

# Increased lipid peroxidation in sera of ALS patients

## A potential biomarker of disease burden

E.P. Simpson, MD; Y.K. Henry, BS; J.S. Henkel, PhD; R.G. Smith, MD, PhD; and S.H. Appel, MD

**Abstract—Background:** Markers of oxidative stress and immune activation are significantly elevated in postmortem ALS CNS tissue, although the relevance to pathogenesis is unclear. **Objective:** To determine the degree and distribution of oxidative stress and immune activation in living ALS patients and whether these levels correlate with the rate of progression or extent of disease. **Method:** Serum and CSF samples from sporadic ALS (sALS) patients were assayed for 4-hydroxy-2,3-nonenal (HNE), a lipid peroxidation product, and monocyte chemoattractant protein-1 $\alpha$  (MCP-1 $\alpha$ ), a  $\beta$ -chemokine, by high-performance liquid chromatography and ELISA and compared with levels measured in disease and normal control subjects by one-way analysis of variance. SALS serum levels were analyzed in relation to rate of progression, stage of disease, and drug therapy. **Results:** HNE levels were significantly elevated in the sera and spinal fluid of sALS patients compared with control populations and positively correlated with extent of disease but not rate of progression. MCP-1 $\alpha$  levels were also elevated in the sera of sALS patients, with the exception of the neurodegenerative disease control subjects, but decreased with advancing disease. CSF MCP-1 $\alpha$  levels were not different between the sampled populations. There was no correlation between serum HNE and MCP-1 $\alpha$  levels in sALS patients and extent of disease. However, an inverse relationship between HNE and MCP-1 $\alpha$  was demonstrable in vitro. Low levels of HNE stimulated release of MCP-1 $\alpha$  from cultured human macrophages, whereas high levels inhibited release of MCP-1 $\alpha$ . **Conclusions:** These data confirm the presence of increased oxidative stress and immune activation in ALS patients. HNE is also suggested as a possible biomarker of disease.

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Oxidative stress and immune/inflammation have been considered as relevant mechanisms in the pathogenesis of ALS, as markers of both are increased in the CNS tissue, spinal fluid, and serum of ALS patients.<sup>1–6</sup> However, it is unclear what initiates or amplifies the pathways involved and to what extent these pathways are truly relevant to neurodegeneration in ALS.

Lipid peroxidation is one of several outcomes of oxidative stress, and as the CNS is enriched with polyunsaturated fatty acids, it is particularly vulnerable to such stress. One of the most toxic products of lipid peroxidation is 4-hydroxy-2,3-nonenal (HNE), an unsaturated aldehyde that can modify proteins by Michael addition to form covalent adducts with cysteine, lysine, or histidine.<sup>7</sup> This initiates conformational and structural changes, resulting in neuronal death.<sup>8,9</sup> Specific mechanisms by which neuronal death occurs are likely related to increased sphingolipid synthesis,<sup>10</sup> impairment of glutamate and glucose transport<sup>11</sup> and function of ion-motive ATPases,<sup>12</sup> cytoskeletal alterations,<sup>13</sup> inhibition of mitochondrial respiration,<sup>14</sup> and increases in intra-

cellular calcium.<sup>9</sup> In ALS, we have previously shown that HNE-modified proteins are increased in the spinal cord and ventral horn motor neurons of ALS patients compared with control subjects,<sup>1</sup> and levels are also increased in ALS spinal fluid, thus linking oxidative damage in the form of lipid peroxidation and motor neuron degeneration.<sup>15</sup> The source of the oxidative stress in ALS and other neurodegenerative disease is unknown but may derive from several processes including excitotoxicity,<sup>16</sup> mitochondrial dysfunction,<sup>17</sup> and iron accumulation.<sup>18</sup> Immune activation is also a significant source of oxidative stress in the form of released oxygen radicals and nitric oxide from immune cells such as microglia and macrophages<sup>19,20</sup> and may have a direct role in generating significant oxidative stress in neurodegenerative diseases such as in ALS in which an immune/inflammatory response is present.<sup>21–23</sup> Conversely, the presence of oxidatively modified proteins can result in the activation of the immune system directed toward the clearing away of these proteins,<sup>24</sup> thus implicating a cycle of injury and inflammation that may be critical to neuronal degeneration. We therefore

From the Department of Neurology (Drs. Simpson, Henkel, Smith, and Appel, Y.K. Henry), Baylor College of Medicine, Houston, and Department of Neurology (Dr. Smith), University of Texas Medical Branch, Galveston, TX.

