [84].

Epidemiological studies have identified several modifiable risk factors associated with chronic migraine, including obesity, depression, female sex, snoring, comorbid pain disorders, stressful life events (e.g. divorce, marriage, or change of employment status) and lower socioeconomic status [85]. However, the ICHD-IIIb reports that the most common risk factor for migraine chronicization is paradoxically represented by the overuse of any one or more antimigraine drugs routinely taken for acute headache pain relief. If some degree of medication

overuse co-occurs in chronic migraine patients, their headache disorder is specifically defined by ICHD-IIIb as *medication overuse headache*.

2.1 Clinical classification

Medication overuse headache (MOH), previously named "rebound headache", "drug induced headache" or "medication-misuse headache", is defined by ICHD-IIIb as a secondary chronic "headache occurring on 15 or more days per month developing as a consequence of regular overuse of acute or symptomatic headache medication (on 10 or more, or 15 or more days per month, depending on the medication) for more than 3 months. It usually, but not invariably, resolves after the overuse is stopped." [17].

Table 5: ICHD-IIIb diagnostic criteria for medication overuse headache [17].

CRITERIA	DESCRIPTION
A:	Headache occurring on ≥ 15 days per month in a patient with a pre-existing headache disorder
B:	Regular overuse for >3 months of one or more drugs that can be taken for acute and/or symptomatic treatment of headache
C:	Not better accounted for by another ICHD-3 diagnosis.

It is now accepted that all symptomatic medications may lead to MOH in susceptible patients. The ICHD-IIIb accurately report diagnostic criteria for each MOH "subtypes" according to the specific drug (or drugs) overused [17].

Table 6: ICHD-IIIb diagnostic criteria for MOH depending on drug(s) overused [17].

MOH SUBTYPE	DIAGNOSTIC CRITERIA
Ergotamine-overuse headache	Regular intake of ergotamine on ≥ 10 days per month for > 3 months.
Triptan-overuse headache	Regular intake of one or more triptans, in any formulation, on ≥ 10 days per month for > 3 months.
Simple analgesic-overuse headache (acetylsalicylic acid or paracetamol)	Regular intake of acetylsalicylic acid on ≥ 15 days per month for >3 months.
Other non-steroidal anti-inflammatory drug (NSAID)-overuse headache	Regular intake of one or more NSAIDs other than acetylsalicylic acid on ≥ 15 days per month for > 3 months.
Opioid-overuse headache	Regular intake of one or more opioids on ≥ 10 days per month for > 3 months
Combination-analgesic-overuse headache (intended as formulations combining drugs of two or more classes, each with analgesic effect or acting as adjuvants)	Regular intake of one or more combination analgesic medications on ≥ 10 days/month for > 3 months.
Medication-overuse headache attributed to multiple drug classes not individually overused	Regular intake of any combination of ergotamine, triptans, simple analgesics, NSAIDs and/or opioids on a total of ≥ 10 days per month for > 3 months without overuse of any single drug or drug class alone.
Medication-overuse headache attributed to unverified overuse of multiple drug classes	Regular intake of any combination of ergotamine, triptans, simple analgesics, NSAIDs and/or opioids on ≥10 days per month for > 3 months. The identity, quantity and/or pattern of use or overuse of these classes of drug cannot be reliably established.

Overall, it is estimated that MOH affects around 1% of general population, with a male to female ratio of 1:3-4 [1]. It's prevalence peaks in the forties and then decrease with older age [86].

Chronic analgesic consumption rarely induce MOH in non-headache patients, suggesting that MOH may results from an interaction between the overuse of acute medications and a susceptible patient [87].
The most common headache types affecting patients before MOH development are migraine (65%), tension-type headache (27%) and mixed/other headaches (8%) [88]. The desire to relieve pain and to normally carry out daily activities may lead these subjects to overuse migraine acute medications. The fear of experiencing severe pain and disability may also foster them to preemptively take acute medications at the first weak warning or, even worse, in anticipation of an attack [89]. In this context, it must be underlined that over-the-counter drugs are the most commonly overused antimigraine medications in primary care while triptans and other more potent, centrally acting, drugs emerged as the most overused ones by secondary e tertiary care patients [86]. Among overused drugs, triptans cause MOH faster and with fewer doses compared with ergots and analgesics [90]. In Europe, MOH is rarely cause by opioids overuse cause their prescription is largely discouraged in the medical environment [91].

Clinical presentations of MOH strongly vary among patients in terms of localization, quality and intensity of headache attacks. A plausible explanation underlying this variability in headache characteristics may consist in the fact that more than 90% of patients tend to concomitantly use different painkillers [88]. Common traits in MOH subtypes are morning headaches (possibly due to overnight drug withdrawal) and accompanying symptoms such as neck pain, cutaneous allodynia, rhinorrhea, lacrimation and gastrointestinal symptoms [92].
Risk factor for migraine transformation to MOH other than anti-migraine drug(s) overuse include tobacco smoke, sedentary lifestyle, low socioeconomic status and gastrointestinal complaints [93]. Chronic musculoskeletal pains, anxiety,

depression and obsessive compulsive disorder are often comorbid with MOH [94] [95].

2.2 Pathophysiology

The pathophysiological mechanisms underlying the development of MOH are not completely known.

As in migraine, the development of MOH seems to be triggered by cortical spreading depression (CSD). Neurophysiological studies demonstrated that an increased in neuronal excitability, at least in somatosensory and visual cortices, is identifiable in MOH patients. Supporting this, the electrical stimulation on the forehead or limb in MOH patients resulted in increased sensory-evoked cortical potentials that reverted/normalized after withdrawal from acute medications [96]. Moreover, chronic paracetamol or ergot use emerged leading to augmented frequency of CSD in rats [97].

Cortical changes are evident in MOH. More precisely, Coppola and colleagues in 2010 reported that MOH patients, especially those overusing NSAIDs, had an increased sensitization of somatosensory cortex [98]. By measuring glucose metabolism with 18-FDG PET, Fumal et al identified several areas of hypometabolism in MOH patients, including bilateral thalamus, orbifrontal cortex and insula/ventral striatum. All dysmetabolic areas recovered to almost normal glucose uptake after withdrawal of analgesics, except for the orbifrontal cortex [99]. Alterations of gray matter volume in cortical areas were also reported by anatomical studies. More precisely, increased volumes of periacqueductal grey, bilateral thalamus and ventral striatum as well as decreased volumes of orbifrontal cortex, left and right insula and precuneus were identified in 29 MOH patients [100].

Another mechanism underlying pathogenesis of MOH is represented by peripheral and central sensitization via the trigeminal pain pathway. De Felice and colleagues demonstrated that rats who overused triptans had cutaneous allodynia (an index of central sensitization in trigeminal nucleus caudalis) and increased CGRP levels [101]. Moreover, chronic ergot and paracetamol administration seem to facilitate trigeminal nociception [102].

MOH may also be considered a bio-behavioral disorder. Patients affected by this condition show dysfunctions in the ventromedial prefrontal cortex of the mesocorticolimbic dopamine circuit, which plays a crucial role in reward circuit and in drug dependence [103].

Serotonin, endocannabinoids, orexin A and corticotrophin-releasing factor neurotransmission systems are altered in MOH patients.

A suppression of serotonin function has been described in MOH. More precisely, MOH patients had lower levels of platelet serotonin (which revert after withdrawal) and higher density of 5-HT2A receptors in platelets [104] [105]. Moreover, Ayzenberg and colleagues in 2008 demonstrated that activity of the platelet serotonin transporter was increased in patients with analgesic- and triptan induced MOH [106]. Serotonin neurotransmission is closely linked to cortical spreading depression and trigeminovascular system. Supporting this, cortical spreading depression emerged leading to an increased 5-HT2A receptors expression in cerebral cortex in rats chronically treated with paracetamol [107]. In addition to this, animals with low levels of serotonin also showed an increase in CGRP expression in trigeminal ganglion as well as an augmented release of CGRP induced by cortical spreading depression [102].

Platelets levels of anandamide and 2-acylglicerol, two endogenous cannabinoids, were decreased in MOH patients compared to controls [108]. The endocannabinoid system seems to inhibit the trigeminovascular system antagonizing the

development of neuronal sensitization [92]. Lastly, augmented concentration of orexin A (a protein involved in regulating sleep cycles) and corticotropin-releasing factors were found in cerebrospinal fluid in MOH patients compared to controls [108].

However, because several alterations herein reported are also observed in patients affected by chronic migraine that did not show medication overuse, it is plausible that these anatomical, functional and biochemical changes may reflect headache worsening not specifically induced by drug overuse.

2.3 Treatment

Due to the lack of knowledge concerning MOH pathophysiology, there's still a lack of mechanism-based therapies aimed to effectively treat patients affected by this pathological condition. Nevertheless, the scientific community agrees that withdrawal from overused drug(s) is the first mandatory approach able to lead to an improvement of headache in the majority of MOH patients, irrespective of the drug(s) previously overused. However, no internationally accepted guidelines for the clinical management of MOH are currently available.

Detoxification protocols broadly vary among centers and include home treatment with simple advice of withdrawal, hospitalization or day hospital. Nevertheless, contrasting results have emerged from clinical studies investigating the efficacy/superiority of each different detoxification settings.

Few evidences suggest that simple advice to withdrawal could be effective [109] [110]. For example, two Italian studies performed on MOH patients showed that the efficacy of simple advice in reverting MOH at 2 months were of 78-92% in MOH patients with low medical needs (previously defined by ICHD-II "simple

MOH") and 60% in MOH patients presenting also psychological problems or medical comorbidities (defined by ICHD-II "complicated MOH") [111] [112].

However, several experts are in favour of a more robust support to patients undergoing detoxification program. Indeed, it must be acknowledged that drug detoxification may result in withdrawal symptoms in the majority of MOH patients. Withdrawal symptoms may last 2-10 days and include rebound headache (described as an initial worsening of headache pain), nausea, vomiting, hypotension, anxiety, tachycardia and sleep disturbances [113]. The duration of rebound headaches varies according to the specific drug overused. More in detail, triptan overuse, when interrupted, may lead to withdrawal headache lasting 4.1 days while ergotamine and NSAIDS detoxification may induce rebound headaches lasting, respectively, 6.7 days and 9.5 days [114]. Pharmacological therapies for withdrawal symptoms are so needed and broadly vary among centers. In this context, the most commonly used drugs include intravenous hydration, NSAIDs with a long duration of action (i.e. naproxen and piroxicam, if not previously overused), corticosteroids (prednisone), antiemetics, benzodiazepines and eventual rescue medication (i.e. other analgesics than overused one) [115] [116].

Among robust detoxification programs, the inpatient setting emerged as the most effective approach, with a 70% rate of reported success [113]. Hospitalization may last from 2 days to 2 weeks and should be preferred to other settings in patients with opioids and barbiturate overuse, in subject with psychiatric comorbidities and, in general, in patients having difficulties in stopping the overuse. On the contrary, motivated patients without psychiatric comorbidities or subjects with NSAIDs- or analgesic-overuse headache seem to better benefit from an outpatient setting [91]. A day hospital regimen also emerged as effective in reducing long-term headache frequency and analgesic intake [117] [118].

Abrupt vs gradual suspension of the overused drug(s) is an another aspect of detoxification protocols largely debated by specialists. Even if no studies directly compared efficacy of abrupt versus gradual withdrawal, clinical practice suggests that abrupt detoxification may be preferred for triptan-, ergot- and NSAID-overuse headache while a gradual washout is indicated for patients overusing barbiturates, opioids, benzodiazepine or compounds containing caffeine [113].

Preventive therapies are often initiated when MOH treatment begins and include the same classes of drugs administered for migraine prophylaxis (i.e. beta-blockers, calcium channel blockers, anticonvulsants, ACE inhibitors) [115]. The most effective prophylactic drugs include topiramate and onabotulinum toxin A, resulting in significantly reduced headache days per month [119] [120]. However, two studies did not found a superior efficacy of withdrawal combined with prophylactic therapy compared to withdrawal alone, suggesting that a preventive therapy should be initiated in MOH patients who did not previously benefit from detoxification alone [109] [121].

Medication overuse headache usually, but not invariably, resolves after the overuse is stopped. At short term, the withdrawal intervention has good outcomes, with only the 25% of patients not responding to the treatment [116]. However, the long term prognosis of patients initially responding to the withdrawal worsens over time. In fact, a consistent proportion of patients, varying from 22 to 75%, relapse again into medication overuse headache within 1 year from an initially effective withdrawal [122][123][124][125]. The risk of relapse fortunately tends to decrease if medication overuse is avoided for at least 12 months after withdrawal. Risk factors for relapse into medication overuse headache include: male sex, primary tension type headache, higher severity of migraine condition, longer duration of overuse, higher number of prophylactic medication previously administered to the

patient, intake of combination analgesic products made up of NSAIDs and caffeine or codeine, smoking, alcohol consumption, poor improvement after drug withdrawal and reduced sleep quality [126] [127]. Moreover, the type of drug overused before withdrawal seems to impact on the risk of relapse: more precisely, triptans emerged as conferring a lower risk of relapse compared to analgesics [125]. Psychiatric comorbidities have also been suggested to be predictors of a poor prognosis after withdrawal [94].

2.4 Genetics and pharmacogenetics of MOH

As previously mentioned, several risk factors for migraine transformation into MOH have been identified in the last years. In this context, the observation that MOH subjects with a familiar history of MOH tended to have a threefold increased risk of developing MOH, fostered the hypothesis that individual genetic background might also contribute to influence MOH susceptibility [128].

Nowadays, the body of knowledge about genetic predisposition to MOH is still relatively recent and sparse. The first study aimed to assess the role of genetic polymorphisms as predictors of migraine transformation into chronic daily migraine accompanied by drug overuse was performed by Cevoli and colleagues in 2006 [129]. More precisely, in the light of alterations in mesocorticolimbic dopamine circuit of MOH patients [103], they conducted a genetic association study aimed to assess the role of 4 dopamine metabolism related genes (DRD4, DAT, MAOA and COMT) in the genetic liability to MOH. As results, they found that the allele 10 of a 40-base-pair tandem repeat in 3' untranslated region of the SLC6A3 gene dopamine transporter (DAT chromosome 5p15-3), was significantly underrepresented in patients with chronic migraine accompanied by drug abuse compared to migraineurs without drug abuse.

Besides dopamine, a number of neurotransmission systems have been postulated to be involved in MOH susceptibility. Among them, serotonin primarily emerged as a neurotransmitter playing a crucial role not only in migraine and headache but also in the sensitization induced by drugs [104] [105] [106] [130]. In this context, four genetic association studies were performed with the aim of identifying potential correlations between polymorphisms in genes involved in serotoninergic neurotransmission and the risk of developing MOH. Cevoli and colleagues in 2010 investigated the role of six SNPs in five serotonin metabolism-related genes (SLC6A4 STin2 VNTR, 5-HT1A A82G, 5-HT1B G861C and T261G, 5-HT2A T102C, 5HT6 C267T) as predictors of MOH susceptibility [131]. As results, genotypic and allelic distribution of all polymorphisms investigated did not differ among MOH patients and healthy controls. A further study performed by our group specifically focused on the analysis of 2 polymorphisms in the serotonin 5HT2A receptor gene (A1438G and C516T) as plausible risks factors for MOH [132]. In this context, we did not find significant differences in genotype distributions between MOH patients and healthy controls. However, C516T polymorphism emerged as a predictor of the number of symptomatic drug doses taken per month by MOH patients. In 2012, we also conducted an association analysis between 3 genetic variants in SLC6A4 (5HTTLPR, STin2 VNTR and rs1042173) and MOH susceptibility [133]. None of the analyzed SNPs were nominally associated with MOH susceptibility but the haplotype-based analysis suggested that the haplotype STin2 VNTR-rs1042173 might have a trend of association with MOH. Lastly, Ishii and colleagues in 2013 investigated the plausible role of 3 SNPs (rs4570625, rs4565946 and rs4341581) in the TPH2 gene as predictors of migraine transformation into MOH [134]. More precisely, TPH2 encodes for tryptophan hydroxylase 2, which is a rate-limiting enzyme in the synthetic pathway for brain serotonin. Despite valid assumptions, genetic distributions of analyzed SNPs did not differ between migraineurs and patients affected by MOH.

On the basis of the aforementioned disparate results concerning genetic susceptibility of MOH, Onaya et al in 2013 analyzed the potential correlation between migraine transformation into MOH and 12 SNPs in 12 candidate genes involved in the onset of migraine and/or depression, including SLC6A4, 5HT2A, 5HT1B, MTHFR and DRD2 [135]. More precisely, genotypic distributions for all analyzed SNPs were compared between 47 migraine patients and 22 MOH patients. Among all studied SNPs, only MTHFR C677T and DRD2 C939T resulted significantly associated with the development of MOH in migraineurs.
Overall, this results are clearly inconclusive and do not support a validated role of specific genetic variants in influencing MOH susceptibility. Further studies with larger sample sizes and independent replication cohorts are so needed to clarify the impact of genetic background on MOH. Moreover, the performance of genome-wide association studies may potentially allow to identify new genetic variants involved in MOH susceptibility, giving the chance to spread light on still unknown pathogenic mechanisms underlying this neurological condition.

If little is known about genetic susceptibility to MOH, then even less has been discovered concerning genetic predictors of the prognosis of MOH patients. Di Lorenzo and colleagues in 2009 evaluated the role of BDNF Val66Met as a worsening factor in the progress of MOH disease [136]. More precisely, due to the fact that MOH shares some pathophysiological mechanisms of drug addiction and that BDNF Val66Met is involved in substance abuse, they hypothesized that this genetic variant in BDNF might influence the number of doses taken by MOH patients. As results, they found that homozygous wild-type subjects for Val66Met (GG) showed a lower consumption of monthly drug doses compared to other genotypes (non-GG).
As previously mentioned, withdrawal from overused drug(s) is to date the best available treatment for MOH patients. Surprisingly, even if several studies have

been conducted with the aim of identifying clinical predictors of short- and long-term outcomes after detoxification, no evidences are now available regarding the influence of genetic background on the response to withdrawal therapy in MOH patients. In our opinion, the identification of genetic predictors for the prognosis of MOH patients underwent withdrawal is strongly needed because it may allow clinicians to identify subjects that may benefit or not from a specific detoxification protocol. In addition to this, the choice of a more or less robust withdrawal setting according to genetic predictors may, in principle, result in improved MOH patients' quality of life and optimized health-care resources.

2.5 Pharmacoepidemiology of triptan-overuse headache

Migraine chronicization into medication overuse headache is a clinical issue of primary importance in the field of therapeutic management of migraine. Indeed, MOH is a costly disease, both in terms of social burden and high direct and indirect economic costs. Recently, a comprehensive estimation of expenditure for headache disorders in Europe was conducted in eight European countries representing the 55% of adult European population. Mean per-person annual costs emerged to be three times higher for MOH (€ 3561, 92% of indirect costs) compared to those estimated for episodic migraine (€ 1222, 93% of indirect costs). What is more is that, despite MOH low prevalence, its annual costs resulted to account for the 21% of total annual expenditure for all headache types (€173 billions) [137]. In the light of the aforementioned results, there is clearly a need of still performing multiple strategies aimed to curb anti-migraine drugs overuse and reduce the risk of MOH onset.

In the interest of lightening migraine and MOH burdens, the analysis of the utilization patterns of anti-migraine drugs in a real setting has represented a key step in drawing a more comprehensive picture of the actual use or overuse of these drugs in unselected populations.

In general terms, the study of the utilization and effects of drugs in large populations is provided by the *pharmacoepidemiology research*, recently defined by the World Health Organization as "the application of epidemiological methods and reasoning to the study of the use and effects/side-effects of drugs in large numbers of people, with the purpose of supporting the rational and cost-effective use of drugs in the population, thereby improving health outcomes". More precisely, the pharmacoepidemiologic description of the extent, quality and determinants of drug use is specifically performed by *drug utilization studies*, which are aimed to facilitate and promote the rational use of drugs, "giving special emphasis on the resulting medical, social and economic consequences" [138].

Hence, drug utilization studies focusing on the dispensing of anti-migraine drugs could allow not only to trace use and overuse of the aforementioned drugs in large communities but also to promote drugs' appropriate use in the interest of patients' quality of life and optimization of health resources.

As previously mentioned in Chapter 2.1, all symptomatic medications for migraine, if overused, can trigger migraine transformation into MOH.

At this stage, the question that arises is whether is possible to trace the use/overuse of all anti-migraine symptomatic drugs available on the market. The response to this question directly depends on the data available for the pharmacoutilization analysis. Indeed, the data sources of drug utilization studies broadly vary among countries depending on the level of record keeping and data collection, and usually include in-field collected data, disease registries and large healthcare databases. Due to their reliability and worldwide diffusion, healthcare databases are nowadays

commonly used in drug utilization studies and are defined as "electronic systems design to store, on an ongoing basis, disease related data (e.g. drug prescription, hospital diagnoses, outpatients visits and so forth) from a well-defined dynamic population" [139]. More precisely, healthcare databases can be classified into:

i) databases collecting information for administrative purposes (healthcare utilization databases - HCU), which were initially born as simple electronic storages of data pertaining patient's demography, healthcare procedures and other health services (e.g. drug prescription/dispensation data, hospital admission and diagnoses, laboratory examinations, surgical and other interventions) for supplying payments to providers of health services;
ii) databases generated by medical records (MRs), which allow physicians to easily collect and retrieve data concerning the clinical picture of their patients.

HCU databases have a major real life clinical practice representativeness compared to MRs, with larger sizes of the covered populations (often up to million patients) and lower costs for the information obtaining. Prescription/dispensation data recorded in HCU databases are particularly suitable for investigating drug-use profiles, in terms of prevalence, incidence and duration of drugs' use. In fact, accurate information regarding drug name, formulation, dose, frequency of administration and duration of treatment may be provided by each prescription form recorded in the database. With respect to Italy, data concerning all prescribed drugs reimbursed by the Italian National Health Service are recorded into administrative databases by regional health authorities.

On the basis of the aforementioned, it's clear that prescription data cannot trace the pattern of use of all routinely used antimigraine drugs. Most of the commonly used non-specific drugs for migraine (i.e. simple or combination analgesics and NSAIDs) are over-the-counter drugs. Their uncontrolled dispensation may

obviously result in unlimited drug consumptions potentially triggering MOH; nevertheless, their pattern of use during time cannot be traced by means of drug prescription data.

Contrary to NSAIDs, triptans are prescribed by general practitioners in a number of countries, including Italy, and so, in principle, the analysis of individual triptan prescription may allow to accurately estimate the real prevalence of triptan use and overuse in large communities. Moreover, since triptans are specific anti-migraine drugs and migraine is their only therapeutic indication, the analysis of triptan use and overuse may surely allow to identify only subjects affected by migraine and potentially at risk of developing triptan-induced MOH. Conversely, other non-specific antimigraine drugs (e.g. NSAIDs and analgesics) cannot be considered unequivocal tracers of migraine condition because they may be used and overused for treating pain conditions other than migraine.

On the basis of the aforementioned reasons, several drug utilization studies have been performed worldwide in the last 20 years with the aim of describing use and overuse of triptans in large and unselected population samples.

Different methods and different threshold have been used to define triptan overuse.

According to the International Classification of Headache Disorders, version III-beta, MOH induced by triptans overuse (named "triptan-overuse headache") is defined as a secondary chronic headache due to a "regular intake of one or more triptans, in any formulation, on ≥ 10 days per month for at least 3 consecutive months" [17]. Assuming that patients takes all the triptans prescribed, but not more than one dose per day, the minimum threshold used to define triptan overuse is the prescription/dispensation of at least 10 DDDs [3] of triptans every month for at least three consecutive month. More stringent definitions of triptan overuse (set on the

[3] DDD (Defined Daily Dose): the assumed average maintenance dose per day for a drug used for its main indication in adults. DDD is a comparison unit and does not necessarily correspond to the recommended dose. According to World Health Organisation, DDD as a measuring unit has become the gold standard for international drug utilization research.

basis of higher thresholds of monthly dispensed DDDs of triptans) have been often used, allowing the identification of more serious triptans overusers.

Overall, the results emerged from these drug utilization studies suggest that the 1-year rate of triptan users among general population ranged from 0.55% to 1.4% of general population. The most commonly used threshold for defining triptan overuse was set at the dispensing of more than 120DDDs per year [140] [141] [142] [143]. On the basis of this definition, the prevalence of triptan overuse in triptan users varied between 3.2 and 14.3%.

Among this studies, two were performed on prescription data recorded by the Italian Regional Health Authority of Tuscany and Emilia-Romagna [142] [143]. The first one was conducted on prescription data covering around 225,000 resident of Tuscany in 2005 [142]. The 0.55% of general population resulted using triptans in that period. Among them, the 3.2% emerged overusing at least 120 DDDs of triptans per year; an even more intense overuse (≥216 DDDs/year) was observed only in the 0.9% of triptan users.

A subsequent study was performed on prescription data recorded by the Regional Health Authority of Emilia Romagna (4,249,533 inhabitants) [143]. In this community, triptan users represented the 0.8% of the population, a percentage consistent with that one reported in the previous study conducted on a Tuscany population. Herein, the rate of users being dispensed more than 120 DDDs/year reached the 14.3% of triptan users and the 7,5% of triptan users showed an overuse of at least 180 DDDs per year.

Overall, the rates of triptan overuse emerged from the studies conducted on these two Italian communities are inconsistent. In addition to this, available data run back in time and so it would be important to have an updated snapshot of triptan use in Italy.

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Chapter 2

Outlines of the thesis

The pharmacogenetics of migraine

Migraine is a highly disabling primary headache disorder that affects around 10% of the worldwide adult population, with a male to female ratio of 1:3. Despite several symptomatic and preventive drugs are currently commonly used in the clinical management of acute migraine, this neurobiological condition still remains under-diagnosed and under-treated, placing a substantial burden on society. The relatively recent advent of triptans in acute migraine treatment has greatly ameliorated the societal impact of the disease to the extent of leading triptans to be considered by many experts as the gold-standard therapy for acute migraine. Nevertheless, clinical evidences have reported a significant interpatient variability in the response to all drugs routinely used for migraine treatment, including triptans. Non-response to each anti-migraine drugs classes was hypothesized not to be fully explained by altered pharmacokinetic parameters or incorrect drug administration, suggesting that genetic individual variability could also account for pharmacokinetic and pharmacodynamic drug variability. Surprisingly a very few pharmacogenetic studies have been performed in migraine. With the aim of comprehensively collecting all available pharmacogenetic evidences in episodic migraine, we conducted in September 2012 a systematic review of literature concerning genetic predictors of response to all available acute or preventive antimigraine drugs. As expected, we reported sparse and inconclusive pharmacogenetic evidences exclusively supporting potential correlations between few genetic variants and triptan response in migraineurs.

In order to fill the lack of pharmacogenetic knowledge into migraine therapeutics, we further performed three candidate gene association studies investigating the role

of common genetic polymorphisms on the response to triptans in large cohorts of migraineurs enrolled at specialized headache centers. More precisely, the polymorphism COMT rs4680 emerged as a potential genetic predictor of triptan response in both an exploratory and a validation cohort of migraineurs. Conversely, genetic variants in GRIA1 (rs548294, rs2195450) and CGRP-related genes (CALCA rs3781719, RAMP1 rs3754701, RAMP1 rs7590387) were found not to be correlated with triptan response in migraineurs.

The pharmacogenetics of medication overuse headache

Medication overuse headache (MOH) is a daily or almost daily type of headache which results from chronicization of episodic migraine or tension-type headache as a consequence of symptomatic drug overuse. Withdrawal of the overused medication/s is at present recognized as the treatment of choice for MOH. At short term, the withdrawal intervention results in good outcomes, with only the 25% of patients not responding to the detoxification. However, the long term prognosis of patients, initially responding to the withdrawal, worsens over time, with the 22-75% of patients relapsing again into MOH within 1 year from the withdrawal. Even if several studies have been conducted with the aim of identifying clinical predictors of short- and long-term outcomes after detoxification programs, no evidences are reported regarding the influence of genetic background on the response to withdrawal therapy in MOH patients. In the light of this, we conducted two exploratory pharmacogenetic studies assessing the role of several polymorphisms as genetic predictors of both short-term prognosis (14 polymorphisms in 8 candidate genes) and long-term prognosis (COMT rs4680, COMT rs6269, SLC6A4 STin2 VNTR) of MOH patients underwent in-patient withdrawal therapy. More precisely, DRD2 NcoI emerged as a plausible genetic determinant of detoxification outcome in MOH patients at 2 months of follow-up. In addition to this, the combination of multiple genetic markers among the tested

ones resulted to be clinically useful for the identification of MOH patients at higher risk for poor short-term prognosis. As regards to the identification of genetic predictors of long-term prognosis, COMT rs4680G allele carriers or subjects with COMT rs6269G-rs4680G haplotype were found to be at lower risk of relapse into MOH within one year from successful detoxification therapy, compared, respectively, to rs4680AA carriers or COMT rs6269A-rs4680A carriers.

The pharmacoepidemiology of migraine chronicization into medication overuse headache

In the interest of lightening migraine and MOH burdens, the analysis of the patterns of triptans utilization in a real setting has represented a key step in drawing a more comprehensive picture of the actual use or overuse of these drugs in unselected populations. In Italy, drug utilization studies performed on prescription data recorded by administrative databases of two Regional Health Authorities suggested that the 0.6-0.8% of general population used triptans during the study period. Among triptan users, the 3.2-14.3% emerged to overuse these drugs, so being at risk of developing MOH. Since this data ran back in time, we aimed to have an updated snapshot of triptan use/overuse in Italy. We performed a drug utilization study on 1-year prescription data (1 Jan 2012- 31 Dec 2012) derived from the drug dispensation monitoring system of the Local Health Authority of Vercelli (about 175,000 inhabitants) and of the Umbria region (about 885,000 inhabitants). Our findings suggested that triptans were used by the 0.7-1.0% of the population and that the 10% of triptan users showed triptan overuse during the study period. Moreover, about two-thirds of triptan users who overused triptan in the first semester of 2012 persisted in this behavior in the following six months. Given the need of reducing prevalence and duration of MOH, our data suggest that an approach based on drug prescription databases could be useful for early identification of patients at higher risk of developing MOH induced by triptans.

Chapter 3

Pharmacogenomics of episodic migraine:

time has come for a step forward

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Abstract

Background: Migraine is a disabling condition characterized by a heterogeneous behavior of response to drug assumption. In the recent years many resources have been invested in studies attempting to unravel the genetic basis of migraine, while the role of genetics in preventive or symptomatic responses to currently available drugs has received less attention.

Methods: We performed a systematic literature search identifying original articles pertaining to pharmacogenetics of episodic migraine.

Results: Seven primary studies on the pharmacogenetics of symptomatic medication and two primary studies on pharmacogenetic aspects of preventive medication in episodic migraine were found. The number of patients studied in the

individual articles ranged between 40 and 130. There was a strong heterogeneity among these studies.

Conclusion: We believe that Pharmacogenetic studies, if properly designed, could give a contribution in optimizing the treatment and reducing the burden of migraine, in turn helping patients and optimizing resources. Yet, our knowledge on the pharmacogenetics of migraine is growing too slowly, and concerted measures should be undertaken to speed up the process.

Keywords

Pharmacogenetics, pharmacogenomics, migraine, triptans, symptomatic treatment, preventive treatment

Introduction

Migraine is a highly prevalent and disabling condition, with high socioeconomic and quality of life impact. In the World Health Organization's study on the global burden of disease, migraine was ranked among the first diseases causing worldwide disability, with a high incidence in more active age [1].

In recent years, a number of studies have focused on attempting to unravel the genetic basis of migraine [2]. These attempts have also included genome-wide association studies [3] which have indeed found an association between common genetic variants and the risk of migraine with or without aura [4-6]. The investigation of the genetic backgrounds that confer a higher or lower possibility to respond to single drugs used in migraine has not, instead, received as much attention. This is surprising, given also the high number of preventive or symptomatic treatments available and the well-known fact that there is a great

inter-patient variability of response. Moreover, migraineurs may have side effects, or adverse events, to treatments, more frequently than other groups of patients [7]. Pharmacogenomic studies in this respect would be warranted to allow for a more tailored therapeutic approach, as well as to unravel sub-populations that at present are inadequately treated by drugs on the market [8,9]. Yet, despite rigorous pharmacogenomic studies have been carried out in many other diseases with interesting and clinical-relevant results, our impression is that in migraine they are very few, with many methodological limitations, different study designs and finally contrasting results. The lack of shared and appropriate "guidelines" to carry out these studies, interpretation the data and write the manuscript, is so deep that even a non-univocal choice of the key words makes it difficult to find these few original studies in PubMed using the keywords (("pharmacogenomics" OR pharmacogenomics") AND migraine").

Objective

The aim of this work was to evaluate, by a systematic review to identify articles dealing with pharmacogenomic studies in episodic migraine, the quality of what has been produced so far in terms of number of manuscripts, methodology, results and translationality. With this in hand, secondary we will propose some suggestion to design proper and shared guidelines for such studies. These should avoid that other efforts will be produced to perform non-comparable, underpowered, or not well-designed studies, which would just increase the "noise" on the topic.

Methods

Search strategy

To perform this systematic review the principles of the PRISMA statement were used. To retrieve the relevant articles, we used the database Pubmed and the last

search was performed on September the 6th 2012. We used three groups of combination of terms: (i) "(pharmacogenetic OR pharmacogenomic) AND migraine"; (ii) a symptomatic treatment (ST) search that combined any drug ranked at least at "level C" or "weak" recommendation in at least one of the most updated European, U.S. and the most recent national (Canadian and Italian) guidelines for the symptomatic treatment of migraine [10-13] AND the terms "(gene OR genetics OR genomics OR polymorphism OR polymorphisms OR SNP OR SNPs) AND migraine"; and (iii) a preventive treatment (ST) search that combined any drug ranked at least at "level C" or "weak" recommendation in at least one of the most updated European, U.S. and the most recent national (Canadian and Italian) guidelines for the preventive treatment of migraine[10-13]) AND the terms "(gene OR genetics OR genomics OR polymorphism OR polymorphisms OR SNP OR SNPs)" AND migraine". A full list of terms used in the symptomatic treatment (ST) and preventive treatment (PT) search is present in Table 1. We also considered articles from the reference lists of relevant papers and reviews on pharmacogenomics of migraine.

Table 1: Search strings used to specify drugs used in symptomatic or preventive treatment of migraine.

