MIGRAINE AND CARDIOVASCULAR DISEASES: ROLE OF ACE AND MTHFR GENES POLYMORPHISMS

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Summary

Genetic factors that increase susceptibility to oxidative stress, endothelial dysfunction and, possibly, stroke include Angiotensin Converting Enzyme gene deletion polymorphism (ACE-DD) and Methylentetrahydropholate reductase genes polymorphism (MTHFR C677T and A1298C). It has been reported that the ACE-DD genotype act in combination with MTHFR-TT genotype to increase migraine susceptibility, with a greater effect in that with aura. The TT polymorphism is also associated to an increate risk of migraine with aura, independently of other cardiovascular risk factors.

The aim of our study was to evaluate the incidence of ACE and MTHFR genes polymorphisms in a consecutive series of migrainous patients and of patients affected by myocardial infarction. We studied a series of 103 migrainous patients **(1)**, whose age was between 13 and 75 years (81 suffering from migraine without aura, MwA, 9 from migraine with aura, MWA, 13 from mixed forms MwA-MWA, according to ICHD-II 2004 criteria) and of 336 patients **(2)** suffering from ischaemic cardiopathy (myocardial infarction, MI). The analysis, based on Polymerase Chain Reaction (PCR) and on reverse-hybridization, showed as follows:

MTHFR (C677T): 60 patients (58%) **(1)** and 186 (56%) **(2)** were heterozygous; 9 patients (9%) **(1)** and 54 (16%) **(2)** were mutated. The result of 1patient **(2)** was unknown.

MTHFR (A1298C): 54 patients (52%) **[1]** and 146 (44%) **[2]** were heterozygous, 7 patients (7%) **(1)** and 33 (10%) **(2)** were mutated. The result of 1 patient **(2)** was unknown.

ACE (evaluated on 101 patients **(1)** and 245 **(2)**): 45patients (43%) **(1)** and 133 (54%) **(2)** had an ID genotype; 42 (41%) **(1)** and 87 (36%) **(2)** had a DD genotype.

So the results of our study confirm the high incidence of ACE and MTHFR genetic polymorphism in migraineurs. These data are also confirmed in the sample of patients suffering from myocardial infarction. Thus highlights a strict relationship between migraine and major cardiovascular diseases, supporting the hypothesis that ACE and MTHFR systems play a crucial role in the pathogenetic model of migraine, as they interfere with the endothelial regulation of vasal tone. An effective role has been assigned to these genetic mutations in the genesis of migraine and in the increased risk of migrainous patients to evolve into an ischemic pathology.

Key words: Migraine, Polymorphism, Stroke

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Introduction

The potential association between migraine and ischaemic stroke risk is an important public health concern, but the causal relationship between them is complex and not fully clear. Migraine and stroke can coexist, stroke may occur with the clinical features of migraine, or it may be induced by migraine. In the last case, a prolonged migraine aura may provoke a condition called "true migrainous infarction" **[1].**

There are good epidemiological proofs about the association of migraine not only with an increased risk of stroke (which is stronger in young adults, but may persist in the elderly) but also with any vascular ischaemic event, myocardial infarction included.

The exact mechanism by which migraine with aura may lead to ischaemic vascular events is still unknown and probably very complex. Prospective data give no evidence that migraine without aura is associated with increate risk of any ischaemic vascular events **[2]**.

Some observational studies showed an increased risk of stroke in people suffering from migraine, some others failed in finding this association. The probable mechanism is thought to be partly a platelet hyperaggregability and a cerebral blood flow reduction, usually occurring in migraine with aura.

On this subject, a meta-analysis was conducted by Etminam *et al*., by systematically searching for english and non-english articles in Medline (1966-June 2004) and Embase (1974-June2004), containing "brain ischemia", "cerebrovascular accidents", "cerebrovascular disorders", "cerebral infarction", "ischemic attack", "migraine" and "oral contaceptives", as both medical subject heading terms and text words.

The intention was to include studies using clear diagnostic criteria for both migraine and ischaemic stroke, controlled for potential confounders and providing odds ratios, relative risks, 95% Confidence Intervals, or enough data to calculate them. In order to quantify the risk of stroke among

migrainers using oral contraceptives, the studies had to provide data for patients who were using oral contraceptives compared to those who weren't.

Studies were qualitatively classified, by using a 10 point scale adapted from a recently published quality scale for observational studies (five criteria ranked 0, 1 or 2) and then stratified by score (a score of 7 or above indicates high quality; a score of 6 or below indicates low quality).

The search resulted in 11 case-control studies, 3 cohort studies and 1 cross sectional study. The last was excluded as it was very difficult to deduce the timing of diagnosis with respect to the development of ischaemic stroke. Six studies provided data on the risk of ischaemic stroke and migraine with and without aura. The age of participants to the studies included ranged from 15 and 84 years **[3]**.

Unlike previous studies, showing no increase in the risk of haemorrhagic stroke in migraineurs, Etminam *et al*. gave evidences about a causal relation between migraine and stroke. The reported increased Relative Risks were 1.8 in migraine without aura, 2.3 in migraine with aura and 8.7 in oral contraceptive users **[4]**.

So, the results of meta-analysis strongly suggest that migraine may be an independent risk factor for stroke, and the entity of this risk is comparable across all studies (case-control and cohort), as well in those providing data on migraine with and without aura and oral contraceptive users. Three studies indicated a very high risk of stroke among oral contraceptive users, while other studies showed that oral contraceptive users with a history of migraine have twice the likelihood of developing an ischaemic stroke compared with those without migraine (Relative Risk 2.15, 95% Confidence Interval 0.85 to 5.45).

Possible mechanisms for this association include irregularities in blood flow, cardiac abnormalities, and abnormal production of prostaglandins as well as noradrenergic or cholinergic transmitters and receptors.

The meta-analysis is subject to several limitations. First, it was considered lacking in accuracy, as it didn't include similar populations and as some important confounders, such as familial history,

smoking, diabetes, hypertension (antihypertensive drugs may be used to prevent migraine attacks as well as future strokes, so their use among people with migraine would probably have decreased the risk of stroke, and this hasn't been considered in the study) and common therapies may not have been evenly controlled for; high risk patients with migrainous symptoms due to other conditions weren't excluded; some studies were characterized by an uncertain diagnosis of both migraine and stroke (sometimes even including transient ischaemic attacks); a distinction between high/low oral contraceptive doses or only progesterone users wasn't made: this may be crucial as risks with the latter are lower or non-existent.

Antiphospholipid antibodies were thought to be linked to stroke and possibly to migraine, but none of the studies included in the meta-analysis provided information on this potential confounder. Migraineurs might be less likely to be diagnosed as having a stroke, as the symptoms of migraine may be confused with those of stroke. Although this remains a possibility, many of the studies included in the meta-analysis used strict criteria to define ischaemic stroke, including duration of symptoms of at least 24 hours, as well as confirmation of diagnosis by brain imaging or autopsy. Finally, it wasn't possible to infer a temporal relation between the onset of migraine from the studies.

What is more serious, these studies didn't provide any information about the influence of age, the most important risk factor for stroke. The absolute risk in young people is very low, so the increased reported relative risks, even in oral contraceptive users, shouldn't be worrying. An increased risk of stroke in older migraineurs, indeed, may confirm another suspected risk factor and offer a new hope of prevention. The risk in old people is due to the influence of several factors and may exceed the sum of their individual relative risks. Older migraineurs should therefore be assessed more carefully **[3,4]**.

Migraine was associated to an unfavorable cardiovascular risk profile. What emerged from the Genetic Epidemiology of Migraine study is that migraineurs are more probably smokers, with a familial history of early myocardial infarction, compared with controls; the same study highlighted

that migraineurs with aura have an unfavorable cholesterol profile, hypertension, frequently report a history of early onset coronary heart disease or stroke and present a two-fold increased 10-year risk of coronary heart disease, compared to the Framingham score, even after adjusting for age **[2]**.

A reduction of cerebral blood flow in some regions and an increased platelet activity, factors contributing to the risk of thrombosis, were noticed in migraine **[4]**. The detailed study of migraine pathophysiology announced that a dysfunction of brain cells and arteries is a major component of this disorder. The involvement of cerebral arteries, in fact, together with the high prevalence of migraine in young people with stroke induced Kurth *et al.* to hypothesize that migraine may be a risk factor for stroke and to find the potential biological mechanisms underlying this connection.

The study envisioned several hypothesis:

1) That migraine might be a direct cause of an ischaemic event (i.e. migrainous infarct);

2) That the pathophysiology of migraine might affect the endothelial function and by this alone or in combination with pre-existing might increase the risk of stroke outside of a migraine attack;

3) That migraine might be associated to an increased prevalence of risk factors for ischaemic vascular events;

4) That the connection might be provoked by migraine-specific drugs;

5) That migraine and ischaemic vascular events might be connected through a genetic component. As regards to ischaemic stroke, some congenital heart defects, such as patent foramen ovale, were discussed as potential biological mechanisms **[2]**.

Several other studies had a similar approach, by analyzing the same aspects, one at a time or in combination and obtaining sometimes similar, otherwise totally different results.

The accepted neurovascular theory of migraine integrates the phenomena of head pain and aura, the focal neurological symptoms that precede or accompany headache in a sizable minority.

Migraine pain was ascribed to a vascular dilatation, a perivascular inflammation and a nociceptor activation, so the trigeminal nerves play a prominent role, emanating from the brainstem and

innervating the vasculature. Migraine aura, once attributed to intracranial vasospasm, is now generally accepted as a consequence of CSD (Cortical Spreading Depression) of Leao, a short-lasting depolarization wave that moves across the cortex at a rate of 3-5 mm/min. The brief phase of excitation of the neuronal and astroglial network is immediately followed by prolonged nerve cell depression. This process induces an efflux of excitatory amino acids from nerve cells, enhanced energy metabolism, and changes in genes, growth factors, neurotransmitters, neuromodulators and inflammatory mediators. Microvascular changes, marked by a brief cortical spreading hyperemia, followed by a longer lasting cortical spreading oligemia, have been observed during the phase of cortical neurons depression **[5,6]**.

