Rev Environ Health 2012;27(1):19–41 © 2012 by Walter de Gruyter • Berlin • Boston. DOI 10.1515/reveh-2012-0002

Are environmental exposures to selenium, heavy metals, and pesticides risk factors for amyotrophic lateral sclerosis?

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Abstract

The etiology of sporadic amyotrophic lateral sclerosis (ALS), the most common form of this degenerative disease of the motor neurons, is still unknown, despite extensive investigation of several genetic and environmental potential risk factors. We have reviewed laboratory and epidemiological studies assessing the role of exposure to neurotoxic chemicals (metalloid selenium; heavy metals mercury, cadmium, and lead; pesticides) in ALS etiology by summarizing the results of these investigations and examining their strengths and limitations. Despite limitations in the exposure assessment methodologies typically used in human studies, we found suggestive epidemiological evidence and biologic plausibility for an association between ALS and antecedent overexposure to environmental selenium and pesticides. The relation with mercury, cadmium, and lead appears weaker.

Keywords: heavy metals; motor neuron disease; neurotoxins; pesticides.

Introduction

Amyotrophic lateral sclerosis (ALS) is a rare and extremely severe human neurodegenerative disease characterized by a degeneration of upper and lower motor neurons in both motor cortex and spinal cord, leading to progressive paralysis and death due to respiratory failure (unless mechanical ventilation is supplied). The etiology of this most common form of motor neuron disease (MND), nearly 140 years after its description by Charcot, still remains unknown. Both environmental and genetic factors have been involved in the familial and in the more common sporadic form, the incidence of which might be increasing according to recent reports (1, 2) and appears to have uneven spatial distribution (3). Environmental risk factors for which some epidemiological and clinical evidence exists include neurotropic viruses, cyanobacterial toxins, magnetic fields, and several chemicals (4–6). In this review, we have summarized and discussed the evidence supporting a role of environmental exposure to chemical substances in ALS etiology, focusing on a metalloid [selenium (Se)], three heavy metals [mercury (Hg), cadmium (Cd), and lead (Pb)], and pesticides.

Selenium

Se is a trace element commonly found in very low concentrations in the environment and occurs naturally in several organic and inorganic species. The element is of paramount importance in both toxicological and nutritional aspects (7–10). The main source of Se intake in humans is the diet, although sometimes ambient air in occupational settings and in areas characterized by large combustion of coal (11, 12) or in drinking water (10, 13) can become relevant sources of exposure. The exact role of Se in human health is still not well defined and highly controversial (10, 14), despite recent advancements in the understanding of its dichotomous involvement in human health and disease (10, 12, 14–17). ALS is among the human diseases suggested to derive from excess Se exposure on the basis of two epidemiological studies that found an increased risk of ALS associated with residence in a seleniferous area or with consumption of drinking water with unusually high levels of inorganic hexavalent Se. Laboratory investigations have provided supportive evidence of a cause-effect relation (18, 19).

Se has long been recognized as a wide-spectrum neurotoxic agent, based on laboratory studies. In the rat, Se compounds (in most cases, in its inorganic tetravalent form, selenite) were observed to enter the central nervous system (CNS) from peripheral blood vessels, showing an enhanced affinity for the spinal cord and the hypothalamus (20). The metalloid can alter dopamine levels in brain, exert hypothermic and nociceptive responses, impair locomotor activity (21), interfere with prostaglandin D synthesis and activity (22), inhibit at very

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Received October 11, 2011; accepted January 5, 2012

low levels δ-aminolevulinate dehydratase in the CNS (23), and affect the latencies and conduction velocity distribution of sciatic nerve fibers (24). Se compounds increase the activity of lactato dehydrogenase and the amount of thiobarbituric acid reactive substances in the rat brain (25), impair glutamate uptake (26, 27), and inhibit succinic dehydrogenase, acetylcholine esterase, and Na/K ATPase (28). In cultured primary mouse cortical neurons (29), Se levels as low as 8 μg/L and even less in its inorganic tetravalent form (selenite) induced apoptosis. Selenite was shown to provoke abnormalities in dopamine metabolites, lipids, and thiobarbituric acid-reactive substances in the striatum (19, 30) and cardiorespiratory effects, hind-limb paralysis, and death after intravenous administration (31). Se may also be responsible for the accumulation of a Hg-Se complex in CNS areas (32), with a consequent possible release of these toxic elements into the CNS over time. Depending on its concentration and chemical form, Se toxicity in cultured nervous system cells might be additive or antagonistic to Hg toxicity (33).

Se compounds are able to damage mitochondrial functions and integrity (34, 35), a pattern that might be involved in ALS etiopathogenesis (36). Several lines of evidence support the view that mitochondria are affected in the course of motor neuron degeneration and that mitochondrial dysfunction may actively participate in the demise of motor neurons. Mitochondrial abnormalities have been described in ALS patients. In vitro studies using rabbit aorta have shown Se compounds to damage vascular smooth muscle reactivity by inhibiting both contracting and relaxing properties (37), which might also be related to the amyotrophic process characterizing ALS. Se also inhibits purified human squalene monooxygenase, a key enzyme for cholesterol biosynthesis (38). Inhibition of this enzyme leads to a peripheral demyelinating neuropathy in rats, which occurs secondary to a systemic block in cholesterol synthesis (39).

All the above-mentioned studies demonstrate the potential neurotoxicity of Se in the experimental animal. Nevertheless, the strongest evidence of a biological plausibility for the Se-ALS association emerges from veterinary medicine studies in swine, which showed that Se intoxication (due to inorganic Se compounds in most cases) results in a selective necrosis of the ventral horns of the spinal cord and other CNS regions (40–45). The induction of symmetrical poliomyelomalacia in pigs was enhanced by inorganic Se compared with organic Se, despite the higher Se tissue content after the administration of the organic form (46). The occurrence of polioencephalomyelomalacia has also been experimentally induced in cattle following the administration of inorganic Se as selenite; neurological signs included trembling of skeletal muscles, enhanced by exercise, which led the treated animals to shiver (47), resembling the blind-staggers originally described in livestock from seleniferous areas in the USA (12). Sodium selenite was found to be toxic to chicks, inducing neuromuscular blockade and tetanic spasm: the compound blocked axonal conduction and directly affected the muscle membrane by releasing Ca^{2+} from the sarcoplasmic reticulum (18). Selenite also inhibited neurotransmitter release and axonal conduction in a mouse phrenic nerve-diaphragm preparation (48). Morgan et al. (49) have shown the capability of long-term Se exposure to impair locomotor activity and even to paralyze the nematode *Caenorhabditis elegans*, likely due to a Se-induced generation of reactive oxygen species (ROS).

Overall, in the light of the above studies, Se appears to exert selective toxicity on the motor neurons in several animal species at least. The same pathological feature characterizes ALS and seems to be a distinctive feature of Se compared with all other chemicals, to the best of our knowledge. Such neurotoxic effects of Se, however, may vary considerably according to the animal species and the chemical forms of the metalloid involved (12, 14, 50).

The key evidence linking Se to ALS, however, is based on epidemiological evidence arising from human studies. The first investigation to suggest a relation between exposure to environmental Se and ALS was a report published in 1977 by a physician in a private practice, Arthur Kilness, and the Harvard neurologist Fred Hochberg, who described the occurrence of a cluster of four cases of ALS among male ranchers within a sparsely populated South Dakota county (around 4000 inhabitants) during an 11-year period (51). The authors also outlined the early veterinary medicine observations that showed the occurrence of the so-called blind-staggers disease in livestock chronically intoxicated with Se, as well as the finding of spinal cord degeneration in malformed embryos of hens characterized by excess Se intake, and remarked on the need to further investigate environmental Se as a potential risk factor for human ALS. This study had some limitations, however, such as the lack of a prespecified area and period of the study and, more importantly, the possibility of ecological bias due to other environmental or genetic factors.

A few case-control studies were subsequently carried out to investigate the role of Se exposure in ALS etiology, although the lack of specific biomarkers of long-term exposure to the metalloid, as well as the severe impairment of health status occurring during early disease progression, substantially hampered the assessment of long-term antecedent exposure in patients. Two studies found higher Se levels in the spinal cord of ALS patients compared with controls (52, 53), whereas in two Italian studies, blood and toenail Se concentrations were not increased in such patients (54, 55). The results of these studies also suggest that the nutritional status of patients might severely alter the circulating Se levels and, therefore, that case-control studies lacking long-term indicators of exposure are unsuitable to investigate the Se-ALS relation. Furthermore, no studies were conducted on the possible relation between the different Se chemical forms and ALS risk, which further hinders our ability to assess this issue, considering the differences in the biological activity and toxicity of the different Se compounds. Other observations from seleniferous areas of China indicated the potential of environmental Se to induce slight to severe motor disorders in humans (56, 57), although no such effect was recently reported in a population the Brazilian Amazon characterized by high Se intake and which also has the highest Hg exposures reported in the world today (58). The authors noted that their results are not necessarily applicable to populations with lower Hg exposure and/or Se status. Signs of neurotoxicity were also detected

in Inuit children from Northern Quebec exposed to high levels of methyl-Hg, polychlorinated biphenyl, and Se, but in that case, the adverse effects were exerted on visual-evoked potentials (59).

