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EPIGENETIC DNA METHYLATION CHANGES IN CHRONIC AND EPISODIC MIGRAINE

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ABSTRACT

Background: According to International Headache classification, migraine is classified in episodic migraine (EM) and chronic migraine (CM) based on the frequency of attacks. Medication-overuse headache (MOH) is a secondary chronic headache disorder defined as a frequent headache (15 days per month or more) induced by the overuse of analgesics. MOH occurs in patients with a previous headache history, usually episodic or chronic migraine. Currently the only treatment options include a detoxification program based on the abrupt withdrawal of overused painkillers and eventually a preventive treatment. Despite these therapeutic efforts, the relapse rate of MOH is high and no valid biomarkers are available to detect among patients who suffer from EM to CM and among chronic migraine those at greatest risk of develop a MOH. According to the data available, certain groups of people are more vulnerable to develop chronic pain conditions and poor adaptation to stress. On the basis of the scientific knowledge, a behavioral model of headache was proposed considering the strong association between pain, homeostatic imbalance and affective behavior. This pain and stress vulnerability is determined by genetic and non-genetic but heritable factors under the effect of environmental exposures as well as stochastic events during development. Differences between individuals' DNA sequences can predispose toward maladaptive behaviors in many disorders comorbid with MOH, conferring a risk for chronic pain and by functioning as a type of molecular memory. Several evidences confirmed that pain vulnerability and attitude to chronic pain sensitivity are heritable via genetic but also epigenetic pathways though changes in DNA expression. Epigenetic mechanisms would have the potential to link early life events, neuro-inflammation and brain plasticity in the aetiology of migraine chronification.

Aim of the study: to identify changes in DNA methylation associated with headache chronification.

Method: This was a pilot, longitudinal, prospective, observational, study. We enrolled patients into three groups: Patients with MOH, EM and healthy control without headache (HC). For MOH patients visits occurred at baseline (T0), at T1 for detox program, then at 2 month (T2) and 6 months (T3) after detox. About group of EM and HC, patients were evaluated at baseline (T0) and 6 months later (T1). At each follow-up visit Genomic DNA was extracted from whole blood and whole blood genome-wide DNA methylation profiles was analysed using the Infinium HumanMethylationEPIC Bead- Chip (Illumina)

Results: A total of 25 patients with MOH were enrolled at baseline (T0), 21 underwent protocol of withdrawal (T1), of these 19 and 18 were followed to visit T2 and T3 respectively. In the group of EM, 20 patients were enrolled and all subjects completed the study to T1. With regard to HC, 13 subjects were enrolled at T0, and 11 completed study at T1. Overall at T3, 6 month later detox program, 4 patients

remained chronic with medication overuse (22%), while 14 (78%) were cured from overuse. Among them 11 (61%) still fulfilled a diagnosis of chronic migraine and 3 (16%) reversed to an EM. We compared 22 MOH samples at T0 vs 13 HC at T0. No differentially methylated regions (DMRs) reached statistical significance after Benjamini–Hochberg correction for multiple test. When considering a nominal threshold of 0.01, we identified 721 DMRs. While most of the DMRs showed very small DNA methylation differences between the two groups, 29 had a delta of at least 0.05 in at least one CpG site. Then we then compared 22 MOH samples at T0 and 19 samples MOH at T1 and MOH samples at T0 and at T3, excluding those subjects (4) that at T0 were still MOH at T3: no DMR reached statistical significance after Benjamini–Hochberg correction.

Discussion: Comparing MOH vs HC group, none differentially methylated regions reached statistical significance, nevertheless 29 Different methylated regions had a delta of at least 0.05 in at least one CpG site. Among these 29 "risk DMRs " there are 4 most significant CpG site located in genes relevant for the possible implication with migraine chronification and drug addiction. Epigenetic alterations and mostly changes in DNA methylation have been previously hypothesized as a possible mechanism of migraine chronification. Our pilot study revealed that major DNA methylation mostly differs between MOH and HC. Data obtained from analysis of different methylated regions seems to support the clinical hypothesis of prominent role of Medication overuse in chronicization risk. Epigenetic mechanisms suggested as involved in migraine chronicization, play a crucial role in processes implicated in controlling dependence and cognitive-emotional regulation of stress. Conversely, these exploratory results lacked to detect differences in DNA methylation profile of MOH in response to treatments of detox and prophylaxis. Our results are preliminary and require then replication and validation in a larger sample.

INTRODUCTION

EPISODIC AND CHRONIC HEADACHE: STATE OF THE ART

Migraine, is a headache disorder characterized by repeated attacks of painful headache with specific associated features such as photophobia and phonophobia according to the International Headache Society (Classification of Headache Disorders, 3rd edition 2013). According to classification, it is classified in two main categories: episodic migraine (EM), defined as less than 15 headache days per month, and chronic migraine (CM), defined as ≥15 headache days per month for the last 3 months, with ≥8 days per month meeting migraine criteria with or without aura. Medication overuse headache (MOH) represents an aggravation of pre-existing chronic headache (Diener HC et al 2004). IHS criteria required regular overuse for more than 3 months of 1 or more drugs for acute and/or symptomatic treatment of headache:

1. Ergotamine, triptans, opioids or combination analgesics on 10 or more days/month

2. Simple analgesics on 15 or more days/month

3. Any combination of acute/symptomatic drugs on 10 or more days/ month without overuse of any single class alone

The majority of MOH patients suffered from migraine or tension-type headache (TTH) as primary headache and rarely cluster headache (Paemeleire K et al 2008). Overall, CM has a significant impact on the population, as each year, about 2.5 % of patients with EM develop new-onset CM, while it is estimated that MOH affects between 1% and 2% of the general population, up to 25-50% of the chronic headache population. (Stovner L et al 2005, Evers S et al 2011) MOH is extremely costly both to the patient and to society because of absenteeism and the burden on the health care services. (Evers S et al 2010) Recently, the Eurolight study estimated the mean per- person annual costs for MOH to €3561, three times the costs of migraine and more than ten times the costs of Tension type headache. (Linde M et al 2012) According to EFNS guidelines, treatment of MOH patients should include: patient's education on the nature of the disease, on risk factors and on treatment options; withdrawal including rescue medication; preventive treatment and a multimodal approach including psychological support, if necessary (Evers S et al 2011). Recent studies confirmed not only headache frequency and acute medication intake, but also disability, depression and anxiety were considerably reduced in patients with MOH by detoxification and prophylactic treatment. (Bendtsen L et al 2014) Actually it is generally agreed that an abrupt withdrawal is considered the gold standard treatment for MOH. (Evers S et al 2011) After an abrupt withdrawal, the patients' headache episodes gradually decrease in frequency over a 4 to

12-week period. (Katsarava Z et al 2001) The role of detoxification programs and the timing to begin prophylactic therapy with respect to detox program is still highly debated (Diener HC 2012, Olesen J 2012). Although these evidences, the relapse rate in MOH after detox program remains high, especially, based on the duration of MOH, the amount and types of drugs overused, the coexistence of psychiatric disorders, and history of relapse after withdrawal to better target the treatment. Patients with migraine as the primary headache, as well as patients who had overused triptans, had fewer relapses, whereas chronic tension-type headache, overuse of opioids and comorbid psychiatric disorders were associated with increased risk of relapse. Patients with a duration of MOH over 1 year, overuse of opioids or more than 1 type of prescription medications, coexisting psychiatric disorders, or a history of relapse or unsuccessful withdrawal have been shown to have a poorer treatment outcome. (Rossi P et al 2009) Chiang et al. summarized the remission and relapse rates after discontinuation in 22 studies, with follow-ups of 2–60 months. The relapse rate varied between 0% and 45%. Overall the majority of studies showing a relapse rate of 25–35%. (Chiang, C. C. et al 2015)

PATHOPHYSIOLOGY AND CLINICAL BIOMARKERS OF CHRONIC HEADACHE

Despite experts have studied CM and MOH for long time, the biological mechanisms of headache chronification are poorly understood and do not exist therapeutic biomarkers enabling clinicians to act preventive approaches. Recent evidences suggest hypothesis that CM cannot be just a complication of EM but the two process coud be independent, probably expression of separate diseases rather than one. (Burshtein R et al 2015) Several studies have tried to identify clinical risk factors that can worsen the natural history of Migraine with increased risk to evolve into CM. Firstly it has been known that an excessive use of acute medication can cause headache to worsen. Prolonged exposure to pain medication seems to be the main force that drives MOH-related alterations in structural and functional properties of the brain. All pain medications, including migraine-specific (triptans and ergotamines) and nonspecific (analgesics, opioids) drugs, can cause MOH. Certain classes of drugs can result in MOH faster and/or with shorter overuse duration than others, suggesting that the pathophysiological mechanisms in MOH might be at least partly specific to the overused drug. (De Felice M. et al 2010) Several observations suggest that analgesic overuse is the cause of chronic headache, not the consequence and MOH results from an interaction between an excessive use of abortive medication and a susceptible patient. Other factors such as stressful events in a patient's life, but also comorbidities such as arterial hypertension, early physiological or surgical menopause and psychiatric diseases (depression, anxiety and bipolar disorder) have been known to promote MOH (Diener HC et al 2016).

