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Clinical Perspective of Oxidative Stress in Sporadic ALS

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Abstract

Sporadic amyotrophic lateral sclerosis (sALS) is one of the most devastating neurological diseases; most patients die within 3 to 4 years after symptom onset. Oxidative stress is a disturbance in the pro-oxidative/anti-oxidative balance favoring the pro-oxidative state. Autopsy and laboratory studies in ALS indicate that oxidative stress plays a major role in motor neuron degeneration and astrocyte dysfunction. Oxidative stress biomarkers in cerebrospinal fluid, plasma, and urine, are elevated, suggesting that abnormal oxidative stress is generated outside of the central nervous system. Our review indicates that agricultural chemicals, heavy metals, military service, professional sports, excessive physical exertion, chronic head trauma, and certain foods might be modestly associated with ALS risk, with a stronger association between risk and smoking. At the cellular level, these factors are all involved in generating oxidative stress. Experimental studies indicate that a combination of insults that induce modest oxidative stress can exert additive deleterious effects on motor neurons, suggesting multiple exposures in real-world environments are important. As the disease progresses, nutritional deficiency, cachexia, psychological stress, and impending respiratory failure may further increase oxidative stress. Moreover, accumulating evidence suggests that ALS is possibly a systemic disease. Laboratory, pathologic, and epidemiologic evidence clearly support the hypothesis that oxidative stress is

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central in the pathogenic process, particularly in genetically susceptible individuals. If we are to improve ALS treatment, well-designed biochemical and genetic epidemiological studies, combined with a multidisciplinary research approach, are needed and will provide knowledge crucial to our understanding of ALS etiology, pathophysiology, and prognosis.

Keywords

sALS; oxidative stress; environmental epidemiology; phenotypic variation; disease prognosis

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is one of the most devastating neurological diseases. Patients with ALS develop relentlessly progressive paralysis that involves all the skeletal muscles, as well as the bulbar and respiratory muscles. This paralysis ultimately leads to the patient's death on average 40 months after symptom onset. Riluzole is the only medication that has received Food and Drug Administration approval, but it has only modest benefits at best (1–3). More than a dozen molecular mutations have been discovered in familial ALS (fALS), which constitutes 5% to 10% of all ALS cases (4–9). In contrast, despite decades of intense research and a number of highly plausible hypotheses (10–12), still little is known regarding factors related to the causes or risks of developing sporadic ALS (sALS), which we focus on in this review.

Our review is specifically focused on clinical, or patient-oriented, research in oxidative stress in sALS. Since discussing oxidative stress on the basic science level is not our objective, we provide a few excellent reviews here for this information (13–15). First, we briefly discuss the effects of oxidative stress on motor neurons and the central nervous system (CNS). Moreover, we review currently available biomarkers that are useful for investigating oxidative stress in sALS and the potential consequences of oxidized products. Then, we examine epidemiological studies and how environmental and lifestyle factors potentially trigger oxidative stress in exposed individuals. We also discuss evidence that oxidative stress is not just an event in the CNS but rather a systemic process, although the CNS and motor neurons may be most vulnerable to systemic oxidative stress. Because oxidative stress results from pro- and anti-oxidative imbalance (15, 16), current knowledge of intrinsic anti-oxidative mechanisms is reviewed along with possible interactions between oxidative stress and modifier genes in sALS. We close with a call for molecular epidemiology studies in ALS. We hope that this review will stimulate more research in patients with ALS that investigates the relationship between sALS pathogenesis and environmental exposures related to oxidative stress, which will ultimately lead to novel therapeutic approaches and better clinical management.

MOTOR NEURON DEGENERATION IN ALS

The CNS as a whole is particularly susceptible to oxidative stress because the neuronal membrane contains a high abundance of polyunsaturated fatty acids, especially arachidonic and docosahexaenoic acids; it consumes oxygen at a high rate; and it contains high concentrations of redox-active transition metals but a relatively (compared to the oxidative stress level) low concentration of antioxidants (17, 18). In sALS, at the cellular level, genetic factors, excitotoxicity, apoptosis, inflammation, mitochondrial dysfunction, protein aggregates, and oxidative stress are among the primary hypotheses put forth to explain motor neuron degeneration (10–12). Among these factors, oxidative stress appears intimately linked to a series of cellular events in motor neurons that contribute to neuronal degeneration and death (19). In fact, analyses of post-mortem neuronal tissue from patients

with sALS consistently show oxidative damage to proteins, lipids, and DNA (20–24), suggesting that oxidative injury is very likely to be one of the principal cellular mechanisms of motor neuron degeneration (25). Several excellent reviews on oxidative stress in ALS are available, mostly focusing on the molecular mechanisms of oxidative stress in neuronal degeneration (25–31). In recent years, however, laboratory studies have revealed that astrocytic glial cells play a key role in neuronal degeneration (32–36). When cultured astrocytes undergo oxidative damage, glutamate transport is impaired, likely resulting in excitotoxic neuronal injury (37). There are a number of *in vitro* studies, suggesting that experimental oxidative stress in astrocytes and oligodendroglia could lead to motoneuronal degeneration (38–41).

OXIDATIVE STRESS BIOMARKERS AVAILABLE FOR STUDIES IN ALS

Whereas oxidative stress appears to be closely associated with motor neuron degeneration in ALS, it still remains unsettled whether oxidative stress is involved outside of the CNS such as in skeletal muscles (28, 42, 43). Reliable oxidative stress biomarkers are the first essential step to ascertaining such extra-CNS involvement (42). Oxidative stress damages critical cellular macromolecules, which can eventually lead to cell death by necrosis or apoptosis (44). The localization and effects of oxidative stress may be determined from analysis of discrete biomarkers of oxidative or nitrosative stress damage in tissues and biological fluids (13, 25, 45). Among the well-studied biomarkers are oxidized DNA, lipids, and proteins. To be truly useful, an oxidative stress biomarker must have some degree of predictive validity, but this relation still must be fully demonstrated for sALS (46), so such markers should be interpreted cautiously in cases of sALS (47). Here, we review the most thoroughly studied and reliable (and thus the most important) oxidative stress biomarkers to date, and how they are used in sALS studies.

DNA is clearly a preferred oxidative stress target. In particular, guanine is easily oxidized (48). 8-Oxo-deoxyguanosine (8-oxoG) is the most abundant type of oxidative DNA damage and is well-studied as an oxidative stress biomarker *in vivo* (49, 50). CNS lipids are also common targets of reactive oxygen species (ROS, e.g., H₂O₂, O₂⁻, RO, OH, HOCl) and reactive nitrogen species (RNS, ONOO⁻, NO₂, NO) (51).

Oxidative stress reactions can significantly alter membrane structure and intracellular lipids, resulting in altered fluidity, permeability, transport, and metabolic processes. Fatty acid peroxides can give rise to a variety of aldehydes, such as 4-hydroxy-2-nonenal (HNE), thiobarbituric acid-reactive substances (TBARS) like malondialdehyde (MDA), and isoprostanes (IsoPs) (52). These products are relatively stable and can diffuse within or exit the cell and attack targets far from the site of the original event. Therefore, they are not only end products and remnants of lipid peroxidation processes but also may act as “second cytotoxic messengers” (53, 54). Lipid peroxidation products can also be absorbed from the diet and excreted in urine. It follows that measurements of hydroxyl fatty acids in plasma total lipids as well as plasma or urinary MDA and HNE can be confounded by diet and should not be used as an index of whole-body lipid peroxidation unless diet is strictly controlled (55).

HNE is considered one of the most toxic molecules known to cause cellular damage and apoptosis (56, 57). Moreover, HNE is capable of disintegrating membranes and can diffuse between cell components (58). HNE interacts with the cell membrane and a wide array of amino acid residues present in receptors, chaperones, electron transport chain proteins, and proteins that repair oxidative damage (56). Other lipid biomarkers of oxidative stress include the F₂-isoprostane family (F₂-IsoPs) (59). F₂-IsoPs are formed non-enzymatically as a result of the free radical-mediated peroxidation of arachidonic acid (60). F₂-IsoPs have been

found in measurable quantities in most of the biological fluids that have been analyzed. Plasma and urine are the sample types that are commonly analyzed, as they are the most convenient to obtain and the least invasive (61). F2-IsoPs are not only biomarkers of oxidative stress but exert biological effects themselves, suggesting they also might function as pathophysiologic mediators of oxidant injury (62). Most current knowledge on the biological actions of F2-IsoPs is limited to 15-F2t-IsoP, which is a potent vasoconstrictor. However, it is not known whether the IsoP concentrations reached locally *in vivo* is sufficient to exert biological effects. No specific inhibitor of their biological action exists (63); however, molecular modeling of phospholipids containing IsoPs reveals remarkably distorted molecules (64, 65). Thus, the formation of these abnormal phospholipids would be expected to exert profound effects on membrane fluidity and integrity, well-known sequelae of oxidative injury (60). Moreover, exposure to IsoPs is associated with neurotoxicity that modulates neuronal apoptosis (66).