Two studies carried out in a peculiar Italian area, where a limited population experienced intake of inorganic hexavalent Se (selenate) for several years, have provided new and suggestive evidence linking environmental Se with ALS (60, 61). The study area was Rivalta, a neighbor of the city of Reggio Emilia in Northern Italy (around 150,000 inhabitants), where during the 1970s and the 1980s, the public water supply system had to be separated from the remaining system due to technical reasons. The Rivalta aqueduct was connected to two local wells providing, as detected several years later, water with an unusually high content of Se (around 8 µg/L) in its inorganic hexavalent form, selenate, the species usually found in underground waters (Figure 1) (62, 63). Consumption of this high-Se drinking water by the local population (2000 to >6000 residents, according to length of drinking water consumption) was unknown to both the population itself and the Municipal Water Authority until 1988 (60, 62, 64). Only then, due to the disclosure of the high Se content, were the two wells disconnected from the municipal aqueduct, and the Rivalta aqueduct shared the water distributed in the remaining municipality, containing <1 µg Se/L. This situation provided the unusual design of a 'natural experiment', a situation of strong interest in epidemiology to investigate adverse health effects of toxicants that cannot be studied in planned experimental studies for obvious ethical reasons. Indeed, the exposed population was unaware of the high Se content of the drinking water they consumed, and no other chemical features differentiated the tap water distributed in Rivalta from the tap water supplied to the remaining municipality. The subjects consuming the high-Se drinking water in Rivalta and the remaining municipal population also appeared to be considerably similar regarding their socioeconomic characteristics (62, 63). This situation provided an optimal setting to perform a cohort study, originally designed under the hypothesis that a higher Se intake in Rivalta might be associated with beneficial effects on cancer and cardiovascular risk (64, 65) but later including other adverse effects, such as ALS incidence in the follow-up (60). Long-term consumption of the high-Se drinking water was associated with a strong increase in ALS risk [4.2, 95% confidence interval (CI) 1.2–10.8], which was even higher when we limited the analysis to the 2065 individuals having the longest exposure (8.9, 95% CI, 2.4–22.8) (60). Several years after this report, an analysis of the Se-ALS relation was repeated in the same locale, with two key methodological variations to the study design: extension of the analysis to any source and period of consumption of high-Se drinking water and choice of a case-control approach. The ALS incidence was assessed among the exposed subjects in the period 1995–2006, immediately subsequent to that examined in the prior cohort study (1986–1994) (61). The

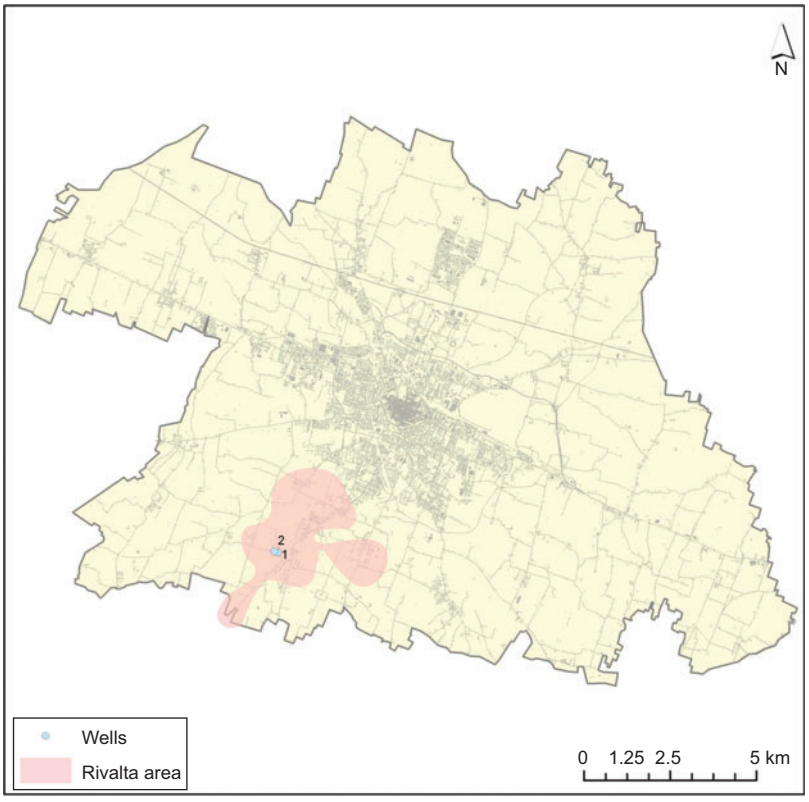

Figure 1 Map of the Rivalta area in the Reggio Emilia municipality (extension, 231.6 km^2; population, ~150,000), where tap water with unusually high content of inorganic hexavalent Se from the two local wells, 1 and 2, was distributed from 1972 to 1988.

results of this investigation confirmed an association between drinking water (inorganic) Se and ALS, showing a relative risk (RR) of 5.4 (95% CI, 1.1–26) for the consumption of tap water with Se ≥1 µg/L after adjustment for potential confounders and evidence of a dose-response relation between the long-term intake of inorganic Se and disease risk (61). Overall, the results confirmed the likelihood of a selective relation between Se and ALS in the municipal population under study, suggesting that major sources of bias were unlikely to occur.

Thus, the epidemiological evidence suggests that Se may cause or contribute to triggering ALS. As high Se areas are detected in various parts of the world (7, 10, 11) and occupational exposures to Se compounds may occur, further epidemiological studies for the ascertainment of this issue appear feasible and should be seriously pursued (12). In line with the usual paradoxes characterizing Se research, we should note that due to the antioxidant activity of the Se-containing enzymes glutathione peroxidases, the metalloid has also been considered a potential tool in the therapy of the disease, but a careful analysis of the epidemiological data has not provided convincing evidence of such an effect (66).

Mercury

The heavy metal Hg exists in a wide variety of physical states: elemental Hg, organic, and inorganic Hg compounds. The neurotoxic effects of elemental Hg (e.g., 'mad hatter syndrome') have been known for centuries, and the first detailed account of the clinical neurotoxic syndrome induced by occupational exposure to organic Hg compounds was published in 1940 (67). A massive epidemic of organic Hg poisoning became known in the 1950s following the Minamata disease outbreak in Japan (68, 69). Subsequent studies have implicated Hg in the etiology of several neurodegenerative disorders, including Alzheimer disease and ALS (70).

Elemental Hg is liquid at cool room temperatures and in this form is less toxic than inorganic or organic Hg compounds. When heated, however, Hg evaporates and becomes highly toxic. In an aquatic environment, elemental Hg may also undergo biomethylation by bacteria and algae: the organic compounds that are obtained, such as methyl-Hg and ethyl-Hg, accumulate in fish, crustaceans, and throughout the food chain to humans. These compounds can adversely affect human health because they are rapidly and completely absorbed from the gastrointestinal tract; they can bind to free cysteine, after which the final complex may replace methionine. Because of this mimicry, organic Hg compounds can be transported freely throughout the body and pass through the blood-brain barrier and the placenta, with consequent harm to the developing fetus (71). Occupational exposure to elemental Hg has also been associated with parkinsonism (72). In animal studies, inorganic Hg-chloride ($HgCl_2$) has been shown to concentrate in the cerebellar gray matter, area postrema, and hypothalamus, whereas organic Hg compounds have a more uniform distribution (73–76). Hg-chloride, in particular, induces evident nervous tissue atrophy, alongside a reduced number of neurons and with the proliferation of astrocytosis, glial fibers, and capillary networks. The disruption of granule cells in rat cerebellum following dimethylmercuric sulfide administration has been reported (77). In addition to the neuronal body, Hg may damage myelin sheaths, which lose their laminar structure (78–80).

This heavy metal may damage blood-brain barrier function due to its capacity to form cross-linkages with cell membrane proteins, thus inducing its permeability (81). Steinwall and colleagues (82–84) demonstrated that this process occurs when Hg is administered at high dosages or is perfused directly into the CNS. Once the blood-brain barrier is disrupted, however, Hg ions, especially in the organic forms (73), may exert various neurotoxic effects on the adult CNS, affecting different intracellular organelles. In the rat, Hg intoxication impairs protein synthesis (85–87). Hg also adversely affects several enzymatic activities, decreasing the activity of sulfhydryl enzymes such Mg-activated ATPase, fructose-diphosphate aldolase, and succinic deydrogenase (85), increasing acid phosphatase activity with a consequent accumulation in the lysosomes (88), impairing the glycolytic pathway, and decreasing ATP levels (89). In rat nerve cells, $HgCl_2$ markedly reduced RNA levels (79, 80), probably by two mechanisms: diminished synthesis of RNA and increased RNA degradation, with a consequent altered RNA turnover. Hg reacts with and depletes free sulfhydryl groups and determines a decline of superoxide-dismutase (SOD) activity leading to oxidative stress, a mechanism implicated in ALS pathophysiology (90). Interestingly, in a mouse ALS model overexpressing the human *SOD type 1* [SOD1 or copper (Cu)-zinc (Zn) SOD] gene, chronic exposure to methyl-Hg induced an early onset of hind-limb weakness (69).

Because ALS is characterized, among other effects, by the dislocation of a DNA-binding protein (TDP-43) from nucleus to cytoplasm, forming inclusions (91–93) for a lengthy period (94, 95), Pamphlett and Jew (94) exposed mice to different concentrations of inorganic Hg (the $HgCl_2$ vapor) for a prolonged time. No TDP-43 inclusions in motor neurons were detected, however, and the exposed mice continued to move and run without presenting weakness or other signs resembling ALS. A single dose of $HgCl_2$ was shown to cause Hg deposition in spinal motor neurons in mice (96). Noteworthy is that in all ALS forms, TDP-43 inclusions are generally found, with the exception of *SOD1* mutation-associated disease, suggesting that Hg might play a role only in the latter (95). Neurophysiologic studies also suggested that Hg slows conduction velocity in dorsal roots and repolarization (97, 98). Also, low concentrations of methyl-$HgCl_2$ caused a stable increase in the threshold for excitation and blockage of action potentials in isolated squid axons without changing their resting membrane potentials, whereas higher concentrations decreased the resting membrane potentials (99). Although these studies were carried out in different experimental systems, the overall results indicate that Hg intoxication in the CNS disrupts cellular metabolism and degrades several cellular constituents, eventually leading to cell death and clinical disease. Probably, the biochemical mechanisms and the clinical pictures of Hg toxicity in the human depend

on several factors, such as individual genetic susceptibility (specific polymorphisms or mutations); the chemical forms of the metal; and the source, length, timing, and amount of exposure during life.