There are functional imaging data to suggest that CSD also occurs in migraine without aura. PET and MRI techniques give evidence that cerebral blood flow during the oligemic phase of CSD remains above the range associated with ischaemic injury **[7].**

Some animal models of CSD demonstrate a release of matrix metalloprotease 9, during the initial neural hyperexcitability phase (depolarization), an immune cell invasion into nervous tissue and a direct nervous tissue injury **[2,6,8]**.

So the vascular complications seen in migraine might be a consequence of migraine attacks themselves, a hypothesis supported by the parallel increase in lesions with attack frequency **[8]**, but at the same time thought to be unlikely, as criteria introduced by the International Headache Society (1988 and 2004) describe a migrainous infarction as characterized by one or more aura symptoms, associated with ischaemic brain lesions, in an appropriate territory demonstrated by neuroimaging, occurring in conjunction with a migraine attack in patients with migraine with aura, except those who have aura symptoms lasting more than 60 minutes.

Other causes for ischaemia have to be ruled out. In addition, migrainous infarcts provoked by a severe hypoperfusion during a migraine attack whit aura are very rare and likely to be over-diagnosed **[2]**. Finally, most ischaemic strokes occur between attacks and not during or shortly after a migraine attack with aura **[2,5]**.

Microcirculatory vasoconstriction (CSD-related oligemia) and intracerebral large vessel spasm are putative ischaemic stroke mechanisms, involving the vasculature not only in headache, but also in migraine-related infarction.

It is still unclear whether endothelial dysfunction may be a cause or a consequence of headache, or whether they coexist for other reasons.

A cross-sectional study of 50 patients with migraine and an equal number of matched controls without migraine showed that brachial artery diameter, as well as brachial artery and femoral artery compliance were decreased in migraineurs, who presented an increased aortic augmentation index **[2]**. A reduction in bioavailability of vasodilatator factors, such as NO and an increase in endothelial-derived contracting factors, with a consequent impairment of the reactivity of the vasculature, are associated with, and predicts, an increased rate of cerebro- and cardiovascular events. Vasospasm, once thought to be the mechanism of migraine aura, results from the ictal release of potent vasoconstrictive substances, such as serotonin and endothelin.

Migraine is thought to be a non traditional risk factor for endothelial dysfunction and one of the most widely accepted biomarkers is vWF (von Willebrand factor), whose levels and activity were found significatively higher in migraineurs than in non-headache controls, during the interictal phase. More evident value differences were highlighted in migraineurs with a history of prior stroke. Several studies conducted on migraineurs showed increased ictal platelet activation and PAF and vWF levels, compared with interictal measurements.

Cerebral endothelial cells release PAF (Platelet-Activating Factor) when challenged by Hypoxia and CGRP (Calcitonin Gene-Related Peptide, released by activated trigeminal endings during migraine). A potent inducer of platelet activation and aggregation, PAF also prompts the release of vWF, a large endothelial-derived glycoprotein indirectly activating the platelet IIb/IIIa receptor, crucial for binding fibrinogen and leading to primary haemostasis **[5]**.

A study conducted by Kozubski *et al*. on migraine patients in the phase between attacks, found that the number and affinity of fibrinogen receptors on their platelets were significatively increased;

Palowska *et al*. reported a considerable increase of GPIIb on the platelet membrane of migraine patients both during and between attacks. The animal model of Moskowitz proposed neurogenic inflammation as a possible mechanism implicated in migraine, with a role of platelet activation leading to the release of Thromboxane A2 (TXA2) and this could explain the efficacy of Aspirin, non-steroidal anti-inflammatory drug and Thromboxane synthetase inhibitors, together with drugs of major importance in migraine prophylaxis, such as β-adrenergic blockers. On the other hand, the efficacy of other drugs, such as triptans, acting by activating 5-HT_1 receptors present on sensory fibers innervating blood vessels in dura-mater, had to be considered; their action didn't deal with platelets **[9]**.

Increasing platelet function and thrombinic markers were documented in migraine patients and thought to be a cause of or an epiphenomenon during attacks. The contribution of hypercoagulability to migraine-related ischaemic disease remains uncertain. Migraine with aura attacks, for example, were associated to thrombocytosis and e polycythemia vera, two conditions associated to an increased ischaemic risk too. The use of antiplatelet drugs to control migraine in case of thrombocytosis and of periodical phlebotomy in the case of polycythemia vera confirmed the causative relationship between migraine and hypercoagulability **[5]**.

In addition to biomarkers, several studies demonstrated both a cerebral and a systemic impaired reactivity in migraine patients. In CADASIL, an hereditary condition notable for small vessel stroke and migraine, cutaneous laser Doppler flowmetry demonstrates an impairment of endothelial dependent vasodilatation; but it's not clear whether or not small cerebral infarcts, including clinically silent lesions in the white matter demonstrated in migraineurs without CADASIL are associated with endothelial dysfunction **[6]**.

Endothelial dysfunction is mediated by an increased oxidative stress, an important promotor of inflammatory processes and inflammation, and it's known that inflammation is involved in the pathogenesis of migraine. Few studies demonstrate the association between migraine and inflammatory markers, but many studies give evidence of the efficacy of anti-inflammatory agents

such as aspirin, ibuprofen, Cox-2. Clinical investigation of markers of oxidative stress in a migraine population during, after and between migraine attacks has supported this association. Compared with migraine-free controls, oxidative markers were higher in migraineurs, even during the interictal period. Within the migraine sample, the same markers were much higher during than between attacks **[5]**.

Antiphospholipid antibodies, which predispose to clotting through an unknown mechanism, are probably not associated with migraine *per se*, but may increase clotting risk in migraineurs, or serve as a marker of endothelial perturbation. A group of antiphospholipid antibodies, anticardiolipin antibodies (aCL), are correlated to silent white matter lesions in migraineurs and to stroke. They're serum immunoglobulins responsible for several neurological diseases: cerebral infarction, transient ischaemic attacks (TIA), vascular dementia, cerebral venous thrombosis, chorea and migraine **[5,9]**. Since 1978 (Brandt and Lessel), several studies evaluated the association between migraine and antiphospholipid antibodies, but there was no evidence that the frequency of aPL syndromes was increased in migraine. By contrast, there seems to be an increased frequency of transient neurological events, some of them having features of migrainous aura, both in primary and in secondary aPL syndromes **[9]**.

Patent foramen ovale (PFO), a risk factor for ischaemic stroke in the young, has been found to be more common in young ischaemic and non-ischaemic stroke patients with migraine. The evidence of an improvement of migrainous symptomatology after PFO closure confirms the relationship. There is no evidence of a possible association between PFO and white matter lesions **[5]**.

The interest towards a potential genetic component of migraine-stroke association was induced by the observation that, in some patients, migraine and stroke occur commonly as a part of a distinct disorder, including CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leucoencephalopathy) and MELAS (Mitochondrial myopathy, Encephalopathy, Lactacidosis and Stroke), autosomal dominant diseases affecting, however, very few migraine patients **[2]**.

The investigation on the susceptibility genes of myocardial infarction started substantially in the‘80s and most efforts were mainly addressed to identify and to evaluate genes involved in those systems already suspected to be implicated in the pathogenesis of CHD, i.e. lipid metabolism, coagulation, fibrinolytic system, membrane receptors platelets, plasma homocysteine levels and vascular tone **[10]**.

Genetic aspects underlying common forms of migraine are not clear, but the wide clinical spectrum of migraine suggests that several polymorphisms may interact to determine its manifestation and gravity, while the effect of a single mutation is thought to be minimal.

Among genetic factors that increase susceptibility to oxidative stress and to endothelial dysfunction, with a consequent increase in the risk of stroke, the Angiotensin-Converting Enzyme (ACE) gene deletion polymorphism and the Methylentetrahydrofolate reductase (MTHFR) C677T gene polymorphism are involved **[6]**.

In the attempt to individuate genes potentially implicated in the etiopathogenesis of migraine, ACE gene is probably the most studied gene **[11]**.

An insertion/deletion (I/D) polymorphism localized within intron 16 of the ACE gene, is due to the presence (allele I-Insertion) or absence (allele D-Deletion) of a 287-bp alu repeat sequence and it can produce three different genotypes:

II insertion in homozygosis

ID insertion/deletion heterozygosis

DD deletion in homozygosis **[12]**.

Several studies reported an association between DD homozygous genotype and the intermediate phenotype (circulating concentrations of the enzyme). DD homozygosis provokes a 56% increase in ACE activity compared with I allele homozygous **[13]**.

A clinical study demonstrated that ACE-DD genotype acts in combination with MTHFR-TT genotype, to increase susceptibility to migraine, particularly migraine with aura. MTHFR-TT

genotype is also associated with an increate risk migraine with aura, independently from other cardiovascular risk factors.

An analysis conducted by Paterna *et al*. compared 302 patients suffering from migraine without aura, with no history of cardiovascular diseases and major risk factors for ischaemic events, with 201 non migrainous controls. evaluation focused on the genotype of ACE gene, plasma ACE activity and the frequency and duration of migraine attacks. Migraineurs showed a higher incidence of ACE DD gene (48,34%) compared to (37,32%) and the frequency of migraine was higher in DD patients (2,11 ± 1,9 average attacks per week) compared to ID ones (1,54 ± 1,44), while no relevant differences were observed in duration of attacks. Plasma activity was higher in patients with a DD genotype, so it was thought to play an important role in determining migraine attacks and in their frequency **[14]**.