Symptomatic treatment	'(sumatriptan OR rizatriptan OR zolmitriptan OR almotriptan OR eletriptan OR naratriptan OR frovatriptan OR 'acetylsalicylic acid' OR aspirin OR NSAID OR 'non-steroidal anti-inflammatory drugs' OR ibuprofen OR diclofenac OR ketoprofen OR naproxen OR paracetamol OR acetaminophen OR ketorolac OR metamizol OR indometacin OR flurbiprofen OR piroxicam OR pirprofen OR 'mefenamic acid' OR butalbital OR ergotamine OR DHE OR dihydroergotamine OR 'ergot alkaloids' OR codeine OR tramadol OR meperidine OR butorphanol OR isometheptene OR lidocaine OR corticosteroids OR dexamethasone OR chlorpromazine OR metoclopramide OR prochlorperazine OR domperidone OR phenazon OR 'tolfenamic acid') AND (gene OR genetics OR genomics OR polymorphism OR polymorphisms OR SNP OR SNPs) AND migraine'
Preventive treatment	'(metoprolol OR propranolol OR timolol OR atenolol OR nadolol OR bisoprolol OR nebivolol OR pindolol OR candesartan OR lisinopril OR clonidine OR guanfacine OR flunarizine OR cinnarizine OR valpro* OR divalproex OR topiramate OR carbamazepine OR amitriptyline OR venlafaxine OR cyproheptadine OR petasites OR butterbur OR naproxen OR acetylsalicylic OR gabapentin OR magnesium OR tanacetum OR feverfew OR parthenolide OR riboflavin OR 'Coenzime Q10' OR methysergide) AND (gene OR genetics OR genomics OR polymorphism OR polymorphisms OR SNP OR SNPs) AND migraine'

Eligibility Criteria

Studies investigating the association between common genetic variants (polymorphisms) and the clinical response (in terms of efficacy and/or tolerability) to symptomatic or preventive medications in episodic migraine patients of any age were retrieved. Only original articles were considered in the analysis while review articles were excluded but their reference list was scanned for further articles of interest. No language, publication date or publication status were imposed. Studies on patients with chronic migraine (with or without medication overuse) or cluster headache were excluded.

Study selection and data extraction

Eligibility assessment of manuscripts was performed independently of each other by two investigators (M.V. and C.D.L) who examined all the abstracts found in the literature search. Disagreement between reviewers was solved by consensus. Whenever the abstract suggested that the publication was relevant, the entire manuscript was retrieved and examined.

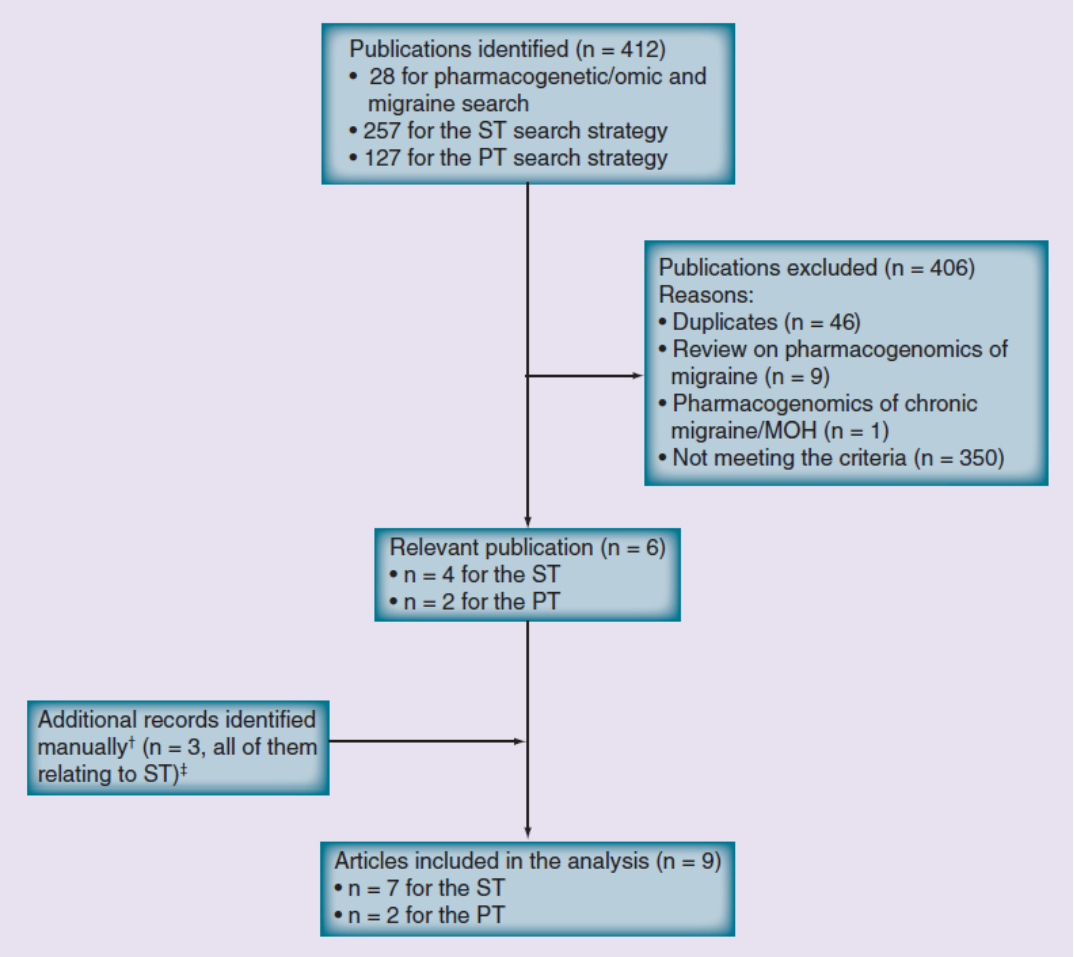
Two investigators (M.V. and C.D.L.) independently extracted data from the articles and entered them into a customized database. In case of disagreement, a consensus agreement was reached by involving the third person (A. A. G.). This was the case in the review of two abstracts. The extracted data included: publication information (authors, year of publication, Country), population characteristics (number of patients, gender distribution, age, BMI, overlapping sample among studies), clinical information (diagnosis - diagnostic system by which the diagnosis was performed, headache frequency, duration of illness, previous exposure to the medication - or class of medications – studied, other concomitant medications), study methodology (prospective/retrospective, exclusion criteria, use of an headache diary, number of attacks treated for the symptomatic medication), genetic polymorphisms, medication(s) studied, clinical endpoint (primary and secondary

outcomes, measures and results), statistical analysis (type of analysis, calculation of Hardy-Weinberg equilibrium, use of effect size measures, application of any post hoc correction) and results of the genotype-phenotype association analysis. We contacted two authors (corresponding authors of 4 manuscripts [14-17]) for further information, in particular relating to the study methodology. Both responded fully to our queries.

Results

The search strategy identified a total number of 412 published studies: 28 for the "(pharmacogenetic OR pharmacogenomic) AND migraine" search, 257 for the ST search and 127 for the PT search (Figure 1). Of these, 46 articles were duplicated in more than one search, nine were reviews on pharmacogenetic/genomics of migraine [18-26], one was an original article concerning a pharmacogenetic study in chronic migraine and/or MOH [27], whereas 350 did not meet the main inclusion criteria. Six articles were considered for a full text reading (4 for ST [15-17, 28] and 2 for PT [29, 30]). The reference list of these six papers and of the nine reviews mentioned above were scanned for further articles, and three additional studies [14, 31, 32] (all relating to ST) were identified. It has to be noted that during this latter search we found also four papers regarding pharmacogenetic studies in MOH [33-36] that were therefore excluded. The nine articles were all in the English language. Key findings of these studies are summarized in Supplementary tables 1 & 2. Only a single eligible study [17] was found by the use of the search terms "(pharmacogenetic OR pharmacogenomic) AND migraine".

Figure 1: Review process



†By checking the references of relevant papers and reviews. ‡Four articles were not taken into account as they were only related to the pharmacogenomics of medication overuse in headache.

MOH: Medication-overuse headache; PT: Preventive treatment; ST: Symptomatic treatment.

Symptomatic Treatment

In all the seven studies the Authors investigated the association between genetic polymorphisms and clinical response to triptans [14-17, 28, 31, 32]. These seven studies, spanning from 1998 to 2012, stemmed from 4 groups. One group performed different genetic analyses from 1998 to 2012 on the same sample of patients that underwent the same clinical trial [15-17]. One group performed

different genetic analyses on overlapping patients [31, 32]. The number of patients studied in the individual articles ranged between 40 and 130. The cumulative number of patients studied was 480, but the unique patients enrolled were 280. All the studies reported the age and gender distribution (see supplementary table 2); none reported data on BMI of the patients. With respect to the diagnosis only the study by Asuni et al [28] was performed on a homogenous sample: 40 patients affected by migraine without aura. In three studies the sample population presented either migraine without aura or migraine with aura [14, 31, 32], while three studies reported that the population was composed by "migraine patients" without further specifications [15-17]. Headache frequency and the age of onset of migraine were lacking in three studies [14, 31,32], the presence of a preventive therapy was reported just in two out of seven studies [31,32], concomitant use of other medications was not reported in any of the papers. Whether patients were naïve to triptan use was not reported in any paper. Exclusion criteria such as the presence of a psychiatric comorbidity or contraindication to the use of a triptan compound were reported only in two studies [14,31]. The symptomatic treatment evaluated was a single triptan in four studies (rizatriptan 10mg per os on 40 patients [28] and sumatriptan 6 mg subcutaneous in other 40 patients [15-17]), while in three studies a pool of different triptans were used: four in one case [14] and six in two studies [31, 32] (in all the three studies each patient used for all the three attacks tested the same triptan).

With respect to the study design of the clinical assessment, four studies were retrospective [14-17] while three were prospective (with a specific diary to fulfill during the attacks) [28, 31, 32].

Polymorphisms studied were related to 5HT1B in five studies [14, 16, 17, 28, 32], SLC6A4 in three studies [14, 28, 31], DRD2 in three studies [14, 28, 31], MAOA in two studies [14, 27, 28], GNB3 in two studies [14, 31], 5HT2A [14], 5HT1F [15], MTHFR, ACE, ESR1, TNF-B in one article [14]. Triptan efficacy was always

included as the primary outcome, yet the clinical measures were different among the studies (including studies performed by the same group). Considering these differences the response rate ranged from 66.9 to 76.6%. Four out of seven studies investigated also an association between gene polymorphisms and the presence of side effects as a secondary endpoint [15-17, 32].

The Hardy-Weinberg equilibrium was not reported in 2 studies [14, 32]. None adopted an a priori power analysis to detect the sample size of the study whereas three studies adopted a post hoc power analysis [17, 31].

Statistical analysis was conducted by Fisher's exact test or chi-square test alone [16, 28, 32] or followed by multivariate logistic regression analysis [14, 31] while two studies did not mention the statistics used [15, 17]. All studies analyzed a single cohort of patients and no replicatory cohort was used.

In these studies, DRD2 rs6275 (also known as C939T or NcoI) and SLC6A4 Stin2 VNTR polymorphisms have been reported as associated to the response to triptans in terms of efficacy [14, 28, 31]. Only one study applied correction for multiple testing [31]. Asuni et al [28] found that rs6275 C/C carriers were associated with a good response to rizatriptan. Yet, in a similar size population sample, Ishii et al [14] found in the multivariate analysis an association of the C/C genotype with a lack of response to triptans as a class. In a third study, no association was found between DRD rs6275 and response to triptans, albeit the patient characteristics, the triptans used, and the efficacy end-point were different [31]. Terrazzino et al [31] found a significant association between the variable number of tandem repeats polymorphism in intron 2 (Stin2 VNTR) of the serotonin transporter gene (SLC6A4) and the clinical response to triptans. However, this finding was not replicated in a subsequent study by Ishii et al [14]. No study found any association between side effects and gene polymorphisms [15-17, 32].

Preventive Treatment

The two studies concerning the pharmacogenetics of preventive treatment (PT) in migraine investigated the role of the angiotensin converting enzyme (ACE) genotype on response to ACE inhibitors/sartans [30] and the role of mtDNA haplogroups on the response to riboflavin [29]. The number of patients was 104 and 64, respectively. Yet, the largest study recruited from two previous randomized, double-blind, placebo-controlled crossover studies and the patients were stratified in two groups: lisinopril (on 47 patients) and candesartan (in 57 patients) in migraine prophylaxis [37, 38]. Both studies reported the age and gender of patients but neither reported the BMI of patients. Both studies included patients suffering from migraine with and without aura. In respect to clinical data, headache frequency was reported in both studies while age of onset of migraine was reported only in the study by Di Lorenzo et al. In the riboflavin study [29] and in the candesartan group of the ACE polymorphism study [30, 37] it was specified that only patients naïve for the tested medication were enrolled while. Just in one study only patients who were not under any chronic medication (except for contraceptive pill) were enrolled [29].

Both studies were prospective and a headache diary was used. As reported above, the candesartan and lisinopril studies were randomized, double-blind, placebo-controlled crossover studies, while the riboflavin study was an open study where the pharmacogenetic side was blinded. The primary outcome measure was the efficacy in migraine prevention but with some differences: in the riboflavin study the Authors used as the reduction in monthly attack frequency of at least 50% between the month preceding inclusion and the fourth month of treatment whereas in the candesartan and lisinopril studies the treatment period was compared to the placebo period (responders being patients with at least 50% reduction of headache days). Responder rates were 62.5% for riboflavin [29], 32.1% for candesartan and 24.4% for lisinopril [30].

The Hardy Weinberg equilibrium was only tested in one paper [30] since it is not required in haplogroup analysis [29]. Power analysis was performed in both studies (a priori analysis in the riboflavin study [29], not specified in candesartan/lisinopril study [30]).

In the study that investigated the association between ACE genotypes and the response to candesartan or lisinopril there was no significant association [30], while in the study by Di Lorenzo et al. riboflavin appears to be more effective in patients with a particular cluster of mitochondrial haplogroups [29]. No replication cohorts were present in these studies and no further studies investigating these polymorphisms were reported in the literature.

Discussion

The data presented above confirms our hypothesis, highlighting a number of key flaws in the field of migraine pharmacogenomic studies.

First, the manuscripts presenting primary data on the pharmacogenomics of migraine is extremely slim. This is highly surprising, given that a tool to tailor therapy to migraineurs is badly needed. Furthermore, it would be expected that a disease with such high incidence would have presented an ideal setting to recruit patients.

Second, all the articles so far published in the field are hypothesis-driven, and there is no GWAS at present available. As this latter technique is costly, this fact may suggest that funding is one of the limiting steps in the pharmacogenetics of migraine. Part of this might be due to the lack of industrial interest given the patent expiry of most molecules used. Yet, it is paradoxical that public funding is limited for a situation that could greatly improve health while decreasing treatment costs.

Third, population sizes in the published manuscripts are small (albeit adequate in most circumstances). Once again, this is surprising, given that migraine has such a

high prevalence and that the study designs are particularly simple and of an observational nature.

Fourth, no manuscript presents a replication cohort, which at present is becoming the norm for pharmacogenetic associations in most fields. The replication cohort improves the quality of the result, reducing significantly false positives. Indeed, when comparing the published studies, the study designs appear largely heterogenous, suggesting that no consensus on how to best approach the problem has been reached.

Fifth, studies facing preventive treatments are substantially absent (no studies on first choice treatments are present in literature), despite the high number of pharmacological classes involved migraine prophylaxis. Pharmacogenomic of migraine prophylaxis is a very interesting field of research, with a high translational impact. In fact, the response to a prophylaxis is often late and patient has to face a long, frequently expensive and non-free of side effects treatment before to understand if the therapy is effective or not.

Sixth, the importance of common comorbid medical conditions is almost neglected by authors, even if they may influence treatment efficacy and side effects. It is, for instance the case of BMI that may influence results of such studies in at least two ways: it could modify the pharmacokinetics of drugs [39] and could worsen frequency, severity and clinical features of migraines [40]. Recent data evidenced that another important comorbidity that has to be taken into account in pharmacogenomics studies is psychiatry. In fact, the genetic background of migraine comorbid to major depressive disorder seem to be different from migraine alone [41]. Different genetic backgrounds could account for different pharmacogenomics consequences.

To summarize all our considerations, we can assert that the field of pharmacogenomics in migraine is still at its year zero. The distance between research in migraine genetics and in pharmacogenetics is at present immense. To

give an example, the most recent high impact paper published on the genetics of migraine without aura included a GWAS on a total of 2300 patients from two separate cohorts and the results were replicated in four independent patient datasets (including 2500 patients) [42].

We feel that the time for a step forward in the search for genetic determinants of drug response in migraine has come. Indeed, it must be acknowledged that, at present, the choice of which preventive and/or symptomatic treatment to administer to a single patient is mainly guided by contraindications, side effects, and personal clinical experience and therefore an index of a priori efficacy would be badly needed. In this respect, it would be helpful to harmonize future studies around a few key pillars, to make studies comparable. This could also help form consortia in the future with similar databases. As an example, a simple scheme as that presented in Box 1 could be a good starting point.

Yet, the use of such restrictive inclusion/exclusion criteria raises a crucial concern: the difficulty in patient recruitment. In fact, some of the authors of this manuscript have started a pharmacogenomic study in symptomatic treatment in migraine (triptans) for 18 months, following these guidelines (Box 1). Yet, out of 2000 patients referred to Mondino Headache Center (tertiary university based center), just 40 patients were eligible and finally enrolled. Wordwide, most of the research in headache is run in tertiary / University based centers. Yet, these are the centers where most complicated cases are seen and therefore they might not be the ideal settings in which to recruit naïve patients. This point is more dramatic in the case of studies regarding over the counter drugs or nonsteroidal anti-inflammatory drugs, since it is virtually impossibile to find naïve patients for these treatments and it is unknown if the prolonged consumption of these drugs will have some influence on triptan response. We feel that best option to reach an adequate number of patients is to perform a multicenter study and to eventually involve local general practitioners in the referral of selected patients.

Box 1: Possible observational protocol for pharmacogenetic studies in episodic migraine.

Collect demographic & clinical information that might affect drug response
Examples: age, duration of illness and BMI, among others.

Collect headache characteristics that might affect drug response
Examples for symptomatic treatment (ST) studies: frequency, attacks upon waking, menstrual-related attacks and delay of intake of the symptomatic medication, among others.
Tool: use a headache diary to record triggers, headache phenotype and patient's behavior/use of symptomatic medication.

Collect pharmacological history of medications that might affect response
Example: concomitant nonmigraine medication and other symptomatic treatment or preventive medication (recommendation for ST studies: only patients without prophylaxis).
Consumption of cigarettes, caffeine, herbs or other substances.

Population
Homogeneous for diagnosis (at least second level of International Classification of Headache Disorders, beta release of 3rd version [46]; e.g., 1.2 code = migraine without aura).
Sample size at least sufficient to detect medium effect sizes (clinically relevant).
Exclude patients with comorbid conditions known to affect response to treatment (i.e., psychiatric disorders).
Only patients naive for the tested medication (it should be better to enroll patients that have never taken drugs of the same pharmacological class of the medicine that is being studied).

Study design
Prospective assessment of the clinical response to drug.
Use a headache diary.
Challenge one medication in one formulation for line of treatment (and consider the patient that had no benefit: nonresponder to that single compound in that formulation). Consider the patient a nonresponder to the whole class when he had no benefit from all the compounds in all the formulations available [42].

Outcome measures
Use a primary end point that is least affected by the placebo effect (i.e., in ST studies, the pain free 2 h in at least two attacks out of three, is more trustworthy than pain relief for 2 h in one attack).
Use a primary end point that is least affected by other variables that cannot be controlled (in studies about efficacy of preventive treatments, it is better to chose as primary outcome the reduction of attack frequency as it is less influenced by use of STs than headache days per month, duration or disability).

Statistical analysis
Appropriate statistics based on the nature of the outcome measured (both univariate and multivariate analyses) and correction for multiple testing if required.

A second crucial issue that still remain in migraine studies, is that in a number of fields pharmacogenomic analysis is now strengthened by side-by-side pharmacokinetic analysis [43]. Whether this would be feasible in migraine remains to be established.

Nonetheless, also by using harmonized, agreed-upon protocols, a few issues in the pharmacogenomic of migraine will remain to be established. For example, most pharmacogenomic studies usually compare groups taking the same medication but exhibiting different effects (e.g. responders vs. non-responders; presence vs. absence of side effects). In a disease such as migraine, in which patient response may be inconsistent intra-patient, which clinical end-point is best and more clinically relevant remains to be established. Furthermore, it must be acknowledged that the placebo effect is relevant in most end-points used, and it remains to be decided whether to consider these patients responders, or whether to control for them with a more complex study design [44].

Certain limitations of the present review should be acknowledged. First, our last PubMed search was performed in September 2012. In that period we have prepared a lecture on this topic for a conference. We have decided to not perform new research because the possible inclusion of a few further studies would not have modified the outline of the situation: the poorness of study in the examined topic. Second, we have limited our observations to only episodic migraine, excluding chronic forms. Chronic migraine is very often complicated by MOH. In a lot of cases, the discontinuation of overuse of symptomatic drugs is enough to revert chronic headache to an episodic form [45]. Therefore, the pharmacogenomics of MOH could be completely different to the pharmacogenomics of chronic migraine without medication overuse: the first concerns the pharmacogenomics of a side effect [46], the second of a drug response. Studies regarding chronic migraine are often not clear about the presence of medication overuse, thus we decided to exclude this topic from our overview. Third, even if it was not our intention to

nullify the positive outcomes of pharmacogenomics studies in this review, discussion of so far published articles was limited to highlight flaws and criticisms. This choice was in accordance with the objectives of this work, which aimed to identify articles dealing with pharmacogenomics studies in episodic migraine and to propose suggestions for designing proper and shared guidelines for future studies.

Conclusions and future perspective

Since our knowledge on the pharmacogenomic of migraine is growing at a very low pace, only concerted measures should be undertaken to speed up the process. By collaborative efforts and through support by adequate funding we will try to move this important field of research forward, actually fading by an excess of indifference, approximation and absence of common guidelines. For this reason, there is a need for a consensus conference and for the birth of specific study groups.

Conflict of interest statement

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Supplementary matherials

Supplementary Table 1. Relevant data from accepted articles about symptomatic treatment. Pt: patient; F: Female: M: Male; Dx: Diagnosis; Ys: Years; MA: Migraine with aura; MO migraine without aura; TRPT: triptan; Suma: Sumatriptan; Zolmi: Zolmitriptan; Ele: Eletriptan; Riza: Rizatriptan; NR: Not reported; R: retrospective; Frova: Frovatriptan; Almo: Almotriptan; P: prospective; *(at time 0, after 2h and if recurrence occurred) indicating headache intensity; ^ (responder with or without headache recurrence and non responders); # and who experienced headache recurrence within 24 hours in less than 1 out of 5 successfully treated attacks (for other clinical groups - patients with headache recurrence, non-responders, patient with chest symptoms, patients without chest symptoms – see paper).

Study: author, year, country	Pts: # (F:M), age	Dx (# of pts); diagnostic criteria	Gene polymorphisms	Acute Medication (# pts)	Preventive Medication (# pts)	Primary outcome	Clinical parameters	Results of clinical outcome	Secondary outcome	Results of genotype-primary outcome association	Overlapping patients
Ishii et al. 2012, Japan [15]	60 (46:14), age: 44.1 ± 10.9 ys	MO (42), MA (18); ICHD-II	SLC6A4 (5-HTTLPR, 5-HTTVNTR), 5-HT2A T102C, 5-HT1B G861C, MAOA (VNTR, T941G), MTHFR C677T, ACE I/D, ESR1 (G325C, G594A), DRD2 C939T, TNF-b G252A, GNB3 C825T	4 TRPTs per os: suma (20), zolmi (8), ele (2), riza (30)	NR	Efficacy	Consistent responder: Improvement of pain to mild within 4 h, or none within 2 h after triptan intake, in at least 2 attacks out of 3	76.6% (40 out of 60)	No	C/C at DRD2 C939T associated with negative response to triptans	No
Terrazzino et al 2010, Italy [32]	130 (102:28), age: 37.6 ± 10.3 ys	MO (117), MA (13); ICHD-II	SLC6A4 (5HTTLPR, STin2NTR), GNB3 C825T, DRD2 (TaqI A, NcoI)	6 TRPTs per os: ele (33), riza (29), suma (20), frova (18), almo (21), zolmi (9).	None (51), flunarizine (32), amitriptyline (25), topiramate (7); others (10), combined (7)	Efficacy	Consistent Response: ≥2 point reduction in a 4 points scale intensity of pain 2 h after triptan administration in at least 2 out of 3 consecutive attacks	66.9% (87 out of 130)	No	Association of STin2 NTR to inconsistent response to triptan.	Yes

Velati et al 2008, Italy [33]	120. 15 dropped out. (82:23), age 39.5 ± 10.6ys [range: 19–65])	MO (92), MA (13), ICHD-II	5HT1B (T-261G, A-161T, G861C)	6 TRPTs per os: almo (12), ele (31), frova (12), riza (24), suma (17), zolmi (9)	None (46), amitriptyline (18), flunarizine (29), propranolol (5), others (7)	Efficacy	Pain relief (improvement after 2 h from triptan administration, i.e. pain intensity changing from severe or moderate to mild or absent) in at least 2 attacks out of the 3	68.5% (72 out of 105)	Headache recurrence and presence and intensity of side effects.	No association	Yes
Asuni et al 2007, Italy [29]	50 (45:5), age: 34.6 ± 8ys. 6 pts not genotyped	MO; "IHS"	DRD2 (C939T, -141C Ins/Del), SLC6A4 5-HTTLPR, 5-HT1B G861C, MAO-A EcoRV	Riza 10mg per os	NR	Efficacy	Pain-free within two hours after rizatriptan administration in at least 4 out of 5 migraine attacks (a 4 points scale intensity of pain was used).	70.4% (31 out of 44)	No	Ncol associated to improved clinical response	No
Mehrotra et al 2007, The Nederlands [18]	40 (35:5), age: 20-69ys	"Migraine patients" (not specified if MO and/or MA), ICHD-I,	5HT1B (F124C, A-161T)	Suma 6mg s.c.	NR	Efficacy ^ and Tolerability	Responders: pts with headache relief within 2 hours after 6 mg s.c. suma in at least 4 out of 5 migraine attacks #	Responders (with or without recurrence): 68.4% (26 out of 38)	Presence of side effects (chest symptoms).	No association	Yes
Maassen Van Den Brink et al 1998, The Nederlands [16]	40 (35:5), age: 20-69ys	"Migraine patient" (not specified if MO and/or MA), ICHD-I,	Mutational analysis of coding region of 5HT1F	Suma 6mg s.c.	NR	Efficacy ^ and Tolerability	Responders: pts with headache relief within 2 hours after 6 mg s.c. suma in at least 4 out of 5 migraine attacks #	Responders (with or without recurrence): 68.4% (26 out of 38)	Presence of side effects (chest symptoms).	No association	Yes
Maassen Van Den Brink et al 1998, The Nederlands [17]	40 (35:5), age: 20-69ys	"Migraine patient" (not specified if MO and/or MA), ICHD-I,	5HT1B (T-261G, G861C)	Suma 6mg s.c.	NR	Efficacy ^ and Tolerability	Responders: pts with headache relief within 2 hours after 6 mg s.c. suma in at least 4 out of 5 migraine attacks #	Responders (with or without recurrence): 68.4% (26 out of 38)	Presence of side effects (chest symptoms).	No association	Yes

Supplementary Table 2. Relevant data from accepted articles about preventive treatment. Pts: patients; F: Female: M: Male; DX: diagnosis; Ys: Years; MA: Migraine with aura; MO migraine without aura; NR: Not Reported; P prospective; * overuse of antimigraine medications, pregnancy or lactation, presence of severe organic or psychiatric comorbidities, and concomitant drug treatment except for the contraceptive pill. § Some of the data were collected by reading the original paper of the two previous randomized, double-blind, placebo-controlled crossover studies that evaluated the efficacy of either lisinopril (on 47 patients) or candesartan (in 57 patients) in migraine prophylaxis; NOS: Not otherwise specified; # *Lisinopril group*: use of prophylactic drugs for migraine in the four weeks before randomization, pregnancy or inability to use contraceptives, decreased renal or hepatic function, hypersensitivity to ACE inhibitors, history of angioneurotic edema, and psychiatric disorder; *Candesartan group*: pregnancy, nursing, or inability to use contraceptives in women, decreased renal or hepatic function, use of prophylactic drugs for migraine in the four weeks before randomization, previous history of angioneurotic edema, hypersensitivity to active substance, psychiatric illness preventing full participation, use of daily migraine prophylactics in 12 weeks, having used more than 1 migraine prophylactic prior to study and cardiac problems or use of diuretics.

Study: author, year, country	Pts: # (F:M), age	Dx # pts; diagnostic criteria	Exclusion criteria	Gene polymorphisms	Preventive medication	Symptomatic medication	Concomitant use of other medication/ xenobiotic	Period of treatment evaluated	Outcome and clinical parameters	Results of clinical outcome	Results of genotype - clinical outcome association
Di Lorenzo et al 2009 Belgium/Italy [37]	64 (49:15), 32.2 ± 12.4 ys	23 MA, 41 MO; ICHD-II	Use of other PT during the previous 3 months, previous treatment with riboflavin *	mtDNA haplogroups	Riboflavin 400mg/die	NR	No, except for the contraceptive pill.	4 months	Efficacy: at least 50% reduction in **monthly attack frequency** between the month preceding inclusion and the fourth month of treatment	62.5% responders (40 out of 64)	Non-H haplogroup is related to riboflavin response
Tronvik et al 2008, Norway [38] §	candesartan: 57 (45F, age 42ys, 12M age 48ys); Lisinopril: 47 (38F age 41 ± 9ys; 9M age 43 ± 5 ys)	candesartan: migraine NOS, Lisinopril: MA and MO NOS	Interval headache not distinguishable from migraine headache; #	ACE I/D	lisinopril 10 mg/die (49 pts), candesartan 16 mg/die (59pts)	Analgesics and triptans	NR	3 months	Efficacy: reduction in **days with headache** of at least 50% in the treatment period compared to the placebo period	candesartan : 32.1% responder (18 out of 58); Lisinopril: 24.4% responders (12 out of 49)	No association

Chapter 4

An opposite-direction modulation of the COMT Val158Met polymorphism on the clinical response to intrathecal morphine and triptans

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Abstract

Genetic variation in the COMT gene is thought to have clinical implications for pain perception and pain treatment. In the present study, we first evaluated the association between COMT rs4680 and the analgesic response to intrathecal morphine in patients with chronic low back pain to provide confirmation of previously reported positive findings. Next, we assessed the relationship between rs4680 and headache response to triptans in two independent cohorts of migraine patients. In patients with chronic low back pain (n=74), logistic stepwise regression analysis showed that age (OR: 0.90, 0.85-0.96, P=0.002) and the presence of the COMT Met allele (vs Val/Val, OR: 0.21, 0.04-0.98, P=0.048) were predictive factors for lower risk of poor analgesic response to intrathecal morphine. Intriguingly, in migraine patients, the COMT rs4680 polymorphism influenced headache response to triptans in the opposite direction. Indeed, in an exploratory

cohort of migraine patients without aura (n=75), homozygous carriers of the COMT 158Met allele were found at increased risk to be poor responders to frovatriptan when compared to homozygous patients for the Val allele (OR: 5.20, 95% CI: 1.25-21.57, P=0.023). In the validation cohort of migraineurs treated with triptans other than frovatriptan (n=123), logistic stepwise regression analysis showed that use of prophylactic medications (OR: 0.43, 95%CI: 0.19-0.99, P=0.048) and COMT Met/Met genotype (vs Val/Val, OR: 4.29, 95% CI: 1.10-16.71, P=0.036) were independent risk factors for poor response to triptans. Perspective: This study highlights the importance of COMT rs4680 in influencing the clinical response to drugs used for chronic pain, including opioid analgesics and triptans. These findings also underline a complex relationship between COMT genotypes and pain responder status.

Keywords: low back pain; morphine; migraine; triptans; response; COMT polymorphism.

Introduction

The catechol-O-methyltransferase (COMT) enzyme metabolizes catecholamines such as dopamine, adrenaline and noradrenaline that are involved in modulation of pain.[35,36,57] Genetic variation in the COMT gene may therefore contribute to the interindividual variability in human pain phenotypes such as pain sensitivity, chronicity, severity and response to analgesics.[1,19] The rs4680G>A variant (Val158Met) in the COMT gene causes a substitution from a valine (Val) to a methionine (Met) at amino acid position 158, leading to a three- to four-fold reduced enzymatic activity and higher dopamine availability (Met/Met > Val/Met > Val/Val).[5,26] The COMT rs4680 variant has been shown to influence efficacy of

morphine used for cancer pain, for which the Met/Met genotype group needs lower morphine doses than Val/Val genotype group,[28,41,43] possibly explained by an increased density of μ-opioid receptors in Met/Met genotype individuals.[4,58] However, some other reports were unable to demonstrate an involvement of rs4680 on the opioid dose requirement in cancer patients.[20,25] Failure to confirm such an association may be explained by several confounding factors which are inherent features of these studies on cancer patients, including the presence of both neuropathic and somatic pain. Hence, pharmacogenetic studies in non-cancer patients may contribute to clarify the relationship between rs4680 and the analgesic response to opioids.

Dopaminergic system hypersensitivity has been suggested in the pathogenesis of migraine on the basis of pharmacological evidences supporting the clinical use of dopamine antagonists in the treatment of acute migraine, either as an adjunct treatment for nausea or for migraine itself.[7,27] Although rs4686 does not appear to be involved in the predisposition to migraine,[51] this genetic factor has been involved in the phenotypic expression of migraine without aura (MwoA), with 158Met-allele carriers displaying a higher pain intensity of headache and a higher incidence of the accompanying nausea/vomiting compared to MwoA patients without 158Met allele.[32] Therefore, it is possible that inter-individual differences in COMT activity might influence efficacy of drugs used for the treatment of migraine pain, including the triptan class of serotonin 5-HT1B/1D receptor agonists.[11,50] While controversial results have been reported on the role of the DRD2 NcoI polymorphism on the variability in the therapeutic effects of triptans,[3,16,53] no data are available as to whether an increased dopaminergic tone, as expected in COMT Met/Met individuals, might affect headache response to triptans in migraine sufferers.