In the epidemiological literature, a few studies have suggested the possibility of an Hg-ALS exposure-effects linkage. A case of an ALS-like syndrome following exposure to organic Hg was described in a farmer, who presented with a 3-year history of progressive muscle weakness and died 8 months after diagnosis (100). The authors of this study concluded that exposure to Pb or Hg or excessive milk ingestion might have been the events leading to disease onset. Barber (101) and Adams (102) reported cases of inorganic Hg intoxication leading to ALS-like symptoms, with subsequent resolution of the disease after removal from exposure. More recently, Praline et al. (103) reported the case of an 81-year-old woman affected by ALS who presented with a high level of Hg in the blood, urine, and spinal cord. Despite a therapeutic trial with a chelating agent, the Hg levels remained elevated, indicating heavy Hg body burden due to severe antecedent chronic Hg intoxication. An association between Hg and ALS was also suggested in the case of a 24-year-old man who injected himself intravenously with elemental Hg in a suicide attempt; he died 5 months later after heroin injection and wrist laceration but without a clinical history of ALS. Postmortem examination showed, however, dense deposits of Hg in large cortical motor neurons but not in other neurons, whereas all glial cells were occupied by Hg deposits.

Two studies did not find an association between Hg exposure and ALS. In a retrospective case-control study carried out on 66 patients and 66 age- and gender-matched controls, Gresham et al. (104) investigated, through a self-administered questionnaire, exposure to nine heavy metals, including Hg and Se. Another 'negative' case-control study was that conducted by Moriwaka et al. (105) in subjects living in the non-endemic area of Hokkaido, Japan: Hg concentrations in plasma, blood cells, and scalp hair were lower in 21 ALS patients than in their 36 controls, although such abnormalities were considered by the authors to be a disease-induced effect.

In conclusion, humans exposed to various forms of Hg may have neurotoxic effects, including in utero and postpartum exposures, but no convincing evidence has emerged for an involvement of this heavy metal in ALS etiology. Too few human studies have, however, been conducted on this issue, and most were affected by methodological limitations concerning exposure assessment and/or confounders control. In addition, the results of epidemiological investigations have been conflicting.

Cadmium

Pure Cd is found naturally in small quantities in air, water, and soil. Cd in the environment occurs as a byproduct of the smelting of other metals, such as Zn, Pb, and Cu, and its distribution is increased due to human activities. The ingestion of foods, such as cereals, seafood, and offal and the inhalation of tobacco smoke are generally considered the main sources of environmental Cd exposure (106). Occupational exposure is frequently due to fume inhalation, working in the nickel (Ni)-Cd battery industry, and exposure to paint pigments. Once absorbed from the gastrointestinal tract, Cd has a long half-life in the body, as it is not biodegradable.

Cd stimulates the formation of metallothioneins (MT), a family of cysteine-rich low-molecular-weight metal-binding proteins. Experimental data support the participation of MT in the detoxification of toxic metals, such as Cd and in scavenging ROS. Cd is red-ox inert as compared with other transitional metals, except when conjugated with MT. The Cd-MT complex is formed in the liver, released into the blood, and transported to the kidneys (107, 108). Acute Cd poisoning causes pulmonary edema and hemorrhage; chronic exposure adversely affects kidney and bone (109). Cd also acts as an endocrine disruptor (110, 111) and may thus affect reproduction and child development (112). In addition, Cd and its compounds are classified by the International Agency for Research on Cancer as a group 1 human carcinogen based on evidence that lung cancer is increased in Cd workers (113). Cd exposure has also been linked to human prostate and renal cancer, although this linkage is weaker than for lung cancer (114). The role of the metal in liver, pancreas, and stomach carcinogenesis is considered equivocal (115).

Some of the toxicological effects of Cd mirror the biochemical mechanisms underlying ALS pathophysiology, thus providing biological plausibility to a Cd-ALS relation. In particular, considering that 20% of familial ALS cases show *SOD1* gene mutations (5), the capacity of Cd to alter SOD1 activity is of considerable interest. Cd can induce MT expression (116), which may act as a protective factor against ROS, but this protein also binds Zn ions in mammalian cells in addition to Cd (117), irreversibly decreasing SOD1 enzyme activity (118). Moreover, Cd can interfere with the secondary structure of the SOD1 protein by decreasing its Zn content and thus enzymatic activity (109) and by inducing misfolding and aggregation of the SOD1 protein (119). These effects were analyzed by spectroscopy, which showed that Cd modifies SOD1 conformation by increasing the α-helix structure and decreasing the random coil domain. Cd also induces SOD1 cytoplasmic inclusions in the proximal axon and neuron cell body, which represent the pathological findings detected in motor neurons and astrocytes from ALS patients (120). Degeneration of the neural tube in Cd-treated embryos of zebra fish has also been demonstrated (121). In particular, if the animal embryos are exposed to Cd before neurulation, a gap in the anterior neural tube is observed, and if exposure follows the closure of the neural tube, upper limb defects may occur (122, 123). Other studies confirmed that Cd induces cell apoptosis, especially in mouse N2A neuroblastoma cells. (109). The mechanism through which Cd induces programmed cell death is not clear, but proteomic studies identified differences of protein expression and aggregation between control and Cd-treated N2A cells, involving structural proteins, stress-related and chaperone proteins, ROS enzymes, apoptosis, and survival signals (109).

In the epidemiological literature, of interest is the observation by Pamphlett et al. (124) of higher blood Cd concentrations in 20 ALS patients compared with controls. Successive studies measuring heavy metal levels in ALS patients have produced contradictory results, but in most investigations, Cd appeared to be increased. Bar-Sela et al. (125) reported in 2001 a case of a 44-year-old patient who died from ALS after 9 years of Cd exposure while working in a Ni-Cd battery plant in Israel. The work conditions and exposures in that plant were extremely hazardous, and the exposure of this patient was considerably more intense than that characterizing his coworkers. In particular, the patient had to shake the barrels to loosen up chunks of Cd, a process releasing Cd-containing fumes. As soon as ALS was diagnosed, the blood Cd level was 8 μg/L, 10-fold higher than in non-smoking Israelis, but this concentration might not reflect the long-term antecedent exposure because blood Cd levels are known to fall rapidly when exposure ends (126). The urinary Cd level, an indicator of cumulative past exposure, was 13 μg/L (125). The epidemiological data are extremely limited, but the available evidence suggests that occupations and workplace Cd exposure may be linked with excess ALS risk, but there could have been a contributory role for solvent. The patient was one of many with neurotoxic type syndromes, and many other effects associated with mixed exposures to Cd, Ni, and solvents in appalling working conditions (127). A case-control study carried out in New England between 1993 and 1996 showed a higher risk of ALS for construction workers [odds ratio (OR)=2.9; 95% CI, 1.2–7.2] and precision metal workers (OR=3.5; 95% CI, 1.2–10.5), but establishing what compounds – including Cd – were specifically involved was impossible (128). In accordance, other research gave support to the hypothesis that ALS patients are more likely to have been exposed to heavy metals compared with controls (129–131).

On the other hand, Pierce-Ruhland and Patten (132) were unable to detect long-term excessive exposure to Cd in ALS patients, although overall exposure to heavy metals, such as Pb and Hg appeared to be related to disease risk. Their study encompassed 80 MND patients and 78 controls using telephonic interviews. In another case-control study from Australia, no correlation between ALS and antecedent occupational exposure to Cd or other metals (as ascertained through questionnaires) was found when analyzing 179 disease cases and a corresponding number of controls (133). Interestingly, in an older study based on autopsy samples carried out on 24 patients with ALS and 18 controls, an elevated level of MT in liver and kidneys of patients was detected (134), which may be of considerable interest for ALS etiology, considering the interrelation between MT and Cd.

In summary Cd, a recognized neurotoxic element, is assumed to be involved in ALS etiology on the basis of specific biological plausibility supporting this view, but epidemiological evidence is still lacking due to the very few number of studies (generally with low statistical power) carried out to date. Additionally, despite various international guidelines from the 1990s that have banned the use of Cd in plastic, PVC, batteries, and electronics and have led to a decrease in Cd occupational exposure, little evidence has emerged of decreased ALS incidence [which, on the converse, might be increasing (2)]. Thus, although Cd might be involved in ALS etiopathogenesis, such a relation is still far from being demonstrated and we need more reliable data to assess it.

Lead

Pb is a naturally occurring metal that is present in small amounts in the earth's crust. In the environment, Pb derives mainly from human activities, including burning fossil fuels, mining, and manufacturing. Pb is used in the production of batteries, ammunition, metal products (solder and pipes), and devices to shield X-rays. Due to health concerns, Pb from gasoline, paints and ceramic products, caulking, and pipe solder has been reduced in recent years; hence, cases of overt Pb poisoning have become less frequent (135). The toxic effects of Pb on the nervous system are well known and include Pb encephalopathy (primarily in children) and a peripheral motor neuropathy (primarily in adults) (136). After absorption from the gut, Pb is deposited in the soft tissues (liver, kidney, erythrocytes), and then transferred to the bones, where it is stored in a biologically inactive form. Bone Pb levels increase with age (135). The half-life of Pb is 1 month in blood, 3–5 years in trabecular bones, such as patella, and 15–25 years in cortical bones, such as tibia. Hence, blood Pb is generally considered to reflect acute exposure, whereas bone Pb is believed to reflect cumulative exposure. Prolonged exposure results in increased bone Pb concentration that persists after the termination of the original exposure (137).