A study of Kowa *et al.* on japanese population confirms that the association between ACE gene polymorphism and migraine increases the risk of developing thrombotic events. 54 patients suffering from migraine with aura (MWA), 122 from migraine without aura (MwA) and 248 healthy controls were examined. The incidence of ACE-DD genotype was significatively higher in patients suffering from MWA (25,9%), compared to controls (12,5%). No difference was found in the other groups [**15**].

Despite previous, the case-control study conducted by Lin *et al*. didn't show a remarkable difference in allelic frequency (I and D) of ACE polymorphism, after examining 240 migrainous patients and 200 healthy controls. Male migraineurs with a DD genotype were even less than male controls, thus suggesting a protective effect of such genotype **[16]**.

Lea *et al*. showed that the DD variant of ACE gene confers a weak independent risk to migraine susceptibility but, in combination with the mutated variant C677T of MTHFR gene, it confers a stronger influence on the disease, in Caucasians. The study was conducted on 270 migraine cases (88% of them with a DD genotype) and an equal number of controls (81% DD) **[17]**.

Tronvik *et al*. investigated on the possibility that the ACE genotype could be a possible risk factor for migraine with and without aura in Norwegians. 347 patients whose age ranged from 18 and 68 years (155 suffering from MWA, 187 from MwA and 5 missing aura subgroup data according to ICHD-II criteria) and 403 healthy controls aged more than 40 years were included. No differences were found between migraineurs and controls, with regard to ACE genotype distribution and ACE genotyping could not predict a clinical response to drugs acting on the Renin-Angiotensin system used as migraine treatment. However these findings should be confirmed by other studies including more patients and among different ethnic groups **[18]**.

Some studies reported as a cause of increased risk of migraine, the common single nucleotide polymorphism of MTHFR gene, wherein the nucleotide thymine replaces nucleotide cytosine at position 677, in the region encoding for the binding site of the enzyme. This "missense" mutation provokes an alanine-to-valine substitution in the final enzyme, thereby becoming thermolabile, reducing its specific activity and producing hyperhomocysteinemia, particularly in low folate level conditions **[19,20]**.

Therefore, this polymorphism is thought to be an important candidate in determining migraine susceptibility, but it's even one of the most studied candidates in stroke **[20]**.

Two independent studies, conducted by Kowa *et al*. and Kara *et al.*, gave evidence of the role of TT genotype as a risk factor for migraine, in particular MWA, in Japaneses and Turks, respectively.

Oterino et al. genotyped 230 patients (152 MwA and 78 MWA) and 204 healthy controls, but they didn't find any relevant association between migraine and the presence of MTHFR-TT polymorphism, although it was more frequent in patients suffering from MWA (42%), compared to MwA (29%) and to healthy controls (36%) **[21-25]**.

Pezzini *et al*. examined a group of non-relative migraine patients and a control group, matched for sex, age, ethnic group and geographical location.

Table 1: *ACE genotype and allele distributions among controls and migraine patients in different studies [18]*

				Genotypes		Alleles
	N	**DD(%)**	**ID(%)**	**II(%)**	**D(%)**	**I(%)**
Controls						
Tronvik	403	92 (26.6)	204 (50.6)	107 (22.8)	388 (48.1)	418 (51.9)
Paterna (ref 8)	201	75 (37.3)	101 (50.3)	25 (12.4)	251 (62.4)	151 (37.6)
Lea (ref 9)	244	76 (31.1)	122 (50.0)	46 (18.9)	274 (56.1)	214 (43.9)
Kowa (ref 10)	248	31 (12.5)	114 (46.0)	103 (41.5)	176 (35.5)	320 (64.5)
Migraine						
Tronvik	347	78 (22.5)	186 (53.6)	83 (23.9)	342 (49.3)	352 (50.7)
Paterna	302	146 (48.3)	129 (42.7)	27 (9.0)	421 (69.7)	183 (30.3)
Lea	250	77 (30.8)	142 (56.8)	31 (12.4)	296 (59.2)	204 (40.8)
Kowa	176	33 (18.7)	86 (48.9)	57 (32.4)	152 (43.2)	200 (56.8)
MwA subgroup						
Tronvik	155	34 (21.9)	87 (56.1)	34 (21.9)	155 (50.0)	155 (50.0)
Paterna	NA	NA	NA	NA	NA	NA
Lea	151	48 (31.8)	85 (56.3)	18 (11.9)	181 (59.9)	121 (40.1)
Kowa	54	14 (25.9)*	26 (48.2)	14 (25.9)	54 (50.0)*	54 (50.0)
MoA subgroup						
Tronvik	187	43 (23.0)	96 (51.3)	48 (25.7)	182 (48.7)	192 (51.3)
Paterna	302	146 (48.3)*	129 (42.7)	27 (9.0)	421 (69.7)	183 (30.3)
Lea	99	29 (29.3)	57 (57.6)	13 (13.1)	115 (58.1)	83 (41.9)
Kowa	122	19 (15.6)	60 (49.2)	43 (35.2)	98 (35.2)	146 (59.8)

* Reported significant finding for genotype or allele frequencies

MTHFR-TT genotype distribution didn't show relevant differences between MwA patients and healthy controls, while there were between MWA patients and controls, as well as between MwA and MWA, always with an overrepresentation in MWA subgroup.

In the second place, it was demonstrated that a familiar history of migraine is associated with an increate risk of stroke, differently according to aura status. Finally, The C677T MTHFR polymorphism appears to be mainly associated with MWA and its influence in predisposing to the risk of stroke is partially mediated by migraine itself **[20]**.

A second mutation in MTHFR gene, A1298C, was associated to a reduced enzymatic activity, (about 60%; about 40% if in association with C677T mutation). This mutation, in effect, provokes an increase of blood homocysteine levels in patients with the C677T mutation **[26-27]**.

Materials and methods

The sample studied was represented by 103 patients suffering from migraine and 336 from ischaemic cardiopathy. Migrainous patients, 73 females and 30 males, aged 13-75 years, were observed to the Headache Center of S. Luca Hospital, Vallo della Lucania (Sa) between 2004 and 2009. In the same period 336 patients, 237 males and 99 females, suffering from ischaemic cardiopathy (myocardial infarction) at the Coronary Unity of S. Luca Hospital were studied too. Headache patients suffered from migraine with and without aura and from mixed forms of migraine, diagnosed on the base of the International Headache Society criteria (IHS-1988, and revisited with the new ICHD-II 2004 criteria.

Exclusion criteria from the study were:

- ✓ positive anamnesis for abuse of analgesics
- ✓ presence of serious medical diseases, which obliged patients to treatments interfering with the study
- ✓ need to take drugs for other disorders

A clinical schedule was compiled for each patient presenting for the first time at the Headache Center. Once reported personal data and information about the general personal and familial physiological anamnesis, in the second part of the questionnaire the semiological characteristics oh headache were identified: familiarity, age of onset, course, frequency, recurrence. Later, in the third part, information about the description of headache, such as seat and kind of onset, type of pain (pulsating, gravative, constrictive, etc.) localization, diffusion, intensity were collected. In order to correctly diagnose headache, the potential presence, localization, kind-order-duration of appearance of visual (flickering lights, spots or lines), sensory, motor, as well as speech symptoms.

Several local and/or general symptoms, associated to migraine, as well as possible trigger factors precipitating headache, i.e. psycho-physical stress (the more frequently reported) were identified. Finally, every patient was asked whether he/she used drugs, particularly analgesics, or what kind of measures he/she adopted during attacks, to reduce pain. This aspect was very important, as most patients, particularly those suffering from MwA, aim to press temples, to reduce pain, while patients who refer photo-phonophobia prefer laying on bed in the dark.

Subsequently, dietary habits were investigated (number of daily meals, quality and quantity of food consumed, liquids daily taken etc.), as well as the existence of clinical signs indicating probable nourishment deficits.

Data collected were integrated with a general clinical and neurological exam, then every patient was given a diary, to report attacks occurring in the period between the first and the second visit (number of attacks, duration and intensity). For fertile female patients, the diary was also useful to verify the probable combination of headache attacks and menstruations.

Patients also received a schedule to search for probable trigger factors and a prescription of tests to complete the diagnosis (supra-aortic trunk doppler, CT and/or brain MR, EEG test, Ocular Fundus, blood tests to determine basal homocysteine (by HPLC), fibrinogen, antitrombina III, folates and vitamin B12 levels).

Finally, a series of test to determine genetic polymorphisms was prescribed. Our study focused on the evaluation of the following polymorphisms:

- ✓ MTHFR (C677T)
- ✓ MTHFR (A1298)
- ✓ ACE I/D

At the end of the first visit, each patient was suggested an attack therapy, based on headache characteristics.

The assay for the identification of genetic mutations associated with cardiovascular diseases was based on polymerase chain reaction (PCR) and reverse-hybridization and included three steps:

1) DNA isolation
2) PCR amplification using biotinylated primer
3) Hybridization of amplification products to a test strip containing allele-specific oligonucleotide probes immobilized as an array of parallel lines. Bound biotinylated sequences were detected using streptavidin-alkaline phosphatase and color substrates.

The assay covered 12 mutations: FV G1691A (Leiden), FV H1299R, prothrombin G20210A, factor XIII V34L, β-fibrinogen -455 G→A, PAI-1 4G/5G, HPA-1, MTHFR C677T, MTHFR A1298C, ACE I/D, ApoB (R3500Q), ApoE2/E3/E4.

Amplification mix, Taq dilution buffer, conjugate solution, wash solution B contained 0.05% NaN_3. Conjugate solution contained streptavidin-alkaline phosphatase. Color developer contained nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

Store all reagents at 2-8°C when not in use. Store Taq polymerase at -20°C.