In the present study we assessed the value of COMT rs4680 as a predictive factor for the response to opioids or triptans, two classes of medication used to assist in

the management of chronic pain. More specifically, we evaluated the association between rs4680 and the analgesic response to intrathecal morphine in patients with chronic low back pain to provide confirmation of previously reported positive findings, while the relationship between rs4680 and headache response to triptans was assessed in two independent cohorts of migraineurs: one exploratory cohort of exclusively MwoA patients treated with frovatriptan and one validation cohort of migraineurs patients treated with other types of triptans.

Methods

Patients with persistent chronic low back pain

Patients suffering from chronic low back pain who received intrathecal morphine were enrolled in this study at the Pain Therapy U.O. of Rimini Hospital. The study was approved by the local Ethical Committee. These patients received intrathecal morphine as a trialing method to evaluate suitability to having an intrathecal drug delivery system implanted.[9,23,38,39] A total of 74 subjects were enrolled between 2008 to 2012 according to the following inclusion/exclusion criteria: Inclusion criteria: a) patient able to read, understand and voluntarily sign the informed consent to participation before undergoing any procedure for the study; b) age 18 years or older, at study entry; c) patient affected by chronic low back pain secondary to spinal stenosis and failed back surgery, and eligible to receive implantation of an intrathecal drug delivery system;[9,23,38,39] d) patient receiving an intrathecal morphine trialing protocol at a dose of 0.030 mg. Exclusion criteria were: a) patient who is pregnant or breast-feeding; b) patient who received an investigational drug within 30 days prior to screening; c) patient with a known hypersensitivity to opioid drugs; d) patient for which the use of opioid analgesia is contraindicated; e) patient with pre-existing history of psychosis; f) patient with a history of drug addiction.

Pain levels were assessed using a visual analog scale (VAS) of 0–10 (0= "No pain", 10= "worst pain possible") based on patient self report at the time of initial assessment (baseline), and at 1 hour after intrathecal administration of morphine. The intrathecal administration of 0.03 mg of morphine has been previously demonstrated to be effective in inducing pain relief in patients with chronic noncancer pain.[14,40] The presence of side effects commonly associated with opioids was also assessed. Patients were considered good responders to intrathecal morphine if pain reduction was ≥ 60 %, moderate responders if it was ≥ 40 % and < 60 %, and poor responders if pain reduction was < 40 %.

Patients with migraine pain

A total of 198 Caucasian migraine outpatients of the Novara and Pavia headache centers were enrolled in the study. All patients were diagnosed by a neurologist after neurological examination and direct interview according to the diagnostic criteria set by the International Headache Society (Headache Classification Subcommittee of the International Headache Society, 2004) for migraine without aura (MwoA) (IHS code 1.1) and migraine with aura (MwA)—typical aura with migraine headache (IHS code 1.2.4). Exclusion criteria were a headache that fulfilled the diagnostic criteria for a probable medication—overuse headache (IHS code 8.2.7) and contraindication to triptan use. Tension type headache patients and patients with double diagnosis were not enrolled in this study. In the first visit, patients were prescribed one of the six triptans commercially available in Italy according to the clinician's judgement and were given a diary on which to record the clinical response to the drug in three consecutive migraine attacks. If indicated, they were also prescribed a migraine prophylactic therapy. For each of the migraine attacks, the patient was asked to record on the diary the intensity of pain (on a scale from 0 to 3; 0=absent pain, 1=mild pain/no disability, 2=moderate pain/partial disability and 3=severe pain/total disability) at the moment of the triptan intake and

after 120 min, and the presence and intensity (on a scale from mild-to-severe) of side effects. The second visit took place after three attacks. Good responders were defined as the migraineurs who experienced a ≥2 point reduction in a 4-point scale intensity of pain from 3 (severe) to 0 (absent) 2 h after triptan administration in at least two attacks out of the three,[54] otherwise patients were defined as poor responders.

This study was approved by the local Ethics Committees of the institutions involved (Istituto C. Mondino Pavia and Ospedale Maggiore della Carità, Novara) and it met the requirements of the Declaration of Helsinki. Informed consent was obtained from all patients before participation in the study.

COMTVal158Met genotyping

Genomic DNA was extracted from peripheral blood by using the QiaAmp DNA Mini Kit (Qiagen Valencia, California, USA). Polymerase chain reactions (PCRs), conducted in a total volume of 30 μl containing 100 ng of genomic DNA, were performed using 0.4 μM of each couple of the following primers: FW: 5'-TCG TGG ACG CCG TGA TTC AGG-3'; Rev: 5'-AGG TCT GAC AAC GGG TCA GGC-3'. After 33 cycles of PCR amplification (denaturation at 94 °C for 30 s, annealing at 55 °C for 30 sec, extension at 72 °C for 30 sec), amplification products of 217 bp in length were electrophoresed in 2% agarose gel and visualized after staining with ethidium bromide. The PCR products (10 μl) harboring the SNPs were digested overnight at 37°C by 2 U of NlaIII (New England Biolabs, Milano, Italy). Wildtype COMT Val/Val was characterized by 136, 81 bp fragments, heterozygotes (Val/Met) by 138, 96, 81 and 40 bp fragments, and homozygotes for the Met allele (Met/Met) by 96,81,40 bp sized fragments. All PCR reactions were set up in a dedicated PCR area with dedicated pipettes and reagents. For quality control purposes, each PCR and restriction enzyme digestion included negative as

well as positive controls. For validation, about 10% of the samples were re-genotyped. The results were reproducible with no discrepancies in genotyping.

Statistical analysis

Data were summarized and presented in the form of mean, standard deviation and percentage as descriptive statistics. The Hardy-Weinberg equilibrium was verified in each patient cohort using the chi-square test as implemented in the Finetti program (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). Patients were dichotomized in two groups on the basis of drug response status: responders (good- and moderate-) and poor responders. In a preliminary analysis, the Armitage test for linear trend in proportions was performed on genotype frequency data to assess the dosage effect of possessing zero, one or two copies of the Met allele (i.e. an additive effect) on drug responses rates (analgesic response to intrathecal morphine or headache response to triptans). Next, the magnitude of the effect (effect size) of categorical or continuous variables (age) on the risk of poor drug responses was evaluated by unconditional logistic regression analysis (univariate analysis). Odds ratios (ORs) and their 95% confidence intervals (CIs) were used as estimates of relative risk. Finally, a binary logistic regression model, weighted for multilevel data and with forward stepwise selection of the variables (with input p-values set at 0.15), was performed to investigate the dependence of drug response status on a set of explanatory variables. A $P<0.05$ was considered statistically significant. All clinical and genotype data were managed with the statistical software package SYSTAT for Windows (version 12; Systat Software Inc., Chicago, IL, USA).

Results

Analgesic response to intrathecal morphine in patients with chronic low back pain.

Of the 74 patients with persistent chronic low back pain (age: 60.7 ± 16.1 years), 34 (45.9%) were males and 40 (54.1%) females (Table 1). The percentage of patients with good, moderate and poor analgesic response to intrathecal morphine was 74.3%, 9.5% and 16.2%, respectively. Distribution of COMT genotypes (Val/Val: n=19; Val/Met: n=44; Met/Met: n=11) was in Hardy–Weinberg equilibrium (P=0.08). The analgesic response rate according to COMT Val/Met genotype distribution is presented in Fig 1A. The analysis on dichotomized responses (good- and moderate- vs poor response) showed a significant better response across the three genotypes according to the number of copies of the Met allele carried (Armitage trend test; P=0.018) with 100% of the patients with Met/Met experiencing response (good or moderate) to intrathecal morphine compared to 68.4% of responders in patients with Val/Val genotype (P=0.037). As none of patients with Met/Met responded poorly to intrathecal morphine, Val/Met and Met/Met genotypes were combined to estimate the impact of COMT genotypes on the risk of poor intrathecal morphine response. The univariate logistic regression analysis (Table 1) showed that patients with poor response to intrathecal morphine differed from responders (good- or moderate) for younger age (OR: 0.91, 95%CI: 0.86-0.96, P=0.001) and lower frequency of the Met allele compared to Val/Val genotype (OR: 0.26, 95%CI: 0.07-0.96, P=0.043). Given that COMT activity may be under hormonal control[17,56] and our cohort was composed by a similar proportion of males and females, we conducted separate analyses for each gender. The sex-specific analysis of the data showed a trend in both male and female carriers of the Met allele towards a lower risk to be poor responders to intrathecal morphine (Table1), but in both groups the effect of COMT genotype did not reach statistical significance, probably due to the small number of patients. The

two-way ANCOVA analysis adjusted for age revealed that the interaction between COMT genotype (Met carriers vs Val/Val) and gender on the analgesic response to intrathecal morphine was not significant (P=0.515). In the logistic stepwise regression analysis (Table 1), age (OR: 0.90, 0.85-0.96, P=0.002) and the presence of the COMT Met allele (vs Val/Val, OR: 0.21, 0.04-0.98, P=0.031) were selected as significant independent predictors for lower risk of poor analgesic response to intrathecal morphine.

Table 1. Logistic regression analysis evaluating the association between COMT rs4680 and clinical variables with analgesic response to intrathecal morphine.

Variable	Total patients n=74 (%)	Responders (good- or moderate-) n= 62 (%)	Poor responders n= 12 (%)	OR (95% CI)	P value
Univariate analysis					
Sex					
Female	40 (54.1)	33 (53.2)	7 (58.3)	1	
Male	34 (45.9)	29 (46.8)	5 (41.7)	0.81 (0.23-2.84)	0.745
Age at study entry (year)					
mean ± SD	60.7 ± 16.1	63.8 ± 14.4	44.4 ± 15.4	0.91 (0.86-0.96)	**0.001**
COMT rs4680 (total sample)					
Val/Val	19 (25.7)	13 (21.0)	6 (50.0)	1	
Val/Met	44 (59.5)	38 (61.3)	6 (50.0)		
Met/Met	11 (14.9)	11 (17.7)	0 (0)	0.26 (0.07-0.96)*	**0.043**
COMT rs4680 (females only)					
Val/Val	11 (27.5)	8 (24.2)	3 (42.9)	1	
Val/Met	21 (52.5)	17 (51.5)	4 (57.1)		
Met/Met	8 (20.0)	8 (24.2)	0 (0)	0.43 (0.08-2.32)*	0.325
COMT rs4680 (males only)					
Val/Val	8 (23.5)	5 (17.2)	3 (60.0)	1	
Val/Met	23 (67.6)	21 (72.4)	2 (40.0)		
Met/Met	3 (8.8)	3 (10.3)	0 (0)	0.14 (0.02-1.06)*	0.057
Multivariate stepwise logistic regression analysis					
Age				0.90 (0.85-0.96)	**0.002**
COMT_Met allele carriers vs Val/Val				0.21 (0.04-0.98)	**0.048**

Note: some percentages may not add up to 100% due to rounding.

*Met allele carriers vs Val/Val.

P values ≤0.05 are in boldface.

Headache response to frovatriptan in patients without aura (MwoA)

Demographic and clinical data of MwoA patients treated with frovatriptan, in the overall cohort (n=75) and after stratification for headache response status, are shown in Table 2. Eighty-four percent of the study population was female (63/75), the average age in the cohort was 40.9 years ± 11.3 and fifty-six percent of patients (42/75) used prophylactic medications. Thirty-four out of the 75 patients (45.3%)

were poor responders to frovatriptan. Distribution of COMT genotypes was in accordance with Hardy-Weinberg equilibrium (P=0.72). Sex, age and use of prophylactic medications were similarly distributed between good and poor responders to frovatriptan (P=0.78, P=0.31, P=0.36, respectively). The headache response rate of MwoA patients to frovatriptan after stratification for COMT Val/Met genotypes is shown in Fig 1B. The Armitage trend test showed a significant worse headache response across the three genotypes according to the number of copies of the Met allele carried (P= 0.017) and 31.6 % of migraine patients with Met/Met experienced response to frovatriptan, while the response rate was higher in the Val/Val group (70.6% of responders, P= 0.019). In the univariate analysis (Table 2), homozygous carriers of the COMT 158Met allele were found at increased risk to be poor responders to frovatriptan when compared to homozygous patients for the Val allele (OR: 5.20, 95% CI: 1.25-21.57, P=0.023). Similar results were obtained when analysis was restricted to women. The relationship between rs4680 polymorphism and poor response to frovatriptan remained significant after adjustments for sex, age and use of prophylactic medications (Met/Met vs Val/Val, OR: 5.73, 95% CI: 1.33-24.67, P=0.019).

Table 2. Univariate logistic regression analysis evaluating the association between COMT rs4680 and clinical variables with response to frovatriptan in migraine patients without aura.

Variable	Total patients n=75 (%)	Responders n=41 (%)	Poor responders n=34 (%)	OR (95% CI)	P value
Sex					
Female	63 (84.0)	34 (82.9)	29 (85.3)	1	
Male	12 (16.0)	7 (17.1)	5 (14.7)	0.84 (0.24-2.92)	0.781
Age at study entry (year)					
mean ± SD	40.9 ± 11.3	41.9 ± 11.1	39.3 ± 11.5	0.98 (0.94-1.02)	0.309
Use of prophylactic medications					
No	33 (44.0)	20 (48.8)	13 (38.2)	1	
Yes	42 (56.0)	21 (51.2)	21 (61.8)	1.54 (0.61-3.88)	0.361
COMT rs4680 (total sample)					
Val/Val	17 (22.7)	12 (29.2)	5 (14.7)	1	
Val/Met	39 (52.0)	23 (56.1)	16 (47.0)	1.67 (0.49-5.67)	0.411
Met/Met	19 (25.3)	6 (14.6)	13 (38.2)	5.20 (1.25-21.57)	**0.023**
COMT rs4680 (females only)					
Val/Val	13 (20.6)	10 (29.4)	3 (10.3)	1	
Val/Met	34 (54.0)	19 (55.9)	15 (51.7)	2.63 (0.61-11.30)	0.193
Met/Met	16 (25.4)	5 (14.7)	11 (37.9)	7.33 (1.38-38.88)	**0.019**

Note: some percentage may not add up to 100% due to rounding. P values ≤0.05 are in boldface.

Headache response to other triptans in migraineurs

In order to validate the generality of our findings we studied an independent cohort of migraine pazients treated with triptans other than frovatriptan. Demographic and clinical data of the second cohort of migraineurs (n=123) are shown in Table 3. Seventy-seven percent of the study population was female (95/123), the average age in the cohort was 38.3 years ± 10.2, 90.2% of which affected by MwoA and 9.8% by MwA. The triptans prescribed were: rizatriptan (n=34), eletriptan (n=34), almotriptan (n=25), sumatriptan (n=21) and zolmitriptan (n=9). Sixty-five of 123 patients (54.2%) were on prophylactic medication, while for three patients the data

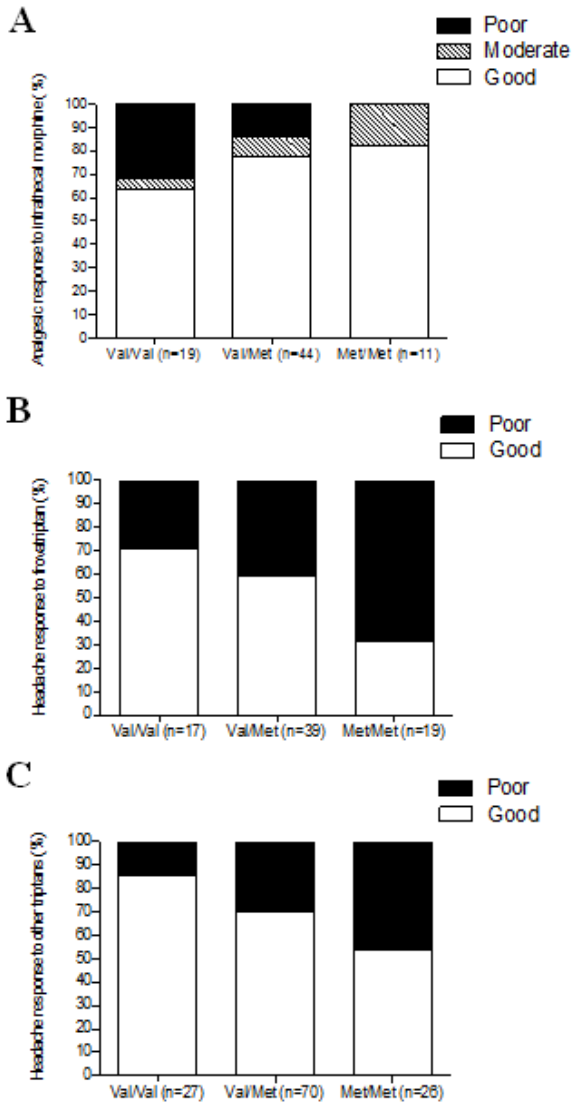
on the use of preventive medication were lacking. Poor response to triptans was observed in 30.1% of migraine patients (37/123). The genotype frequency distribution of rs4680 was in accordance with Hardy-Weinberg equilibrium expectations (P=0.12). Fig 1C shows headache response rates after stratification for the COMT Val/Met genotypes, in the validation cohort of migraineurs. The analysis revealed again a significant worse headache response across the three genotypes according to the number of copies of the Met allele carried (Armitage trend test; P= 0.013) and 53.8 % of the patients with Met/Met experienced response to triptans other than frovatriptan while the response rate was higher in the Val/Val genotype (85.5% of responders, P= 0.013). In the univariate analysis (Table 3), patients undergoing prophylactic treatment (n=120) were found at lower risk to be poor responders, as compared to patients who were not on prophylactic treatment (OR: 0.44, 95% CI: 0.2-0.99, P=0.046). All other demographic and clinical variables considered were similarly distributed when comparing good and poor responders to triptans (Table 3). In addition, homozygous carriers of 158Met allele were more frequently poor responders to triptans when compared to homozygous patients for the Val allele (OR: 4.93, 95%CI: 1.33-18.31, P=0.017) and similar results were obtained when analysis was limited to women (Table 3). In the logistic stepwise regression analysis (Table 3), use of prophylactic medications (OR: 0.43, 95%CI: 0.19-0.99, P=0.048) and COMT Met/Met genotype (vs Val/Val, OR: 4.29, 95%CI: 1.10-16.71, P=0.036) were selected as independent risk factor for poor response to triptans (Table 3).

Table 3. Logistic regression analysis evaluating the association between COMT rs4680 and clinical variables with response to triptans other than frovatriptan in migraine patients.

Variable	Total patients n=123 (%)	Responders n=86 (%)	Poor responders n=37 (%)	OR (95% CI)	P value
Univariate analysis					
Sex					
Female	95 (77.2)	68 (79.0)	27 (73.0)	1	
Male	28 (22.8)	18 (21.0)	10 (27.0)	1.40 (0.57-3.41)	0.461
Age at study entry (year)					
mean ± SD	38.3 ± 10.2	38.0 ± 10.3	38.8 ± 10.4	1.007 (0.97-1.04)	0.715
Diagnosis					
MwoA	111 (90.2)	79 (91.9)	32 (86.5)	1	
MwA	12 (9.8)	7 (8.1)	5 (13.5)	1.76 (0.52-5.97)	0.362
Triptan					
Rizatriptan	34 (27.6)	21 (24.4)	13 (35.1)	1	
Eletriptan	34 (27.6)	27 (31.4)	7 (18.9)	0.42 (0.14-1.23)	0.115
Almotriptan	25 (20.3)	17 (19.8)	8 (21.6)	0.76 (0.26-2.26)	0.621
Sumatriptan	21 (17.1)	13 (15.1)	8 (21.6)	0.99 (0.32-3.05)	0.992
Zolmitriptan	9 (7.3)	8 (9.3)	1 (2.7)	0.20 (0.02-1.81)	0.152
Use of prophylactic medications (n= 120)					
No	55 (45.8)	34 (40.0)	21 (60.0)	1	
Yes	65 (54.2)	51 (60.0)	14 (40.0)	0.44 (0.2-0.99)	**0.046**
COMT rs4680 (total sample)					
Val/Val	27 (22.0)	23 (26.7)	4 (10.8)	1	
Val/Met	70 (56.9)	49 (57.0)	21 (56.8)	2.46 (0.76-8.00)	0.134
Met/Met	26 (21.1)	14 (16.3)	12 (32.4)	4.93 (1.33-18.31)	**0.017**
COMT rs4680 (females only)					
Val/Val	23 (24.2)	20 (29.4)	3 (11.1)	1	
Val/Met	52 (54.7)	38 (55.9)	14 (51.9)	2.46 (0.63-9.56)	0.195
Met/Met	20 (21.1)	10 (14.7)	10 (37.0)	6.67 (1.49-29.79)	**0.013**
Multivariate stepwise logistic regression analysis					
Prophylaxis_Yes				0.43 (0.19-0.99)	**0.048**
COMT_Val/Met				2.27 (0.69-7.51)	0.180
COMT_Met/Met				4.29 (1.10-16.71)	**0.036**

Note: some percentage may not add up to 100% due to rounding. P values ≤0.05 are in boldface.

Fig 1: A) Analgesic response rate to intrathecal morphine according to COMT Val158Met genotype distribution in patients with chronic low back pain. Comparison of responders (good and moderate) with Armitage trend test across the three genotypes (P=0.018). B) Headache response rate to frovatriptan according to COMT Val158Met genotypes in migraine patients without aura (Armitage trend test across the three genotypes, P= 0.017). C) Headache response to triptans other than frovatriptan in an independent cohort of migraineurs (Armitage trend test; P= 0.013).



Discussion

Experimental pain studies have consistently shown that individuals with low COMT activity have low tolerance to pain. For instance, healthy volunteers with the COMT Met/Met genotype displayed higher sensory and affective ratings of pain and a higher regional density of μ-opioid receptors in the brain as measured by ligand-PET (positron emission tomography).[58] Moreover, in a functional neuroimaging study, homozygous subjects for the Met-allele exhibited a higher blood oxygen level-dependent response in the anterior cingulate cortex to painful laser stimulation compared to carriers of the Val-allele.[29] In chronic clinical pain, the effect of COMT on pain sensitivity and modulation has been suggested to depend on the pain conditions.[51] Indeed, in neuropathic and cancer-related pain, COMT variation does not play a large role, [2,41,45] while in chronic musculoskeletal pain and migraine low COMT activity appears to increase incidence and/or pain symptoms.[19,32] On the other hand, the genetic background may also influence the analgesic response to various pharmacotherapies, however the specific genetic variations underlying inter-individual differences in analgesic drug responses remain poorly elucidated. As genetic variation in the COMT gene may have clinical implications not only for pain perception but also for pain treatment, in the present study we have addressed a possible contribution of rs4680 in the COMT gene to the individual variability in the response to morphine or triptans, two classes of medication used to control pain in patients with chronic low back pain and migraine, respectively.

Our results provide evidence in patients with chronic low back pain that rs4680 significantly influences the response to intrathecal morphine, with the analgesic outcome being inversely proportional to the enzyme activity: better response rate in patients with lower COMT activity (Met/Met), worse response in patients with higher COMT activity (Val/Val). These results support a higher efficacy of intrathecal morphine therapy in patients with Met/Met genotype. Therefore, our

findings are in the same direction of previous studies reporting that cancer patients with Met/Met genotype require less morphine than patients with Val/Val genotype to achieve the same level of analgesia.[28,41,43]

The use of intrathecal drug delivery systems in chronic non-malignant pain is indicated in those patients in which traditional administration routes are poorly effective or in those who cannot tolerate high doses because of systemic side-effects.[9,23,39] However, the efficacy of intrathecal morphine treatment is hampered by the large variability and unpredictability in individual response. Although the factors explaining variability in opioid efficacy are still largely unknown, clinical features and types of pain,[8,38] as well as polymorphisms in genes encoding drug targets,[47] drug metabolizing enzymes, and/or drug transporters,[48] have been suggested to contribute to the large interindividual variability in the efficacy of intrathecal morphine administration. At present, there is no agreement regarding the intraspinal screening method that will be most predictive of patients' long term response to intrathecal morphine. Thus, given the results presented here, we propose that COMT Val158Met polymorphism should be evaluated further to investigate whether it can predict efficacy of chronic intrathecal morphine therapy.

We also provide for the first time evidence that allelic variation of the COMT rs4680 polymorphism affects headache response to triptans in patients with migraine pain. Intriguingly, the impact of rs4680 on headache response to triptans was in the opposite direction. Indeed, frovatriptan-treated patients with the Met/Met genotype showed a poorer headache response than patients with the Val/Val genotype and similar results were obtained in a second cohort of migraineurs treated with other types of triptans. Altogether, our results highlight a role of rs4680 as response-modifying gene variant in relation to morphine or to triptan therapy. In addition, our study suggests that the COMT rs4680 variant, affecting catecholaminergic neurotransmission, may influence the individual response to different classes of drugs used for chronic pain, irrespective of their

primary molecular target. The better response to opioids in Met/Met carriers has been previously explained by an increased amount of regional μ-opioid receptors[4,58] as a compensatory mechanism in response to lower content of enkephalin within the peripheral neurons of these individuals.[22,41] In contrast, the lower rate of response to triptans in migraineurs with Met/Met genotype is an entirely novel finding, for which data on possible molecular mechanisms are missing. We can speculate that, in migraine subjects, the lower activity of COMT is associated with a reduced metabolization of cathecolamines, such as norepinephrine and epinephrine, thereby leading to a potentiation of pain signaling through the downstream stimulation of β_2- and β_3-adrenergic receptor pathways.[31] The more aggressive phenotype described by Park et al[32] in Met/Met migraineurs may therefore represent a consequence of a genetic predisposition, and the poorer response to triptans just reflects the failure to control more intense attacks. Alternatively, a complex interplay between enhanced adrenergic and dopaminergic activity in different parts of the nociceptive system might explain the complicated actions of low COMT.[1,19] On the other hand, the possible contribution of COMT rs4680 in migraine pain therapy stems from reports supporting the usefulness of dopamine antagonists in the treatment of acute migraine, either as an adjunt treatment for nausea or for the migraine itself.[7,27,49] Given that COMT inactivates NE and DA, but not 5-HT, our data support the possibility that triptans are less effective in migraine patients with a higher catecholaminergic tone, as expected in patients with Met/Met genotype. Noteworthy is that the combination of sumatriptan with the dopaminergic antagonist metoclopramide has been reported to provide relief in some migrainers who failed to achieve adequate relief with a triptan alone.[46] It is therefore tempting to speculate that COMT rs4680 genotyping could be useful to identify patients at higher risk of poor response to triptan monotherapy which can benefit from a combination therapy (triptan + DRD2 antagonist).[34]

Although the similarities of 5-hydroxytryptamine (5-HT) $_{1B/1D}$ receptor agonists outweigh their differences, important differences exist in the pharmacokinetic profile of triptans. For instance, bioavailability of oral formulations ranges between 14% (sumatriptan) and 69% (almotriptan), and their elimination half-life ranges from 2 h (sumatriptan and rizatriptan) to 26 h (frovatriptan).[37] In addition, the beneficial effect of triptans in patients with migraine may be related to their multiple mechanisms of action at either peripheral and/or central sites implicated in the pathophysiology of migraine.[13] In this regard, triptans as a class display a poor brain-blood barrier penetration with brain/plasma partition coefficients ($K_{p,brain}$) well below 1, when compared with typical CNS marketed drugs (e.g. diphenhydramine with a $K_{p,brain}$ of 9).[18,33] In contrast, the relatively hydrophilic triptan, sumatriptan, has been regarded either to be incapable of crossing the brain-blood barrier or to cross it to a lower extent compared to other triptans.[55] Given the wide variety of drug treatments received by migraine patients due to the naturalistic setting of our study, it was not possible to conduct a rigorous analysis of the possible differential effect of COMT rs4680 on headache response to the different triptans. However, it should be noted that the effect size of COMT genotype in patients treated with to the long-acting triptan (frovatriptan) was similar to that observed in patients treated with the fast-acting triptans (eletriptan, rizatriptan, almotriptan, sumatriptan, zolmitriptan). In addition, the significance of COMT genotype was retained in both univariate (Met/Met vs Val/Val, OR: 5.04, 95% CI: 1.87-13.60, P=0.001) and fully adjusted multivariate analysis (Met/Met vs Val/Val, OR: 4.09, 95% CI: 1.43-11.67, P=0.008), when patients receiving sumatriptan were excluded from the combined analysis of the two migraine cohorts.

We recognize some limitations in our study. First, the COMT Val158Met polymorphism alone cannot fully account for the variation in enzyme activity as COMT haplotypes have been shown to influence COMT function[30] and to explain the effects on pain perception or opioid efficacy to a greater extent than rs4680

alone.[10,42,52] In addition, rs740603 and haplotypes containing SNPs in intron 1, but not rs4680, have been associated to adverse effects of morphine.[45] Thus, further studies in larger populations in which COMT haplotype analyses can be better evaluated are required to replicate and extend the current findings. In addition, we also recognize that polymorphisms in other genes encoding for drug metabolizing enzymes, drug transporters or drug targets may be also involved in the individual variability of clinical response to opioids or triptans.[6,12,21,24,44] Therefore, approaches based on multiple genetic markers, along with demographic and clinical characteristics of patients, are required to characterize the joint effects of multiple genes in predicting the clinical response to opioid analgesics or triptans. Another potential limitation of this study is the absence of placebo-treated groups. Since we do not know the rate of non-specific or non-drug attributable responses, we cannot exclude the possibility that some patients in the responder group were subjected to a placebo effect, which in a very recent paper has also been observed with rs4680.[15] Nonetheless, given the confirmatory nature of the study conducted in morphine-treated patients and the consistent association emerged in the exploratory/validation study of triptan-treated migraineurs, we feel that the presence of placebo groups may not have significantly affected our results. In addition, the observational design of the study conducted in triptan-treated patients reflects the conditions of migraine managing in primary care, in which triptans are the first-line treatment and placebo is not used. Finally, given the limited number of male patients in our cohorts, larger studies are required to evaluate gender-specific effects of COMT Val158Met polymorphism on the efficacy of morphine or triptans.

In conclusion, the current results highlight the importance of COMT rs4680 genotype in influencing the clinical response to drugs used for chronic pain including opioid analgesics and triptans. The opposite direction of rs4680 effect on the clinical response to these classes of drugs in two different pain conditions

reveals a complex relationship between COMT genotypes and pain responder status which appears to be drug-specific and likely to reflect the multifaceted interaction between different pain states and the cathecolaminergic neurotransmission.

Conflict of interest statement

None of the authors have any conflicts of interest.

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Chapter 5

Lack of association between GRIA1 polymorphisms and haplotypes with migraine without aura or response to triptans

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Abstract

The present study was designed to replicate previous findings reporting a significant association between the rs548294 polymorphism at the glutamate receptor subunit GluR1 gene (GRIA1) with migraine without aura, either as a single marker or in haplotype combination with rs2195450. In addition, the role of GRIA1 polymorphisms and haplotypes was evaluated in migraine patients without aura as predictive factors for consistency in headache response to triptans. Analysis of rs548294 and rs2195450 polymorphisms of GRIA1 was conducted by Real-time PCR allelic discrimination assay in 186 migraine patients without aura and 312 healthy controls, respectively. In the logistic regression analysis adjusted for gender and age, genotype and haplotype frequencies for the two polymorphisms did not significantly differ between migraine patients without aura and controls. In addition, no evidence of association was found between GRIA1 polymorphisms/haplotypes and consistent response to triptans. This study failed to

replicate previously reported association between GRIA1 rs548294 and migraine without aura, either as single marker or when analysed in haplotype combination with rs2195450. In addition, no evidence was found for a relevant role of GRIA1 polymorphisms and haplotypes as modulating factors of headache response to triptans.

Keywords: migraine, susceptibility, triptans, response, GRIA1, polymorphisms.

Introduction

Migraine is a common, painful and debilitating disorder with a strong genetic basis. About 50% of affected individuals have a first-degree relative also suffering from migraine [1-3], with estimates of heritability from family and twin studies ranging between 34 and 57% [2,4,5]. The involvement of glutamate in trigemino-vascular activation, cortical spreading depression and central sensitization [6,7] as well as the effectiveness of glutamate receptor-subtype antagonists in preclinical studies [8,9] argue for a primary role of the glutamatergic neurotransmission in the pathophysiology of migraine. Recent results of genome–wide association studies support a correlation between migraine and variants in glutamatergic system genes [10-12]. However, the effect estimates of all associations with genome-wide significance confer a small to moderate change in risk for migraine, a result which suggests that only a small part of the genetic background of migraine has been established so far.

Glutamate exerts its actions through activation of ionotropic and metabotropic receptors [4]. Great interest was therefore aroused by results of a case-control study showing that rs548294 and rs2195450 SNPs of the glutamate receptor subunit

GluR1 gene (GRIA1) are significantly associated with migraine, either as single markers or in haplotype combination [13]. Intriguingly, stratified analysis of migraine subtype revealed that the rs548294 SNP was primarily associated with migraine without aura (MwoA), while rs2195450 was found associated with aura, but not with MwoA. More precisely, in the subgroup analysis comprising 109 MwoA patients and 260 control healthy subjects, carriers of the minor allele of rs548294 were found at higher risk of developing MwoA than homozygotes wild-type carriers (OR: 2.7, 95%CI: 1.6-4.3). Given that these GRIA1 SNPs are located in the same linkage disequilibrium (LD) block [13], in the present study we undertook a case-control association study on rs2195450 and rs548294 to validate their role, either as single SNPs or haplotypes, as genetic determinants of migraine without aura (MwoA). In addition, since the majority of glutamatergic neurons in trigeminal ganglion carry serotonin 5-$HT_{1B/D/F}$ receptors and glutamate has been implicated in the response mechanisms of 5-$HT_{1B/1D}$ agonists (triptans) [14], we also assessed whether GRIA1 polymorphisms and haplotypes may be predictive factors for consistency in headache response to triptans in MwoA patients.

Materials and methods

Patient selection

All patients were diagnosed for migraine without aura (MwoA) (IHS code 1.1) by two headache specialists (M.V. and D.M.) after neurological examination and direct interview according to the diagnostic criteria set by the International Headache Society (Headache Classification Subcommittee of the International Headache Society, 2004). In the first visit, patients were prescribed one of the six triptans commercially available in Italy according to the clinician's judgement and were given a diary on which to record the clinical response to the drug in three consecutive migraine attacks. If indicated, they were also prescribed a migraine

prophylactic therapy. For each of the migraine attacks, the patient was asked to record on the diary the intensity of pain (on a scale from 0 to 3; 0= absent pain, 1= mild pain/no disability, 2= moderate pain/partial disability and 3= severe pain/total disability) at the moment of the triptan intake and after 120 min. The second visit took place after three attacks. Consistent responders to triptans were defined as the migraineurs who experienced a ≥2 point reduction in a 4-point scale intensity of pain from 3 (severe) to 0 (absent) 2h after triptan administration in at least two attacks out of the three, otherwise patients were defined as inconsistent responders [15]. Controls were randomly selected from a population of same regional background (northwest Italy) to minimize population heterogeneity and stratification. This study was approved by the local Ethics Committees of the institutions involved (Istituto C. Mondino Pavia and Ospedale Maggiore della Carità, Novara) and met the requirements of the Declaration of Helsinki. Informed consent was obtained from all patients before participation in the study.