With regard to ALS, the mechanisms underlying Pb neurotoxicity make the relation of Pb to ALS biologically plausible because Pb neurotoxicity depends on the same mechanisms suggested for the pathogenesis of this disease. The pathophysiological mechanisms underlying the development of ALS appear to be multifactorial, with emerging evidence of a complex interaction between genetic and environmental factors. In the last years, in addition to the SOD1 gene, new genes have been discovered (TDP43, FUS, VCP, etc.) that can act synergistically with environmental factors through different pathogenetic mechanisms. Such mechanisms include glutamate neurotoxicity, oxidative stress, mitochondrial or axonal transport dysfunction, autophagy, and protein misfolding. An excessive activation of these postsynaptic receptors by glutamate, known as glutamate-induced excitotoxicity, can incite neurodegeneration through the activation of calcium-dependent enzymatic pathways. Glutamate-induced excitotoxicity can also result in the generation of free radicals, which in turn can cause neurodegeneration by damaging intracellular organelles and up-regulating proinflammatory mediators (138). Oxidative stress might also be caused by the toxic gain of function of the SOD1 enzyme following SOD1 mutation (139). In addition, structural abnormalities of mitochondria, dysfunction of the sodium/potassium ion pump, autophagy, and disrupted axonal transport systems have all been implicated in ALS pathogenesis (138). Non-neuronal cells, such as astrocytes and microglia, might also directly contribute to neurodegeneration through mechanisms including insufficient

release of neurotrophic factors, secretion of neurotoxic mediators, and modulation of glutamate receptor expression (known as noncell autonomous neurodegeneration) (140). Another possible pathogenetic mechanism involves protein misfolding. SOD1 mutations induce conformational instability and misfolding of the SOD1 peptide, resulting in the formation of intracellular aggregates that inhibit normal proteosomic function, disrupting axonal transport systems and vital cellular functions. The TAR DNA binding protein 43 (TDP-43) has also been recognized as a major component of ubiquitinated cytoplasmic protein aggregates in almost all patients with sporadic ALS (sALS) (141). Aggregates of another protein, the fused-in sarcoma protein (FUS), were found in ALS but not in patients with pathological changes in TDP-43 or SOD1, indicating a novel disease pathway (142). Pb can also substitute for calcium in many intracellular reactions, damage mitochondria, and amplify glutamate excitotoxicity (143). Animal studies have suggested that ALS onset may be related to motor neuron function, whereas progression is regulated by neuroglia (144).

Surprisingly, Pb might injure motor neurons and at the same time stimulate glial cells to provide trophic support to neurons and therefore delay cell death. Experimental evidence supports the view that astrocytes can sequester and buffer Pb in the CNS, preventing further diffusion of the metal to the neuronal compartment and subsequent neurotoxicity or altered synaptic transmission (145, 146). In particular, astrocytes are the cells that preferentially induce cytoprotective and antioxidant gene expression in response to Pb (147). When pretreated with low, non-toxic Pb concentrations, astrocytes can induce neuroprotective mechanisms, such as the up-regulation of VEGF (vascular endothelial growth factor) gene expression, and can down-regulate neuroinflammation, as shown by a dramatic reduction of GFAP-immunoreactive astrocytes (148). Accordingly, Barbeito and colleagues (149) found that Pb exposure prolonged survival in SOD1 transgenic mice.

Hence, Pb exposure could potentially have opposing actions during the course of ALS, initially promoting the degeneration of motor neurons but later abrogating damage and neuroinflammation mediated by dysfunctional glia (148). Additionally, Pb decreases SOD activity in Pb-exposed rats (150) and stimulates antioxidant enzyme hemoxygenase 1 expression in astrocytes (147), mechanisms that could contribute to neuronal protection. Alternatively, another possibility is that factors associated with better survival are also associated with higher Pb levels in the population, such as gender (men have higher Pb levels and longer ALS survival) but not age because older individuals have higher Pb levels but shorter ALS survival (151). Another possibility is that Pb exposure is related not to ALS risk but rather to longer survival, favoring the higher participation of Pb-exposed cases in epidemiological studies, thus being a source of selection bias (151). Another possibility is that of reverse causality, i.e., Pb levels are a result of ALS due to the decline in physical activity in ALS patients, leading to bone demineralization and the release of Pb from bone into blood, as also suggested by the direct correlation between blood Pb and disability due to the disease in a case-control study (54). Nevertheless, the associations of blood and bone Pb in ALS patients were not appreciably changed by adjusting for physical activity levels (143, 152, 153) or by bone turnover (152).

In epidemiological studies, Pb exposure has long been investigated as a potential ALS risk factor and as a factor affecting survival in this disease (154). In the first report on progressive muscular atrophy by Aran (155) in 1850, 3 of 11 cases had contact with Pb, and in 2 cases, Pb poisoning was diagnosed. A number of cases with lower motor neuron signs and pyramidal involvement can be dated at the first years of the 20th century. More recently, Oh et al. (156) reported a case of ALS in a worker who was exposed to Pb while working in electronic parts manufacturing. Besides single case reports, a number of case-control studies and registry-based case-control or cohort studies have dealt with this topic. The majority of the case-control studies (104, 131, 132, 143, 152, 154, 157–159) found some evidence for an association of ALS with Pb exposure as estimated through the administration of questionnaires. In 1970, Campbell et al. (154), through a case-control study on 74 patients with MND and 74 age- and gender-matched controls, found a history of extensive Pb exposure in 15% of patients and in 5.4% of the controls and a history of bone disease or fracture in 25% of patients and in 9.4% of the controls. The authors also found a 54% 5-year survival rate in MND patients previously exposed to Pb vs. 16% in patients without antecedent Pb exposure. Increased Pb exposure and increased frequency of bone fractures could suggest a role of toxic Pb reservoir for the bone, which may release Pb after traumatic events, thus contributing to ALS onset. Yet, the authors did not find different bone Pb levels in the two groups. Felmus et al. (157) studied the antecedent events of Pb exposure in 25 patients with ALS and 50 controls, detecting a higher exposure to Pb, Hg, athletics, and consumption of milk in patients compared with controls (157). This study was replicated in 1981 with confirmation of the results (132). Armon et al. (158) found that men with ALS had worked more frequently at welding and soldering, suggesting an association between ALS in men and exposure to Pb vapors. In a study from the Scottish MND Register carried out on 103 ALS patients and their matched controls, history of fractures (OR=1.3), manual occupation (OR=2.6), and exposure to Pb (OR=5.7), and solvents/chemicals (OR=3.3) was more frequent in patients (131). Other studies, however, did not confirm these results (104, 160, 161). Nevertheless, the validity of the early data is still debated. These studies include occupational exposures that epidemiologists often use as a surrogate to assess potentially toxic exposures, and many studies relied on self-reporting through questionnaire use, an approach at risk of recall bias. Indeed, McGuire et al. (159) reported that Pb exposure based on expert evaluation of self-reported occupational histories by a panel of industrial hygienists was not associated with ALS (OR=1.1), suggesting that recall bias might explain findings obtained on the basis of self-reported data. In another study (143), occupational Pb exposure based on review of self-reported occupational history was associated with ALS, and the RR was similar to that computed for self-reported Pb exposure. More

recently, blood Pb levels were found to be increased among US veterans affected by ALS compared with controls (152), suggesting that Pb exposure might at least in part explain the higher risk of ALS noted for military personnel, who can be exposed to Pb from firing and other practices. Residential and recreational Pb exposure might also represent a risk factor for ALS, but such exposures are generally lower than those experienced in occupational settings and therefore more difficult to investigate due to a high risk of misclassification. In the Italian province of Modena, ALS rates were higher in residents in the 'ceramic district', where several paving-tile factories were located and induced a severe environmental Pb pollution than in the unexposed population (162). However, this excess incidence was not confirmed in following studies carried out in the same province (163) or in the neighboring province of Reggio Emilia (164), although the possibility of delayed effects of such extensive Pb contamination cannot yet be ruled out.

Several studies examined the Pb-ALS relation through an analysis of biomarkers of exposure, but their results were inconsistent. Studies from one group reported that, compared with controls, ALS cases had higher Pb levels in plasma (165), cerebrospinal fluid (166), and muscle (165). Kurlander and Patten (167) reported that even after chelation therapy, postmortem concentration of tissue metals was consistently increased. Other studies yielded null results (168–172), including the population-based investigation by Bergomi et al. (55) on toenails trace elements concentrations in 22 ALS patients and 44 matched controls. In this study, no evidence of an association between ALS risk and toenails trace elements (including Pb) was detected.

All the above-mentioned studies have limitations, including small size, possible confounding by uncontrolled factors affecting Pb levels, and potential bias introduced by the use of hospital-referred controls in some cases. Several factors related to blood and bone Pb levels, including age, gender, cigarette smoking, alcohol use, and education, are known to be potential confounders in the Pb-ALS relation. A recent study, however, combined biological measures and interviews to analyze this issue (143). The authors studied 109 cases and 256 controls and found that ALS risk was associated to self-reported occupational Pb exposure (OR=1.95) and also with increased blood and bone Pb levels, with OR=1.9 for each µg/dL increase in Pb blood levels, OR=3.6 for each unit increase in log-transformed patella Pb, and OR=2.3 for each unit increase in log-transformed patella Pb. The same group also found that Pb exposure was associated with longer survival in ALS cases (151), an observation apparently contrasting with the previous observation of an excess disease risk in Pb-exposed subjects (143). The same conflicting results had been previously reported by Campbell et al. (154) in the 1970s, which may indicate that Pb exposure might have different effects on ALS onset and progression.