COMPONENTS:	
✓ Lysis Solution	50 ml
✓ Gen^{x} Tract Resin (resuspended each time, immediately before removing an aliquot)	5 ml
✓ Amplification Mix A (yellow cap)	500 ml
✓ Amplification Mix B (green cap)	500 ml
✓ Taq Dilution Buffer (transparent cap)	500 ml
✓ DNAT (containing 1,6% NaOH, blue cap)	1.5 ml
✓ Typing Trays	
✓ Teststrips	
✓ Hybridization Buffer	25 ml
✓ Wash Solution A (white cap)	80 ml
✓ Conjugate Solution	25 ml
✓ Wash Solution B	80 ml
✓ Color Developer	25 ml
✓ Taq DNA Polymerase 5 U/µl (red cap)	125 U

✓ Adjustable Microcentrifuge capable of 3000-12000 rpm
✓ 1.5 ml Microtubes with screw cap
✓ Incubator (heating block, water bath) capable of 56^{o} C and 98^{o} C (±2^{o}C)
✓ Thermocycler and suitable thin-walled plastic reaction tubes-strips
✓ Waterbath with shaking platform and adjustable temperature (45^{o}C ± 0.5^{o}C)
✓ Vacuum aspiration apparatus
✓ Shaker (rocker or orbital).

DNA isolation

Use fresh or frozen blood with EDTA or citrate anticoagulant: avoid blood containing heparin. Do not store blood for more than 3 days at ambient temperature or more than 1 week at 2-8°C before use. Blood which has been kept frozen for more than one year, or gone through more than three freeze-thaw cycles is unsuitable to be used in this procedure.

Bring blood samples to room temperature. Mix well by carefully inverting blood collection tubes several times. Repeat mixing each time before withdrawing an aliquot of blood.

Allow Lysis solution and GEN^{X}TRACT Resin to reach room temperature.

- ✓ Pipette 100 µl blood sample into a 1.5 ml micro tube with screw cap.
- ✓ Add 1 ml lysis solution, close tube and mix by inverting several times.
- ✓ Let stand for 15 minutes at room temperature.
- ✓ Centrifuge for 5 min at 3000 rpm in a microcentrifuge.
- ✓ Remove and discard the upper 1 ml supernatant.
- ✓ Add 1 ml lysis solution, close tube and mix by inverting several times.
- ✓ Centrifuge for 5 min at 12000 rpm.
- ✓ Remove and discard the supernatant except for approximatively 50µl of a visible soft pellet.
- ✓ Resuspend Gen^{x}Tract resin by swirling the bottle thoroughly.
- ✓ Add 200 µl Gen^{x} Tract resin to the pellet. Close tube and vortex for 10 sec.

N.B: The resin sediments quickly. Repeat resuspension each time immediately before removing another aliquot.

- ✓ Incubate for 20 min at 56°C. Vortex for 10 sec.
- ✓ Incubate for 10 min at 98°C. Vortex for 10 sec.
- ✓ Centrifuge for 5 min at 12000 rpm. Cool on ice.

The resulting supernatant contains DNA template suitable for immediate use in OCR. For further storage, the supernatant should be transferred into a fresh tube and kept refrigerated (2^{o}- 8°C up to one week) or frozen at 20°C.

In vitro amplification (PCR; 2 separate reactions per sample)

Keep all PCR reagents and DNA templates refrigerated throughout. Perform all steps until start of the thermal cycling program on ice (0-4^{o}C).

- ✓ Prepare a fresh working dilution (1:25, final conc. 0.2 U/µl) of Taq DNA polymerase in Taq dilution buffer.
- ✓ Prepare 2 reaction tubes for each sample to be amplified. Place tubes on ice.
- ✓ For each sample prepare 2 final PCR reaction mixes (A and B on ice:

Amplification Mix A	15 µl	Amplification Mix B	15µl
Diluted Taq DNA polymerase (1U)	5 µl	Diluted Taq DNA polymerase (1U)	5µl
DNA template	5 µl	DNA template	5 µl

- ✓ Cap tubes tightly. Preheat the thermocycler at 94°C.
- ✓ Insert reaction tubes and run the following thermocycling program:

1. pre-PCR — 94°C/2 min (inizial denaturation)
2. thermocycling — 94°C/15 sec (denaturation)
   58°C/30 sec (annealing)
   72°C/30 sec (extension); (35 cycles)
3. final extension — 72°C/3 min

Store amplification products on ice at 2°- 8°C for further uses.

Optional: Analyze amplification products by 2% agarose gel electrophoresis coloured with ethidium bromide. Lines should be visible at 134/156/173/202/223/254/297/324 bp (amplification product A) or at 225/248/283/346 bp (amplification product B).

Hybridization (45°C; shaking waterbath)

Adjust the water level of the waterbath to approximatively ½ of the height of the typing tray. Heat the waterbath to 45°C (±0.5°C) and check it with a calibrated thermometer. Prewarm hybridization buffer and wash solution A to 45°C (take care that all precipitates formed at 2-8°C become completely dissolved). Allow teststrips, DNAT, conjugate solution, wash solution B and color developer to reach room temperature. Remove one teststrips for each sample using clean

tweezers (touch teststrips with gloes only!). Label teststrips outside of the marker lines with a pencil.

✓ Pipette 20 µl DNAT into the lower corner of each lane to be used in the typing trays (one lane per sample).

✓ Add 10 µl amplification product A into the corresponding drop of DNAT.

✓ Add 10 µl amplification product B into the same drop. Mix thoroughly with a pipette. The solution will remain blue.

✓ Let stand for 5 min at room temperature.

✓ Add 1 ml hybridization buffer (prewarmed to 45°C) into each lane. Gently agitate tray. The blue color will disappear.

✓ Insert teststrips with marked side up (lines visible!) into the respective lanes. Submerge completely.

✓ Incubate for 30 min at 45°C on the shaking platform of the waterbath. Set moderate shaking frequency (approx. 50 rpm) to avoid spilling. Keep the cover of the waterbath closed to avoid variations in temperature.

✓ At the end of incubation remove hybridization solutions by vacuum aspiration. Proceed immediately. Do not allow teststrips to run dry during the entire procedure.

Stringent wash (45°C; shaking waterbath)

✓ Add 1 ml wash solution A (prewarmed to 45°C). Rinse briefly (10 sec). Remove liquids by vacuum aspiration.

✓ Add 1 ml wash solution A.

✓ Incubate for 15 min at 45°C in the shaking waterbath. Remove the liquid.

✓ Add 1 ml wash solution A.

✓ Incubate for 15 min at 45°C in the shaking waterbath. Remove the liquid.

Color development (room temperature)

✓ Add 1 ml conjugate solution.

✓ Incubate for 15 min at room temperature on a rocker or orbital shaker. Remove liquids by vacuum aspiration.

✓ Add 1 ml wash solution B. Rinse briefly (10 sec). Remove liquids by vacuum aspiration.

✓ Add 1 ml wash solution B.

✓ Incubate for 5 min at room temperature on a rocker or orbital shaker. Remove liquids by vacuum aspiration.

✓ Add 1 ml wash solution B.

✓ Incubate for 5 min at room temperature on a rocker or orbital shaker. Remove liquids by vacuum aspiration.

✓ Add 1 ml color developer.

✓ Incubate for 15 min at room temperature in the dark on a rocker or orbital shaker.

✓ A purple staining will appear upon positive reaction.

✓ Wash teststrips several times with distilled water. Let strips dry in the dark on absorbent paper. Do not expose teststrips to intense light after color development.

Interpretation of results

The genotype of a sample was determined using the enclosed "Collector" sheet. The red marker line (top) and the green marker line (bottom) are useful to correctly align the strip and the control line. Staining intensities of positive lines may vary. This in of no significance for the results. A positive reaction of the uppermost Control line indicates the correct function of conjugate solution and color developer. This line should always stain positive.