Genotyping

Genomic DNA was extracted from peripheral blood by using the QiaAmp DNA Mini Kit (Qiagen Valencia, California, USA). Genotyping of rs548294 and rs2195450 SNPs of the GRIA1 gene was performed by real-time PCR using Applied Biosystems TaqMan Pre-Designed SNP Genotyping assays [rs548294 Assay ID: C_318751_20; rs2195450 assay ID: C_15850372_10]. Real-time PCR amplification and detection was conducted on genomic DNA in 48-well PCR plates using a MiniOpticon Real–Time PCR Detection System (Bio-Rad, Milan, Italy). Thermal cycling was initiated with a denaturation step of 10 min at 95 °C, followed by 50 cycles of 15 s at 95 °C and 90 s at 60 °C. After PCR was completed, allelic discrimination was analyzed using the Bio-Rad CFX Manager Software (Version 2.1, Bio-Rad). Genotype assignment was determined by plotting the end point relative fluorescent units (RFU) for one fluorophore (allele 1 on the x-axis) against

the RFU for the other fluorophore (allele 2 on the y-axis) on the allelic discrimination plot. All reactions of real-time PCR were set up in a dedicated PCR area with dedicated PCR pipettes and reagents. For quality control purposes, each Real-time PCR included negative as well as positive controls for the three genotypes. For validation, about 10% of the samples were re-genotyped. The results were reproducible with no discrepancies in genotyping.

Statistical analysis

Data were summarized and presented in the form of mean, standard deviation and percentage as descriptive statistics. Each polymorphism was tested for deviation from the Hardy-Weinberg equilibrium (HWE) by use of Pearson's chi-square test as implemented in the Finetti's program (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). The association between GRIA1 SNPs and clinical endpoints was assessed by logistic regression analyis with adjustment for confounding covariates using the SNPStats software [16]. Odds ratios (ORs) and their 95% confidence intervals (CIs) were used as estimates of relative risk. The SNPStats software was also used for the calculation of linkage disequilibrium (measured as Lewontin's D′-values) between GRIA1 SNPs, for the estimation of haplotype frequencies and for the evaluation of haplotype association with the two clinical endpoints considered. As the haplotype test was a *post-hoc* analysis, we considered it to be an additional test. Therefore, for a Bonferroni correction on the P values we used P= 0.05/3 (total 2 polymorphisms + 1 haplotype) = 0.017 as a threshold for significance. Power calculations were performed using Quanto version 1.2.4 (http://hydra.usc.edu/gxe/). Meta-analysis was performed with Open Meta-Analyst available at http://tuftscaes.org/open_meta (Joseph Lau, Boston, Massachusetts). Data were combined using random-effects (DerSimonian and Laird) models which incorporate the between-study heterogeneity and allow for a different effect in each population [17]. We estimated the between-study heterogeneity by using the Cochran's Q chi-square test

(significant for $P < 0.10$) [18]. We also reported the I^2 index, which quantifies heterogeneity irrespective of the number of studies (range, 0-100%; values ≥75% imply extreme heterogeneity).

Results

This study included a total of 186 Caucasian MwoA patients (79.0% of women) with a mean age of 39 years (standard deviation: 10.6) and 312 population-based controls (62.8% of women) with a mean age of 56 years (standard deviation: 18.1). Genotypic distributions of rs548294 and rs2195450 polymorphisms in the GRIA1 gene in MwoA patients and control subjects are shown in Table 1. The two polymorphisms analyzed were in HWE both in MwoA patients ($P_{HWE\ rs548294}$= 0.73; $P_{HWE\ rs2195450}$ = 0.25) and in control subjects ($P_{HWE\ rs548294}$= 0.57; $P_{HWE\ rs2195450}$ = 0.46). In addition, in control subjects, the observed minor allele frequencies (MAFs) of both SNPs (MAF of rs548294: 0.34; MAF of rs2195450: 0.27) were similar to those reported in HapMap-CEU population (MAF of rs548294: 0.36; MAF of rs2195450: 0.28). After adjusting for age and gender, rs548294 and rs2195450 polymorphisms did not emerge as factors significantly associated to MwoA when analyzed as single locus variants, either under the dominant model of inheritance (Table 1) or other genetic models (codominant, recessive, over-dominant and log-additive). Pooled meta-analysis of the two studies (the present study and [13]) also found no statistically significant associations between rs548294 polymorphism and MwoA risk in the genetic dominant, codominant, recessive and additive models (Fig 1).

As haplotype association analysis of polymorphisms in strong LD has more power than single locus tests to detect gene–disease associations, an approach based on haplotype combination of GRIA1 gene polymorphisms was also used to detect association with MwoA. To this end, we first estimated LD between the two polymorphisms of the GRIA1 gene. Haplotype analysis in both patients and

controls revealed a strong pairwise LD between rs548294 and rs2195450 polymorphisms (D'= 0.86). In the logistic regression analysis adjusted for age and gender, no association was found between GRIA1 haplotypes and MwoA (Global haplotype association P-value: 0.34, Table 1).

Table 1. Genotype distribution of GRIA1 SNPs in MwoA patients (n = 186) and control subjects (n= 312) and association results as single markers or haplotypes (rs548294 - rs2195450).

SNP	**Controls n (%)**	**Cases n (%)**	**OR[*] (95% CI)**	**P-value**
GRIA1 rs548294 (G>A)				
GG	135 (43.3)	85 (45.7)	1 (ref)	
GA	137 (43.9)	83 (44.6)		
AA	40 (12.8)	18 (9.7)	D: 0.89 (0.60-1.34)	0.59
GRIA1 rs2195450 (C>T)				
CC	162 (51.9)	104 (55.9)	1 (ref)	
CT	122 (39.1)	74 (39.8)		
TT	28 (9.0)	8 (4.3)	D: 0.93 (0.62-1.39)	0.72
Haplotype [†]	**Haplotype frequency**		**OR[*] (95% CI)**	
	Controls	**Cases**		
G-C	0.381	0.449	1 (ref)	
A-C	0.334	0.309	0.77 (0.55-1.07)	0.12
G-T	0.271	0.231	0.79 (0.55-1.14)	0.21
A-T	0.014	0.011	0.63 (0.10-3.99)	0.63

Abbreviations: D, dominant model of inheritance. [*]Logistic regression analysis adjusted for age and gender. [†]Global haplotype association P-value: 0.34.

Next, we assessed the association between GRIA1 SNPs and haplotypes and consistent response to triptans in MwoA patients. Consistent response to triptans was observed in 64.5% of patients with MwoA (120/186). Patients receiving triptans other than frovatriptan (n=111, 59.7%) displayed higher consistent response rates than patients treated with frovatriptan (OR: 2.05, 95%CI: 1.11-3.78,

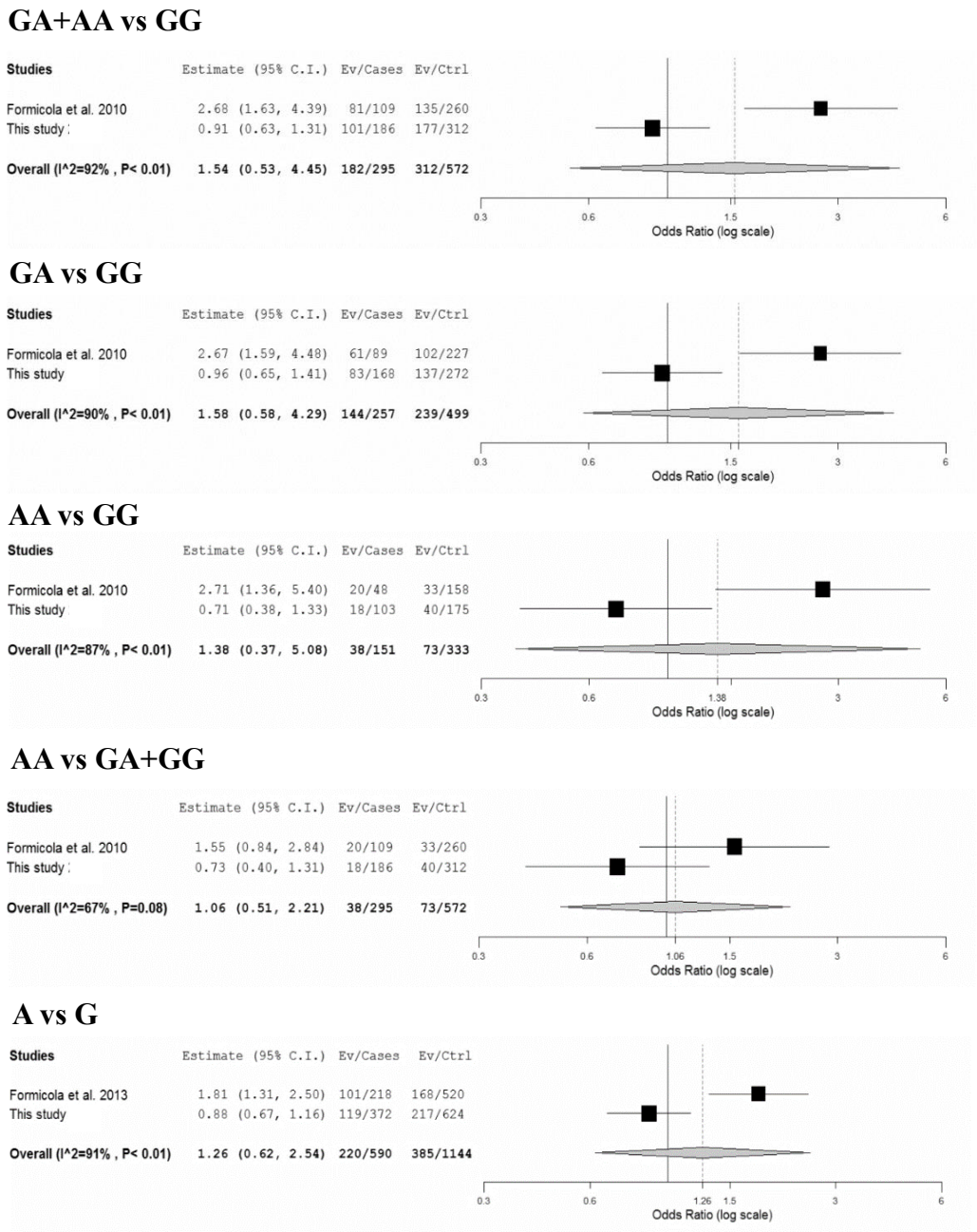
P=0.022, Table 2). All the other demographic and clinical variables were similarly distributed when comparing consistent responders with inconsistent responders. In the logistic regression analysis adjusted for triptan (frovatriptan vs other triptans), rs548294 and rs2195450 SNPs of the GRIA1 gene were not found significantly associated to response to triptans, either as single markers or when analysed in haplotype combination (Table 2).

Table 2. Clinical variables and genotype distribution of GRIA1 polymorphisms in MwoA patients with consistent (CR) and inconsistent response (IR) to triptans and association results of SNPs as single markers or haplotypes (rs548294-rs2195450).

Variable	IR n=66 (%)	CR n=120 (%)	OR* (95% CI)	P value
Sex				
Female	52 (78.8)	95 (79.2)	1 (ref)	
Male	14 (21.2)	25 (20.8)	0.98 (0.47-2.04)	0.95
Age at study entry, year				
Mean (SD)	39.4 (10.6)	39.4 (10.7)	1.00 (0.97-1.029)	0.97
Triptan				
Frovatriptan	34 (51.5)	41 (34.2)	1 (ref)	
Rizatriptan	11 (16.7)	18 (15.0)	Others 2.05 (1.11-3.78)	0.022
Eletriptan	5 (7.6)	24 (20.0)		
Almotriptan	8 (12.1)	17 (14.2)		
Sumatriptan	7 (10.6)	13 (10.8)		
Zolmitriptan	1 (1.5)	7 (5.8)		
Use of prophylactic medications (n= 183)				
No	29 (45.3)	49 (41.2)	1 (ref)	
Yes	35 (54.7)	70 (58.8)	1.18 (0.64-2.18)	0.59
GRIA1 rs548294 (G>A)				
GG	32 (48.5)	53 (44.2)	1 (ref)	
GA	25 (37.9)	58 (48.3)	D: 1.13 (0.61-2.08)	0.70
AA	9 (13.6)	9 (7.5)	R: 0.52 (0.19-1.40)	0.20
GRIA1 rs2195450 (C>T)				
CC	35 (53.0)	69 (57.5)	1 (ref)	
CT	30 (45.5)	44 (36.7)	D: 0.76 (0.41-1.41)	0.39
TT	1 (1.5)	7 (5.8)	R: 3.68 (0.44-31.00)	0.17
Haplotype †	**Haplotype frequency**		**OR# (95% CI)**	
	IR	**CR**		
G-C	0.446	0.450	1 (ref)	
A-C	0.311	0.308	0.92 (0.54-1.56)	0.76
G-T	0.228	0.233	0.91 (0.50-1.63)	0.74
A-T	0.015	0.009	0.59 (0.04-9.95)	0.72

Abbreviations: D, dominant model of inheritance; R, recessive model of inheritance. *****Crude logistic regression analysis. #Logistic regression analysis adjusted for triptan (frovatriptan vs other triptans). †Global haplotype association P-value: 0.960.

Figure1. Forests plot for the association of GRIA1 rs548294 with MwoA risk in the genetic dominant (GA + AA vs GG), codominant (GA vs GG or AA vs GG), recessive (AA vs GA + GG) and allele models (A vs G). Pooled estimates (OR, odds ratio) are from the random-effects model.



Discussion

The GWASs so far conducted have identified eight genetic variants that are associated with genome-wide significance with migraine [10-12,19]. Among these, rs1835740 is located between MTDH and PGCP genes, which are both involved in glutamate homeostasis, and rs11172113 is located within the LRP1 gene which may interact with neuronal glutamate receptors [20]. Together, these findings support a role of glutamatergic gene variants in the pathophysiology of migraine. In line with this hypothesis are therefore previous results showing that rs548294 and rs2195450 polymorphisms at the GRIA1 are significantly associated with migraine (with and without aura) [13]. Although a number of case-control studies have reported associations between particular candidate gene polymorphisms and migraine susceptibility, replication studies to confirm previous findings are generally lacking [21].

In the present study, we aimed to validate the role of GRIA1 rs548294 as genetic determinant of migraine without aura (MwoA), either as single marker or in haplotype combination with rs2195450. However, we were unable to confirm previous association of rs548294 SNP of GRIA1 gene as susceptibility factor for MwoA [13]. In addition, results of the haplotype combination of rs548294 and rs2195450 polymorphisms also provide evidence against an association of the GRIA1 gene with MwoA. Failure to confirm a previously identified association is not unusual in the search for genetic determinants of complex traits and multifactorial diseases such as migraine. Probably, the identification of genetic variants in migraine susceptibility remains challenging for several reasons, including population heterogeneity, environmental effects, and low sample size. Assuming a dominant model of inheritance, our study has >80% statistical power to detect an association of rs548294 with the effect size (OR) of 2.2 (α level of 0.017, with a 34% of MAF in controls) or to detect an association of rs2195450

with an effect size of 2.1 (α level of 0.017, with a 27% of MAF in controls). Thus, the failure to confirm the association of rs548294 with MwoA in the dominant model is not probably due to insufficient statistical power of our study. A possible alternative reason of these contrasting findings may be the result of choice of controls, however genotype frequencies of control patients in the present study (GG: 43.3%, GA: 43.9%, AA: 12.8%) did not differ compared to the previous one (GG: 48.1%, GA: 39.2%, AA: 12.7%). In addition, when the two studies were combined for pooled analysis, none of the genetic models tested provided a significant association between rs548294 polymorphism and MwoA risk. While no obvious explanation exists for these discrepant results, it should be noted that in our study the two GRIA1 SNPs were found in HWE both in controls and in MwoA patients, while rs2195450 was not previously found in HWE either in controls and in MwoA patients [13], suggesting the possibility in the previous study of inappropriate population stratification and selection or other confounding factors. It should be also acknowledged that, in the time of writing this article, a further study reported no evidence of association between rs548294 or rs2195450 and risk of migraine, either with or without aura [22]. Pooled results of the three studies so far conducted [the present study and 13, 22] excluded a role for rs548294 or rs2195450 as risk factors for migraine, either with or without aura (see Supplementary Material).

Several hypothetical ways have been suggested by which triptans might affect glutamate neurotransmission such as affecting the glutamate receptors binding site, inhibiting glutamate release, increasing the glutamate uptake by glial cells and/or by neuronal transporters, or decreasing the neuronal firing in the trigeminal nucleus [14,23-25]. In view of the facts that triptans may work in part by reducing extracellular glutamate as reflected by the decrease in CSF levels [14], and glutamate plays a significant role in the transmission of nociceptive information in the sensory thalamus [26,27], functional polymorphisms in glutamate receptors

binding sites including GRIA1 may affect the clinical response to triptans. However, our results obtained of either single SNP and haplotype-based analysis of rs548294 and rs2195450 exclude a possible involvement of GRIA1 SNPs in triptan response mechanisms in MwoA patients. Nonetheless, it should be noted that we did not perform a detailed LD-based association analysis with tagging SNPs covering a maximum amount of genetic variation in the GRIA1 gene, thus we cannot exclude the possibility that SNPs located in other LD blocks of the GRIA1 gene might be involved in the development of MwoA or headache response to triptans.

Conclusion

The present results do not confirm previous association of rs548294 with MwoA, either as single marker or in haplotype combination with rs2195450 polymorphism. Our findings also do not support a role of GRIA1 SNPs and haplotypes in modulating the clinical response to triptan therapy. Although the presence of other risk variants in the gene studied cannot be excluded, the present study highlights the importance of replication before accepting an association between genetic variation and any complex trait. In addition, the present study provides further insight to the search of pharmacogenetic determinants in the field of triptan therapy in migraine.

Conflict of interest

The authors report no conflict of interest.

Aknowledgements

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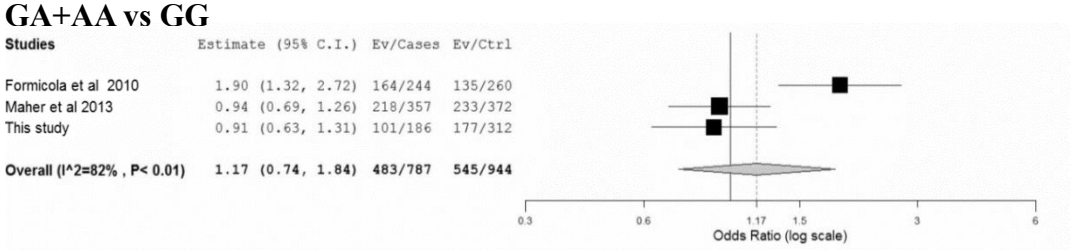
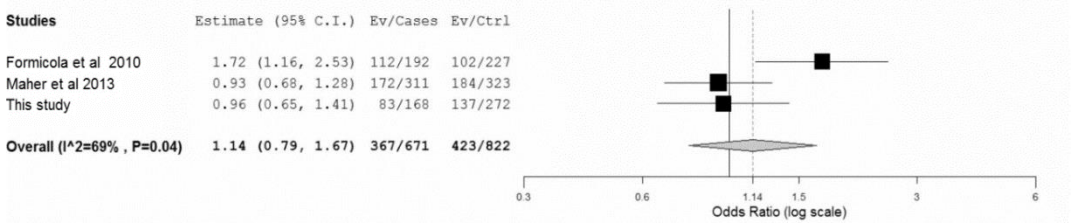
Supplementary material

Supplementary Figure 1. Forest plots for the association of GRIA1 rs548294 with migraine, either with or without aura, in the genetic dominant (GA+AA vs GG), codominant (GA vs GG or AA vs GG), recessive (AA vs GA+GG) and allele models (A vs G). Pooled estimates (OR, odds ratio) are from the random-effects model.

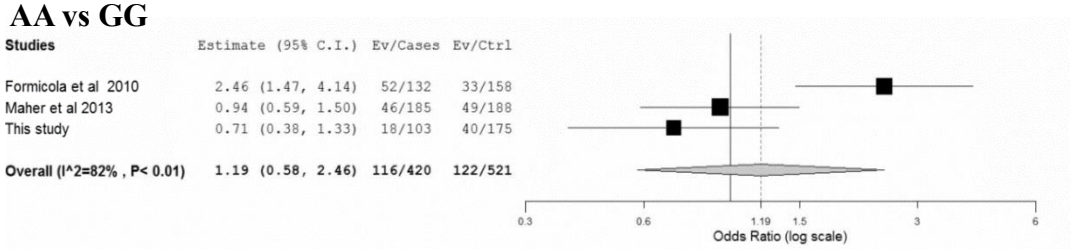
GA+AA vs GG



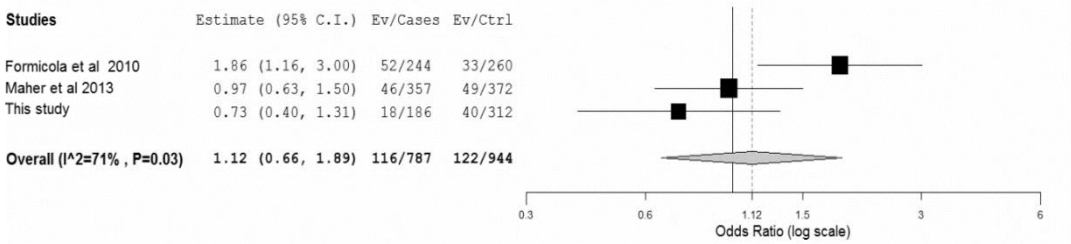
GA vs GG



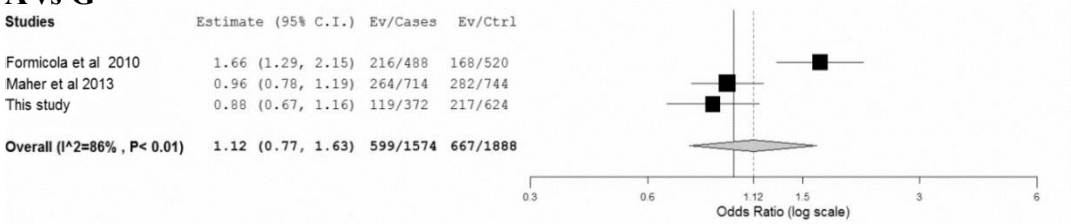
AA vs GG



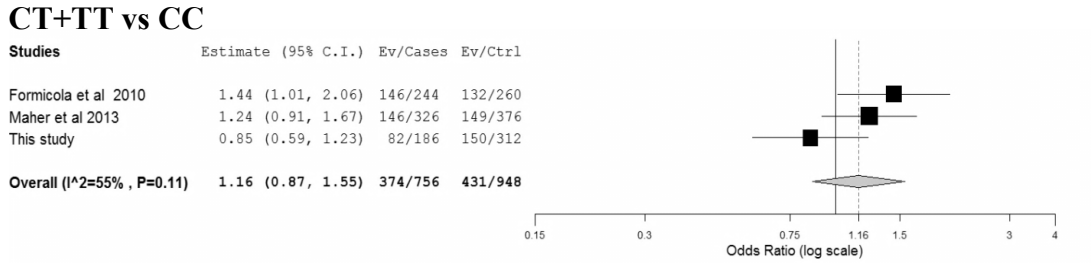
AA vs GA+ GG

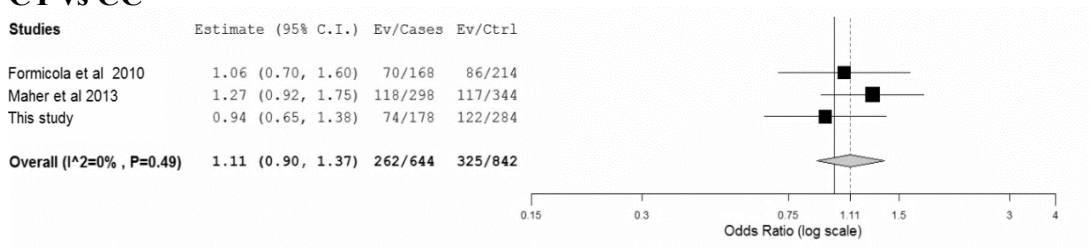


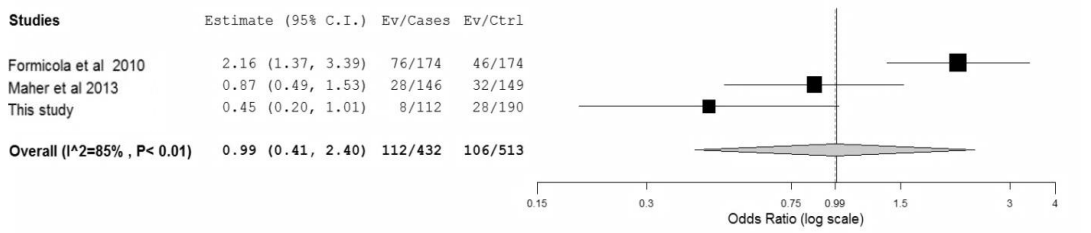
A vs G

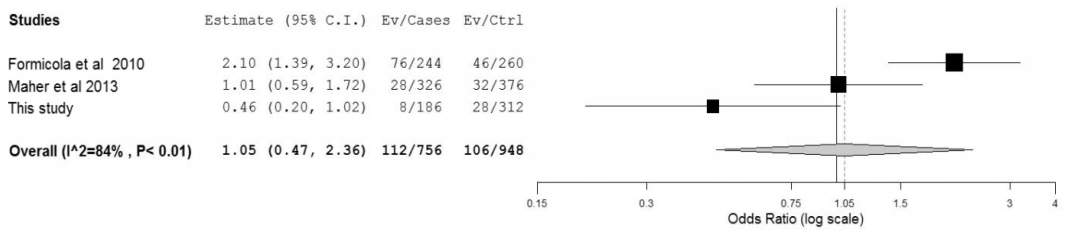


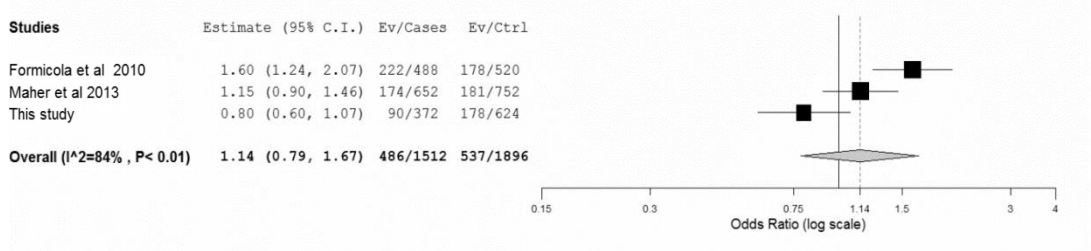
Supplementary Figure 2. Forest plots for the association of GRIA1 rs2195450 with migraine, either with or without aura, in the genetic dominant (CT+TT vs CC), codominant (CT vs CC or TT vs CC), recessive (TT vs CT+CC) and allele models (T vs C). Pooled estimates (OR, odds ratio) are from the random-effects model.











Chapter 6

Association of *RAMP1* rs7590387 with the risk of migraine transformation into medication overuse headache

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Abstract

Objectives. We herein investigated the role of polymorphisms in calcitonin gene-related peptide (CGRP)-related genes looking at the association of rs3781719 (T>C) in the calcitonin gene-related polypeptide-alpha (*CALCA*) gene and of rs3754701 (T>A) and rs7590387 (C>G) at the receptor activity modifying 1 (*RAMP1*) locus with triptan response in patients with migraine without aura (MwoA). In addition, their role was evaluated as risk factors for transformation of episodic migraine into medication overuse headache (MOH). **Background**. The calcitonin gene-related peptide (CGRP) has a central role in the pathogenesis of migraine, however few information is currently available concerning the role of polymorphisms in CGRP-related genes as determinants of clinical response to anti-migraine drugs or as risk factors for migraine chronification. **Methods.** Genotyping

was conducted retrospectively by Real-time PCR allelic discrimination assay in 219 patients with MwoA and 130 with MOH in whom migraine was the primary headache type. Gene variants association was evaluated by logistic regression analysis adjusted by confounding factors. The threshold of statistical significance was set according to the total number of polymorphisms analysed in the current study and in previous publications arising from overlapping datasets. **Results.** No evidence of association was found between the three polymorphisms tested and triptan response in MwoA patients. Conversely, carriers of *RAMP1* rs7590387GG displayed a lower risk of episodic migraine transformation into MOH (vs C allele carriers, OR: 0.27, 95%CI: 0.13-0.57, P=0.0002; threshold of significance set at P< 0.0029). When genotype distribution for *RAMP1* rs7590387 was compared between healthy controls (n=209) and MOH patients, carriers of rs7590387GG were found at lower risk of developing MOH (OR: 0.43, 95%CI: 0.22-0.85, P= 0.011). **Conclusion**. These results suggest that *RAMP1* rs7590387 may have a role in the transformation of episodic migraine into MOH.

Keywords: migraine, medication overuse headache, susceptibility, triptans, CGRP-related genes, polymorphisms.

Abbreviations

CALCA calcitonin gene-related polypeptide-alpha; CI confidence interval; HWE Hardy-Weinberg equilibrium; ICHD International Classification of Headache Disorders, MAF minor allele frequency, MOH medication overuse headache; MwoA migraine without aura; RAMP1 receptor activity modifying 1; RFLP restriction-fragment length polymorphism, OR odds ratio; SNP single nucleotide polymorphism.

Introduction

The neuropeptide calcitonin gene-related peptide (CGRP), a potent vasodilator agent involved in pain transmission, is recognised to play a central role in the pathogenesis of migraine.[1] Clinical evidence shows that CGRP levels are elevated during migraine and reduced by triptans through action on 5-hydroxytryptamine (5-HT)1B/1D presynaptic receptors.[2,3] Although triptans represent the standard of care for migraine patients with mild-to-moderate attacks, up to 40% of treated patients do not respond to triptan therapy.[4,5] Genetic factors have been postulated to be involved in interindividual variability of the therapeutic effects of triptans,[6] however no information is currently available on the role of single nucleotide polymorphisms (SNPs) in CGRP-related genes as pharmacogenetic determinants.

On the other hand, the frequent use of triptans or other anti-migraine agents can lead to medication overuse headache (MOH) which is a daily or almost daily headache resulting from chronicization of episodic migraine or tension-type headache as a consequence of symptomatic drug overuse.[7] The process of migraine chronification (i.e., progression from episodic to chronic migraine) is complex and involves comorbid risk factors such as depression, obesity, hypertension and stressful life events.[8-11] Although genetic factors have been postulated to have a role in migraine chronification, only a few studies with limited number of patients have investigated the association with polymorphic gene variants.[12-14] The observation of increased blood levels of CGRP outside migraine attacks in women with chronic migraine compared to women with episodic migraine[15] raises the possibility that polymorphic genes in the signaling pathway of CGRP may be implicated in migraine chronification. Among these are the calcitonin gene-related polypeptide-alpha (*CALCA*) gene which encodes the two peptide hormones calcitonin and α-CGRP, and the receptor activity modifying protein-1 (*RAMP1*)

gene encoding for the subunit of the CGRP receptor required for trafficking to the cell surface and for CGRP binding.[16,17]

Given the central role of CGRP in migraine pathophysiology, we hypothesized that single nucleotide polymorphisms (SNPs) of CGRP-related genes may have an impact on triptan response rates or be involved in episodic migraine transformation into MOH. To test these hypotheses, in the present study we first investigated the effect of rs3754701 and rs7590387 at the *RAMP1* locus and of rs3781719 in the *CALCA* gene on triptan response in patients with episodic migraine without aura (MwoA). Then, in the context of an association case-control study design, the role of the three SNPs was assessed as risk factors for trasformation of episodic migraine into MOH.

Methods

Study Subjects

Adult patients with migraine without aura (MwoA), and patients with MOH referred to Headache Center of Mondino Institute of Pavia and the Headache Center of "Maggiore della Carità" University Hospital of Novara were eligible to participate. MwoA patients have been enrolled from May 2004 to March 2014 while MOH patients have been recruited from March 2005 to September 2008. The inclusion criteria for enrollment of subjects with MwoA were i) headache fulfilling ICHD-II criteria for migraine without aura from at least one year, ii) age between 18 and 65 years; exclusion criteria were i) previous or current diagnosis of MOH, ii) contraindications to be prescribed with triptans, iii) history of psychiatric disorders. The triptan response was assessed as previously reported.[18] Briefly, MwoA patients were given a specific diary to record the clinical response to the triptan intake for three consecutive migraine attacks. For each of the migraine

attacks, the patient was asked to record on a diary the intensity of pain (on a scale from 0 to 3; 0= absent pain, 1= mild pain/no disability, 2= moderate pain/partial disability and 3= severe pain/total disability) at the moment of the triptan intake and after 120 min. Consistent responders to triptans were defined as the migraineurs who experienced a ≥2 point reduction after triptan administration in at least two out of three consecutive attacks, otherwise MwoA patients were defined as inconsistent responders. The inclusion criteria for enrollment of subjects with MOH were i) headache fulfilling ICHD-2 criteria for MOH[19,20] with migraine as primary headache type, ii) accurate compilation of the diary for a 3-month pre-withdrawal period; exclusion criteria were: iii) history of psychiatric disorders that could sustain a chronic pain condition (psychotic and/or somatoform disorders; most patients also underwent an interview with a skilled psychologist and was administered the Minnesota Multiphasic Personality Inventory Test; MMPI-2 Italian Version), iv) other major medical conditions. Control subjects were matched with MOH patients by age and sex and were randomly selected from general population of same ethnic background (north-west Italy) to minimize population heterogeneity and stratification. As no clinical evaluation has been performed on the control group, we cannot rule out the possibility that some individuals present in the control sample might be affected by MOH. However, this fraction of affected individuals is unlikely to be higher than that observed in the general population (1-2%).[7] This study was approved by the local Ethics Committees of the institutions involved ("C. Mondino" Institute, Pavia and "Maggiore della Carità" University Hospital, Novara) and it met the requirements of the Declaration of Helsinki. Informed consent was obtained from all patients before participation in the study.

