Vol. XXXI - No. 1 - January/March 2016 - Trimestrale - ISSN 0393 - 5264
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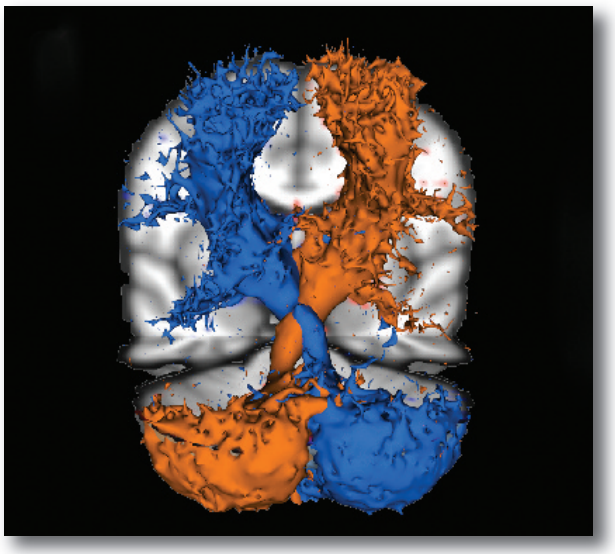

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Quarterly published by
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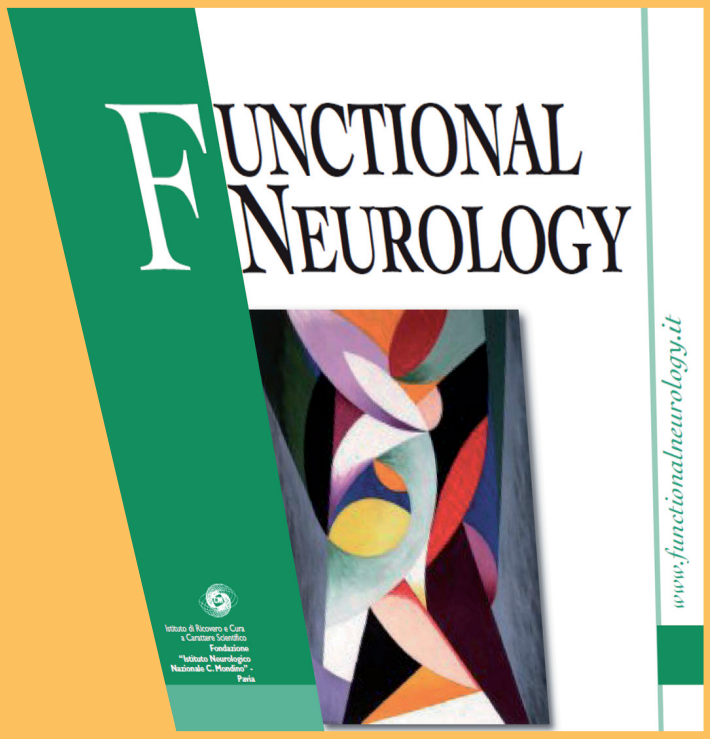
FUNCTIONAL NEUROLOGY *online*



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Publisher
CIC Edizioni Internazionali srl
Lungotevere Michelangelo 9
00192 Rome, Italy
Tel. +39 06 8412673
Fax +39 06 8412688
E-mail: info@gruppocic.it
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Cover: *Detail of the decussation area for left (orange) and right (blue) mean cerebello-thalamo-cortical pathways across 15 healthy subjects without threshold.*

Functional Neurology is supported by a grant from the C. Mondino National Institute of Neurology Foundation, IRCCS, Pavia, and by the University Consortium for Adaptive Disorders and Head pain (UCADH): Pavia, Varese, Rome "La Sapienza", Pisa, Ferrara, Novara, University of Calabria at Rende (Cosenza).

Contributions appearing in this journal are abstracted and indexed in: PubMed, PubMed Central, Journal Citation Reports, Neuroscience Citation Index, Science Citation Index-Expanded, Current Advances in Neuroscience, Medline - Index Medicous Physicians' Silverplatter Neurology, Proquest, Embase - Excerpta Medica, Data Base "Medicine", Research Alert, PsycINFO, The Academy of Sciences of the Russian Federation, Index Copernicus, Google Scholar, Scirus, Scopus, Web of Science, Scimago, DOAJ.

IMPACT FACTOR 2013: 1.855

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- Annual subscription (4 issues): Italy: € 70,00 - Other countries: € 90,00 - Institutional subscription: € 100,00
- One single current issue: € 17,50, $ 22,50; One back issue: double cost

L'IVA, condensata nel prezzo di vendita, è assolta dall'Editore ai sensi dell'Art. 74, primo comma, lett. C), D.P.R. 633/72 e D.M. 29.12.89

BUSINESS OFFICE: CIC Edizioni Internazionali srl - Lungotevere Michelangelo, 9 - 00192 Rome, Italy

ISSN 0393-5264

Autorizzazione del Tribunale di Roma n. 221/85 del 19-4-1985

N. di registrazione al R.O.C.: 6905/71821

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functional neurology
and new trends in interventional neurosciences

AIMS AND SCOPE

The rapid development of the neurosciences creates a need for continuous updates over a wide range of disciplines, ultimately converging towards a general understanding of the working of the central nervous system (CNS) and of its possible mechanisms of impairment and recovery. For this reason *Functional Neurology* has introduced an E-pub ahead of print system.
Experimental and clinical studies are making enormous progress and novel findings are published in specialized journals practically every day. However, because of the intrinsic multi-level organization of the CNS and the corresponding variety of experimental approaches, it is often difficult to set these numerous investigations in a coherent framework.
We feel that it is urgent to develop an integrative perspective in order to reinforce the links between experimental and clinical studies, and thus favor transitions from basic to applied research, and from the laboratory to the clinic, and vice versa. *Functional Neurology* intends to provide an ideal interdisciplinary platform for new developments in brain science, with special attention to frontier technology and translational research.

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Decision making in MS: factors affecting engagement in treatment choices

Multiple sclerosis (MS) is an autoimmune disorder of the central nervous system, and the most common chronic neurological condition affecting young and middle-aged adults. The majority of people diagnosed with MS (85%) will initially experience a 'relapsing-remitting' course, with periods of increased symptom activity followed by full or partial resolution (Ebers, 2001). Over time, physical, cognitive and sensory function can worsen. Disease-modifying therapies (DMTs) have been found to be effective in relapsing-remitting MS, reducing new lesion formation and disease progression (Kieseier et al., 2011). The number of DMTs available for MS has increased over the past 20 years, and each offers a distinct side-effect profile and, potentially, differences in clinical benefit. Complex decisions therefore need to be made not only about whether to start treatment, but also which treatment to choose.

Involving patients in treatment decisions is increasingly encouraged (e.g. Department of Health, 2005; Institute of Medicine, 2001) and has been linked to a number of positive outcomes. It can improve clinical outcomes, leading, for example, to better treatment adherence and possibly better health outcomes (Hibbard and Greene, 2013), reduced healthcare consumption, with fewer diagnostic tests and referrals, and decreased use of healthcare services (Rieckmann et al., 2015). From the perspective of those with MS, shared decision making can improve satisfaction with treatment (Little et al., 2001) and MS knowledge (Stacey et al., 2014). Yet, despite these benefits, some choose not to engage in decision-making processes.

In a neat, clear study in this issue of *Functional Neurology*, D'Amico and colleagues examine the role of clinical and demographic variables in patient preferences regarding engagement in treatment decisions. Consecutive newly diagnosed people with MS were invited to take part, and 100 were enrolled. Each indicated whether they preferred active involvement, collaborative involvement or passive involvement in medical decision making. Sixty wanted either an active or a collaborative role in their treatment decisions. The 25 people preferring to be actively involved had greater physical disability (as rated using the EDSS) and had experienced more relapses than the other two groups. This was in line with the view that those who experience more disease activity may become more involved in their own healthcare. No group differences were seen in age, disease duration or years of education.

After identifying the factors predicting engagement, we then need to consider how people with MS can make the best, most informed treatment decisions. In this, they can be supported through patient information and educational initiatives (including the excellent patient-focused treatment information provided by many national MS societies) and effective clinician-patient communication (Coulter, 2012). Greater understanding of how MS affects risk evaluation is also becoming increasingly important. Previous work has suggested that some people with MS, particularly those with secondary progressive MS or with greater physical or cognitive impairment, may show decision-making deficits, and so may struggle, for example, to adjust to different levels of risk (Kleeberg et al., 2004; Muhlert et al., 2015; Radomski et al., 2015). This may have implications for adherence, as those who fail to adhere to DMTs tend to devalue treatment efficacy and inflate treatment risks (Bruce et al., in press). Evaluation of risk/benefit ratios of treatment in MS can however improve following educational programs (Heesen et al., 2011). Further work is needed to assess whether those most likely to experience MS-related changes in risk perception benefit most from these programs, and what influence they have on treatment adherence and patient engagement in the long term.

In summary, the findings by D'Amico et al. help to indicate those most likely to engage in treatment decisions. In addition, they shed light on those who could receive more information about the benefits of engaging at an early stage. Risk evaluation may be affected at later stages in the disease. Combined, these studies suggest that educational interventions aimed at those with low activity early in the disease course, or that focus on optimizing risk evaluations later in the disease course, may prove effective strategies to improve patient engagement and patient outcomes.

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Amyotrophic lateral sclerosis and environmental factors

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Summary

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder that affects central and peripheral motor neuron cells. Its etiology is unknown, although a relationship between genetic background and environmental factors may play a major role in triggering the neurodegeneration. In this review, we analyze the role of environmental factors in ALS: heavy metals, electromagnetic fields and electric shocks, pesticides, β-N-methylamino-L-alanine, physical activity and the controversial role of sports. The literature on the single issues is analyzed in an attempt to clarify, as clearly as possible, whether each risk factor significantly contributes to the disease pathogenesis. After summarizing conflicting observations and data, the authors provide a final synthetic statement.

KEY WORDS: ALS, BMAA, electromagnetic fields, metals, pesticides, sports.

Introduction

Amyotrophic lateral sclerosis (ALS) is a relentless neurodegenerative disease that affects the corticospinal tract, leading to upper and lower motor neuron death. It is a rare condition, having a mean incidence rate of 2.8/100,000 in Europe and 1.8/100,000 in North America, and a mean prevalence rate of 5.40/100,000 in Europe and 3.40/100,000 in North America (Chiò et al., 2013). The observation, from the first decades of the twentieth century, of some high-prevalence areas (the island of Guam in the West Pacific and the Kii Peninsula in Japan) led to the suspicion that an environmental cause could be involved in ALS pathogenesis.

The majority of cases are sporadic (SALS), while 5 to 10% are familial (FALS).

Muscle weakness and hypotrophy, fasciculations and cramps, variously associated with pyramidal signs, such as spastic hypertonus and hyperreflexia, are the main clinical manifestations. Patients can also develop bulbar signs, like dysarthria, dysphagia and respiratory weakness. Electromyographic findings allow confirmation of the diagnosis.

Non-motor signs, especially behavioral disturbances and dysexecutive impairment, can also be present, and frontotemporal dementia (FTD) can be found in up to 15% of cases.

ALS includes different phenotypes, defined by various combinations of upper and lower motor neuron involvement, and by heterogeneous symptom distribution (spinal and/or bulbar): classic, progressive muscular atrophy, primary lateral sclerosis, progressive bulbar palsy, flail arm syndrome, flail leg syndrome (Table I).

The prognosis is typically severe, with a mean survival of 2 to 4 years from onset (Chiò et al., 2013), which may be even worse in bulbar forms, particularly in those with early respiratory involvement (median survival: 1.5 years).

Unfortunately, the therapeutic prospects are not encouraging, as the only drug that demonstrates survival benefit is riluzole. This molecule counters the excitotoxic damage in ALS by inhibiting persistent Na(+) current and thus repetitive firing. Moreover, enhancement of calcium-dependent K(+) current and inhibition of neurotransmitter release and of fast Na(+) current have been reported (Bellingham, 2011).

Despite huge interest in the etiology of this disease, it remains unknown. Familial cases allowed the identification of gene alterations implicated in the neurodegeneration; these have occasionally been detected in sporadic cases, too. Several genes associated with ALS have been identified; of these SOD1, FUS/TLS,

TARDBP-43 and the recently described C9orf72, are the most frequent. Mutation of C9orf72, an intronic hexanucleotide repeat expansion in open reading frame 72 on chromosome 9, occurs in about 30% of FALS and 5% of SALS, making it one of the most important associated genes. It is also present in 25% of familial FTD cases, strengthening the hypothesis that classical ALS and FTD represent the extremes of a single spectrum (Bäumer et al., 2014).
ALS can reasonably be considered a multifactorial disease, in which the interaction between genetic background and external factors is thought to play major role. Our aim in this review is to focus on environmental factors that have been associated with ALS. We evaluate the role of exposure to heavy metals, pesticides, β-N-methylamino-L-alanine (BMAA), and physical activity (professional football in particular) as risk factors for ALS.

Heavy metals

The role of exposure to heavy metals, such as lead, selenium, mercury, cadmium and iron, as a risk factor for ALS has long been studied, and the results produced are contradictory. The potential role of several heavy metals as contributors to molecular mechanisms leading to motor neuron degeneration has been widely explored but only partially characterized (Trojsi et al., 2013).
A recent systematic review analyzed 50 studies dealing with the relationship between metals and ALS and found only three studies that suggested an association. One study reported significantly higher ALS risk for individuals exposed to selenium (risk estimate 5.72, 95% CI 1.46–15.57). Another study concerning the effect of lead, mercury, aluminum, cadmium, chromium and manganese revealed no significant associations (Sutedja et al., 2009).

Heavy metals and genetic susceptibility

The hypothesis that host genetic background can modify the response to xenobiotics has long been postulated. Polymorphisms have been found in the genes encoding for delta-aminolevulinic acid dehydratase (ALAD) and for vitamin D receptor (VDR), while an impaired ability to detoxify pesticides and heavy metals, due to polymorphisms on metallotionein (MT), transcription factor (MTF-1) and glutathione synthetase (GSS) genes, was detected in several ALS patients (Morahan et al., 2007).
With regard to heavy metals, the ALAD and VDR genes may be the major ones involved in determining genetic susceptibility (Kamel et al., 2003).
ALAD (9q34) encodes for an enzyme implicated in heme group synthesis in erythrocytes and is the major Pb-binding protein inside these cells. A single nucleotide mutation, located in position 177 of the ALAD gene-coding region (K59N), is able to enhance the protein's affinity for lead and can alter the consequences of exposure to it.
Although a positive correlation seemed to emerge in the first studies (Kamel et al., 2003), a later paper (Fang et al., 2010) failed to detect the same correlation.
Vitamin D can influence lead absorption and distribution. Even though various studies have attempted to validate this assumption, the evidence in the literature does not confirm the involvement of the VDR gene (12q) in a genetic susceptibility to lead in ALS (Kamel et al., 2003).

Heavy metals: conclusion

Lengthy and in-depth study of the relationship between Pb exposure and genetic susceptibility has given conflicting and inconclusive results. There has been considerable interest in the possible role of sele-

Table I - Genotype-phenotype correlations in ALS.

	Clinical findings	Prognosis: median survival time
Classic ALS	Onset in upper or lower limbs; presence of pyramidal signs with lower motor neuron signs	2.5 years (Diamanti et al., 2013)
Progressive muscular atrophy	Selective involvement of lower motor neurons	7 years (Diamanti et al., 2013)
Primary lateral sclerosis	Selective involvement of upper motor neurons	More than 10 years (Diamanti et al., 2013)
Progressive bulbar palsy	Involvement of bulbar upper and lower motor neurons with dysarthria, dysphagia, tongue wasting; no peripheral spinal involvement for the first 6 months	2 years (Diamanti et al., 2013)
Flail arm	Involvement of upper limbs, predominantly proximal; no other district involved for the first 12 months	4 years (Diamanti et al., 2013)
Flail leg	Involvement of lower limbs, predominantly distal	3 years

nium, particularly in South Dakota and in Northern Italy (Reggio Emilia) on account of the presence of seleniferous drinking water in these areas (Trojsi et al., 2013). Evidence based on animal and cell culture studies confirmed that mercury is involved in ALS pathogenesis, however, epidemiological case-control studies showed no relationship between Hg exposure and ALS incidence (Trojsi et al., 2013).
Likewise, there exists *in vivo* but not epidemiological evidence of neurotoxic properties of cadmium and iron in the human population.

Electromagnetic fields and electric shocks

A remarkable number of articles suggest a relationship between neurodegeneration and occupational or residential exposure to electromagnetic fields (EMFs), especially extremely low-frequency electromagnetic fields (ELF-EMFs), and electric shocks.
ELF-EMFs have frequencies ranging from 3 Hz to 3,000 Hz. Electricians, electrical and electronic equipment repairers, train drivers, telephone installers or repairers and machinists are constantly exposed to them (Zhou et al., 2012).
Håkansson et al. (2003) assessed the impact of ELF-EMFs in a cohort of Swedish engineering industry workers and highlighted an increased risk of Alzheimer's disease (relative risk, RR 0.4; 95% CI 1.4–11.7) and of ALS (RR 2.2; 95% CI 1.0–4.7). They suggested that further studies were needed.
Similar results were reported by Huss et al. (2014), comparing ALS mortality in 2000–2008 and job exposure to ELF-EMFs and electric shocks in the Swiss National Cohort.
In 2012 an interesting meta-analysis of seventeen studies (Zhou et al., 2012) found an increased risk of ALS in pooled studies (RR 1.29, 95% CI 1.02–1.62), and in case-control studies, with an odds ratio (OR) of 1.39 (95% CI 1.05–1.84), but not in cohort studies, in which the RR was 1.16 (95% CI 0.80–1.69). The authors affirmed that the RR value was not high enough to exclude the possibility of biases.
In 2014, Seelen et al. (2014) criticized previous studies and stated that they reported null findings, since they were based on registry data, and did not consider clinical data. They performed a large population-based case-control study collecting detailed phenotypic data to evaluate the relationship between ELF-EMF residential exposure and the risk of ALS. The study included 1,139 ALS patients and 2,864 frequency-matched controls, derived from a large population study performed in the Netherlands from January 2006 to January 2013. Distance between participants' residence and electromagnetic sources was used to estimate ELF-EMF exposure. Power lines were classified as high voltage (50 kV, 100 kV and 150 kV) and very high voltage (220 kV and 380 kV). The study found no increased ALS risk among people living near to ELF-EMF sources.
A recent study (Vergara et al., 2014) used 1991–1999 US mortality data to investigate the relationship between ALS and occupational exposure to electric shocks and magnetic fields. The results showed an association between ALS and electrical occupations, as suggested by previous studies. However, statistical analysis of the collected data demonstrated that occupational exposure to neither electric shocks nor magnetic fields could explain it.
A recent meta-analysis study (Capozzella et al., 2014) investigated ALS risk related to occupational exposure to physical (ELF-EMFs) and chemical agents (solvents, pesticides and heavy metals). The study analyzed 750 publications from the period 1980 to April 2013. As regards ELF-EMFs, only a low level of association was found.

Electromagnetic fields and electric shocks: conclusion

Residential and occupational exposure to EMFs and electric shocks has long been investigated as a possible risk factor for ALS. Despite encouraging initial results, recent evidence (Zhou et al., 2012; Seelen et al., 2014; Vergara et al., 2014) tends to deny this hypothesis.

Cyanotoxins

Exposure to the neurotoxic non-protein amino acid β-N-methylamino-L-alanine (BMAA) may play a role in neurodegenerative processes.
BMAA is produced across the cyanobacterial order and it has been detected in several aquatic and terrestrial environments worldwide, suggesting that it is ubiquitous.
Cyanobacteria produce several cyanotoxins, divided into neurotropic (e.g. BMAA) and hepatotropic, such as cycasin, whose carcinogenic potential is well documented. A considerable BMAA concentration is present in cycad seeds (Cycas micronesica), used by the Guam indigenous population to produce flour, even though Duncan and coworkers report that it is largely removed during the washing of the seeds (Duncan et al., 1990): these authors analyzed 30 flour samples and revealed that most of the neurotoxic amino acid (almost 87%) was removed during processing.
The hypothesis that BMAA may have a role in neurodegenerative diseases was initially based on reports of elevated rates of ALS, Parkinson's disease and dementia in the island of Guam, a finding which gave rise to the term Western Pacific ALS-Parkinson Dementia Complex (ALS-PDC). A remarkable incidence of these neurodegenerative diseases was first noticed there in 1904, and in 1945 the ALS-PDC incidence rate in Guam was 50 to 100 times higher than overall world incidence. Recent studies have shown a decreasing incidence rate in the area in the last four decades, even though it remains three times higher than the overall one (Banack et al., 2010).
Subsequently, *in vivo* experiments were developed: in

1987, Spencer and collaborators conducted one in which they fed BMAA (100 to 250 mg/kg) to macaques for 12 weeks; they observed numerous neurological symptoms, which suggested the presence of an active neurodegenerative process (Chiu et al., 2011).

In spite of these epidemiological data, this hypothesis was initially challenged because the amount of BMAA contained in the dietary flour of the Guam population was considered insufficient to trigger neurodegeneration.

However, the role of BMAA as a risk factor for ALS has since been reconsidered on the strength of the finding that various animals consuming cycad seeds, such as flying foxes, pigs and deer, contribute to biomagnification of BMAA through the food chain in Guam (Cox et al., 2003).

Moreover, the BMAA concentration at autopsy in the nervous tissues of deceased Chamorro indigenous people affected by ALS/PDC, together with its absence in indigenous people who died for other reasons, strengthens the hypothesis of a role for BMAA in ALS/PDC in Guam.

Increased brain levels of BMAA were later confirmed by Pablo et al. (2009), who also found similar BMAA levels in the brains of ALS patient in Florida, but not in the brains of healthy subjects. Pablo et al. (2009) evaluated BMAA in neuroproteins of post-mortem brain tissues of 13 ALS patients, 12 Alzheimer's disease patients, eight Huntington's disease patients and 12 controls who died of non-neurological causes. BMAA quantification was performed using the fluorescent high performance liquid chromatography method previously used to detect BMAA in ALS/PDC patients in Guam. Tandem mass spectrometric analysis was carried out to confirm the identification of BMAA in neurological specimens. The concentrations of BMAA were below what had been reported previously in Chamarro ALS/PDC patients, suggesting that BMAA can, in any case, considered a risk factor.