Carbonylation and other ROS-associated post-translational modifications readily inactivate proteins, including enzymes (67, 68). Oxidation of the protein backbone is initiated by hydroxyl radical-mediated extraction of the α -hydrogen atom from the amino acid residues, forming a carbon-centered radical (67). All amino acid residues can be modified, resulting in the oxidation of amino acid side chains and protein-protein cross-linkages. Methionine and cysteine residues are more prone to oxidation than others due to the presence of sulfur. Tryptophan is also readily oxidized. Direct oxidation of lysine, arginine, tyrosine, proline, and threonine residues give rise to carbonyl derivatives, which, because they are easily detected, are most often used as a marker of proteins modified by oxidative stress (69, 70).

Peroxynitrite also affects protein activity by oxidizing amino acid residues as seen in nitrotyrosine, which is found in the CNS in human sALS, fALS and ALS-mouse models (71–74). 3-nitrotyrosine (3-NT) is often used as a marker of nitrosative stress in CNS (cerebrospinal fluid, CSF) and peripheral blood samples (74, 75). Peroxynitrite-mediated tyrosine nitration has been suggested as a potential mechanism for the induction of neuronal degeneration in several neurodegenerative disorders including ALS (76, 77); however, the exact mechanisms underlying how free nitrotyrosine can cause neuronal degeneration remain to be elucidated (78).

Investigating the effects of oxidative stress on molecular targets and identifying reliable biomarkers involved in oxidative stress are two great challenges in sALS. A compelling study by Bogdanov et al (42), investigating 65 patients with newly diagnosed ALS (60 with sALS), 63 subjects without neurological disorders, and 37 patients with other neurological disorders, examined levels of 8-oxodG in plasma (n= 57, 21, and 14 patients, respectively), urine (n= 59, 56, and 35 patients, respectively) and CSF (n= 23, 19, and 10, respectively). They found that 8-oxodG was elevated in the CSF and urine of ALS patients compared to both control groups. Furthermore, plasma levels in ALS were significantly higher than in the group with no neurological disorders but not in the group with other neurological disorders. In all subjects, plasma and CSF 8-oxodG concentrations increased with age, confirming that oxidative damage has a role in normal aging. Plasma and urine 8-oxodG concentrations increased significantly with time (9 months from the baseline) within the ALS group (31 subjects had follow up) and the increase in urinary 8-oxodG was significant in the ALS group compared to the neurological disorders-free group (42). Our preliminary studies of oxidative stress in sALS progression have also revealed increased urinary 8-oxodG (43).

In 2004, Simpson et al (79) measured HNE in both serum and CSF in 63 patients with sALS, comparing the findings to 19 patients with other neurodegenerative disease, 14 patients with neurologic but non-neurodegenerative disease, and 16 healthy controls (serum only). They found that serum and CSF HNE levels were significantly elevated in sALS

cases compared to both disease and healthy control groups. Further, HNE levels were 2.3-fold greater in CSF than serum in the sALS patients ($P = 0.00005$). In 42 of the patients with sALS, serum HNE was measured throughout the disease course; HNE concentration increased over time and positively correlated with advancing disease ($r = 0.17$, $P = 0.03$).

Chromatographic mass spectrometry has revealed increased CSF F2-IsoP concentration in Alzheimer disease but not in ALS (80). However, F2-IsoPs have been found to be significantly increased in urine as measured using an immunoassay technique in a cross-sectional pilot study of 50 participants with sALS compared to 46 healthy control subjects ($P = 0.002$) (43). Using logistic regression and controlling for sex and age, a positive association between sALS and the natural log of urinary F2-IsoP concentration (adjusted for creatinine) was found ($OR = 3.24$; $95\%CI = 1.40-7.55$; $P = 0.006$) for a doubling in the level of urinary F2-IsoPs (43).

Table 1 lists additional studies of potential oxidative stress biomarkers in sALS (74, 81–90), not been described above.

In summary, biomarkers of oxidative stress and the methods used to measure them vary among sALS studies, making comparison of study findings difficult. Furthermore, whether oxidative stress is an inciting or a downstream event during the already ongoing degenerative process remains to be determined. Yet, it is clear that oxidative stress is likely to be a major player in the damage that occurs to DNA and pathologic changes in lipid and protein metabolism (19).

ENVIRONMENTAL AND LIFESTYLE RISK FACTORS ASSOCIATED WITH sALS

In this section, we review environmental exposures and life style factors that have been associated with ALS that also may be mechanistically involved with underlying oxidative stress. Table 2 summarizes potential relationships among environmental and lifestyle factors and sALS and oxidative stress.

Agricultural chemicals

Organophosphate pesticides, until their recent ban, were among the most widely used synthetic chemicals for controlling domestic and agricultural pests. Several epidemiological studies suggest that exposure to organophosphate pesticides is associated with sALS (91–102). In 2003, a new evidence-based medicine method to evaluate the quality of ALS epidemiological studies was proposed (103). It includes assessment of 1) general methodological criteria (selection of control group, high response rate, blinding, recall bias, quantification of exposure, accounting for confounding and bias, and appropriate analytical approach); 2) the diagnostic certainty of ALS based on established criteria; and 3) temporal precedence of exposure and disease onset (103). Based on these criteria, Armon identified two population-based, one case-control, and one mortality surveillance study (91, 98, 101, 102). Further stringent criteria and a new method to qualify exposure accuracy and reproducibility have subsequently been developed based on 1) self-reported exposures, 2) assigned exposure by an expert or computer-based assessment of exposure levels, and 3) evidence of external exposure, internal exposure (e.g., for metals) or a biomarker of exposure (usually a metabolite) of suspected agents (e.g., for pesticides) (104). When one includes these critical assessment techniques, only two studies, both population-based, meet these criteria (91, 94). Both demonstrate that the risk for sALS is associated with pesticides exposure.

One of these two investigations (91) is a population-based case-control study of 174 cases and 348 controls in Washington state, matched according to sex and age, during 1990 through 1994, when the state had a population of approximately 2.5 million (105). After adjustment for age and education, exposure to agricultural chemicals was found to be associated with sALS (OR = 2.0, 95%CI = 1.1 – 3.5); this association was observed for men (OR = 2.4, 95%CI = 1.2 – 4.8) but not for women (OR = 0.9, 95%CI = 0.2 – 3.8) (91). Park et al (94) conducted a mortality surveillance in a population over 2 million in 22 states during 1992–1998 and analyzed the association of mortality with neurodegenerative disease, including motor neuron disease (MND), in occupational groups previously described (99) and occupations with probable pesticide exposure: 1) all farming occupations, 2) farming occupations with likely pesticide exposure (farm workers, horticultural specialty workers etc.), and 3) farmers alone (99). Risk for MND was strongest for farmers (OR = 1.23, 95% CI = 1.03 – 1.46). However, in other occupations where pesticide use might be expected (e.g., farm workers, horticultural specialty workers), significant elevations were not observed (94).

Although not fulfilling the strict criteria described by Sutedja et al (104), two case-control studies (93, 95), originating from Europe (93) and Oceania (95), showed that pesticide exposure was positively associated with ALS. The first one showed that the number of incident cases of ALS in which the patient's 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) (93). A case-control study performed in Oceania showed a higher risk of developing sALS with exposure to industrial herbicides, pesticides, or both (OR = 4.18, 95% CI = 1.79–9.74, P = 0.006) but not for farm-based herbicides or pesticides (95).

A population-based case-control study (97) and a case-control study (96), both European and published after the critical assessment techniques described above were developed, showed very similar ORs for pesticide exposure (OR = 3.6; 95% = CI 1.2–10.5, and OR = 3.04; 95% CI = 1.1–7.7, respectively). Moreover, the American Cancer Society's recent prospective Cancer Prevention Study II, with nearly 1.2 million participants (106), evaluated the relationship between regular exposure to 11 different chemical classes or x rays and sALS mortality (92). Follow-up from 1989 through 2004 identified 617 deaths from ALS among men and 539 among women but found no statistically increased risk of ALS mortality by self-reported exposure to pesticides, herbicides, or both (OR = 1.07; 95%CI = 0.79 – 1.44). Regarding the duration of exposure, ORs for those reporting < 4 years' exposure were 0.62 (95% CI = 0.09 – 4.45), for 4 to 10 years, 1.92 (95% CI = 0.71 – 5.19), and for >10 years of exposure, 1.48 (95% CI = 0.82 – 2.67) (92).

Therefore, epidemiological evidence of an association between exposure to agricultural chemicals and sALS is present but modest and thus is, at best, suggestive. If the association between agricultural pesticides and sALS is small to modest and if a specific genotype modifies the association, then even large-scale case-control and cohort studies may not be effective in finding consistent associations between organophosphate-induced oxidative stress and sALS.

Most agricultural organophosphates can be clearly linked to oxidative stress (107–110). Among these, chlorpyrifos, parathion, and malathion have been shown to impair GSH homeostasis by decreasing GSH levels (111). GSH is a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine) with multiple functions in living organisms (112). It can be directly oxidized by hydroxyl radical (HO•) and peroxynitrite (ONOO-) or indirectly during GSH-dependent peroxidase-catalyzed reactions (113). Increased oxidative stress biomarkers (urinary 8-oxodG, malondialdehyde), reduced GSH levels and DNA damage (COMET assay) are observed in organophosphate-treated lymphocyte cell cultures (109, 114).