Genotyping

Genomic DNA was extracted from peripheral blood by using the QiaAmp DNA Mini Kit (Qiagen Valencia, California, USA). Genotyping was performed by real-

time PCR using Applied Biosystems TaqMan Pre-Designed SNP Genotyping assays [*CALCA* rs3781719 Assay ID: C_2698777_10; *RAMP1* rs3754701 assay ID: C_27496443_10; *RAMP1* rs7590387 assay ID: C_26481962_10]. Real-time PCR amplification and detection was conducted on genomic DNA according to previously described methods.[21] For validation, about 10 % of the samples were re-genotyped. The results were reproducible with no discrepancies in genotyping. *CALCA* rs3781719 was genotyped in all MOH patients also by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), as previously described.[22]

Statistical analysis

Data were summarized and presented in the form of mean, standard deviation, and percentage as descriptive statistics. Each polymorphism was tested for deviation from the Hardy-Weinberg equilibrium (HWE) by use of the exact test implemented in the online Finetti's program (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). The association between SNPs and the two clinical endpoints (risk of inconsistent response to triptans and risk of trasformation of episodic migraine to MOH, respectively) was assessed by logistic regression analysis with adjustment for confounding clinical covariates (cut-off of P-value <0.1 from univariate analyses). In addition, *SLC6A4* STin2 VNTR and *COMT* Val158Met were included as covariates in the adjusted analyses, being found associated with triptan response in previous publications arising from an overlapping dataset.[18,23] For all the selected polymorphisms, we considered either a log-additive, a dominant or a recessive mode of inheritance. The log-additive genetic model is a trend test for the genotypes, similar to the allele model, but comparisons are among subjects (N) instead of chromosomes (2N). In the log-additive model, estimates are based on a logistic regression model that coded the genotypes as 0,1, or 2 to reflect the number of minor alleles. Clinical and genotype data were managed with the statistical

software package SYSTAT for Windows (version 12; Systat Software Inc., Chicago, IL, USA) and SNPStats software.[24] The SNPStats software was also used for the calculation of linkage disequilibrium (measured as r^2-value) between *RAMP1* SNPs. Due to the exploratory nature of this study, we reported nominal statistical associations (P value <0.05). Adjusted P values based on the Bonferroni correction were also considered to avoid chance findings due to multiple testing, and the threshold of statistical significance was adjusted according to the total number of polymorphisms analysed in the current study and in any previous publication arising from an overlapping dataset. Specifically, the significance level for the risk of inconsistent response to triptans was lowered to P<0.0035 to account for a total of 14 polymorphisms analysed, of which eleven in previous publications.[18,21,23,25] The threshold of significance for the risk of episodic migraine transformation into MOH was set at P < 0.0029 to account for a total of 17 SNPs analysed including those reported in previous publications.[26-28] Given our sample size of 138 MwoA patients with consistent response to triptans and 81 MwoA patients with inconsistent response and assuming a power of 80% and a nominal level of significance of 0.05, for the investigated gene variants (Minor allele frequency: 0.30 to 0.45) the minimal detectable odds ratio for inconsistence response to triptans was 1.8 under the log-additive model of inheritance, and ranged from 2.3 to 2.8 under the dominant model and from 2.6 to 3.1 under the recessive model of inheritance. The minimal detectable odds ratio for transformation of episodic migraine (n=207) into MOH (n=130) was 1.6 under the log-additive model, and ranged from 1.9 to 2.1 under the dominant and from 2.1 to 2.5 under the recessive model. Power calculations were performed using Quanto software (www.hydra.usc.edu/gxe/).

Results

This study included a total of 219 MwoA patients (79.9% of women) with a mean age of 38.4 years (standard deviation: 10.6), 130 MOH patients (75.4% of women) with a mean age of 47.8 years (standard deviation: 11.1) and 209 healthy subjects (75.6% of women) with a mean age of 50.0 years (standard deviation: 15.5). Among MOH patients, 20 (15.4%) overused exclusively 1, or more, types of triptan, 39 (30.0%) exclusively NSAIDs (1, or more, types of non-steroidal anti-inflammatory drugs), 22 patients (16.9%) overused exclusively combination drugs (a single pharmaceutical product that contains more than 1 active principle), 47 (36.2%) overused 2 or more classes of drugs (which we called association drugs), 1 patient overused exclusively ergotamine (0.8%), and 1 patient exclusively opiates (0.8%).

Clinical variables and genotypes distribution in the whole cohort of MwoA patients and after stratification according to their consistency status of headache response to triptans are shown in Table 1. Inconsistent response to triptans was observed in 36.9% of patients with MwoA (81/219). Patients receiving triptans other than frovatriptan (n=108, 49.3%) displayed a lower risk of inconsistent response rates than MwoA patients treated with frovatriptan (OR: 0.49, 95%CI: 0.28-0.86, P=0.013, Table 1). All the other demographic and clinical variables were similarly distributed when comparing consistent responders with inconsistent responders. In MwoA patients, *CALCA* rs3781719, *RAMP1* rs7590387 and *RAMP1* rs3754701 were in HWE (P_{HWE} rs3781719 = 0.52; P_{HWE} rs3754701= 0.77; P_{HWE} rs7590387= 0.41). The observed minor allele frequencies (MAFs) of the three SNPs (MAF of rs3781719: 0.30; MAF of rs3754701: 0.37; MAF of rs7590387: 0.45) were similar to those reported in Hap-Map-CEU population (MAF of rs3781719: 0.32; MAF of rs3754701: 0.37; MAF of rs7590387: 0.49). In addition, in our cohort of MwoA patients rs3754701 and rs7590387 were not found in linkage disequilibrium (LD)

(r^2=0.006, p=0.092), a result in accordance with Caucasian HapMap LD data (r^2=0.005).

In the logistic regression analysis adjusted for triptan (frovatriptan vs other triptans), and for *SLC6A4* STin2 VNTR and *COMT* val158met polymorphisms, which were found associated with triptan response in previous publications,[18,23] *CALCA* rs3781719, *RAMP1* rs3754701 and *RAMP1* rs7590387 were found not significantly associated to headache response to triptans, either under the log-additive, the dominant or the recessive model of inheritance (Table 1). Given the confounding effect of frovatriptan on headache response rates and its unique pharmacokinetic profile, we also carried out subgroup analyses according to the use of frovatriptan. Similar results were obtained when analysis was conducted separately on patients treated with the long-acting triptan (frovatriptan) and patients treated with the fast-acting triptans combined (eletriptan, rizatriptan, almotriptan, sumatriptan, and zolmitriptan) (Table 2).

In order to extend previous results showing lack of association of *CALCA* rs3781719, *RAMP1* rs3754701 and *RAMP1* rs7590387 with migraine susceptibility,[22] we evaluated their role as risk factors for episodic migraine transformation into MOH. In MOH patients, *RAMP1* rs3754701 and *RAMP1* rs7590387 but not *CALCA* rs3781719 were found in HWE (P_{HWE} rs3754701= 0.56; P_{HWE} rs7590387= 0.086; P_{HWE} rs3781719= 0.009). Confirmation of genotypes for rs3781719 by PCR-RFLP in all MOH patients excluded that its deviation from HWE was a result of genotyping errors. The association of SNPs in *CALCA* and *RAMP1* genes with the risk of episodic migraine transformation into MOH was assessed by comparing genotypes distribution in the whole cohort of MwoA patients and MOH patients. Given that the mean age of the MOH group was significantly higher than that in the MwoA group (P<0.001), we used the logistic regression method for adjustment of data to remove the effect of age variation as a confusing factor. No association was found with *CALCA* rs3781719 and *RAMP1*

rs3754701. Conversely, the *RAMP1* rs7590387G allele (log-additive model: OR 0.60, 95%CI 0.42-0.86, P=0.004) and carriers of rs7590387GG genotype (recessive model: 0.27, 95%CI: 0.13-0.57, P=0.0002) were found at lower risk of episodic migraine transformation into MOH (Table 3). It is noteworthy that the significance of rs7590387 under the recessive model of inheritance was retained even after lowering P-value threshold at < 0.0029 to account for a total of 17 SNPs analysed including those reported in previous publications.[26-28] When analysis was restricted to MOH patients that, after withdrawal therapy, displayed chronic headache despite the interruption of the drug overuse and patients with chronic headache that still overused acute medication at the 2-month follow-up, conformation to HWE was observed for all three SNPs in this subgroup of MOH patients (n=30) (P_{HWE} rs3781719= 0.18; P_{HWE} rs3754701= 1; P_{HWE} rs7590387= 0.69), and the nominal significance of *RAMP1* rs7590387 was retained (recessive model, OR: 0.21, 95%CI: 0.05-0.95, P=0.015). When comparing genotype distribution for *RAMP1* rs7590387 between healthy controls (n= 209, P_{HWE} rs7590387= 0.16) and MOH patients, carriers of rs7590387GG genotype were found at lower risk of developing MOH (OR: 0.43, 95%CI: 0.22-0.85, P= 0.011, Table 4). Conversely, controls did not differ from MwoA patients in the distribution of rs7590387 genotypes (Table 4), confirming previous results showing lack of association between *RAMP1* rs7590387 and migraine susceptibility.[22]

Table 1. Clinical variables and genotype distribution of SNPs in CALCA and RAMP1 genes in the whole set of MwoA patients and after stratification according to their consistency status of headache response to triptans.

Variable	All patients n (%)	CR n=138 (%)	IR n=81 (%)	OR* (95% CI)	P value
Sex					
Female	175 (79.9)	113 (81.9)	62 (76.5)	1 (ref)	
Male	44 (20.1)	25 (18.1)	19 (23.5)	1.39 (0.71-2.71)	0.34
Age at study entry (year)					
mean ± SD	38.4 (10.6)	38.6 (10.6)	38.1 (10.7)	0.99 (0.97-1.02)	0.73
Triptan					
Frovatriptan	111 (50.7)	61 (44.2)	50 (61.7)	1 (ref)	
Rizatriptan	29 (13.2)	18 (13.0)	11 (13.6)	Others 0.49 (0.28-	0.013
Eletriptan	27 (12.3)	23 (16.7)	4 (4.9)	0.86)	
Almotriptan	24 (11.0)	16 (11.6)	8 (9.9)		
Sumatriptan	20 (9.1)	13 (9.4)	7 (8.6)		
Zolmitriptan	8 (3.7)	7 (5.1)	1 (1.2)		
Use of prophylactic medications (n= 209)					
Yes	103 (49.3)	68 (51.5)	35 (45.5)	1 (ref)	
No	106 (50.7)	64 (48.5)	42 (54.5)	1.27 (0.73-2.24)	0.40
SNP	**All patients n (%)**	**CR n=138 (%)**	**IR n=81 (%)**	**Genotypic model OR# (95% CI)**	**P value**
CALCA rs3781719 (T>C)					
TT	109 (49.8)	71 (51.5)	38 (46.9)	A: 0.96 (0.62-1.48)	0.84
TC	88 (40.2)	52 (37.7)	36 (44.4)	D: 1.04 (0.59-1.85)	0.88
CC	22 (10.0)	15 (10.9)	7 (8.6)	R: 0.80 (0.30-2.11)	0.65
RAMP1 rs3754701 (T>A)					
TT	87 (39.7)	53 (38.4)	34 (42.0)	A: 0.90 (0.60-1.37)	0.63
TA	100 (45.7)	65 (47.1)	35 (43.2)	D: 0.73 (0.41-1.32)	0.30
AA	32 (14.6)	20 (14.5)	12 (14.8)	R: 1.19 (0.53-2.67)	0.66
RAMP1 rs7590387 (C>G)					
CC	69 (31.5)	41 (29.7)	28 (34.6)	A: 1.11 (0.75-1.65)	0.60
CG	102 (46.6)	71 (51.5)	31 (38.3)	D: 0.84 (0.46-1.54)	0.57
GG	48 (21.9)	26 (18.8)	22 (27.2)	R: 1.69 (0.86-3.30)	0.12

Abbreviations: CR, consistent responders to triptans; IR, inconsistent responders; A, log-additive model of inheritance; D, dominant model of inheritance; R, recessive model of inheritance. *****Univariate logistic regression analysis. #Logistic regression analysis adjusted for triptan (frovatriptan vs other triptans), and for SLC6A4 Stin2 VNTR and COMT val158met polymorphisms which were found associated with triptan response in previous publications arising from an overlapping dataset [18,23]

Table 2. Association analysis of SNPs in CALCA and RAMP1 genes in MwoA patients treated with the long-acting (frovatriptan) and fast-acting triptans (eletriptan, rizatriptan, almotriptan, sumatriptan, and zolmitriptan), respectively.

SNPs	CR n (%)	IR n (%)	Genotypic model OR* (95% CI)	P value
MwoA patients treated with frovatriptan (n=111)				
CALCA rs3781719 (T>C)				
TT	27 (44.3)	20 (40.0)	A: 0.94 (0.51-1.71)	0.83
TC	27 (44.3)	26 (52.0)	D: 1.10 (0.51-2.40)	0.80
CC	7 (11.5)	4 (8.0)	R: 0.70 (0.19-2.58)	0.59
RAMP1 rs3754701 (T>A)				
TT	22 (36.1)	17 (34.0)	A: 1.03 (0.57-1.85)	0.93
TA	32 (52.5)	27 (54.0)	D: 1.02 (0.46-2.27)	0.96
AA	7 (11.5)	6 (12.0)	R: 1.08 (0.33-3.53)	0.90
RAMP1 rs7590387 (C>G)				
CC	20 (32.8)	19 (38.0)	A: 1.08 (0.64-1.81)	0.78
CG	31 (50.8)	18 (36.0)	D: 0.77 (0.35-1.70)	0.51
GG	10 (16.4)	13 (26.0)	R: 1.75 (0.69-4.47)	0.24
MwoA patients treated with triptans other than frovatriptan (n=108)				
CALCA rs3781719 (T>C)				
TT	44 (57.1)	18 (58.1)	A: 1.04 (0.54-2.02)	0.89
TC	25 (32.5)	10 (32.3)	D: 1.06 (0.44-2.54)	0.90
CC	8 (10.4)	3 (9.7)	R: 1.02 (0.24-4.23)	0.98
RAMP1 rs3754701 (T>A)				
TT	31 (40.3)	17 (54.8)	A: 0.75 (0.41-1.39)	0.37
TA	33 (42.9)	8 (25.8)	D: 0.52 (0.21-1.24)	0.14
AA	13 (16.9)	6 (19.4)	R: 1.30 (0.43-3.92)	0.65
RAMP1 rs7590387 (C>G)				
CC	21 (27.3)	9 (29.0)	A: 1.14 (0.61-2.12)	0.68
CG	40 (52.0)	13 (41.9)	D: 0.92 (0.35-2.40)	0.87
GG	16 (20.8)	9 (29.0)	R: 1.57 (0.60-4.15)	0.36

Abbreviations: CR, consistent responders to triptans; IR, inconsistent responders; A, log-additive model of inheritance; D, dominant model of inheritance; R, recessive model of inheritance. *****Logistic regression analysis adjusted by COMT Val158Met and SLC6A4 STin2 VNTR polymorphisms.

Table 3. Association of SNPs in CALCA and RAMP1 genes with risk of episodic migraine transformation into MOH.

SNP	MwoA n(%)	MOH, n(%)	Genotypic model OR[#](95% CI)	P-value
CALCA rs3781719 (T>C)				
TT	109 (49.8)	73 (56.1)	A: 0.97 (0.69-1.37)	0.87
TC	88 (40.2)	40 (30.8)	D: 0.81 (0.50-1.29)	0.37
CC	22 (10.1)	17 (13.1)	R: 1.47 (0.71-3.04)	0.30
RAMP1 rs3754701 (T>A)				
TT	87 (39.7)	59 (45.4)	A: 0.81 (0.58-1.15)	0.24
TA	100 (45.7)	55 (42.3)	D: 0.75 (0.47-1.21)	0.24
AA	32 (14.6)	16 (12.3)	R: 0.79 (0.39-1.60)	0.51
RAMP1 rs7590387 (C>G)				
CC	69 (32.0)	48 (36.9)	A: 0.60 (0.42-0.86)	0.004
CG	102 (47.0)	70 (53.9)	D: 0.71 (0.43-1.16)	0.17
GG	48 (22.0)	12 (9.2)	R: 0.27 (0.13-0.57)	0.0002

Abbreviations: A, log-additive model of inheritance; D, dominant model of inheritance; R, recessive model of inheritance. [#]Logistic regression analysis adjusted for age. The threshold of significance for Bonferroni correction was set at $P < 0.0029$ to account for a total of 17 SNPs analysed including those reported in previous publications.[26-28]

Table 4. Association of RAMP1 rs7590387 (C>G) with migraine susceptibility or MOH risk.

Genotypes	Controls n(%)	MwoA, n(%)	Genotypic model OR[#] (95% CI)	P-value
CC	54 (25.8)	69 (31.5)	A: 0.93 (0.69-1.26)	0.66
CG	115 (55.0)	102 (46.6)	D: 0.66 (0.41-1.06)	0.082
GG	40 (19.1)	48 (21.9)	R: 1.35 (0.81-2.27)	0.25
Genotypes	**Controls n(%)**	**MOH, n(%)**	**Genotypic model OR[*] (95% CI)**	**P-value**
CC	54 (25.8)	48 (36.9)	A: 0.61 (0.43-0.86)	0.004
CG	115 (55.0)	70 (53.9)	D: 0.60 (0.37-0.95)	0.032
GG	40 (19.1)	12 (9.2)	R: 0.43 (0.22-0.85)	0.011

Abbreviations: A, log-additive model of inheritance; D, dominant model of inheritance; R, recessive model of inheritance. [#]Logistic regression analysis adjusted for age. [*]Univariate logistic regression analysis. The threshold of significance for Bonferroni correction was set at $P < 0.0029$.

Discussion

Despite clinical data supporting the involvement of CGRP in the clinical response to triptans in migraine patients[29,30] or in migraine chronification,[15] the possible contribution of polymorphisms in CGRP-related genes as pharmacogenetic determinants or as risk factors for chronification of episodic migraine has been poorly investigated. In the present study, we assessed the impact of rs3754701 and rs7590387 at the *RAMP1* locus and of rs3781719 in the *CALCA* gene on triptan response rates in MwoA patients, as well as their role as risk factors for migraine transformation into MOH. We acknowledge that our study is underpowered to detect small genetic main effects, however it has sufficient power to detect medium-large effect sizes of clinical relevance. While our results exclude a clinically relevant impact of the SNPs tested on headache response to triptans, our findings suggest that *RAMP1* rs7590387 may have a role in the transformation of episodic migraine into MOH.

A few studies have addressed the genetic basis for variability in the therapeutic effects of triptans.[6] Among the most interesting findings are associations with polymorphic variants in the *SLC6A4*,[18] *COMT*[23] and *DRD2*[31] and genes, however none of these associations has been independently validated. Polymorphisms in CGRP-related genes may be plausible pharmacogenetic candidates for interindividual variability of triptan response since administration of sumatriptan is effective in reversing the CGRP-induced migraine[32] and elevation of salivary levels of CGRP predicts responsiveness to rizatriptan.[30] In the present study, we found no association of the three SNPs in *CALCA* and *RAMP1* genes on triptan response, either in the overall cohort of MwoA patients and in patients treated with the most frequently prescribed frovatriptan (a long-acting triptan). However, given the limited number of MwoA patients enrolled and the wide variety of triptans prescribed due to the naturalistic setting of our study, it was not possible to conduct

a stratified analysis of *CALCA* and *RAMP1* gene SNPs according to all the triptans administered.[33] Thus, further studies would be needed to evaluate the impact of the three SNPs tested on headache response to a specific fast-acting triptan.

While polymorphisms in dopaminergic system genes have been involved with the prognosis of MOH patients after withdrawal therapy,[28,34] the genetic basis for transformation of episodic migraine into MOH is not known. Early evidence of a genetic component for MOH comes from epidemiological studies showing a three-fold increased risk of developing MOH in subjects with a family history of MOH.[35] Furthermore, MOH appears to share some pathogenic mechanisms with other kinds of addictive disorders[36,37] and genetic factors are established contributors to drug addiction.[38] Although serotonin has been implicated in the predisposition to substance-related disorders,[39,40] no evidence of correlation has been reported between serotonergic gene variants and MOH risk.[25-27,41] Despite lack of association between variants in *CALCA* and *RAMP1* genes and migraine susceptibility,[22,42,43] polymorphisms at these loci could have a role in migraine chronification. This possibility has been raised in a recent study with a three-stage design evaluating 144 SNPs selected from 48 candidate genes.[44] Of these, eight SNPs including *CALCA* rs2956 and *RAMP1* rs302680 were found nominally associated with chronic migraine in the two-stage discovery phase, although none was significant in the replication stage.[44]

In the present study, we provide for the first time evidence that carriers of the major allele for rs7590387 are at higher risk for transformation of episodic migraine into MOH. Our findings are strengthened by confirmation of this association when analysis was restricted to MOH patients who continued to have daily headaches after drug withdrawal. However, it should be acknowledged that MwoA patients were younger than MOH subjects, therefore we cannot exclude that a percentage of included MwoA patients may develop MOH later. Although no *in vitro* or *in vivo* expression/functional data exists regarding the three SNPs analysed,

it should be noted that rs3781719 and rs3754701 are located in the promoter region of *CALCA* and *RAMP1*, respectively, while rs7590387 is localized 1.4 kb downstream of the *RAMP1* gene. Thus, rs7590387 is not expected to be the true causal variant, and the association here reported may be due to linkage disequilibrium of rs7590387 with an unknown functional polymorphism which is the actual determinant factor for migraine transformation. Therefore, further studies are warranted, based on haplotype analysis of tightly linked SNPs in *RAMP1* gene, to provide more conclusive evidence of association with migraine transformation. It is also noteworthy that a significant interaction has been reported between *BDNF* and *CALCA* genes in migraine susceptibility, showing an increased risk for the AT-genotype of rs2049046 and the GC-genotype of rs1553005 for migraineurs.[45] Thus, an approach based on SNP-SNP interaction analysis could also provide further insights in the genetic dissection of migraine transformation into MOH. Also noteworthy is the observation of lack of association beween rs7590387 and triptan response despite a correlation with migraine transformation. This deserves further study to investigate whether an inadequate response to triptans could entail the transformation of episodic migraine into MOH.

In conclusion, although the present results exclude a clinically relevant impact of rs3754701 and rs7590387 at the *RAMP1* locus and of rs3781719 in the *CALCA* gene on headache response to triptans, our findings support a role of *RAMP1* rs7590387 in the transformation of episodic migraine into MOH. However, the single institution and retrospective nature of the present study require our findings to be validated in larger, preferably multi-institutional prospective studies. In addition, further investigation based on haplotype analysis of tightly linked SNPs or on SNP-SNP interaction analysis is warranted to provide conclusive evidence of association of *RAMP1* rs7590387, or of a linked functional SNP, with migraine transformation into MOH.

Conflict of interest

The authors report no conflict of interest.

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Chapter 7

Combined effect of common gene variants on response to drug withdrawal therapy in medication overuse headache

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Abstract

Purpose: No information is currently available on genetic determinants of short-term response to drug withdrawal in medication overuse headache (MOH). In the present study we aimed to evaluate the role of 14 polymorphisms in 8 candidate genes potentially relevant for drug addiction (OPRM1, DRD2, DBH, COMT, BDNF, SLC6A4, 5HT2A and SLC1A2) as predictors for detoxification outcome of MOH patients at 2 month of follow-up**.**

Methods: Genotyping was conducted by PCR, PCR-RFLP analysis or real-time PCR allelic discrimination assay on genomic DNA extracted from peripheral blood. The association between gene variants and risk of unsuccessful

detoxification was evaluated by univariate and multivariate logistic regression analyses.

Results: One hundred and eight MOH patients with effective drug withdrawal therapy and 65 MOH patients with unsuccessful detoxification were available for the analysis. In the multivariable logistic regressions analysis, triptan overuse (OR: 0.271, 95% CI: 0.083–0.890, P= 0.031) and TT genotype carriage of DRD2 NcoI (OR: 0.115, 95% CI: 0.014–0.982, P= 0.048) emerged as independent predictors for unsuccessful detoxification. In addition, carriers of at least 4 of the 6 top-ranked gene variants (P<0.10) were found at higher odds for unsuccessful detoxification than patients with ≤ 3 high risk genotypes (OR: 3.40, 95%CI: 1.65-7.01, P=0.001).

Conclusion: This exploratory study suggests that DRD2 NcoI may be a genetic determinant of detoxification outcome in MOH patients. Our findings also show that an approach based on the combination of multiple genetic markers could be clinically useful for identification of MOH patients at higher risk for unsuccessful detoxification.

Keywords: medication overuse headache; drug withdrawal therapy; detoxification; outcome; risk; gene polymorphisms.

Introduction

Medication overuse headache (MOH) is a chronic secondary headache due to overuse of symptomatic headache drugs, including single and combination analgesics, barbiturates, opioids, ergot alkaloids and triptans [1, 2]. MOH has a prevalence of 1-2% in the general population [3] and a high economic burden on society, being estimated per-person annual costs three times higher than those of migraine and ten times higher than those of tension-type headache [4, 5]. Although

drug withdrawal therapy is performed very differently within and across countries, it represents the first choice treatment for MOH [6, 7]. In most MOH patients, chronic headache is resolved or reverted to its previous pattern within 2 months after drug withdrawal [8-10]. Despite a number of studies focusing on clinical predictors for detoxification outcome or evaluating brain metabolic changes in pain processing structures after drug withdrawal [9, 11], to date no information is available concerning the role of common genetic variants as determinants of short-term outcome of MOH patients after drug detoxification treatment.

A genetic susceptibility to MOH has been suggested on the basis of epidemiological studies showing a threefold increased risk of developing MOH in subjects with a family history of MOH or other substance abuse such as drug or alcohol abuse [12, 13]. Increasing evidences also suggest that MOH shares some pathogenetic mechanisms with other kinds of drug addiction [14], which has been postulated to be a complex phenotype influenced by either environmental and genetic factors [15]. The dopaminergic system has been most extensively studied in addiction research due to its strong association with reward and the observation that most drugs of abuse enhance dopamine levels [16]. For instance, the -141C Ins/Del polymorphism of the dopamine receptor D2 (DRD2) and the ANKK1/DRD2 Taq1A variant have been related to alcohol dependence [17, 18]. Moreover, the -1021C>T variant of the dopamine beta–hydroxylase (DBH) gene has been linked with the psychotic effects caused by cocaine [19], while a higher frequency of the Val allele of the catechol-O-methyltransferase (COMT) val158Met polymorphism has been found in polysubstance abusers [20] or methamphetamine users [21]. On the other hand, several studies have shown that 118G allele carriers of the μ-opioid receptor gene **(**OPRM1**)** 118A>G SNP are at higher risk of alcohol or opiate dependency [22], while BDNF val66met has been linked to methamphetamine or heroin abuse [23]. Among serotonergic genes, the serotonin 2A receptor (5HT2A) -1438A>G gene polymorphism has been related to

alcoholism [24], while the STin2VNTR of the serotonin transporter (SLC6A4) has been reported to have an influence on treatment outcome in alcohol-dependent patients [25]. In addition, a dysregulation of excitatory glutamatergic system has been hypothetized to play a key role in the neuroadaptations associated with repeated drug use [26]. Although it is generally accepted that polymorphic genes may play a role in development of addictive diseases, only few studies have addressed the question of whether genetic variants may also contribute to treatment outcome in detoxification.

Recently, we reported that COMT rs4680, alone or in haplotype combination with rs6269, is a determinant for relapse of MOH patients within the first year of follow-up after successful drug withdrawal, suggesting a genetic basis for long-term outcome of successfully detoxified MOH patients [27]. In this exploratory ‘‘hypothesis-generating’’ study, we evaluated the role of 14 polymorphic variants in 8 candidate genes (OPRM1, DRD2, DBH, COMT, BDNF, SLC6A4, 5HT2A and SLC1A2), potentially relevant for drug addiction, as predictive factors for unsuccessful detoxification of MOH patients 2 months after medication withdrawal. In addition, their role as risk factors for developing MOH was evaluated in the context of a case-control association study.

Methods

Partecipants

The medical records of 227 MOH patients who underwent withdrawal therapy between March 2005 and September 2008 at the inpatient Headache Unit of C. Mondino National Neurological Institute’ (Pavia, Italy) and who had given their informed consent to genetic analysis were retrospectively reviewed. Inclusion and exclusion criteria for MOH patients have been previously described [28]. All patients underwent a standard in-patient withdrawal protocol which was carried on according to previously described procedures [29]. At the 2-month follow-up visit,

the pattern of the headache was evaluated, on the basis of the information recorded by the patient in the diary, the number of days with headache and the use of symptomatic drugs. Withdrawal therapy was considered "successful" if, after 2 months, the patients reverted to an episodic pattern of headache, stopped their overuse of symptomatic drugs and did not overuse another symptomatic medication (intake of NSAID/paracetamol on ≥15 days/month or other symptomatic medication or combination of symptomatic medications ≥10 days/month). On the other hand response to withdrawal therapy was labeled "unsuccessful" if medication overuse had ceased within the last two months but headache had not resolved or reverted to its previous pattern or if overuse medication has not yet been withdrawn or if there was an overuse of another symptomatic medication (see above). The control sample consisted of 312 unrelated subjects [196 women (62.8%) and 116 man (37.2%)] with a median age of 56 (range 18–100). Control subjects were randomly selected from general population of same ethnic background (north-west Italy) to minimize population heterogeneity and stratification. As no clinical evaluation has been performed on the control group, we cannot rule out the possibility that some individuals present in the control sample might be affected by migraine or MOH. However, the fraction of control subjects that may be affected by MOH is unlikely to be higher than that observed in the general population (1-2%).

This study was approved by the local Ethics Committee and it met the requirements of the Declaration of Helsinki. Written informed consent was obtained from all patients before participation in the study.

Genotyping

The SNPs selected and their National Center for Biotechnology Information (NCBI) dbSNP ID (rs) were the following: DRD2 -141C Ins/Del (rs1799732); DRD2 TaqI A (rs1800497); DRD2 NcoI (rs6275); DBH -1021C>T (rs1611115);

COMT rs6269; COMT val158met (rs4680), BDNF val66met (rs6265); OPRM1 118A>G (rs1799971); 5HT2A 516C>T (rs6305); 5HT2A -1438A>G (rs6311); SLC6A4 5-HTTLPR; SLC6A4 STin2 VNTR, SLC6A4 rs1042173; SLC1A2 -181A>C (rs4354668). Genomic DNA was extracted from peripheral blood by use of the QiaAmp DNA Mini Kit (Qiagen Valencia, CA). Except COMT rs6269 and SLC1A2 rs4354668 polymorphisms, which were determined by real-time PCR allelic discrimination assay using Applied Biosystems TaqMan Pre-Designed SNP Genotyping assays [rs6269 Assay ID: C_2538746_1; rs4354668 Assay ID: C__27142767_10], genotyping was performed according to PCR- RFLP methods by using the primers and restriction enzymes listed in Table S1 of Supplementary Material. All reactions of PCR/real-time PCR were set up in a dedicated PCR area with dedicated pipettes and reagents. For quality control purposes, each PCR run included negative as well as positive controls for the three genotypes. For validation, about 10% of the samples were re-genotyped. The results were reproducible with no discrepancies in genotyping. Genotyping was done blinded to MOH patient's outcome status.

Statistical analysis

Data were summarized and presented in the form of mean, standard deviation and percentage as descriptive statistics. For each polymorphism an exact test for Hardy–Weinberg equilibrium was performed for deviation from Hardy–Weiberg equilibrium by using the SNPstat software (available at http://bioinfo.iconcologia.net/snpstats/start.htm) [30]. The effect of clinical and genetic variables on the risk of unsuccessful detoxification was first evaluated by univariate logistic regression analysis. Odds ratios (ORs) and their 95% confidence intervals (CIs) were used as estimates of relative risk. The following clinical parameters were considered: gender, age at study entry, age of primary headache onset, familiarity for headache, primary headache diagnosis, drugs of abuse,

monthly drug number, headache per month and duration of MOH disease before withdrawal therapy. For all the selected polymorphisms, we considered either a log-additive, a dominant or a recessive mode of inheritance. The Akaike information criterion (AIC) was used to select the genetic model that best fits the data (i.e., the model with the lowest AIC score was the best fitting). The explanatory variables with a cut-off of P-value <0.1 from univariate analyses were used and included into the multivariate logistical regression model to identify independent predictors of unsuccessful detoxification. Clinical and genotype data were managed with the statistical software package SYSTAT for Windows (version 12; Systat Software Inc., Chicago, IL, USA) and SNPStats software. Due to the exploratory nature of this study, we reported nominal statistical associations (P value <0.05). Adjusted P-values based on the Bonferroni correction were also considered to avoid chance findings due to multiple testing of the 14 polymorphisms, and the significance levels were lowered to P= 0.00357 (P= 0.05/14). Details on power calculations are provided in Supplementary Material.

Results

Medical charts of 227 MOH patients undergoing inpatient drug withdrawal therapy were retrospectively evaluated. Distribution of clinical and demographic characteristics of MOH patients in the whole cohort and after stratification according to their detoxification status at 2 months of follow-up are shown in Table 1. Outcome data was available in 173 MOH patients, of which 108 were MOH patients with effective drug withdrawal therapy and 65 were MOH patients with unsuccessful detoxification. In the univariate association analysis, triptan overuse (OR: 0.27, 95% CI 0.09–0.83, P= 0.022) and primary headache diagnosis (mixed type vs migraine type, OR: 2.06, 95% CI 1.04–4.10, P= 0.039) were found associated with unsuccessful detoxification. No other clinical factor was

univariately correlated to unsuccessful detoxification with a cut-off of P <0.1 (Table 1).

Table 1. Clinical and demographic characteristics in the whole set of MOH patients and after stratification according to their detoxification status at 2-months of follow-up.