Possible mechanisms of chronicization include alteration of cortical neuronal excitability and central

sensitization involving the trigeminal nociceptive system. Cortical hyperexcitability is the main factor underlying the transformation of episodic migraine into chronic migraine as confirmed by results of electrophysiological and functional imaging studies. Chronic analgesic exposure leads to hyperexcitability in cortical neurons and an increase in cortical spreading depression. Studies using transcranial magnetic stimulation data have demonstrated that occipital cortex is in a state of hyperexcitability in patients with chronic migraine (Aurora, S.K 2009). As well as evidences from neuroimaging studies confimed dysmodulation of the Pain System in the Brainstem: in chronic migraine, there are structural and functional dysfunctions in cerebral areas localized in the brainstem and in the lateral and medial pain pathways. Advances technique of volumetric magnetic resonance imaging (MRI; voxel-based morphometry) showed reduction in the grey and white matter in brain areas of the pain network and increased density of the structures of the brainstem were in patients with chronic migraine (Aurora, S.K 2009, Chiapparini L, 2010). The results of electrophysiologic and functional imaging studies indicate that chronic migraine is associated with abnormalities in the periaqueductal gray matter (PAG) that may be progressive (Aurora, S.K 2009). Several nuclei located in this area, namely PAG, nucleus raphe, and locus coeruleus are known to be pivotal in the modulation of sensory information. Therefore, derangement of this complex network can result in abnormal sensory perception (eg, throbbing headache, photophobia, and phonophobia) as seen during the attacks of migraine. Chronic alteration of this system can lead to an increase in headache frequency (Srikiatkhachorn A, 2010) and is involved in sensory processing and pain (Chudler EH,1995). Similarly, clinical and preclinical studies have consistently demonstrated increased excitability of neurons in the cerebral cortex and trigeminal system after medication overuse, promoting the process of peripheral and central sensitization. These changes are mostly due to changes in serotonergic and dopaminergic pathways including the endocannabinoid system. Increased expression of excitatory cortical 5-HT_{2A} receptors may increase the susceptibility to developing cortical spreading depression, an analog of migraine aura. A reduction of diffuse noxious inhibitory controls may facilitate the process of central sensitization, activate the nociceptive facilitating system, or promote similar molecular mechanisms to those involved in kindling. Low 5-HT levels also increase the expression and release of calcitonin gene-related peptide from the trigeminal ganglion and sensitize trigeminal nociceptors. Thus, derangement of central modulation of the trigeminal system as a result of chronic medication use may increase sensitivity to pain perception and foster or reinforce medication overuse headache. (Srikiatkhachorn A, 2014). Functional imaging studies also support to the hypothesis of alteration in cortical excitability in MOH. Using fludeoxyglucose (F18) position emission tomography, Fumal et al demonstrated several areas of hypometabolism, including the bilateral thalamus, orbitofrontal cortex, anterior cingulate gyrus, insula/ventral striatum, and right inferior parietal lobule, in patients with MOH. (Fumal et al, 2006). The gray matter volume was found to be increased in the PAG,

bilateral thalamus, and ventral striatum, and decreased in the frontal regions, including the orbitofrontal cortex, anterior cingulate cortex, the left and right insula, and the precuneus. (Riederer F, 2012) Because these areas are involved in pain perception, these observed abnormalities suggest an alteration in pain modulatory networks in patients with MOH.

INTER-INDIVIDUAL PAIN AND STRESS VULNERABILITY AND EPIGENETIC MODIFICATIONS

A behavioural model of headache was proposed by Montagna (Montagna P, et al 2008), based on the association between pain, homeostatic imbalance and affective behavior. This theory is strictly linked to current concept of pain vulnerability. Pain is a fundamental experience comprising both sensory and emotional components, which in turn are intensively linked to cognitive factors such as expectations and previous experiences. While acute pain is biologically adaptive by signaling potential or actual tissue damage to evoke protective behavior, in some cases it becomes maladaptive and chronic. The nociception is not always synonymous with pain, which is experienced as a conscious percept, but it might trigger brain responses without necessarily causing the feeling of pain. (Lee MC, et al 2009) During a migraine attack, the nociceptive phase is mediated by the trigeminovascular pathway (Noseda R et al. 2011) but the pain conscious perception of pain occurs through a network known as ‘‘pain matrix”. (Tracey I et al. 2007) Within the pain matrix, the anterior cingulate cortex is involved in the affective (cognitive–evaluative) component of pain, while the insula is situated at the interface of the cognitive, homeostatic, and affective systems of the human brain, providing a link between stimulus-driven processing and brain regions involved in monitoring the internal milieu. (Cortelli P et al. 2013) Hence “Pain matrix” is not standalone entity, but is a substrate continuously subject to modulation dependent upon the interplay of homoeostatic and environmental factors. These complex systems acts simultaneously and influence the individual perception of pain. (Tracey I et al. 2007) Stressful life events, emotions, motivations, and consequent behaviors acting on the pain coming in turn, inducing a vicious circle of constant brain remodeling which influence the neuronal architecture and molecular processes of the brain structures involved in the perception of pain. (Ritter C et al 2009) Several evidences confirmed that pain vulnerability and attitude to chronic pain sensitivity per se are heritable via genetic and epigenetic pathways though changes in DNA expression. There are evidence from twin and population-based studies that genetic risk factors can explain some of the individual differences in pain perception and the etiology of chronic pain conditions. Epigenetic mechanisms would have the potential to link early life events, neuro-inflammation and estrogen activities in the etiology of migraine and in its chronification (Montagna

P 2008) and pharmaco-epigenetics could be implicated in the wide spectra of different drug treatment responses (Montagna P 2008).

In recent years it has become evident that epigenetic processes play an important role in a range of multifactorial diseases (Yan H et al.2016). Epigenetics encompasses changes to the DNA structure without changing the genetic code, resulting in chromatin remodelling and consequently affecting transcriptional potential and expression of genes. The main epigenetic modifications or 'marks' are post-translational modifications of the tails of histone proteins and DNA methylation, collectively comprising the epigenome. Epigenetic marks are tissue specific, can be dynamic but can also be stably inherited through cell divisions. Therefore, epigenetic processes enable cell and developmental stage-specific regulation of gene expression, but also play an important role in programming lasting responses to environmental cues. A main epigenetic mechanism is DNA methylation, the covalent addition of a methyl group to the 5th carbon of cytosine residues, which is typically associated with gene silencing. Due to the ability of DNA methylation changes to shift a biological system from one stable state to another, their implication in headache chronification is of particular interest. (Eising E et al. 2013) It has been hypothesized that frequent headache attacks may lower the threshold for subsequent headache attacks through epigenetic mechanisms resulting in a feed-for-ward loop. (Eising E et al. 2013) Similarly, psychological acute and chronic stress and female sex hormones, which have been implicated in migraine genesis, are known to exert their physiological effects partly through epigenetic mechanisms (Griffiths BB et al 2014, Labruijere S et al 2014). The effects of female hormones are predominantly transmitted via nuclear receptors that adjust epigenetic programming of their target genes (Green CD et al 2011). Sex hormones (estrogen, progesterone or testosterone) alter brain function. Estrogens can modulate neuronal activity electrophysiologically and morphologically, potentially through estrogen receptors that are widely distributed throughout the brain, with high concentrations in the hypothalamus (Laflamme et al., 1998). Taken together, gonadal hormonal feedback to the hypothalamus and other brain regions (McEwen et al., 2012) has significant impact on behaviors or neurological adaptations through specific neurotransmitters (Scharfman and MacLusky, 2008). One such system is the serotoninergic system (Hamel, 2007). Serotonin-producing neurons are found in the mid-and hindbrain regions, and project to forebrain, limbic, diencephalic (rostral 5-HT nuclei), and the spinal cord (caudal 5-HT nuclei) (Bethea et al., 2002), all of which contain both estrogen and progesterone receptors. Thus, aside from changes that may influence migraine circuits per se, estrogen–5-HT interactions may influence behaviors including mood playing a role in chronicization. About sensitization effect, this is the result of vasodilation and the release of pro-inflammatory cytokines. Preliminary results showed prolonged inflammatory pain was shown to promote pain sensitivity by causing histone hypoacetylation at the Gad2 gene, which is involved

in GABAergic signaling (Zhang Z et al 2011). Therefore, migraine-related pain may cause sensitization of certain pain pathways via inflammation-induced changes in epigenetic gene regulation. A role for epigenetics has been suggested firstly for depression and other neuropsychiatric disorders, many of them comorbid with MOH (such as panic, obsessive-compulsive disorders, generalized anxiety, phobia). (Peña CJ et al 2014, Radat F et al 2005) Interestingly, depression also shares modulatory factors with migraine, such as female hormones and chronic stress, the latter of which is an established risk factor for depression. The main proof for a role of epigenetic mechanisms in depression is evident from animal models for major depressive disorder that show large changes in epigenetic programming of stress related genes (for example, BDNF) that could be reversed by antidepressant treatment. (Wilkinson MB et al 2009). Finally drug addiction can be viewed as maladaptive neural plasticity that occurs in vulnerable individuals in response to repeated exposure to a drug of abuse. Although there are no studies in MOH, some evidences showed that this vulnerability is partly determined non-genetic factors which include environmental exposures as well as stochastic events during development which act through epigenetic mechanisms. (Nestler EJ. 2014)

In recent years the technologies for studying nucleic acids have literally exploded. The reduction of nucleic acids sequencing costs and the availability of cost effective microarray solutions for the analysis of DNA methylation has favoured the implementation of epigenomic studies, in particular DNA methylation microarray has been thoroughly used providing new insight regarding the variability and the role of such epigenetic agent. DNA methylation, miRNA and histone modifications have proven to be a potential source of powerful and robust biomarkers. Taken together both the new genetic and epigenetic omic approaches have the potential to provide new molecular insight in the aetiology of migraine chronicization, patient stratification, and therapy. (Garagnani P et al 2015). Recently the first genome-wide study of DNA methylation in headache chronification was published. Several potentially implicated loci and processes were identified but in the combined meta-analysis the strongest associated CpG sites were related to SH2D5 and NPTX2, two brain-expressed genes involved in the regulation of synaptic plasticity. Both proteins are highly expressed in the adult human brain. H2D5 gene, encodes the SH2 Domain Containing 5 protein which regulates synaptic plasticity through the control of Rac-GTP levels. The second strongest associated CpG site was 76kb downstream from the nearest gene NPTX2, which encodes the Neuronal Pentraxin II protein an inhibitor of excitatory synapses, through binding and clustering of glutamatergic AMPA receptors. (Winsvold BS et al 2017)

AIM OF THE STUDY

Aim of the study was to identify changes in DNA methylation associated with headache chronification comparing healthy controls without migraine, episodic migraineurs and patients suffering from chronic migraine with medication overuse headache. For all selected subjects, genome-wide DNA methylation levels were characterized at baseline and longitudinally during follow-up.