Although the molecular mechanism underlying organophosphate induction of oxidative stress remains to be fully clarified (115), active metabolites (various oxon compounds), that are enzymatically converted from organophosphates and able to deplete intrinsic antioxidants, have been identified in the human liver, lung, and brain tissues (116–118). Paraquat, a common herbicide, can induce GSH depletion in the CNS (119). In a neuron-microglia culture system, paraquat was toxic to neurons even at low concentrations (120). In this system, paraquat activates microglial NADPH oxidase generating superoxide, which is well studied in the microglia (120, 121). Moreover, organophosphate uptake into the mitochondria across the mitochondrial inner membrane leads to reduction by complex I, resulting in redox cycling and mitochondrial toxicity (122). Paraquat not only weakly inhibits complex I but impairs complex III function as well, and the respiratory chain disruption generates more oxidants (121, 123).

Lead exposure

Exposure to metals is an intriguing potential suspect in the cause of sALS, with many case reports linking different metals to sALS (124). Furthermore, some investigators have found higher levels of certain metals in the blood, bone, CSF, urine, or spinal cords of patients with sALS compared to controls [reviewed in (125)]. The role of metals exposure has also been investigated in epidemiological studies (92, 94, 126–130). Despite many investigations, the role, if any, of these metals in the pathogenesis of sALS remains unclear [reviewed in (104)]. A study by Kamel et al (128) is particularly noteworthy, however, because they longitudinally assessed lead exposure in sALS. In a case-control study, they recruited 109 ALS cases from two major referral ALS centers in New England from 1993 to 1996 along with 256 controls (matched to cases by age, sex, and region of residence), identified by random-digit dialing. They collected information on occupational, residential, and recreational exposure to lead using a structured interview. In addition, they measured blood and bone lead concentrations (blood was collected from 107 cases and 39 controls; bone lead was measured at two sites, the mid-tibial shaft and the patella, in 104 cases and 41 controls). The risk of ALS was associated with self-reported occupational exposure to lead (OR = 1.9; 95% CI = 1.1 – 3.3). Moreover, risk of ALS was associated with elevations in both blood and bone lead levels: the OR was 1.9 (95% CI = 1.4 – 2.6) for each µg/dL increase in blood lead, 3.6 (95% CI = 0.6 – 20.6) for each unit (µg /g) increase in log-transformed patella lead, and 2.3 (95% CI = 0.4 – 14.5) for each unit (µg /g) increase in log-transformed tibia lead.

Genotyping for known SNPs in the δ -aminolevulinic acid dehydratase (*ALAD*) gene (129) revealed that the *ALAD* 2 allele (177G to C; K59N) was associated with decreased lead concentration in both patella and tibia, although not in blood; it showed a trend toward an increased ALS risk (OR = 1.9; 95% CI = 0.60 – 6.3), although the confidence interval was wide, and the association was not statistically significant. Further, a previously unreported *ALAD* SNP at an *MspI* site in intron 2 (IVS2+299G>A) was associated with decreased bone lead concentration and a non-significant trend toward a decreased ALS risk (OR = 0.35; 95% CI = 0.10 – 1.2). The *ALAD* enzyme is the principal lead-binding site in erythrocytes, and the *ALAD* 2 protein binds lead more tightly than the *ALAD* 1 protein (131). This change in intron 2 alters the lead toxicokinetics and may modify the risk associated with lead exposure (132).

More recently, Kamel et al. (130) obtained follow-up data for 91% of the patients who participated in the original case-control study (128). Surprisingly, lead exposure appeared to directly correlate with longer survival time among ALS cases (OR = 0.5; 95% CI = 0.2 – 1.0).

Environmental lead exposure markedly reduces GSH levels and induces caspase 3 and prostaglandin E2 activation in neuroblastoma cells (133). In astroglia, lead also causes neuronal oxidative injury by interfering with copper homeostasis (134) and by inducing glial heme oxygenase synthesis (135). Collectively, this evidence suggests that lead alone or in combination with other biochemical stressors leads to oxidative injury (136, 137). Further laboratory studies have shown that astrocytes can sequester and buffer lead in primary co-culture with neurons, modulating its diffusion (138). In particular, astrocytes induce neuronal cytoprotective and antioxidant gene expression in response to lead exposure (139). In immortalized human fetal astrocytes, this expression results in increased vascular endothelial growth factor (VEGF) (135) and suggests that VEGF protects motor neurons in culture and in organotypic culture (140–142). However, further studies are clearly needed. Recent evidence has shown that lead exposure can cause epigenetic changes, which can lead to alterations in the transcription and subsequent translation rates of genes and proteins throughout the genome (143).

Military deployment

Although controversial, two studies suggest an approximately 2-fold increase in the risk of sALS among veterans of the 1991 Gulf War (144, 145). A study involving only Gulf War veterans under the age of 45 also found an elevated risk of sALS in this population (146).

Subsequently, a secondary analysis of these data showed that the increased risk of ALS was limited to the decade following the war and also to deployed military personnel only, suggesting that exposure to potential neurotoxic agents occurred over a relatively brief period of time (147). However, previous case-control (148) and prospective (149) studies found no association between deployment and ALS among Gulf War veterans.

In 2005, Weisskopf et al (150) prospectively assessed the relation between military deployment and sALS mortality among participants in the American Cancer Society Prevention Study II, a cohort that included more than 500,000 men (106). They compared cause-specific mortality between 281,874 deployed military personnel, excluding Gulf War veterans (military service was ascertained in 1982), and 126,414 men who did not serve in the military. Of 280 deaths due to ALS, 217 occurred in the military group and 63 in the group who had not served, indicating that those who served in the military had an increased death rate from ALS (OR = 1.53; 95% CI = 1.12 – 2.09). Furthermore, when the relation between ALS mortality and the different branches of military service were analyzed, mortality was greater among those who served in the Army or National Guard (OR = 1.54; 95% CI = 1.09 – 2.17), the Navy (OR = 1.87; 95% CI = 1.28 – 2.74), Air Force (OR = 1.54; 95% CI = 0.99 – 2.39), and Coast Guard (OR = 2.24; 95% CI = 0.70 – 7.18), not in the Marines. The increased risk of ALS mortality was similar among those who served in World War II (OR = 1.60; 95% CI = 1.12 – 2.30), Korea (OR = 1.54; 95% CI = 0.92 – 2.60), or Vietnam (OR = 1.44; 95% CI = 0.47 – 4.47) compared with those who had never served.

In a recent study, various factors (demographics, medical history, major traumatic injury, smoking status) and aspects of military service [(cumulative time of military service, primary branch of service (Air Force, Army, Coast Guard, Marines, or Navy), and deployment (in Korea, Vietnam, or the Persian Gulf regions)] were investigated to evaluate their influence on the survival in a group of more than 1000 military veterans from across the US enrolled in the National Registry of Veterans with ALS (151). In this group, 14% reported past deployment to Korea, 21% to Vietnam, and 5% to the Persian Gulf; 4% reported deployment to more than one location. Past deployment in Vietnam compared to other locations was associated with shorter survival in ALS (OR = 1.73; 95%CI = 1.36 – 2.19), suggesting the possibility that patients who were deployed in Vietnam shared some common exposure (e.g., virus, toxin) that worsened the effects of ALS once it developed.

One of the biases of this study is that only veterans alive in 2003 and beyond are in the ALS registry.

Obviously a single individual may be exposed to multiple different potential risk factors. A 2012 case-control study of 184 veterans with ALS and 194 control subjects from the National Registry of US Veterans (152) found that blood lead concentration was higher among ALS cases compared with controls ($P < 0.0001$, adjusted for age) and a doubling of blood lead was associated with a 1.9-fold increased risk of ALS (95% CI = 1.3 – 2.7) (153). However, it is possible that the wasting associated with ALS leads to increased bone turnover and lead leaching from bone into the blood. Thus, higher blood lead levels may be possible in ALS without being causal.

A recent cohort study of more than 2,000 medical records of US veterans from April 2003–September 2007 (152) included data on 241 incident ALS cases and 597 controls. The study authors found that veterans who had experienced head injuries during the 15 years before the reference date (the date of the ALS diagnosis for cases and the date of the interview for controls) had an adjusted (age, sex, race/ethnicity and education) OR of 2.33 (95% CI = 1.18 – 4.61) (154). Moreover, a gene-environment interaction analysis of 221 cases and 476 controls that included cigarette smoking (never vs. ever), head injury (reported by 79 cases and 183 controls at least one time), and apolipoprotein E (*ApoE*) genotype (homozygous *ApoE-3* carriers vs. *ApoE-2* carriers vs. *ApoE-4* carriers) reported weak evidence for the interaction; the association between ALS and head injury was stronger in *ApoE-4* carriers (likelihood ratio test of 2.7 1 df, $P = 0.10$) than in *ApoE-2* carriers (0.2; 1 df, $P = 0.68$) (154). However, the very small sample size pertaining to the ApoE analysis is a limitation.