The latest *in vivo* studies confirm that BMAA can be incorporated into nerve cell proteins, causing misfolding, aggregates and cell death (Dunlop et al., 2013; Trojsi et al., 2013).

In an interesting review, Chui et al. (2011) summarized the principal mechanisms of action of BMAA: BMAA crosses the blood-brain barrier and, after reacting with bicarbonate ions, forms β-carbamate, which can bind to glutamate receptors and stimulate noradrenaline release. Moreover, this amino acid enhances oxidative stress through glutathione depletion and stimulates cytochrome-c release from the mitochondria, inducing cellular apoptosis.

Dunlop et al. (2013) state that "motor neurons, as they are post-mitotic cells, are particularly vulnerable to aggregated protein damage since they are unable to reduce it by distributing [aggregates] among daughter cells". Moreover, they state that misfolded proteins, resulting from misincorporation of non-protein amino acids, can be transmitted within the brain through a prion-type mechanism; this may trigger the neurodegenerative cascade. In the same study, Dunlop et al. (2013) analyzed the effects of BMAA exposure in cultured cells (fibroblasts, human neuroblastoma cell line and human endothelial cells), focusing in particular on the incorporation of BMAA into human proteins. They demonstrated that BMAA is incorporated by a protein synthesis-dependent mechanism, since the incubation of human cells with BMAA and a protein synthesis inhibitor significantly reduced BMAA incorporation. It was also found that incorporation of BMAA into cell proteins was inhibited in the presence of L-serine in a concentration-dependent manner, while the same effect was not observed with D-serine. The incorporation of BMAA into proteins induced apoptosis in neuroblastoma cells *in vitro*, an effect previously reported with other non-protein amino acids. Moreover, autofluorescence developed in perinuclear and cytosolic regions of the cells incubated with 300 mM BMAA, suggesting the presence of protein aggregation. This phenomenon was prevented by co-incubation with an equimolar concentration of L-serine.

The authors of a recent retrospective study (Masseret et al., 2013) evaluated BMAA exposure in ALS cases in whom the disease was diagnosed between 1994 and 2009 in the ALS center of Montpellier; their study included cluster analysis and evaluation of BMAA sources in this area. One significant ALS cluster was observed in the area surrounding the Thau lagoon, one of the main areas of shellfish production and consumption on the French Mediterranean coast: 68 cases were detected there (as opposed to 33.7 expected cases), leading to a standardized incidence ratio (SIR) of 2.02 and RR of 2.24 (p=0.0024). The BMAA concentration in mussels and oysters was also measured and it was found that the level was higher during the summer, when cyanobacteria, considered the source of this amino acid, bloom.

BMAA: conclusions

BMAA remains a possible, non-proven risk factor for ALS/PDC in Guam. Although there is some *in vivo* evidence that validates its neurotoxic properties, epidemiological evidence is substantially lacking and further investigations are necessary to prove the role of BMAA in sporadic ALS throughout the world.

Pesticides

The main classes of pesticides are insecticides, fungicides, herbicides and rodenticides. Exposure to these substances occurs frequently, and not only in professional farmers, through the oral, dermal and inhalation routes. Some of them, particularly organophosphate pesticides, can cause neurological damage, due to inhibition of acetyl cholinesterase, the enzyme responsible for terminating the biological activity of acetylcholine. Moreover, most of these chemical compounds are known for their ability to induce oxidative stress, mitochondrial dysfunction, α-synuclein storage and neuronal loss.

Interest in pesticides grew following the observation of an increased incidence of ALS among Gulf War veterans, albeit in the absence of sufficient epidemiological evidence on this population. Furthermore, pesticides seem to be implicated in the pathogenesis of other neurodegenerative disorders, particularly PD. Indeed, a higher incidence of neurodegenerative disorders, such as PD and ALS, among subjects from rural areas is documented in the literature (Sutedja et al., 2009).
In view of the above data and the well-known pro-oxidant property of pesticides, they feature among ALS environmental risk factors, as summarized in table II.
McGuire et al. (1997), in a population-based case-control study, evaluated ALS risk associated with agricultural chemicals, solvents and heavy metals, and reported an OR of 2.4 (95% CI 1.2–4.8). In the same paper they denied a higher risk of motor neuron disease associated with exposure to solvents and heavy metals. The same data also emerged from another cohort study, which investigated ALS mortality among employees of a chemical industry (Burns et al., 2001) (Table II).
Other case-control studies highlighted an increased risk associated with exposure to pesticides (Qureshi et al. 2006), revealing ORs of 1.57 for overall pesticides and herbicides, and 5.58 for industrial pesticides and herbicides (Morahan and Pamphlett, 2006).
Another prospective study (Weisskopf et al., 2009), which assessed ALS risk and pesticide exposure in a large cohort, detected only a slight but not statistically significant increased ALS risk.
The hypothesis of a relationship between pesticides and motor neuron diseases is also supported by other case-control studies (Bonvicini et al., 2010; Malek et al., 2012; Pamphlett, 2012).
A systematic review of the MEDLINE, EMBASE, CINAHL and Cochrane databases up to March 2007 found that seven out of 37 studies dealing with chemicals and pesticides had sufficient methodological and exposure assessment quality. A higher risk for motor neuron diseases was revealed by two studies, with risk estimates of 2.5 and 1.2 respectively (Sutedja et al., 2009).
Kamel et al. (2012) published an interesting paper describing their meta-analysis study of the involvement of pesticides in ALS and, in the second part, reporting data from a large cohort study. The meta-analysis revealed an association between overall pesticides and ALS, with an OR of 1.9. Nevertheless, on the basis of the cohort study data it was concluded that evidence suggesting an involvement of these chemical compounds in ALS pathogenesis was present, but not statistically significant.
A more recent meta-analysis (Malek et al., 2012), which evaluated 1,517 ALS deaths reported in a retrospective cohort study and 589 ALS cases reported in five case-control studies, and calculated the sex-specific pooled ORs, found a pesticides-related increased risk of ALS for male cases (OR 1.88).
A recent meta-analysis study, which analyzed 750 publications from 1980 up to April 2013, found an association between ALS and pesticides only in men, with a dose-response relationship (Capozzella et al., 2014).

Pesticides and genetic susceptibility

Several studies have underlined the importance of genetic predisposition to pesticide-induced damage and the long-term consequences of exposure to pesticides. There has been a growing interest in the role of the paraoxonase gene cluster, of which PON1 is the most intensively studied. It codifies for A-esterase paraoxonase-1, which is able to detoxify organophosphate pesticides. PON1 is able to hydrolyze organophosphate pesticides and this ability is largely dependent on its different genetic variants; indeed, some PON1 genetic polymorphisms may determine decreased detoxifying activity. Given this wide variation in hydrolytic activity, it is possible that PON1 mutation predisposes to ALS by reducing pesticide hydrolysis and promoting oxidative stress processes. Therefore this protein cluster has long been a focus of studies aiming to highlight its possible role in ALS susceptibility.
Slowik et al. (2006) reported a case-control study that investigated the presence of PON1, PON2 and PON3 polymorphisms in ALS cases and healthy controls and found an association between ALS risk and Q192R (PON1 gene) and C311S (PON2 gene) polymorphisms.
Ticozzi et al. (2010) stated that six studies showed an association between ALS risk and PON1 variants, even though a meta-analysis failed to detect this relationship. They studied 260 FALS patients, 188 SALS patients and 188 healthy controls; they found eight heterozygous rare variants from nine FALS and three SALS cases. To verify whether these SNPs were benign polymorphisms they genotyped each of the eight variants in 1,159 control DNA samples and in an additional 996 SALS DNA samples: five of the eight variants were not found in control samples, suggesting that they were linked with ALS pathogenesis.
A recent article (Gagliardi et al., 2013) showed that PON2 genes are down-regulated in the central nervous system in ALS patients compared with controls.
We demonstrated the involvement, in ALS susceptibility, of another gene cluster implicated in xenobiotic detoxification: the flavin-containing monooxygenases (FMOs). Indeed, FMO expression was generally higher in human spinal cord from ALS subjects than in control tissues and in the G93A transgenic mice model (Gagliardi et al., 2013, 2011). Moreover, we found a significantly higher frequency of two polymorphisms in the 3'UTR region of FMO1, exclusively in the female population, in SALS patients compared to controls ($p<0.01$) (Cereda et al., 2006), which suggests that FMO1 is involved in genetic susceptibility to sex-related pesticide-induced damage.

Pesticides: conclusion

Strong evidence indicates that pesticides play a role in ALS. This, in addition to the involvement of pesticides in other neurodegenerative diseases such as Parkinson's disease, leads to the conclusion that they are a reliable risk factor for neurodegeneration.

Table II - Pesticide exposure and ALS.

Authors and Setting	Design	Subjects/Materials	Methods	Results
McGuire et al., 1997 Dept of Epidemiology, School of Public Health and Community Medicine, Univ. of Washington, Seattle, USA	Population-based case-control study	ALS cases (n=174), diagnosed in 1990–1994, and age- and sex-matched controls (n=348)	Assessment of detailed lifetime job history for exposure to metals, solvents and agricultural chemicals	Agricultural chemicals: association with ALS risk in men (OR 2.4; 95% CI 1.2–4.8) but not in women (OR 0.9; 95% CI 0.2–3.8). Solvents and metals: no association with ALS.
Burns et al., 2001 Department of Epidemiology, Midland, USA	Cohort study	Male employees of the Dow Chemical Company from 1945 to 1994	Evaluation of mortality causes among employees exposed to the herbicide 2,4-dichlorophenoxyacetic acid (2,4 D)	ALS cases: n=3 Comparison with other company employees led to a RR of 3.45 (95% CI 1.1–11.11), thus suggesting a link between pesticide exposure and ALS risk.
Morahan & Pamphlett, 2006 Dept of Pathology, Univ. of Sydney, Australia	Case-control study	SALS cases (n=179) and age-, ethnicity-, sex-matched controls (n=179)	Questionnaire to verify exposure to solvent/chemical substances and to herbicides/pesticides	Solvent/chemical substances: OR 1.92 (95% CI 1.26–2.93) Overall herbicides/pesticides: OR 1.57 (95% CI 1.03–2.41); Industrial herbicides/pesticides: OR 5.58 (95% CI 2.07–15.06). Exposure to herbicides/pesticides shows a dose-response effect.
Qureshi et al., 2006 Neurology Clinical Trial Unit, Massachusetts General Hospital, Charlestown, USA	Case-control study	ALS cases (n=95), healthy controls (n=106)	Questionnaire to analyze risk factors	Significant risk associated with lead (p=0.02) and pesticide (p=0.03) exposure.
Weisskopf et al., 2009 Dept of Environmental Health, Harvard School of Public Health, Boston, USA	Prospective study	414,493 males and 572,736 females from the Cancer Prevention Study-II cohort of the American Cancer Society (period 1989–2004)	Assessment of exposure through questionnaire	617 ALS deaths during 5,473,411 person-years among men; 539 ALS deaths during 8,104,402 person-years among women RRs for <4 years, 4–10 years, >10 years of exposure to pesticides were 0.62 (95% CI 0.09–4.45), 1.92 (95% CI 0.71–5.19) and 1.48 (95% CI 0.82–2.67) respectively. Slight, not statistically significant, suggestion of increased risk of ALS mortality with self-reported exposure to pesticides.
Sutedja et al., 2009 Dept of Neurology, Institute for Risk Assessment Sciences, Division of Environmental Epidemiology, Utrecht, Netherlands	Systematic review	Systematic review according to MOOSE guidelines. Search was performed in MEDLINE, EMBASE, CINAHL and Cochrane databases up to March 2007	Inclusion criteria: 1) design: case-control or cohort study; 2) exposure: pesticides or metals; 3) outcome: sporadic ALS; 4) language: restricted to English, French, German, Dutch	7 of 37 studies dealing with chemicals and pesticides had sufficient methodological and exposure assessment quality. Significantly increased risk for pesticide exposure reported in two studies (risk estimate 2.5 and 1.2 respectively). Conclusion: pesticides are potential risk factors for ALS.

Table II *continued*

Bonvicini et al., 2010 Dept of Public Health Sciences, Univ. of Modena and Reggio Emilia, Italy	Population-based case-control study	ALS patients (n=41) diagnosed in 1995–2006, healthy controls (n=82)	Questionnaire to assess exposure to environmental factors	ALS associated with pesticides (RR 3.6; 95% CI 1.2–10.5); this association persisted after inclusion of potential confounders in the statistical analysis.
Kamel et al., 2012 National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA; Division of Neurology, Dept of Medicine, Duke University Medical Center, Durham, NC, USA	Meta-analysis + cohort study	Meta-analysis: published studies on exposure to pesticides as a group Cohort study: 52,394 private pesticide users and 32,345 spouses to evaluate risk associated with specific pesticides, using data from the Agricultural Health Study	Exposure to pesticides as a group: meta-analysis of published studies collected through December 31, 2011. Data were used to estimate ORs using mixed-model analysis of variance or fixed effects analysis of variance (in the event of study-to-study heterogeneity). Exposure to specific pesticides: questionnaire administration and assessment of ALS cases	Meta-analysis: pesticides as a group are associated with ALS (OR 1.9, 95% CI 1.1–3.1). Cohort study: no association with pesticides as a group, but association with organochlorine insecticides (OR 1.9; 95% CI 0.8–3.5), pyrethroids (OR 1.4; 95% CI 0.6–3.4), herbicides (OR 1.6; 95% CI 0.7–3.7) and fumigants (OR 1.8; 95% CI 0.8–3.9). None of these associations was statistically significant.
Pamphlett, 2012 Dept of Pathology, Univ. of Sydney, Australia	Case-control study	ALS patients (n=787), non-related controls (n=778)	Questionnaire to assess pesticide exposure	OR 1.77 (95% CI 1.30–2.39). This supports previous reports that exposure to pesticides enhances ALS risk.
Malek et al., 2012 Dept of Neurosciences, College of Medicine, Medical University of South Carolina, Charleston, USA	Meta-analysis	1,517 deceased ALS cases (males) from one retrospective cohort study, 589 ALS or motor neuron disease cases from five case-control studies	Calculation of sex-specific pooled ORs with random effect model	Evidence of an association of exposure to pesticides and ALS risk compared to controls: OR 1.88 for male cases (95% CI 1.36–2.61).
Malek et al., 2014 Dept of Neurosciences, College of Medicine, Medical University of South Carolina, Charleston, USA	Case-control study	ALS cases (n=66), age-sex- race-matched controls (n=66)	Occupational exposure to metals and pesticides assessed through questionnaire	Occupational exposure to pesticides led to an OR 6.95 (95% CI 1.78–23.77).

Abbreviations: ALS=amyotrophic lateral sclerosis; OR=odds ratio; RR=relative risk; 95% CI=95% confidence interval.

Physical activity and football

Physical activity and soccer have been postulated as risk factors for ALS, ever since an Italian prosecutor, Raffaele Guariniello, ordered an inquiry to investigate the main causes of death in soccer players. This inquiry was set up in response to the complaint of a football coach, worried about the use of illegal drugs among players. In a large retrospective cohort study, Belli and Vanacore (2005) considered 24,000 professional soccer players who had played, in the period 1960–1996, in Italy's three top leagues (A, B and C). They found a surprisingly high prevalence of ALS: eight cases among the entire cohort, giving a SPMR (standardized proportionate mortality ratio)[1] of 11.58.

Subsequently, several epidemiological studies attempted to confirm these findings, as summarized in table III. Chiò et al. (2005) performed a retrospective incidence study in a rigorously defined cohort of Italian professional soccer players, playing in *Serie* A and *Serie* B in the period 1970–2001. They found a total of five ALS cases during the 137,078 person-years follow-up (the number of expected cases for this period was 0.77); the consequent SMR was 6.5 (95% CI, 2.1–15.1), revealing a significant relationship between ALS and professional soccer activity. Moreover, a dose-response relationship between the duration of activity and ALS risk was observed. In a subsequent article (Chio et al., 2009), the follow-up was extended to include the period 2001–2006, and another three

[1]Ratio between the number of subjects who have died from a disease and the number of subjects expected to die from the same disease.

Table III - Soccer/football playing and ALS.

Authors and Setting	Design	Subjects/Materials	Methods	Results
Belli & Vanacore, 2005 Dept of Environment and Primary Prevention, Rome, Italy; National Center for Epidemiology, Surveillance and Health Promotion, Rome, Italy	Cohort study	24,000 Italian soccer players (1960–1996)	Ascertainment of causes of death in subjects included in studied cohort; ascertainment of ALS cases in studied cohort (n=8); calculation of SPMR	SMR 1158 (95% CI 672–1998). A high risk of ALS among Italian soccer players was observed.
Chiò et al., 2005 Dept of Neuroscience, University of Turin, Italy; Dept of Pharmacology and Biochemistry, University of Pavia, Italy	Cohort study	7,325 Italian professional soccer players (1970–2001)	Ascertainment of ALS cases through: death certificates obtained from ISTAT, archives of major ALS centers, self-reports by ALS patients or relatives (ALS cases n=5 versus expected cases n=0.77). Calculation of SMR	SMR 6.5 (95% CI 2.1–15.1). An association between ALS and football was found.
Valenti et al., 2005 Section of Medical Statistics and Epidemiology, University of L'Aquila, Italy; Neurology, Sant'Andrea Hospital, Rome, Italy; Dept of Neuroscience, Ancona, Italy; Institute of Molecular Medicine, 'La Sapienza' University, Rome, Italy	Case-control study	300 cases and 300 matched controls	Collection of exposure data (questionnaire); conditional logistic regression analysis to establish risk associated with the various exposures.	None of the exposure variables was significantly associated with risk of ALS.
Vanacore et al., 2006 National Center for Epidemiology, Surveillance and Health Promotion, Rome, Italy	Case report	Italian soccer player affected by sporadic ALS		
Abel, 2007 Department of Neurology, Wayne State University, USA	Cohort study	3,891 American soccer players who played after 1960	Retrospective analysis to detect ALS cases (n=8) and calculate ALS prevalence in this cohort	The prevalence was 206 cases per 100,000 versus 5 cases per 100,000 in the general USA population; a 40-fold higher prevalence in the cohort studied (p<0.001).
Wicks et al., 2007 Centre for Neurodegeneration Research, King's College, London, UK	Case report	Three amateur league soccer players from the same part of southern England developed ALS simultaneously.		
Chiò et al., 2009 Dept of Neuroscience, University of Turin, Italy; Dept of Physiological-Pharmacological Sciences, University of Pavia, Italy; Dept of Neurorehabilitation, Salvatore Maugeri Foundation, IRCCS, Pavia, Italy	Cohort study	Follow-up (period considered: 2001–2006) of the previously studied cohort of 7,325 soccer players (Chiò et al. 2005) plus basketball players (n=1,973) and cyclists (n=1,701)	Ascertainment of ALS cases from death certificates obtained from ISTAT, archives of major ALS centers, self-reports by ALS patients or relatives; calculation of SMR	ALS cases n=5 versus expected cases: 0.77. Soccer players: 3 new ALS cases vs expected cases: 1.24. SMR 6.45 (95% CI 2.70–12.70; p<0.00001); basketball players: no ALS cases (expected cases: 0.14); cyclists: no ALS cases (expected cases: 1.82).

Table III *continued*

Lehman et al., 2012 Centers for Disease Control and Prevention, The National Institute for Occupational Safety and Health, Division of Surveillance, Hazard Evaluations and Field Studies, Cincinnati, OH, USA	Cohort study	3,439 National Football League players who played between 1959-1988	Cohort divided into two groups: non-speed players and speed players. Ascertainment of neurodegenerative disorders (ALS-AD-PD) and comparison with the USA population (SMR). Internal comparison between speed and non-speed players used SRRs	SMR for neurodegenerative disorders as a group: 3.26 (95% CI 1.90–5.22); SMR for ALS: 4.31 (95% CI 1.73–8.87); SRR for neurodegenerative disorders as a group: 3.29 (95% CI 0.92–11.7); SRR for ALS: 3.88 (95% CI 0.47–32.2).
Savica et al., 2012 Mayo Foundation for Medical Education and Research	Cohort study	Male students who played football in Rochester high schools between 1946 and 1956 (n=512) versus a non-football-playing referent group of students (n=203)	Evaluation of neurodegenerative disorders through the Rochester Epidemiology Project. Comparison between football players and control group through SIR	ALS cases: 2 in football players, 1 in control group. HR: 0.52 (95% CI 0.05–5.68). SIR in football players: 3.15 (95% CI 0.38–11.33); SIR in control group: 6.44 (95% CI 0.16–35.7). American football does not increase ALS risk.
Huisman et al., 2013 Department of Neurology, Rudolf Magnus Institute of Neuroscience, University Medical Centre, Utrecht, The Netherlands	Case-control study	636 sporadic ALS 2166 controls	Questionnaire on lifetime history of occupations, sports and hobbies.	ALS patients had higher levels of leisure time physical activity compared with controls (OR 1.08, 95% CI 1.02–1.14, p=0.008). No significant difference for vigorous physical activities (marathon/triathlons/hard occupational activity).
Hamidou et al., 2014 Institute of Neuroepidemiology and Neurology, University of Limoges, France	Systematic review	Databases: PubMed (MEDLINE), SCOPUS, ScienceDirect, IngentaConnect, Refdoc, Cochrane Database.	Inclusion criteria: meta-analyses/literature reviews of epidemiological studies; editorials, letters to the editor; works in *in vitro*, *in vivo* or cell models; conference proceedings. 37 articles were considered.	Practice of sports and physical activity is not a risk factor for ALS (level A evidence). Football/soccer may be considered a possible risk factor for ALS (level C).

Abbreviations: ALS=amyotrophic lateral sclerosis; SPMR=standardized proportionate mortality ratio; SMR=standardized morbidity ratio; 95% CI=95% confidence interval; ISTAT=Italian Statistics Bureau; SRR= standardized rate ratio: SIR=standardized incidence ratio; HR=hazard ratio

ALS cases were found (SMR 6.45; 95% CI 2.70–12.70; p<0.00001). Moreover, the authors compared ALS incidence in soccer players with that recorded basketball players and cyclists: the fact that they did not observe ALS cases in the latter two cohorts strengthened the soccer/ALS hypothesis.

Subsequently, similar studies conducted in National Football League players in the United States (Abel, 2007; Lehman et al., 2012) confirmed an apparent increased risk of neurodegenerative disorders, in particular ALS, in this cohort. Nevertheless, a retrospective case-control study published in 2012 (Savica et al., 2012) failed to confirm the association.