MATERIALS AND METHODS

STANDARD PROTOCOL APPROVALS AND PATIENT CONSENTS

The study was conducted in agreement with principles of good clinical practice and the study protocol was approved by the Local Ethic Committee of the local health service of Bologna, Italy. All patients gave their written informed consent to study participation.

PARTICIPANTS

Patients from the Headache Centre of IRCCS of Neurological Sciences of Bologna, Italy were recruited consecutively. We enrolled patients into three groups:

- Group A: Patients with chronic migraine and medication overuse headache (MOH): Patients who met criteria for MOH as defined by the International Headache Society 3rd edition (beta version) : Headache present on 15 or more days/month in a patient with a pre-existing headache disorder; Regular overuse for more than 3 months of 1 or more drugs that can be taken for acute and/or symptomatic treatment of headache:

- Ergotamine, triptans, or combination analgesics on 10 or more days/month
- Simple analgesics on 15 or more days/month
- Any combination of acute/symptomatic drugs on 10 or more days/ month without overuse of any single class alone

Exclusion criteria was opioids overuse.

- Group B: Episodic Migraineurs: Patients suffered from episodic migraine with or without aura according to International Headache Society 3rd edition (beta version): we selected patients with low frequency of attacks (maximum 6 attacks/month). An exclusion criterion was presence of prophylaxis therapy at baseline.

- Group C: Healthy controls who do no suffer form headache: Subjects were screened for headache disorder present or not according to the HARDSHIP questionnaire (Headache-Attributed Restriction, Disability, Social Handicap and Impaired Participation. HARDSHIP is a modular instrument incorporating demographic enquiry, diagnostic questions based on ICHD-3 beta criteria, and enquiries into each of the following as components of headache-attributed burden (Steiner TJ et al 2014). From

Hardship questionnaire we used questions number 12: “Have you ever had a headache in your lifetime?” and number 13 “Have you had a headache during the last 12 months?”. Were enrolled only subjects who responded “no” to question 13.

Overall, all patients were eligible if they were ≥18 years of age, were able to give verbal and written informed consent. Exclusion criteria included pregnancy and breast-feeding, secondary headaches, history of any types of addiction (such as alcohol, sedative, cannabis and psychoactive substances), as well as any serious ongoing physical or psychiatric illness. Secondary headaches were ruled out by clinical examination, laboratory testing and neuroimaging studies, when indicated.

STUDY DESIGN

This was a pilot, longitudinal, prospective, observational, study. An exploratory GWAS longitudinal study of DNA methylation was performed for understanding Epigenetic gene regulatory abnormalities in MOH, Episodic migraineurs (EM) and Healthy Controls (HC) with the collaboration of Department of Experimental, Diagnostic and Specialty Medicine - Bologna University.

Fig. 1 illustrates the study design. In Group A, MOH patients, unresponsive to prophylaxis lasted three-month period based on clinical history and previous headache diary were enrolled at baseline (T0). At T0 patients were educated about the MOH diagnosis and received advice to stop overused drugs and were asked to stop prophylaxis therapy; Education consisted in a brief explanation about the nature of the disease and about the consequences of too frequent intake of medication to treat headache attacks. At T1, patients still fulfilling the diagnosis of MOH underwent the inpatient 5-day withdrawal program (T1). During pharmacological withdrawal program patients received paracetamol 2000 mg at 8.00 a.m., 1000 mg at 2.00 p.m., and 1000 mg at 8.00 p.m. Allowed rescue therapies were: metoclopramide 10 mg i.m. and lorazepam 1 mg or 2.5 mg cap. T2 and T3 were follow-up visits. Overall visits occurred at baseline (T0), 3 months after baseline (T1), then at 2 month (T2) and 6 months (T3) after inpatient withdrawal program (Fig. 1). About group B and C, patients were evaluated at baseline (T0) when they were enrolled and 6 months later (T1). Fig. 1. A clinical diary in which patients recorded all headaches attacks and the drugs taken for headache during all the study period was given at the preliminary visit and checked at every follow-up visit. Patients recorded the number of days of headache attacks, the headache intensity (classified in a range from 1 = mild to 10 = severe), the number and type of painkiller used and the number of days treated . Healthy survey, Depressive and anxious symptoms (SF-12, Beck Depression scale and Beck anxiety scale) (Ware J Jr, et al 1996, Beck A 1961, Beck A. T 1988) and degree of disability (Migraine Disability Assessment Score, MIDAS, D'Amico D et al 2001) were evaluated at T0,

T1,T2,T3 in MOH, and T0 and T1 in EM and HC . Patients were interviewed and examined by neurologists expert in headaches.

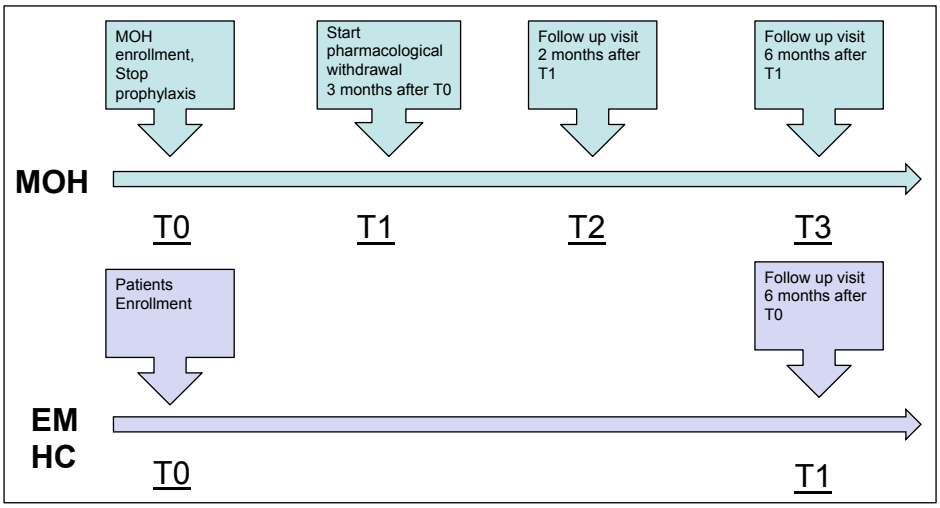

Figure 1: Study design. MOH = medication overuse headache; EM= Episodic migraineurs; HC= Healthy controls

GENOME-WIDE QUANTIFICATION OF DNA METHYLATION

Genomic DNA was extracted from whole blood using the QIAamp DNA Mini Kit (QIAGEN) DNA was bisulfite-converted using the EZ DNA Methylation-Gold Kit (Zymo Research) and analysed using the Infinium HumanMethylationEPIC Bead- Chip (Illumina) following the manufacturer's instructions. Signal intensity IDAT files were extracted using the minfi Bioconductor package. 4773 probes were identified as failed positions according to their detection p-value (>0.05) and were hence discarded. After removing the 4773 probes, Quality Control plot reported 5 samples with bad quality which were discared. Therefore statistical analysis was carried on 22 MOH samples at T0, 19 at T1, 18 at T2, 18 at T3, 18 EM at T0 and 17 at T1, 13 HC at T0 and 11 at T1. Stratified quantile normalization was performed on beta values using the function preprocessQuantile implemented in the R packege minfi.

DNA methylation differences between the different groups of samples were assessed using the analytical pipeline described in (Bacalini et al.2015) Briefly, we firstly classified CpG probes according to their genomic localization and density in the region. We identified 41356 genomic regions mapping in CpG islands, shores or shelves and including at least 3 probes, for a total of 254568 probes. To identify statistically significantly differentially methylated regions (DMRs) (regions in which multiple adjacent CpG probes differ in the comparison under investigation) we applied a multivariate analysis of variance (MANOVA) on sliding windows including three adjacent CpG sites.

STATISTICS

Quantitative variables were expressed as the mean ± standard deviation (SD) while categorical variables were described by their absolute and/or relative frequencies. We compared categorical variables using Chi square test. Kruskal-Wallis Tests was performed to compare continuous variables with an asymmetrical (non-normal) distribution. Significance level was set at $p \leq 0.05$. Data analysis was performed with SPSS® version 22.

RESULTS

CLINICAL RESULTS

A total of 25 patients with MOH were enrolled at baseline (T0), 21 underwent protocol of withdrawal (T1), of these 19 and 18 were followed to visit T2 and T3 respectively. In the group B, EM, 20 patients were enrolled and all subjects completed the study to T1. With regard to HC, 13 subjects were enrolled at T0, and 11 completed study at T1. (Fig. 2) Clinical and Demographic baseline characteristics for the MOH, EM and HC patients who completed the study are presented in Table 1 and 2. Of the 25 MOH patients enrolled, 21(84%) were females, 18 (90%) in EM and 8 (62%) in HC group; mean age ± standard deviation (SD) was 50 ± 9 years, 44 ± 10, 54 ± 12 ($p = 0.008$) in MOH, EM, HC respectively. All headache participants suffered from migraine without aura at onset, with a mean age at onset of 16 ± 6 years in MOH and 17 ± 8 in EM. Chronification age was 36 ± 9 years with duration of MOH of 14 ± 9 years (Table 1). In MOH group overused medications included triptans (48%), non steroidal anti-inflammatory drugs (NSAID, 28%), paracetamol (4%) and combination analgesics (20%). Overused opiates ere excluded. In EM group, painkiller used included triptans (40%), NSAID (45%), paracetamol (5%) and combination analgesics (10%). No statistical difference was found in acute medication used between the two groups. In the MOH group 15 patients (60%) received preventive monotherapy while 10 (15 %) received polytherapy (a combination of 2 drugs). Main prophylactic medications included anti-epileptics (40%), beta-blockers (28%), 25 % antidepressants and 12% calcium channel blockers. The three groups did not differ in associated comorbidities with exception in association with depression and anxiety that were more prevalent in MOH patients: 16 (64%) in MOH vs EM 4 (20%) and HC 1 (7%) ($p < 0.05$). (Table 2). Table 3 summarizes comparison in number of days of headache per month, days treated with acute medication and total painkiller used monthly as well as intensity of pain in MOH and EM at baseline T0. Data confirmed days with headache/month were significant higher in MOH both at T0 (28 ± 3) vs EM (5 ± 1 $p < 0.05$). Total number of painkiller used substantiate a value significantly higher in MOH 48 ± 20 at T0 when compared to EM (5 ± 1, $p < 0.05$) while no difference was observed in intensity of pain between groups. About the evaluation of disability, the average MIDAS score was significantly higher in MOH vs EM (96 vs 27, $p = 0.002$). With regard to average score of BAI and BDI, results showed MOH patients fulfilled the criteria for depression and anxiety disorder with statistical significance ($p = 0.002$). At baseline, physical status evaluated through SF 12 resulted significantly worse in MOH group compared vs EM and HC ($p = 0.001$), while no differences were found in Mental status. (Table 4)