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Address correspondence and reprint requests to Dr. S.H. Appel, Department of Neurology, NB 302, 6501 Fannin, Houston, TX 77030; e-mail: sappel@bcm.tmc.edu

**Table 1** Patient demographics

Data	Sporadic ALS	Familial ALS	Neurodegenerative disease	Nonneurodegenerative disease	Normals
No., serum/CSF					
HNE	75/65	10/4	19/19	14/14	16
MCP-1 $\alpha$	65/30	14/1	8/9	12/16	22
Gender					
HNE	24F	3F	11F	6F	8F
MCP-1 $\alpha$	17F	6F	5F	7F	16F
Age, y	51.04 $\pm$ 13.7	43.97 $\pm$ 15.03	57.3 $\pm$ 13.3	48.76 $\pm$ 10.32	49.52 $\pm$ 11.3
% bulbar onset	20	22			
% patients taking immunomodulatory therapy	38	22			
Rate of progression, points/mo	3.42	5.58			

HNE = 4-hydroxy-2,3-nonrenal; MCP-1 $\alpha$  = monocyte chemoattractant protein-1 $\alpha$ .

sought to investigate whether indexes of increased oxidative stress and immune activation were present in ALS patients and if they correlated with disease stage, progression, and drug therapy.

**Patients and methods.** *Patients and controls.* Inclusion criteria for 122 ALS subjects (108 sporadic [sALS] and 14 familial [fALS]) included a diagnosis of definite or probable ALS according to El Escorial criteria of the World Federation of Neurology. Functional disability and rate of progression in ALS patients were measured by the Appel Scoring System.<sup>25</sup> The Appel score (AS) is the sum of five groups of functional scores: bulbar function, respiration, muscle strength, upper extremity functions, and lower extremity functions. The normal score for each functional group is 6, making 30 the AS for a completely normal person. As a patient's condition worsens, the score increases to a final value of 164. In this study, clinical disease is characterized as early (AS: 30 to 49), mid (AS: 50 to 119), and late (AS: 120 to 155) based on this scoring system. Patients in the early stage (AS: 30 to 49) are able to maintain their activities of daily living (ADL) without assistance. Patients in midstage of disease (AS: 50 to 119) require varying degrees of assistance within or outside the home but are still able to function in one or several areas of their lives, that is, job, home, travel, etc. Late-stage ALS patients are fully dependent or require assistance for all ADL.

The patient populations were evaluated at the Department of Neurology at Baylor College of Medicine (Houston, TX) from 1990 to present. Sera was collected and stored from patients followed from 1990 to 2001. CSF collected at initial visit was available from archived storage from 99 (88%) of all patients entered into the study. Normal control (NC) subjects consisted of nonrelated caregivers or spouses. All subjects were included in the study after informed consent.

There were 108 sALS patients in total. The different measurements were done in different patients at different time points. In summary, 75 patients had serum HNE and 65 had CSF HNE (63 had both) measurements, whereas 65 had serum monocyte chemoattractant protein-1 $\alpha$  (MCP-1 $\alpha$ ) and 30 had CSF MCP-1 $\alpha$  (23 had both) measurements (table 1).

For patients whose sera were analyzed at multiple time points, the average rate of progression was 3.4  $\pm$  2.7 points/month and the average duration of disease was 58.4 months. Thirty-two percent of sALS patients (n = 34) had multiple samples for MCP-1 $\alpha$  measurement (time points: 3.6  $\pm$  1.5; time intervals: 8.22  $\pm$  7.1 months). Thirty-nine percent of sALS patients (n = 42) had multiple samples for HNE measurement (time points: 4.1  $\pm$  1.8; time interval: 7.7  $\pm$  5.6 months). Thirty-four of 41 sALS patients whose samples were assayed for both HNE and MCP-1 $\alpha$  had samples

collected at multiple time points (time points: 3.2  $\pm$  1.1; time intervals: 7.7  $\pm$  6.51 months).