Trauma

Although an association between head trauma and sALS is tenuous (101, 155–164), the report in US veterans discussed above suggests that the potential relationship to brain injury may deserve further exploration. The finding of a very high incidence of sALS among Italian professional footballers (165–167) and American football players (168) has led to speculation over whether head injury caused by collision is a relevant cause. A rigorous population-based study of sALS in western Washington state found that sALS was not associated with previous fractures, head injuries, or hospitalizations (101). In a recent cohort study, the adjusted rate ratio for sALS after head injury was 1.5 (95% CI = 1.1 – 2.1), but this elevated risk was found only within the first year after injury, possibly suggesting that very early subclinical weakness can increase the risk of head injury due to falls and fractures in sALS (164). A recent review evaluated the epidemiological literature regarding the association between head trauma and ALS published between 1980 and October 2010 (169), using the American Academy of Neurology evidence-based classification of evidence for inferring causality and assigning level of conclusion (170). Twelve articles (one with Class II evidence, three with Class III evidence, and eight with Class IV evidence) met the inclusion criteria. Analysis showed that a causal relationship between a single instance of head trauma or head injury and occurrence of ALS is unproven or unsupported (Level U conclusion) (169).

During a traumatic brain injury, the brain and spinal cord undergo shear deformation, which transiently elongates axons. Traumatic axonal injury also perturbs the cytoskeleton, causing microtubule and neurofilament dissolution and pathologic reorganization of neurofilament proteins (171). Trauma to the CNS triggers stress responses that include oxidative stress due to ROS generation (172). Necrotic events due to cellular disintegration resulting from an acute injury are associated with release of cellular contents, eliciting an inflammatory response and oxygen radical production.

Repetitive head injury is associated with the development of chronic traumatic encephalopathy (CTE), a tauopathy characterized by neurofibrillary tangles throughout the brain in the relative absence of β -amyloid deposits. McKee et al. (173) described 12 cases of CTE, 10 of which had widespread TAR DNA-binding protein-43 (TDP-43) inclusions (TDP-43 proteinopathy). Among these cases, 3 were athletes who developed progressive MND that clinically resembled an ALS-like syndrome. TDP-43 seems to be critical in mediating the response of the neuronal cytoskeleton to axonal injury (174). It is intrinsically prone to aggregation, and TDP-43 expression is upregulated after experimental axotomy in mouse spinal motor neurons (175). Conceivably, traumatic axonal injury may also accelerate TDP-43 accumulation, aggregation, and translocation to the cytoplasm and thereby enhance its neurotoxicity. Even though the number of cases was small, the coexistence of any MND phenotype and CTE neuropathology has raised interest in the ALS community.

The importance of TDP-43 in ALS quickly became evident when researchers discovered TDP-43 inclusions in ALS and frontotemporal dementia (FTD) (176, 177). TDP-43 inclusions are now known to be present in the majority of ALS cases, including ALS-FTD, sALS, non-SOD1 fALS, and the ALS-Parkinson disease (PD) complex in Guam (178–181). Recent studies suggest that oxidative stress may have a close relationship to TDP-43 and its pathologic alteration. Cohen et al. (182) demonstrated that oxidative stress promotes TDP-43 cross-linking via cysteine oxidation and disulphide bond formation, leading to decreased TDP-43 solubility. Moreover, prostaglandin metabolites, which are major players in oxidative stress and inflammation, can affect TDP-43 proteolysis, solubility, and subcellular localization (183). Chronic exposure of cultured neuron-like (SH-SY5Y and NSC34) cells to ROS and RNS via paraquat and ethacrynic acid administration revealed TDP-43 C-terminal phosphorylation, insolubility, C-terminal fragmentation, and translocation of nuclear TDP-43 to the cytosol (184, 185). Therefore, it appears that oxidative stress may play an important role in TDP-43 proteinopathy, further suggesting an intriguing relationship to sALS disease mechanisms.

Strenuous exercise and physical exertion

Strenuous exercise diminishes lymphocyte GSH content, resulting in oxidative stress-induced apoptosis, and increases urinary excretion of 8-oxodG, whereas moderate exercise attenuates lymphocyte apoptosis, possibly by improving antioxidative capacity (186). Strenuous exercise generates a host of complex biological changes; however, evidence is sufficient to suggest that it increases free radical production that leads to oxidative stress (187).

A study of aerobic exercise (incremental workload on a cycloergometer) in 10 patients with ALS, 8 of whom had sALS, assessed oxidative stress by measuring blood lipoperoxides (188). Values were significantly higher in patients with ALS ($P < 0.05$) than in control subjects ($n = 5$ with various chronic neuropathies) at rest, during exercise, and 30 minutes afterward. Moreover, the increase in lipoperoxide concentration during exercise strongly and positively correlated with lactate accumulation ($r = 0.94$, $P < 0.01$), a finding suggesting impaired mitochondrial function (188).

Epidemiological studies investigating an association between strenuous physical activity and sALS suggest that more evidence is needed to establish a possible causative role. In a case-control study of 279 patients with ALS and 152 with other neurologic diseases, a greater number of subjects with ALS reported they had been varsity athletes (OR = 1.7, 95%CI = 1.04 – 2.76) and had always been slim (OR = 2.21, 95%CI = 1.40 – 3.47) compared to disease controls (189). A recent matched case-control study has been performed in a large cohort of more than 680,000 Swedish male subjects born between 1951 and 1965 (190). Data on body weight and height, physical fitness, resting heart rate, and isometric strength

measured at military conscription (age 18–19 years) were collected. The population was followed through December 2006, and 85 men died from ALS during this period. The authors found that not physical fitness per se but rather weight-adjusted physical fitness, one of the strength measures, was a risk factor for ALS (OR = 1.98, 95% CI = 1.32 – 2.97). They raised the possibility that unknown genetic factors that are involved in muscle function and modulate human strength and endurance could be the link between weight-adjusted physical fitness and ALS (190). In Italian professional soccer players, a higher risk for sALS was found among midfielders (standardized morbidity ratio of 12.2, 95% CI = 3.3 – 31.2), a role characterized by great aerobic effort and a higher proportion of lean body mass (165). But an absence of sALS cases in professional road cyclists and basketball players, who also have a high level of aerobic fitness and higher proportion of lean body mass (191), suggests that physical exertion or fitness may not explain a causal relationship with sALS. Further studies are clearly needed (166, 192).

Diet

A population-based, case-control study in western Washington state from 1990 to 1994 showed that increased dietary fat intake was associated with a greater risk of ALS (OR = 2.7, 95% CI = 0.9 – 8.0) as was glutamate intake (OR = 3.2, 95% CI = 1.2 – 8.0), whereas dietary fiber intake was associated with a decreased risk (OR = 0.3, 95% CI = 0.1 – 0.7) (193). In 2008, Morozova et al (194) examined the relation between diet and risk of ALS among participants in the American Cancer Society Prevention Study II cohort (a population of nearly 1.2 million subjects) (106). No association was found. In a more recent case-control study, 153 sALS and 306 gender- and age-matched controls randomly selected from the general population in the Tokai area of Japan completed a self-administered food frequency questionnaire to estimate pre-illness intake of food groups and nutrients. A high intake of carbohydrates was significantly associated with an increased risk of ALS (OR = 2.14, 95% CI = 1.05 – 4.36) (195).

One micronutrient, vitamin E, deserves brief discussion as an extrinsic anti-oxidant. It is a fat-soluble hydrocarbon and an important component of cell membranes and lipoproteins. (196). Its antioxidant properties are attributed to the inhibition of lipid peroxidation and free-radical formation, because vitamin E can attack the peroxyl radical more rapidly than can polyunsaturated fatty acids, donating its phenolic hydrogen atom to the radical and converting it to a more stable product (197). Vitamin E might have a potential protective role in preventing the development of ALS (198) while also slowing disease progression (199). A mortality surveillance study conducted in a well-defined population [participants in the American Cancer Society Prevention Study II (106)] indicated that regular use of vitamin E supplements for 10 years or more is associated with a lower risk of dying of ALS (relative risk 0.38, 95% CI 0.16–0.92, based on trend analysis, $P=0.004$) (198). Further, a recent pooled analysis of mortality data from 5 different prospective cohort studies (among 1,055,546 participants, 805 developed ALS) revealed a positive trend ($P=0.01$) for a decline in ALS progression within patients (231 cases) with longer duration of vitamin E supplementation but found no overall protective role for vitamin E (199).

Smoking

Several studies, population-based and case-controlled, have established a probable relationship between sALS and cigarette smoking, as shown in Table 3 (154, 200–209), although some results have been inconsistent (210, 211). Methodological differences and heterogeneity in studied populations could account in part for these disparities (103, 212).

Cigarette smoking is associated with oxidative stress, inducing protein oxidation, DNA damage, and cell death (213, 214). It is hypothesized that cigarette smoking causes lipid

peroxidation via formaldehyde formation (215) and increases cadmium and lead levels in seminal fluid, plasma and blood; all of which may increase oxidative damage (216).

A recent pooled analysis prospectively examined the relation between smoking and ALS in 5 well-established large cohorts with study-specific follow-up, ranging from 7 to 28 years (205). Among 562,804 men and 556,276 women, 832 ALS cases were documented. Smokers had a higher risk of ALS than those who never smoked, with age- and sex-adjusted relative risks of 1.44 (95% CI = 1.23 – 1.68) for former smokers and 1.42 (95% CI = 1.07 – 1.88) for current smokers (205).