With regard to treatment in MOH patients, of 25 subjects, 21 completed the detoxification protocol. 19 were followed to visit T2 and 18 to visit T3. Patients who dropped out become unable to reach the headache Center for follow-up visits due to personal employment or personal reasons. In total, headache days reduced from 28 ± 3 to 15 ± 9 ($p = 0.002$) with a significant reduction in both number of days treated with acute medication and total numbers of painkillers/month (Table 5). No difference was observed in intensity of pain. A significant reduction in disability was registered by MIDAS (mean values at T0 96 vs 61, $p < 0.05$) and depression score at BAI (21 ± 7 vs 15 ± 5, $p < 0.05$). No statistical difference was observed in anxiety score and evaluation of Healthy mental and Physical status. (Table 5) Overall at T3, 6 month later detox program, 4 patients remained chronic with medication overuse (22%), while 14 (78%) were cured from overuse. Among them 11 (61%) still fulfilled a diagnosis of chronic migraine and 3 (16%) reversed to an EM.

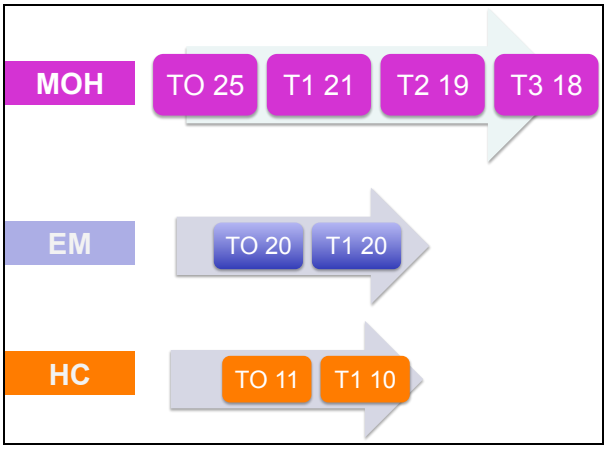

Figure 2: Flow chart of patients included in the study

	MOH	EM	HC	p
Gender (F, %)	21 (84%)	18 (90%)	8 (62%)	0.03
Age (mean ± sd)	50 ± 9	44 ± 10	54 ± 12	0.008
Age onset Headache (mean ± sd)	16 ± 6	17 ± 8		ns
Age of Chronicization (mean ± sd)	36 ± 9			
Duration of MOH (mean ± sd)	14 ± 9			
Acute medication used				
Triptans	12 (48%)	8 (40%)		ns
NSAID	7 (28%)	9 (45%)		ns
Paracetamol	5 (20%)	1 (5%)		ns
Combination analgesic	1 (4%)	2 (10%)		ns

Table 1: Demographic and baseline clinical characteristics of the study sample. NSAID = non steroidal anti-inflammatory drugs.

	MOH	EM	HC	p
Comorbidities				
Autoimmune disease	6 (20%)	3 (15%)	2 (15%)	ns
Neoplastic disease	8 (2%)	2 (10%)	2 (15%)	ns
Anxiety and depression	16 (64%)	4 (20%)	1 (7%)	< 0.05
Insomnia	8 (36%)	2 (10%)	1 (7%)	ns
Hypertension	2 (8%)	0	2 (15%)	ns
Other disease	2 (8%)	0	2 (15%)	ns

Table 2: Clinical Comorbidities characteristics of the study sample.

	MOH	EM	p
T0 days with headache last month	28 ± 3	5 ± 1	0.003
T0 days treated with painkillers/last month	27 ± 4	5 ± 1	0.001
T0 Total number of painkiller/month	48 ± 20	5 ± 1	0.001
T0 intensity of pain	7 ± 2	6 ± 1	0.021

Table 3: Baseline characteristics of headache in MOH and EM at T0

	MOH	EM	HC	p
MIDAS (Migraine disability assessment score)	96 (46 -180)	27 (15 – 52)		0.002
BDI (Beck depression score)	21 ± 7	7 ± 5	4 ± 5	0.002
BAI (Beck Anxiety score)	10 ± 8	4 ± 5	2 ± 5	0.002
SF 12 Health survey (Physical status)	37 ± 10*	46 ± 10	55 ± 5	0.001
SF 12 Health survey (Mental status)	41 ± 11	47 ± 11	53 ± 6	ns

Table 4: Disability, anxiety and depression baseline clinical characteristics of the study sample.

	T0 (25)	T1(20)	T2 (19)	T3 (18)	p
Days with headache last month	28 ± 3	27 ± 5	19 ± 9	15 ± 9	0.002
Days treated with painkillers/last month	27 ± 4	26 ± 5	15 ± 10	15 ± 9	0.001
Total number of painkiller/month	48 ± 20	42 ± 18	18 ± 18	14 ± 13	0.001
Intensity of pain	7 ± 2	7 ± 1	6 ± 2	6 ± 2	ns
MIDAS (mean)	96 (46 -180)			61 (30 – 115)	< 0.05
BDI (Beck depression score)	21 ± 7			15 ± 5	< 0.05
BAI (Beck Anxiety score)	10 ± 8			8 ± 7	ns
SF 12 Health survey (Physical status)	37 ± 10			40 ± 8	ns
SF 12 Health survey (Mental status)	41 ± 11			43 ± 10	ns

Table 5: Clinical Characteristics of MOH patients at baseline and during follow-up after detox program

RESULTS OF GENOME-WIDE QUANTIFICATION OF DNA METHYLATION

We used the Infinium MethylationEPIC BeadChip to evaluate whole blood genome-wide DNA methylation profiles of the subjects included in the study.

First, we conducted principal component analysis to identify major traits of variation between samples. No clear separation between the groups was observed neither along the first component or the second component (Figure 3).

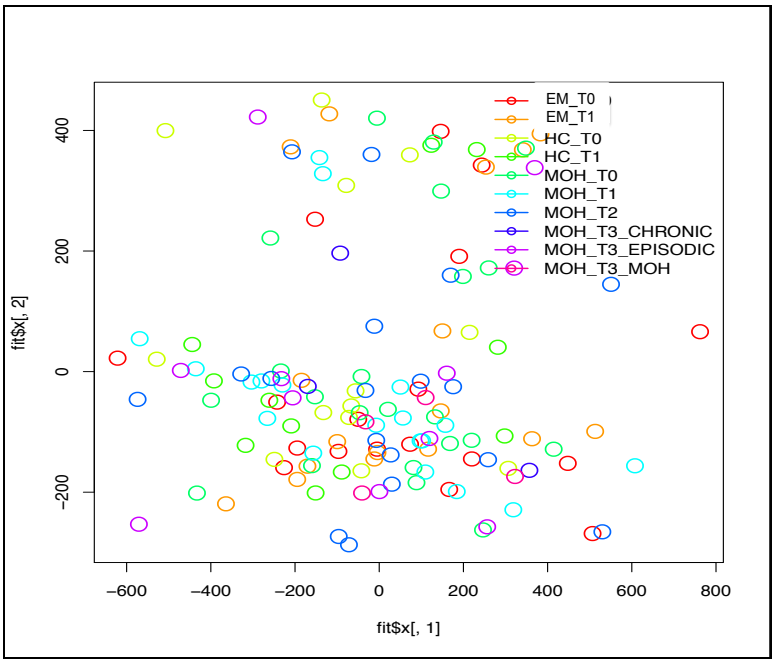

Figure 3: PCA on DNA methylation values (beta values) from the samples included in the study.

To identify subtler epigenetic differences underling the effect of the pathology and the treatment, we then compared the three groups focusing on the following comparisons:

1. MOH T0 vs HC T0
2. MOH T0 vs MOH T1
3. MOH TO vs MOH T3

In these comparisons, we focused on the identification of differentially methylated regions (DMRs), that is genomic traits including multiple adjacent CpG sites that are concordantly affected by the phenotype under investigation. Compared to the analysis of single CpG sites, this approach has been demonstrated to be more effective in identifying biologically relevant epigenetic patterns. The discovery of the DMRs was performed using the analytical pipeline described in Bacalini et al. 2015, using as input only the CpG sites located in CpG islands and surrounding regions (shores and shelves) associated to a gene.

1. T0 MOH vs HC

First, we compared 22 MOH samples at T0 vs 13 HC at T0. No DMR reached statistical significance after Benjamini–Hochberg correction for multiple test. When considering a nominal threshold of 0.01, we identified 721 DMRs. While most of the DMRs showed very small DNA methylation differences between the two groups, 29 had a delta of at least 0.05 in at least one CpG site (Table 6). Of these, 2 DMRs mapping in COMTD1 and ACSF3 genes had at least 2 adjacent CpG sites with a DNA

methylation difference between MOH T0 and HC T0 of at least 0.05 (Figure 4).

To evaluate the capability of the 29 DMRs to distinguish HC T0 and MOH T0 samples, we performed a multidimensional scaling on the DNA methylation values of the most significant CpG site within each DMR. As shown in Figure 5, the MOH DNA methylation signature is not able to completely distinguish the disease from healthy controls, although a trend towards a separation is observed.

Finally, we considered the DNA methylation of the 29 DMRs in EM samples at T0. Interestingly, for many of the DMRs, EM displayed DNA methylation values intermediate between HC and MOH (see Figure 6 for an example). Accordingly, in the MDS EM tended to cluster between HC and MOH (Figure 7).