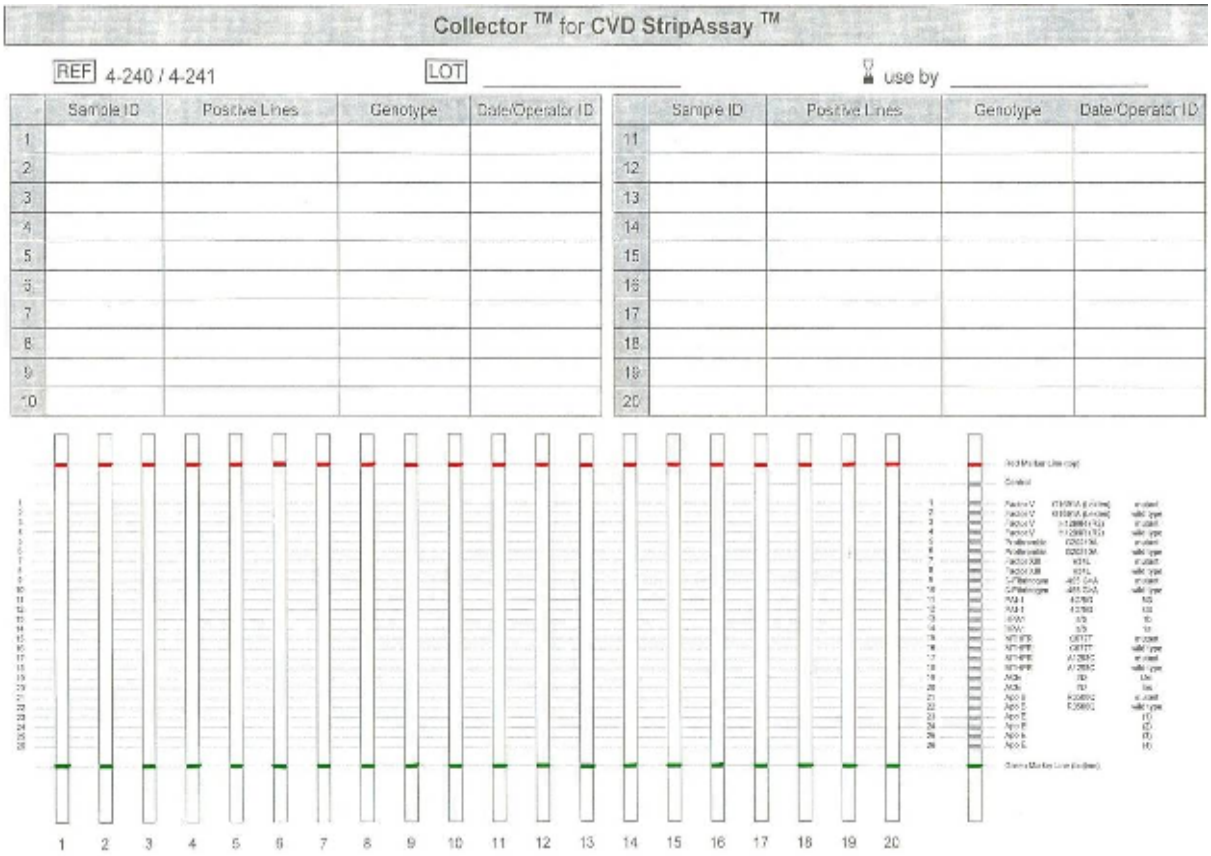

Figure 1: Collector for interpretation of results

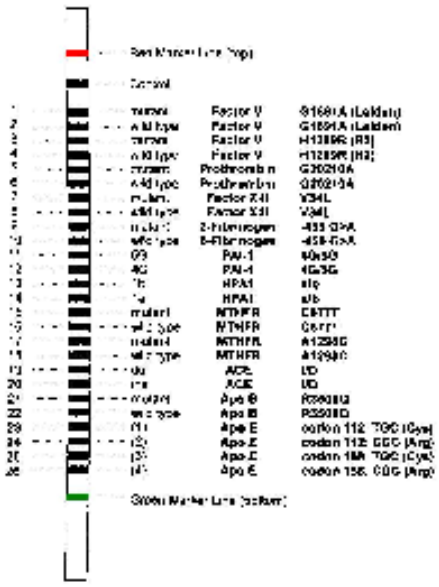

Figure 2: Polymorphic positions and related genotypes

For each polymorphic position, one of the following staining patterns should be obtained:

1. Wild type line only → Normal genotype
2. Wild type and mutant lines → Heterozygous genotype
3. Mutant line only → Homozygous mutant genotype

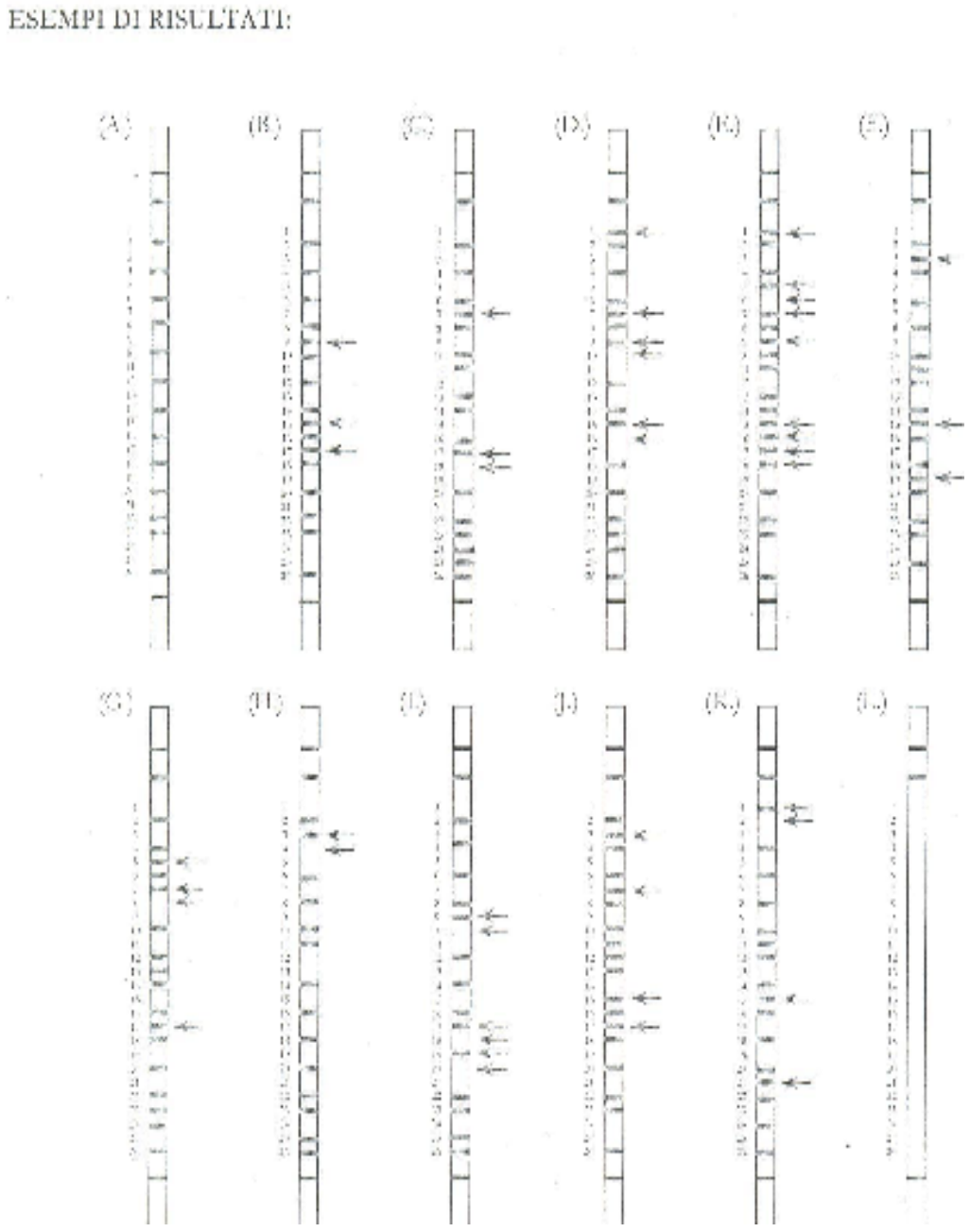

Figure 3: Examples of results

	FV L	FV R2	PTH	FXIII	FGB	PAI-1	HPA1	M677	M1298	ACE	ApoB	ApoE
(A)	norm	norm	norm	norm	norm	4G/4G	a/a	norm	norm	I/I	norm	E3/3
(B)	norm	norm	norm	norm	etero	4G/4G	a/a	etero	etero	D/D	norm	E3/3
(C)	norm	norm	norm	etero	norm	5G/5G	a/b	norm	omo	I/D	norm	E2/4
(D)	etero	norm	norm	etero	omo	4G/4G	a/a	omo	norm	I/D	norm	E3/4
(E)	etero	norm	omo	etero	etero	5G/5G	a/b	etero	etero	I/D	norm	E3/3
(F)	norm	etero	norm	norm	norm	4G/5G	a/a	etero	norm	I/D	norm	E2/2
(G)	norm	norm	etero	omo	norm	4G/4G	a/b	norm	etero	I/I	norm	E3/4
(H)	norm	omo	norm	norm	norm	5G/5G	a/a	norm	norm	I/D	norm	E2/3
(I)	norm	norm	norm	norm	omo	4G/4G	a/a	norm	omo	D/D	norm	E2/3
(J)	norm	etero	norm	etero	norm	4G/5G	b/b	etero	etero	I/D	norm	E3/3
(K)	omo	norm	norm	norm	norm	4G/5G	a/a	etero	norm	I/I	etero	E4/4
(L)	controllo negativo o errore sperimentale											

Figure 4: Interpretation of results in Figure 3

Results

Migrainous patients came almost consecutively at our observation. 81 of them suffered from MwA (79%), 9 from MWA (9%) and 13 were affected by mixed forms MwA-MWA (12%).

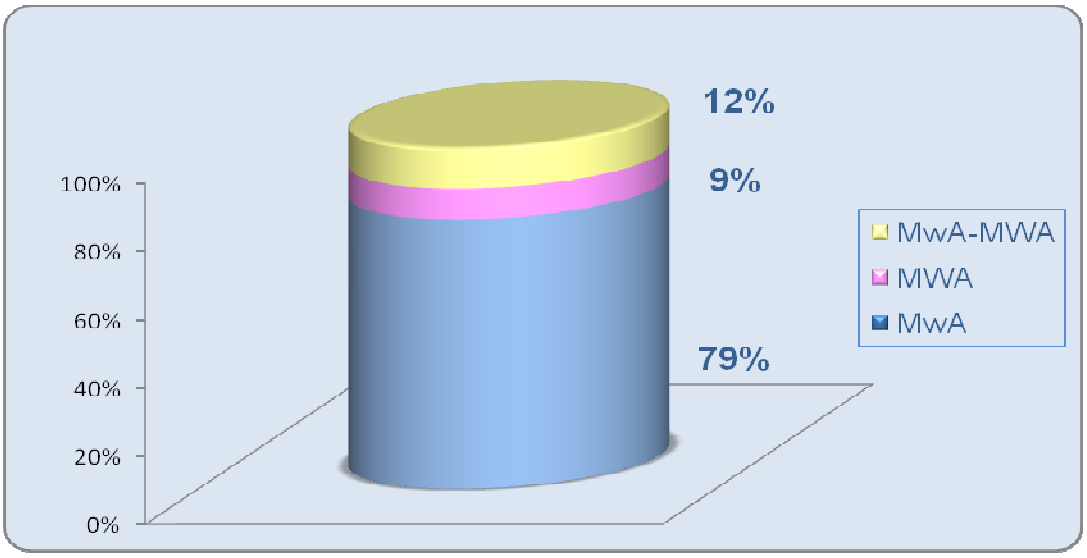

Figure 5: Distribution of different forms of migraine in the examined sample

40 patients (38.9%) referred that one relative at least suffered from an unknown form of headache and 7 (6.8%) had a positive familial history for vascular diseases.