Combined Effects of Multiple Stressors

Undoubtedly, concurrent exposure to environmental and lifestyle factors occurs. In experimental models, combined exposure to glutamate and lead as compared to either agent alone increased neuronal cell death via mechanisms involving an elevation in the production of oxygen radicals and a decrease in intracellular GSH defenses against oxidative stress (217). Exposure of mouse embryonic (day 12) spinal cord and dorsal root ganglion cells to low levels of paraquat, exogenous glutamate, and thermal stress, both singly and in combination (218), revealed that paraquat administered alone was associated with cell death in a dose-dependent manner, an effect that was multiplied by heat shock. This model is particularly intriguing because a combination of low-level oxidative stress factors was associated with motor neuron vulnerability to environmental stressors. Such low-level cumulative stress may, in part, explain the mid- to late-life symptom onset in both familial and sALS patients. Other investigations (219) found various combinations of stressors differentially affected enzymatic and non-enzymatic antioxidant defense systems, protein oxidation, and lipid peroxidation in the brain.

These experiments are important because they attempt to mimic the human situation in which multiple stressors are likely to be present, resulting in additive or multiplicative interactions that contribute to abnormal oxidative stress. Thus, multidisciplinary epidemiological studies are needed that consider joint associations between demographic, biologic and genetic risk factors to better understand the disease mechanisms and eventually identify markers of susceptibility in patients with sALS.

INTRINSIC ANTIOXIDANTS

Little is known about the role of intrinsic antioxidants in sALS. Serum total antioxidant status, a measure of peroxyl adduct-scavenging capacity, has been reported to be significantly ($P < 0.05$) higher in ALS patients ($n = 28$) compared with that of healthy controls ($n = 20$) but did not correlate with ALS onset phenotype, disease duration, or clinical state (220). More recently, the TBARS concentration and antioxidants such as SOD1, catalase, GSH peroxidase, GSH reductase, and glucose-6-phosphate dehydrogenase were assessed in the erythrocytes of 20 sALS patients and 20 controls (221). In sALS, the TBARS concentration was significantly increased with respect to that of controls ($P < 0.001$); moreover, TBARS started to increase and antioxidant molecule concentration began to decrease as sALS progressed over 6 to 24 months, suggesting a correlation between these variables and duration of disease (221). In a second study, anti-oxidative stress markers were examined in 31 patients with ALS, 24 patients with PD, and 30 healthy subjects. SOD activity was significantly decreased in ALS ($P = 0.001$), whereas GSH peroxidase activity was decreased in PD ($P = 0.001$), suggesting that the affected antioxidant system might differ between ALS and PD (222).

SOD1

SOD1, or Cu/Zn-SOD, was the first identified and more comprehensively studied antioxidant enzyme of the SOD family; mitochondrial Mn-SOD2 and extracellular Cu/Zn-SOD3 also exist [for review see (25, 223)]. SOD1 catalyzes dismutation of O_2^- to H_2O_2 and O_2 in a two-step redox reaction involving the reduction and reoxidization of the copper ion in the active site of the dimeric protein.

The role of SOD1 in ALS pathogenesis has received great attention since the first description of mutations in the gene encoding SOD1 (224). Since then, more than 160 mutations have been reported. They account for approximately 15% – 20% of fALS cases, and thus SOD1 mutations are the second most frequent known cause of fALS after C9ORF72 (8, 9, 225). How mutant SOD1 leads to the death of motor neurons has not been established, although a number of hypotheses have been presented (25, 226–229). Importantly, the cause of motoneuron degeneration in *SOD1* mutation carriers is not the result of a loss of anti-oxidative function of the SOD1 enzyme, but instead, a gain of function appears to be pathogenic. We refer here to several excellent papers since this topic is beyond the scope of this review (25, 229, 230).

Although *SOD1* mutations can cause fALS, wild-type SOD1 protein might also have a role in sALS pathogenesis. First, oxidized wild-type SOD1 occurs in lymphocytes of sALS patients, resulting in mitochondrial dysfunction (231). Moreover, misfolded SOD1 is found not only in sALS motor neurons (232, 233), but also in the motor neurons of FUS- or TDP43-linked fALS cases - these are cases with no associated *SOD1* mutation (234). The mechanisms underlying how wild-type SOD1 is misfolded in these ALS cases remain to be clarified (229, 235). On the other hand, if this process is a frequent phenomenon in sALS or other types of fALS, SOD1 misfolding might explain a common molecular event between fALS and sALS (234).

Paraoxonase

Paraoxonase (PON) is a potential intrinsic antioxidative enzyme (236, 237). PON hydrolyzes paraoxon, which is a metabolite of parathion – a highly toxic organophosphate; exposure can lead to acute cholinergic crisis and subacute or chronic neurotoxicity. PON is a family of 3 independent enzymes: PON1, PON2, and PON3. The genes are located on chromosome 7 (238). PON1 is synthesized in the liver, and a portion is secreted into the plasma as a component of HDL (239).

Despite its name, PON1 is not very efficient in hydrolyzing paraoxon, which accounts in part for its toxicity. The mechanisms of organophosphate hydrolysis by PON1 have been well studied in laboratory animals, and oxidative stress occurs because organophosphate detoxification consumes GSH (240). On the other hand, oxidative stress may reduce PON1 activity (241). In fact PON1 is highly susceptible to hydroxyl radicals produced from metal (Cu^{2+} or Fe^{2+}) -catalyzed oxidation, and the modification of some histidine residues results in the decrease of its antioxidant activity (241).

PON1 has been extensively studied in cardiovascular disease because of its ability to protect against atherosclerosis (242). Certain SNPs in *PON1* have been associated with differential susceptibility to organophosphate exposure (243): two SNPs, *Q192R* (*rs662*) and *L55M* (*rs854560*) (244) have the greatest paraoxon hydrolytic activity but less effectively protect against low-density lipoprotein (LDL) oxidation (245). Decreased PON1 activity is associated with an increased risk of coronary heart disease (246) and organophosphate toxicity (247). In laboratory animals, PON1 decreases oxidation of LDL by hydrolyzing hydroperoxides into less reactive hydroxides (242). Moreover, PON1 has hydrolytic

activities on aromatic and long-chain aliphatic lactones, including arachidonate and lipid mediators generated from the oxidation of polyunsaturated fatty acids and exogenous lactone-containing drugs, such as prulifloxacin. Statin lactones (simvastatin, lovastatin) and the diuretic spironolactone, previously reported to be hydrolyzed by PON1, are metabolized by PON3 (248). PON2 and PON3 lack the ability to hydrolyze organophosphates but have lactonase or peroxidase-like activity or both (249, 250). Like PON1, PON3 is also found in HDL and serum. PON2 is expressed in a number of tissues, including the brain (251, 252). A human cell line overexpressing PON2 had significantly less intracellular oxidative stress (as measured by a nonspecific fluorometric assay for oxidative stress) following treatment with H₂O₂ (251). Serum PON1 activity in a given human population can vary by 40-fold. Although a portion of this variation is explained by extensive genetic variation in the PON1 coding regions (more than 160 SNPs have been described), activity may be largely influenced by environmental and perhaps even epigenetic factors (253). Age plays the most relevant role, at least early in life, as PON1 activity is very low before birth and gradually increases during the 1 to 2 years after birth in humans (254). PON1 activity may also decline with aging, possibly because oxidative stress increases with age (255). A sex effect has also been proposed, as females have higher PON1 activity (253). A variety of other factors (environmental chemicals, drugs, smoking, alcohol, diet, disease conditions) are suspected to reduce PON1 activity (253). On the other hand, statins increase PON1 expression and activity *in vitro* studies (256, 257). PON1 activity increased by ~13% in patients who took low-dose acetylsalicylic acid (258).

PON1 SNPs have been reported in ALS (259, 260), but subsequent results have been conflicting (261–268). A meta-analysis of 10 published studies and 1 unpublished study on *PON* SNPs found no significant association with sALS (269). A recent case-control study involving 1,160 sALS patients and 1,240 control subjects of Dutch descent that examined *PON1* and *PON3* genetic variants showed that the SNP frequencies did not differ between the two groups (268). Thus, work to date indicates that *PON1* SNPs contributing to ALS either do not exist or, if they do, seem to not be easily detected. If SNP variation is a factor, it may only have an effect in a susceptible minority of patients. A different genetic and epidemiological approach may be required to detect such abnormalities (270).

MODIFIER GENES ASSOCIATED WITH sALS AND OXIDATIVE STRESS

In addition to *PON1*, other possible genetic factors modifying sALS disease expression have been reported: *ApoE*, survival motor neuron (*SMN*), inducers of angiogenesis [such as *VEGF* and angiogenin (*ANG*)], and other genes involved in the regulation of many cellular processes, including some of the known RNA processes (e.g., transcription and post-transcriptional and translational regulation).

The role of ApoE in sALS is uncertain. It was suggested that the $\epsilon 4$ allele has a deleterious effect on ALS survival (271, 272) and association with younger age at ALS onset (273), but these findings were not subsequently confirmed (274). ApoE is a widely distributed, well-characterized cholesterol transport protein that circulates in the plasma after being synthesized by the liver, spleen, and kidneys (275). It is the major apolipoprotein in the CNS, where it is synthesized by glia, macrophages, and neurons (276). Animal studies showed that ApoE might have a role in promoting neuron general health and survival (277). How the *ApoE-4* genotype might confer vulnerability to a wide variety of neurodegenerative processes is not clear. It is hypothesized that this effect may result from impaired neuronal remyelination and axonal regeneration in *ApoE-4* carriers. A possible link to oxidative stress could be due to the fact that ApoE itself has antioxidant activity, which varies depending on the isoform -E-2 has the highest antioxidant activity and E-4 the lowest (278).