Clinical features	All sample (n=227)	Successful withdrawal therapy		OR (95% CI)	*P* value
		Yes n (%)	NO n (%)		
Gender					
Women	175 (77.1)	79 (73.1)	53 (81.5)	1 (Ref)	
Men	52 (22.9)	29 (26.9)	12 (18.5)	0.62 (0.29–1.31)	0.21
Age at study entry, years	47.5 (11.7)	47.5 (12.0)	47.4 (12.2)	1.00 (0.97–1.03)	0.98
Age of primary headache onset, years (n=218)					
>20	63 (28.9)	31 (30.1)	17 (27.4)	1 (Ref)	
10-19	77 (35.3)	39 (37.9)	19 (30.6)	0.89 (0.40–1.99)	0.77
<10	78 (35.8)	33 (32.9)	26 (41.9)	1.44 (0.66–3.14)	0.36
Familiarity for headache (n=217)					
No	82 (37.8)	39 (37.9)	20 (31.7)	1 (Ref)	
Yes	135 (62.2)	64 (62.1)	43 (68.3)	1.31 (0.67–2.54)	0.42
Primary headache diagnosis					
migraine type	144 (63.4)	74 (68.5)	39 (60.0)	1 (Ref)	
mixed type[a]	71 (31.3)	23 (21.3)	25 (38.5)	2.06 (1.04–4.10)	**0.039**
other/unknown	12 (5.3)	11 (10.2)	1 (1.5)	0.17 (0.02–1.39)	**0.098**
Drugs of abuse					
Triptans[b]					
No	195 (85.9)	87 (80.6)	61 (93.8)	1 (Ref)	
Yes	32 (14.1)	21 (19.4)	4 (6.2)	0.27 (0.09–0.83)	**0.022**
NSAIDS[c]					
No	148 (65.2)	75 (69.4)	40 (61.5)	1 (Ref)	
Yes	79 (34.8)	33 (30.6)	25 (38.5)	1.42 (0.74–2.71)	0.28
Combination drugs[d]					
No	190 (83.7)	93 (86.1)	51 (78.5)	1 (Ref)	
Yes	37 (16.3)	15 (13.9)	14 (21.5)	1.70 (0.76–3.80)	0.19
Association drugs[e]					
No	151 (66.5)	70 (64.8)	45 (69.2)	1 (Ref)	
Yes	76 (33.5)	38 (35.2)	20 (30.8)	0.82 (0.42–1.58)	0.55
Monthly drug number[f]	42.4 (27.3)	41.8 (26.9)	45.3 (27.7)	1.00 (0.99–1.02)	0.41
Headache days per month[f]	25.2 (6.2)	25.06 (6.2)	25.5 (6.4)	1.01 (0.96–1.06)	0.67
Duration of MOH desease (months)[f]	32.7 (40.9)	33.1 (46.8)	27.8 (34.3)	1.00 (0.99–1.01)	0.45

[a]Migraine associated with episodic tension-type headache; [b]1, or more, types of triptan. [c]1, or more, types of NSAIDs; [d]a single pharmaceutical product that contains more than 1 active principle, [e] 2 or more drugs of the classes above; [f]before withdrawal therapy. *P* values ≤0.1 are in boldface.

All the polymorphisms analyzed were in Hardy–Weinberg equilibrium both in MOH patients (n=227) and control subjects (n=312) (all P > 0.05). Among MOH patients, at univariate analysis, carriers of COMT rs4680 AA (OR: 0.40, 95%CI: 0.16–0.099, P= 0.047), carriers of DRD2 NcoI TT (OR: 0.14, 95% CI: 0.02–1.09, P = 0.061), DBH rs1611115 T allele carriers (OR: 0.57, 95%CI 0.30–1.09, P=0.091) and BDNF val66met in the log-additive genetic model (OR: 0.60, 95%CI: 0.36–1.02, P=0.060) were found at lower risk of withdrawal therapy failure, while OPRM1 118G allele carriers (OR: 2.19, 95%CI 1.11–4.30, P=0.023) and 5HT2A 516T allele carriers (OR: 2.22, 95%CI 0.90–5.48, P=0.084) displayed a higher risk of unsuccessful detoxification (Table 2).

Table 2. Association analysis of top ranked gene variants (P≤ 0.1) with the risk of MOH patients for unsuccessful detoxification: univariate logistic regression analysis.

SNP	Successful_withdrawal therapy		Genetic model	OR (95% CI)	P value
	Yes	NO			
OPRM1 118A>G (rs1799971)					
AA	84 (77.8)	40 (61.5)	Dominant	2.19 (1.11–4.30)	0.023
AG	17 (15.7)	21 (32.3)			
GG	7 (6.5)	4 (6.2)			
COMT val158met (rs4680)					
GG	29 (26.9)	16 (24.6)	Recessive	0.40 (0.16–0.099)	0.047
GA	54 (50.0)	42 (64.6)			
AA	25 (23.1)	7 (10.8)			
BDNF val66met (rs6265)					
Val/Val	58 (53.7)	43 (66.2)	Log-additive	0.60 (0.36–1.02)	0.060
Val/Met	40 (37.0)	20 (30.8)			
Met/Met	10 (9.3)	2 (3.1)			
DRD2 NcoI (rs6275)					
CC	50 (46.3)	32 (49.2)	Recessive	0.14 (0.02–1.09)	0.061
CT	47 (43.5)	32 (49.2)			
TT	11 (10.2)	1 (1.5)			
5HT2A 516C>T (rs6305)					
CC	98 (90.7)	53 (81.5)	Dominant	2.22 (0.90–5.48)	0.084
CT	9 (8.3)	12 (18.5)			
TT	1 (0.9)	0 (0)			
DBH -1021C>T (rs1611115)					
CC	59 (54.6)	44 (67.7)	Dominant	0.57 (0.30–1.09)	0.091
CT	41 (38.0)	15 (23.1)			
TT	8 (7.4)	6 (9.2)			

None of the other polymorphisms investigated was found related by univariate analysis to treatment outcome with a cut-off of P-value <0.1 (Table S2, Supplementary Material). In both univariate and multivariate analyses, no polymorphism remained of statistical significance when the conservative Bonferroni correction was used for multiple testing (Bonferroni P value threshold <0.0036). Nevertheless, in the multivariable logistic regressions model including clinical and genetic factors found to be significant on univariate analysis at a P-value <0.1, triptan overuse (OR: 0.271, 95% CI: 0.083–0.890, P= 0.031) and DRD2 NcoI (TT vs CT+CC, OR: 0.115, 95% CI: 0.014–0.982, P= 0.048) emerged as nominal independent predictors of unsuccessful detoxification (Table 3).

Tables 3. Predictive factors for unsuccessful detoxification in MOH: multivariate logistic regression analysis.

Variables	OR (95%CI)	P value
Triptan overuse[a]		
No	1 (Ref)	
Yes	0.271 (0.083–0.890)	0.031
DRD2 NcoI		
CC+CT	1 (Ref)	
TT	0.115 (0.014–0.982)	0.048

[a]of 1, or more, types of triptan.

In order to evaluate the cumulative effect of the six top ranked gene variants (P<0.1 in the univariate analysis) on the risk of unsuccessful detoxification, the number of high risk genotypes for each patient was considered. In the logistic regression analysis adjusted for triptan overuse and primary headache diagnosis, carriers of 4 or more high risk genotypes were at higher odds for unsuccessful detoxification than were patients with ≤ 3 high risk genotypes (OR: 3.40, 95%CI: 1.65-7.01, P=0.001, Table 4). Noteworthy that the cumulative effect of the six high risk

genotypes remained significant after Bonferroni's correction (threshold of significance required for Bonferroni correction for 14 polymorphisms analyzed, P< 0.00357). In addition, when only the 3 dopaminergic gene variants were considered in the combined analysis (COMT val158met, DRD2 NcoI, DBH -1021C>T), nominal significance of this joint analysis was retained (3 vs ≤2 high risk genotypes, OR 2.316, 95%CI: 1.20-4.47, P=0.012, Table 4). The patients that displayed an unsuccessful detoxification were mainly composed of patients with chronic headache despite the interruption of the drug overuse (n=22) and patients with chronic headache that still overused acute medication at the 2-month follow-up (n=35). The cumulative effect of genetic risk variants was significant in both groups when analysed separately (Table S3, Supplementary Material).

Table 4. Cumulative effects of genetic risk variants on the risk of MOH patients for unsuccessful detoxification.

N° of high risk genotypes	**Successful withdrawal therapy**		**OR*** **(95% CI)**	***P* value**
	YES	**NO**		
	n (%)	**n (%)**		
Combined analysis of six high risk genotypes				
≤3	54 (50.0)	14 (21.5)	1 (Ref)	
4-6	54 (50.0)	51 (78.5)	3.403 (1.652–7.011)	0.001
Combined analysis of three dopaminergic gene variants (COMT val158met, DRD2 NcoI, DBH -1021C>T)				
≤2	66 (61.1)	27 (41.5)	1 (Ref)	
3	42 (38.9)	38 (58.5)	2.316 (1.199–4.472)	0.012

*Logistic regression analysis adjusted for triptan overuse and primary headache diagnosis.

We have previously reported a lack of association between -1438A>G and 516C>T SNPs of 5HT2A with the risk for developing MOH [31] and a nominally significant association with SLC6A4 rs1042173 but not with SLC6A4 5HTT-LPR or SLC6A4 VNTR STin2 [28]. In the present study, we extended this association analysis to the other 9 gene variants investigated (OPRM1 118A>G, DRD2 NcoI, DRD2 -141C Ins/Del, DRD2 Taq1A, DBH -1021C>T, COMT rs6269, COMT

val158met, BDNF val66met and EAAT2 -181A>C). None of these polymorphic variants emerged as factor associated with the risk for developing MOH (Table S4, Supplementary Material).

Discussion

In the present study we found that 37% of MOH patients responded poorly to drug withdrawal therapy, a rate which is comparable to that reported by previous reports [9, 32]. It is not yet known why only about two-thirds of patients with MOH improve after drug withdrawal therapy, whereas the remainder continue to have chronic headache and/or to overuse symptomatic medication inspite of drug detoxification. A number of studies have been conducted so far to identify clinical predictors of short-term outcome of MOH patients after detoxification [9-11, 29]. However, the possibility of a genetic basis for variability in the response of MOH patients to medication withdrawal has not been investigated yet and, in our opinion, this issue is of clinical relevance as a personalized approach to individual patients could be implemented if the relative risk was known. The importance of this issue is also highlighted by the fact that, although in the new ICHD-III improvement after withdrawal is no longer required for the diagnosis of MOH [33], according to the previous ICHD-II criteria only patients that had undergone a successful detoxification were classified as "true" MOH patients, while nonresponders to detoxification were diagnosed as "probable" MOH (pMOH) [34].

We here reported that MOH patients overusing triptans are at lower risk of unsuccessful detoxification, a finding consistent with previous results [9]. In addition, we showed for the first time that common gene variants influence short-term outcome of MOH patients after medication withdrawal. Specifically, DRD2 NcoI was found to be an independent predictor of unsuccessful detoxification, with T allele carriers of DRD2 NcoI found at lower risk compared to C allele homozygotes carriers. In addition, results of the analysis comprising the six top-

ranking polymorphisms highlighted the notion that common gene variants with small effects can be clinically relevant when analysed in combination, being their cumulative effect found significant even after Bonferroni's correction. Amongst the top-ranking gene variants are 5HT2A C516T and BDNF Val66Met, which have been reported to influence drug consumption in MOH patients [28, 35], and COMT rs4680, which we recently reported to be associated with relapse risk within the first year after successful detoxification therapy [27]. Furthermore, we found an increased risk of an unsuccessful detoxification in MOH patients carrying the OPRM1 118G allele, a result in line with previous observations showing a better response to detoxification among alcohol dependent patients with the 118AA genotype [36]. In addition, a role of dopaminergic pathway gene polymorphisms in detoxification of MOH patients is also suggested by the involvement of DBH -1021 C>T, which has been previously reported to influence response to disulfuram's efficacy in cocaine dependence [37]. It can be argued that the association of the aforementioned polymorphisms with detoxification outcome could be related to their possible involvement in MOH susceptibility. However, we failed in detecting evidence for a role of the polymorphisms investigated in the present study as risk factors for MOH. This lack of association confirms a previous finding for COMT rs4680 and patients with chronic daily headache associated with drug abuse (CDHDA) [38]. Intriguingly, the same article reported an underrepresentation of a particular dopamine transporter gene polymorphism in CDHDA patients, strengthening the link between MOH and dopamine.

Several studies reported dysfunctions in the mesocorticolimbic dopamine circuit and in other pain-processing-related areas of MOH patients [39-42]. While most dysmetabolic regions have been shown to normalize after medication withdrawal, a persistent hypometabolism has been found in the orbitofrontal cortex (OFC) of MOH patients [11, 43]. A possible role of OFC in MOH is also supported by its implication in drug addiction [44, 45] and by the recent observation showing a

correlation between a decreased volume of gray matter of OFC at baseline and poor response of MOH patients to detoxification [46]. On the other hand, gene variants including COMT Val158Met [47], BDNF Val66Met [48], and a DRD2 SNP linked to NcoI [49] have been reported to influence regional gray matter volumes and cognitive performances in healthy subjects. Therefore, it is tempting to speculate that the risk genotypes identified in the present study may act cumulatively to affect gray matter volume of OFC, predisposing MOH patients to a higher risk of unsuccessful detoxification. Further preclinical and clinical investigations are warranted to test this hypothesis.

Results of the present study should be interpreted in the light of the following considerations. First, its retrospective and exploratory nature require findings validation in a well-powered prospective study. Second, although our case-control study was sufficiently powered to detect large, clinically relevant effect of common gene variants, we cannot exclude small effect sizes of the polymorphisms investigated for conferring an increased risk of MOH**.** Finally, further investigations using a pathway-based approach on a larger number of candidate polymorphic genes or using a genome-wide approach are strongly warranted for identification of the minimal set of polymorphisms that could be clinically useful for the identification of MOH patients at higher risk of unsuccessful detoxification.

In conclusion, our results support the possibility that response of MOH patients to medication withdrawal may be a polygenic trait dependent on the combined effect of several polymorphic genes and suggest that an approach based on multiple genetic markers could be clinically useful for identification of MOH patients more likely to respond poorly to drug withdrawal therapy. Nonetheless, further larger prospective studies are needed to validate our findings and to verify the clinical utility of a multigene approach for prediction of MOH patients at higher risk of unsuccessful drug detoxification treatment.

Conflict of interest

The authors report no conflict of interest.

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Supplemetary materials

Methods

Power calculations

Given our sample size of 108 MOH patients successfully detoxified and 65 MOH patients with unsuccessful detoxification, and assuming a power of 80% and a level of significance of 0.05, the minimal detectable risk for the investigated gene variants (Minor allele frequency: 0.07 to 0.49) ranged from an odds ratio of 1.9 to 2.7 under the log-additive model of inheritance, from 2.5 to 3.3 under the dominant model and from 2.5 to 5.7 under the recessive model of inheritance (MAF: 0.17 to 0.49). The association between gene polymorphisms and risk for developing MOH was evaluated by using logistic regression analysis adjusted for age and gender. Our sample size of 227 MOH patients and 312 control subjects has power of 80 ($\alpha < 0.05$) to detect an odds ratio for MOH risk ranging from 1.5 to 1.85 under the log-additive model (MAF: 0.07 to 0.49), from 1.6 to 1.9 under the dominant model (MAF: 0.07 to 0.49) and from 1.75 to 3.1 under the recessive model (MAF: 0.17 to 0.49). Power calculations were performed using Quanto software (www.hydra.usc.edu/gxe/).

Table S1. Primers and restriction enzymes used for genotyping.

Polymorphism	Primer sequence (5'-3')	Product (bp)	Restriction enzyme	Allele phenotypes (bp)
5HT2A 516C>T	F: CATAGGGTACCGGTGGCCTCT R: GTCCAAACAGCAATGATTTTCA	198	Sau96AI	C: 109, 87 T: 198
5HT2A -1438A>G	F: AAGCTGCAAGGTAGCAACAGC R: AACCAACTTATTTCCTACCAC	468	MspI	A: 468 G: 244,224
SLC6A4 5-HTTLPR	F: GGCGTTGCCGCTCTGAATGC R: GAGGGACTGAGCTGGACAACCAC	528 484	-	L: 528 S: 484
SLC6A4 STin2 VNTR	F: TGGATTTCCTTCTCTCAGTGATTGG R: TCATGTTCCTAGTCTTACGCCAGTG	390 360 345	-	Allele 12: 390 Allele 10: 360 Allele 9: 345
SLC6A4 rs1042173	F: ATATTCCCATGGTAGACTGTG R: TTTCATTTTAGCTTCTTACATCTT	357	ApoI	G: 269, 88 A: 357
DRD2 -141C Ins/Del	F: ACTGGCGAGCAGACGGTGAGGACCC R: TGCGCGCGTGAGGCTGCCGGTTCGG	303	BstNI	I: 160, 144 D: 303
DRD2 TaqI A	F: CACGGCTGGCCAAGTTGTCTA R: CACCTTCCTGAGTGTCATCAA	304	TaqI	A2: 176,128 A1: 304
DRD2 NcoI	F: TTGTCCGGCTTTACCCA R: ATCCTGCAGCCATGG	445	NcoI	C: 445 T: 278,175
DBH -1021C>T	F: GGAGGACACGTTCTAGTCC R: CACCTCTCCCTCCTGTCCTCTCGC	131	HhaI	C: 109, 22 T: 131
COMT val158met	F: TCGTGGACGCCGTGATTCAGG R: AGGTCTGACAACGGGTCAGGC	217	NlaIII	Val: 138, 81 Met: 96, 81, 40
BDNF val66met	F: CCCCATGAAAGAAGCAAACA R: TTTGTCTGCTGCCGTTACC	403	NlaIII	Val: 245 Met: 168
OPRM1 118A>G	F: CCGTCAGTACCATGGACAGCAGCGGTG R: GTTCGGACCGCATGGGTCGGACAGAT	154	MboI	A: 154 G: 128, 26

Table S2. Association analysis of polymorphic gene variants (P>0.1) with the risk of MOH patients for unsuccessful detoxification at 2-months of follow-up: univariate logistic regression analysis.

SNP	Successful_withdrawal therapy		Genetic model	OR (95% CI)	P value
	Yes	NO			
DRD2 -141C Ins/Del (rs1799732)					
II	90 (83.3)	59 (90.8)	Log-additive	0.63 (0.25–1.55)	0.30
ID	18 (16.7)	5 (7.7)	Dominant	0.51 (0.19–1.36)	0.16
DD	0 (0)	1 (1.5)	Recessive	NC	NC
DRD2 Taq1A (rs1800497)					
CC	70 (64.8)	42 (64.6)	Log-additive	0.91 (0.52–1.59)	0.74
CT	33 (30.6)	22 (33.9)	Dominant	1.01 (0.53–1.92)	0.98
TT	5 (4.6)	1 (1.5)	Recessive	0.32 (0.04–2.82)	0.25
COMT rs6269					
AA	33 (30.6)	15 (23.1)	Log-additive	1.18 (0.75–1.86)	0.48
AG	55 (50.9)	38 (58.5)	Dominant	1.47 (0.72–2.98)	0.28
GG	20 (18.5)	12 (18.5)	Recessive	1.00 (0.45–2.20)	0.99
EAAT2 -181A>C (rs4354668)[#]					
TT	28 (26.2)	21 (32.3)	Log-additive	1.03 (0.65–1.64)	0.89
TG	64 (59.8)	30 (46.1)	Dominant	0.74 (0.38–1.46)	0.39
GG	15 (14.0)	14 (21.5)	Recessive	1.68 (0.75–3.76)	0.21
5HT2A -1438A>G (rs6311)					
AA	25 (23.1)	14 (21.5)	Log-additive	1.23 (0.80–1.90)	0.35
AG	56 (51.9)	29 (44.6)	Dominant	1.10 (0.52–2.30)	0.81
GG	27 (25.0)	22 (33.9)	Recessive	1.53 (0.78–3.01)	0.21

NC, not calculated. [#]One genotype is lacking due to DNA exhaustion.

Table S2. Association analysis of polymorphic gene variants (P>0.1) with the risk of MOH patients for unsuccessful detoxification at 2-months of follow-up: univariate logistic regression analysis.

Variant	Successful_withdrawal therapy		Genetic model	OR (95% CI) value	P
	Yes	NO			
SLC6A4 5HTT-LPR					
LL	27 (25.0)	23 (35.4)	Log-additive	0.80 (0.52–1.24)	0.32
LS	57 (52.8)	28 (43.1)	Dominant	0.61 (0.31–1.19)	0.15
SS	24 (22.2)	14 (21.5)	Recessive	0.96 (0.46–2.02)	0.92
SLC6A4 VNTR STin2[#]					
12/12	49 (45.4)	23 (35.4)	Log-additive	1.20 (0.76–1.87)	0.44
12/s	44 (40.7)	34 (52.3)	Dominant	1.52 (0.80–2.86)	0.20
s/s	15 (13.9)	8 (12.3)	Recessive	0.87 (0.35–2.18)	0.77
SLC6A4 rs1042173					
GG	30 (27.8)	13 (20.0)	Log-additive	1.23 (0.78–1.93)	0.37
GT	55 (50.9)	37 (56.9)	Dominant	1.54 (0.73–3.22)	0.25
TT	23 (21.3)	15 (23.1)	Recessive	1.11 (0.53–2.32)	0.78

[#]The STin2.9 allele was accumulated with STin2.10 as one unit termed short allele (s), as previously reported [28].

Table S3. Cumulative effect of top six gene variants on the risk of unsuccessful withdrawal therapy at 2-months of follow-up: subgroup analysis.

N° of high risk genotypes	Successful_withdrawal therapy		OR[*] (95% CI)	*P* value
	Yes n (%)	NO[#] n (%)		
Subgroup analysis in patients with chronic headache with drug abuse (n=22)				
≤3	54 (50.0)	4 (18.2)	1 (Ref)	
4-6	54 (50.0)	18 (81.8)	4.081 (1.227–13.572)	0.022
Subgroup analysis in patients with chronic headache without drug abuse (n=35)				
≤3	54 (50.0)	8 (22.9)	1 (Ref)	
4-6	54 (50.0)	27 (77.1)	3.283 (1.358–7.939)	0.008

[*]Logistic regression analysis adjusted for triptan overuse and primary headache diagnosis. [#] Eight patients were not included in the subgroup analysis due to lacking of relevant data.

Table S4. Distribution of gene variants in MOH patients (n = 227) e control subjects (n = 312) and association analysis with MOH risk.

SNP	Controls	MOH	Genetic model	OR* (95% CI)	P value
OPRM1 118A>G (rs1799971)					
AA	216 (69.2)	161 (70.9)	Log-additive	1.02 (0.73–1.43)	0.89
AG	87 (27.9)	55 (24.2)	Dominant	0.91 (0.62–1.36)	0.66
GG	9 (2.9)	11 (4.8)	Recessive	2.06 (0.79–5.37)	0.14
COMT val158met (rs4680)					
GG	84 (26.9)	60 (26.4)	Log-additive	0.95 (0.73–1.23)	0.69
GA	154 (49.4)	121 (53.3)	Dominant	1.03 (0.68–1.55)	0.89
AA	74 (23.7)	46 (20.3)	Recessive	0.83 (0.54–1.29)	0.41
BDNF val66met (rs6265)					
Val/Val	187 (59.9)	133 (58.6)	Log-additive	1.22 (0.91–1.65)	0.18
Val/Met	109 (34.9)	77 (33.9)	Dominant	1.20 (0.83–1.74)	0.33
Met/Met	16 (5.1)	17 (7.5)	Recessive	1.70 (0.81–3.58)	0.16
DRD2 NcoI (rs6275)					
CC	147 (47.1)	109 (48.0)	Log-additive	0.93 (0.70–1.24)	0.63
CT	136 (43.6)	102 (44.9)	Dominant	0.99 (0.69–1.42)	0.96
TT	29 (9.3)	16 (7.0)	Recessive	0.70 (0.36–1.38)	0.30
DBH -1021C>T (rs1611115)					
CC	195 (62.5)	137 (60.4)	Log-additive	1.21 (0.90–1.62)	0.21
CT	101 (32.4)	72 (31.7)	Dominant	1.16 (0.80-1.68)	0.43
TT	16 (5.1)	18 (7.9)	Recessive	1.80 (0.86-3.78)	0.12

*Logistic regression analysis adjusted by sex and age.

Table S4. Distribution of gene variants in MOH patients (n = 227) e control subjects (n = 312) and association analysis with MOH risk (cont'd).

SNP	Control	MOH	Genetic model	OR* (95% CI)	P value
DRD2 -141C Ins/Del (rs1799732)					
II	273 (87.5)	200 (88.1)	Log-additive	1.03(0.60–1.74)	0.92
ID	38 (12.2)	26(11.4)	Dominant	0.98 (0.57–1.71)	0.95
DD	1 (0.3)	1 (0.4)	Recessive	NC	NC
DRD2 Taq1A (rs1800497)					
CC	219 (70.2)	148 (65.2)	Log-additive	1.30 (0.93–1.83)	0.13
CT	86 (27.6)	72 (31.7)	Dominant	1.34 (0.91–1.98)	0.14
TT	7 (2.2)	7 (3.1)	Recessive	1.47 (0.49–4.42)	0.49
COMT rs6269					
AA	96 (30.8)	64 (28.2)	Log-additive	1.05 (0.80–1.36)	0.74
AG	157 (50.3)	122 (53.7)	Dominant	1.12 (0.75–1.66)	0.58
GG	59 (18.9)	41 (18.1)	Recessive	0.99 (0.62–1.57)	0.95
EAAT2 -181A>C (rs4354668)#					
TT	99 (31.7)	69 (30.7)	Log-additive	0.97 (0.74–1.27)	0.82
TG	157 (50.3)	121 (53.8)	Dominant	1.01 (0.69–1.50)	0.94
GG	56 (17.9)	35 (15.6)	Recessive	0.88 (0.54–1.44)	0.62

*Logistic regression analysis adjusted by sex and age. NC, not calculated. #Two genotypes in MOH patients are lacking due to DNA exhaustion.

Chapter 8

Functional polymorphisms in COMT and SLC6A4 genes influence the prognosis of patients with medication overuse headache after withdrawal therapy

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Abstract

Background: It is currently unknown if common genetic variants influence the prognosis of patients with medication overuse headache (MOH). We herein evaluated the role of two common SNPs in the COMT gene (rs4680 and rs6269), as well as the STin2 VNTR polymorphism in the SLC6A4 gene, as predictors for long-term outcomes of MOH patients after withdrawal therapy.

Methods: Genotyping was conducted by PCR, PCR-RFLP analysis or real-time PCR allelic discrimination assay on genomic DNA extracted from peripheral blood. Gene variants association was evaluated by logistic regression analysis adjusted for clinical confounding factors, and the threshold of statistical significance for multiple testing was set at $P < 0.012$.

Results: Sixty-five MOH patients with unsuccessful detoxification and 83 MOH patients with effective drug withdrawal therapy were available for the analysis.

rs4680G allele carriers or the COMT rs6269G-rs4680G haplotype were found associated to a lower risk of relapse within the first year after successful detoxification therapy, in comparison to homozygous rs4680A allele carriers (OR: 0.17, 95%CI: 0.05-0.61, P=0.007) or to the COMT rs6269A-rs4680A haplotype (OR: 0.19, 95% CI 0.06-0.54, *P*= 0.003), respectively. In addition, carriers of the STin2 VNTR short allele were found at higher odds for the composite poor outcome including unsuccessful withdrawal therapy and relapse within 12 months of follow-up after successful detoxification (OR: 2.81, 95%CI: 1.26-6.25, P=0.009).

Conclusions: Our results indicate that genotyping for COMT rs4680 and SLC6A4 STin2VNTR could be useful for the identification of MOH patients at higher risk of poor prognosis after drug withdrawal.

Keywords: medication overuse headache; detoxification; relapse; COMT; SLC6A4; polymorphisms

Introduction

Medication overuse headache (MOH) is a daily or almost-daily type of headache which results from chronicization of episodic migraine or tension-type headache as a consequence of symptomatic drug overuse [1]. Long-term prospective studies indicate that 30-45% of MOH patients relapse into overuse after successful withdrawal therapy, the majority of them within the first year of follow-up [2]. Despite the growing number of studies focusing on the role of clinical factors as predictors of long-term outcome [3,4], to date no study has investigated the contribution of common genetic variants as determinants of relapse of MOH patients after successful withdrawal therapy.

The rs4680 (G>A) polymorphism at the COMT locus at chromosome 22q11 causes a four-fold decrease in enzyme activity, which in turn leads to higher dopamine levels in the synaptic cleft [5]. Also known as valine158methionine (Val158Met), rs4680 contributes to inter-individual differences in pain sensitivity [6], being the Met/Met genotype associated with increased pain sensitivity in human pain studies [7,8]. Given the occurrence of dysfunctions in the mesocorticolimbic dopamine circuit and in other pain-processing-related areas of MOH patients [9], we hypothesized that an alteration of COMT activity, as expected in Met/Met patients, may have an impact on the prognosis of MOH patients. On the other hand, the SLC6A4 gene locus on chromosome 17q11.2 encodes the serotonin transporter, which is a key synaptic regulator acting in the fine-tuning of brain serotonergic neutrotrasmission [10]. Recently, we reported in MOH patients a relationship between the STin2 VNTR polymorphism of the SLC6A4 gene and the number of monthly headache days before withdrawal therapy [11]. On the basis of this observation, we herein re-analysed STin2 VNTR genotype data in MOH patients to evaluate its value as predictor of long-term outcome after successful withdrawal therapy.

Given the potential role of polymorphisms in monoamine neurotransmitter related genes in pain modulation, the primary aim of the present study was to evaluate the role of COMT (rs4680G>A and rs6269A>G) and SLC6A4 (STin2 VNTR) gene polymorphisms as determinants of relapse of MOH patients within the first year after successful drug withdrawal. The secondary objective of the study was to investigate the role of the aforementioned polymorphisms as predictive factors for prognosis of MOH patients after withdrawal therapy.

Materials and methods

Partecipants

The medical records of 227 MOH patients who underwent withdrawal therapy between March 2005 and September 2008 at the inpatient Headache Unit of IRCCS 'C. Mondino National Neurological Institute' Foundation (Pavia, Italy) and had given their informed consent to genetic analysis were retrospectically reviewed. Inclusion and exclusion criteria for MOH patients have been previously described [11]. Patients that had completed a detoxification program conducted according to previously described procedures [12] or had follow-up visits after 2, 6 and 12 months after successful detoxification were included in the analysis. The following items were assessed during follow-up visits: (i) frequency and clinical characteristics of headache; (ii) types of preventive medication currently used, and compliance with the prescribed therapy (regularity of intake); and (iii) types of symptomatic medication currently used and frequency of their use. Relapsers were defined as those patients who were no longer overusers at the 2-month follow-up, as a result of detoxification, but reverted back into overuse during the subsequent observation period.

This study was approved by the local Ethics Committee and it met the requirements of the Declaration of Helsinki. Written informed consent was obtained from all patients before participation in the study.

Genotyping

Genomic DNA was extracted from peripheral blood by using the QiaAmp DNA Mini Kit (Qiagen Valencia, California, USA). Of the several single nucleotide polymorphisms (SNPs) of COMT gene, rs4680 and rs6269 were selected based on the observation that these two SNPs in combination are sufficient to differentiate between the three most common COMT activity haplotypes [13]. COMT rs6269 genotype was determined by real-time PCR using Applied Biosystems TaqMan

Pre-Designed SNP Genotyping assays [Assay ID: C_2538746_1]. Genotyping of COMT rs4680 was performed by PCR-RFLP according to previously described procedures [14]. For quality control purposes, each PCR run included negative as well as positive controls for the three genotypes. Genotyping was done blinded to the outcome status of patients.

Statistical analysis

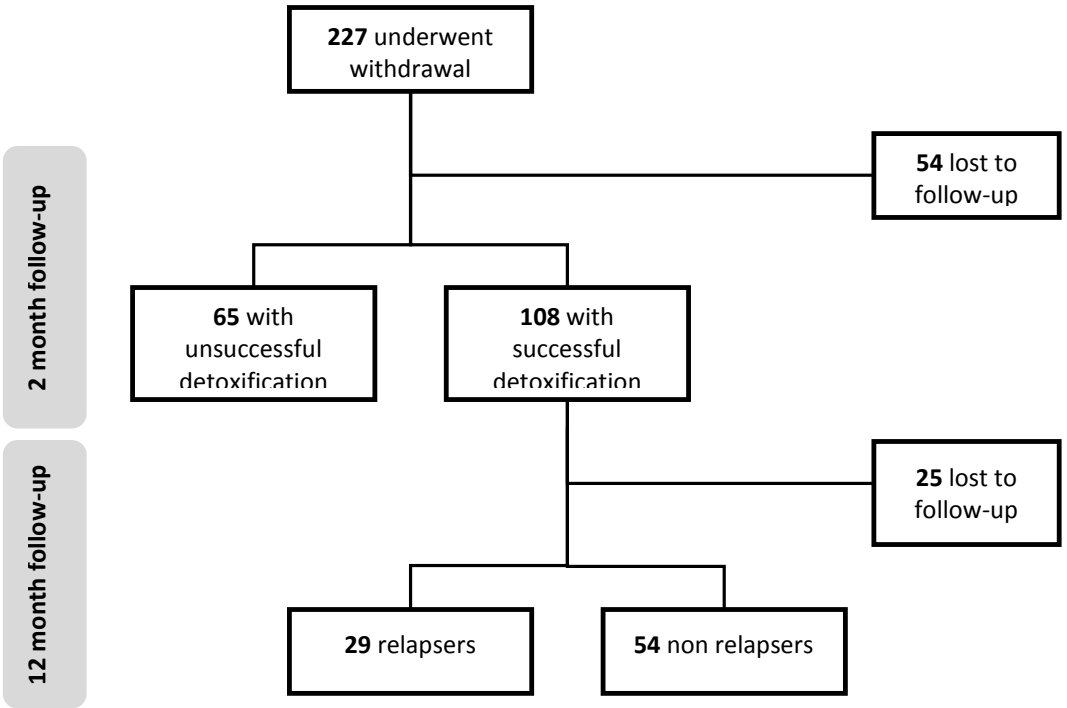
Each COMT polymorphism was tested for deviation from the Hardy-Weinberg equilibrium (HWE) by use of Pearson's chi-square test as implemented in the Finetti's program (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). The association between gene variants and relapse risk was evaluated in a binary logistic regression model adjusted for clinical variables with $P \leq 0.1$ upon univariate correlation analysis. The SNPStats software [15] was used for the calculation of linkage disequilibrium (measured as Lewontin's D′-values) between COMT SNPs, for the estimation of COMT haplotype frequencies and for the evaluation of haplotype association with relapse risk. As the haplotype test was a *post-hoc* analysis, we considered it to be an additional test. Therefore, for a Bonferroni correction on the *P* values we used $P < 0.012$ as a threshold for significance [0.05/4 (total 3 polymorphisms + 1 haplotype)]. Given the sample size of our cohort (54 relapsers and 29 non-relapsers) and assuming a power of 80% and a level of significance of 0.012, the minimal detectable risk for the investigated gene variants (minor allele frequency: 0.38-0.49) ranged from an odds ratio of 3.1 to 3.3 under the log-additive model of inheritance. Power calculations were performed using Quanto software (www.hydra.usc.edu/gxe/).