2. MOH T0 vs MOH T1 and vs MOH T3

   We then compared 22 MOH samples at T0 and 19 samples MOH at T1. Also in this case no DMR reached statistical significance after Benjamini–Hochberg correction for multiple test. When considering a nominal threshold of 0.01, we identified 783 DMRs, but only 1 of them, located in the island of the RHOV gene, showed a DNA methylation difference of at least 0.05 between the two groups (Figure 8).

3. We compared MOH samples at T0 and at T3, excluding those subjects (4) that at T0 were still MOH at T3. As in the previous analysis, no DMR reached statistical significance after Benjamini–Hochberg correction, and of the 461 DMRs with a nominal p-value lower than 0.01 only 1, mapping in the island of NBL1/MINOS1 gene, had a CpG site with a DNA methylation difference of at least 0.05 between the two groups (Figure 9).

   Nor RHOV nor NBL1 genes show significant differences between MOH at T0 and HC at T0 (Figures 8 and 9). For the significant CpG sites in the genes RHOV and NBL1 we also evaluated the longitudinal changes in DNA methylation at T0, T1 and T3 in the subjects that at T3 changed to chronic or episodic (red lines) and in those that at T3 were still MOH (black lines). However, no clear trend towards hypermethylation or hypomethylation was observed (Figures 10 and 11).

Finally, we assessed if the 29 DMRs selected in the comparison MOH at T0 vs HC at T0 were included in the list of DMRs in the comparisons MOH at T0 vs MOH at T1 and MOH at T0 vs MOH at T3. Only 1 DMR, the one mapping in the island of the gene IER3, was hypermethylated in MOH at T1 compared to MOH at T1 (p-value=0.0038, DNA methylation difference=0.04) (Figure 12).

DMR	GeneName	p-value	MOH respect to HC	Gene function
chr8:10586613-10586886*Island	SOX7	8.06E-05	HYPO	Family of transcription factors involved in the regulation of embryonic development
chr4:111539906-111540127*S_Shelf	PITX2	0.000189786	HYPER	Acts as a transcriptional regulator involved in basal and hormone-regulated activity of prolactin.
chr6:127664192-127664755*Island	ECHDC1	0.000724248	HYPER	Ethylmalonyl-CoA Decarboxylase 1 is a Protein Coding gene. Among its related pathways are Propanoate metabolism and Fatty Acid Biosynthesis
chr10:76993892-76995953*S_Shore	COMTD1	0.000903274	HYPER	Catechol-O-methyltransferase is important in the metabolism of catecholamines, including the neurotransmitters dopamine, epinephrine, and norepinephrine.
chr2:135475698-135476993*Island	TMEM163	0.001022101	HYPER	Transmembrane Protein 163) is a Protein Coding gene. May bind zinc and other divalent cations and recruit them to vesicular organelles.
chr13:114774812-114775134*Island	RASA3	0.001214778	HYPER	Encodes a protein that binds inositol 1,3,4,5-tetrakisphosphate and stimulates the GTPase activity of Ras p21
chr5:131607018-131607889*Island	PDLIM4	0.001412643	HYPO	Encodes a protein which may be involved in bone development.
chr19:18543828-18549161*Island	ISYNA1	0.001886107	HYPO	Encoded protein plays a critical role in the myo-inositol biosynthesis pathway. Diseases associated Whipple Disease.
chr20:23330636-23332046*Island	NXT1	0.001956097	HYPO	The protein encoded by this gene is located in the nuclear envelope. It has protein similarity to nuclear transport factor 2. Diseases associated with NXT1 include St. Louis Encephalitis
chr19:44645494-44646069*N_Shore	ZNF234	0.002092452	HYPER	ZNF234 (Zinc Finger Protein 234) is a Protein Coding gene. Among its related pathways are nucleic acid binding and transcription factor activity, sequence-specific DNA binding.
chr12:95941906-95942979*Island	USP44	0.002234688	HYPO	Encodes a protease that functions as a deubiquitinating enzyme. It is thought to help regulate the spindle assembly checkpoint by preventing early anaphase onset.
chr6:30071225-30071428*S_Shelf	TRIM31;TRIM31-AS1	0.002395454	HYPER	Encodes a protein that functions as an E3 ubiquitin-protein ligase. This gene shows altered expression in certain tumors and may be a negative regulator of cell growth.
chr12:72665683-72667551*S_Shore	TRHDE;TRHDE-AS1	0.002973925	HYPER	Encodes a member of the peptidase M1 family, an extracellular peptidase that specifically cleaves and inactivates the neuropeptide thyrotropin-releasing hormone
chr1:231003631-231004655*Island	C1orf198	0.003147764	HYPO	Chromosome 1 Open Reading Frame 198) is a Protein Coding gene
chr3:62304514-62304780*Island	PTPRG-AS1;C3orf14	0.003181439	HYPO	Encodes a member of the protein tyrosine phosphatase (PTP) family. PTPs are known to be signaling molecules that regulate a variety of cellular processes including cell growth, differentiation, mitotic cycle, and oncogenic transformation.
chr14:51410614-51411464*S_Shore	PYGL	0.003407722	HYPO	Encodes a homodimeric protein that catalyses the cleavage of alpha-1,4-glucosidic bonds to release

				glucose-1-phosphate from liver glycogen stores. Humans have three glycogen phosphorylase genes that encode distinct isozymes that are primarily expressed in liver, brain and muscle, respectively.
chr2:201450526-201451027*Island	AOX1	0.004314493	HYPO	Aldehyde oxidase produces hydrogen peroxide and, under certain conditions, can catalyze the formation of superoxide.
chr16:11348541-11350803*Island	SOCS1	0.004627827	HYPO	Encodes a member of the STAT-induced STAT inhibitor (SSI). SSI family members are cytokine-inducible negative regulators of cytokine signaling.
chr12:132603367-132603646*N_Shelf	EP400NL	0.00496126	HYPER	EP400NL (EP400 N-Terminal Like) is a Pseudogene
chr16:89167763-89167991*S_Shore	ACSF3	0.005537377	HYPO	Encodes a member of the acyl-CoA synthetase family of enzymes that activate fatty acids by catalyzing the formation of a thioester linkage between fatty acids and coenzyme A.
chr4:57521621-57522703*S_Shore	HOPX	0.005766163	HYPER	Encodes a homeodomain protein that lacks certain conserved residues required for DNA binding. It was reported that choriocarcinoma cell lines.
chr7:99063423-99063624*S_Shore	ATP5J2;ATP5J2-PTCD1	0.006142224	HYPER	Encodes ATP Synthase, H+ Transporting, Mitochondrial Fo Complex Subunit F2)
chr6:30710307-30712440*Island	IER3	0.006796733	HYPER	Involeved in the protection of cells from Fas- or tumor necrosis factor type alpha-induced apoptosis.
chr17:43393891-43394938*Island	MAP3K14	0.006812384	HYPO	Encodes mitogen-activated protein kinase kinase 14, which is a serine/threonine protein-kinase. It participates in an NF-kappaB-inducing signalling cascade common to receptors of the tumour-necrosis/nerve-growth factor (TNF/NGF) family and to the interleukin-1 type-I receptor.
chr11:45686160-45687495*Island	CHST1	0.007419375	HYPO	Encodes a member of the keratin sulfotransferase family of proteins. The encoded enzyme catalyzes the sulfation of the proteoglycan keratin.
chr12:110433797-110434205*Island	GIT2	0.008816633	HYPO	Encodes a member of the GIT protein family, which interact with G protein-coupled receptor kinases and possess ADP-ribosylation factor (ARF) GTPase-activating protein (GAP) activity. GIT proteins traffic between cytoplasmic complexes, focal adhesions, and the cell periphery,
chr17:1952919-1962328*Island	HIC1;MIR132;MIR212	0.009069263	HYPO	This gene functions as a growth regulatory and tumor repressor gene. Hypermethylation or deletion of the region of this gene have been associated with tumors and the contiguous-gene syndrome, Miller-Dieker syndrome
chr2:25354283-25354777*Island	EFR3B	0.00939227	HYPO	EFR3B probably acts as the membrane-anchoring component.
chr10:77155128-77169600*Island	ZNF503-AS2	0.009643908	HYPO	Is an RNA Gene.

Table 6: DMRs sites which showed significant methylation differences (delta of at least 0.05) in at least one CpG site comparing 22 MOH samples at T0 vs 13 HC at T0. Bold refers to most relevant genes.

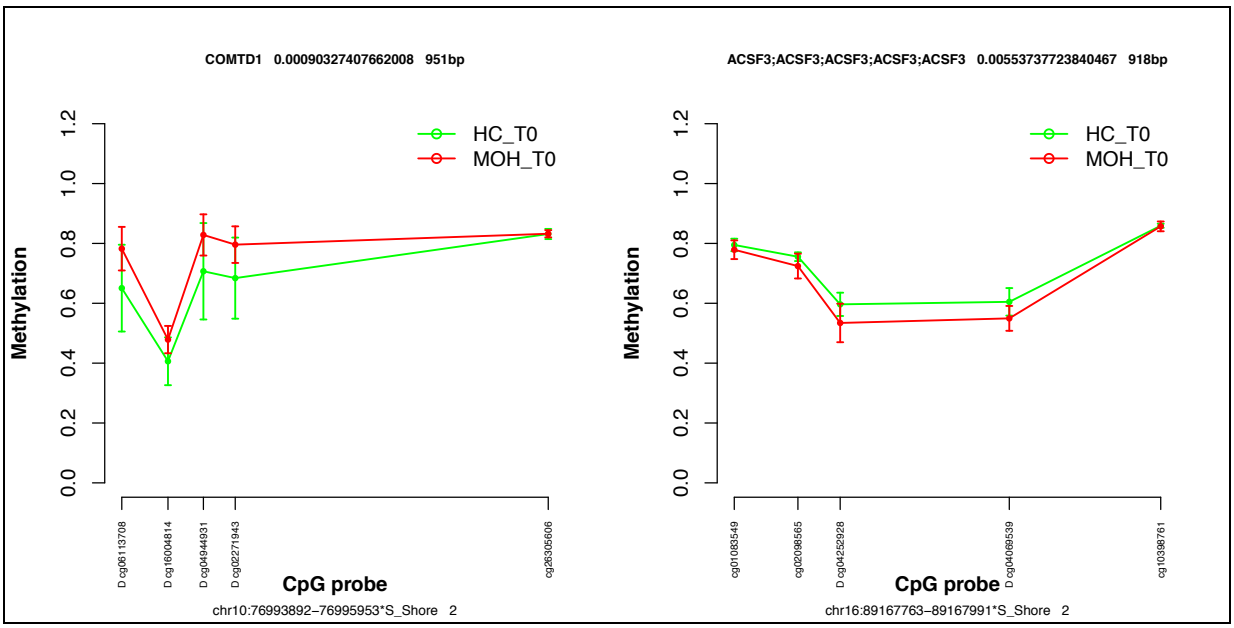

Figure 4; DNA methylation profile of COMTD1 and ACSF3 DMRs in HC and MOH at T0.