Other genes, such as *SMN*, *VEGF*, and *ANG*, have been associated with sALS as potential disease modifiers, but the associations and underlying mechanisms remain to be established (279–281). Duplications of *SMN1* were shown to be a risk factor for sALS (280). Yet, to date how *SMN* can modify neuronal survival is not fully understood (282). Similarly, *VEGF* is known disease modifier for sALS, but its contribution is at best unsettled (279). Another angiogenesis factor, *ANG*, has just been reported as a susceptibility gene for sALS (283). *ANG* is a secreted ribonuclease that cleaves tRNA to initiate a stress-response program in mammalian cells; it possesses properties that are both angiogenic and cytoprotective against apoptotic inducers, such as oxidative stress, although the mechanisms have yet to be fully elucidated. Secreted *ANG* enters cells via receptor-mediated endocytosis and translocates to the nucleus to promote ribosomal RNA transcription and cellular proliferation (284, 285).

Dysregulation of iron homeostasis may also be involved in sALS motor neuron degeneration. As iron is a transition element, the ionic form is prone to participate in one-electron transfer reactions, making it a source of oxidative stress (286). Because iron pathway dysfunction has been identified in sALS (increased stored iron, increased levels of serum ferritin, and similar pathologies), some studies have examined the genes involved in iron metabolism, like *HFE*, the gene mutated in hereditary hemochromatosis. An association of the *HFE* alleles *H63D* and *C282Y* with ALS has emerged in several investigations [reviewed in (287)]. A recent case-control study analyzed the possible role of an intronic SNP in *SLC11A2* (288), a gene that encodes an iron transporter in cerebral endosomal compartments. Comparing 579 ALS to 517 controls showed that the *SLC11A2* genotype did not correlate with disease presence but that the *C* allele was associated with shorter disease duration in patients who also had lower limb onset (OR = 1.5, 95% CI = 1.1–2.1) (288). This finding suggests that *SLC11A2* could be a genetic modifier in sALS. The metallothionein (MT) protein family is involved in pathways that detoxify heavy metals through intracellular sequestration of free toxic metals (289). A study examining a possible silencing of *MT-Ia* and *MT-IIa* promoters failed to show any modifications, however, in patients with ALS (290).

A recently discovered gene expressed in mitochondria, oxidation resistance 1 (*OXR1*) is induced by oxidative stress and can prevent oxidative damage to DNA in neuronal cells, although the full mechanism is not known (291). A recent study showed that *Oxr1* is overexpressed in ALS spinal cord samples compared to age-matched controls. *Oxr1* is susceptible to oxidation by peroxides, resulting in a loss of function that underscores how oxidative stress can be a vicious, self-sustaining process (292).

Gene mutations in fALS other than SOD1

Most gene mutations found in fALS cause mutated protein accumulation, aggregation, or both, that is likely to result in endoplasmic reticulum (ER) stress, a new area of ALS research (293). Particularly, vesicle-associated protein-associated protein B (VAPB), whose mutation causes of a rare form of fALS, is required for ER activation during ER stress and participates in intracellular vesicle transport. Other rare mutations occur in valosin-containing protein (VCP), essential for ubiquitin-dependent protein degradation (294, 295). The ER is a well-orchestrated protein-folding machine composed of protein chaperones, proteins that catalyze protein folding, and mechanisms to detect misfolded or unfolded proteins. Discrepancies between the demand and capacity of ER function lead to ER stress, leading to more misfolded proteins, which are also considered a potential cause of sALS [see in review (296)]. Although the mechanisms still remain to be clarified, persistent oxidative stress and protein misfolding may play a predominant role in the pathogenesis of these fALS cases (297). Further studies are clearly needed.

DISEASE PROGRESSION AND OXIDATIVE STRESS

The mean survival in sALS is approximately 40 months after symptom onset, but the duration of survival and functional prognosis vary widely, as approximately 10% of patients live beyond 10 years after symptom onset (298). The reasons for this variability are enigmatic. The most consistent finding is that the longer the duration between symptom onset and diagnosis, the longer the survival, a relationship that may be partly attributable to biological variability: the slower the disease progression, the longer it takes for the diagnosis to be established. Longer survival is associated with younger age of onset, pure upper neuron involvement (primary lateral sclerosis), or pure lower motor neuron involvement (progressive muscular atrophy) (298). It is possible that during the disease course, oxidative stress continues to accumulate and may hasten disease progression.

Malnutrition and cachexia

Cachexia is defined as a complex metabolic syndrome associated with underlying illness and characterized by loss of muscle with or without loss of fat mass and is associated with poor clinical outcome (299). Classically, cachexia is associated with chronic infections and malignant conditions, such as advanced cancer, severe chronic obstructive pulmonary disease, kidney failure, and HIV infection, all of which have associated oxidative stress (300). Malnutrition and cachexia occur in sALS (301), and weight loss carries a 7.7-fold greater risk of death (302). Malnutrition is common among patients with sALS, and in some cases can be attributed to increased resting energy expenditure (303). Rats subjected to protein caloric restriction had greater oxidative stress (304). In humans, most data have come from studies performed in patients undergoing maintenance hemodialysis for kidney failure (305). Frequently, they have protein-energy malnutrition, and show elevated serum markers of oxidative stress may act synergistically with other factors, such as inflammatory processes, to involving the entire body in a vicious self-sustaining process (306).

Respiratory failure and hypoxia

Most patients with sALS develop progressive respiratory muscle weakness and die of respiratory failure. Noninvasive ventilation significantly prolongs survival and is advised when forced vital capacity (FVC) falls below 50% (307). Some patients in whom sleep disorders develop also experience intermittent hypoxia or possibly chronic hypoxia, both of which are associated with induction of oxidative stress (308).

In the CNS, O₂ must cross the blood-brain barrier to enter the interstitial space and subsequently the intracellular space (309). Within the CNS, O₂ distribution is heterogeneous (310). This heterogeneity is the result of differences in vascularization, tissue diffusion, and cell-specific oxygen consumption and indicates that CNS cells have a surprisingly high degree of flexibility and tolerance for variation in O₂ concentration (311). Many studies in *in vitro* models of cerebral ischemia have shown that hypoxia induces oxidative stress in the brain (312). During hypoxia, mitochondria release superoxide anion (O₂⁻). The mechanism for this is not well understood but probably involves respiratory chain dysfunction and a decrease in ATP production that leads to greater intracellular Ca²⁺, NOS activation, and thus, •NO production with a possible enhancement of RNS production (313).

Psychological stress and distress

Although major depressive illness is relatively uncommon in patients with sALS (314), the patients who do experience hopelessness, depression, and mental stress have shorter survival (315). A positive mood is associated with a 6-month longer survival time (316). In diseases other than ALS, depression and anxiety disorders, as well as perceived stress levels, are associated with increased oxidative stress, lower telomerase activity, and shorter telomere

length (317). Trait anxiety and depressive symptoms in the absence of clinical disorders have been related to variations in gene expression and SNPs in healthy subjects (318). Stressor-induced alterations in proinflammatory cytokines (interleukin 1, TNF- α , interferon- γ) affect neuronal functioning through processes involving apoptosis, excitotoxicity, oxidative stress, and metabolic derangement (319). Overall, psychopathology and recent or current perceived stress may help to explain biological changes, including oxidative stress, that are associated with onset and perhaps progression of a wide range of medical conditions, including sALS. Although determining the temporal precedence of the association might be difficult, a longitudinal analysis of the temporal relationship between sALS progression and psychological stress may provide important clues to factors influencing the disease prognosis.

sALS AS A SYSTEMIC DISEASE

Although motor neurons are selectively affected in sALS, the possibility that sALS might be part of a systemic disease in which motor neurons are especially vulnerable has been postulated for some time (320, 321). A number of well-established abnormalities in sALS occur in the immune system, skin and skeletal muscle tissue, and lipid metabolism (28, 321–325). Concentrations of proinflammatory cytokines, such as MCP-1 (79, 326) and IL-6 (327), are elevated in sALS CSF and serum, as is serum TNF- α (328). More recently, Zhang et al (324) found significantly numbers of CD4 T lymphocytes in sALS compared to normal controls ($P < 0.05$) but no differences in CD8 T-cell numbers and the CD4/CD8 ratio. The same group found significantly higher levels of plasma lipopolysaccharide and greater monocyte activation (directly related to level of lipopolysaccharide) in sALS compared to healthy individuals ($P < 0.05$) (323).