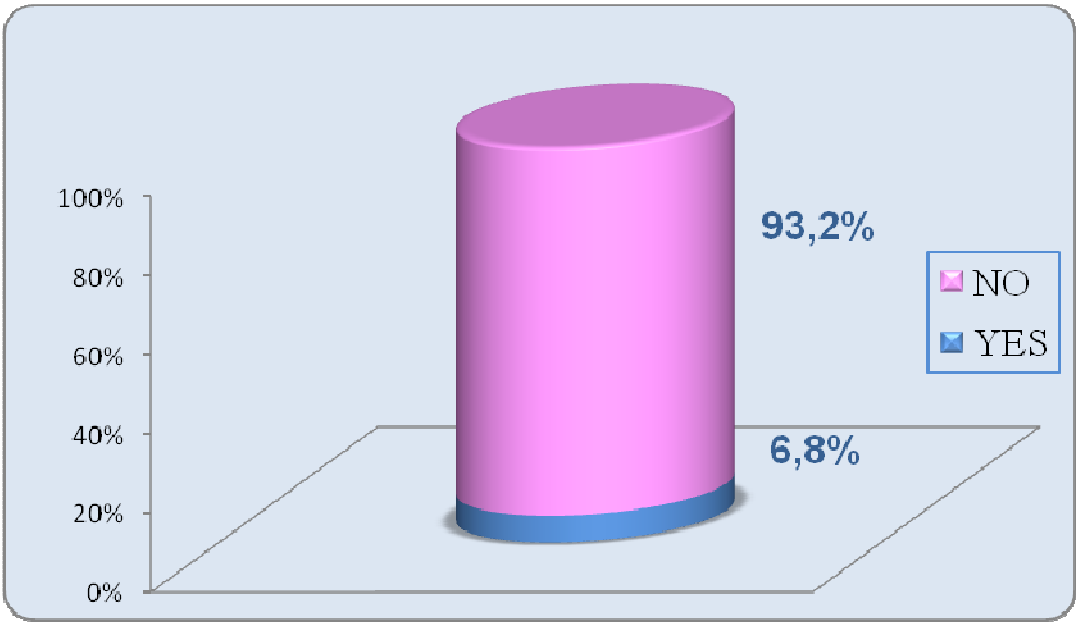

Figure 6: Familiarity for headache

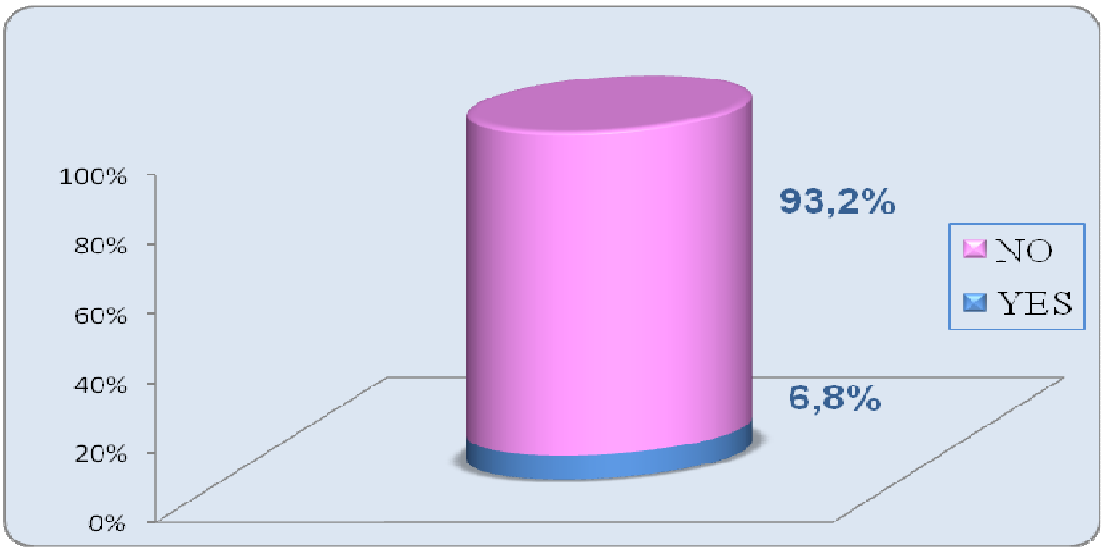

Figure 7: Familiarity for vascular events

MTHFR C677T mutation was found in 9 patients (9%), while the heterozygous genotype was found in 60 patients (58%), and the normal one in 34 patients (33%).

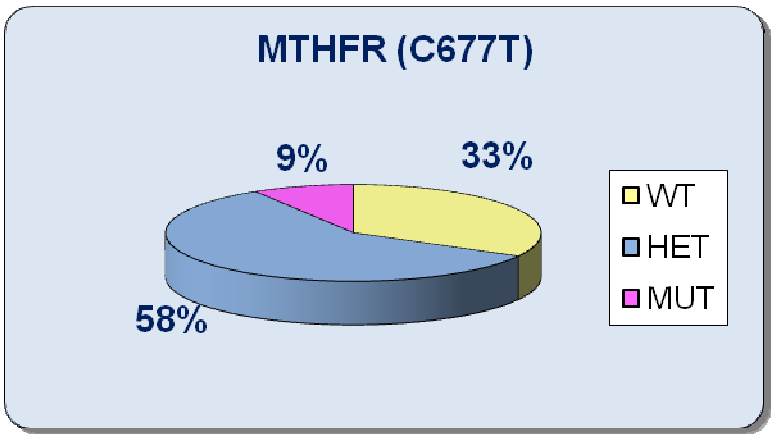

Figure 8: MTHFR (C677T) polymorphism in migraine group

7 patients (7%) were mutated for the A1298C variant of MTHFR, 54 (52%) were heterozygous and 42 (41%) were normal.

Finally, ACE polymorphism, evaluated on 101 patients (98%), was in its I/I form in 14 patients (13%), I/D in 45 patients (43%) and D/D in 42 patients (41%).

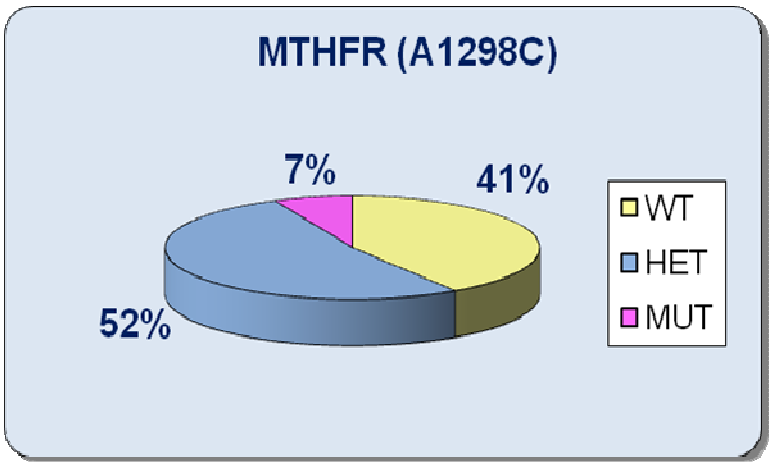

Figure 9: MTHFR (A1298C) polymorphism in migraine group

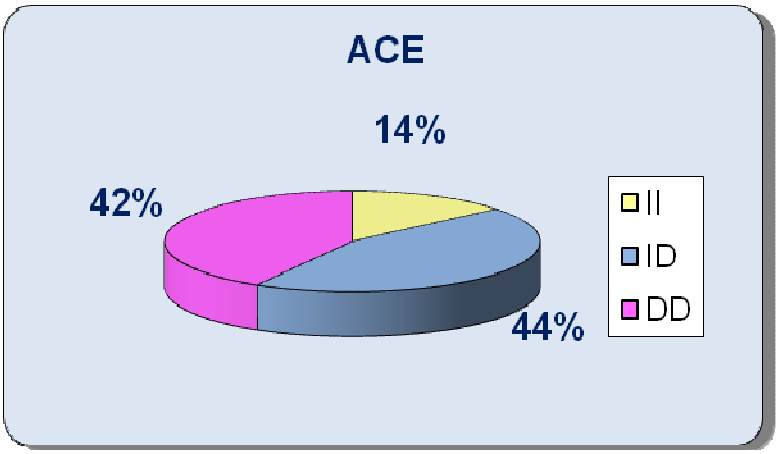

Figure 10: ACE polymorphism in migraine group

The control sample was composed by 336 patients suffering from Acute Myocardial Infarction (IMA), admitted, in the same period, at the Intensive Coronary Care Unit of S.Luca Hospital, in Vallo della Lucania. They underwent the same series of screening for genetic polymorphisms, and results were the following:

The evaluation of C677T variant of MTHFR identified 54 mutated (16%), 186 (56%) heterozygous and 95 (28%) normal patients. The result of 1 patient was unknown.