Results

Association of clinical variables with relapse risk

The flow-chart of MOH patients is shown in Figure 1. A total of 227 MOH patients undergoing drug withdrawal therapy were retrospectively evaluated. Sixty-five MOH patients with unsuccessful detoxification and 83 MOH patients with effective drug withdrawal therapy were available for the analysis. Seventy-nine subjects (34.8%; 65 females and 14 males; mean age 46.2±11.3) were lost to follow-up mainly due to patients' decision of not returning to the centre or failure to comply with the protocol. Among these, 54 patients dropped out at the 2-month follow-up and 25 patients at 1 year after successful detoxification. No significant differences in demographic data, clinical history and genotype distribution were found between available patients and those lost to follow-up (data not shown).

Figure 1: Flow-chart of MOH patients.



Clinical and demographic characteristics of 83 MOH patients with successful drug withdrawal treatment are displayed in Table S1. Twenty-nine MOH patients (34.9%) with successful drug withdrawal relapsed within the first year of follow-up. In the univariate logistic regression analysis triptan overuse (OR: 0.19, 95% CI 0.04-0.91, *P* = 0.038), onset of primary headache before 10 years of age (vs >20 years, OR: 3.69, 95% CI 0.96-14.21, *P*= 0.057) and higher number of headache days per month before detoxification (OR: 1.07, 95% CI 0.99-1.16, *P* = 0.091) were found related to relapse risk with a cut-off of $P \leq 0.1$ (Table S1).

Association of COMT and SLC6A4 gene variants with relapse risk

The genotype frequency distributions of the three polymorphisms analysed were in accordance with Hardy-Weinberg equilibrium ($P > 0.05$). The distribution of COMT SNPs after stratification of MOH patients according to the 1-year relapse status is shown in Table 1. The major allele at rs6269 (A) (OR 2.53, 95% CI 1.27-5.03, *P* = 0.007) or the variant allele at rs4680 (A) (OR 3.51, 95% CI 1.77-6.95, *p* = 0.0002) were more frequently found in patients who relapsed within the first year after successful drug withdrawal. For each COMT SNP, the proportion of relapsed subjects increased with the number of risk alleles carried by an individual subject, as shown by the Armitage's trend test (rs6269: *P* = 0.006; rs4680: *P* = 0.0002, Table 1). To control for triptan overuse as potential confounding factor in the effect of COMT SNPs on relapse risk, we conducted an analysis limited to triptan nonoverusers (n=66). The significance of P-values for both COMT SNPs was retained when patients overusing triptans alone were excluded from the analysis (Table 1). As none of MOH patients with rs6269GG relapsed within the first 1 year after drug withdrawal, adjusted OR for confounding clinical factors was calculated for rs4680 only. In the logistic regression analysis adjusted for confounding clinical factors, rs4680G allele carriers were found at lower risk of relapse compared to patients with rs4680AA genotype (OR: 0.17, 95%CI0.05-0.61, P=0.007, Table 2).

In contrast to COMT SNPs, no difference in distribution of SLC6A4 STin2 VNTR was observed between relapsing and not relapsing MOH patients (Table 2). As expected, haplotype analysis of COMT SNPs revealed a strong pairwise linkage disequilibrium between rs6269 and rs4680 (D' = 0.988). In the logistic regression analysis adjusted for confounding clinical factors, the G-G haplotype was found at lower risk to relapse within the first year of follow-up, compared to the most common A-A haplotype (OR: 0.19, 95% CI 0.06-0.54, *P*= 0.003, Table 2).

Table 1. Association of COMT alleles with relapse risk of MOH patients during the first year of follow-up after successful withdrawal treatment.

SNP	Outcome at 1 year		OR (95% CI)	*P* value	Armitage's trend test *P* value
	No relapse n (%)	Relapse n (%)			
All patients (n=83)					
rs6269 A>G					
AA	14 (25.9)	13 (44.8)	2.53 (1.27-5.03)*	0.007	0.006
AG	27 (50.0)	16 (55.2)			
GG	13 (24.1)	0 (0)			
rs4680 G>A					
GG	19 (35.2)	1 (3.5)	3.51 (1.77-6.95)#	0.0002	0.0002
GA	26 (48.1)	15 (51.7)			
AA	9 (16.7)	13 (44.8)			
Triptan non-overusers[a] (n=66)					
rs6269 A>G					
AA	9 (23.1)	12 (44.6)	3.19 (1.52-6.72)*	0.002	0.002
AG	17 (43.6)	15 (55.6)			
GG	13 (33.3)	0 (0)			
rs4680 G>A					
GG	15 (38.5)	1 (3.7)	3.80 (1.81-7.97)#	0.0003	0.0004
GA	18 (46.1)	14 (51.9)			
AA	6 (15.4)	12 (44.4)			

[a]1, or more, types of triptan. *Allele 1 vs Allele 2 (ref); #Allele 2 vs Allele 1 (ref). For multiple comparisons, the Bonferroni correction was used with a significant *P* value being <0.012.

Table 2. Association of SLC6A4 STin2VNTR, COMT rs4680 and COMT haplotype (rs6269-rs4680) with relapse risk of MOH patients during the first year of follow-up after successful withdrawal treatment.

SNP	Outcome at 1 year		OR* (95% CI)	*P* value
	No relapse n (%)	Relapse n (%)		
SLC6A4 STin2 VNTR#				
12/12	30 (55.6)	12 (41.4)	1 (Ref)	
12/s	19 (35.2)	12 (41.4)	D: 1.71 (0.60-4.86)	0.31
s/s	5 (9.3)	5 (17.2)	R: 2.05 (0.40-10.62)	0.39
COMT rs4680 G>A				
AA (158Met/Met)	9 (16.7)	13 (44.2)	1 (Ref)	
GA+GG	45 (83.3)	16 (55.2)	0.17 (0.05-0.61)	0.007

COMT haplotype (rs6269-rs4680)	Outcome at 1 year		OR* (95% CI)	*P* value
	No relapse (%)	Relapse (%)		
A-A	0.41	0.69	1 (Ref)	
G-G	0.49	0.26	0.19 (0.06-0.54)	0.003
A-G	0.10	0.04	0.16 (0.01-2.03)	0.16

*Logistic regression analysis adjusted for age of primary headache onset, triptan overuse and headache days per month before withdrawal therapy. #The STin2.9 allele was accumulated with STin2.10 as one unit termed short allele (s), as previously reported [11]. D, dominant model of inheritance; R, recessive model of inheritance. The threshold of significance required for Bonferroni correction was $P < 0.012$.

Association of COMT and SLC6A4 gene variants with clinical outcome of MOH patients after withdrawal therapy

From a clinical stand-point it could be of interest to include patients that have undergone an unsuccessful detoxification, thereby comparing MOH patients with good clinical outcome (i.e. patients with successful detoxification and not relapsing) to MOH patients with poor prognosis (i.e unsuccessful detoxification and relapse within 12 months of follow-up after successful detoxification. Indeed, this would include all patients classified as MOH in the new ICHD-III guidelines [16]. Clinical and demographic characteristics and comparisons of these two groups are shown in Table S2. After adjusting for confounding clinical variables, homozygous rs6269G allele carriers were found at lower odds to be MOH patients

with poor composite outcome in comparison to rs6269A carriers (OR: 0.37, 95% CI: 0.14-0.97, *P* value = 0.043), while rs4680A allele carriers displayed an increased risk to be patients with poor prognosis in comparison to rs4680GG homozygotes (OR: 2.59, 95%CI: 1.14-6.34, *P* = 0.036, Table 3). However, *P*-values for both COMT SNPs did not reach the significance required for Bonferroni correction (P<0.012) and the association of their haplotype combination did not reach nominal statistical significance (G-G vs A-A haplotype, OR=0.60, 0.33-1.11, *P*=0.11, Table 5). Conversely, carriers of the STin2 VNTR short allele were found at higher odds to be MOH patients with poor prognosis in comparison to carriers of STin2 VNTR 12.12 genotype and the statistical significance did not exceed that required for Bonferroni correction (OR: 2.81, 95%CI: 1.26-6.25, P=0.009). When the combined effect of COMT rs4680 and SLC6A4 STin2VNTR genotypes was examined, carriers of 1 risk genotype (OR: 10.97, 95%CI: 2.036-59.10, P=0.005) or 2 risk genotypes (OR: 20.04, 95%CI: 3.48-115.40, P= 0.001) were found at higher odds for the development of poor prognosis than were patients without risk genotypes (Table 3).

Table 3. Association of SLC6A4 STin2 VNTR and COMT SNPs with risk for the composite poor outcome including unsuccessful detoxification and relapse within 12 months of follow-up after successful detoxification.

Genetic marker	Composite outcome		OR* (95% CI)	*P* value
	Good (n=54)	Poor (n=94)		
SLC6A4 STin2 VNTR				
12/12	30 (55.6)	35 (37.2)	1 (Ref)	
12/s	19 (35.2)	46 (48.9)		
s/s	5 (9.3)	13 (13.8)	D: 2.81 (1.26-6.25)	0.009
COMT rs6269 A>G				
AA	14 (25.9)	28 (29.8)	1 (Ref)	
AG	27 (50.0)	54 (57.5)		
GG	13 (24.1)	12 (12.8)	R: 0.37 (0.14-0.97)	0.043
COMT rs4680 G>A				
GG	19 (35.2)	17 (18.1)	1 (Ref)	
GA	26 (48.1)	57 (60.6)		
AA	9 (16.7)	20 (21.3)	D: 2.59 (1.06-6.34)	0.036
Number of high risk genotypes†				
0	12 (22.2)	3 (3.2)	1 (Ref)	
1	25 (46.3)	46 (48.9)	10.97 (2.04-59.10)	0.005
2	17 (31.5)	45 (47.9)	20.04 (3.48-115.41)	0.001
COMT haplotype (rs6269-rs4680)	**Composite outcome**		**OR* (95% CI)**	***P* value**
	Good (%)	**Poor (%)**		
A-A	40.7	51.0	1 (Ref)	
G-G	49.1	40.9	0.60 (0.33-1.11)	0.11
A-G	10.2	7.5	0.66 (0.20-2.12)	0.49

*Logistic regression analysis adjusted for age of primary headache onset, primary headache diagnosis, familiarity of primary headache, number of headache days per month, triptan overuse and NSAIDS overuse. D, dominant model of inheritance; R, recessive model of inheritance. †STin2 VNTR 12/s or s/s and rs4680GA or AA. The threshold of significance required for Bonferroni correction was $P < 0.012$.

Discussion

In the present study we found that about 35% of MOH patients with successful drug withdrawal were found to relapse within the first year of follow-up, a relapse rate which is comparable to previous results [17,18]. In addition, MOH patients overusing triptans alone were less likely to relapse compared to patients overusing other types of analgesics, a result also consistent with prior studies [4,18]. The present study shows for the first time that common genetic variants of COMT and

SLC6A4 genes affect clinical long-term outcome of MOH patients receiving detoxification therapy. More specifically, the COMT rs4680AA genotype (158Met/Met) confers a higher risk of relapse within the first year of follow-up after successful drug withdrawal. In addition, carriers of the STin2 VNTR variant allele were found at higher odds for the composite poor outcome including unsuccessful detoxification and relapse within 12 months of follow-up after successful detoxification.

It has been argued that rs4680 alone cannot fully account for the variation in enzyme activity since COMT haplotypes formed by rs4680 and tightly linked SNPs may influence COMT function and explain the effects on pain perception to a greater extent than rs4680 alone [19]. Indeed, three common COMT haplotypes have been identified corresponding to three levels of pain sensitivity, with the rs4680G (Val) allele being part of both the high- and low- pain sensitive haplotype, whereas the haplotype containing the rs4680A (Met) allele is associated to intermediate pain sensitivity [19]. Intriguingly, an inverse correlation has been reported between pain sensitivity and COMT activity, meaning that the haplotype corresponding to the high pain sensitivity represents the low COMT activity haplotype, whereas the haplotype corresponding to the low pain sensitivity represents the high COMT activity haplotype [20]. Our data shows that the high COMT activity haplotype (rs6269G-rs4680G) confers a lower risk of relapse when compared to the intermediate COMT haplotype activity (rs6269A-rs4680A). It could therefore be that the fact that MOH patients with high COMT activity haplotype have a decreased risk of relapse is correlated with their pain sensitivity. On the other hand, given that the intermediate COMT haplotype is the only haplotype corresponding to methionine at position 158 of the COMT protein, our results support that rs4680 genotyping alone could be sufficient in the clinical setting to identify MOH patients at higher risk of relapse. As the effect size is large, studies are warrented to prospectively evaluate the clinical utility of rs4680

genotyping for the identification of the subgroup of MOH patients who may benefit from a stricter follow-up. The importance of our findings is also strengthened by the observation that SLC6A4 STin2VNTR alone, or in combined analysis with COMT rs4680, allows the identification of MOH patients at higher risk for the composite poor outcome that includes unsuccessful detoxification and relapse within 12 months of follow-up after successful detoxification. Since SLC6A4 STin2VNTR correlates with the composite poor outcome but not with relapse risk, our findings suggest a complex relationship between SLC6A4 and COMT gene variants and modulation of pain-related phenotypes.

Our findings should be interpreted in the light of the following limitations. First, its single-institution and retrospective nature require its findings to be validated in larger, preferably multi-institutional prospective studies. Second, the relatively high incidence of patients lost at follow-up (34.8%) could limit the generalizability of our study. However, no significant differences of COMT and SLC6A4 genotype distribution were found between available patients and those lost to follow-up, thus excluding a possible bias on our results. In addition, a similar dropout rate has been reported in follow-up of MOH patients, ranging from 20% to 48% [21], a result highlighting that effective interventions and therapeutic approaches are required to address this specific issue. Third, the wide variety of different types of acute drug overused and the limited sample size of our MOH patient cohort limited the possibility to conduct a stratified analysis of COMT SNPs and haplotypes according to the different type of drug being abused. Finally, since COMT activity has been reported to be under estrogen control [22], larger studies are required to evaluate gender-specific effects of COMT SNPs and haplotypes on the relapse rates of MOH patients.

In conclusion, the current results highlight the importance of COMT rs4680, alone or in haplotype combination with rs6269, as determinant for relapse of MOH patients with successful drug withdrawal. In addition, the combined analysis of

COMT rs4680 with SLC6A4 STin2 VNTR allows the identification of MOH patients more likely to experience the composite poor outcome of non-response to detoxification and relapse within 12 months of follow-up after successful detoxification. Nonetheless, further genetic and functional studies are needed to validate our findings and to clarify the complex relationship between COMT and SLC6A4 gene interaction, pain sensitivity and prognosis of MOH patients.

Conflict of interest

The authors report no conflict of interest.

Aknowledgements

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Supplementary materials

Table S1. Clinical and demographic characteristics of MOH patients with successful withdrawal treatment in the whole cohort and after stratification according to the 1-year relapse status.

Clinical features	All patients (n=83)	Outcome at 1 year		OR* (95% CI)	*P* value
		No relapse n (%)	Relapse n (%)		
Gender					
Women	57 (68.7)	37 (68.5)	20 (69.0)	1 (Ref)	
Men	26 (31.3)	17 (31.5)	9 (31.0)	0.98 (0.37-2.60)	0.97
Age at study entry, years	48.9 (11.7)	48.8 (11.7)	49.1 (12.0)	1.00 (0.96-1.04)	0.90
Age of primary headache onset, years (n=79)					
>20	20 (25.3)	16 (29.6)	4 (16.0)	1 (Ref)	
10-19	34 (43.0)	25 (46.3)	9 (36.0)	1.44 (0.38-5.47)	0.59
<10	25 (31.6)	13 (24.1)	12 (48.0)	3.69 (0.96-14.21)	**0.057**
Familiarity for headache (n=79)					
No	38 (48.1)	28 (52.8)	10 (38.5)	1 (Ref)	
Yes	41 (51.9)	25 (47.2)	16 (61.5)	1.79 (0.69-4.66)	0.23
Primary headache diagnosis					
migraine type	60 (72.3)	41 (75.9)	19 (65.5)	1 (Ref)	
mixed type[a]	16 (19.3)	10 (18.5)	6 (20.7)	1.29 (0.41-4.08)	0.66
other/unknown	7 (8.4)	3 (5.6)	4 (13.8)	2.88 (0.58-14.15)	0.19
Drugs of abuse					
Triptans[b]					
No	66 (79.5)	39 (72.2)	27 (93.1)	1 (Ref)	
Yes	17 (20.5)	15 (27.8)	2 (6.9)	0.19 (0.04-0.91)	**0.038**
NSAIDS[c]					
No	60 (72.3)	42 (77.8)	18 (62.1)	1 (Ref)	
Yes	23 (27.7)	12 (22.2)	11 (37.9)	2.14 (0.78-5.74)	0.131
Combination drugs[d]					
No	71 (85.5)	46 (85.5)	25 (86.7)	1 (Ref)	
Yes	12 (14.5)	8 (14.5)	4 (13.3)	0.92 (0.25-3.36)	0.90
Association drugs[e]					
No	53 (63.9)	36 (65.5)	17 (56.7)	1 (Ref)	
Yes	30 (36.1)	18 (34.5)	12 (43.3)	1.41 (0.56-3.58)	0.47
Monthly drug number[f]	43.0 (28.5)	41.5 (28.0)	42.2 (23.2)	1.00 (0.98-1.02)	0.91
Headache days per month[f]	24.9 (6.2)	24.1 (6.6)	26.5 (5.0)	1.07 (0.99-1.16)	**0.091**
Duration of MOH desease (months)[f]	34.1 (51.0)	37.3 (60.0)	27.9 (25.8)	0.99 (0.98-1.01)	0.45

Prophylactic drugs during follow-up (n=78)					
Beta-blockers					
No	62 (79.5)	40 (75.5)	22 (88.0)	1 (Ref)	
Yes	16 (20.5)	13 (24.5)	3 (12.0)	0.42 (0.11-1.63)	0.21
Antidepressants					
No	34 (43.6)	23 (43.4)	11 (44.0)	1 (Ref)	0.96
Yes	44 (56.4)	30 (56.6)	14 (56.0)	0.98 (0.37-2.54)	
Antiepileptics					
No	63 (80.8)	45 (84.9)	18 (72.0)	1 (Ref)	
Yes	15 (19.2)	8 (15.1)	7 (28.0)	2.19 (0.69-6.92)	0.18
Others					
No	59 (75.6)	41 (77.4)	18 (72.0)	1 (Ref)	
Yes	19 (24.4)	12 (22.6)	7 (28.0)	1.33 (0.45-3.93)	0.61
Symptomatic drugs during follow-up (n=78)					
Triptans					
No	66 (84.6)	43 (81.1)	23 (92.0)	1 (Ref)	
Yes	12 (15.4)	10 (18.9)	2 (8.0)	0.37 (0.07-1.85)	0.23
NSAIDS					
No	11 (14.1)	8 (15.1)	3 (12.0)	1 (Ref)	
Yes	67 (85.9)	45 (84.9)	22 (88.0)	1.30 (0.31-5.40)	0.37
Others					
No	75 (96.2)	51 (96.2)	24 (96.0)	1 (Ref)	
Yes	3 (3.8)	2 (3.8)	1 (4.0)	1.06 (0.09-12.30)	0.96

[a]Migraine associated with episodic tension-type headache; [b]1, or more, types of triptan. [c]1, or more, types of NSAIDs; [d]a single pharmaceutical product that contains more than 1 active principle, [e] 2 or more drugs of the classes above; [f]before withdrawal therapy. Categorical data are number and percentages of total subjects, whereas continuous data are expressed as mean (SD). [*]Univariate logistic regression analysis. *P* values ≤0.1 are in boldface.

Table S2. Association between clinical variables of MOH patients and risk for the composite poor outcome including unsuccessful detoxification and relapse within 12 months of follow-up after successful detoxification.

Clinical features	Composite outcome		OR[#] (95% CI)	*P* value
	Good (n=54)	Poor (n=94)		
Gender				
Women	37 (68.5)	73 (77.7)	1 (Ref)	
Men	17 (31.5)	21 (22.3)	0.63 (0.29-1.33)	0.22
Age at study entry, years	48.8 (11.7)	48.0 (12.1)	0.99 (0.97-1.02)	0.69
Age of primary headache onset, years				
>20	16 (29.6)	21 (22.3)	1 (Ref)	
10-19	25 (46.3)	28 (29.8)	0.85 (0.37-1.99)	0.71
<10	13 (24.1)	38 (40.4)	2.23 (0.90-5.51)	**0.083**
Not known	0 (0)	7 (7.4)	NC	
Familiarity for headache				
No	28 (52.8)	30 (31.9)	1 (Ref)	
Yes	25 (47.2)	59 (62.8)	2.20 (1.01-4.42)	**0.026**
Not known	0 (0)	5 (5.3)	NC	
Primary headache diagnosis				
migraine type	41 (75.9)	58 (61.7)	1 (Ref)	
mixed type[a]	10 (18.5)	31 (33.0)	2.19 (0.97-4.96)	**0.060**
other/unknown	3 (5.6)	5 (5.3)	1.18 (0.27-5.21)	0.83
Drugs of abuse				
Triptans				
No	39 (72.2)	88 (93.6)	1 (Ref)	
Yes	15 (27.8)	6 (6.4)	0.18 (0.06-0.49)	**0.001**
NSAIDS				
No	42 (77.8)	58 (61.7)	1 (Ref)	
Yes	12 (22.2)	36 (38.3)	2.17 (1.01-4.67)	**0.047**
Combination drugs				
No	46 (85.2)	76 (80.9)	1 (Ref)	
Yes	8 (14.8)	18 (19.1)	1.36 (0.55-3.38)	0.51
Association drugs				
No	36 (66.7)	62 (66.0)	1 (Ref)	
Yes	18 (33.3)	32 (34.0)	1.03 (0.51-2.10)	0.93
Monthly drug number before withdrawal	41.5 (28.0)	44.4 (26.3)	1.00 (0.99-1.02)	0.53
Headache days per month before withdrawal	24.1 (6.6)	25.8 (5.99)	1.04 (0.99-1.10)	**0.10**
Months of MOH desease before withdrawal	37.3 (60.0)	27.8 (31.7)	0.99 (0.99-1.00)	0.24

[*] [#]Univariate logistic regression analysis. NC, not calculated. P values ≤ 0.1 are in boldface.

Chapter 9

Triptan use in Italy: insights from administrative databases

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Abstract

Introduction: In this drug utilization study, we aimed at assessing the pattern of triptan use in Italy by means of the drug prescription databases of two Local Health Authorities, accounting for approximately 1 million citizens.

Methods: The study population included all residents aged 18 to 84 years in the Vercelli province (about 175,000 inhabitants) and in the Umbria region (about 885,000 inhabitants), who had at least one dispensation for triptans in 2012. A frequent-user, which might be at risk of medication overuse headache (MOH), was defined as a patient being dispensed at least 10 defined daily doses (DDD) of triptans every month for at least 3 consecutive months.

Results: Triptans were used by 0.7-1% of the population. While most patients were dispensed less than 60 DDDs per year, about 10% of all triptan users were

classified as frequent users. In both areas, patients below the age of 29 were less likely to be frequent users while the 40-49 year-old population was the most affected, with no sex difference. About two thirds of frequent users persisted in this behavior for an additional 3-month period in the following 6 months.
Conclusions: Our data indicate that approximately 10% of all triptan users in the Italian population are potentially at risk for MOH. An approach based on drug prescription databases could be useful to identify patients at risk of MOH.

Keywords
Migraine, triptans, medication overuse headache, prescription data, pharmacoepidemiology.

Introduction

According to the International Classification of Headache Disorders (ICHD-III edition beta version) medication-overuse headache (MOH) is a chronic headache occurring as a consequence of regular overuse of acute or symptomatic headache medications on at least 10 days per month (15 days for simple analgesics and NSAIDs) for at least 3 consecutive months (1). While the previous definition of the International Headache Society (HIS) included the resolution or improvement of the headache after withdrawal of symptomatic treatment (2), the latest criteria have excluded this parameter although it is mentioned that chronic headache usually resolves after the overuse is stopped (1). In brief, therefore, the cause of MOH is the headache treatment itself. Even though every new agent that arrived on the market raised the hope that it would not lead to MOH, it is now accepted that all symptomatic treatments for migraine can trigger chronic headache. Indeed,

ergotamine, triptans, non-steroidal anti-inflammatory drugs, opioids and combination treatments, are all listed in the ICHD-III beta as causative factors (1).

The therapy of MOH entails the discontinuation of the abused drugs, which, according to the severity of MOH and to different clinical practices, can be achieved with a variety of means ranging from simple advice (3) to hospitalization (4). Nonetheless, MOH is a costly disease in terms of social burden as well as from a pharmacoeconomic perspective (*e.g.* increased medicine prescriptions, increased costs for headache clinics and hospitalization, etc.) (5,6). Furthermore, while the condition is well known to neurologists and to experts in headache clinics, it is probably less known to general practitioners and pharmacists. Epidemiological data indicate that MOH affects approximately 1% of the general population, with a similar prevalence in Europe, North America and Asian countries (7). However, given that most analgesics are over-the-counter drugs, it is also difficult to have accurate figures, because patients overusing medications not reimbursed by the NHS may not be caught in the statistics. On the other hand, in a number of countries, including Italy, triptans, which are considered by many experts as the gold standard therapy for acute treatment of migraine, are prescribed by General Practitioners and are reimbursed by the NHS. In principle, therefore, it is possible to identify individual patients' drug intake to estimate the prevalence of triptan use. In a study conducted in the Netherlands, prevalence of triptan use in the Dutch population was 1.3%, and 10.4% of these patients could be considered overusers following the IHS criteria (8). In Italy, data collected in selected areas suggested a slightly lower use of triptans (0.6-0.8% of the population) (9-12) but a higher proportion of patients at risk of MOH using the IHS criteria (14.3%) (12). These data, nonetheless, run back in time and it would be important to have an updated snapshot of triptan use in Italy.

According to sales statistics published by the Italian medicines agency (AIFA), almost the entire amount of triptan use in the 2001-2011 decade (around 95%) was

covered by the NHS (13). Thus, when individual prescriptions are recorded, it is possible to identify almost all triptan users. To quantify the prevalence of MOH related to triptan use and to assess the role of potential risk factors, we carried out a drug utilization study in two Italian areas: the Province of Vercelli (about 175,000 inhabitants) and the Umbria Region (about 885,000 inhabitants). The use of two separate databases allowed us to investigate which clinical and demographic factors were consistent across populations.

Methods

Triptans are reimbursed by the Italian National Health Service (NHS) and data relevant to each prescription are recorded for administrative purposes (the reimbursement of retail pharmacies). The following data were used in this study: drug code, number of dispensed packages, encrypted patient identification number and date of prescription. From the drug code we were able to identify the information on substances, expected duration of the prescription (on the basis of the Defined Daily Doses – DDD - contained in each package), and ATC (Anatomical, Therapeutic and Chemical classification).

Prescribing data were derived from the drug dispensation monitoring system of the Local Health Authority of Vercelli and of the Umbria Region. A descriptive drug utilization study was conducted on subjects living in Vercelli (exploratory cohort) and in the Umbria Region (validation cohort). All subjects aged 18 to 84 years who received at least one triptan (ATC N02CC) prescription in the period from 1 January 2012 to 31 December 2012 were included in the study. Patient demographic data were obtained by means of automated record-linkage with the regional health office database in which all individuals assisted by the NHS are listed. The record-linkage procedure is based on the NHS patient identification

code, which does not contain any sensitive data related to patient identity, thus ensuring confidential treatment of personal data.

Triptans are reimbursed by the Italian National Health Service (NHS) and related prescription details are entirely recorded in regional health authorities databases. All triptans available in 2012 on the Italian market (almotriptan, rizatriptan, frovatriptan, sumatriptan, zolmitriptan and eletriptan) were included in the analysis. A "*triptan user"* was defined as a patient who was prescribed and dispensed at least one triptan during the study period. A *"frequent user"* was categorized as a patient being dispensed at least 10 DDDs of triptans every month for at least 3 consecutive months. Assuming that the patient takes all the triptans prescribed, but not more than one dose per day, this definition would parallel that of the International Headache Society for MOH. DDDs used herein are updated to 2011 and refer to the indications of Durg-Italia, a scientific association affiliated with the European Drug Utilization Research Group (Euro-Durg) (Supplementary Table 1).

We also identified a subgroup of patients, *"consistently frequent users"*, as subjects who were classified as frequent users between January and June 2012, and who were exposed to an additional 3-month period of frequent use between July and December 2012.

Triptan users in the study populations were described in terms of age, sex, administered triptan and months of treatment. Drug consumption was evaluated in terms of: prevalence of use (by dividing the number of drug users by the overall resident population) and DDDs per 1,000 users/inhabitants per day (the mean number of doses consumed every day by 1,000 patients included in the study/resident population). The risk of frequent use was estimated for each triptan found in the observation period.

Concomitant drug prescriptions were also retrieved for frequent users to identify comorbidities and consumption of migraine prophylactic medications. Supplementary Table 2 illustrates the categories of drugs that were considered as

indicators of comorbidities. With regard to migraine prophylactic medications, given that no information on the indication is recorded in the administrative databases, it was impossible to establish whether these drugs were prescribed for migraine or for other indications. However, we assumed that the following drugs might be prescribed as prophylactic medicines: metoprolol (ATC C07AB02), propranolol (C07AA05), topiramate (N03AX11), valproic acid (N03AG01), timolol (C07AA06), atenolol (C07AB03), amitriptyline (N06AA09), venlafaxine (N06AX16), and pizotifen (N02CX01).

Statistical analysis

Clinical characteristics of frequent users and regular users and the difference for continuous variables are presented with 95% CIs. The two groups were compared using Mann-Whitney U- test for median age and prophylactic medications. To evaluate the differences among triptans, we estimated the odds ratio (OR) using almotriptan, the most frequently used drug, as reference. Adjusted odds ratios, with 95% confidence interval, were assessed by a logistic regression model. ORs were adjusted for age, gender and prophylactic medications. IBM® SPSS® software (version 22) was used for the statistical analyses.

Results

Triptan use in Italy

First, we referred to a comprehensive database on drugs bought by the National Health system, updated to 2012 (13). The prescription of triptans has steadily increased in the last decade in Italy (Supplementary figure 1A). In 2011, 0.76 DDDs/1,000 inhabitants per day were prescribed/dispensed. The prescription pattern differs significantly from Region to Region, ranging from 40% below (e.g. Umbria, Campania) to 40% (e.g. Sardegna, Valle d'Aosta) above the national

average (Supplementary figure 1B). Unlike many other drug classes (13), there is no North-to-South trend in the number of prescriptions and there is little variability in the regional expenditure per DDD since prices of various triptans are fairly similar.

Triptan use in the two Italian areas in 2012

Having gathered a general picture of triptan use in Italy, we then proceeded in analyzing triptan use in the Vercelli province and in the Umbria region. The prevalence of triptan use, stratified by age, sex and different substances, was fairly similar in the two areas, even though the level of use observed in Vercelli was approximately 30% higher than in Umbria (Table 1). This observation parallels the one obtained with the national database.

Table 1. Characteristics of the triptan users in the two areas

	Province of Vercelli			**Umbria Region**		
	Male	**Female**	**Total**	**Male**	**Female**	**Total**
Prevalence of use, N (%)						
Total	**299 (0.4)**	**1141 (1.5)**	**1440 (1.0)**	**1043 (0.3)**	**3614 (1.0)**	**4657 (0.7)**
Age, N (%)						
18-29	34 (0.3)	103 (1.1)	137 (0.7)	108 (0.2)	337 (0.6)	445 (0.4)
30-39	48 (0.4)	223 (2.0)	271 (1.2)	198 (0.3)	706 (1.2)	904 (0.7)
40-49	85 (0.6)	359 (2.6)	444 (1.6)	302 (0.5)	1218 (1.8)	1520 (1.1)
50-59	72 (0.6)	273 (2.2)	345 (1.4)	224 (0.4)	842 (1.4)	1066 (0.9)
60-69	44 (0.4)	126 (1.1)	170 (0.8)	141 (0.3)	362 (0.7)	503 (0.5)
≥70	16 (0.1)	57 (0.4)	73 (0.3)	70 (0.1)	149 (0.2)	219 (0.2)
almotriptan, N(%)	66 (0.1)	303 (0.4)	369 (0.3)	288 (0.1)	1014 (0.3)	1302 (0.2)
rizatriptan, N (%)	81 (0.1)	309 (0.4)	390 (0.3)	291 (0.1)	1010 (0.3)	1301 (0.2)
frovatriptan, N (%)	56 (0.1)	285 (0.4)	341 (0.2)	205 (0.1)	837 (0.2)	1042 (0.1)
sumatriptan, N (%)	71 (0.1)	179 (0.2)	250 (0.2)	192 (0.1)	582 (0.2)	774 (0.1)
zolmitriptan, N (%)	17 (0)	72 (0.1)	89 (0.1)	89 (0)	331 (0.1)	420 (0.1)
eletriptan, N (%)	34 (0)	112 (0.2)	146 (0.1)	78 (0)	278 (0.1)	356 (0)
1 triptan	275 (0.4)	1037 (1.4)	1312 (0.9)	953 (0.3)	3235 (0.9)	4188 (0.6)
2 or more triptans	24 (0)	104 (0.1)	128 (0.1)	90 (0)	379 (0.1)	469 (0.1)

Median age, year	48	47	47	47	46	46
Expenditure per capita (€)	0.93	3.47	2.24	0.60	1.97	1.31
Expenditure per user (€)	213.6	224.4	222.1	198.1	201.5	200.7
DDD/1,000 inhab. per die	0.54	1.95	1.27	0.27	1.04	0.67
Months in which dispensations occurred, median (SD)	3 (3.1)	4 (3.1)	4 (3.1)	3 (3.1)	4 (3.4)	4 (3.3)

DDD:defined daily dose

As expected, most users were dispensed less than 60 DDDs per year (Table 2). Seven to ten percent (Vercelli and Umbria, respectively) of patients were dispensed an average of at least 10 DDDs/month (120 DDDs/year), while a small proportion (3% in Vercelli and 2% in Umbria) were dispensed 18 DDDs/month (216 DDDs/year) or more.