Abnormalities reported in lipid metabolism in patients with sALS raise highly intriguing questions concerning disease mechanisms and prognosis. In a cohort of 369 patients with ALS and 286 healthy controls, Dupuis et al (329) first reported that patients with ALS had high cholesterol levels, particularly a high LDL/HDL ratio compared to the controls. Within the ALS group, patients with a high LDL/HDL ratio (defined on the basis of the LDL/HDL ratio mean value) had a substantially longer (greater by ~12 months) median survival than those with a lower ratio. Subsequent studies regarding the role of lipid metabolism in ALS provided conflicting results. No independent association between lipid levels and survival was found in 658 Italian patients ALS as compared to 658 healthy controls, but patients with respiratory impairment had a significantly lower blood lipid concentration, particularly the LDL/HDL ratio (330). In contrast, in another study investigating 488 ALS patients, a triglyceride concentration above the median of 1.47 mmol/L was associated with a 14-month longer survival (median survival, 45 months, $P = 0.006$), compared to patients with a serum triglyceride level < 1.47 mmol/L (median survival 31 months). In addition, patients with hypercholesterolemia (serum cholesterol > 5.23 mmol/L) had a prolongation in life expectancy of 11 months (median survival, 42 months, $P = 0.047$), compared to patients with normal serum cholesterol (< 5.23 mmol/L, median survival = 31 months) (331). Two more recent studies failed to find that hyperlipidemia was significantly associated with survival in sALS patients (332, 333). Sutedja et al (334) conducted a case-control study of more than 300 ALS patients recruited at a tertiary referral clinic in the Netherlands and 500 controls recruited from participants enrolled previously in two prospective studies in the same area (335, 336). Compared to controls, patients with ALS used cholesterol-lowering agents less frequently (OR = 0.6, $P = 0.008$), had a lower BMI (OR = 0.9, $P = 0.001$), a lower LDL/HDL ratio (women: OR=0.5, $P < 0.001$; men: OR = 0.4, $P < 0.001$), and a lower homocysteine concentration (women: OR=0.9, $P = 0.02$; men: OR = 0.9, $P < 0.001$). The mean LDL and total cholesterol concentrations were significantly lower among patients with a lower percent predicted FVC. In univariate analysis, a higher LDL/HDL ratio correlated

with increased survival (OR = 0.9, $P = 0.04$), but this association disappeared in the multivariate analysis, after adjusting for age, site at onset, and FVC. Because results vary depending on where the study was performed, geographic (such as diverse diet habits) or genetic variability might be present. Further studies are clearly needed.

Skeletal muscle mitochondria generate a large amount of oxidants and thus may affect oxidative stress in sALS (28, 321). A number of studies suggest skeletal myocyte mitochondria are abnormal in ALS (337–341).

Various dermatologic abnormalities in patients with ALS have been reported, including a decrease in the amount and diameter of collagen fibrils, a marked increase in amorphous material in the ground substance, increased collagen solubility, changes in collagen cross-linking, decreased type IV collagen, increased hyaluronic acid, and increased expression of neurotrophic factors such as ciliary neurotrophic factor, insulin-like growth factor, vascular endothelial growth factor, TDP-43, ubiquitin, and recently, the accumulation of valosin-containing protein, which is involved in the proteasome degradation pathway (325, 342–346). Although the mechanisms of these cutaneous changes in ALS remain unknown, they all suggest systemic abnormalities are present outside of the CNS. Skin fibroblasts derived from sALS patients were more sensitive to oxidative stress induced by H_2O_2 and 3-morpholiniosydnonimine hydrochloride than were fibroblasts from patients with fALS and the SOD1 mutation (347). We clearly need further studies to determine whether other cells, in addition to motor neurons, can be useful in studying the sALS disease process.

Other evidence suggesting that sALS is a systemic disease include ultrastructural abnormalities in hepatocyte mitochondria (348) and reduced GSH and GSH peroxidase activity in erythrocytes (89). Although oxidative stress biomarkers are most likely generated in the CNS because of its high oxygen consumption, some are undoubtedly produced outside of the CNS (42, 349). Particularly, lipid peroxidase products such as acrolein, HNE, and the IsoPs are highly biologically active, causing further cellular damage via apoptosis or nucleophilic action (58). These neurotoxic lipid peroxidase products can cause cellular damage far from their site of production because they can diffuse throughout the cell. In primary motor neuron cultures, ApoE protein isoforms differentially detoxified HNE, with ApoE-2 detoxifying more HNE than ApoE-4 (350). This suggests that lipid metabolism may be strongly associated with pro- and antioxidative processes. Following the oxidative stress profile throughout the disease course could give us much information about the adaptive changes of the individual and their ability to respond to oxidative stress. Oxidative stress biomarkers are not just an end product of oxidative stress but are bioactive and potentially neurotoxic themselves. Further studies are needed in this area.

WHAT IS THE NEXT STEP?

Although the evidence for oxidative damage in sALS pathogenesis is extensive, the ultimate trigger(s) that causes increased ROS levels is still unknown, leading to speculation as to whether oxidative stress is a primary cause of disease or merely a secondary consequence. Furthermore, it is always possible that there may be unrecognized alternative mechanisms that can, in part, explain the pathogenic changes described above. Prospective studies have shown that smoking, a cause of oxidative stress, is associated with sALS, whereas the evidence for other factors is, at best, equivocal. Smoking is relatively common in the general population, but other factors, such as agricultural or heavy metal exposure, are less common; thus, statistically significant relationships are more difficult to find. The results may depend heavily on the populations and locations studied. Many epidemiological studies have failed to demonstrate strong relationships, in part due to methodological shortcomings (104, 351, 352). Yet, the reality also is that it is difficult to find strong associations between rare events

and a rare multifactorial disease such as sALS. Although a prospective epidemiological study to search for a positive causative factor in sALS is desirable, such a study would involve following a large population for an extended period of time. Such studies are expensive and not feasible.

We may be able to take an alternative approach to identifying common factors in patients that cause oxidative stress. Low levels of oxidative stress induced by various environmental or lifestyle factors may converge to produce a cumulative, measurable degree of oxidative stress, as discussed above. If sALS is indeed a multifactorial disease, oxidative stress may be an underlying biological basis for multiple, apparently diverse unrelated stressors. Oxidative stress can further accumulate throughout the disease course because of malnutrition, respiratory failure, and psychological stress. Moreover, if sALS is truly a systemic disease, oxidative stress not only affects the patient systemically but also affects motoneuron integrity. Lipid peroxidase products, such as HNE or IsoPs, which are highly neurotoxic, may be generated centrally and peripherally due to oxidative stress. However, they can affect the vulnerable motor neuron in the CNS because those lipid peroxidase products can damage the cerebral microvascular endothelial cells of the blood brain barrier, leading to their breakdown (57). Almost certainly, the real picture is far more complex than what we describe here, because individual genetic susceptibility and epigenetic mechanisms, which further modify gene-environment interactions, are likely to influence disease onset, disease progression, and even disease phenotypes. Furthermore, such studies are in their infancy. Nevertheless, we believe that a molecular epidemiological study in sALS, focusing on one biological process – namely, oxidative stress – may be an alternative and perhaps a more effective approach to identify potential factors in sALS.

Answering these simple but fundamental questions requires well-planned, well-designed epidemiological studies that recruit patients from multiple centers to achieve adequate sample sizes. Moreover, a multidisciplinary approach is essential, because both molecular and environmental risk factors are involved. Clinical investigators who define the disease, epidemiologists from different disciplines (general, nutritional, occupational, genetic epidemiology), laboratory scientists who are experts in oxidative stress biology, molecular geneticists, experts in epigenetics, clinical psychologists, and biostatisticians must participate in such studies, because their combined expertise is essential to uncover the etiology of a complex disease like sALS. In addition, the appropriate collection and processing of biospecimens is critical for success.

We believe that this approach will provide solid biological explanations for disease onset, phenotypic changes, and disease severity in sALS. If it is determined that oxidative stress is a key factor in the pathomechanism(s) of sALS, we will need to devise innovative clinical trials to successfully investigate oxidative stress and develop more effective treatments and prevention strategies. Although several past clinical trials that were intended to counteract presumed oxidative stress failed to demonstrate any clinical benefits in ALS (353–357), these failures do not mean that the hypothesis of oxidative stress is wrong. These medications with anti-oxidative properties have limited ability to cross the blood brain barrier, and further, oxidative stress may be involved in different biological steps of cellular metabolism. With a more thorough understanding of these mechanisms of cellular oxidative stress, the treatment and management of sALS will be biologically based, and proactive and appropriate treatments can be developed that slow or even stop progression of this devastating neurodegenerative disease.

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Abbreviations

ALAD	δ -aminolevulinic acid dehydratase
ALS	Amyotrophic Lateral Sclerosis
fALS	familial Amyotrophic Lateral Sclerosis
sALS	sporadic Amyotrophic Lateral Sclerosis
ANG	angiogenin
ApoE	apolipoprotein E
CNS	central nervous system
CSF	cerebrospinal fluid
CTE	chronic traumatic encephalopathy
ER	endoplasmic reticulum
FVC	forced vital capacity
IsoPs	F2-isoprostanes
FTD	frontotemporal dementia
GSH	glutathione
HDL	high-density lipoprotein
HNE	4-hydroxy-2-nonenal
IsoPs	isoprostanes
LDL	low-density lipoprotein
MDA	malondialdehyde
MND	motor neuron disease
MT	metallothionein
NADPH	nicotinamide adenine dinucleotide phosphate
NOS	nitric oxide synthetase
3-NT	3-nitrotyrosine
8-oxoG	8-oxo-deoxyguanosine
OXR1	oxidation resistance 1
PD	Parkinson disease
PON	paraoxonase 1
RNS	reactive nitrogen species

ROS	reactive oxygen species
sALS	sporadic ALS
SMN	survival motor neuron
SNP	single nucleotide polymorphism
SOD1	superoxide dismutase 1
TBARS	thiobarbituric acid-reactive substances
TDP-43	TAR DNA-binding protein
VAPB	vesicle associated protein-associated protein B
VCP	valosin-containing protein
VEGF	vascular endothelial growth factor

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Highlights

- Oxidative stress is a key pathogenic mechanism of motoneuronal degeneration in ALS.
- Oxidative stress biomarkers are increased in CSF, plasma and urine in ALS.
- Many environmental risk factors in ALS can cause systemic oxidative stress.
- Malnutrition and respiratory and psychological stress may cause oxidative stress.
- Increased systemic oxidative stress may quicken ALS disease progression.