The supposed relationship between football and ALS is also supported by several case-report studies (Vanacore et al., 2006; Wicks et al., 2007).

Valenti et al. (2005) reported a case-control study conducted to assess the trustworthiness of this association: 300 ALS cases and an equal number of matched controls were enrolled, and exposure data concerning physical activity were collected through a questionnaire. The results did not show a role for sports, particularly soccer, as risk factors for ALS.

A similar case-control study (Huisman et al., 2013) also failed to detect a statistically significant association.

In a recent article, Hamidou et al. (2014) reported a literature review conducted according to the Meta-analysis of Observational Studies in Epidemiology guidelines, in which the authors considered data drawn from six databases (PubMed, SCOPUS, ScienceDirect, IngentaConnect, Refdoc, Cochrane Database): of the 37 papers included, two (5.5%) provided class I evidence and five (13.5%) class II evidence. The others offered class III (21.6%), IV (43.2%) and V (16.2%) evidence. The results were stratified according to the type of exposure: i) occupation, ii) soccer and American football, iii) physical

activity related to sport and work, iv) proxies of physical activity (PA).
They found nine studies relating to soccer and American football: two class V, one class IV, four class III, one class II and one class I. They concluded that football may be considered a possible risk factor for ALS (level C evidence).
A literature review by Armon (2007) did not confirm the excess of ALS in Italian soccer players (level C evidence, two class III articles; the evidence level of the conclusions is based on re-analysis of the data, rather than on the class and level of the original methodology).

Possible risk factors in football players

The cause of this supposed association is not well-defined, but several hypotheses have been postulated to explain the epidemiological findings that seem to indicate its existence. Football players may be exposed to various risk factors that could contribute to neurodegenerative processes, namely excessive PA, repeated head injuries, exposure to pesticides and dietary supplements or illegal substances.

Heavy physical activity

It has been supposed that heavy physical stress could enhance the production of reactive oxygen species, leading to nucleic acid damage (Julien, 2001), particularly in association with dietary supplements (branched-chain amino acids, BCAAs) and drugs.
Several epidemiological investigations have been conducted on the basis of this assumption.
Veldink et al. (2005) in their case-control study denied any association between PA and motor neuron disease risk.
Huisman et al. (2013) found a weak association between leisure time PA and ALS (OR 1.08, 95% CI 1.02–1.14). "The lack of association with occupational PA and the absence of a dose-response relationship" led them to affirm that "not PA per se but rather a genetic profile or lifestyle promoting fitness increases ALS susceptibility".
Pupillo et al. (2014) recently published a European population-based case-control study, whose results allowed them to assert that PA is not a risk factor and may be considered protective against the disease.
In a recent literature review of six databases (PubMed, SCOPUS, ScienceDirect, IngentaConnect, Refdoc, Cochrane Database), Hamidou et al. (2014) stated that PA is not a risk factor for ALS (level A evidence).

Repeated head injuries

The possibility of a link between head trauma and ALS risk has been analyzed repeatedly over more than a century. However, retrospective case-control studies often offer only equivocal results, due to selection bias or because of imprecise definition of trauma, its gravity and temporal relationship with ALS onset.
This hypothesized link was examined in a meta-analysis study (Chen et al., 2007), which showed a moderately elevated ALS risk associated with a history of head injuries (OR 1.7; 95% CI 1.3–2.2).
Armon (2007) stated that head trauma is probably not a risk factor for ALS (level B evidence).
A recent European population-based case-control study (Pupillo et al., 2014) evaluated several environmental risk factors for ALS, including head trauma, and denied any association with motor neuron disease.

Exposure to pesticides and dietary supplements/illegal substances

Pesticides are widely employed on soccer and football fields. As previously seen (pesticide paragraph), pesticide exposure is involved in triggering neurodegenerative diseases, particularly in predisposed individuals. However, no epidemiological study has shown direct involvement of pesticides in increasing ALS among soccer and football players.
Exposure to food toxicants or to illegal substances used to enhance physical performance have been considered possible risk factors since the first study (Belli and Vanacore, 2005). Creatine monohydrate, growth hormone (GH) and BCAAs are the dietary supplements that have been the main focus of attention.
Creatine monohydrate supplements, often used by athletes, were initially thought to be capable of worsening motor neuron function, but experimental results disproved this hypothesis. Conversely, oral administration of creatine monohydrate in murine models has been shown to improve motor neuron performances and demonstrates oxidative stress reduction (Klivenyi et al., 1999).
In a multicenter double-blind study 107 ALS patients were randomized to receive either daily creatine monohydrate (5g/day) or placebo and were followed up for nine months, during which the authors monitored their clinical status and ALS functional rating scale scores. The results showed that creatine monohydrate did not significantly improve motor, respiratory or functional capacity. A trend toward improved survival in patients was noticed, as affirmed in another published report (Rosenfeld et al., 2008).
In a phase I study, Atassi et al. (2010) remarked that creatine monohydrate has been shown to be able to cross the blood-brain barrier. Indeed oral administration of creatine monohydrate,15g, to six patients recruited in the study was associated with increased *in vivo* brain creatine concentrations and decreased glutamate concentrations.
Growth hormone is often taken by athletes, alone or in association with other anabolic steroids, to increase muscle mass and strength. GH was not found to be dangerous for motor neuron survival; rather, it showed a trophic effect on the nervous system. Moreover, GH deficiency in ALS patients has been reported in the literature (Saccà et al., 2012; Pellecchia et al., 2010).
A deficiency in GH secretion similar to that seen in human ALS has been reported in hSOD1 (G39A) transgenic mice; moreover, a reduction in the expres-

sion of the IGF-1 receptor α-subunit in skeletal muscle and lumbar spinal cords suggested impaired signaling within these tissues (Steyn et al., 2012).
However, a recent clinical trial failed to detect clinical and survival improvement in ALS patients administered GH (Saccà et al., 2012).
Branched-chain amino acids (leucine, isolucine and valine) are widely used among athletes to stimulate muscle protein synthesis, improve physical resistance and reduce delayed onset muscular soreness. BCAAs were initially evaluated as a possible therapeutic option for ALS patients but the results obtained were discouraging (Testa et al., 1989).
They have therefore been investigated as risk factors for ALS. Piscopo et al. (2011) examined the effect of a diet enriched with BCAAs in C57Bl/6J mice and demonstrated that BCAAs are able to down-regulate the expression of some antioxidant genes and can alter oxidative stress pathways in the brain; the authors concluded that caution should be exercised in the use of these dietary supplements by athletes.
Venerosi et al. (2011) demonstrated that BCAA supplements can exacerbate motor deficits in G39A mice. Electrophysiological tests on mouse brain slices showed impaired synaptic function, but no increased glutamate toxicity. Although these results do not confirm a role for BCAAs in ALS etiology in mice models, they indicate a complex effect on the central nervous system, depending on genetic background, and suggest that they should be used with caution.

Physical activity and football: conclusions

The literature in this field shows that an increased ALS incidence may be present only among soccer players, while professionals from other sports do not demonstrate a higher risk for ALS. Analysis of single risk factors for soccer players has given negative results. A potentiating effect of a number of risk factors acting together cannot be excluded in soccer players. Studies on the effect PA in the general population show that it is not a risk factor and it may be considered protective against the disease.

Concluding remarks

Despite remarkable progress in ALS genetic and environmental studies, the pathophysiological mechanisms responsible for the neurodegeneration remain unclear. Environmental risk factors for ALS, on account of their variety and heterogeneity, are an intriguing research field. However, the identification of such risk factors is difficult for several reasons. Indeed, environmental factors are numerous and exposure to them should be monitored for a long time; instead, previous studies concerning dietary xenobiotics have considered only limited periods of time. Furthermore, this type of research, which generally requires long and detailed interviews (sometimes with the assistance of medical staff), is time-consuming. It would take a considerable investment, over a long period of time, to reach a high number of recruited subjects, as seen in genetic studies. Finally, there is a growing interest in genetic susceptibility to toxicant-induced damage; indeed, it is believed that results cannot be interpreted correctly without knowledge of the genetic background.
In this context, we analyzed the literature on environmental factors in ALS to evaluate the reliability of epidemiological data and *in vivo* experiments. In spite of great interest over several decades, evidence of a role for heavy metals in ALS pathogenesis appears incomplete, as shown in previous systematic reviews (Sutedja et al., 2009).
We evaluated residential and occupational exposure to electromagnetic fields and electric shocks as a possible risk factor for ALS. Despite encouraging initial results, recent evidence (Zhou et al., 2012; Seelen et al., 2014; Vergara et al., 2014) does not seem to support this hypothesis.
Evidence emerging from the literature seems to be strengthening the assumption of an involvement of pesticides in ALS neurodegeneration. Indeed, we can affirm that pesticide exposure is the only environmental factor for which the literature evidence is less conflicting. Moreover, pesticides are also considered to be risk factors for other neurodegenerative diseases (Alzheimer's and Parkinson's disease), as confirmed in several epidemiological studies.
On the strength of these epidemiological data, in addition to the known pro-oxidant properties and long-term neurotoxicity of pesticides (reminiscent of the pathophysiological mechanisms hypothesized in ALS), pesticide exposure can be considered one of the most reliable risk factors for ALS.
β-N-methylamino-L-alanine also shows important neurotoxic properties, as highlighted in several *in vivo* studies. Guam cluster analysis reveals that this non-protein amino acid may be responsible for ALS/PDC clusters, as confirmed by the detection of high levels of BMAA in the brains of deceased ALS/PDC patients. Moreover, the demonstration of BMAA biomagnifications through the food chain, and of its worldwide presence – it has been found in Florida, the UK and France – makes it ubiquitous and suggests that it should be investigated in further epidemiological studies. Despite encouraging results from *in vivo* studies, epidemiological evidence remains scarce.
The first reports of young football players with ALS prompted considerable media coverage and led to further investigations in this field. However, the latest study (Hamidou et al., 2014) failed to detect any association between football and ALS, and the same conclusion was drawn in another paper (Armon, 2007). Moreover, intense physical activity, dietary supplements and head injuries have emerged as unreliable risk factors among soccer players.
In conclusion, the influence of environmental factors in ALS is not yet completely defined and, for most of them, is truly slight or null. Instead, genetic research on ALS is discovering more and more genes involved in FALS cases and in a growing amount of SALS patients. Most of the genes have been found to be

involved in possible pathophysiological pathways and it can be hypothesized that ALS is the final common result of a single gene mutation or of polygenic pathogenetic pathways. The relationship between genetic background and xenobiotics may be the avenue to pursue in order to clarify the influence of environmental factors in ALS.

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Disability may influence patient willingness to participate in decision making on first-line therapy in multiple sclerosis

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Summary

Patient autonomy is a concept that implies variable degrees of patient participation in different aspects of health and healthcare, including the choice of therapy. This study, conducted in patients with multiple sclerosis (MS), examined several factors in relation to the patient's role in the therapeutic decision-making process.
One hundred newly diagnosed patients with MS attending their first ever specialist consultation at the MS center of Catania, Italy, were consecutively enrolled in a single-center, open, observational study. Clinical and demographic data were collected as part of this routine first consultation. Through administration of the Control Preferences Scale, we ascertained the patients' willingness to participate in the decision-making process on their first-line treatment, classifying them, on the basis of their attitude, as passive, collaborative or active.
Of 100 patients with MS, 40 had a passive attitude, while 35 were willing to collaborate and 25 wanted to play an active role in the decision-making process. The patients showing an active attitude had a significantly higher Expanded Disability Status Scale score and a significantly higher number of relapses ($p<0.5$ for both) than those who showed other attitudes.
Persons with MS prefer to know the benefits and risks related to the first-line treatment. Those with higher disability prefer to be active in the decision-making process.

KEY WORDS: first ever consultation, multiple sclerosis, multiple sclerosis therapy, shared decision-making process

Introduction

Patient-centered care is a complex approach within the healthcare system that focuses on the provision of care that is respectful of and responsive to individual patient preferences, needs and values, and which seeks to ensure that patient values guide all clinical decisions (Wilson et al., 2014). Shared decision making (SDM) is a cornerstone of patient-centered care: health decisions should be made jointly by the health professional and the patient, and they should be based on the best available evidence and on patient values (Charles et al., 1997). In recent years, increasing evidence has shown that patient-centered care and SDM are associated with increased patient satisfaction and empowerment, less decisional conflict, less treatment non-compliance, less medical litigation, and a stronger patient-physician alliance (Heesen et al., 2007).
Multiple sclerosis (MS), a chronic disease of the central nervous system that typically occurs in young adults, and affects women two to three times more frequently than men, is a paradigmatic disease for the study of patient-centered care and SDM, because patients with MS are highly aware of their disease (Wilson et al., 2014; Heesen et al., 2013).
We assessed the willingness of newly diagnosed patients with MS to participate in the treatment decision-making process.

Materials and methods

From January 2013 to February 2014, a total of 115 patients with MS were consecutively admitted to the tertiary MS center of Catania, Italy, for a first ever MS consultation. At the time of the consultation, all the patients were naive to any licensed therapy for MS (Wingerchuk and Carter, 2014). MS was diagnosed according to the 2005 McDonald criteria (Polman et al., 2005). Patients eligible for inclusion in this study were aged 18 years or older and able to understand and give informed consent.
As part of the first consultation, clinical and demographic data, including details of education and employment status, were collected. Participating patients were administered the Control Preferences Scale (CPS). The CPS was developed to evaluate the

preference of an individual regarding his/her involvement in healthcare decisions (Degner et al., 1997). It consists of five cards, each illustrating, through a cartoon and a short descriptive statement, a different role in decision making: the examiner asks the subject to choose his/her preferred card, which is then covered up; the examiner then asks the subject to choose his/her preferred card from the remaining four cards. The procedure continues (for a total of four choices) until one card is left. If the second preference is incongruent with the first (non-adjacent pairing, such as card A with card C), then the test is explained again, and re-administered. In the event of further incongruences the test is abandoned. The test has six possible results, based on the person's two most preferred roles. These results are collapsed to: "active'' (active-active or active-collaborative), "collaborative" (collaborative-active or collaborative-passive), or "passive" (passive-collaborative or passive-passive). We used the Italian version of the CPS (Giordano et al., 2008). Disability was assessed using the Expanded Disability Status Scale (EDSS) (Kurtzke, 1983), administered by a trained neurologist, and the number of relapses since clinical onset was recorded.

All the patients in the study sample gave their written consent to participate. The protocol was approved by the local ethics committee.

Statistical analysis

The Shapiro-Wilk test was used to verify the normal distribution of the continuous variables. Descriptive statistics were calculated in the total sample of patients with MS and in the sample divided into two subgroups on the basis of preferred level of participation in the decision-making process on the first-line MS treatment (passive and collaborative-active). Demographic (gender, age, education, marital status, employment status) and clinical characteristics (age at onset, age at diagnosis, disease duration, MS phenotype, EDSS score, number of relapses) were compared between the two subgroups using the chi-square test or the Mann-Whitney U test as appropriate. The main results were reported by means of frequency distribution graphs. Statistical analyses were performed in SPSS Statistics, Version 22. Significance was accepted as $\alpha \leq 0.05$.

Results

Of the 115 patients approached, 15 refused to participate in the study. Lack of time was the most frequent reason given for refusal. Table I shows the demographic and clinical characteristics of the whole group of participating patients, and of the patients divided into three subgroups (passive, collaborative, active) on the basis of their preferred level of participation in the decision-making process prior to the start of the first-line MS treatment. Demographic characteristics were similar across the three subgroups (Table I).

Forty of the 100 patients with MS preferred to be passive in the decision-making process regarding the first-line treatment, 35 preferred to share in the process ("co-participation"), while the remaining 25 expressed a willingness to have an active role. The subgroup analysis of the passive, collaborative and active groups showed that the active group had a higher median EDSS score and a higher mean number of relapses than the other groups (Table I).

Discussion

Patients with MS should be regarded as partners in medical decision making. Our study investigated the patients' willingness, at the first specialist consultation, to participate in the therapeutic decision-making process. Interestingly, factors such as years of education and employment status did not seem to influence willingness to receive health information (another aspect investigated by the CPS). A higher EDSS score and higher mean number of relapses influenced the taking of an active role in SMD. Clinicians know that patients with more "active MS" appear more worried about their disease, possibly because they are more fearful regarding its evolution; this may lead them to show more engagement in their own healthcare. More studies seeking to shed light on the issues that influence patient engagement in MS are needed.

Previously, a collaborative role was found to be preferred by Italian MS patients (who varied in clinical and general characteristics) (Giordano et al., 2008). The patients' cognitive styles and personality traits were not routinely investigated in depth. However, no patient suffered from psychiatric symptoms or cognitive decline or showed limitations in daily social functioning. In advanced stages of MS, when patients may present cognitive deficits and/or altered decision-making abilities and emotional reactivity or psychiatric symptoms, patient participation in decision making may seem impossible. Further studies are needed to clarify the impact of decision-making abilities and emotional reactivity on MS treatment decisions.

These interesting data need to be confirmed in prospective studies that could evaluate the importance of worsening disability (and its management) and relapses (in terms of frequency and severity) as modulators of MS patients' willingness to be involved in SDM.

Patient autonomy has been stressed as the ideal concept for medical decision making. MS could represent a prototypical condition for studying this approach, both because the disease affects young people who are highly disease aware, and also because the available treatments can have side effects (particularly in second- and third-level therapy) (Utz et al., 2014).

In recent years several studies have outlined communication and information deficits in the care of patients with MS (Wilson et al., 2014). Tools such as question-

Table I - Demographic and clinical characteristics of participants by total group (A) and decision-making subgroups (B).

	Total group	Decision-making subgroups			p-value
	n=100	Passive (n=40)	Collaborative (n=35)	Active (n=25)	
Sex M/F	40/60	18/22	14/21	8/17	n.s.
Age (yrs)	33.4±10.3	33±9	33±10	34±12	n.s.
Age at onset (yrs)	29.7±9	30±8	30±7	29±10	n.s.
Age at diagnosis (yrs)	32.9±10.2	33±9	33±8	33±12	n.s.
Disease duration (yrs)	4.8±3.9	4±3	4±4	4±5	n.s.
Education(yrs)	11.9±4.3	11±4	11±5	12±5	
Years of education, n					
≤ 8	38	18	10	10	n.s.
8-13	39	16	13	10	
> 13	23	6	12	5	
Marital status, n					
Single	47	18	15	14	n.s.
Married	53	22	20	11	
Employment status					
Unemployed	41	18	13	10	n.s.
Working	59	22	22	15	
Type of MS, n					
RMS	89	36	30	23	
PMS	11	4	5	2	n.s.
Relapses	3.4±2.5	2.8±1.6	2.3±1.8	4.1±3.0	p<.05
Median EDSS score	1.7	1.2	1.4	2.1	p<.05
EDSS score subgroups, n					
< 4.0	90	30	25	18	n.s.
≥ 4.0	10	10	10	7	

Abbreviations: M=male; F=female; n=number; yrs=years; MS=multiple sclerosis; RMS= relapsing MS; PMS= progressive MS; EDSS= Expanded Disability Status Scale. Data are frequencies or mean ± SD.

naires designed to assess knowledge of the disease and the feeling of involvement and participation in medical processes have been developed for MS patients (Wilson et al., 2014). Clinical trials have been performed to validate batteries of tools designed to analyze different steps in SDM in MS in order to help to benchmark knowledge and compare the decision-making process in different healthcare settings or different countries and cultures. The SIMS-Trial was performed in five Italian centers and it showed that information in newly diagnosed MS patients improves disease knowledge and satisfaction with the patient-physician relationship (Borreani et al., 2014).

However, it is not entirely clear whether patients really want to participate, whether they really do share decisions, or whether they just want to feel that they are involved. We also do not yet know which other clinical factors (related to the MS itself) influence the decision-making process. Our findings may be helpful in addressing prospective randomized clinical trials in order to better study DSM in patients with MS.

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Effects of treadmill training on cognitive and motor features of patients with mild to moderate Parkinson's disease: a pilot, single-blind, randomized controlled trial

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Summary

The aim of this pilot randomized controlled trial was to evaluate the effects of treadmill training on cognitive and motor performance in patients with Parkinson's disease (PD). Seventeen persons with mild to moderate PD were enrolled. Nine patients were allocated to the Intervention group and received twelve 45-minute sessions of treadmill training: one session a day, three days a week, for four consecutive weeks. Eight patients were allocated to the Control group; these patients did not undergo physical training but were required to have regular social interactions, following a specific lifestyle program. All the patients were evaluated at baseline and one month later. The primary outcome measures were the Frontal Assessment Battery-Italian version (FAB-it) and the 6-minute walking test (6MWT). At the one-month evaluation significant differences were found between the groups in their performance on the FAB-it (p=0.005) and the 6MWT (p=0.018). Our findings support the hypothesis that treadmill training might effectively improve cognitive and motor features in patients with PD.

KEY WORDS: basal ganglia, executive function, gait, movement disorders.

Introduction

Parkinson's disease (PD) is a common idiopathic neurodegenerative disorder characterized by a progressive loss of dopaminergic neurons in the substantia nigra pars compacta (Monchi et al., 2004). Patients with PD are typically debilitated, presenting with symptoms of muscular rigidity, impaired movement, loss of balance and tremor at rest (Pothakos et al., 2009). This results in abnormal gait patterns mainly characterized by reduced gait speed and shortened stride length (Picelli et al., 2010). As well as motor impairment, about 25% of newly diagnosed, non-demented people with PD show some cognitive deficits involving attention, memory and visuospatial and executive functions (Elgh et al., 2009; Mamikonyan et al., 2009).

To date, a wide range of physical therapy modalities has been proposed and employed to treat motor impairment in PD (Ceravolo et al., 2001; Picelli et al., 2012a,b; Carda et al., 2012; Picelli et al., 2013, 2015; Smania et al., 2013; Tomlinson et al., 2013). In particular, the implementation of physical activity programs for people with PD has resulted in improvements in daily activities, motor performance, ambulation and overall functional independence (Lau et al., 2011). Furthermore, in experimental animal models of PD, aerobic exercise has been shown to improve neurochemical and mitochondrial function, with a positive impact on cognitive and emotional aspects of behavior (Pothakos et al., 2009; Pietrelli et al., 2012; Tuon et al., 2012, 2014)

Cognitive-motor relationships have been explored in patients with PD (Domellöf et al., 2011; Poletti et al., 2012). Recently, a cross-sectional pilot study investigated, in depth, the relationship between cognitive deficits and motor dysfunctions in PD; balance and gait skills were found to show significant correlations with some cognitive features in parkinsonian patients (Varalta et al., 2015). These observations could have

implications for rehabilitation, offering perspectives for clinical treatment protocols based on cognitive-motor relationships in patients with PD. In view of these considerations, the present study was conducted to evaluate the effects of treadmill training on cognitive and motor performance in patients with mild to moderate PD.