Thirty-eight percent of sALS patients were receiving immunomodulatory therapy during one or more time points used in this analysis. For the purpose of our study, immunomodulatory therapy is defined as treatments that have selective or general effects on the activation or suppression of immune activation and associated pathways. In our patient population, these include corticosteroids, IV immunoglobulin, plasma exchange, cyclophosphamide, cyclosporine, hydroxychloroquine,<sup>26</sup> selegiline,<sup>27</sup> and allopurinol.<sup>28,29</sup>

There were 22 NC patients, 6 men and 16 women (age 49.52  $\pm$  11.3); 14 fALS patients, 8 men and 6 women (age 43.97  $\pm$  15.03); 19 neurodegenerative disease (ND) patients, 9 men and 11 women (age 57.3  $\pm$  13.3); and 14 nonneurodegenerative disease (NND) control patients, 8 men and 6 women (age 48.76  $\pm$  10.32) (see table 1). ND control subjects included patients diagnosed with Parkinson disease, Alzheimer disease and frontal lobe dementias, and Parkinson-plus disorders. NND control subjects included patients with acquired peripheral neuropathies, acquired cerebral ischemic disease, myasthenia gravis, multiple sclerosis, cervical myelopathy, and acquired seizure disorders.

*Measurement of HNE.* *Sample preparation.* Serum samples and standards were extracted using mammalian protein extraction reagent buffer (Pierce Chemical, Rockford, IL). The supernatant and pellet were separated after centrifugation. Cyclohexanedione derivatization was done on both the supernatant and the methanol-suspended pellet, and the combined value was reported for the total HNE.<sup>30</sup>

*Chromatography.* Samples were injected (50  $\mu$ L) via a Rheodyne valve (Rohnert Park, CA) using a manual injection technique. The analytical column was Symmetry C<sub>18</sub> (3.9  $\times$  150-mm column; Waters Corp., Milford, MA). The samples were then analyzed on a Waters 625 high-performance liquid chromatograph using a Hewlett-Packard Chemical Station (Palo Alto, CA). A 1046A programmable fluorescence detector was used at an excitation wavelength of 380 nm and emission of 446 nm. Standards of HNE were derivatized, and the analysis of pure standards was used to generate a standard curve and verify peak identify. Sample concentrations were calculated by comparison of peak heights with those obtained by standards after subtraction of appropriate blanks.

*Measurement of cytokines and chemokines.* Serum and CSF samples were stored at -80  $^{\circ}$ C until assayed. Duration of storage had no effect on assay results. Assays were performed on sera collected from ALS patients at multiple time points throughout disease course and from ND, NND, and NC patients collected at a single time point. Serum concentrations of MCP-1 $\alpha$ , MIP-1 $\alpha$ , tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), interleukin-6 (IL-6), interferon- $\gamma$  (INF $\gamma$ ) inducible protein (IP-10), monokine induced by INF $\gamma$

**Table 2** Cytokines and chemokine serum levels in studied populations

Serum levels	ALS	DC	NC
<b>Cytokines*</b>			
IFN $\gamma$ (OpiEIA)	43.99 $\pm$ 138.1	5.90020 $\pm$ 8.760086	16.55 $\pm$ 23.12
IL-6 (OpiEIA)	10.16 $\pm$ 8.77	7.34 $\pm$ 7.82	13.02 $\pm$ 22.1
GMCSF (OpiEIA)	0.147 $\pm$ 0.0285	0.0005 $\pm$ 0.0036	-0.0405 $\pm$ 0.01711
TNF $\alpha$ (Duo Set)	0.067 $\pm$ 0.0402	0.039 $\pm$ 0.014	0.05 $\pm$ 0.018
<b>Chemokines*</b>			
GRO- $\alpha$ (DuoSet)	43.41 $\pm$ 65.6	74.20 $\pm$ 103.93	25.13 $\pm$ 22.7
IP-10 (OpiEIA)	115.7 $\pm$ 64.3	117.54 $\pm$ 68.8	139.6 $\pm$ 60.1
MIG (OpiEIA)	660 $\pm$ 204.4	925 $\pm$ 263.3	591.3 $\pm$ 200.2
MIP-1 $\alpha$ (DuoSet)	0.081 $\pm$ 0.10	0.09 $\pm$ 0.06	0.06 $\pm$ 0.07