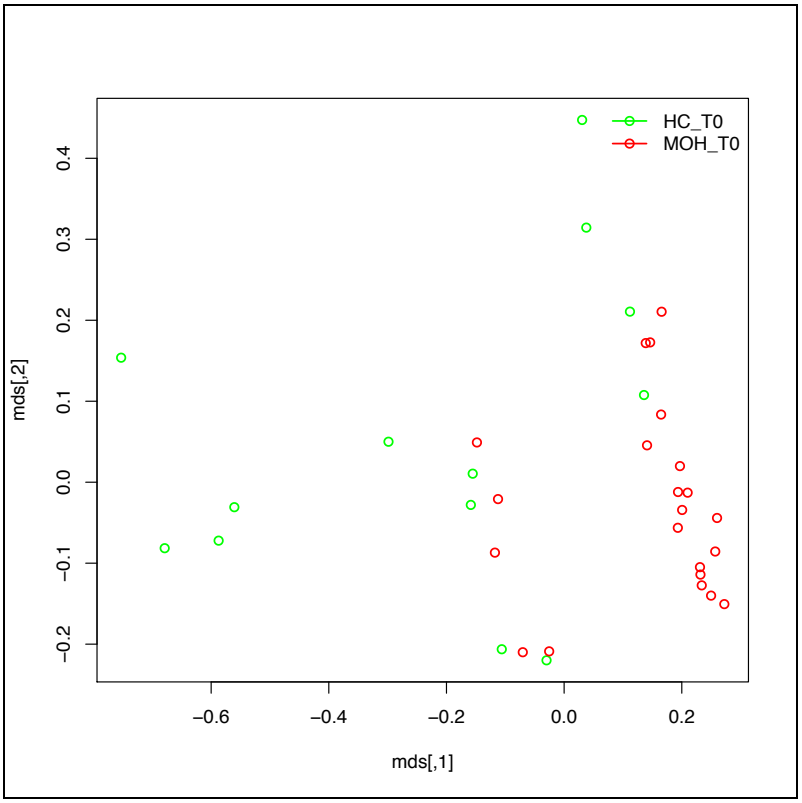

Figure 5: MDS of HC and MOH at T0 samples calculated on DNA methylation values of the selected 29 DMRs between HC and MOH at T0.

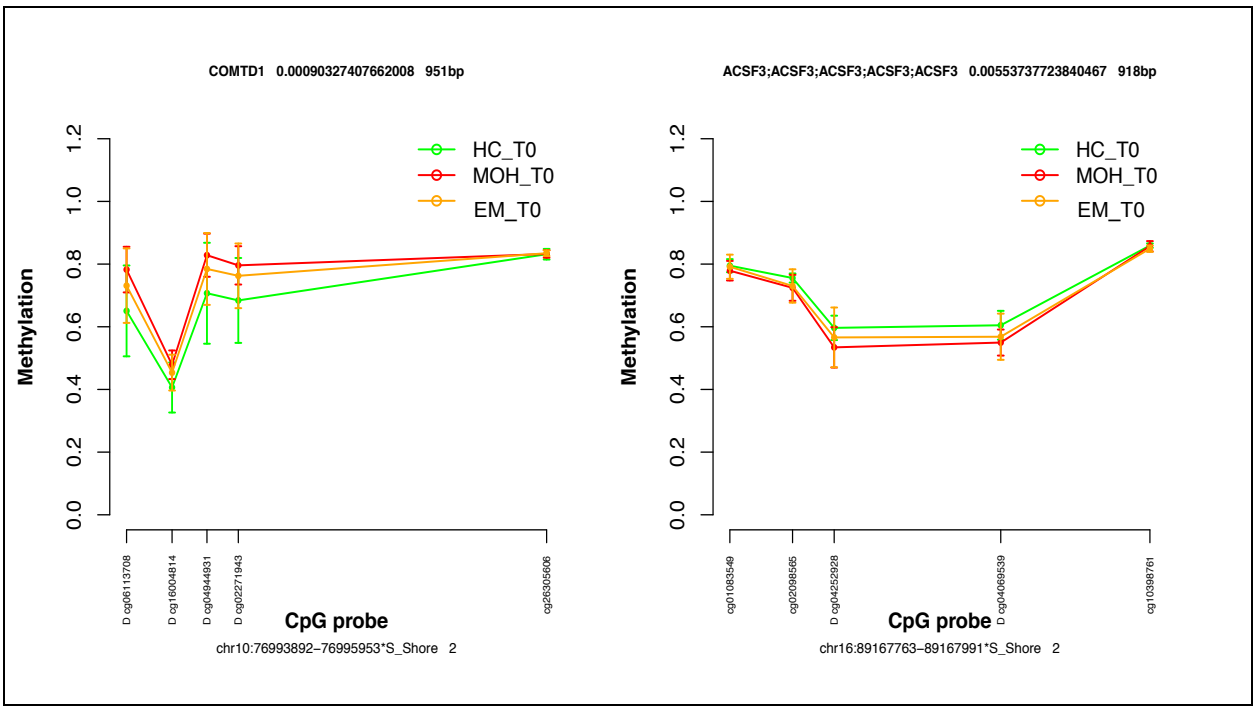

Figure 6: DNA methylation profile of COMTD1 and ACSF3 DMRs in HC, MOH and EM at T0.

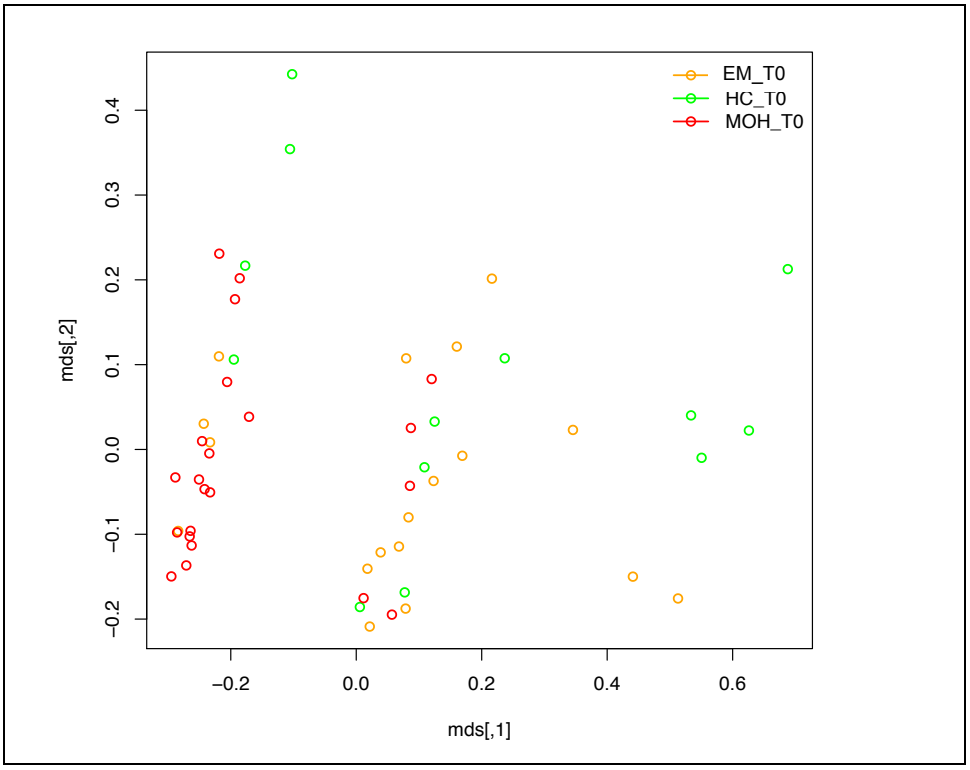

Figure 7: MDS of HC, MOH and EM at T0 samples calculated on DNA methylation values of the selected 29 DMRs between HC and MOH at T0.