Materials and methods

This was a pilot, single-blind, single-center, randomized controlled trial. The inclusion criteria were a confirmed diagnosis of idiopathic PD according to the UK Brain Bank Criteria (Hughes et al., 1992); Hoehn and Yahr stage 3, determined in the "on" phase (Hoehn and Yahr, 1967); and a Mini-Mental State Examination score greater than 24 (Folstein et al., 1975). The exclusion criteria were: severe dyskinesias or "on-off" fluctuations; important modifications of PD medication during the study (i.e. drug changes); deficits of somatic sensation involving the lower limbs (as assessed by means of a physical and neurological examination); vestibular disorders or paroxysmal vertigo; other neurological or orthopedic conditions involving the lower limbs (musculoskeletal diseases, severe osteoarthritis, peripheral neuropathy, joint replacement); and cardiovascular comorbidity (recent myocardial infarction, heart failure, uncontrolled hypertension, orthostatic hypotension).
All the participants were outpatients and gave their informed written consent to participate in the current study, which was carried out in accordance with the Declaration of Helsinki and approved by the local ethics committee. Prior to testing, eligible participants were allocated, in a one-to-one ratio, to one of two arms according to a balanced (restricted) randomization scheme: a group that performed treadmill training without body-weight support and a group that received no physical treatment. The investigator (V.V.) who decided whether a subject was eligible for inclusion in the trial was unaware, when making this decision, of which group the subject would be allocated to (allocation was by sealed opaque envelopes). Another investigator (V.Z.) checked correct patient allocation according to the randomization list. After unmasking at the end of the study, we checked that no errors had been made in the allocation process. During the study, participants were instructed to take their PD medications regularly and were tested and trained during the on phase, 1 to 2.5 hours after taking their morning dose. The participants did not perform any type of rehabilitation in the three months leading up to the study, or undergo any form of physical therapy other than that scheduled in the study protocol.

Treatment procedures

Patients allocated to the Intervention group performed treadmill training without body-weight support using the Jog Now 500MD (Technogym, Cesena, Italy). The training program consisted of twelve 45-minute sessions (including rest periods): one session a day, three days a week (Monday, Wednesday, Friday), for four consecutive weeks (Picelli et al., 2013, 2015). Each training session comprised three parts with a 5-minute rest after each. First, patients were trained at a speed of 1.0 km/h for 10 minutes; then, at 1.5 km/h for 10 minutes; finally, at 2.0 km/h for 10 minutes (Picelli et al., 2013, 2015). Patients unable to maintain the established pace were excluded.
Patients allocated to the Control group did not perform physical training; however, they were instructed to have regular social interactions, according to a specific lifestyle program, during the study period in order to ensure that they had social interactions with the same frequency and of the same duration as the Intervention group attending the rehabilitation center.

Evaluation procedures and outcomes

The patients were evaluated at baseline (T0) and one month later (T1). To avoid facilitating the Intervention group, the T1 evaluation was not conducted at the training center. All the patients were evaluated by the same rater (C.M.), who was blinded to the group allocation. Asking the assessor to make an educated guess tested the success of the blinding.

Primary outcomes

The primary cognitive outcome measure was the Frontal Assessment Battery-Italian version (FAB-it) (Appollonio et al., 2005). The FAB-it assesses executive functions such as conceptualization, mental flexibility, programming, sensitivity to interference, inhibitory control and environmental autonomy. It consists of six tests (similarities, lexical fluency, Luria motor series, conflicting instructions, go no-go, prehension behavior), each rated on a scale from 0 to 3 points. The total score is the sum of all the items, and it ranges from 0 (worst performance) to 18 (best performance) (Appollonio et al., 2005).
The primary motor outcome was the 6-minute walking test (6MWT) (Enright, 2003), which was used to assess walking capacity. The subjects were asked to cover as much ground as possible in six minutes (walking continuously at their fastest possible speed without using walking aids) along a marked 40-meter circuit. The distance covered was recorded.

Secondary outcomes

Secondary cognitive outcomes were the Montreal Cognitive Assessment (MoCA), the trail making test (TMT) and a memory with interference (MI) test. The MoCA investigates a patient's skills in seven domains: visuospatial/executive, naming, memory, attention, language, abstraction and orientation. The total score is the sum of all the items, with a maximum score of 30 (best performance) (Nasreddine et al., 2005). Attention capacity was evaluated with the TMT (parts

A and B), specifically to assess selective attention, psychomotor speed and sequencing skills. Part B also investigates the ability to switch attention between two rules or tasks. The time taken to complete the trails is recorded (longer = worse performance) (Giovagnoli et al., 1996). Working memory was assessed with the MI test. In this test, subjects are asked to recall a consonant trigram after a ten-second interval during which they were required to count forward starting from a three-digit number randomly presented by the examiner immediately after the trigram. The maximum score is 9 (best performance) (Mondini et al., 2011).

The secondary motor outcome was the 10-meter walking test (10MWT), selected as a measure of gait speed (Bohannon et al., 1996). The subjects were asked to walk on a flat, hard floor at their fastest speed for 10 meters without assistance or the use of walking aids. A 10-m walkway was marked by two lines on the floor at 2 m and at 8 m. In order to minimize the effect of acceleration and deceleration, gait speed was measured in the 6 meters between the two marks (timing started when the toes of the leading foot crossed the 2-m mark and stopped when the toes of the leading foot crossed the 8-m mark) (Bohannon et al., 1996). The time taken was measured using a handheld stopwatch.

In addition to cognitive and motor skills, the patients were also administered the Beck Depression Inventory (BDI), to evaluate mood, and the Unified Parkinson's Disease Rating Scale (UPDRS), to assess their disease course. The BDI, which focuses on psychological aspects of depression, consists of 21 items, each rated on a four-point scale of severity. The total score is the sum of all the items; the maximum score is 63 (worst mood) (Beck et al., 1961). The UPDRS has four subsections and its score ranges from 0 to 147 (higher scores = worse performance) (Song et al., 2009).

Statistical analysis

Statistical analysis was carried out using the Statistical Package for the Social Sciences (SPSS) software, version 20.0, for Macintosh (SPSS Inc., Chicago, Illinois). We assessed all the patients who were randomized (intention-to-treat principle). We used the Mann-Whitney U test to assess the homogeneity of the sample before the study and compare the effect of treatment between groups (to determine this, we computed the differences between T1-T0 performances for all outcomes). Within-group comparisons were performed using the Wilcoxon signed-ranks test. Descriptive analysis was used to evaluate the effect size measures between groups (Cohen's d calculation) and the 95% confidence intervals (Benjamini and Hochberg, 1995). The alpha level for significance was set at $p<0.05$.

Results

Seventeen persons (12 men and 5 women; mean age: 70.0 years) presenting with mild to moderate idiopathic PD (mean disease duration: 9.9 years) were recruited from among 45 outpatients referred to our research center. The enrollment period was from January 2014 to February 2015. Nine patients were allocated to the Intervention group and 8 patients were allocated to the Control group. No drop-out was observed and no adverse events occurred during the trial in either of the groups. The flow diagram of the study is shown in figure 1.

Baseline

No significant difference was observed between the groups with regard to age (p=0.771), primary outcome measures (FAB-it: p=0.293; 6MWT: p=0.923) and secondary outcomes (MoCA: p=0.772; TMT-A: p=0.885; TMT-B: p=0.664; MI: p=0.286; 10MWT: p=0.178; BDI: p=0.961; UPDRS: p=0.962) at T0. The patients' demographic and clinical features are detailed in table I.

Primary outcomes

The FAB-it showed a significant difference between the groups at T1 (p=0.005; z=–2.791; effect size=0.63). Within-group comparisons showed a significant improvement at T1 versus T0 only in the Intervention group (p=0.011; z=–2.530). As for the 6MWT, a significant difference was found between groups at T1 (p=0.018; z=–2.360; effect size=0.53). Within-group comparisons showed a significant improvement at T1 versus T0 only in the Intervention group (p=0.008; z=–2.668). Group data and within-group comparisons are detailed in table II.

Secondary outcomes

The MoCA showed no significant difference between the groups at T1 (p=0.365; z=–0.906; effect size=0.30). As regards the TMT, the assessment at T1 revealed a significant difference between the groups in both TMT-A (p=0.027; z=–2.213; effect size=–0.51) and TMT-B (p=0.009; z=–2.600; effect size=–0.33) scores. Significant between-group differences at T1 were also found in the MI (p=0.014; z=–2.459; effect size=0.57), 10MWT (p=0.001; z=–3.418; effect size=–0.80), BDI (p=0.009; z=–2.616; effect size=–0.61),

Table I - Demographic and clinical features of the patients.

	Intervention group (n=9)	Control group (n=8)
Age mean (SD)	71.2 (9.2) y	71.6 (7.2) y
Gender, m/f	5/4	4/4
Disease duration, mean (SD)	11.2 (5.6) y	10.8 (4.1) y

Abbreviations: SD=standard deviation; n=no. of patients; y=years

and UPDRS (p=0.022; z=–2.294; effect size=–0.54) scores. Group data and within-group comparisons are reported in table II.

Discussion

This pilot, randomized, controlled trial was conducted to evaluate the effects of treadmill training on cognitive and motor performance in mild to moderate PD. We found significant improvements in cognitive performance (as measured by the FAB-it, the TMT and the M test) and motor performance (as measured by the 6MWT and the 10MWT) in patients with mild to moderate PD who underwent a training program consisting of four weeks of treadmill training without body-weight support. Furthermore, the PD patients who underwent treadmill training also showed significant mood and disease course improvements (as measured by the BDI and the UPDRS).

Conventionally, exercise is thought to produce an overall benefit in terms of physical fitness and mental stimulation, to slow down the aging process, and to help prevent the onset of chronic disease (Lau et al., 2011; van Praag et al., 2005; Pereira et al., 2007). Furthermore, the impact of exercise in promoting brain angiogenesis and neurogenesis has been well established, supporting the notion that exercise can act to slow decline of cognitive and memory function during the course of normal aging (van Praag et al., 2005; Pereira et al., 2007). In PD, physical activity has been found to potentially reduce the risk of further neurological impairment (Lau et al., 2011; Tuon et al., 2012). In particular, long-term treadmill exercise training has been shown to protect against neurotoxin-induced protein oxidation (by reducing the level of striatal carbonylated proteins), impaired mitochondrial function (by restoring mitochondrial respiration, adenosine triphosphate and superoxide dismutase levels in the striatum), and loss of dopaminergic neurons and transmission (by increasing striatal dopamine receptors); furthermore, it has been found to elevate nigrostriatal neurotrophic factors in chronic experimental models of PD (Lau et al., 2011; Tuon et al., 2012). The internal generation of movements depends on a decision-making process (i.e. the selection of an action, among several alternatives, for the performance of a task) (Nagano-Saito et al., 2014). The basal ganglia, whose activity is mostly modulated by dopaminergic projections, seems to play an important role in mediating cognitive and motor modules, and thus in allowing the selection and generation of an appropriate action for the task in hand (Nagano-Saito et al., 2014). Patients with PD, in whom the dopaminergic projec-

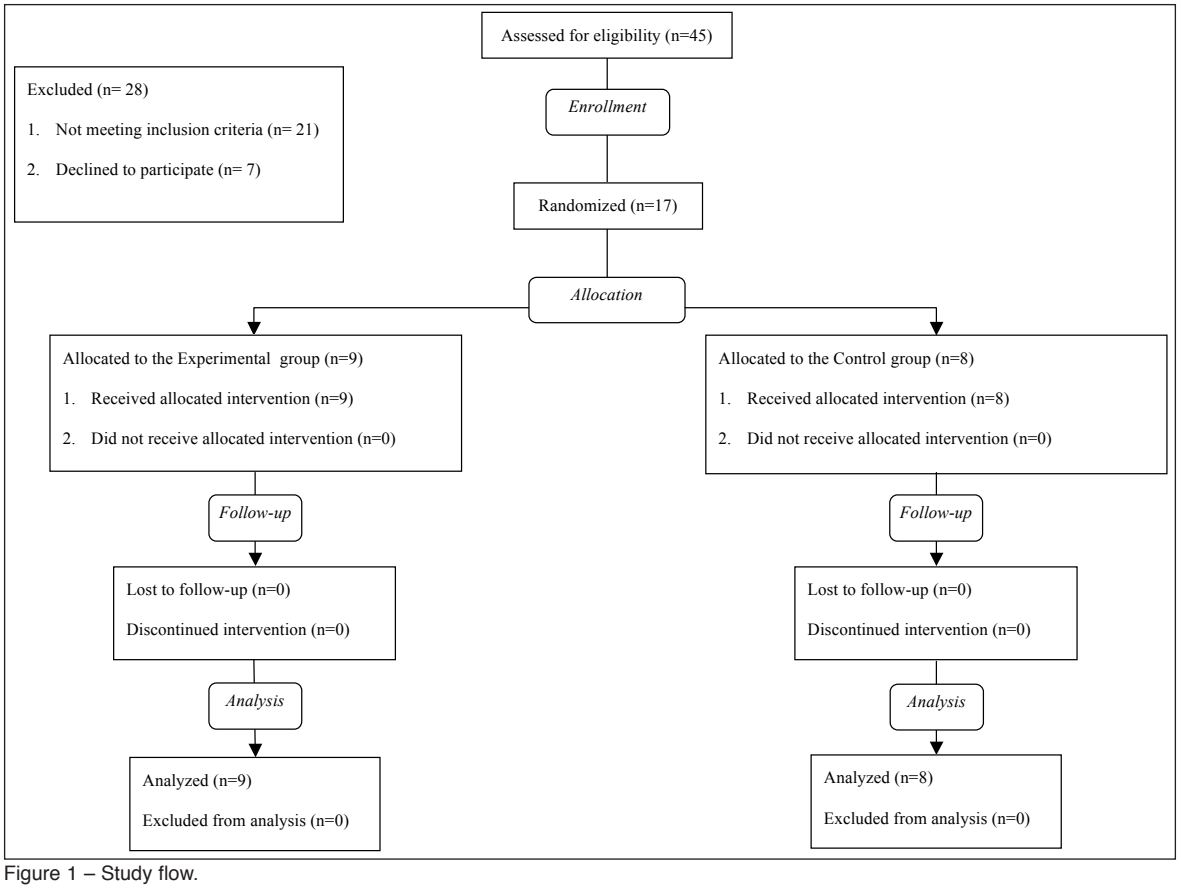

Figure 1 – Study flow.

tions to the striatum are significantly reduced, show some difficulties in performing internally generated movements as well as cognitive deficits that are often manifested as impaired executive functions (Nagano-Saito et al., 2014; Varalta et al., 2015). On these bases, it has been suggested that dopamine, first processed in cognitive brain networks, is involved in the transfer of information toward motor-related networks, and thus in task performance (Nagano-Saito et al., 2014). In short, the dopaminergic projections of the basal ganglia may be involved in the formation of an ideal network, combining the cognitive and motor networks in the brain, for the conducting of a series of tasks (Nagano-Saito et al., 2014). Our findings are in line with these concepts. Indeed, our observation of significant improvements in motor and cognitive performances after four weeks of treadmill training in the Intervention group provides further confirmation of the close relationship between impaired cognitive performance and motor dysfunction in patients with PD (Varalta et al., 2015; Kelly et al., 2015). These findings have relevance to rehabilitation, considering that PD-associated cognitive deficits are important features of the disease, contributing to reduced quality of life and an increased risk of disability and mortality (Kelly et al., 2015). In our view, these findings not only highlight the possibility of obtaining improvements in cognitive performance through motor training in PD, but also suggest a role for new rehabilitation approaches integrating both cognitive and motor training. In particular, it would be interesting to use physical aerobic exercise (i.e. treadmill training) in order to prime cognitive rehabilitation in people with PD, in line with what is already proposed for patients with other neurological disorders such as stroke (Mang et al., 2013).

Investigation of neuropsychiatric symptoms has revealed the presence of comorbid depression in a high percentage of patients with PD (Elgh et al., 2009; Mamikonyan et al., 2009; Tuon et al., 2014). This may be explained by the fact that mood symptoms are related to alterations in serotoninergic pathways, which are known to interact with the dopaminergic degeneration associated with PD (Jellinger, 2015). Physical exercise was recently found to prevent depressive symptoms in PD by increasing the levels of brain-derived neurotrophic factor and preventing neurodegeneration (Tuon et al., 2014). Accordingly, in the present pilot study we observed some positive effects on depression in patients who performed treadmill training. As regards walking, gait in PD is

Table II – Group data and within-group comparisons.

Outcome	Group	Baseline	One month	Within-group comparisons
				One month vs baseline p value (95% CI)
FAB-it (0-18) median (IQR)	Intervention	14.00 (11.00; 15.00)	16.00 (14.00; 17.00)	0.011 (1.16; 3.95)*
	Control	14.50 (14.00; 16.00)	14.00 (14.00; 15.75)	0.705 (–1.09; 1.09)
6MWT (meters) mean (SD)	Intervention	310.22 (83.28)	346.67 (80.70)	0.008 (18.99; 53.90)*
	Control	298.75 (101.31)	307.25 (89.47)	0.362 (–9.92; 26.92)
MoCA (0-30) median (IQR)	Intervention	24.00 (18.50; 27.00)	25.00 (20.50; 28.00)	0.017 (0.53; 2.58)*
	Control	23.00 (20.25; 26.00)	24.50 (22.00; 26.75)	0.227 (–0.71; 1.96)
TMT-A (seconds) mean (SD)	Intervention	141.00 (113.99)	120.67 (104.59)	0.018 (–37.32; –3.35)*
	Control	123.50 (101.27)	124.75 (108.55)	0.735 (–10.14; 12.64)
TMT-B (seconds) mean (SD)	Intervention	200.00 (80.19)	149.56 (69.33)	0.008 (–79.75; –21.14)*
	Control	195.25 (93.92)	181.13 (78.96)	0.345 (–67.68; 39.43)
MI (0-9) median (IQR)	Intervention	4.00 (2.00; 6.00)	7.00 (5.00; 9.00)	0.010 (1.53; 3.58)*
	Control	6.50 (3.25; 8.75)	5.50 (4.00; 8.75)	1.000 (–1.84; 1.84)
10MWT (seconds) mean (SD)	Intervention	10.20 (1.52)	7.61 (1.50)	0.008 (–3.28; –1.90)*
	Control	9.41 (3.02)	9.30 (2.42)	0.624 (–0.95; 0.72)
BDI (0-63) median (IQR)	Intervention	11.00 (8.00; 25.00)	6.00 (3.00; 16.50)	0.012 (–8.24; –1.98)*
	Control	13.00 (9.25; 20.25)	13.00 (7.00; 19.75)	0.914 (–2.18; 3.18)
UPDRS (0-147) median (IQR)	Intervention	40.00 (33.50; 45.50)	37.00 (30.50; 43.00)	0.013 (–5.58; –1.46)*
	Control	42.00 (33.75; 43.75)	40.50 (34.25; 42.75)	0.285 (–2.10; 0.85)

Abbreviations: IQR=interquartile range; SD=standard deviation; CI=confidence interval; FAB-it=Frontal Assessment Battery-Italian version; 6MWT=6-minute walking test; MoCA=Montreal Cognitive Assessment; TMT=trail making test; MI=memory with interference test; 10MWT=10-meter walking test; BDI=Beck Depression Inventory; UPDRS=Unified Parkinson's Disease Rating Scale. * = statistically significant (p<0.005).

characterized by reduced speed, a shortened stride length and a longer double support phase, leading to mobility problems, instability and falls, and thus a reduction in quality of life and mental well-being. Treadmill training without body-weight support has been shown to effectively improve walking ability in patients with PD (Mehrholz, 2010; Carda et al., 2012; Picelli et al., 2013, 2015). Our results confirm previous findings about the usefulness of treadmill training for promoting mobility (in particular walking capacity and gait speed) in PD through restoration of stride length, gait rhythmicity and a more stable walking pattern.

This study has several limitations. First, the sample size was small. We estimated that a total of 66 patients (33 per group) would provide a power of 80% to detect a between-groups difference of 1.26 points (standard deviation 1.81 points) on the FAB-it (Lima et al., 2008). Second, the Control group did not perform any specific training during the study period. Thus, we cannot exclude that changes observed in the Intervention group might be consequent to a placebo effect. Third, no long-term follow-up was considered. Fourth, we did not test participants "off" medication and thus cannot draw conclusions on the unmedicated state. Fifth, even though we did not include patients with severe fluctuation, since possible levodopa effects were not controlled we cannot exclude some degree of fluctuation in our patients.

In conclusion, our preliminary findings support the hypothesis that aerobic physical exercise consisting of treadmill training without body-weight support may improve some cognitive and motor features in non-demented patients with mild to moderate PD. Properly-sized randomized controlled trials are needed to further validate these findings.

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Melatonin 4 mg as prophylactic therapy for primary headaches: a pilot study

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Summary

There is growing evidence that headaches are connected to melatonin secretion. Our aim was to assess the potential effectiveness of melatonin for primary headache prevention.
Forty-nine patients (37 with migraine and 12 with chronic tension-type headache, TTH) were prescribed oral melatonin, 4 mg, 30 minutes before bedtime for six months. Forty-one (83.6%) of the 49 patients completed the study, while eight dropped out for personal reasons.
A statistically significant reduction in headache frequency was found between baseline and final follow-up after six months of treatment (p=0.033 for TTH patients and p<0.001 for migraineurs). The Headache Impact Test score was significantly reduced in both groups of headache patients (p=0.002 and p<0.001, respectively).
At baseline, melatonin levels, measured both during a headache attack and a pain-free period, did not differ between patients with TTH and migraineurs (p=0.539 and p=0.693, respectively), and no statistically significant differences in Hamilton Depression Rating Scale scores were found between the two groups.
This pilot study shows promising results, in terms of headache frequency reduction and daily quality of life improvement, in both groups.