\* No difference between ALS and control populations.

DC = neurologic disease controls (familial ALS, neurodegenerative disease, nonneurodegenerative disease patients); NC = normal control patients; IFN $\gamma$  = interferon- $\gamma$ ; IL-6 = interleukin-6; GMCSF = granulocyte/macrophage colony-stimulating factor; TNF $\alpha$  = tumor necrosis factor- $\alpha$ ; GRO- $\alpha$  = growth-related oncogene- $\alpha$ ; IP-10 = IFN $\alpha$  inducible protein; MIG = monokine induced by IFN $\alpha$ ; MIP-1 $\alpha$  = macrophage inflammatory protein.

(MIG), growth-related oncogene- $\alpha$  (GRO- $\alpha$ ), granulocyte/macrophage colony-stimulating factor (GMCSF), and INF $\gamma$  were assayed by ELISA according to the manufacturer's protocol (table 2). CSF concentrations of MCP-1 $\alpha$  were also assayed by ELISA according to the manufacturer's protocol. For analysis of HNE's effect on monocytes in culture, concentrations of MCP-1 $\alpha$  in the media were measured by ELISA. Cell number was used for standardization of MCP-1 $\alpha$  levels.

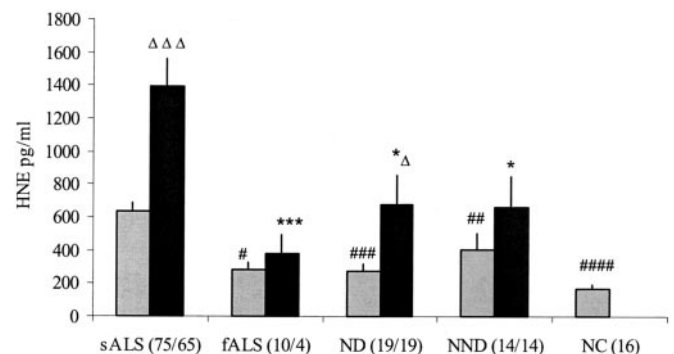
**Treatment of cultured human macrophages with HNE.** Human peripheral blood monocytes, isolated by the Ficoll centrifugation and adherence method,<sup>31</sup> were grown in RPMI media supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 10 ng/mL of GMCSF at 37 °C in a humidified incubator with 5% CO<sub>2</sub>. Cells were exposed to 0.001, 0.1, and 10 pM, 1.0 and 100 nM, and 1 and 10  $\mu$ M HNE (Cayman Chemical, Ann Arbor, MI) or phosphate-buffered saline in the same medium without serum supplementation, and the aldehyde concentrations were maintained at constant levels by supplying HNE every 10 minutes for 1 hour of exposure. Supernatants were then collected and stored at -80 °C until assayed for MCP-1 concentration.

**Statistical analysis.** Statistical comparisons were made by one-way analysis of variance (ANOVA; OriginPro; OriginLab Corp., Northampton, MA) with post-hoc analysis with two-tailed Student *t*-test (Microsoft XP Excel software, Redmond, WA). Results were expressed as means  $\pm$  SEM. Linear regression analyses (OriginPro) were performed to evaluate correlation of HNE and MCP-1 $\alpha$  with stage of disease and rate of progression as measured by the AS. Effects of immunomodulatory therapy upon HNE and MCP-1 $\alpha$  levels were compared by two-tailed Student *t*-test. Significance is set at *p* < 0.05 for all statistical analyses. Sample values that were 3 SD from the mean were excluded from analysis.

