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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