KEY WORDS: Hamilton Depression Rating Scale, Headache Impact Test (HIT-6), melatonin, primary headaches, prevention

Introduction

All organisms depend on the maintenance of a state of dynamic equilibrium or homeostasis, which is threatened by intrinsic and environmental stimuli. Environmental triggers have always been at the forefront of headache research. Since the pineal gland is considered to be a transducer of environmental stimuli into the neuroendocrine system, a pineal gland abnormality may be involved in headache etiology and homeostasis disruption. Levels of the pineal hormone melatonin have been found to be low in migraine (Claustrat et al., 1989; Murialdo et al., 1994; Brun et al., 1995) and in cluster headache (Leone et al.,1995). Melatonin can act as a chronobiotic (being a circadian pacemaker of sleep) (Cajochen et al., 2003), an antioxidant, an antihypertensive, an anxiolytic and a sedative (Yousaf et al., 2010). Experimental studies have indicated a dose-dependent analgesic effect of melatonin in animals (Pang et al., 2001; Naguib et al., 2003; Noseda et al., 2004; Tu et al., 2004; Mantovani et al., 2006; Wang et al., 2006). Oral melatonin has shown promising results in migraine prevention (3 mg, 30 minutes before bedtime) (Peres et al., 2004) and in cluster headache prophylaxis (10 mg) (Leone et al., 1996). The current study evaluates whether melatonin 4 mg has a beneficial effect as prophylactic therapy in tension-type headache (TTH) and migraine (the most disabling forms of headache), and whether it can play a role in the improvement of daily quality of life.

Materials and methods

Study population

The study population comprised subjects of both genders, aged 18-75 years, affected by primary headache (migraine or TTH) fulfilling the diagnostic criteria of the latest International Headache Society (IHS) headache classification (Headache Classification Committee of

the International Headache Society, 2013). The main exclusion criteria were secondary headaches and severe psychiatric and sleep disorders. Informed consent was obtained from all the patients. The study was approved by the local ethics committee. The procedures followed were in accordance with the ethical standards of the Helsinki Declaration of 1975, as revised in 1983.

Study design

The study was conducted at the outpatient Headache Clinic of Athens Korgialenio Benakio Hospital from January 2010 to June 2012. The recruited patients were classified in two groups: i) migraine with and without aura present for at least three months with at least four migraine attacks per month; ii) TTH, with over 15 headache days per month for at least three months. Patients on systemic prophylactic therapy were instructed to continue this therapy throughout the duration of the trial and, during attacks, to take (as needed) the symptomatic therapy they usually used as rescue therapy. A detailed headache history was obtained from all the patients. Physical, neurological and ophthalmological examinations were conducted and, when indicated, specific neuroimaging (MRI) investigations were performed.

The study period for every patient was six months. The scheduled visits were one initial (baseline) visit and two follow-up visits: at two and six months after initiation of melatonin prophylaxis.

At the baseline visit, the diagnosis was confirmed and a clinical and neurological examination was performed. Blood samples, for measurement of melatonin levels, were taken both during a headache attack and during a pain-free period. Both blood samples were taken at the same time of day (11 p.m.) under low light to avoid bias due to the normal circadian fluctuation of melatonin levels in blood. Blood was collected into a heparinized tube and immediately centrifuged at 4°C, 2000 g for 20 minutes; plasma was decanted and stored at –20°C until the radioimmunoassay test was performed (Graham et al., 1998). To allow us to identify, at the beginning of the study, any differences between these two main primary headaches, all patients completed the Hamilton Depression Rating Scale (HAMD), the Epworth Sleepiness Scale (ESS) and the Headache Impact Test (HIT-6). As mentioned, any current prophylactic headache therapy was continued and all patients were instructed to record the duration and frequency of their headache attacks in diaries. After completion of the baseline assessment, the subjects included in the study started melatonin prophylactic therapy consisting of 4 mg/day of melatonin (Circadin®, a prolonged release melatonin formulation) administered orally, 30 minutes before bedtime.

Visit 2 took place eight weeks after initiation of melatonin prophylactic therapy and comprised an interview with the patient during which progress with the prophylactic therapy was discussed and the headache diaries were reviewed. Possible side effects of the melatonin treatment were also recorded.

At visit 3 (final visit), six months after the initiation of the melatonin prophylaxis, all headache diaries were collected and HIT-6 was repeated. Visit 3 marked the official end of the study, and the patients were informed of this, but the melatonin prophylactic treatment was continued. Patients had to have adhered to the treatment for at least six months to be included in the analysis of the data.

The HIT-6 is a self-administered, six-item questionnaire that measures the impact of headache in several domains: social functioning, role functioning, vitality, cognitive functioning and psychological distress. Each question is presented as a five-point Likert item measured on a scale anchored at never (6) and always (13). The results of the six items were summed to give the total score, which thus ranged from 36 to 78. The impact of headache, according to HIT-6 scores, was graded as follows: a score ≤49 describes little or no impact; 50–55, some impact; 56–59, substantial impact; and ≥60 severe impact. A score >56 was considered clinically significant. The HIT-6 shows good internal consistency (0.89) and test-retest reliability (0.90), construct validity and responsiveness in general headache patients (Kosinski et al., 2003).

The HAMD is a 17-item scale that evaluates depressed mood, vegetative and cognitive symptoms of depression, and co-morbid anxiety symptoms. It provides ratings on current DSM-IV symptoms of depression, with the exception of hypersomnia, increased appetite and impaired concentration/indecision. The 17 items are rated on either a five-point (0–4) or a three-point (0–2) scale. The five-point scale items use the following ratings: 0 = absent; 1 = doubtful to mild; 2 = mild to moderate; 3 = moderate to severe; 4 = very severe. A rating of 4 is usually reserved for extreme symptoms. The three-point scale items use the following ratings: 0 = absent; 1 = probable or mild; 2 = definite. Applying the established clinical cut-offs, HAMD scores are classified in three groups (absent, mild and definite depression) (Zimmerman et al., 2013).

The ESS (Johns, 1991) is a brief self-administered questionnaire that measures average daytime sleepiness. Patients rate their probability of falling asleep on a scale from 0 to 3 for eight different situations. The scores for the eight questions are added together to obtain a single total (a number between 0 and 24). A number in the 0–9 range is considered to be normal while a number in the 10–24 range indicates excessive sleepiness. The internal consistency reliability of the test ranges from 0.74 to 0.88.

Statistical analysis

Continuous variables are expressed as mean and standard deviation (SD). Quantitative variables are expressed as absolute and relative frequencies. For the comparison of proportions, Fisher's exact tests were used. For the comparison of quantitative study

factors between the TTH and migraine group, the non-parametric Mann-Whitney test was computed for non-normal variables. Changes in attack frequency and HIT-6 scores during the follow-up period in the two groups (TTH and migraine) were evaluated using the Wilcoxon signed-rank test. The Spearman correlation coefficient was used to explore the association of two continuous variables. All p values reported are two-tailed. Statistical significance was set at 0.05 and analyses were conducted using SPSS statistical software (version 19.0).

Results

A total of 49 patients (12 TTH, 37 migraine) met the inclusion criteria and were included in this study. Forty-one (83.6%) of the 49 patients completed the study, while eight dropped out for personal reasons. The baseline characteristics of the 49 participants are described in table I.

As shown in table II which shows the results of the intervention, oral melatonin 4 mg had a favorable effect on attack frequency, which was found to be reduced in both the migraine and the TTH patients after six months of prophylactic therapy. No patient reported an increase in headache frequency.

The HIT-6 score (Table II) was statistically significantly reduced in the migraineurs after the treatment ($p<0.001$). A significant benefit of melatonin on headache impact was also recorded in the TTH group ($p=0.002$) at the end of the study period.

The main secondary outcome, melatonin levels measured both during a pain-free period and during a headache attack, did not show any statistically significant differences between the migraine and TTH patients ($p=0.693$ and $p=0.539$ respectively, mean values are shown in table I) or between males and

Table I - Demographic and baseline characteristics of the total study population.

	TTH n=12	Migraine n=37	p-value[1]
Sex			
Male	4 (33.3%)	7 (18.9%)	0.427
Female	8 (66.7%)	30 (81.1%)	
Age, years (mean ± SD)	45.25 ± 10.25	38.65 ± 11.77	0.012**
Duration of headache attacks, hours (mean ± SD)	13.25 ± 12.22	11.86 ± 8.90	0.852**
Drug therapy for headache attacks			
NSAIDs	9 (75%)	29 (78.4%)	0.743*
NSAIDS+triptans	1 (8.3%)	6 (16.2%)	
NSAIDS+antidepressants	1 (8.3%)	0 (0)	
NSAIDs+anxiolytics	1 (8.3%)	1 (2.7%)	
Triptan	0	1 (2.7%)	
Mean duration of prophylactic drug therapy before inclusion in the study, months (mean ± SD)	4.75 ± 2.05	4.24 ± 2.41	0.461**
Mean ESS score ± SD	4.41 ± 2.71	3.87 ± 2.92	0.249**
Hamilton Depression Rating Scale			
Absent (0–7)	7 (58.3%)	26 (70.27%)	0.098*
Mild (8–10)	2 (16.7%)	10 (27.02%)	
Definite (>10)	3 (25%)	1 (2.71%)	
Mean Mel Inter(pg/ml), mean ± SD	11.05 ± 7.46	10.58 ± 4.5	0.693**
Mean Mel Attack(pg/ml), mean ± SD	7.61 ± 4.3	6.65 ± 3.83	0.539**

Abbreviations: TTH=tension-type headache, NSAIDs=nonsteroidal anti-inflammatory drugs; Mel Inter=melatonin level during a pain-free period; Mel Attack=melatonin level during headache attack; ESS=Epworth Sleepiness Scale.
[1] Difference of frequencies tested with Pearson's chi-square, difference of means with Student's t-test and non-parametric Mann-Whitney U applied in each type of headache. p significant at <0.05; *Fisher's exact test; ** Mann-Whitney test.

Table II - Outcomes in melatonin intervention period compared to baseline.

Type of headache	AF Baseline mean±SD	AF 6 months mean±SD	p-value[1]	HIT-6 Baseline mean±SD	HIT-6 6 months mean±SD	p-value[1]
TTH	15.91 ± 6.88	5.33 ± 2.57	0.033	57.66 ± 8.42	36.7 ± 22.5	0.002
Migrane	4.72 ± 0.73	2.18 ± 0.84	<0.001	63.51 ± 5.43	44.37 ± 23.94	<0.001

Abbreviations: AF=attack frequency (mean per 4 weeks); TTH=tension-type headache; HIT=Headache Impact Test; SD=standard deviation. p significant at <0.05; [1]Wilcoxon signed rank test.

females (pain-free period mean±SD: 10.4±7.1 vs 10.7±4.7, p=0.853; headache attack mean±SD: 5.9±3.8 vs 7.1±3.9, p=0.351). In the total headache group, melatonin levels during headache attack were not significantly correlated with HAMD score (r=–0.11, p=0.465) or ESS score (r=0.10, p=0.475). The two headache groups showed no statistically significant difference in either HAMD (p=0.098) or ESS (p=0.249) scores at baseline.

Discussion

The results of this pilot study support our hypothesis that oral melatonin 4 mg has a positive role on headache frequency in patients suffering from TTH and migraine. In addition, the HIT-6 score was significantly reduced, indicating an improvement in daily quality of life, both in patients with migraine and in those with TTH. We also showed a non-significant relationship between melatonin levels and HAMD score during both headache attacks and pain-free periods.

There are certain limitations in our study that could be improved in future studies. The main ones are the open-label design and the lack of a control group. Another issue to address is the size of the sample, and especially the small number of patients with TTH. Crossover studies with established headache prophylactic therapeutic agents would require a longer follow-up period in order to determine whether melatonin works as prophylactic therapy when used in the long term, but crossover trials are vulnerable to the effects of subject withdrawal. The eight withdrawals in our study probably did not have a major impact on its results. In addition, our findings are limited by the lack of pre- vs post-intervention comparisons of HAMD and ESS scores. These two scales, used simply for the purpose of detecting possible differences between the two groups of headache patients at baseline, were administered only in the initial phase. That said, this trial was not primarily designed to assess the quality of sleep as the majority of subjects did not have insomnia.

Two double-blind placebo-controlled studies have documented a significant effect of melatonin on sleep quality and morning alertness in patients with insomnia (Lyseng-Williamson, 2012). In a randomized, double-blind, placebo-controlled trial, sleep quality assessed using the Pittsburgh Sleep Quality Index did not improve during melatonin treatment in migraineurs (Alstadhaug et al., 2010).

Serum melatonin levels are reduced in patients with cluster headache, particularly during a cluster period (Pringsheim et al., 2002). As regards plasma melatonin levels in migraine, the limited published data available derive from three small studies, the results of which suggested that female migraine sufferers have decreased nocturnal plasma melatonin levels both overall and during migraine attacks (Claustrat et al., 1989; Brun et al., 1995; Murialdo et al., 1994). Our study was not able to confirm these findings as we did not include a control group. Abnormal melatonin levels could reflect a global sympathetic hypofunction. Furthermore, migraine sufferers without depression had lower nocturnal plasma melatonin levels than controls, and migraine patients with superimposed depression exhibited the greatest deficiency of melatonin compared with the control group (Claustrat et al., 1989).

Similarly, there is little published literature on melatonin treatment in other primary headaches and, accordingly, only a few studies with small numbers of participants have reported benefits of melatonin treatment in primary headaches. A double-blind, placebo-controlled study of 20 patients showed the efficacy of oral melatonin 10 mg for cluster headache prophylaxis (Pringsheim et al., 2002). In an open-label trial conducted in 34 patients, melatonin 3 mg reduced headache frequency and intensity (Peres et al., 2004). Another study (Alstadhaug et al., 2010) provided Class I evidence that 2 mg of prolonged release melatonin given one hour before bedtime failed to replicate the results of the above-mentioned open-label study with 3 mg of oral melatonin. It can be hypothesized that the benefit of melatonin in migraine sufferers is dose dependent, but the authors of these studies stated that the dose of melatonin was appropriate, and they actually suggest that lower doses have a greater phase-shifting effect (Peres et al., 2004; Alstadhaug et al., 2010). Therefore, no clear relation exists between physiological melatonin levels and its pharmacological doses. Neither of the above studies assessed the effect of melatonin treatment on quality of life or depression symptoms.

Although it was not statistically significant, this is the first study showing a trend toward higher melatonin levels and decreased HAMD score during headache attacks, which suggests an antidepressive effect of melatonin. In accordance with the present results, previous studies have also found no toxicological effect that could compromise the use of melatonin.

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Global fractional anisotropy and mean diffusivity together with segmented brain volumes assemble a predictive discriminant model for young and elderly healthy brains: a pilot study at 3T

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Summary

Several parameters of brain integrity can be derived from diffusion tensor imaging. These include fractional anisotropy (FA) and mean diffusivity (MD). Combination of these variables using multivariate analysis might result in a predictive model able to detect the structural changes of human brain aging.
Our aim was to discriminate between young and older healthy brains by combining structural and volumetric variables from brain MRI: FA, MD, and white matter (WM), gray matter (GM) and cerebrospinal fluid (CSF) volumes.
This was a cross-sectional study in 21 young (mean age, 25.71±3.04 years; range, 21-34 years) and 10 elderly (mean age, 70.20±4.02 years; range, 66-80 years) healthy volunteers. Multivariate discriminant analysis, with age as the dependent variable and WM, GM and CSF volumes, global FA and MD, and gender as the independent variables, was used to assemble a predictive model.
The resulting model was able to differentiate between young and older brains: Wilks' $\lambda = 0.235$, χ^2 (6) = 37.603, p = .000001. Only global FA, WM volume and CSF volume significantly discriminated between groups. The total accuracy was 93.5%; the sensitivity, specificity and positive and negative predictive values were 91.30%, 100%, 100% and 80%, respectively.
Global FA, WM volume and CSF volume are parameters that, when combined, reliably discriminate between young and older brains. A decrease in FA is the strongest predictor of membership of the older brain group, followed by an increase in WM and CSF volumes. Brain assessment using a predictive model might allow the follow-up of selected cases that deviate from normal aging.

KEY WORDS: aging, brain, diffusion tensor imaging, discriminant analysis, volume, white matter.

Introduction

An approach that integrates structural and volumetric biomarkers could be adopted in an attempt to explain some of the discrepancies in the current literature on the aging process of the human brain (Abe et al., 2008); for example, it is known that white matter (WM) changes exceed gray matter (GM) changes during aging (Allen et al., 2005), meaning that human WM is more vulnerable than GM, especially in late-myelinating regions such as the frontal and temporal lobes. Gender has also been associated with the brain aging process: cortical GM declined more steeply with age in men than women, but cortical WM volumes remained stable across the adult age span in both genders (Sullivan et al., 2004). Knowledge of the parameters of brain aging is essential in order to understand what underlies the cognitive declines associated with normal aging and how these deficits differ from those related to pathological conditions such as mild cognitive impairment (MCI) or Alzheimer's disease (AD).
Diffusion tensor imaging (DTI) allows the calculation of several biomarkers of structural integrity of brain tissue (Vernooij et al., 2008). These include fractional anisotropy (FA) and mean diffusivity (MD) (Abe et al., 2008; Hsu et al. 2008). Previous studies have proved the usefulness of a global (whole-brain) approach when using DTI-derived biomarkers in the detection of GM and WM changes (Kochunov et al., 2011). A lower FA value represents a decrease in diffusion directionality due to a loss of microstructural integrity (Vernooij et al., 2008); this is thought to correspond to adecrease in water movement along (the same) axonal tracts. Age-related changes in FA and MD should be presented using a global rather than a regional approach, as a global approach allows a comprehensive quantitation of a tract or group of tracts and not

only a partial measurement of fibers (as is the case when using a regional, or structure-based, approach). Previous studies using a regional approach showed: that FA in the temporal and occipital regions was not correlated with age (Hsu et al., 2008); that FA was negatively correlated in the frontal and temporal WM regions (Abe et al., 2008); that FA measurements did not reveal significant differences, with aging, between the temporal and posterior WM regions (Salat et al., 2005). They also showed the existence of significant differences in FA between cross-sectional normal WM tracts (Nusbaum et al., 2001; Roldan-Valadez et al., 2012). Furthermore, correlations between FA and MD reportedly change depending on the brain region considered: lower FA and higher MD values have been found in the WM of older versus younger subjects, while basal ganglia FA and MD measurements were higher in older than in younger subjects (Pfefferbaum et al., 2010). These variations might be due to methodological differences in image analyses or acquisition, selection and placement of regions of interest, and/or study populations (Jenkinson et al., 2012).

The primary aim of this study was to evaluate global measures of diffusion (i.e. global FA and MD), selected volumes of WM, GM and cerebrospinal fluid (CSF), and gender, assembling a model that allows us, through a multivariate analysis, to discriminate between normal younger and normal older brains.

This global approach offers an integrative model that allows quantitative depiction of normal aging using a series of biomarkers that have previously been used separately in the diagnosis of other neurological diseases, but not as an integrative model of normal brain aging.

Materials and methods

Subjects

A cross-sectional study was performed in right-handed healthy volunteers, divided into young adults and elderly persons. The young subjects were consecutively recruited from a group of medical residents; the elderly subjects were recruited from the Geriatric Unit at Medica Sur Clinic & Foundation, in the period from July 2011 to August 2012. The young adults underwent detailed health examinations performed by a general practitioner; the elderly subjects underwent complete physical and geriatric examinations performed by a board certified geriatrician. Participants were excluded if they had a history of major neurological, psychiatric or cardiovascular disease. The local institutional review board approved the study (protocol #2011.043), and all the participants gave their written informed consent.

MRI scans showing structural abnormalities, such as tumors or stroke, anatomical variations (e.g., mega cisterna magna, cavum septum pellucidum), or technical artifacts, were excluded. WM hyperintensities, as observed on T2-weighted or fluid-attenuated inversion recovery (FLAIR) images, were rated by a radiologist using the age-related WM changes (ARWMC) score (Wahlund et al., 2001). We excluded elderly subjects with regional ARWMC scores higher than 1. For all participants a preliminary neuropsychological evaluation included the Wechsler Adult Intelligence Scale-III intelligence quotient (IQ) test validated for Mexican patients (considering an average IQ of 100 and a standard deviation of 15) (Wechsler, 1997), and a validated, modified version of the Mini-Mental State Examination (MMSE) in Spanish, adjusted for age and level of education (a score of 25 points or more was taken as normal) (Reyes-de-Beaman et al., 2004). Elderly participants with MCI or AD were excluded on the basis of diagnostic criteria established in accordance with the diagnostic guidelines of the National Institute on Aging-Alzheimer's Association work groups (Albert et al., 2011; McKhann et al., 2011).

Brain image acquisition

MRI evaluations of the brain were performed using a 3.0T Signa HDxt scanner (GE Healthcare, Waukesha, WI) and a high-resolution eight-channel head coil (Invivo, Gainesville, FL). Contraindications to MRI were the presence of a pacemaker or metallic implant and claustrophobia. All participants were included.

The examination included standard clinical sequences: sagittal T1-weighted FLAIR (TE/TR = 9.9/2500 ms) with a 5/3-mm slice thickness/gap and 24 x 24 cm field of view (FOV); axial fast spoiled gradient-echo (FSPGR) (TE/TR = 3.9/9.4 ms) with a 1.3/0-mm slice thickness/gap and FOV of 24 x 18 cm; coronal T2-weighted fast spin-echo (TE/TR = 164.1/2617 ms) with a 3/0-mm slice thickness/gap and FOV of 22 x 16 cm; and axial FLAIR (TE/TR = 115.8/11002 ms) with a 5/1-mm slice thickness/gap and FOV of 22 x 22 cm. The DTI sequence resulted in 50 axial slices covering the entire brain and brainstem with 1.7 x 1.7 x 3.0 mm^3 voxel size, acquired with 25 non-collinear diffusion directions with a b-value of 1,000 s/mm^2, and one with a b-value of 0 s/mm^2.

Volumetric data analysis

MRI data of the T1-weighted FSPGR sequence were transferred to a Linux-based workstation. Individual brain atlas-based volumetry was performed using the IBASPM software version 1.0 (Alemán-Gomez et al., 2006), a toolbox for structure segmentation of structural MRI images implemented in MATLAB 7.0 (MathWorks, Natick, MA). This software uses the spatial normalization and segmentation routines of the Statistical Parametric Mapping software version 2 (SPM2) (Roldan-Valadez et al., 2012). A description of the method for volume measurement was recently published elsewhere (Roldan-Valadez et al., 2013).