In conclusion, the data on Pb as a possible risk factor for ALS are conflicting and inconsistent, and methodological limitations of the epidemiological studies hamper reaching definitive conclusions about this issue. Nevertheless, the possibility that Pb exposure increases ALS survival deserves further evaluation.

Pesticides

The four major classes of pesticides include insecticides, herbicides, fungicides, and rodenticides, based on target species, plus other pesticides included in narrower classifications. In the human, exposure to pesticides can occur through the oral, dermal, or inhalation route. Although some pesticides are neurotoxic, these are not generally classified as such. Among pesticides, the organophosphates (OPs, as insecticides) have been associated with ALS following the report of increased incidence of ALS in Persian Gulf War veterans, but no conclusive epidemiological evidence supports this apparent increased risk (69). Returning 1991–1992 veterans were reported to have received prophylactic treatment containing cholinergic inhibitors to protect them against nerve gas and insect pests, and plausibly, such pretreatment could have exacerbated an underlying genetic polymorphism or unmasked other factors that increase the risk of motor neuron effects. The OPs have wide agricultural and home uses.

The primary toxicity of OPs is associated with the acute inhibition of acetyl cholinesterase, the enzyme responsible for the destruction and termination of the biological activity of the neurotransmitter acetylcholine (173–175). A second distinct manifestation of OP poisoning is a paralytic condition called the intermediate syndrome, which consists of a sequence of neurological signs appearing about 24–96 h after the acute cholinergic crisis but before the onset of delayed neuropathy. The major effects are muscle weakness, primarily affecting muscles innervated by the cranial nerves (neck flexors, muscle of respiration) as well as those of the limbs. Cranial nerve palsies are common, respiratory depression and distress are not responsive to atropine or oximes, and death may occur. A third syndrome is the OP-induced delayed neurotoxicity (or OP-induced delayed polyneuropathy), in which the agent binds with neurotoxic esterase without effects on ChE (176).

Other clinical signs and symptoms from impairment of ChE in muscarinic, nicotinic, and central sites are headache, dizziness, paralysis, ataxia, bradycardia, miosis, weakness, anxiety, excessive sweating, fasciculations, vomiting, diarrhea, abdominal cramps, dyspnea, salivation, tearing, pulmonary edema, and confusion.

No studies have been designed to specifically investigate the potential association of OPs with ALS. Morahan et al. (177) analyzed the potential of pesticides, heavy metals, and chemicals to cause sALS and showed that an impaired capacity of patients to detoxify these toxicants could be related to differences in the MT family of genes, metal transcription factor 1 and glutathione synthetase.

Other studies examined paraoxonase (PON) genes in the investigation of the potential association as described by Johnson and Atchison (69). PON is an A-esterase based on its ability to detoxify the prototype OPs, paraoxon. PON1 detoxifies OPs and has several variants, i.e., PON1, PON2,

and PON3. PON hydrolyses OPs, and its ability to detoxify them is largely determined by different variants of PON1 as opposed to other variants. Genetic variations have been found in the coding and promoter region of the human PON1 locus that determines the catalytic activity and enzyme levels. Therefore, theoretically, the risk of developing ALS in an individual could increase if the mutation of the PON1 gene increases and if the body concentration of PON1 is suboptimal. Thus, mutations that impair the ability of PON1 to detoxify OPs could increase sensitivity to the toxicity of these pesticides and possibly of Pb, favoring ALS development, as well as the other effects specifically associated with anticholinesterases. No consistency has emerged, however, in establishing a causal link between OPs exposure and mutations in the PON1 gene, based on epidemiological and animal studies. Wills et al. (178) showed that the polymorphism of PON1 genes did not reduce enzyme activity, suggesting that this gene product is unlikely to be involved in ALS etiology. Other studies suggest that among veterans with Gulf War syndrome, US veterans have significantly lower serum concentrations of one form of PON1 allozyme, whereas British veterans have lower concentrations of both allozymes (Q and R allozymes), when compared with healthy Gulf War veterans (178). A report of two cases has shown massive increases in CPK – a measure of the breakdown of muscle tissue – in persons with occupational exposure to highly toxic anticholinesterases (179). The authors' suggestion that medical programs for Gulf War veterans with Gulf War syndrome should include surveillance for elevated CPK, abnormalities of neuromuscular conduction, and genetic susceptibility to guide diagnosis and care may also be of value in helping to sort out the possibility of mixed causes.

A case of a slowly progressive MND following chronic exposure to pyrethroids that was indistinguishable from ALS was reported in a 44-year-old woman who, as a food shop proprietor, had been using cans of pyrethroid insecticides containing imiprothrin, phenotorin, D-T80-resmethrin, and D-T80-phthalthrin almost every day for 3 years in an unventilated room (180). Further description follows:

> Initially, she experienced tongue numbness, nausea, and rhinitis while using the insecticides. Two years after beginning to use the insecticides, she noticed difficulty lifting heavy objects with her left arm, and her symptoms steadily worsened over the next 8 months. Three months before admission, she developed slurred speech, gait disturbance, and generalized muscle weakness. On admission, neurological examination revealed dysarthria, nasal voice, and dysphagia with fasciculating atrophied tongue, moderate muscle weakness with fasciculation in both upper limbs, predominantly on the left side, and fasciculation in the trunk and both lower limbs. Jaw jerk was hyperactive, and hyperreflexia was seen in all limbs without pathological reflexes. Sensory and autonomic systems were all normal. The patient showed upper and lower motor neuron signs in bulbar, cervical, and lumbosacral regions, consistent with signs in bulbar, cervical, and lumbosacral regions, suggesting clinically definite ALS based on El Escorial criteria. Neurophysiologic studies also indicated both upper and lower motor neuron involvement. Her illness was thought to be caused by pyrethroids for two reasons: [1] the usual manifestations of pyrethroid intoxication, such as tongue numbness, nausea, and rhinitis, preceded the motor dysfunction; [2] the motor symptoms and ongoing denervation potentials partially improved after the cessation of pesticide usage. The case indicates that chronic pyrethroid intoxication may cause an ALS-like disorder in humans, similar to Pb and domoic acid intoxications. Pyrethroids are synthesized from chrysanthemum extracts, and they disturb ion channels, such as voltage-dependent sodium channels and voltage-sensitive chloride channels, and readily induce neuronal excitation by current prolongation. Moreover, deltamethrin, one of the pyrethroids, is reported to impair axonal transport and then degenerate axons in rats, with impaired axonal flow causing motor neuron death in various animal models of MND. Although pyrethroids have low toxicity, due in part to their rapid detoxification via ester hydrolysis in mammals, some human populations are thought to be poor metabolizers of pyrethroids, whereas carboxyesterase inhibitors can enhance pyrethroid toxicity. Therefore, chronic exposure to pyrethroids may cause MND through disturbance of either ion channels or axonal flow, especially in poor metabolizers (180).

A case simulating MND closely associated with overexposure to a pyrethrin and chlordane-based insecticide has also been reported (181, 182). For pyrethrins, such as pymethrin, the elimination of metabolites 4′-hydroxy-3-phenoxy benzyl alcohol or 4′-hydroxy-3-phenoxy benzoic acid occurs by sulfate conjugation, which is the major metabolic pathway in the rat. MND patients have a defect in their ability to convert cysteine into inorganic sulfate and also show a poor capacity to form the sulfate conjugate of paracetamol. Therefore, these two metabolites may be responsible for neurotoxic effects, resulting in MND-type illness.

In human populations, various investigators have examined the potential association of pesticide exposure with ALS risk. Studies have been conducted in different countries, including Italy (Ferrara, Reggio Emilia, and Sardinia), France (Britanny), USA (states of Massachusetts, Michigan, Minnesota, and Washington), Scotland, Sweden (Scaraborg), Australia (all states), and Greece (Athens), as summarized in Table 1. Included are reports that referred to MND and ALS interchangeably, reports that include ALS as one type of MND, and reports that referred to all MND with no mention of ALS, plus reports that are literature reviews of some of the studies. Table 1 also summarizes the methodological strengths and the weaknesses of these studies. The human studies reported on the potential association of pesticides to ALS, or the lack thereof, are also shown in Table 2. 'Potential association' is based on increased rates or risk in the exposed subjects, suggesting a possible but not definitive role of pesticides, taking into account occupation in an agricultural environment, being

Table 1 Overview of studies on possible association of amyotrophic lateral sclerosis (ALS) with exposure to pesticides.