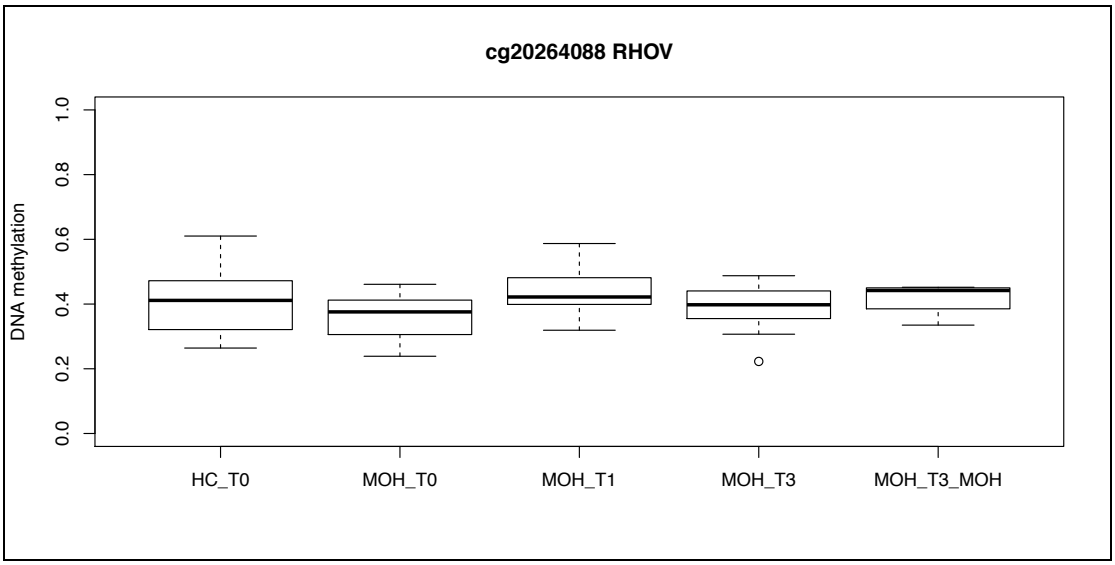

Figure 8: DNA methylation of cg20264088 in the Island chr15:41165847-41166571 of RHOV gene

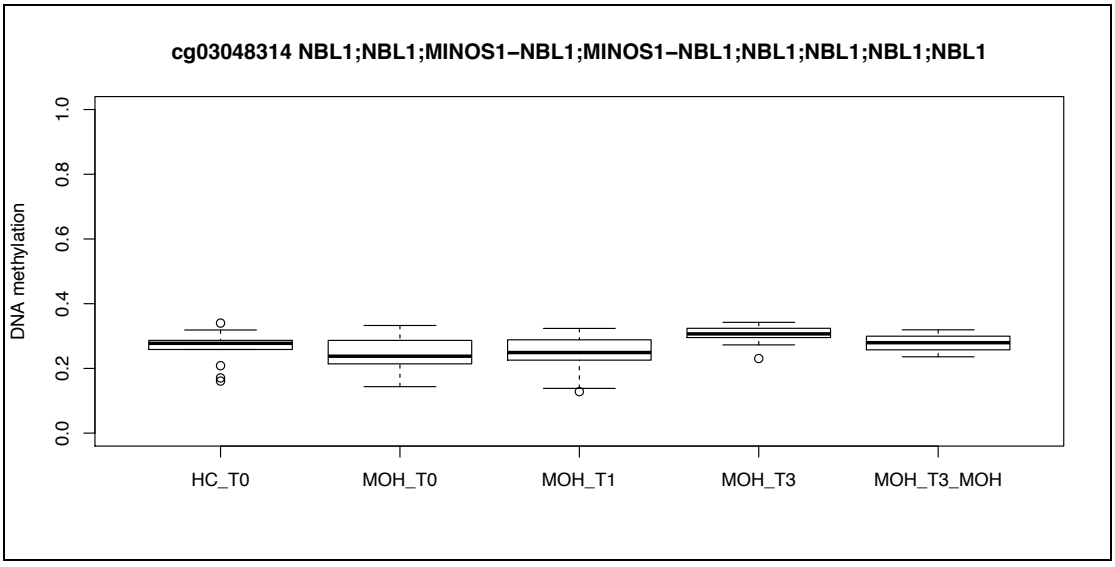

Figure 9: DNA methylation of cg03048314 in the Island chr1:19970255-19971923 of NBL1 gene

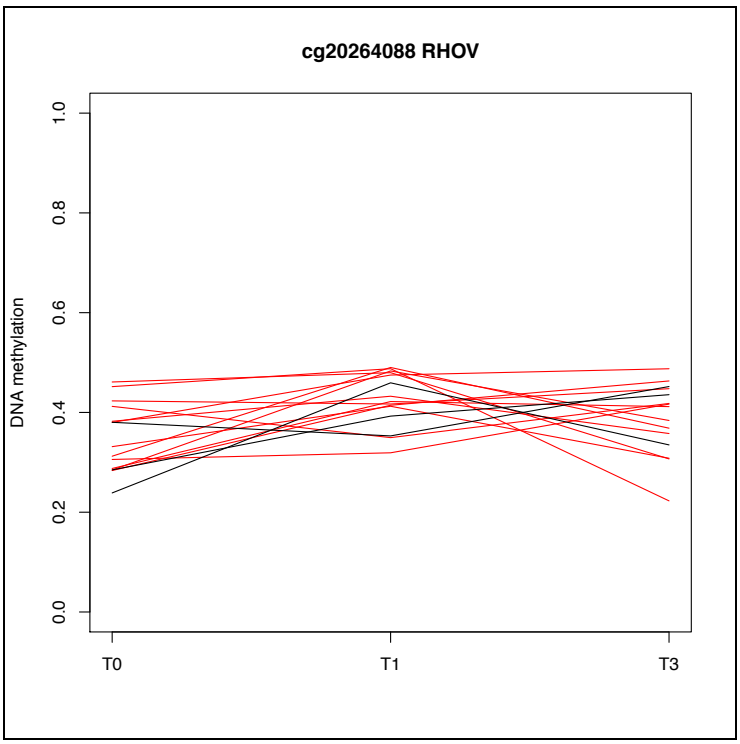

Figure 10: DNA methylation profile of cg20264088 in the Island chr15:41165847-41166571 of RHOV gene, assessed longitudinally at T0, T1 and T3.

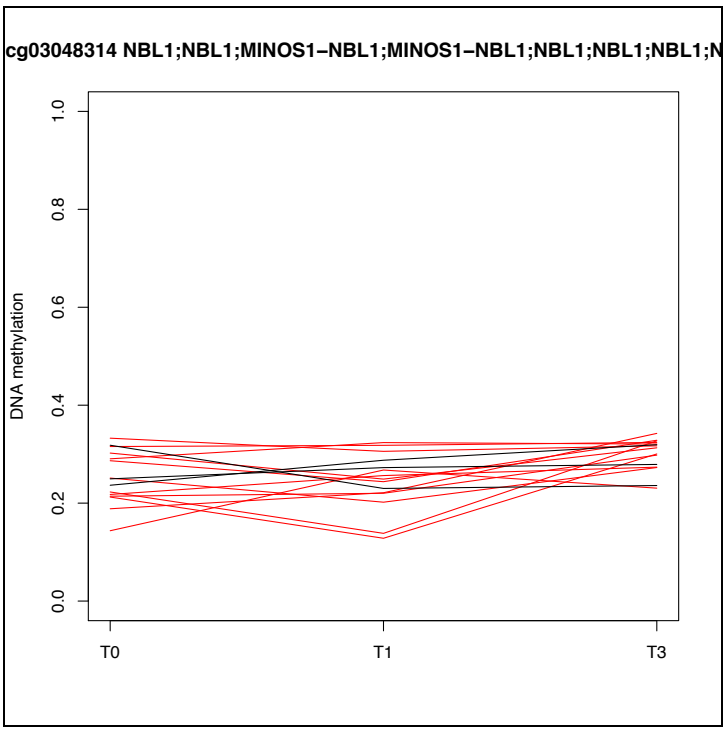

Figure 11: DNA methylation profile of cg03048314 in the Island chr1:19970255-19971923 of NBL1 gene, assessed longitudinally at T0, T1 and T3.

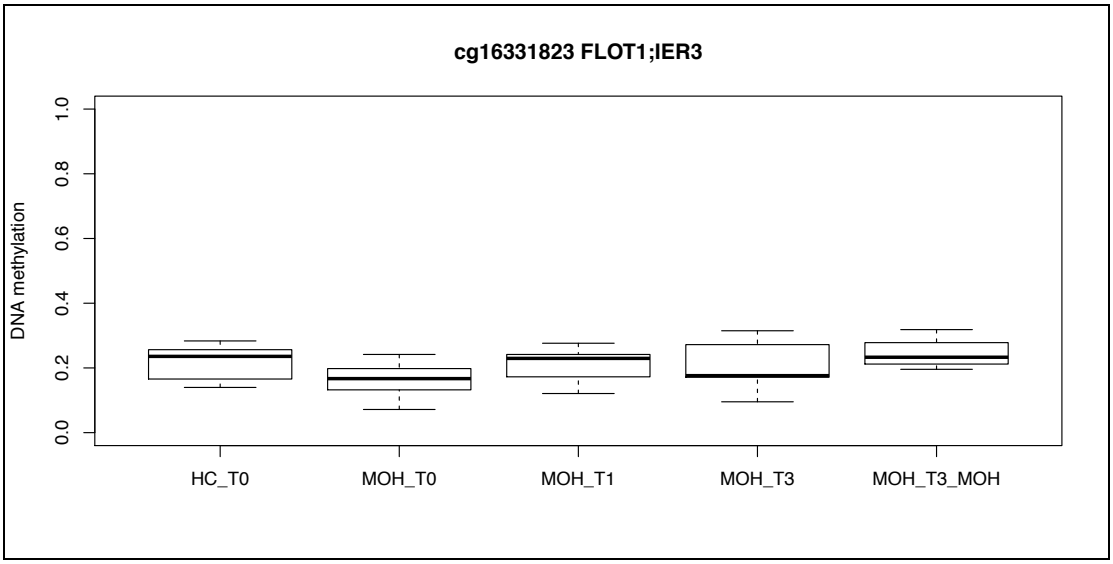

Figure 12: Figure 10: DNA methylation of cg16331823 in the Island chr6:30710307-30712440 of IER3 gene

DISCUSSION

The main aim of this study was to analyse whole blood genome-wide DNA methylation profiles to identify markers of migraine chronicization. Using a prospective and longitudinal case-control design, DNA methylation patterns in the peripheral blood were examined across the genome comparing patients affected from chronic migraine with medication overuse headache versus patients with episodic migraine and healthy controls headache free. Comparing MOH vs HC group, none differentially methylated regions reached statistical significance after correction for multiple test considering a nominal threshold of 0.01. Nevertheless most of the DMRs showed very small DNA methylation differences between the two groups, 29 had a delta of at least 0.05 in at least one CpG site. Among these 29 "risk DMRs " there are 4 most significant CpG site located in genes relevant for the possible implication with migraine chronification and drug addiction. One of the most significant CpG site is at the chr10:76993892 island, which maps in the COMT (catechol-O-methyltransferase) gene and resulted hypermethylated in MOH. The COMT gene encodes the COMT enzyme protein that breakdowns neurotransmitters, such as dopamine, epinephrine, and norepinephrine important enzymes produced by many tissues included nerve cells. The COMT enzyme plays a crucial role in dopamine degradation. In the brain, COMT is particularly important in the prefrontal cortex, a region involved with personality, planning, inhibition of behaviors, abstract thinking, emotion, and working (short-term) memory. The dopaminergic reward system acts an important role in substance use and addiction (Robinson & Berridge 1993) and frequent substance abuse is associated with altered dopamine levels in the brain reward system (Wanat MJ et al. 2009). Studies on genetic variation suggest COMT hyperactivity in substance users (Beuten et al. 2006) From the studies in general population, we know nicotine dependence was related to higher *MB-COMT* promoter methylation, suggesting lower *COMT* gene activity and thus less dopamine degradation in smokers (Xu Q. et al. 2010). In schizophrenia patients, alcohol use was associated with increased *MB-COMT* promoter methylation (Abdolmaleky et al. 2006). Recent study showed that methylation of the *MB- COMT* promoter was associated with non-daily smoking in adolescents. (Van der Knaap LJ et al 2014). Although studies on genetic variation suggest COMT hyperactivity in substance users, there are still few preliminary studies about the association between *COMT* gene methylation and substance abusers with controversial results.