DTI analysis and global MD and FA measurements

We used the *dcm2nii* software (Rorden et al., 2011) (http://www.mccauslandcenter.sc.edu/mricro/mricron/dcm2nii.html) and tools from the FMRIB Software

Library (FSL, www.fmrib.ox.ac.uk/fsl) version 4.1.9 (Smith et al., 2004), as follows. DTI images were extracted using the Brain Extraction Tool (BET) version 2.1 (Smith, 2002). Eddy currents were corrected using the Diffusion Toolbox version 2.0; the Reconstruct Diffusion Tensor (DTIFIT) and the fslmaths tool generated the eigenvector and eigenvalue maps for each tensor metric. The fslstats tool calculated the scalar measures (mean values) of global FA and MD. Evidence of the clinical application of global DTI-derived tensor metrics for brain imaging has recently been published (Roldan-Valadez et al., 2014).

Statistical analysis

Sample size

Considering that this was a pilot/feasibility study, in accordance with the considerations and recommendations of others we chose to include at least 10 subjects per group (Hertzog, 2008), and to have a minimum overall sample size of 30 (Lancaster et al., 2004). The statistical analysis was focused on the calculation of 95% confidence intervals (CIs) according to contemporary definitions (Pfister and Janczyk, 2013); a boot strapping method with bias corrected and accelerated confidence estimates was performed with 1000 bootstrap resamples (Henderson, 2005). Differences between groups (normal young and normal older brains) for each variable were tested using the Mann-Whitney U test; the value of z was used to calculate an approximate value of r as a measure of effect size (r = z/square root of N where N = total number of cases); effect sizes of 0.1, 0.3 and 0.5 were termed small, medium and large, respectively (Cohen, 1988).

Multivariate discriminant analysis

Multivariate discriminant analysis (DA) (Tabachnik and Fidell, 2013) included continuous and categorical variables to identify specific volumetric and structural attributes in young and older brains. The dependent variable (DV) was age group, with subjects classified as young adults or healthy elders. The independent variables (IVs) comprised: three volumes (cm^3): GM, WM and CSF; two DTI-derived measurements: MD (mm^2s^{-1}) and FA (dimensionless number), and one categorical variable (gender: male or female). The effect-size measure for DA was calculated using the squared canonical correlation as the equivalent of the R^2 in regression. By convention, effect sizes of 0.02, 0.15 and 0.35 were termed small, medium and large, respectively (Cohen, 1988).

Diagnostic model evaluation

The cross-validated contingency table generated by the DA was used to evaluate the diagnostic performance of the DA model; we reported values of sensitivity and specificity, positive and negative likelihood ratios, and positive and negative predictive values, with their corresponding CIs. Statistical significance was indicated by a p-value < 0.05.

Software

DA analyses were carried out using the IBM® SPSS® Statistics software (version 22.0.0.0, IBM Corporation, Armonk, NY, USA). Diagnostic performance was assessed using MedCalc® (version 14.8.1 MedCalc Software bvba, Mariakerke, Belgium). Reporting of diagnostic performance tests followed the STARD initiative (Bossuyt et al., 2003).

Results

Subjects

The study was conducted in 31 subjects: 23 females and eight males, distributed in two age groups: 21 young adults (mean age, 25.71 ± 3.04 years; range, 21–34 years) and 10 healthy elders (mean age, 70.20 ± 4.02 years; range, 66–80 years). Table I shows the gender distribution in each age group.
FA values were higher in younger than in older brains, with a significant difference and a large effect size: U = 17.0, z = –3.719, p < .001, r = .66. Significantly lower volumes of CSF were found in young versus older brains, this finding also showed a large effect size: U = 136.0, z = –32.916, p = .004, r = .52. No significant differences between the groups were found for MD (p = .186), GM volume (p = .087) and WM volume (p = .072). Table II presents the mean values, SD and CI for each age group.

Discriminant analysis

The DA was performed by entering the measurements of the six IVs for each of the 31 brains — five continuous (WM, GM and CSF volumes; FA and MD) and one categorical (gender) — for a total of 186 measurements. DA revealed one discriminant function. The assumption of homogeneity of variance-covariance matrices was interpreted as non-significant (Box's M value = 62.335, p = 0.002), assuming the covariance matrices between the groups were equal (Huberty and Petroskey, 2000). This discriminant function significantly differentiated the young and older brains: Wilks'

Table I - Gender distribution between the young and older brains.

Group	Gender	n	%
Young brain	Male	5	16.13
	Female	16	51.61
Older brain	Male	3	9.68
	Female	7	22.58
Total		31	100.00

$\lambda = 0.235$, χ^2 (6) = 37.603, p = .000001. By indicating the significance of the discriminant function, Wilks' λ explained a low proportion (only 23.55%) of the total variability not explained by the model. A canonical correlation of .8743 suggested that the model explains 76.45% of the variance in the final model.

Summary of discriminant functions

The tests of equality of group means provided statistical evidence of significant differences between means of the groups (young adult and elderly) for only three of the IVs (FA, WM and CSF volumes) with FA producing the highest F-test variance ratio value (Table III).
Standardized canonical discriminant function coefficients provided an index of the importance of each predictor of diagnosis with the sign indicating the direction of the relationship. A significant decreased value for FA was the strongest diagnostic predictor of older brains, while a significant increase in CSF volume was next in importance. The variables with large coefficients stand out (for these data) as those that strongly predict allocation to the young or elderly group. On the basis of these coefficient scores the rest of the variables were decreasingly strong as diagnostic predictors (Table IV A).
The structure matrix table provides another way of indicating the relative importance of the diagnostic predictors by showing the correlations (Pearson's coefficients) of each variable with each discriminate function. Many researchers consider structure matrix correlations more accurate than standardized canonical discriminant function coefficients (Field, 2009). By identifying the largest loadings for each discriminate function it is possible to see a different pattern of variables. Here we have FA (a unitless structural measurement) and CSF (measured in cm^3), which account for the largest loadings for the functions that discriminate between the young and elderly groups. A value of 0.30 is taken as the cut-off between important and less important variables (Field, 2009) (Table IV B).
The canonical discriminant function coefficients table shows the unstandardized coefficients (b) that are used to create the discriminant function (equation), operating just like a regression equation. In this study we observed:

D = 17.601(Constant) – (8,842.143 x MD) – (70.806 x FA) + (0.000281 x GM) + (0.012860 WM) + (0.015526 x CSF) + (1.262 x Gender)

The categorical variable gender was classified as: male = 1 and female = 2. The discriminant function coefficients (b) indicate the partial contribution of each variable to the discriminate function controlling for all other variables in the equation (Table IV C).
Group centroids table: we also described each group in terms of its profile, using the group means of the predictor variables called centroids. The mean of the two centroids is considered the cut-off value; if the discriminant score of the function is less than or equal to the cut-off, the case is classed as 1 (young brain), whereas if it is above, it is classed as 2 (older brain). In our study, young brains had a mean of –1.203 while elder brains produced a mean of 2.526 (Table V).

Table II - Mean values, standard deviations and confidence intervals of the structural and volumetric biomarkers in each age group.

Variable	Young brains (20-35 years)				Older brains (60-85 years)			
	Mean	SD	95% CI		Mean	SD	95% CI	
			Lower	Upper			Lower	Upper
GM volume (cm^3)	638.166	69.102	606.377	666.678	591.725	62.345	549.884	628.896
WM volume (cm^3)	382.034	33.501	367.440	395.757	414.978	49.903	386.179	447.963
CSF volume (cm^3)	359.397	68.103	331.930	389.319	477.271	105.053	414.772	543.323
MD (mm^2/s)	0.001224	0.000082	0.001191	0.001262	0.001272	0.00014	0.001183	0.001360
FA (dimensionless)	0.294860	0.012442	0.289698	0.299928	0.266776	0.015586	0.257207	0.276734

Abbreviations: GM=gray matter; WM=white matter; CSF=cerebrospinal fluid; MD=mean diffusivity; FA=fractional anisotropy; SD=standard deviation; 95% CI=bootstrap 95% confidence intervals.

Table III - Multivariate analysis showing the statistical effect ofeach independent variable included in the analysis.

Variable	Wilks' Lambda	F ratio	p-value
Fractional anisotropy	.497	29.330	< .001
Cerebrospinal fluid volume	.671	14.210	.001
White matter volume	.859	4.753	.038
Gray matter volume	.899	3.247	.082
Mean diffusivity	.953	1.437	.240
Gender	.996	.127	.724

We finished the DA by performing a classification phase, using the cross-validated set of data to present the power of the discriminant function. The classification results revealed that 93.5% of the patients were classified correctly in the "young adult brain" or "older brain" groups, this value corresponded to the overall predictive accuracy of the discriminant function; additional results of diagnostic tests are presented in table VI. The average D scores for each group and the group centroids help us to see the effectiveness of the discriminant function. Histograms and box plots of the average D scores for each group were used as visual demonstrations of the power of the discriminant function, the absence of overlap of the plots revealed an excellent discriminant function (Fig.1).

Table IV - Independent variables included in the discriminant analysis.

A Standardized canonical discriminant function coefficients		B Structure matrix		C Canonical discriminant function coefficients	
Variable	Function		Function		Function
1		Variable 1		Variable 1	
MD	–918	FA	–558	MD	–8842.143
FA	–956	CSF volume	.388	FA	–70.806
GM volume	.019	WM volume	.225	GM volume	.000281
WM volume	.506	GM volume	–186	WM volume	.012860
CSF volume	1.264	MD	.124	CSF volume	.015526
Gender	.570	Gender	–037	Gender	1.262

Abbreviations: MD=mean diffusivity; FA=fractional anisotropy; GM=gray matter; WM=white matter; CSF=cerebrospinal fluid
A) ordered by their standardized canonical discriminant function coefficients (variables with larger coefficients stand out as those that strongly predict allocation to each diagnosis). B) Within-groups correlation matrix depicts the participant variables ordered by absolute size of correlation (Pearson coefficients) within function. A value of 0.30 is considered the cut-off between important and less important variables. C) Unstandardized coefficients used to create a discriminant function operating just like a regression equation. Coefficients indicate the partial contribution of each variable to the discriminate function controlling for all other variables in the equation.

Table V – Means of the predictor variables (centroids) used to describe each group in terms of its profile.

Age group	Functions at group centroids
Young adults (20-35 years)	–1.203
Elders (> 60 years)	2.526
Cut-off value	.060

The cut-off value is considered the mean of the two centroids; if the discriminant score of the function is less than or equal to the cut-off a new case can be classed as 1 (young adult), whereas if it is above, it is classed as 2 (elderly).

Table VI – Results of diagnostic tests of the discriminant model.

Test	Value (%)	95% CI
Sensitivity	91.30	71.96–98.93
Specificity	100.00	63.06–100.00
+ likelihood ratio	-	-
– likelihood ratio	0.09	0.02-0.33
+ predictive value	100.00	83.89–100.00
– predictive value	80.00	44.39-97.48

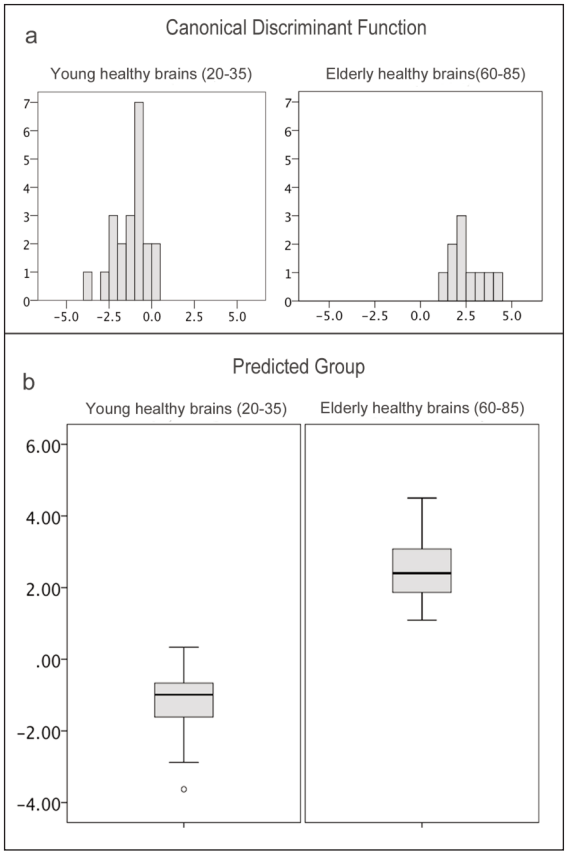

Fig. 1 - Visual demonstration of the effectiveness of the discriminant function.
a) histograms showing the distribution of discriminant scores for young and older brains. b) box plots of the average D scores. Both illustrate the distribution of the discriminant function scores for each group. The absence of overlap between groups constitutes a visual demonstration of excellent discrimination.

Discussion

There is cumulative evidence showing that brain aging is a complex and heterogeneous process characterized by a pattern of age-related preservation and selective loss and associated with a high degree of inter-individual variability (Gunning-Dixon et al., 2009). In this study we investigated whether global (whole-brain) measures of MD and FA, together with segmented WM, GM and CSF volumes and gender, are able to document microstructural brain changes during normal aging.

Our assembled model showed 93.5% accuracy for discrimination between young and elderly brains, with FA topping the ranking of significant discriminant variables, followed by CSF volume and WM volume; such a ranking of MRI global parameters as part of a predictive model has not previously been reported in clinical settings to the best of our knowledge.

Surprisingly, MD, GM volume and gender were not significant parameters for classifying between groups even though they have been reported in the literature as biomarkers of the brain aging process.

In our view the clinical importance of this study lies in the assembling of a multivariate predictive model, which combines global (whole-brain) measures of MD and FA (which are easier to calculate and understand) with segmented WM, GM and CSF volumes and gender, and allows the building of a profile of the healthy elderly brain in normal aging. The adoption of a predictive model might supplement the assessment of brain structure and function in different circumstances: when brain measures in a new patient deviate from the expected parameters of normal aging, as well as in the follow-up of selected cases. Although radiologists and researchers have previously used and reported these parameters separately, the literature lacks a model that integrates them and is able to document microstructural brain changes during normal aging on a day-to-day basis.

Our evidence pointing to decreased FA as the best biomarker for classification of young vs older brains is in agreement with the findings of a previous study in normal aging (Michielse et al., 2010). FA values for WM continue to increase until the third to fourth decade of life, but they start to decline during aging (after 40 years of age) (Kochunov et al., 2011). These findings further support the idea that decreased FA might be interpreted as an expression of degeneration of the axonal myelin sheath (demyelination) and/or replacement of axonal fibers with other cells (gliosis) (Smith et al., 2006), and that it may precede atrophy in many regions of the brain (Hugenschmidt et al., 2008).

On the other hand, MD reflects the physicochemical properties of the nervous system (e.g., viscosity and temperature) as well as its structural components (macromolecules, membranes, and intracellular organelles) (Di Paola et al., 2010). In our study, MD depicted the smallest correlation and its values were not significant for discriminating between groups.

A possible explanation for this apparent difference with previous findings is that in our study we used a global measure of MD instead of a local or regional measure, and it seems that MD changes remarkably depending on the brain region considered. This may suggest that MD is a weak global biomarker of normal aging. Further research on aging and its related biomarkers should focus on FA rather MD, however we acknowledge that MD might still have important applications outside of the aging process.

Gender had a non-significant influence as a biomarker in our study, a finding that agrees with the equivalent disruption of regional WM microstructure between men and women found by Sullivan et al. (2001). It is possible, therefore, that the pattern of transition from the young to the older brain in men and women is equivalent.

An additional interesting finding in this study was that increased WM volume significantly predicted membership of the older brain group; this finding is in line with the continued production of 'redundant myelin' that has been observed in human adults (Allen et al., 2005; Salat et al., 2009) and suggested to be a compensatory mechanism for myelin degeneration.

The observed decrease in GM volume in elderly brains was non-significant, in contrast to earlier findings of a gradual linear decrease of GM, 5% per decade of age, from early adulthood (Smith et al., 2007). These findings suggest that GM loss progresses gradually, whereas WM loss starts later and progresses more precipitously (Raz et al., 2005); these findings might indirectly explain the significant increase in CSF volume in the older brains in our study and its key role in the model. This interplay between segmented WM, GM and CSF volumes remains unclear and could be investigated in further studies including ratios of WM, GM and CSF instead of absolute values. Our model follows the recommendation of Abe et al. (2008) to assess FA and brain volumes together, as complementary indices of brain aging. Despite the non-significant participation of MD and GM volume in our model, we recommend keeping track of their changes during follow-up studies, and also of changes in the other structural and volumetric biomarkers (FA, CSF and WM volumes), until more evidence helps validate their role in integrative models.

Several limitations in our study need to be addressed: our DA model, represented by an equation, behaves like a regression model and is strictly valid only within the range of the observed data on the explanatory variables. Our sample size, although small, was statistically valid for evaluating the diagnostic performance of the predictive model (Cortez-Conradis et al., 2013); this study is a starting point for a research line focused on MRI biomarkers of aging in the normal brain and in degenerative brain diseases. Further studies could increase the homogeneity of the sample in terms of gender, as well as the number of subjects, thus increasing the statistical power for generalizing the findings.

Segmentation algorithms and intensity thresholds of GM, WM and CSF may differ across laboratories, producing variable results. We acknowledge that alternative software like Freesurfer (Han et al., 2006) allows the calculation of surface-based cortical thickness measures. We did not use that software because our aim was to limit the computational costs of our study; also, we aimed to choose variables (brain volumes) which we could compare with previous studies. We believe it is necessary to reach a consensus of standardized software algorithms and measurements able to guarantee that all measurements are conducted within the same algorithms in all patients; in this way, variations in the results would be a reflection only of the distribution of the selected biomarkers. For example, a recent study has proposed the use of machine learning, albeit in a younger age group (8-22 years) (Erus et al., 2015). Despite the initially steep learning curve of the open-source software packages used in this study, they are suitable for use on a day-to-day basis in MRI units, for example those supporting geriatric or family medicine studies, and not only in clinical research. We acknowledge that further studies examining the changes, with age, in the biophysical properties of the DTI signal are necessary, as well as the inclusion of additional brain volume correlates; both groups of variables could supplement the study of neurodevelopment, healthy aging and brain disorders (Roldan-Valadez et al. 2014, 2015).

The increased availability of open source software in MRI units around the world would allow these measurements to become low-cost and commonly used biomarkers. By calculating multivariate discriminant models, further studies will help to rank the influence of these parameters in physiological brain aging. Eventually, similar reports would lead to the generalization and acceptance of multivariate-integrative models by clinicians (geriatricians, neurologists, psychiatrist, neuroscientists, etc.).

In summary our study shows that FA, CSF volume and WM volume are reliable imaging parameters that can depict microstructural changes during normal brain aging by using a global and integrative approach.

Acknowledgments

This study was supported in part by Medica Sur Clinic & Foundation. Ernesto Roldan-Valadez was Coordinator of Research at the MRI Unit of Medica Sur Clinic and Foundation from 2010 to April 2015; Haydee Garcia-Lazaro and David Cortez-Conradis were enrolled as research fellows at the MRI unit of Medica Sur Clinic & Foundation for this project from 2011 to 2012.

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Does chronic exposure to mobile phones affect cognition?

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Summary

Mobile phones form an integral part of our modern lifestyle. Following the drastic rise in mobile phone use in recent years, it has become important to study its potential public health impact. Amongst the various mobile phone health hazards, the most alarming is the possible effect on the brain. The aim of the present study was to explore whether chronic exposure to mobile phones affects cognition. Ninety subjects aged 17-25 years with normal hearing were recruited for the study and divided into three groups according to their duration of mobile phone use. No significant differences in N100, P200, N200, P300 latencies or N2-P300 amplitude were observed. Our results suggest that chronic mobile phone exposure does not have detrimental effects on cognition.

KEY WORDS: cognition, event-related potentials, mobile phones

Introduction

Mobile phones form an integral part of our modern lifestyle. While cell phones provide an efficient means to communicate with friends and family, their overuse may have deleterious effects on health. The drastic increase in the number of mobile phone connections in our country has resulted in a sharp increase in the intensity of electromagnetic radiations in the environment. The potential electromagnetic radiation-related health risks associated with mobile phone overuse are a pressing environmental health issue and warrant investigation.

An emerging body of multidisciplinary studies has been carried out in recent years to investigate and monitor health risks due to electromagnetic emissions from mobile handsets. However, these studies yielded either inconclusive or contradictory results.

Numerous studies have focused on common health problems like headache, irritability, a burning sensation in the ears, and chronic pain and inflammation of joints – the latter due to responding to messages at a rapid speed on smart phones. Back pain is seen in cell phone users who hold their phone between their head and shoulders, especially when multi-tasking. Digital eye strain, eye redness, itching and dry eyes are also common. As the mobile phone antenna is in close proximity to the user's head, the brain is exposed to more electromagnetic fields (EMFs) than the rest of the body. A greater distance between head and handset is therefore essential to reduce radiofrequency exposure (Stefanics et al., 2008).

The effects of the electromagnetic radiation can be thermal (due to thermal interaction between radiation and tissues), producing skin burns, cataracts, seizures (Balbani and Montovani, 2008), or non-thermal (due to attraction between different types of cells in the presence of an electric field), producing changes in the electrophysiology of living cells (Kaprana et al., 2008).

Possible adverse effects of mobile phones on the central nervous system are of growing concern.

Various non-invasive methods are now used in quantitative evaluation of neurophysiological functions (Bastianello et al., 2008; Anjana et al., 2010; Vaney et al., 2011). Event-related potentials (ERPs) are the EEG potentials that are evoked by the perception of or the preparation for events and they include an early sensory evoked potential and a late cognitive response (P300 component) (Goodin, 1992). ERPs, which can be recorded through the scalp, are generated by neural activity associated with specific sensory, cognitive and motor processes. The N100 and P200 components are believed to reflect the activity occurring in neural areas that are activated by sensory stimuli and they are independent of the subject's attention (Picton and Hillyard, 1974). The N200 component is related to the degree of unexpectedness of the stimulus (Ritter et al., 1984), while the P300 wave is

believed to reflect cognitive processes underlying attention allocation and memory updating (Picton, 1992).

Whereas numerous studies have highlighted the effects of acute mobile phone exposure on ERPs of the human brain, there is little available evidence on how chronic exposure to mobile phones affects cognition. With this in mind, the primary aim of this work was to assess the effects on cognition of chronic exposure to electromagnetic radiation emitted by mobile phones.

Materials and methods

The present study was conducted at the Electrophysiology Laboratory in the Department of Physiology of UCMS, Delhi.