Table 1

Oxidative stress (OS) biomarker in ALS (other than described in the text)

Study	ALS cases (n)	Controls	Results
Tohgi et al. 1999(81)	19 sALS	19 HC	↑CSF 3-NT and 3-NT/T ratio in sALS
Tohgi et al, 1999 (82)	18 sALS	14 HC	↑NO3 in sALS, ↑GSSG/GSH ratio in sALS
Bonnefont-Rousselot et al, 2000 (83)	167	62 HC	↑TBARS in ALS, ↑ SOD1 activity in ALS, no other significant differences
Ryberg et al, 2004 (84)	14	19 HC, 17 AD	No increasing in ALS
Sohmiya et al, 2005 (85)	20 sALS	20 HC	↑ oxidized form of coQ10 in sALS and correlation with disease duration, no others significant differences.
Yoshino et al, 2006 ^a (74)	20	/	↓ CSF 3NT levels at the end of antioxidant therapy
Siciliano et al, 2007 (86)	44 sALS, 4 fALS	8 OND	↓ FRA in ALS CSF ↑ AOPP in CSF and plasma ALS
Murata et al, 2008 (87)	30 sALS	17 HC	↑ percentage of oxidized CoQ10 in CSF sALS, correlation oxidized CoQ10 and duration of sALS, not correlation with the ALS score
Keizman et al, 2009 (88)	86	86 HC	↓ ALS uric acid level; correlation serum uric acid levels and rate of progression ALSFRS-R
Cova et al, 2010 (89)	88 sALS	50 HC	↓ all assayed enzymes and correlation with progression rate in sALS, plasma ROS significantly lower in sALS
Mendonça et al, 2011 (90)	10	6 HC	No increased levels in ALS

AD = Alzheimer disease, sALS = sporadic amyotrophic lateral sclerosis, fALS = familial amyotrophic lateral sclerosis, ALSFRS-R = Amyotrophic Lateral Sclerosis Functional Rating Scale- revised, AOPP = advanced oxidation protein products, CAT = catalase, CoQ10 = coenzyme Q10, CSF = cerebrospinal fluid, FRA = ferric reducing ability, GPX = glutathione peroxidase, GR = glutathione reductase, GSH = glutathione, GSSG = Glutathione disulfide, GST = glutathione transferase, G6PD = glucose-6-phosphate dehydrogenase, HC = healthy controls, HNE = 4-hydroxy-2,3-nonenal, F2-IsoPs = F2-isoprostanes, ND = neurodegenerative diseases, NND = non-neurodegenerative disease, ROS = reactive oxygen species, SOD = superoxide dismutase, T = tyrosine, TAS = serum total antioxidant status, TBARS = thiobarbituric acid-reactive substances, 3-NT = 3-Nitrotyrosine, 8-oxodG = 8-oxo-deoxyguanosine

^aPhase II clinical trial: 20 subjects with ALS received either 30 mg (5 subjects) or 60 mg (15 subjects) of edaravone (free radical scavenger) via intravenous drip once per day. Two weeks of administration was followed by a two-week observation period, for six times. In almost all patients in the 60 mg group, the level of CSF 3NT, was reduced at the end of the sixth cycle of administration.

Table 2**Environmental and Lifestyle Factors: Relationship to ALS and Mechanisms of OS**

Factor	Strength of the Association	Mechanisms of Oxidative Stress Toxicity
Agricultural chemicals	Uncertain (91–99, 104, 193)	Apoptosis and ROS-induced DNA damage (114) <i>PON1</i> genotype (263); protein carbonyl production (116) mitochondrial damage (122)
Lead, other heavy metals	Uncertain (92, 94, 104, 127, 128, 130, 353)	↓ cellular GSH (133); ↓ copper homeostasis (134); combined toxicity with other metals (136, 137)
Military service	Uncertain (144–146, 148–151, 153)	Head trauma and ApoE4 genotype (154)
Trauma	Uncertain (101, 154–164, 169)	Possibly via free radical production (ROS) (172)
Strenuous physical activity	Uncertain (189)	↓ GSH and ↑ apoptosis (186); ↑ free radicals (187, 354)
Professional football	Suspected (165–168)	Physical activity per se may not be the cause (166, 190)
Diet (high in fat, glutamate)	Uncertain (193–195)	↑ OS, inflammation, and NF-κB activation in the cerebral cortex (304, 306)
Smoking	Suspected (154, 156, 200–209)	Protein and DNA damage, cell death (213), ↑ heavy metal levels (216)
Malnutrition/Cachexia	Clinically observed (301, 303)	↑ OS (300)
Hypoxia	Clinically observed (307)	ROS production via mitochondrial dysfunction (308); ↑ DNA, lipid, protein oxidation (312, 313)
Psychological stress	Clinically observed (315, 316)	OS and shorten telomere length (317); induction of cytokines, apoptosis, and OS (319)

ApoE = Apolipoprotein E, GSH = glutathione, OS = oxidative stress, PON1 = Paraoxonase 1, ROS = reactive oxygen species.

Table 3

Evidence-based Reviews of Smoking and ALS^a

Author year of publication	Study Population	Study Design	Number of Subjects (case/controls)	Case ascertainment	Adjustment variables	OR (95% CI)
Kamel et al, 1999 (202)	Two-centers study	Case-control	110/256	ALS Clinic	age, gender, education, region	1.70 (1.00 – 2.80)
Nelson et al, 2000 (201)	Population based ^b	Case-control	161/321	Multiple case ascertainment sources	age, gender, education, alcohol	2.00 (1.30 – 3.20)
Weiskopf et al, 2004 (200)	Mortality surveillance ^c	Cohort	291/638,849 (women)	National Death Index	age, alcohol, education	1.67 (1.24 – 2.24)
Weiskopf et al, 2004 (200)	Mortality surveillance ^c	Cohort	330/459,360 (men)	National Death Index	age, alcohol, education	0.69 (0.49 – 0.99)
Fang et al, 2006 (208)	Construction workers ^d	Cohort	160/280,558	Registry	age, area of residence	0.80 (0.60 – 1.10)
Sutedja et al, 2007 (204)	Single center study	Case-control	364/392	ALS clinic	education, occupation, age	1.6 (1.00 – 2.50)
Gallo et al, 2009 (203)	Multicenter ^e	Cohort	118/517,890	Death certificates	age, gender, education, center of recruitment	1.89 (1.14 – 3.14)
Okamoto et al, 2009 (207)	Multicenter	Case-control	153/306	ALS clinic	age, gender	1.00 (0.60 – 1.30)
Schmidt et al, 2010 (154)	Military veterans	Case-control	241/597	Registry	age, gender, race, education	0.90 (0.56 – 1.46)

Author year of publication	Study Population	Study Design	Number of Subjects (case/controls)	Case ascertainment	Adjustment variables	OR (95% CI)
Alonso et al, 2010 (206)	Cohort ^f	Case- control	1143/11,371	Death certificates	age at diagnosis, gender, diagnostic classification n	1.04 (0.80 – 1.34)
Wang et al, 2011 (205)	Cohort ^g	Case- control	832/1,119,080	Death certificates, registries	age, gender, physical activity, education, body mass index	1.42 (1.07 – 1.88)
de Jong et al, 2012 (209)	Population based incidence cohort	Case- control	494/1,599	Multiple sources (ALS clinics, rehabilitation centers, patient support associations)	age, gender, education	1.38 (1.02 – 1.88)

^aStudies of more than 100 ALS patients unless they are population-based

^bThree counties of western Washington state (King, Pierce, and Snohomish) in the US

^cParticipants were recruited by American Cancer Society in 50 States; families with at least one member over the age of 45 and other family members over the age of 30 were invited to participate (Cancer Prevention Study II cohort). Vital status of the study participants has been determined by automated linkage with the National Death Index through December 31, 1998.

^dSwedish Construction Workers Cohort

^eSubjects (aged 35–70) recruited from the general population residing in a given geographical area from 1991 to 2001, in 23 centers across 10 European countries (European Prospective Investigation into Cancer and Nutrition cohort)

^fGeneral Practice Research Database, a computerized clinical database in the United Kingdom

^gStudy population comprised of participants in the following: the Nurses' Health Study, the Health Professionals Follow-up Study, the Cancer Prevention Study -II Nutrition Cohort, the Multiethnic Cohort, and the National Institutes of Health American Association of Retired Persons Diet and Health Study (NIH-AARP)