Location-subjects	Method	Findings	Conclusion	References	Remarks
Reggio Emilia, Italy 150,000 inhabitants 41 ALS patients diagnosed 1995–2006 (at least 6 months residency), 82 age- and sex-matched randomly sampled population controls. El Escorial diagnostic criteria for probable or definite ALS.	Population-based case-control study. Questionnaire on occupational history and leisure time habits. Hospital discharge data, death certificates, prescription of riluzole (an orphan drug specific for ALS). Matched controls from annual directories of residents available through General Registry Office of the Region. Involvement in agricultural work and other pesticide-related professional activities for at least 6 months considered as exposed to pesticides. Estimated RR from OR estimated from conditional logistic regression bivariate and multivariate models.	More cases than controls expose to pesticides for at least 6 months due to agricultural work activities (31.7% vs. 13.4%). Excess ALS risk associated with pesticide exposure (conditional regression model, RR 3.6, 95% CI, 1.2–10.5), even with inclusion of confounders. Association present in both males and females, stronger in males. The excess risk is higher in the oldest age group (median age of age 68 at disease onset used as cut-off).	Appears to indicate that occupational exposure to pesticides is a risk factor for ALS.	Bonvicini et al., 2010 (183)	Strengths of the study: 1) Use of multiple sources of data in identifying new cases; 2) Accuracy in confirming diagnosis of ALS; 3) Methodology to identify the matched controls. Limitations of the study: 1) Small sample size (low statistical stability of the risk estimates); 2) "Crude measure of pesticide exposure". Identified need for further in-depth investigation and pesticide exposure assessment.
Britanny, France 2006–2008 108 sporadic ALS cases matched by age and sex to 122 controls in a well-delimited rural population. More than 1-year residence. El Escorial diagnostic criteria for probable or definite ALS.	Prospective case-control study. Questionnaire. Controls from orthopedic service of a hospital with minor trauma.	Multivariate analysis showed a positive association between agricultural workers and ALS (OR=2.919; p=0.01). Bulbar onset (not previously reported in agricultural workers) predominated in patients who are farm workers vs. other occupations.	Results suggest a potential role of exposure to agricultural chemicals or contact with animals linked to agricultural work.	Furby et al., 2010 (184)	Further investigation needed to identify specific risk factors in agricultural practices, and to elucidate why bulbar motor neurons are primary involved in farm workers with ALS.
USA Over 1 million participants in the American Cancer Society's Cancer Prevention Study 1989–2004. Prospectively followed 414,493 male and 572,736 female CPS-II cohort participants who were alive as of January 1, 1989.	Self-report (baseline questionnaire) of regular exposure to 11 different chemical classes or X-rays and ALS mortality among participants. Follow-up from 1989 through 2004 identified 617 deaths from ALS among men and 539 among women. The vital statuses of the participants were determined by automated linkage with the National Death Index. Death certificates (1989–1992) or codes for cause of death (1993–2004) were obtained for over 98% of known deaths. ALS deaths were defined as an underlying or contributing cause of death on death certificates of ICD-9 (1989–1998) code 335.2 or ICD-10 (1999–2004) code G12.2 (MND). Adjusted for age, sex, smoking, military service, education, alcohol intake, occupation, vitamin E use and all other chemical classes.	The baseline multivariate adjusted rate ratio (RR) of ALS for ALS mortality among individuals exposed to pesticides/herbicides compared with that among unexposed individuals was 1.07 (95% CI, 0.79–1.44), but somewhat higher after excluding those with missing duration of pesticides exposure (number of cases among exposed=18) (RR=1.44; 95% CI, 0.89–2.31; p=0.14).	There was little evidence for any association between pesticides/herbicide exposure and ALS.	Weisskopf et al., 2009 (185)	Because of the longitudinal design, this result is unlikely to be due to bias, but it should nevertheless be interpreted cautiously.

(Table 1 continued)

Location-subjects	Method	Findings	Conclusion	References	Remarks
Italy Five-year prospective extension of an Italian soccer players cohort survey that previously found an increased risk for ALS in Italian professional soccer players actively engaged between 1970 and 2001 (n=7325). The soccer cohort included all male professional soccer players who were engaged by professional football teams in the period September 1, 1970 (1970–1971 playing season) to June 30, 2002 (2001–2002 playing season). Athletes not born in Italy were excluded.	A prospective follow-up of the original cohort to 2006. Investigated the risk of ALS in two other cohorts of professional athletes, basketball players (n=1973) and road cyclists (n=1701). Sources used to identify ALS cases: 1) Death certificates, obtained from the Italian Statistics; 2) The archives of the largest Italian ALS centers; 3) The database of the Italian ALS Association; 4) Information provided by media and websites; 5) Self-reports by ALS patients or their relatives. Only cases with definite or probable ALS were included. Mortality rates were calculated using 5-year age- and gender-specific incidence rates for the whole population and for each subgroup, using as reference rates ALS incidence in the Piemonte and Valle d'Aosta Register (northern Italy) and in the Puglia ALS register (southern Italy).	Among soccer players three new cases of ALS were identified, reaching a total of eight ALS cases (mean age of onset, 41.6 years). The number of expected cases was 1.24, with an SMR of 6.45 (95% CI, 2.78–12.70; p<0.00001). Confirmed the highly significant risk in Italian soccer players of developing ALS, the young age of onset, the dose-effect risk, and a particular predilection for midfielders.	The investigators believe that the predilection for soccer players to develop ALS derives from a complex interplay between a genetic predisposition for physical endurance and external factors, such as drugs and herbicides. A distinctive feature of soccer players is the continuous contact with grass. Therefore toxins used for the maintenance of soccer fields (i.e., large leaf herbicides, defoliants, pesticides, fertilizers) could be related to ALS onset in subjects with a genetic predisposition.	Chio et al., 2009 (186)	Addressed multiple gene-environment interactions that govern several biochemical pathways that may be linked to development of ALS.
Boston, MA, USA 95 subjects with ALS and 106 healthy control subjects. The ALS subjects were identified through the weekly Neuromuscular Clinic at Massachusetts General Hospital. Healthy controls were either non-blood relatives (such as spouses), friends, or unrelated to the ALS subjects. An effort was made to age-match the unrelated controls to their respective cases. All study subjects were at least 18 years of age.	The two-stage study design, case-control, and a longitudinal prospective cohort, included comparison of cases and controls at enrollment for riskfactor ascertainment and subsequent measurement of disease progression in cases to examine risk factors and predictors of disease progression for ALS. Questionnaire. The ALS subjects were prospectively followed for 1 year to define factors that influence the rate of disease progression, measured by rate of change in percent predicted forced vital capacity (%FVC) and the ALS functional rating scale (ALSFRS) score. The association of each potential risk factor with ALS was determined using univariate logistic regression. A random slope model was used to determine the association of each risk factor with disease progression.	Significant risk factors for ALS included reported exposure to Pb (p=0.02) and pesticides (p=0.03). Factors found to have a significant positive association with ALS were a history of toxin exposure, fewer years of education, lack of military service and age at quitting smoking. Subjects who had been exposed to one or more toxins were three times more likely to develop ALS (OR=2.8, p=0.001). After adjusting for smoking, welding, gender, age, and military history, approximately 45% of the ALS subjects, but only 23% of controls, had been exposed to a toxic compund. Of the 95 subjects in the ALS group, the toxin exposure most commonly reported was exposure to pesticides (n=18), followed by Pb (n=15), industrial solvents (n=15), Hg (n=55), and miscellaneous toxins. The ALS subjects were more likely than the controls to report exposure to pesticides (p=0.03) and Pb (p=0.02).	In this analysis of 34 possible ALS susceptibility factors, four showed a statistical association:reported exposure to Pb or pesticides, fewer years of education, absence of a history of military service and age at cessation of smoking.	Qureshi et al., 2006 (187)	Acknowledged possible methodological limitations: 1) A small sample size; 2) May have been subject to referral or selection bias and recall bias. Pertinent Variables not associated with either causation or progression of ALS included physical activity, cigarette smoking, and a history of physical trauma or other clinical disorders.

(Table 1 continued)

Location-subjects	Method	Findings	Conclusion	References	Remarks
Australia Case-control study analyzed epidemiological data on 179 SALS cases and 179 age-, ethnicity-, and sex-matched controls. The sALS patients (125 men, 54 women, mean age±SD: 60±11 years) donated DNA samples and questionnaire data to the Australian Motor Neuron Disease DNA Bank. Controls (125 men, 54 women, mean age±SD: 61±10 years) were made up of 141 individuals unrelated to patients and 38 individuals related to sALS patients. The unrelated controls consisted of 96 patient spouses, 35 community volunteers and, 10 patient acquaintances.	Patients with a recent diagnosis of ALS were recruited for this bank through ALS associations in each state of Australia via regular notices in associations' newsletters. Patients included in the study fulfilled either the probable or the definite modified El Escorial criteria for ALS. Binary (yes/no) responses were analyzed using χ^2-tests and OR with 95% CI. Bonferroni corrections were used to adjust for multiple testing. Representative responses were collectively analyzed using binary logistic regression. Self-reporting questionnaires.	SALS was associated with overall herbicide/pesticide exposure (OR=1.57, 95% CI, 1.03–2.41). A higher dose (regular exposure) was associated with both the total sALS group (OR=4.65, 95% CI. 1.82–11.87) and male SALS patients (OR=4.20, 95% CI, 1.58–11.20).	Exposure to herbicides and pesticides was associated with the total sALS group and more strongly with male sALS patients.	Morahan and Pamphlett, 2006 (133)	Strength: Patient's population Limitations: 1) Self-reporting, lack of awareness of exposure (no objective classification of exposure); 2) Recall bias. Given the methodological limitations noted above, this study cannot claim to be definitive.
Ferrara, Italy 1964–1998 Demographic survey in the local health district (LHD).	Compared number of observed ALS cases and the number of expected ALS cases according to the level of urbanization and usual occupation under the assumption of a homogeneous distribution of ALS. Reviewed clinical files and medical records, contacted first-level relative, used data from Department of Demographic Statistics and Chamber of Trade, Industry, Agriculture, and Handicrafts.	Based on the occupational pattern, the number of incident cases of ALS whose usual occupation was in agricultural work exceeded the expected number (observed ALS cases=22, 95% Poisson CI 13.8–32.3, expected ALS cases 6.0).	Rural residence itself does not influence the risk of ALS, whereas agricultural activities could influence the risk of ALS, with occupational exposure to agricultural chemicals playing a possible role.	Govoni et al., 2005 (188)	Although the number of ALS incident cases in the LHD of Ferrara in 1964–1998, whose usual occupation was in agricultural work, was higher than that expected (22 out of 91 incident cases, 24.2%), the large majority of the incident cases of ALS did not work in agriculture (that is, 69 out of 91 incident cases of ALS, 75.8%). No data available about exposure to agricultural chemicals or elevated levels of agricultural chemicals in body fluids or pathological specimens. The present hypothesis only emerged from the reported separation of agricultural work from rural residence since only agricultural work would seem to increase the risk of ALS whereas rural residence does not. Therefore, the present findings should be considered with caution.