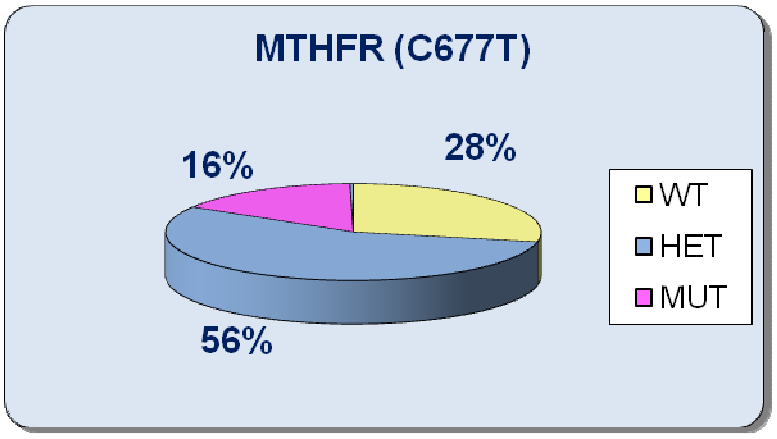

Figure 11: MTHFR (C677T) polymorphism in cardiopathic group

The normal genotype of MTHFR (A1298C) was present in 156 patients (46%); 146 (44%) were heterozygous and 33 (10%) were mutated. The result of 1 patient was unknown.

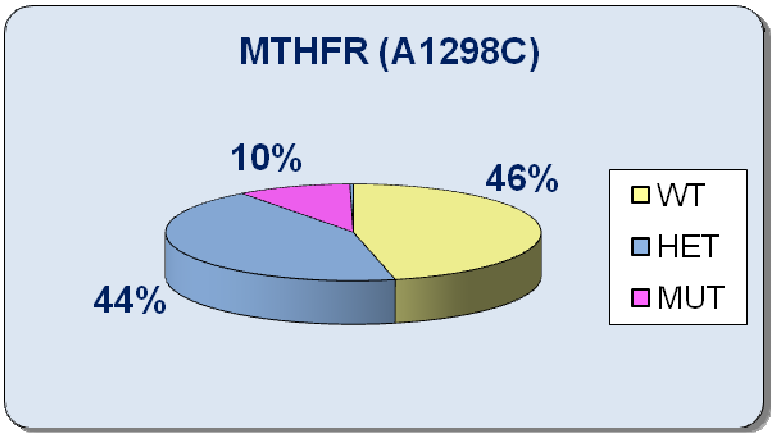

Figure 12: MTHFR (A1298C) polymorphism in cardiopathic group

ACE polymorphism, evaluated on 245 patients, was in its I/I form in 25 patients (10%), I/D in 133 patients (54%) and D/D in 87 patients (36%).

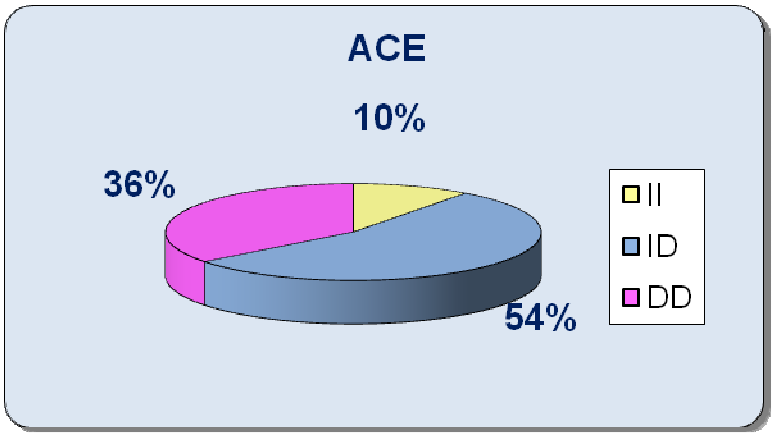

Figure 13: ACE polymorphism in cardiopahic group

A comparison of results is shown in the following graphs:

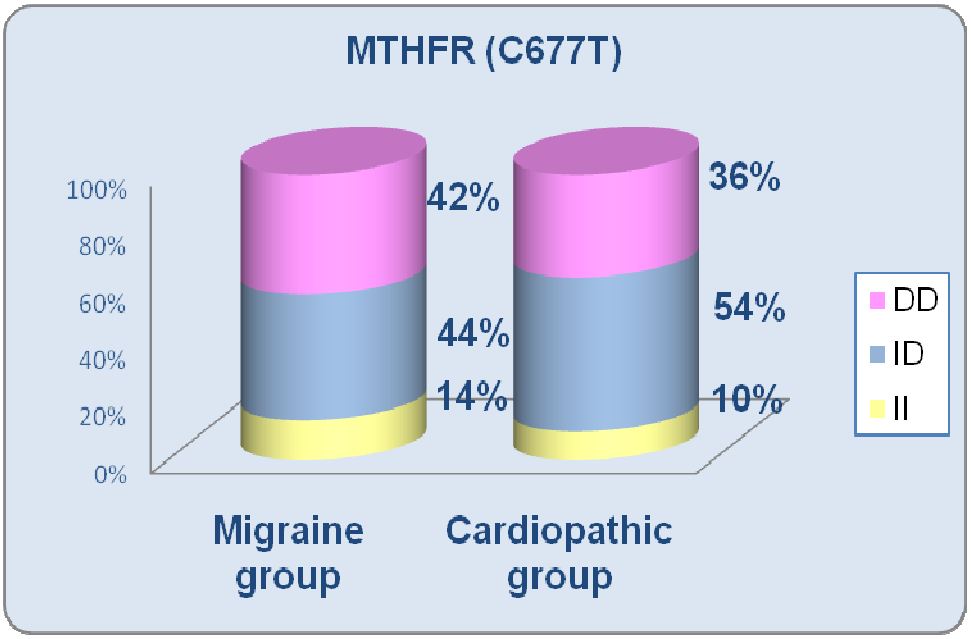

Figure 14: Comparison of MTHFR (C677T) polymorphisms

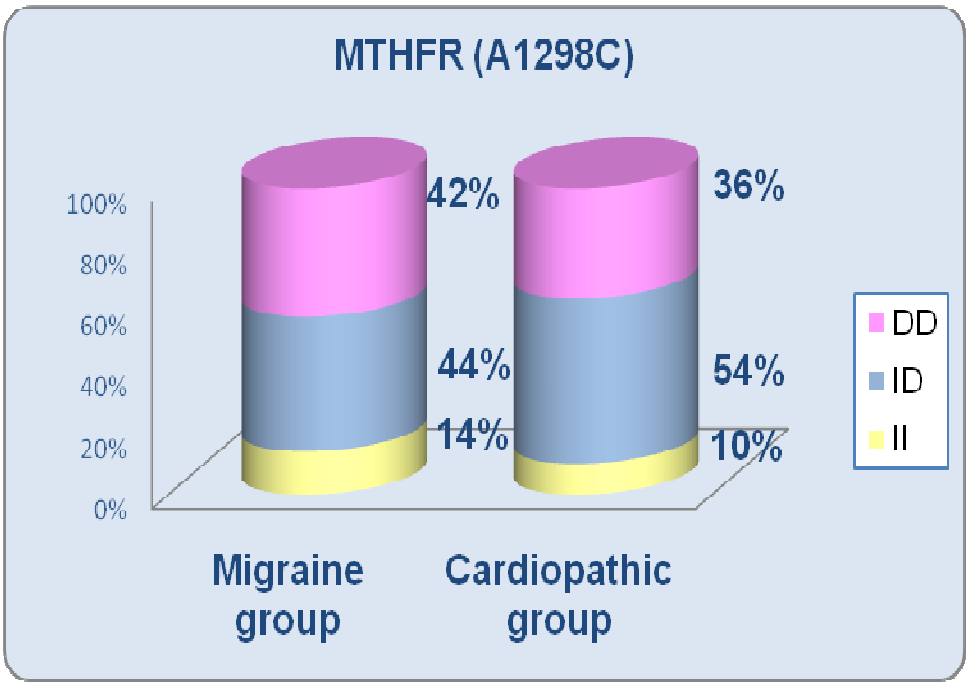

Figure 15: Comparison of MTHFR (A1298C) polymorphisms

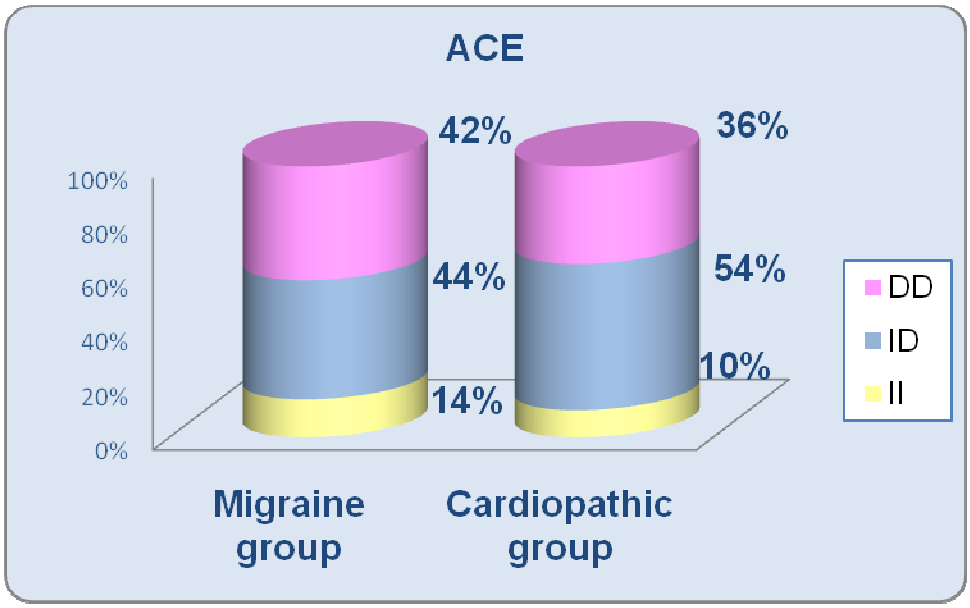

Figure 16: Comparison of ACE polimorphisms

Conclusions

Migraine is a common neurovascular disorder. During the last decade, many clinical studies have given evidence of an association between migraine, particularly migraine with aura, and ischaemic stroke. There are several pathophysiological mechanisms implicated in the genesis of ischaemic events in migrainous patients **[6]**.