The study was carried out in 90 subjects aged between 17 and 25 years. Both males and females were included in the study. The subjects were divided into three groups according to the duration of their mobile phone use: 30 subjects who had used mobile phones for less than five years (Group 1); 30 subjects who had used mobile phones for more than five years (Group 2); 30 subjects who had never used a mobile phone (Group 3). Since this is a pilot study, a sample size of 30 subjects per group was required.

Selection of subjects

Students of UCMS college and neighboring colleges were invited to take part in this study. Only interested volunteers aged between 17 and 25 years were selected for the study. Subjects with a history of hearing impairment, use of ototoxic drugs and having previous ear infection or familial hearing disorder were excluded from the study. Among the college students there were just three who had never been exposed to mobile phones. Hence the subjects for Group 3 were selected from cleaners and other class IV employees of our college who had never been exposed to mobile phones. All the participants in Groups 1 and 2 filled in a questionnaire regarding their mobile phone use. These subjects were using mobile phones primarily to make phone calls, while text messaging and use of the internet constituted less than 20% of their total mobile phone use. The study was approved by the institutional ethics committee and informed consent was taken. The recording procedure was explained to the participants. All the subjects were tested under similar laboratory conditions having first been familiarized with the experimental and environmental (laboratory) conditions.

Recording of event-related potentials

The ERP recordings were done in a soundproofed room and the subjects underwent a trial session before the recording to familiarize them with the stimuli and the recording procedure. Recordings were done from the scalp of the subjects using an Octopus 4 M/C NCV/EMG/EP system (Biostar Health Care, India). Silver-silver chloride disk electrodes were placed at standard scalp locations according to the 10-20 international system. The electrodes were placed at the vertex of the skull (reference electrode Cz), at the forehead (ground electrode FPz), and at the ear lobes (active electrodes A1 and A2), after cleaning the scalp or skin sites with alcohol and then applying skin preparation gel and Elefix™ EEG paste. The skin electrode impedance was kept below 5 kΩ.

The auditory ERPs were recorded using an "oddball paradigm" wherein two stimuli (target and non-target) were presented in a random order through headphones. The target stimulus was a 2 kHz click sound with 20% occurrence and the non-target stimulus was a 1 kHz beep with 80% occurrence. The frequency of the auditory stimuli was 0.9 Hz (about 100 responses were collected and averaged), while their intensity was 90 dB SPL, with 60 dB SPL white noise masking of the contralateral ear.

Procedure

On hearing target stimuli delivered through headphones, the subject pressed a button on the response pad with the thumb of his/her dominant hand. During the recording session, the subject was instructed to close his/her eyes to avoid blink artifacts. Data for two trials were obtained, stored and averaged by computer. The peak latencies of the ERPs were measured from stimulus onset (stimulus artifact) to the peak point of the particular wave, i.e. the point of greatest amplitude. Amplitude was measured as the distance of the corresponding peak from the baseline. Peak latencies of N100, P200, N200 and P300 and amplitude of N2-P300 were recorded.

Statistical analysis

The statistical analysis was done using the SPSS 20 statistical package. The three groups were compared by one-way ANOVA and post-hoc Tukey HSD test at 5% level of significance. Data are presented as mean ± SD. A p-value <0.05 was considered significant.

Results

The latencies of the ERPs, i.e. N100, P200, N200, P300 were compared in the three groups. No significant differences in latencies were observed. Similarly, the N2-P300 amplitude did not differ significantly in the three groups (Table I). Representative ERP waves in the three groups are shown in figure 1.

Discussion

Mobile phone use is ubiquitous as it has become an integral part of modern telecommunications. About

half the population worldwide uses mobile phones, which constitute a rapidly growing market. It has thus become important to study the potential public health impact of mobile phone exposure. Previous ERP studies of mobile phone-related EMF exposure have given varying results. The present research was conducted to study the effect on ERPs of chronic mobile phone exposure. The subjects were divided into three groups according to their duration of mobile phone use.

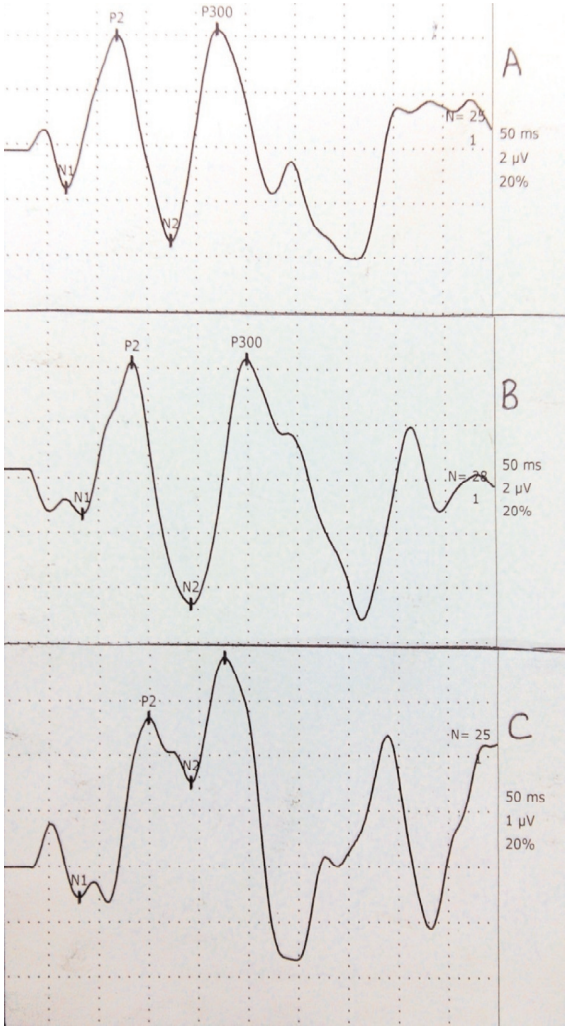

Figure 1- representative ERP waves in Group 1 (<5 years of mobile phone use, A) Group 2 (>5 years of mobile phone use, B) and Group 3 (no previous mobile phone use, C).

The results of present study revealed no significant changes in the latencies of N100, P200, N200 and P300 or in the amplitude of N2-P300 (Table I). ERPs, which can be recorded from the scalp, are generated by neural activity associated with specific sensory, cognitive and motor processes. The different waves of the auditory ERP have been found to reflect cognitive processes associated with different aspects of selective attention. The N100 component is thought to represent the initial extraction of information from the sensory analysis of the stimulus or the excitation associated with allocation of a channel for information processing in the primary cortex (Hansen and Hillyard, 1988). The P200 component may represent inhibition of further processing of the sensory input (Hegerl and Juckel, 1993), possibly via automatic stimulus identification and discrimination (Oades, 1998). As mentioned, it is thought to reflect the activity occurring in neural areas that are activated by sensory stimuli and it is independent of the subject's attention (Picton and Hillyard, 1974). The N200 component is thought to represent an endogenous mismatch detection process related to stimulus discrimination. This component has been associated with response identification (Snyder and Hillyard, 1976) and response selection. Our subjects showed no deficits in information processing as reflected in their ERP findings.

The P300 component of the ERP is associated with psychological processing. It is generated from various sites of the brain including the cortical and subcortical areas, particularly the auditory cortex, hippocampus, amygdala, brainstem and thalamic structures (Smith et al., 1990). The P300 wave is believed to reflect cognitive processes underlying attention allocation and memory updating (Picton, 1992), and its amplitude indicates the amount of difficulty encountered in differentiating target from non-target stimuli in the ERP oddball paradigm (Donchin and Coles, 1988). The lack of significant P300 latency and amplitude differences between the three groups in our study suggests that there were no deficits in the allocation of processing resources to task-relevant stimuli during the auditory oddball paradigm.

Table I - Mean latencies and amplitude of event-related potentials in subjects divided according to mobile phone use: <5 years (Group 1), >5 years (Group 2), none (Group 3).

		Group 1 (n=30)	Group 2 (n=30)	Group 3 (n=30)	p-value
Latency (ms)	N100	75.85 ± 17.21	90.27 ± 89.85	70.25 ± 25.65	0.350
	P200	136.65 ± 23.85	126.86 ± 27.69	124.76 ± 28.62	0.192
	N200	182.22 ± 23.32	186.40 ± 37.38	184.34 ± 27.56	0.865
	P300	239.73 ± 33.05	249.78 ± 37.75	240.68 ± 34.27	0.475
Amplitude (µV)	N2-P300	3.39 ± 2.20	3.09 ± 1.59	3.42 ± 1.54	0.736

Our results are consistent with those of Kleinogel et al. (2008) and Stefanics et al. (2008) who studied the effects of acute mobile phone exposure on ERPs. Kleinogel et al. tested the effects of EMFs on visually evoked occipital P100, the P300 of a continuous performance test, auditory evoked central N100 and the P300 during an auditory odd ball task, and on reaction time. They concluded that as the ERP and behavioral parameters were not changed, EMFs did not affect stimulus reception and processing. Stefanics et al. investigated the effect of 20 minutes of 3G mobile phone radiation on ERP components and early gamma synchronization in an auditory odd ball paradigm. They, too, observed no effect of mobile phone EMF exposure on above-mentioned parameters, however they underlined that since their results were obtained from young, healthy university students, further studies should be performed, focusing on other healthy subgroups, e.g. children, adolescents, elderly, and on groups of neurological patients who are more vulnerable due to specific deficits.

Hamblin et al. (2004) examined the effects of acute mobile phone exposure on human ERPs and performance during an auditory task. They observed decreased N100 amplitude, shortened N100 latency and delayed P300 latency during real as compared to sham exposure. They thus concluded that EMFs emitted by GSM mobile phones may alter human brain activity. In another study (Bak et al., 2010) of the effects on ERPs of acute exposure to GSM signals, no significant changes in the latencies of the N100, N200, P200, P300 waves were found but the P300 amplitude was significantly reduced. All these studies of acute exposure reported a high intensity of radiation.

An important point to consider when interpreting these data is that duration (in years) and frequency (number or duration of calls) of mobile phone use are imprecise measures of microwave exposure. The specific absorption rate (SAR) varies according to the make and model of the handset, the transmission system technology (analog or digital), the distance between the user's head and the handset antenna, and the distance between a mobile phone and its base station (Moulder et al., 2005). The maximum legally permitted levels of mobile phone radiation exposure in the United States and Europe are are ~1.6 W/kg and 2.0 W/kg respectively. Generally, exposure due to the use of hand-held mobile phones falls below these limits (Kesari et al., 2013). In the present study about 47% of the subjects used their mobile phone for just 30 to 60 minutes per day. This may be one of the reasons for the non-significant changes in our study.

All the mobile phone users in the present study used GSM mobile sets of various models. The SAR values of all these were below 1.6 W/kg, i.e. within safe exposure limits (Kesari et al., 2013). Moreover, all these subjects were using handsets and none used Bluetooth. Our subjects were using mobile phones primarily to make phone calls, while text messaging or internet functions constituted less than 20% of their total mobile phone use. Thus it can be concluded that emissions respecting the limits set for GSM mobile phones do not significantly impair cognition. Our study deals only with mild exposure. Further studies on a long-term exposure and in a larger sample size are recommended.

A limitation of our study is the fact that the exact radiation exposure/SAR could not be measured with precision. However, the radiation exposure/SAR values were below 1.6 W/kg, i.e. within safe exposure limits.

On the basis of the findings of the present study (the electrophysiological parameters did not show significant changes), it can be concluded that chronic mobile phone exposure does not have detrimental effects on cognition. However, further studies in larger numbers of subjects and with a longer duration of exposure are needed to substantiate these results.

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Andrographis Paniculata shows anti-nociceptive effects in an animal model of sensory hypersensitivity associated with migraine

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Abstract

Administration of nitroglycerin (NTG) to rats induces a hyperalgesic condition and neuronal activation of central structures involved in migraine pain. In order to identify therapeutic strategies for migraine pain, we evaluated the anti-nociceptive activity of *Andrographis Paniculata* (AP), a herbaceous plant, in the hyperalgesia induced by NTG administration in the formalin test. We also analyzed mRNA expression of cytokines in specific brain areas after AP treatment. Male Sprague-Dawley rats were pre-treated with AP extract 30 minutes before NTG or vehicle injection. The data show that AP extract significantly reduced NTG-induced hyperalgesia in phase II of the test, 4 hours after NTG injection. In addition, AP extract reduced IL-6 mRNA expression in the medulla and mesencephalon and also mRNA levels of TNF-alpha in the mesencephalic region. These findings suggest that AP extract may be a potential therapeutic approach in the treatment of general pain, and possibly of migraine.

KEY WORDS: cytokines, Andrographis Paniculata*, formalin test, migraine, nitroglycerin.*

Introduction

Experimental research has led to considerable advances in understanding of migraine pain mechanisms, and several options for both acute and prophylactic treatment have emerged (Olesen and Ashina, 2011). However, alternative therapeutic approaches are still needed because of tolerability issues and/or high percentages of non-responders. The popularity of medicinal herbs has grown significantly over the past decade and several reports have focused on the therapeutic potential of various plant-derived compounds, particularly molecules derived from natural leads with anti-inflammatory activities. Bioactive chemical constituents from medicinal herbs, namely sesquiterpene lactones, monoterpenes/diterpenes and flavonoids, are known to elicit a variety of pharmacological activities. Some of these compounds act as enzyme inhibitors and antioxidants, and have been reported to have anti-inflammatory properties (Middleton et al., 2000; Havsteen, 2002). The inflammatory response is associated with increased production of nitric oxide (NO) and activation of proinflammatory mediators (Moilanen et al., 1999; Bogdan, 2001). Although the exact mechanisms remain elusive, certain flavonoids and diterpenoids down-regulate NO production in response to inflammatory stimuli (Díaz-Viciedo et al., 2008). Experimental data support the idea that compounds inhibiting expression or activity of NO synthase (NOS) and nuclear factor-kappa B (NF-κB) are potential anti-migraine agents (Reuter et al., 2001). NO is involved in pain transmission, hyperalgesia, chronic pain, inflammation and central sensitization, mostly in a cyclic guanosine monophosphate-dependent way (Neeb and Reuter, 2007). Systemic nitroglycerin (NTG), an NO donor, activates brain nuclei involved in pain transmission as well as in neuroen-

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docrine and autonomic functions in rats. These changes are considered significant for migraine, because NTG consistently induces delayed migraine-like attacks only in migraineurs (Sances et al., 2004). Additionally, in rats, NTG injection in the formalin test (a model of inflammatory pain) induces a state of hyperalgesia that is detectable as an increase in nociceptive behavior (Greco et al., 2014, 2015). Various evidence points to the existence of a condition of trigeminal sensitization in migraineurs, which results in hyperalgesia, allodynia and cognitive dysfunction during and between episodes (Noseda and Burstein, 2013). Therefore, NTG-induced hyperalgesia in rodents is used as an experimental model for sensory hypersensitivity associated with migraine (Tassorelli et al., 1999; Greco et al., 2014, 2015). *Andrographis Paniculata* (AP), also known as "King of Bitters", is a herbaceous plant belonging to the Acanthaceae family; AP has been widely used in traditional Asian medicines for centuries. The traditional uses and pharmacological aspects of AP have been well documented, and its major constituents are diterpenoids, flavonoids and polyphenols (Jarukamjorn and Nemoto, 2008). Phytochemical studies have identified a specific bioactive diterpene, known as andrographolide, as the main component of AP (Sareer et al., 2014). Andrographolide and its derivatives and synthetic analogs have been shown to have a broad range of pharmacological properties, including anti-inflammatory, immunostimulatory and anticancer activities (Akbar, 2011; Varma et al., 2011). In addition, the components of AP extract exhibit anti-inflammatory effects by interfering with the production of inflammatory mediators, in particular cyclooxygenase (COX) enzymes and pro-inflammatory cytokines (Parichatikanond et al., 2010). Notably, these effects are intimately associated with the modulation of the NF-κB signaling network and the down-regulation of pro-inflammatory responses (Lim et al., 2012). Moreover, another critical target for the inflammation suppressive activity regulated by andrographolide is the NO/inducible NOS (iNOS) pathway (Lim et al., 2012). The NO/iNOS system is involved in pain transmission, hyperalgesia, chronic pain, neuroinflammation and central sensitization (Barbanti et al., 2014). Despite the potent anti-inflammatory properties exhibited by AP, its anti-nociceptive and analgesic effects are still largely unexplored. However, recent studies reported an anti-nociceptive effect of AP in the acetic acid-induced writhing, hot-plate and formalin tests (Sulaiman et al., 2010; Adedapo et al., 2015).

The aim of the present study was to evaluate the anti-nociceptive activity of AP and its potential effect on the mRNA expression of specific cytokines in an animal model of hyperalgesia induced by NTG administration (Tassorelli et al., 2003; Greco et al., 2014). This experimental model has been tested over the years with different drugs and is considered a reliable animal model of migraine (Tassorelli and Joseph, 1995; Bergerot et al., 2006; Greco et al., 2011).

Materials and methods

Animals

Adult male Sprague-Dawley rats (weight 250-270 g) were evaluated in the present experiments. The principles of the Helsinki declaration and IASP's guidelines for pain research in animals were rigorously applied (Zimmermann, 1983). The rats were housed two per cage, at 20–22 °C on a 12-h light-dark cycle, with food and water *ad libitum* at the Centralized Animal Facility of the University of Pavia. All procedures were approved by the local Animal Care Committee. All the rats were acclimatized to the test chamber before testing began.

Drugs

The ethanolic extract of aerial parts of AP (Product: L100235L, purified by high performance liquid chromatography; andrographolide average content 10%) was purchased from Truffini and Reggè Farmaceutici, Milan, Italy. The extract was dissolved in saline (used as vehicle) and administered to the animals as intraperitoneal (i.p.) suspensions. Suspensions were freshly prepared every day (Sulaiman et al., 2007, 2010). Nitroglycerin (Bioindustria L.I.M. Novi Ligure (AL), Italy) was prepared from a stock solution of 5.0 mg/1.5 ml dissolved in 27% alcohol and 73% propylene glycol (PG). For the i.p. injections, NTG was further diluted in saline to reach the final concentration of alcohol 6% and PG 16% and administered at a dose of 10 mg/kg. The vehicle control used in these experiments was 16% PG, 6% alcohol and 0.9% saline (NTG vehicle).

Formalin test: experimental design

The rats were randomly divided into groups of 6–8 animals, according to the following treatment schedule:

1) control group (n=8): saline (30') + NTG vehicle, 12 ml/kg, i.p.
2) saline + NTG group (n=8): saline (30') + NTG, 10 mg/kg, i.p.
3) AP 1 + NTG group (n=6): AP extract (30'), 50 mg/kg, i.p. + NTG;
4) AP 2 + NTG group (n=6): AP extract (30'), 25 mg/kg, i.p. + NTG.

In addition, to evaluate the possible interaction of AP extract with NTG vehicle we included another experimental group:

5) AP 1 + NTG vehicle group (n=6): AP extract (30'), 50 mg/kg, i.p. + NTG vehicle

AP or saline was administered 30 minutes (30') before NTG or NTG vehicle injection. The formalin test was performed 4 hours after NTG or vehicle administration. The investigators responsible for the behavioral testing were blind to treatment group assignment. For the formalin test, a 100 μl volume of 1% formalin (formaldehyde diluted in 0.9% saline) was adminis-

tered by intraplantar injection. Pain-related behavior was quantified for 1 h by counting spontaneous flinches and shakes of the injected paw: over 60-s periods for the first 5 min (min 1, 2, 3, 4 and 5) and thereafter following 4-min pauses, for 60-s periods up to the hour. Phase I was defined as the period from 1 to 5 min, phase II was defined as the period from 15 to 60 min inclusive. Phase I is considered the result of chemical activation of nociceptors, whereas phase II reflects the inflammatory reaction and central processing (Tjølsen et al., 1992).

Detection of mRNA expression of cytokines: experimental design

The rats were randomly divided into four groups of 5–6 animals, according to the following treatment schedule:

1) Control group (n=6): saline (30') + NTG vehicle, 12 ml/kg, i.p.
2) saline + NTG group (n=6): saline (30') + NTG, 10 mg/kg, i.p.
3) AP 1 + NTG group (n=5): AP extract (30'), 50 mg/kg, i.p. + NTG
4) AP 1 + NTG vehicle group (n=5): AP extract (30'), 50 mg/kg, i.p. + NTG vehicle

On the basis of the distribution of the nuclei that are known to be activated by NTG and involved in migraine pain, the following discrete brain areas were dissected out 4 hours after NTG or vehicle administration and used for analysis: the medulla (bregma, –13.30 to –14.60 mm), containing the nucleus trigeminalis caudalis (NTC), nucleus tractus solitarius and area postrema; the mesencephalon (bregma, –4.8 to –6.04 mm), containing the ventrolateral column of the periaqueductal gray and parabrachial nucleus.

Real-time polymerase chain reaction

Interleukin-6 (IL-6) (forward primer: TTCTCTCCGCAAGAGACTTC; reverse primer: GGTCTGTTGTGGGTGGTATC) and tumor necrosis factor alpha (TNF-alpha) (forward primer: CCTCACACTCAGATCATCTTCTC; reverse primer: CGCTTGGTGGTTTGCTAC) mRNA expression was analyzed by a real-time polymerase chain reaction (RT-PCR). Total RNA was isolated from the cerebral samples with Trizol reagent in accordance with the method of Chomczynski and Mackey (1995). RNA was quantified by measuring the absorbance at 260/280 nm. cDNA was generated using the iScript cDNA Synthesis kit (Bio-rad, Milan) following the supplier's instructions. Gene expression was analyzed using the Fast Eva Green supermix (Bio-rad). As regards the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH; forward primer: AACCTGCCAAGTATGATGAC; reverse primer: GGAGTTGCTGTTGAAGTCA) was used. The expression of the housekeeping gene remained constant in all the experimental groups considered. The amplification was performed through two-step cycling (95–60°C) for 45 cycles in a light Cycler 480 Instrument RT-PCR Detection System (Roche, Milan) following the supplier's instructions. All samples were assayed in triplicate. Gene expression was calculated using the ΔCt method.

Statistical analysis

Data are expressed as mean ± SEM. The total number of flinches/shakes evoked by formalin injection was counted separately for phase I and for phase II, as described above. An a priori power analysis was conducted to determine the minimal sample size needed to obtain a statistical power of 0.80 at an alpha level of 0.05. In our previous studies, evaluating the difference in nociceptive response in the second phase of the formalin test (total number flinches/shakes) between rats injected with NTG and rats injected with vehicle (Tassorelli et al., 2006), we calculated a standardized effect size of 2.38 for this variable. The analysis estimated a sample size of at least five rats per group. We used 5–8 rats per experimental group. The data were tested for normality using the KS normality test and considered normal. The differences between groups were analyzed by unpaired t test or one-way analysis of variance (ANOVA) followed by the Newman-Keuls Multiple Comparison Test when more than two groups were compared. The minimum level of statistical significance was set at $p<0.05$.