(Table 1 continued)

Location-subjects	Method	Findings	Conclusion	References	Remarks
Italy (soccer players). 7325 professionals who played in Italy's top two divisions between 1970 and 2001.	Turin University survey. The university team monitored the health of the players.	Uncovered five cases of ALS. The average incidence for the same general population size is 0.77 cases. The average age at disease appearance: in soccer players=43 years; in general population=63.	Soccer players are 6.5 times more likely to get the disease than the rest of the population. Suggested that midfielders and older players are more prone to the disease.	See Chio et al., 2009. (186)	Posted on Medscape, and noted as posted June 13, 2005, in ALS research. The title referred to 'pesticides implicated,' but there is no information/data on pesticides in the text.
Midland Michigan, USA Male employees (1517 males of 1567 males and females) of The Dow Chemical Company who manufactured or formulated 2,4-dichlorophenoxyacetic acid (2,4-D) any time from 1945 to the end of 1994.	Cohort (previously identified). Compared mortality experience with national rates and with more than 40,000 other company employees who worked at the same location. Cumulative dose expressed as very low, low, medium, and high exposure using number of years as the indicator.	The internal comparison with other Dow employees showed a RR of 2.63 (95% CI, 0.85–8.33). Death was attributed to ALS for three cohort members. Compared with the other company employees, the RR was 3.45 (95% CI, 1.10–11.11). The cases were employed in the manufacture or formulation of 2,4-D at different periods (1947–1949, 1950–1951, and 1968–1986), and for varying durations of time (1.3, 1.8, and 12.5 years).	Although not an initial hypothesis, an increased RR of ALS was noted (three cases). The authors noted that this finding is unsupported by other animal and human studies.	Burns et al., 2001 (189)	The finding of higher than expected number of cases of ALS is not consistent with previous human or animal studies and may be unrelated to exposure to 2,4-D. Limitations of the study: 1) Finding the residential data for employees that have left the company; 2) Unavailability of potentially confounding lifestyle factors; 3) Mortality endpoint (influenced by many factors). Strengths of the study: 1) Follow up of the cohort was nearly 100% complete; 2) Internal comparison population; 3) Potential exposure to 2,4-D estimated from industrial hygiene monitoring data.
.			The finding of Burns et al., 2001 (ALS/2,4-D association) is consistent with several previous studies and warrantsserious attention in future studies.	Freedman, 2001 (190)	Review and critique of Burns et al., 2001
			His points: 1) Weight of the evidence to be placed upon 'non-statistically significant' increase of non-specific exposures found in human studies of ALS;	Burns 2001 (189)	Response to Freedman 2001, (above).

(Table 1 continued)

Location-subjects	Method	Findings	Conclusion	References	Remarks
			2) The associations found in the case-control studies have limited biological plausibility; 3) Improved exposure assessment needed.		
USA Seven of 38 studies concerning exposure to chemicals (case-control or cohort studies. sALS only, not endemic).	Two systemic reviews (chemical agents and metals) of literature performed according to the MOOSE guidelines for performing and reporting a meta-analysis or systemic review of observation studies. Screened for case-control and cohort studies. Studies appraised according to Armon's classification system for ALS risk factor studies, and newly developed classification system for exposure assessment.	Two studies showed strong associations between increased ALS risk and pesticides exposure. Risk estimates (Forest plots and E-Table V) presented for agricultural chemicals (insecticides, fungicides, herbicides, other pesticides) (McGuire 1977) and occupation (farming) potentially exposed to pesticides (Park 2005). Risk estimates: McGuire 1997 Insecticides, OR=1.0–2.5 Herbicides, OR=1.7–3.0 Other, OR=0.3–0.7 Any, OR=2.5 Park 2005 Farm related, OR=1.2 Farmers, OR=1.23 Related jobs, OR=1.2 Burns 2001 2,4-D, RR=3.45–8.04	Difficulty in obtaining a high level of evidence due to lack of high quality of methodological and exposure assessment components. Pesticides are a potential environmental risk factor for ALS, but additional well-designed studies are clearly needed.	Sutedja et al., 2009 (4)	See McGuire et al., 1997 and Park 2005 (159) for details (study population size, patients, controls, 95% CI)
Washington State, USA (three western counties) Associations between workplace exposures and the risk of ALS.	Population-based case-control study. Cases (n=174) were all newly diagnosed with ALS by neurologists during 1990–1994. Controls (n=348), were matched according to age (±5 years) and sex, identified via random-digit dialing or Medicare enrollment files. Four industrial hygienists blindly assessed detailed lifetime job histories for exposures to metals, solvents, and agricultural chemicals. Case-control comparisons were made for jobs held between 15 and 10 years prior to the cases' dates of diagnosis.	After adjustment for age and education, exposure to agricultural chemicals was associated with ALS (OR=2.0, 95% Cl, 1.1–3.5); this association was observed separately in men (OR=2.4, 95% Cl, 1.2–4.8) but not in women (OR=0.9, 95% Cl, 0.2–3.8). Among men, the OR for low exposure to agricultural chemicals (below the median level for exposed controls) relative to no exposure was 1.5 (95% Cl, 0.4–5.3), and for high exposure, it was 2.8 (95% Cl, 1.3–6.1) (p trend=0.03).	These findings suggest an association between ALS and agricultural chemicals in men	Mcguire et al., 1997 (159)	

(Table 1 continued)

Location-subjects	Method	Findings	Conclusion	References	Remarks
County of Skaraborg, Sweden. All cases of MND with onset during the study period 1961–1990. Identified 168 cases, 107 men and 61 women.	Retrospective study. Fifty percent of cases with MND were alive 2 years after onset. An epidemic-like cluster was compared with the MND morbidity in a neighboring county.	The number of MND cases in consecutive 5-year intervals during the study period was shown to be elevated for males in the period 1981–1985 (Knox test disjoint procedure, p=0.02). During the period 1973–1984, 70 males had onset of MND, corresponding to an average annual incidence of 4 per 100,000 person-years. This epidemic-like cluster was compared with the MND morbidity in a neighboring county and was shown to be elevated. Agricultural work was more common among the cases.		Gunnarsson et al., 1996 (191)	All MND. ALS not mentioned.
Minnesota, USA Evaluated 74 selected patients with ALS and 201 matched controls for risk factors for ALS.	A case-control design and a sequential questionnaire/interview technique to quantitate biographic data. Analyzed occupational and recreational data only for 47 male patients and 47 corresponding 'patient controls'.	Did not report an association.		Armon et al., 1991 (158)	Data for women were insufficient. Used non-parametric analyses to evaluate five primary comparisons of ALS patients with controls, one of which was more years lived in a rural community.
Athens, Greece (Athens University, Eginition Hospital) 315 cases of ALS hospitalized over a 25-year (1964–1988) period at University Department of Neurology. Random control group of 360 hospitalized same period Majority of patients with chronic neurological disease in Southern Greece treated in this hospital at least once.	Emphasis given to occupation of patients and geographical distribution of disease. Reviewed clinical files of patients diagnosed with ALS or MND (regardless of diagnosis). Criteria for inclusion: signs of upper and lower motor neuron involvement, no sensory or sphincter disturbance, positive electromyographic findings, and no X-ray or biochemical findings of other disease. Perfecture is the administrative geographic unit. Study included 31 in southern Greece out of 51 perfectures total in Greece.	Over-representation of farmers among patients and aggregation of cases in the region of Cephalonia. Farmers are over-represented in the population of ALS patients (patients 65.8%, controls 52.5%, p=0.0005). Correlation of number of ALS cases to number of ALS cases for every perfecture (r_s=0.396035, p<0.05).	A high percentage of farmers in the population of ALS patients studied, supported by the positive correlation between the percentage of rural population and the number of ALS cases per prefecture.	Kalfakis et al., 1991 (192)	The results are not easy to interpret. There seems to be an association between agricultural occupation and ALS which warrants further study. The clustering of cases needs further investigation. Do not support other studies reported.
Sweden All cases of ALS (died from ALS) during 1970–1983 (1961 cases among four million) were compared with an age-stratified random sample of 2245 individuals from the Swedish population. About 250 individuals drawn from 5-year-wide birth cohort from 1896–1900 to 1936–1940.	Residency and occupation information from census. All cases were traced through the mortality statistics for Sweden.	The male cases were found to be heterogeneously distributed over occupational groups. More male cases than expected were found among office workers (OR=1.8; 34 cases) as well as among farm workers (OR=1.7; 56 cases). There was a cluster of male cases in agricultural work in one south-western county (OR=3.4; 25 cases).	There seems to be an association between ALS and certain occupations among males.	Gunnarsson et al., 1991 (193)	True associations could be missed by misclassification of occupation, but this would not create bias in terms of increased risk ratios. Census information is not particularly precise from a longitudinal point of view, i.e., the occupation obtained for an individual relates only to a particular point in time. On the other hand, there was no risk of recall bias. A combination of factors might be required for ALS development.