In addition to its involvement in drug abuse mechanisms, *COMT* gene has been studied as a candidate gene for both neuropsychiatric illness and cognitive dysfunction, moreover a functional SNP in codon 158 (Val158Met) of *COMT* has been shown to modulate pain perception and contribute to differences of pain perception (Zubieta JK, et al. 2003) Evidences from animal and human studies suggests that hyper- and hypo- dopaminergic states contribute to mania and depression respectively (Pathak G et al 2015). A

recent review which analyses the interactions between variation in candidate genes and environmental factors in the aetiology of schizophrenia and bipolar disorder. Authors found that polymorphisms in COMT were among the most significant involved in interaction with early life stress and dependence and consequently able to influencing disease outcome. (Misiak B et al 2017) On the same line, a recent study confirmed epigenetic modulation of the expression of the COMT Val158Met polymorphism and subsequent effects on the relationship between traumatic life events and cognition in schizophrenia. (Green MJ et 2014). Beside COMT, in our study GIT2 resulted hypomethyled at CPG site chr12:110433797-110434205*Island. GIT2 encodes a member of the GIT protein family, which interact with G protein-coupled receptor kinases and possess ADP-ribosylation factor (ARF) GTPase-activating protein (GAP) activity. GIT proteins regulate cytoskeletal dynamics and participate in receptor internalization and membrane trafficking. This gene has been shown to repress lamellipodial extension and focal adhesion turnover, and is thought to regulate cell motility. GIT2 is one of the hypermethylated genes recently associated to increased schizophrenia susceptibility (LEE SA. Et al 2016). Another rilevant CpG site is chr19:44645494-44646069*N_Shore which maps in ZNF234 gene and encodes for a Zinc Finger Protein. ZNF differential gene expression in peripheral blood cells from bipolar disorder patients had been previously reported for some of the risk genes identified, including *ZNF641*and *ZNF234*, members of the zinc-finger family of genes, of which *ZNF804A* has been associated with bipolar disorder and schizophrenia in genome-wide association study. Although most of the risk genes had not been previously shown to directly confer risk for bipolar disorder, many of them are within pathways previously implicated in bipolar disorder. (Hess JL et al 2015) Finally, with regard to CPG site chr16:11348541-11350803*Island which maps in the gene SOCS1, a recent study shows that loss of balance among various members of the SOCS family proteins may contribute to pathophysiology of multiple sclerosis. (Toghi M. et al 2017) SOCS1 encodes a member of the STAT-induced STAT inhibitor (SSI), a cytokine-inducible negative regulators of cytokine signalling. These evidences confirmed its involvement in differentiation, maturation and survival of a wide range of cells, including cells of the immune system and suggested a possible role in different cerebral process included migraine chronification.

Although it remains unclear whether psychiatric comorbidities are risk factors for or consequences of MOH, depression and anxiety are more common in MOH patients than in people with episodic. (Diener HC et al 2016) In our sample results from BAI and BDI score confirmed MOH patients fulfilled the criteria for depression and anxiety disorder with statistical significance with respect to EM and HC. Moreover an association has also been described for MOH and subclinical obsessive-compulsive disorders and mood disorders. (Diener HC et al 2016)

Overall these four differently methylated regions resulted relevant because located in genes involved in drug addiction mechanisms and neuropsychiatric illness comorbid with MOH and for their implication in mechanisms of autoimmune control. Moreover, although preliminary, these evidences stand out the value of epigenetic modulation in expression of COMT gene and suggesting its role in inducing an increased susceptibility to develop MOH. After all, several evidences from pharmacogenomics studies showed that The Val (108/158) Met variation of the *COMT* gene is among the most studied polymorphisms associated with response to antidepressants treatment in patients with major depressive disorder. (Biernacka JM et al 2015). Noteworthy according to one study, patients with MOH who are rs4680A homozygous or carry the rs4680A– rs6269A haplotype are at a lower risk of relapse within the first year after successful detoxification than are individuals with other COMT genotypes (Cargnin, S. et al.2014). In our sample, analysis of comparison in MOH group between T0 and T3 (6 month after detox treatment) considering only patients cured from medication overuse (EM and Chronic Migraineurs without Medication overuse) did not revealed statistical difference in methylated regions included CPG site located in COMT gene. The only CpG site with a DNA methylation difference of at least 0.05 between T0 and T3 in the two subgroups mapping in the island of NBL1/MINOS1 gene. NBL1 encodes for the neuroblastoma 1 a protein which acts as BMP (bone morphogenetic protein) involved in growth and development and linked to homonym disease. To confirm at CpG sites in the gene NBL1, longitudinal analysis of changes in DNA methylation at T0, T1 and T3 in the subjects that at T3 changed to chronic or episodic and in those that at T3 were still MOH did not detect any difference. As well as comparison conducted between MOH patients at T0 and T1 in order to detect differences in DNA methylation profile associated with prophylaxis therapy did not find statistical significance in relevant different methylated regions. Although in our study the rate of patients cured from MOH was in line with data from literature, (Chiang, C. C. et al 2015) we hypothesized the small number of sample resulted in limited statistical power. This datum may have influenced detected differences in DNA methylation levels due to treatment in both conditions. With regard to differences in DNA methylation profile due to prophylaxis, apart form small sample, it cannot even be excluded a time-depending effect. In our study design, prophylaxis were stopped for three months before starting detox program. It remains to consider the hypothesis that a period longer that three months would be necessary to detect differences in DNA methylation due to prophylaxis treatment of MOH profile. To our knowledge no previous studies analysed changes in DNA methylation with respect to two treatments approved in MOH (detox and prophylaxis therapy). Due to the limit of this exploratory study, although results of this study did not detect any differences in DNA methylation profile in MOH in response to treatments, we suggest it is necessary to extend the sample size to increase statistical significance and replicate the study in order to confirm or to argue reasons for the absence of these evidences.

Epigenetic alterations and mostly changes in DNA methylation have been previously hypothesized as a possible mechanism of migraine chronification (Eising E et al 2013). Cortical spreading depression has been associated to increased neuronal activity which can result in DNA methylation changes in brain-specific genes related to neuronal plasticity (Guo JU et al 2011). Consequently the suggested hypothesis is that neuronal activity can cause epigenetic changes altering synaptic plasticity and the frequent migraine attacks in a feed-forward loop may promote stable epigenetic changes which altering synaptic plasticity supporting migraine chronification. Recent evidences supporting this hypothesis, come from the study of Winsvold and colleagues (Winsvold BS et al 2017). Our study seems to provide some evidences supporting a role in MOH of epigenetic processes involved in aberrant immune-inflammatory responses and deregulation of dopaminergic neurotransmission theoretically implied in mechanisms of brain plasticity which control drug addiction and cognitive-emotional processes. From this point of view drug addiction can be viewed as maladaptive neural plasticity that occurs in vulnerable individuals in response to repeated exposure to a drug of abuse. (Nestler EJ 2013), and it well known that Medication overuse is a major risk factor for headache chronification (Rossi P et 2009). Notably, unlike previous studies, we did not find statiscal differences in the methylation profile between patients with MOH and patients with EM (data do not shown). Interestingly, a subanalysis of the DNA methylation of the 29 DMRs in EM samples at T0 displayed, for many of the DMRs, values of DNA methylation intermediate between HC and MOH suggesting EM tended to cluster between HC and MOH.

The major strengths of this study include the genome-wide assessment of DNA methylation sites, the clinical longitudinal follow-up that allow confirming the clinical diagnosis and the correct selection of the patients. To these regard it was notable the comparison of DNA methylation level with a group of healthy controls whitout headache which confirmed themselves headache free after 6 months of follow-up. On the other hand our study has clear limitations. First, this is a preliminary longitudinal analysis with a small sample size. Accordingly, our results must be seen as exploratory and one needs to consider that statistically empowered studies are now required for replication and validation. Moreover our search for different methylated regions was performed in whole blood that do not necessarily represent a proxy of brain expression and methylation. Nevertheless, there is evidence showing good concordances between DNA methylation levels in blood with DNA methylation levels in one or more brain regions. (Davies MN et al 2012). Finally, in order to detect reproducible longitudinal epigenetic effects,

data at T0, T1 and T3 should be re-analysed using a statistical approach that specifically assess intraindividual changes in DNA methylation of adjacent CpG sites.

CONCLUSION

In summary, our pilot study revealed that peripheral DNA methylation mostly differs between MOH and HC. Data obtained from analysis of different methylated regions seems to support the clinical hypothesis of prominent role of Medication overuse in chronicization risk. Epigenetic mechanisms hypothesized to be involved in migraine chronicization, play a crucial role in processes implicated in controlling dependence and cognitive-emotional regulation of stress. Conversely, these exploratory results lacked to detect differences in DNA methylation profile of MOH in response to treatments of detox and prophylaxis. Our results are preliminary and require replication and validation in a larger sample, especially considering that chronic migraine is a complex and multidimensional disorder in which several biochemical, cognitive, behavioral and neuro-structural pattern contribute simultaneously.

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