According to Moskowitz theory, trigeminovascular neurons release substance P and other neurotransmitters in response to various triggers. Substance P is associated with vasodilatation, mast cell degranulation, increase in vascular permeability and edema of the meninges, events that configure the so-called "neurogenic inflammation". Excessive trigeminal discharge and neurovascular inflammation of the meninges ensue in migraine headache **[27]**.

Recent studies assess the role of migraine as a risk factor for endothelial dysfunction, responsible not only for a reduced availability of vasodilatators and for an increase of vasoconstrictor agents,

but also for a release of procoagulant, proinflammatory and proliferative factors, predisposing migraineurs to atherogenesis.

Endothelial dysfunction is due to an increate oxidative stress, promotor of inflammatory processes, proposed as implicated in the pathogenesis of migraine **[6]**.

High homocysteic acid levels, provoked by hyperhomocysteinemia, have marked excitatory effects on neurons; by acting as an endogenous agonist of NMDA receptors, in fact, homocysteic acid contributes to the excitability of CNS and has a predominant role in the initiation, propagation and duration of cortical spreading depression involved in migraine pathogenesis. It can furthermore, sensitize dura mater and cerebral arteries, and/or promote trigeminovascular system activation, predisposing subjects to migraine attacks or increasing their gravity **[27]**.

Among the potential mechanisms of hyperhomocisteynemia-induced vascular damage, very relevant is the hypothesis that reactive oxygen species are generated during this amino acid metabolism, and they're implicated in the development of atherosclerotic processes.

So there's a strict proportionality between plasma Hcy levels and its oxidant power, in addition to the correlation between homocysteinemia and Nitric Oxide production, a crucial risk factor for vasculopathies and at the same time implicated in migraine genesis **[28]**.

Several studies showed that Ang-II exerts important pro-inflammatory effects on vessel wall, inducing ROS, inflammatory cytokines and adhesion molecole production.

Ang-II increases blood monocytes migration and differentiation into macrophages, in atherosclerotic plaque; by interacting with AT_1 receptors, it stimulates NADPH-oxidase system and promotes ROS production in vascular cells and in macrophages, notely activators of cytoplasmic signaling cascades, such as NFkB and of other mechanisms increasing oxidative stress in vessel wall and leading to an activation of redox-sensitive genes, i.e. those encoding for proinflammatory cytokines (IL-6). Other mechanism by which RAS may induce the development of atherosclerosis include PAI-1 mediated thrombotic mechanisms and proinflammatory cytokines stimulation **[29]**.

Among genetic factors that increase susceptibility to oxidative stress and endothelial dysfunction, polymorphisms of ACE and MTHFR genes may, through their influence on plasma Ang-II and Homocysteine levels, respectively, play a key role both in migraine and in cardiovascular diseases pathogenesis.

Starting from these hypothesises, our study focused on the identification of ACE and MTHFR (C677T and A1298C) genotypes in a group of migraineurs and in a control group suffering from vascular diseases.

Our results showed essentially comparable frequencies of the three polymorphisms, thus confirming a common etiopathogenesis.

Table 2: Comparison of MTHFR polymorphisms

POLYMORPHISMS	MIGRAINE GROUP			STROKE GROUP		
	% WT	% HET	% MUT	% WT	% HET	% MUT
MTHFR (C677T)	33	58	9	28	56	16
MTHFR (A1298C)	41	52	7	46	44	10

Table 3: Comparison of ACE polymorphisms

POLYMORPHISM	MIGRAINE GROUP			STROKE GROUP		
	% II	% ID	% DD	% II	% ID	% DD
ACE ID	14	44	42	10	54	36

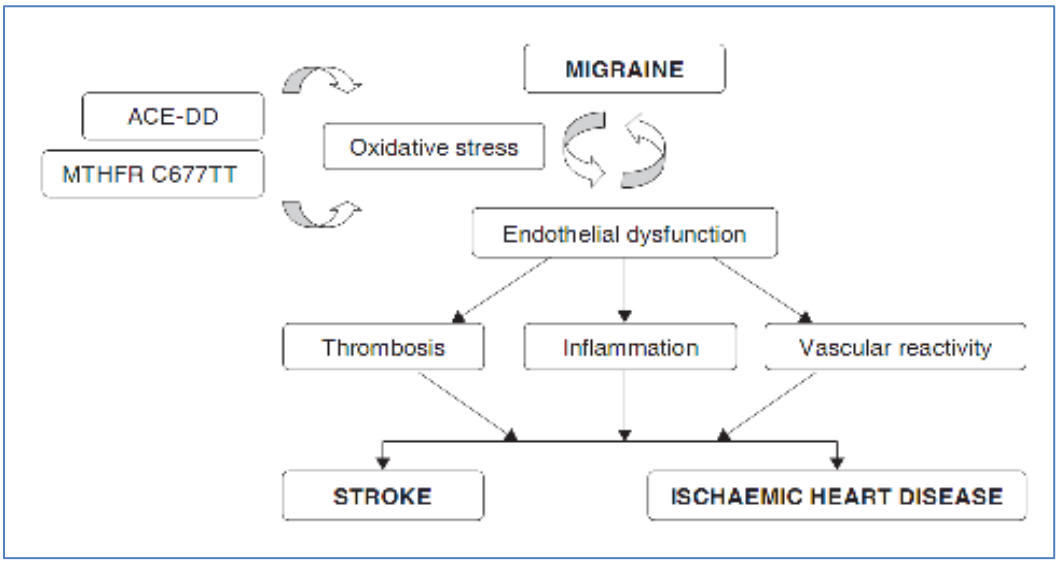

Figure 17: Potential mechanisms of stroke in migraine

On the basis of our data, the pathogenetic model of migraine was integrated with genetic polymorphism, for their capability to interfere with endothelial function.

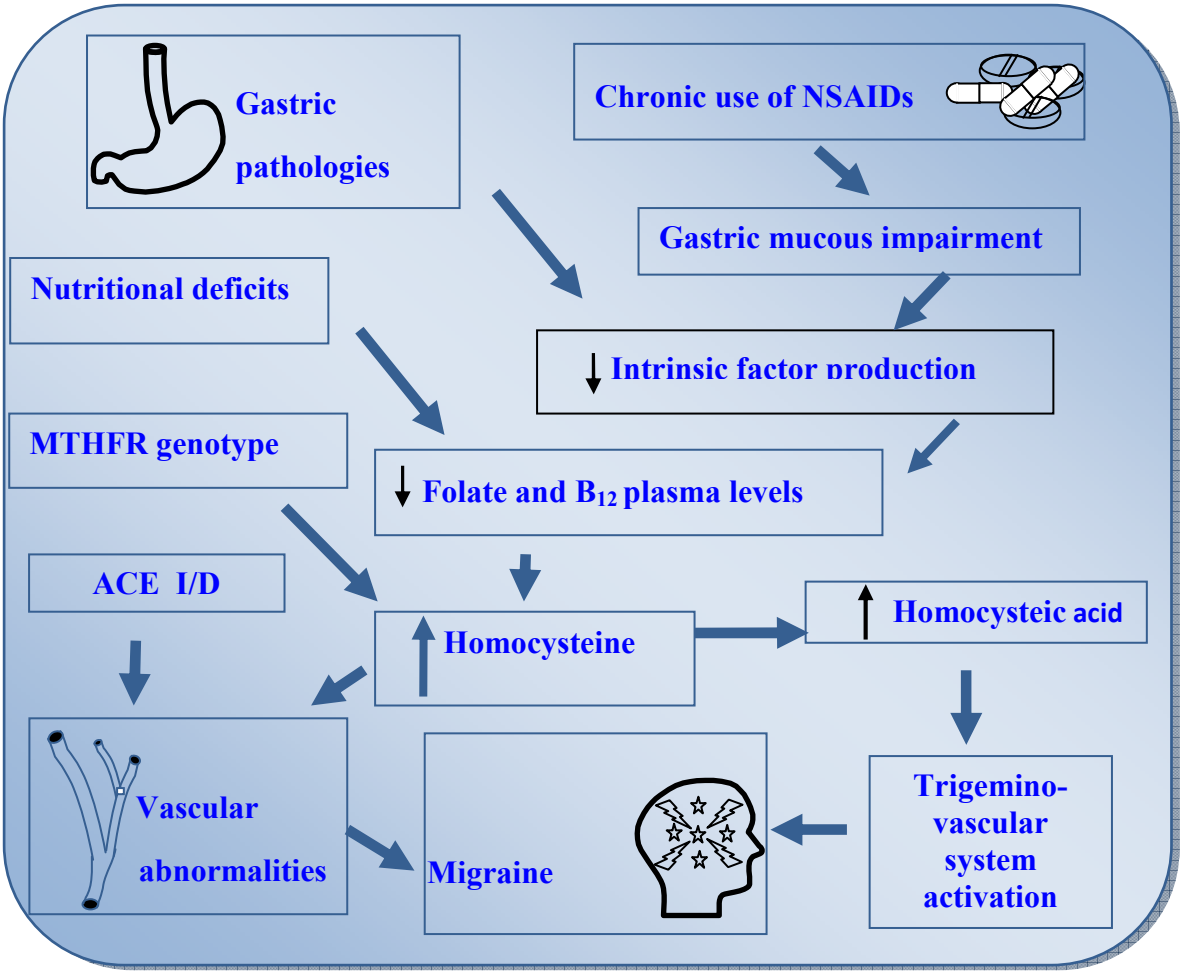

Figure 18: Integrated pathogenetic model of migraine

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