(Table 1 continued)

Location-subjects	Method	Findings	Conclusion	References	Remarks
Sardinia, Italy 182 cases of ALS 1957–1980. ALS diagnosis: (i) Symptoms of a lesion of the upper and lower motor neuron; (ii) Absence of objective disturbances of sensation of loss of control of sphincters; (iii) Normal CSF values and no signs of cervical myelopathy.	Case records from the archives of the Neurological Departments of Cagliari, Sassari, Ozieri, and Nuoro (operating since 1920, 1920, 1993, and 1992, respectively). All ALS patients in Sardinia hospitalized in one of the above 4 departments. Death certificates not considered, as initial cause of death (ALS) was often not specified.	Mean ALS incidence higher in farm workers and shepherds. The ratio between the incidence in farmers and in the other two occupational groups is 5.42 The mean yearly ALS incidence per 1,000,000 population according to occupational status: Farming=2.39 Housewives=0.5 Other non-active population=0.28. Mean incidence=0.51/100,000/year.	Sardinia has a mean annual incidence of 0.51 per 100,000 inhabitants and a prevalence rate of 3.65 for 100,000 inhabitants (prevalence day 21 October 1971). Incidence in various areas of the island was divergent. The common form was more frequent, had earlier onset and greater median survival rate.	Giagheddu et al., 1983 (194)	In the present series, 4.4% of the cases were familial.

a farmer, or environmental risk factors, such as contact with soccer fields assumed to have been treated with herbicides. The pattern relating ALS to occupational exposure is inconsistent, as, in addition to agriculture and farming, some studies reported associations of ALS with different occupations, such as heavy metal work, electric trauma, use of pneumatic tools, employment in the plastics industry, exposure to heavy metals, acids, and animal carcasses, and truck driving (193). Even when farmers were identified, the subjects might not be the traditional thinking of farmers who use pesticides (e.g., pesticide applications for agricultural crops), but rather sheep breeders as in the case reported by Rosati et al. (196). Of the 18 reports, 13 suggest a weak potential association, and five do not report any association. The potential association is examined mainly in terms of occupation and environmental risk factors, generally using existing records of ALS patients along with control populations and questionnaire surveys. A major weakness of these studies is inadequate exposure assessment due to insufficient information on the actual chemicals involved or about their use (amount, application method), exposure pattern (air, dermal; intermittent; short, medium, or long-term; job category), frequency (hours, daily, monthly), duration (total time period), level of exposure (measured concentrations), industrial hygiene (protective measures). Much of the potential association is assumed (e.g., assumed exposure to pesticides because of agriculture-related work), with no specific pesticide products identified. That the studies were conducted in the different parts of the world offers some level of 'consistency', which is a key aspect of causal inference, although no cause-effect relation was clearly identified. Nevertheless, a suggestion of consistent results can be seen across studies from different areas and different researchers, using different methods and subjects. Other limitations of these studies include small sample size (183, 187), unavailable information about confounders, such as lifestyle (e.g., diet, smoking), and other chemical exposures (e.g., chemicals used on farms, in hobbies). Another limitation is the self-reporting nature of certain studies, which might be a subjective process and also may carry recall and selection biases (133, 185, 187). Therefore, that the increased ALS incidences observed in these studies were actually caused by pesticides cannot be definitively confirmed, although the overall evidence is suggestive of an association between increased ALS risk and pesticide exposure.

As noted above, one potential relation of pesticides to MND is the neurotoxic properties of several pesticides, such as OPs pesticides, and the genetic susceptibility of individuals to such toxicity. As PONs are involved in the detoxification of this group of pesticides, studies have been conducted to examine the potential association between sALS and *PON* gene cluster variants. Saeed et al. (197) investigated the association between sALS and *PON* gene cluster variants in a large North American Caucasian family-based and case-control cohort. Clinically definite and probable ALS was diagnosed according to the revised El Escorial criteria, exclusion of family history of ALS, and *SOD1* mutation analysis. Single-nucleotide polymorphism (SNP) genotyping was done using TaqMan gene expression assay. A haploblock of high linkage

Table 2 Summary of reports on human studies on potential association of pesticides to ALS.

References[a]	Potential association[b]	Characteristics used to link study subjects to pesticides[c]
Bonvicini et al., 2010 (183)	+	Agricultural work
Furby et al., 2010 (184)	+	Contact with agricultural chemicals
Weisskopf et al., 2009 (185)	–	No background information (e.g., occupation). American Cancer Society Cancer Prevention study participants self-reporting on exposure to 11 different chemicals classes or X-rays
Chio et al., 2009 (186)	+	Soccer players
Qureshi et al., 2006 (187)	+	No background information (e.g., occupation). Neuromuscular clinic patients self –reporting on toxic exposures.
Morahan and Pamphlett, 2006 (133)	+	No background information (e.g., occupation). ALS patients self-reporting on pesticide/herbicide exposures
Govoni et al., 2005 (188)	+	Occupation in agricultural work
Burns et al., 2001 (189)	+	Manufacture or formulation of 2,4-D
McGuire et al., 1997 (159)	+	Exposure to agricultural chemicals (in men)
Gunnarsson et al. 1991 (193)	+	Farm workers (and office workers)
Giagheddu et al., 1983 (194)	+	Farm workers
Holloway et al., 1982 (195)	+/– (frequency of exposure 9% in patients and 5% in the control population)	Farmers and agricultural workers (including three clinical variants of MND: progressive muscular atrophy, progressive bulbar palsy, and ALS).
Rosati et al., 1977 (196)	+	Agricultural workers, mainly farmers (majority sheep breeders)

[a]The limitations of the studies are summarized in Table 1 and discussed in the text. [b]'Potential association' (+) is based on increased incidence or RR when the exposed population is compared with the unexposed one, suggesting a possible but not a definitive role of pesticides. [c]'Potential association of pesticides' to ALS is inferred or assumed based on potential association of increased incidence or RR with characteristics that linked study subjects to pesticides, such as occupation in an agricultural environment, being a farmer, or reports on environmental risk factors (e.g., contact with soccer fields assumed to have been treated with herbicides).

disequilibrium (LD) spanning *PON2* and *PON3* was associated with sALS. The SNPs rs10487132 and rs11981433 were in strong LD and associated with sALS in the trio (parents-affected child triad) model. The association of rs10487132 was replicated in 450 nuclear pedigrees comprising trios and discordant sibpairs. No association was found in case-control models, and their haplostructure was different from that of the trios with overall reduced LD. Resequencing identified an intronic variant (rs17876088) that differentiates between detrimental and protective sALS haplotypes. The authors concluded that there is an association of variants in the PON gene cluster with sALS, which is compatible with the hypothesis that environmental toxicity in a susceptible host may precipitate ALS.

Some PON1 promoter polymorphisms may predispose to sALS, possibly by making motor neurons more susceptible to OP-containing toxins. To determine whether an impaired ability to break down OPs underlies some cases of sALS, Morahan et al. (177) studied 143 sALS patients and 143 matched controls and compared frequencies of PON1 polymorphisms, investigating gene-environment interactions based on information on self-reported pesticide/herbicide exposure. The PON1 promoter allele 108t, which reduces PON1 expression, was strongly associated with sALS. Overall, promoter haplotypes decreasing PON1 expression were associated with sALS, whereas haplotypes increasing expression were associated with controls.

Wills et al. (178) tested the hypothesis of an association that correlates with functional changes in PON1 (PON1, MIM 168820) within a case-control study. Sera from 140 ALS patients and 153 matched 'healthy' controls and CSF samples from 15 patients and 15 controls were tested for PON, diazoxonase, and arylesterase activities. Participants with ALS were genotyped using tagging SNPs across the PON locus, and survival data and enzyme activity were correlated with genotype. A trend toward increased PON activity was noted in ALS compared with controls, which correlated with increased frequency of the homozygous arginine (RR) variant of PON1Q192R. Contrary to expectations, PON1 protein, PON1, diazoxonase, and arylesterase activities were not reduced in ALS patients. OP hydrolysis rates had no effect on ALS survival. The authors noted that the increase in PON1R192 frequency in ALS in this study supported previous genetic susceptibility studies, and the findings suggested that the influence of PON1 polymorphisms on ALS susceptibility was not due to reduced OPs hydrolysis. A limitation of this study, however, is the lack of a population-based design for cases and controls, an approach that might have been responsible for selection bias.

Conclusions

Overall, our analysis supports the view that Se and pesticides are likely to be involved, either as triggering factors or as causal agents, in ALS etiology, based on epidemiological evidence and some biological plausibility. The evidence is particularly impressive for Se due to its selective toxicity to motor neurons. Less suggestive is the involvement of Hg, Cd, or Pb in disease etiopathogenesis due to the limited evidence from

both epidemiological and laboratory studies and because the studies carried out thus far are inconclusive. Apart from the PONs genes, little is known about the possible role of genetic susceptibility in mediating the effects of environmental toxins in triggering ALS onset. Various methodological limitations of the studies, such as low statistical power, exposure misclassification, and inadequate control of confounders, might explain the conflicting results found in the literature on these issues. Further in-depth investigation of the involvement of Se, heavy metals, and pesticides in ALS etiology is warranted.

Acknowledgments

Financial support to this study was provided by Associazione Italiana Sclerosi Laterale Amiotrofica (AISLA), by Pietro Manodori Foundation of Reggio Emilia, and by the Local Health Unit of Reggio Emilia.

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