Table 2. Number of defined daily doses (DDDs) per user in 12 months.

	Vercelli			**Umbria Region**		
DDDs	**Male N (%)**	**Female N (%)**	**Total N (%)**	**Male N (%)**	**Female N (%)**	**Total N (%)**
<60	224 (75)	846 (74)	1070 (74)	869 (83)	2811 (78)	3680 (79)
60-119	46 (15)	183 (16)	229 (16)	115 (11)	553 (15)	668 (14)
120-180	20 (7)	68 (6)	88 (6)	35 (3)	154 (4)	189 (4)
181-215	1 (0)	15 (1)	16 (1)	7 (1)	36 (1)	43 (1)
≥216	8 (3)	29 (3)	37 (3)	17 (2)	60 (2)	77 (2)
Total	**299 (100)**	**1141(100)**	**1440 (100)**	**1043 (100)**	**3614 (100)**	**4657 (100)**

About 13% of all users in the Vercelli population and 10% in the Umbria Region fell in the category of frequent users (Table 3). Younger patients were less likely to be frequent users (with patients below the age of 29 being significantly less at risk), while the most affected age group in both regions was the 40-49 year old category, with no sex difference. Frequent users were older than regular users; the difference

was slight, but statistically significant in both geographical areas. Frequent users had a significantly higher prescription of prophylactic medications for migraine in Umbria, though this finding was not confirmed in Vercelli. What was confirmed and significant was a strong increase in drug expenditure of frequent users in both Vercelli and Umbria, although this result was obvious given the increased amount of bought drugs. Frequent users, which accounted for 10-13% of the analyzed population, were responsible for about 40% of total triptans and total drugs expenditure.

Table 3. Characteristics of frequent triptan users compared with regular users.

	Total		**Regular users**		**Frequent users**		**Frequent users vs Regular users difference, N (95% IC)**	
	VC	**U**	**VC**	**U**	**VC**	**U**	**VC**	**U**
Male, N (%)	299 (21)	1043 (22)	259 (21)	956 (23)	40 (22)	87 (19)	1 (-5,5;7,2)	-4 (-8,1;-0,6)
Female, N (%)	1141 (79)	3614 (78)	995 (79)	3231 (77)	146 (78)	383 (81)	-1 (-7,2;5,5)	4 (0,6;8,1)
Total, N (%)	**1440 (100)**	**4657 (100)**	**1254 (87)**	**4187 (90)**	**186 (13)**	**470 (10)**		
Median age	47	46	47	46	48	48	*p=0,009*[*]	*p<0,001*[*]
Prophylaxis (%)	14	13	14	12	14	21	*p=0,876*[*]	*p<0,001*[*]
Expenditure per user (€)	222	201	139	131	782	820		
DDD/1000 Ut die	128	105	78	68	466	434		
Age, N (%)								
18-29	137 (10)	445 (10)	129 (10)	424 (10)	8 (4)	21 (4)	-6 (-9,4;-2,6)	-6 (-7,7;-3,6)
30-39	271 (19)	904 (19)	242 (19)	857 (20)	29 (16)	47 (10)	-4 (-9,4;1,9)	-10 (-13,4;-7,5)
40-49	444 (31)	1520 (33)	376 (30)	1315 (31)	68 (37)	205 (44)	7 (-0,8;13,9)	12 (7,5;16,9)
50-59	345 (24)	1066 (23)	305 (24)	941 (22)	40 (22)	125 (27)	-3 (-9,2;3,5)	4 (-0,1;8,3)
60-69	170 (12)	503 (11)	143 (11)	447 (11)	27 (15)	56 (12)	3 (-2,2;8,5)	1 (-1,8;4,3)
≥70	73 (5)	219 (5)	59 (5)	203 (5)	14 (8)	16 (3)	3 (-1,1;6,8)	-1 (-3,2;0,3)

[a]Mann-Whitney U test.

VC: Province of Vercelli; U: Umbria region; CI: confidence interval.

When analyzing the relative risk of high frequency use given by the single triptans (Table 4), there was a trend for eletriptan and sumatriptan to confer an increased risk and of rizatriptan and frovatriptan to confer a lower risk compared to almotriptan. However, no drug appeared to carry a distinctly elevated or decreased risk of high frequency in both areas, weakening any conclusion that could be drawn.

Last, we verified whether the status of frequent user was transitory or persisted in time: it is noteworthy that about two thirds of identified frequent users qualified as 'consistently frequent user' (Table 5).

Table 4. Risk of overuse for each triptan in the two areas.

	Non-overusers		**Overusers**		**OR (95% IC)***	
Triptan	**VC**	**U**	**VC**	**U**	**VC**	**U**
almotriptan, N	282	1004	36	99	1 (Ref)	1 (Ref)
rizatriptan, N	308	995	25	47	0.64 (0.37-1.10)	0.45 (0.32-0.65)
frovatriptan, N	261	775	19	62	0.55 (0.31-0.99)	0.78 (0.56-1.09)
sumatriptan, N	163	528	35	72	1.53 (0.91-2.57)	1.22 (0.88-1.70)
zolmitriptan, N	54	301	19	33	2.54 (1.33-4.83)	1.00 (0.65-1.52)
eletriptan, N	93	226	17	36	1.37 (0.73-2.56)	1.55 (1.02-2.34)

*Adjusted by age, gender and prophylaxis; VC, Vercelli; U, Umbria Region. In the analysis were considered users of only triptan in the period.

Table 5. Consistently frequent users in the two areas by age and sex.

	Frequent users identified Jan-Jun		Consistently frequent users	
	VC	**U**	**VC (%)**	**U (%)**
Gender				
Male, N (%)	28	57	18 (64)	37 (64)
Female, N (%)	93	240	57 (61)	168 (70)
Total, N (%)	**121**	**297**	**75 (62)**	**205 (69)**
Age, N (%)				
18-29	4	12	3 (75)	8 (67)
30-39	17	32	14 (82)	17 (53)
40-49	41	125	24 (59)	86 (69)
50-59	28	75	12 (43)	51 (68)
60-69	19	42	14 (74)	30 (71)
≥70	12	14	8 (67)	13 (93)

VC, province of Vercelli; U, Umbria Region.

Discussion

Our analysis suggests that about 10% of triptan users are prescribed and dispensed a number of monthly doses that, if taken on separate days, would lead them to meet the IHS criteria for medication overuse headache. These frequent users are responsible for 40% of the total cost of triptans. Focusing medical attention on this relatively small proportion of migraineurs might save and optimize a significant part of NHS resources. With respect to our study design, by using two separate populations, we were able to use the two databases as both exploratory and confirmatory and to find common determinants of this pattern of use. While a number of factors (*e.g.* type of triptan) showed a consistent trend in both populations, only age correlated significantly with frequent use. The finding that relatively older patients (40-49 age category) appeared at higher risk is consistent with the data reported in a meta-analysis of 29 studies comprising a total of 2612 patients with MOH, where patients had a mean duration of primary headache of around 20.4 years (14).

With respect to the prescription of prophylactic medications we found conflicting results in the two areas, with a higher level of use in the Umbria population. It must be acknowledged that migraine prophylaxis is difficult to monitor using NHS databases, as most medications are not solely prescribed for migraine and many are not dispensed by the NHS. For example, the prescription of flunarizine, which in a recent study was found to be the most used preventive medication in two provinces (Novara and Pavia) (15) bordering with the Vercelli province could not be monitored as flunarizine is not covered by NHS. In a broader study, involving 10 headache centers from all over Italy, flunarizine was the third used preventive medication (16). Nonetheless, our study suggests that only 14-21% of frequent users of triptans are prescribed a prophylactic medication than can be monitored and these data are in line with a recent Dutch study on migraine preventive medications (17), It would therefore be important to design a study to assess the use of preventive medication in Italy.

The main strength of our study is represented by the fact that triptans in Italy are all reimbursed via the NHS and therefore our census covers the entirety of triptan use in the population of two geographic areas. The very limited amount of triptans that are purchased out of pocket (around 5% of total prescriptions) cannot modify our estimates.

The study also faced several limitations: (i) we focused solely on triptan use and were unable to monitor over-the-counter drugs or other prescription drugs that are not dispensed by the NHS. It is likely that including these drugs would increase significantly the number of patients which might meet IHS criteria for medication overuse headache; (ii) we defined as frequent user a patient that is dispensed more than 10 DDDs per month for three consecutive months. It must be acknowledged that the relation “one DDD one attack” might not hold, as guidelines suggest that patients might decide to take a second dose for the same attack in the same day. It is possible, therefore, that triptan overusers are less than frequent users, as defined

in this study; (iii) as in all pharmacoepidemiological studies, this report is unable to discriminate between dispensation and use, and some factors (*e.g.* sharing of the same prescription among family members, accumulating the drug for future use) might contribute to overestimating the phenomenon.

A number of other studies have been performed in Europe in the last 20 years, using different methods and different thresholds to define a triptan overuser (8,9,12,18-24, see Table 6). Our data are in line with prevalence data obtained in most of these studies and suggest that MOH is not a culture-bound disorder. Yet, probably most important, our data suggest that administrative databases would in principle be able to identify those at higher risk of suffering from this disorder, giving them earlier access to specialized care. This approach might significantly reduce the prevalence and duration of this chronic headache as well as the impact in terms of social and economic burden.

Table 6. Prevalence of frequent triptan users in population studies.

Study [Ref]	Country/Year	Methodology	Population	Period (months)	Triptan users (%)	Females %	Frequent triptan users (% among triptan users)	Females %
Gaist [18][#]	Denmark 1992/1994	Prescription data of the Odense Pharmacoepidemiological Database (OPED) derived from the County of Funen, Denmark	465,000	27	2,878 (0.62)	78.4	127 (4.4)[a] 45 (1.6)[b]	76.4 68.9
Gaist [19][#]	Denmark 1994/1995	Nationwide prescription data of the Register of Drug Statistcs (RDS)	4,243,942 (1994) 4,253,960 (1995)	24	43389	79.4	1726 (4.0)[a] 507 (1.2)[b]	73 63
Sondegaard [20]	Denmark 2000	Dispensing data of 22 community pharmacies in the County of Funen, Denmark	472,000	3	2,463 (0.52)	82.8	88 (3.6)[f]	-
Lohman [21]	Netherlands 2001/2002	Dispensing data from 18 community pharmacies of Sittard, Netherlands	168,000	12	2,343 (1.4)	78	117 (5.0)[c] 45 (1.9)[d]	- -
Lugardon [22]	France 2002	Prescription data of National French Health Insurance System in the Midi-Pyrenees area	1,550,000	6	13,860 (0.89)	80.6	866 (10.0)[g] 166 (1.9)[h]	80.66
Perearnau [23]	France 2003/2004	Prescription data of five of the French National Health's local health agencies in Alsace	1,793,000	12	20,686 (1.1)	78.5	399 (1.9)[e]	-
Dekker [8]	Netherlands 2005	Prescription data of the Dutch Drug Information System/Health Care Insurance Board (GIP/CVZ)	6,704,627	12	85,172 (1.3)	83	8844 (10.4)[c] 2787 (3.3)[d]	84.0 82.0
Pavone [9]	Italy 2005	Prescription data of a Regional Health Authority (Tuscany)	224,065	12	1,238 (0.55)	77.9	40 (3.2)[c] 12 (0.9)[d]	77.5
Biagi [12]	Italy 2007	Prescription data of a Regional Health Authority (Emilia-Romagna)	4,249,533	12	34,915 (0.8)	77.4	4983 (14.3)[c] 2627 (7.5)[i]	80.2 77.5
Braunstein [24]	France 2010/2011	French reimbursement database in two French administrative regions (PACA† and Corse)	-	20	95540	-	2243 (2.3)[j]	-

[#] Data refer to use and frequent use of sumatriptan only; [†]Provence-Alpes-Côte d'Azur. Criteria to define frequent triptan users: [a] 30-59 DDDs/month; [b] ≥60 DDDs/month; [c] users of 120 DDDs or more per year; [d] users of 216 DDDs or more per year; [e] >244 DDD/year; [f] ≥15 doses/month; [g]15-29 DDD/month among new users of triptans (n=8625); [h] >29 DDD/month among new users of triptans (n=8625); [i] >180 DDDs/year; [j] >20 DDD/month for more than 3 months.

Public Health relevance

- In the present study, a descriptive drug utilization study was conducted by means of the drug prescriptions databases of two Local Health Authorities in Italy to evaluate triptan use
- About 10% of identified triptan users are dispensed at least 10 DDDs of triptans every month for at least 3 consecutive months. If these doses were actually taken on different days, these patients would meet the IHS criteria for medication overuse headache.
- An approach based on drug prescription databases could be useful for early identification of patients at higher risk of MOH. Such approach might also reduce the prevalence and duration of MOH as well as decrease its social and economic burden.

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Supplementary matherials

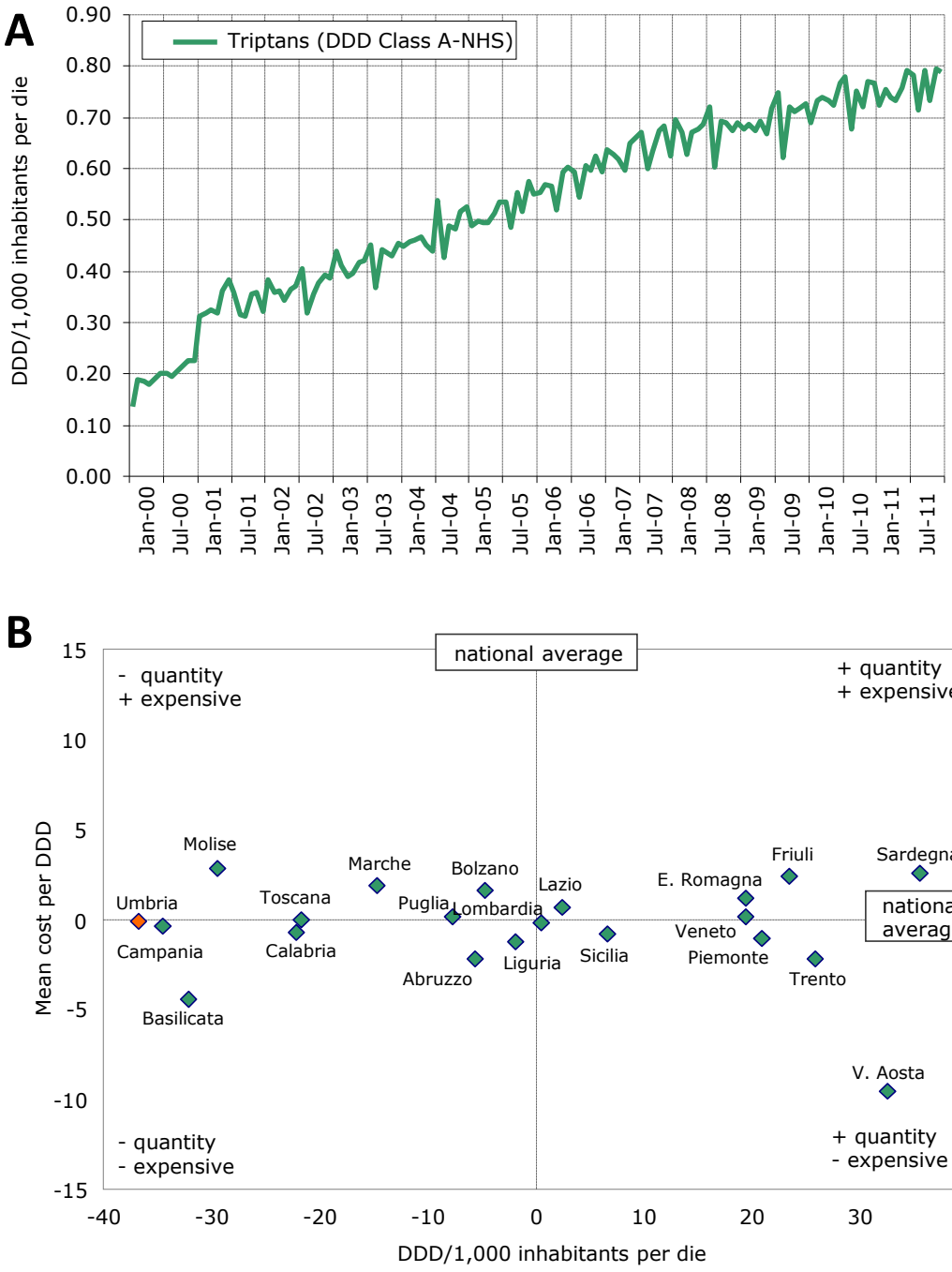
Supplementary Table 1. Medications covered by the analyses and related Defined Daily Dose designated by World Health Organization (WHO).

Active	ATC	Dose	WHO DDD
almotriptan	N02CC05	3, 6 tablets of 12.5 mg	12.5 mg
eletriptan	N02CC06	3, 6 tablets of 40 mg	40 mg
		3 tablets of 20 mg	
frovatriptan	N02CC07	2, 6 tablets of 2.5 mg	2.5 mg
rizatriptan	N02CC04	3, 6 tablets of 10 mg	10 mg
		3 tablets of 5 mg	
sumatriptan	N02CC01	4 tablets of 50 and 100 mg	50 mg
		2 syringes of 6 mg	6 mg
		2 nasal spray	20 mg
		2 suppositories of 25 mg	25 mg
zolmitriptan	N02CC03	2, 3, 6 tablets of 2.5 mg	2.5 mg

Supplementary Table 2. Therapeutic categories considered in the analysis.

Drugs tracer of comorbidity	**ATC code**
Antiacids	A02
Antidiabetics	A10
Antiplatelet/anticoagulant agents	B01A
Other cardiovascular drugs	C01-C03; C07-C09
Lipid-lowering agents	C10
Drugs used in benign prostatic hypertrophy	G04C
Drugs for obstructive airway diseases	R03
Antiepileptics	N03A
Antipsychotics	N05A
Antidepressives	N06A
NSAIDs	M01A; N02B
Osteoporosis medications	M05; A12A
Pain medications	N02A; N02BE

Supplementary Figure 1. Defined Daily doses (DDD) of triptans dispensed in Italy during the last decade (A) and regional variability of triptans utilization according to quantity and average cost of day care (B).



Chapter 10

Discussion

In 2000, Allen Roses published on Nature a comprehensive review concerning the potential application of pharmacogenetic knowledge in clinical practice and drug development. In the introduction, he emphasized how the medical thinking has radically changed during last century in parallel with new available genetic information.

> ‘ “*If it were not for the great variability among individuals, medicine might as well be a science and not an art*.” The thoughts of Sir William Osler in 1892 reflect the view of medicine over the past 100 years. The role of physicians in making the necessary judgements about the medicines that they prescribe is often referred to as an art, reflecting the lack of objective data available to make decisions that are tailored to individual patients. Just over a hundred years later we are on the verge of being able to identify inherited differences between individuals which can predict each patient’s response to a medicine. Sir William Osler, if he were alive today, would be re-considering his view of medicine as an art not a science.’ [1].

Significant advances in genetic technologies have been actually made in the last decades, resulting in a consistent enrichment of the pharmacogenetic and genomic knowledge of several diseases. It is often argued that personalizing treatment may improve the prognosis of patients underwent a pharmacological treatment and help optimizing the use of health care resources. Given the high burden of migraine, in terms of high prevalence, disability and healthcare costs, in principle, one would expect that many efforts must have been made for identifying genetic predictors of migraine pharmacological treatment too. In spite of this, our systematic review

showed that only sparse and contrasting pharmacogenetic evidences have been reported in migraine. In addition to this, we highlighted many consistent methodological limitations in several hitherto performed studies. Among the aforementioned, the small sample size of populations included in the studies stands out and this is remarkable if we consider that migraine has a high prevalence in the general population. Furthermore, in some studies, the size of samples was arbitrarily decided, without a *priori*- or *post hoc* analysis supporting the adequacy of population samples included in the association analysis. Therefore, the conduction of pharmacogenetic studies on large population samples of migraineurs is strongly warranted, aiming to reduce the risk of reporting false-negative findings and that uncontrolled variables influencing migraine severity may not, by chance, be equally distributed in each genotype.

In addition to this, none of the studies included in the systematic review had a replication cohort. Validation of observed effects is a key issue in pharmacogenetic and genomic research. The strength and replicability of results obtained in the exploratory cohort have to be tested in independent large cohorts of subjects characterized by the same phenotype outcomes and pharmacological treatment (i.e. the same drug/s or, if clinically correct, drug/s belongings to the same therapeutic class) of subject included in the exploratory cohort. For instance, when we identified a significant correlation between COMT rs4680 and frovatriptan response in migraineurs (n=75), we replicated our results in a validation cohort of migraineurs (n=123) treated with triptans other than frovatriptan [2].

As expected, besides being a crucial step in the field, results validation often represents a challenge. Enrollment of additional patients for replication cohorts can be a lengthy process for several reasons (e.g. low disease prevalence, specific exclusion criteria, dropouts), so the conduction of multicenter studies ensuring patients' recruitment at many centers may overcome this obstacle. In the light of these limitations, pharmacogenetic and genomic researchers may also focus on

validating potential association reported in previous studies. This is the case, for example, of our replication study published in 2010 that failed to confirm a previously reported correlation between DRD2 rs6275 and triptan response in migraineurs [3] [4] [5]. The same validation approach is obviously applicable in the field of genetic and genomic research. In this context, we were unable to confirm the correlation between migraine susceptibility and polymorphisms in GRIA1 gene [6][7].

Given the growing amount of human pharmacogenetic evidences, it's common that several studies may have attempted to answer similar pharmacogenetic questions. Often, many of the individual studies might report contrasting results in terms of correlation between a specific genetic variant and the response to a given drug or, alternatively, might all fail to show a significant association because of insufficient statistical power. In the light of the need to validate available evidences, the combining of all results concerning a specific association may be effective to verify the strength and replicability of results obtained in different studies. The statistical technique aimed to quantitatively combine results obtained by independent studies is called *meta-analysis*. Nowadays, meta-analyses are a hallmark of evidence-based medicine, offering a rational and helpful way of i) collecting all relevant studies (published or not) on a specific topic, ii) assessing the methodological quality of the design and execution of each included study, and iii) offering an unbiased synthesis of the available empirical data. It's clear that pharmacogenetic studies included in a meta-analysis may differ from each other in terms of study design (e.g. prospective/retrospective, multicenter/single center, cross sectional/longitudinal), patient's characteristics (e.g. ethnicity, disease severity, comorbidities, exclusion and inclusion criteria) and drug administration (e.g. dosages, administered molecule/s). However, the heterogeneity induced by these systematic differences may be tested by meta-analysis technique, which applies a specific statistical analysis method that can take into account the absence/presence

of heterogeneity among studies. Due to the lack of evidences concerning migraine pharmacogenetics, we were unable to perform a systematic review of the literature followed by a meta-analytic quantification of obtained results. However, we recently performed several meta-analysis aimed to assess the clinical validity of extensively studied genetic variants as genetic predictors of drug-induced adverse reactions [8] [9] [10], drug response [4] or prognosis of patients underwent a specific intervention [11] [12] in subjects other than migraineurs.

Lastly, no genome-wide association studies (GWAS) were performed with the aim of exhaustively assessing the role of the whole individual genetic background on the response to symptomatic or preventive anti-migraine drugs. It is known so far that pharmacogenomic GWAS are generally markedly fewer than GWA studies investigating disease risk [13]. A plausible explanation to this observation may lie in the major complexity of pharmacogenomic GWAS study design compared to that one of disease susceptibility GWAS. For instance, the recruitment of controls samples for pharmacogenomic GWAS requires the enrollment of subjects taking the same drug/s of cases but not experiencing the same phenotype; conversely, controls included in disease risk GWAS may simplistically be individuals from geographically matched population not experiencing the studied disease. Moreover, contrary to disease susceptibility GWAS, the collection of medical data concerning populations included in pharmacogenetic GWAS is specific for each study. However, it is undeniable that an enormous distance exists between the pharmacogenetic knowledge of migraine and the study of the genetic basis of migraine susceptibility. Given that the cost of genotype for a GWAS is at least one order of magnitude higher than the cost required to genotype several candidate genes, funding may be one of the limiting factors in migraine pharmacogenomics

[4] "BDNF genetic variation and clinical response to antipsychotic drugs: a systematic review and meta-analysis". Authors: Cargnin S, Massarotti A, Terrazzino S. Accepted for publication on European Psychiatry journal.

research. In this context, it's paradoxical that limited economical resources are destined to study such a common and highly costly disease.

It is acknowledged that, when a GWAS in a given disease or in populations treated with specific drugs has not yet been performed, it is still cost-effective to conduct a candidate gene/pathway association study. In the light of the aforementioned, we performed several candidate gene association studies aimed to enlarge pharmacogenetic knowledge in both migraine and medication overuse headache.
We identified COMT Val158Met (rs4680) as a potential genetic predictor of individual response to different classes of drugs used for chronic pain, such are opioids and triptans, irrespective of their primary molecular target. More precisely, COMT rs4680 Met/Met individuals affected by chronic low back pain showed better response to intrathecal morphine compared to Val/Val and Val/Met subjects. Intriguingly, the impact of the same genetic variant on triptan response in migraineurs was in the opposite direction, being Met/Met carriers at higher risk of being poor responders compared to Val/Val and Val/Met subjects. If, on the one hand, the overexpression of MORs (μ-opioid receptors) detected in Met/Met patients [14] may explain a consistent response to morphine, on the other hand the molecular mechanisms underlying COMT rs4680 Met/Met and poor triptan response are missing. On the basis of recent evidences suggesting a more aggressive phenotype in Met/Met migraine patients [15], we hypothesized that poorer response to triptans in these subjects may reflect the failure to control more intense migraine attacks compared to Val/Val or Val/Met migraineurs. Even if our data in migrainerus represent an interesting and entirely novel finding, our results must be viewed in the light of some limitations, such as: i) the need of being further replicated in larger and, preferably, prospective studies; ii) due to the fact that rs4680 cannot fully account for the variation of COMT enzyme activity, additional COMT haplotypes analyses are required to extend the current findings; iii)

approaches based on multiple genetic markers may allow to more accurately shed light on the plausible joint effect of multiple genes in predicting clinical response to morphine or triptans.

Subsequently, we investigated the influence of genetic variants in GRIA1 gene (rs548294 and rs2195450) on triptan response but we were unable to find a significant correlation between the two SNPs (either as single markers or in haplotype combination) and drug response in 186 migraineurs without aura. Furthermore, we failed to confirm a previously reported association of these variants with migraine susceptibility. Nevertheless, we cannot definitely exclude a plausible enrollment of GRIA1 gene in determining variability in triptan response and migraine susceptibility because, in principle, polymorphisms in other linkage disequilibrium groups may potentially be, almost in part, responsible for migraine development and headache response to triptans.

In the same way, we did not found a significant correlation between three SNPs of CGRP-related genes (CALCA rs3781719, RAMP1 rs3754701, RAMP1 rs7590387) and triptan response in 219 patients affected by migraine without aura. We acknowledge that our study was underpowered to detect small genetic main effects; however, it had sufficient power to detect medium-large effect sizes of clinical relevance. In this context, our results suggested that even if these SNPs were not correlated with drug response in migraineurs, the genetic variant RAMP1 rs7590387 might have a role in the transformation of episodic migraine into MOH. Given that CGRP blood levels are higher in women with chronic migraine compared with women affected by episodic migraine, the plausibility of this observation is strong, representing the first evidence of CGRP involvement in the genetic basis of migraine transformation into MOH. However, being rs7590387 localized 1.4 kb downstream of the RAMP1 gene, we can hypothesize that rs7590387 may not be that true causal variant but that, more probably, an unknown functional polymorphism in linkage disequilibrium with rs7590387 may be the

actual determinant factor for migraine transformation. Also noteworthy is the observation of a lack of association between rs7590387 and triptan response despite a correlation with migraine transformation. This deserves further study to investigate whether an inadequate response to triptans could entail the transformation of episodic migraine into MOH.

As previously mentioned, no information concerning genetic predictors of MOH patients' prognosis was reported in the literature. We showed for the first time that common genetic variants may influence short-term outcome of MOH patients after withdrawal. Specifically, DRD2 NcoI was found to be an independent predictor of unsuccessful detoxification, with TT genotype carriers of DRD2 NcoI being at lower risk of not responding to withdrawal compared to C allele carriers. Moreover, results of the analysis comprising the six top-ranking polymorphisms highlighted the notion that common gene variants with small effects can be clinically relevant when analyzed in combination, being their cumulative effect found significant even after Bonferroni's correction. So, further investigations using a pathway-based approach on a larger number of candidate polymorphic genes or using a genome-wide approach are strongly warranted for the identification of the minimal set of polymorphisms that could be clinically useful for the identification of MOH patients at higher risk of unsuccessful detoxification.

We further identified COMT rs4680 (as single marker or in haplotype combination with rs6269) and SLC6A4 STin2 VNTR variants as potential genetic determinants of long-term prognosis in MOH patients underwent inpatient withdrawal. More precisely, COMT rs4680AA genotype (158Met/Met) emerged to confer a higher risk of relapse within the first year of follow-up after successful drug withdrawal. Furthermore, we considered interesting, from a clinical perspective, comparing COMT rs4680 and SLC6A4 STin2 VNTR genotypes' distributions between MOH patients with good clinical outcome (i.e. patients with successful detoxification and not relapsing) and MOH patients with poor prognosis (i.e. unsuccessful

detoxification and relapse within 12 months of follow-up after successful detoxification). In this context, carriers of the STin2 VNTR variant allele were found to be at higher odds for the composite poor outcome after successful detoxification. Since SLC6A4 STin2 VNTR correlates with the composite poor outcome but not with relapse risk, our findings suggest a complex relationship between SLC6A4 - COMT gene variants and modulation of pain-related phenotypes.

Overall, our results demonstrate that genetic background seems to play a relevant role in determining variability in both antimigraine drug response and prognosis of MOH patients underwent drug withdrawal. The contribution of pharmacogenetic testing for tailoring pharmacological therapy of migraine and MOH could be therefore still considered of primary relevance in the light of the need to optimize patient's clinical outcomes and healthcare resources.

Given the necessity to curb MOH development among episodic migraineurs, there is a growing interest in actually identifying the proportion of subjects at risk of developing MOH in a real scenario, outside the context of selected patient samples enrolled in clinical trials. Drug utilization studies performed by means of administrative databases have represented a good tool for describing the pattern of use/overuse of triptans in general population. In this context, administrative databases offer the chance of studying patterns of drug use/overuse in unselected, heterogeneous and large populations, including vulnerable patients usually excluded by randomized clinical trials. Second, contrary to randomized clinical trials, data recorded in administrative databases are free from the so-called Hawthorne effect, which is the behavioral distortion that occurs when human beings know to be under observation. Third, beyond low cost of pharmacoepidemiologic investigation, the profile of drug use can be in principle determined over prolonged follow-ups and a comprehensive healthcare history of

each beneficiary of healthcare system may be available [16]. In the light of the need to update available pharmacoepidemiological evidences in migraine, we performed a drug utilization study aimed to quantify and describe triptan users and overusers in two vast Italian geographical areas by means of the analysis of administrative database's data. We reported that the 0.7-1% of the general population used triptans during the study period and that around the 10% of them showed an episode of triptan overuse. What is clinically relevant is that the two-thirds of overusers persisted in this behavior over the studied year. Then, focusing medical attention on this relatively small proportion of migraineurs may obviously result in optimization of a significant part of NHS resources. Yet, probably most important, an approach based on drug prescription databases could be useful for early identification of subjects at higher risk of developing MOH, giving them a prompt access to specialized care. Nevertheless, our results might be interpreted in the light of some limitations: i) given that we focused solely on triptan use and that we were unable to monitor over-the-counter drugs or other prescription drugs that are not dispensed by the NHS, the proportion of MOH subject might be underestimated; ii) as in all pharmacoepidemiological studies, this report was unable to discriminate between dispensation and use, and some factors (e.g. sharing of the same prescription among family members, accumulating the drug for future use) might contribute to overestimating the phenomenon.

In principle, many of the results obtained in drug utilization research are important for supporting or modifying a rational drug policy at both national and local levels [17]. In this context, a drug utilization study recently performed by Amadio and colleagues reported considerable variations in triptans usage patterns across several provincial jurisdictions in Canada characterized by different public drug reimbursement criteria [18]. Overall, their results suggest that areas without limits in triptan prescriptions (i.e. Alberta and Ontario) showed significantly higher rates

of subjects at risk of developing MOH compared with other jurisdictions with prescribing restrictions. An exceeding of triptans dispensations was also observed in some areas where prescription quantity limits were imposed by health authorities. However, given that MOH rates in regions without prescription limits were comparable with those ones reported in other countries worldwide without imposed limitations in triptan prescription (including Italy), we consider reasonable to assume that restrictions in triptan prescription at national level may be useful to curb the development of MOH in triptan users and to reduce related costs.

The urgent, presently unmet need for early detection of non-responders to antimigraine drugs and of patients at risk of developing MOH is a strong motivation for a multidisciplinary approach based on the integration of pharmacogenetics and pharmacoepidemiology. On the one hand, pharmacoepidemiology may gather heterogeneity in drug-related outcomes among large and not-selected populations. On the other hand, pharmacogenetics may explain the aforementioned heterogeneity in drug response, both in terms of drug efficacy and safety. In 2010, Şardaş introduced the neologism *pharmacogenovigilance*, defined as a discipline integrating pharmacogenetics and pharmacovigilance, the latter including pharmacoepidemiology, which is its active branch of research [19]. In this context, the merging of this complementary disciplines may significantly enhance the collection and the interpretation of clinically useful pharmacogenetic and genomic data. Moreover, we strongly believe that such a holistic approach may offer the chance to have more discerning insights into drug surveillance research compared to traditional pharmacovigilance.

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Chapter 11

List of publications

Cargnin S, Massarotti A, Terrazzino S. **BDNF Val66Met and clinical response to antipsychotic drugs: a systematic review and meta-analysis**. *European Psychiatry 2015*. Accepted for publication.

Cargnin S, Quaglia M, Canonico PL, Stratta P, Terrazzino S. **Impact of recipient ACE I/D genotype on kidney function in renal transplant patients: a meta-analysis of cross-sectional and longitudinal studies.** *Pharmacogenomics.* 2015;16(16):1887-1902.

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