Results

Formalin test

In the control group (saline + NTG vehicle), the formalin injection induced an initial acute phase of nociception within the first 5 min (phase I), followed by a prolonged tonic response from 15 to 60 min after formalin injection (phase II). As expected, NTG administration significantly increased the nocifensive behavior in phase II of the formalin test, compared with the control group (Fig.1), confirming previous data (Tassorelli et al., 2006). AP ethanolic extract (25 mg/kg) significantly reduced the nocifensive behavior in phase II of the test after NTG administration. The effect was more evident when AP ethanolic extract was administered at a higher dose (50 mg/kg) before NTG administration (Fig.2). No significant effect on nocifensive behavior was observed after AP extract (50 mg/kg) pre-treatment in the experimental group treated with NTG vehicle (Fig.3).

mRNA expression of cytokines

IL-6 and TNF-alpha mRNA expression was significantly increased in the medulla and in the mesencephalon of rats treated with NTG, compared with the control group (saline + NTG vehicle) (Fig.s 4 and 5). AP ethanolic extract pre-treatment significantly reduced

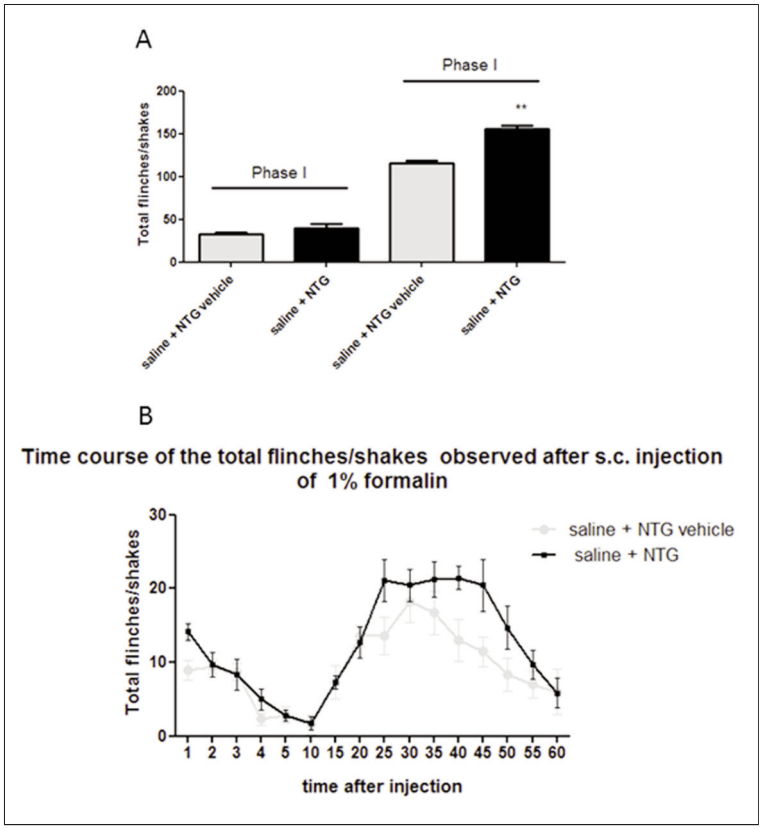

Figure 1 - Nitroglycerin-induced hyperalgesia in the formalin test.
(A) NTG administration significantly increased the total number of flinches/shakes during phase II of the test, when compared with saline + NTG vehicle group (control group). No significant effect was reported in phase I. (B) Time course of the nocifensive behavior in the different groups. Data are expressed as mean ± SEM. Unpaired t test, **p<0.01 vs saline + NTG vehicle.

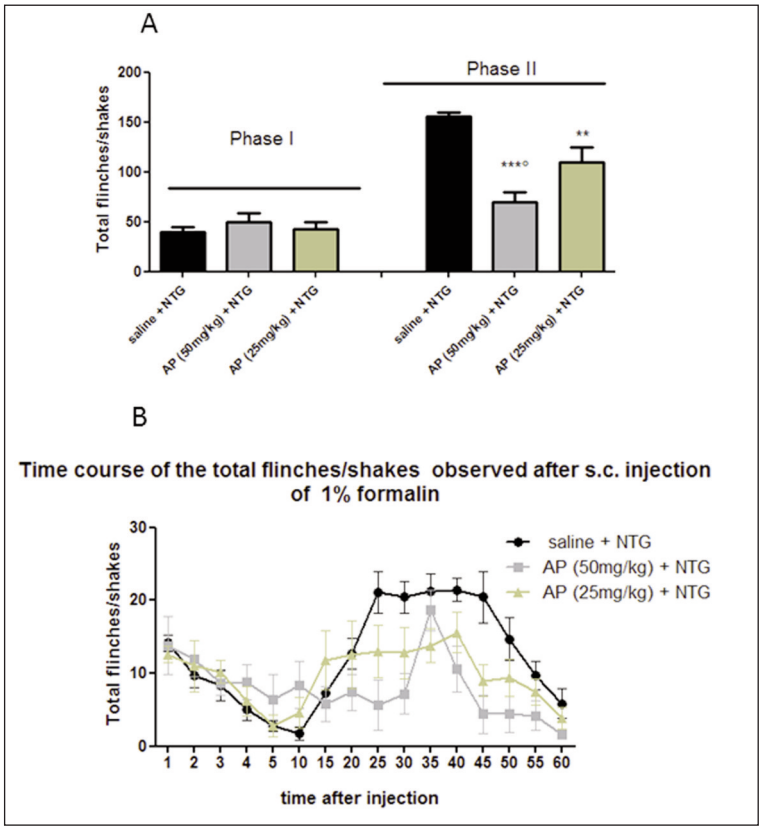

Figure 2 – Effects of *Andrographis Paniculata* (AP) on nitroglycerin-induced hyperalgesia in the formalin test.
(A) The histograms illustrate the total number of flinches and shakes per phase of the test. AP ethanolic extract (50 mg/kg or 25 mg/kg) is effective to counteract NTG-induced hyperalgesia during phase II of the test, compared with saline + NTG group. (B) Time course of the nocifensive behavior in different groups. Data are expressed as mean ± SEM. ANOVA followed by Newman-Keuls Multiple Comparison Test; ***p<0.001 vs saline + NTG; **p< 0.01 vs saline + NTG; °p<0.05 vs AP (25 mg/kg).

the expression of IL-6 mRNA in all cerebral areas. The level of TNF-alpha mRNA was reduced only in the mesencephalon (Fig.s 4 and 5). No significant effect in cytokine mRNA expression was observed after AP extract pre-treatment in cerebral areas of rats treated with saline + NTG vehicle (Fig.s 4 and 5).

Discussion

Systemic NTG activates brain nuclei involved in nociceptive transmission as well as in neuroendocrine and autonomic functions in rats (Tassorelli et al., 1999). These changes are considered relevant for migraine because NTG consistently provokes spontaneous-like migraine attacks in migraineurs (Sances et al., 2004). NTG is able to induce a long-lasting hyperalgesic state in rats, detectable as an increase in the nociceptive behavior evoked by the formalin test (Tassorelli et al., 2006; Greco et al., 2015). This is probably due to complex mechanisms involving NO synthesis but also to sensitization of the trigeminovascular system at the meningeal and central level (Galeotti and Ghelardini, 2013). The formalin test was introduced as a model of tonic pain several years ago, and has since been used extensively in rats and mice (Tjølsen et al., 1992). In this study, we investigated the anti-hyperalgesic effect of AP ethanolic extract in baseline conditions and in NTG-induced hyperalgesia in the formalin test. In addition, in order to evaluate whether pretreatment with AP may modulate expression of pro-inflammatory cytokines, we also examined IL-6 and TNF-alpha mRNA expression in brain areas specific for migraine pain.

AP ethanolic extract proved capable of counteracting NTG-induced hyperalgesia in the second phase of the formalin test but did not alter phase I. This was also the case when a lower dose was used. Lack of phase I responses suggests that AP ethanolic extract does not alter basal pain but rather reduces pain transmission and alters nocifensive behavior in phase II compared with the control group (saline + NTG vehicle). Although several studies have reported a significant analgesic effect of AP in animal models of inflammatory pain, this discrepancy in the results may be due to differences in timing and route of administration (Sulaiman et al., 2007; Adedapo et al., 2015). It should also be pointed out that the possible mechanisms underlying NTG-induced hyperalgesia are currently elusive. However, it is becoming increasingly evident that NTG exerts its activity through a direct effect on the production of NO and pro-inflammatory mediators at central level (Tassorelli et al., 2006, 2007), or indirectly via inflammation mediated by NO-dependent mechanisms at the meningeal level as a consequence of sensitization of the trigeminovascular system (Reuter et al., 2001). The second phase of the formalin test reflects the activation of mediators of the inflammatory response and the activation of dorsal horn neurons (Tjølsen et al., 1992). Therefore, it is possible that the clear-cut anti-hyperalgesic effect observed following the AP administration reflects an

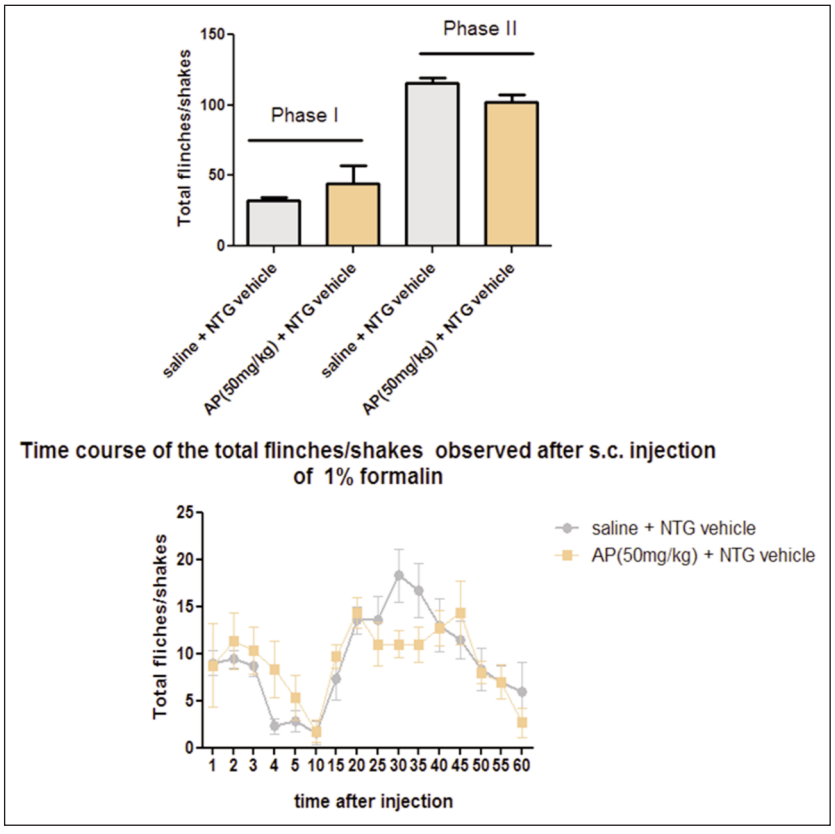

Figure 3 - *Andrographis Paniculata* (AP) effects in baseline conditions (without NTG)
(A) The histograms illustrate the total number of flinches and shakes per phase of the test. After AP extract (50 mg/kg) pre-treatment no significant effect was observed on the nocifensive behavior in the experimental group treated with NTG vehicle.
(B) Time course of the nocifensive behavior in different groups. Data are expressed as mean ± SEM. Unpaired t test.

interaction at the periphery as well as at central level. Cytokines play a key role in inflammatory responses, modulation of the pain threshold and also in trigeminal nerve fiber sensitization. These mediators are related to neurogenic inflammation in the pathogenesis of migraine. Moreover, it is known that increased expression of cytokines is implicated in the development and maintenance of sensitization of peripheral nociceptors and second order neurons (Miller et al., 2009). Migraine patients indeed show higher serum levels of IL-1beta and IL-6, and lower levels of IL-10 than healthy subjects (Uzar et al., 2011). By contrast, TNF-alpha levels increase after migraine pain onset and decrease progressively over time (Perini et al., 2005).

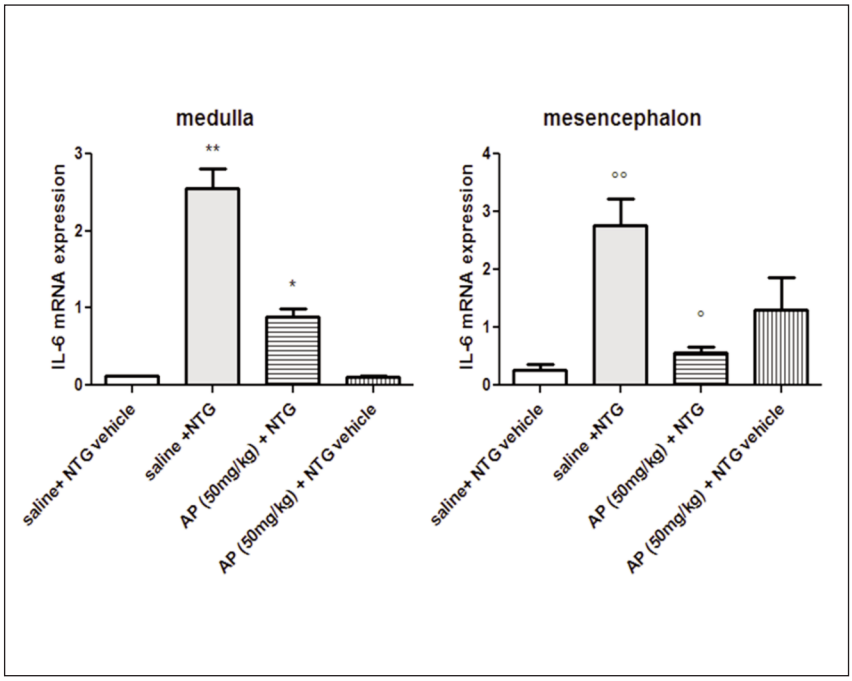

Figure 4 - IL-6 mRNA expression in specific brain areas.
NTG administration significantly increased IL-6 mRNA expression in the medulla and mesencephalon compared with saline + NTG vehicle group. AP pre-treatment significantly reduced IL-6 mRNA expression in both brain areas, compared with saline + NTG group. Data reported as mean ± SEM, ANOVA followed by Newman-Keuls Multiple Comparison Test, **p<0.01 vs saline + NTG vehicle and AP (50 mg/kg) + NTG vehicle; * p<0.05 vs saline + NTG, saline + NTG vehicle and AP (50 mg/kg) + NTG vehicle; °°p<0.01 vs saline + NTG vehicle; °p<0.05 vs saline + NTG.

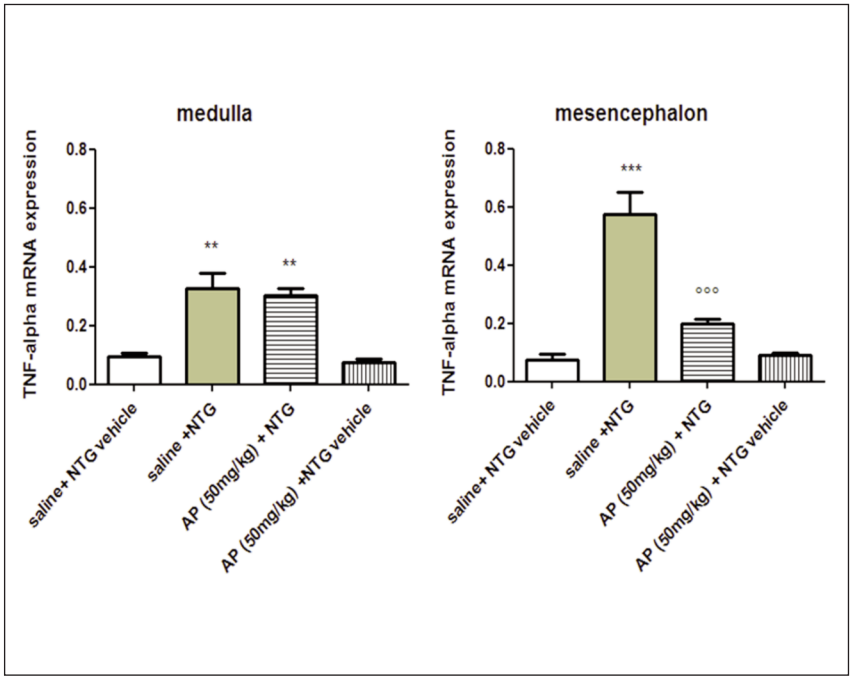

Figure 5 - TNF-alpha mRNA expression in specific brain areas.
NTG administration significantly increased TNF-alpha mRNA expression in the medulla and mesencephalon, compared with saline + NTG vehicle group. AP pre-treatment significantly reduced TNF-alpha mRNA level in the mesencephalon, compared with saline + NTG group. Data reported as mean ± SEM, ANOVA followed by Newman-Keuls Multiple Comparison Test. **p<0.01 vs saline + NTG vehicle and AP (50 mg/kg) + NTG vehicle; ***p<0.001 vs saline + NTG vehicle and AP (50 mg/kg) + NTG vehicle; °°°p<0.001 vs saline + NTG.

Our findings show that NTG significantly increased IL-6 and TNF-alpha expression in all cerebral areas examined. These findings demonstrate for the first time that NTG administration may induce changes in cerebral cytokine gene expression and not only at the dural level (Reuter et al., 2001). A recent study reported a significant increase of pro-inflammatory cytokines in the trigeminal ganglia and in spinal trigeminal nuclei following capsaicin injection in the eyebrow regions (Vause and Durham, 2012). In this study, pre-treatment with AP ethanolic extract significantly reduced IL-6 mRNA expression in all cerebral areas, while a significant reduction of TNF-alpha mRNA expression was found only in the mesencephalon. In accordance with these findings, Parichatikanond et al. (2010) demonstrated that components of AP extract induce a down-regulation of genes involved in inflammatory responses, including IL-6 and TNF-alpha. Andrographolide is one of the main ingredients of AP, and it was reported to possess anti-inflammatory activity (Akbar, 2011, Lim et al., 2012) and to inhibit NO production in lipopolysaccharide-stimulated murine macrophage (Liu et al., 2007). In a previous study, Hidalgo et al. (2005) reported the ability of andrographolide to inhibit NF-κB binding to DNA, which then leads to reduced expression of proinflammatory proteins, including COX-2 and cytokines. The capacity of AP to reduce inflammatory pathways by preventing binding of NF-κB to DNA thus represents a potential mechanism for its anti-hyperalgesic effect. It is noteworthy that NTG increases the transcriptional activity of NF-κB in the dura and medulla of rats (Reuter et al., 2001; Greco et al., 2005) and these changes may also be associated with increased COX-2 expression in the medulla (Tassorelli et al., 2007). The medulla is the area containing the NTC, one of the most important relay stations for pain transmission in the brain. In this area, we found increased IL-6 and TNF-alpha mRNA levels.

Taken together, these observations provide evidence that AP ethanolic extract has anti-hyperalgesic activity in an experimental animal model of sensory hypersensitivity associated with migraine. AP ethanolic extract seems to act through mechanisms that may be related to direct or indirect inhibition of pro-inflammatory responses in specific brain areas involved in migraine pain transmission.

Acknowledgments

This work was funded by FB Health S.p.A. (Ascoli Piceno – Italy)

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FORTHCOMING EVENTS

13-15 April 2016: Venice, Italy
25TH EUROPEAN STROKE CONFERENCE (ESC) 2016
http://eurostroke.eu/esc-venice-2016

15-21 April 2016: Vancouver, Canada
AMERICAN ACADEMY OF NEUROLOGY (AAN) ANNUAL MEETING 2016
www.aan.com/conferences

21-24 April 2016: Budapest, Hungary
31ST INTERNATIONAL CONFERENCE OF ALZHEIMER'S DISEASE INTERNATIONAL (ADI)
www.adi2016.org

1-5 May 2016: Amsterdam, the Netherlands
14TH INTERNATIONAL CHILD NEUROLOGY CONGRESS (ICNC)
www.icnc2016.org

9-11 May 2016: Coex, Seoul, Korea
4TH INTERNATIONAL CONFERENCE ON MOLECULAR NEURODEGENERATION (ICMN 2016)
www.icmn2016.org

10-13 May 2016: Philadelphia, Pennsylvania, USA
9TH WORLD CONGRESS FOR NEUROREHABILITATION (WCNR 2016)
http://wcnr2016.org

28-31 May 2016: Copenhagen, Denmark
2ND CONGRESS OF THE EUROPEAN ACADEMY OF NEUROLOGY (EAN) 2016
www.eaneurology.org

7-9 June 2016: London, UK
THE 2016 ALZHEIMER'S DISEASE CONGRESS
http://lifescienceevents.com/Alz2016

8 -10 June 2016: Rome, Italy
38TH ITALIAN LEAGUE (LICE) NATIONAL EPILEPSY CONGRESS
www.ptsroma.it/lice2016

17-18 June 2016: Warsaw, Poland
5TH INTERNATIONAL CONFERENCE "ADVANCES IN CLINICAL NEUROIMMUNOLOGY" (ACN)
www.acn2016.eu

2-6 July 2016: Copenhagen, Denmark
10TH FORUM OF NEUROSCIENCE (FENS)
http://forum2016.fens.org

11-16 July 2016: Maastricht, The Netherlands
21ST MEETING OF THE INTERNATIONAL SOCIETY FOR THE HISTORY OF THE NEUROSCIENCES (ISHN)
www.ishn.org

11-15 September 2016: Prague, Czech Republic
12TH EUROPEAN CONGRESS ON EPILEPTOLOGY
www.epilepsyprague2016.org

14-17 September 2016: London, UK
32ND CONGRESS OF THE EUROPEAN COMMITTEE FOR TREATMENT AND RESEARCH IN MULTIPLE SCLEROSIS (ECTRIMS)
www.ectrims-congress.eu

20-23 September 2016: Portland, Oregon, USA
4TH WORLD PARKINSON CONGRESS
www.wpc2016.org

12-16 October 2016: Mannheim, Germany
12TH MEETING OF THE EUROPEAN ASSOCIATION OF NEURO-ONCOLOGY (EANO)
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