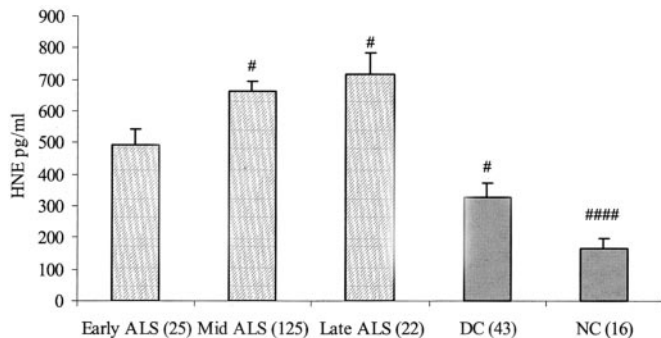
**Results. Serum HNE.** To determine whether indexes of oxidative stress are widespread in ALS patients, serum HNE levels were measured from samples collected at first ALS clinical visit from all patients (sporadic and familial) who had received a diagnosis of probable or definite ALS at a prior screening evaluation. Clinic samples were also available from ND and NND control patients and random samples from NC subjects. The population means were different for serum HNE levels (*p* = 0.0000009) and CSF HNE levels (*p* = 0.0091) by one-way ANOVA. Two-tailed Student *t*-tests showed that sALS HNE serum and CSF levels were elevated above all control values, including those of fALS patients (figure 1). Serum and CSF HNE

levels were not statistically different between fALS, ND, and NND patient samples. Serum HNE levels from fALS and NND patients were elevated above NC levels (*p* < 0.05). Within sampled populations, CSF HNE levels were elevated above serum levels in sALS patients (*p* = 0.00005) and ND control subjects (*p* = 0.03), although CSF HNE levels did not correlate with serum HNE levels in these populations (data not shown). Averaged HNE levels were unchanged for patients receiving immunomodulatory therapy (690.8  $\pm$  64 pg/mL; *n* = 23) or not (613.2  $\pm$  66.4 pg/mL; *n* = 52) at time of baseline clinic visit (*p* = 0.89).

HNE levels were grouped according to clinical categories of disability (early: independent ADL; mid: some assistance with ADL; late: full assistance with ADL). In our



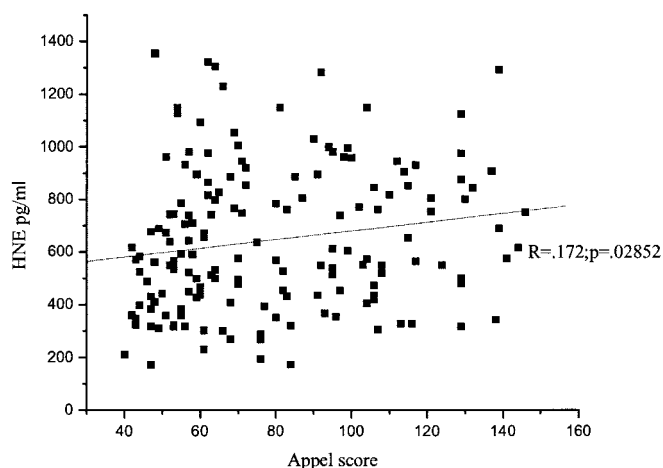
**Figure 1.** Average 4-hydroxy-2,3-nonenal (HNE) levels in serum (gray columns) and CSF (black columns) of patients and normal control subjects (NC) measured at baseline clinical visit and random sampling. Serum HNE levels from sporadic ALS (sALS) patients vs controls, Student *t*-test: #*p* < 0.05, ##*p* < 0.001, ###*p* < 0.0001, ####*p* < 0.00001; CSF HNE levels from sALS patients vs controls, Student *t*-test: \**p* < 0.05, \*\*\**p* < 0.0001; serum HNE vs CSF HNE levels within sALS and neurodegenerative control (ND) populations, Student *t*-test:  $\Delta$ *p* < 0.05,  $\Delta\Delta$ *p* < 0.0001. fALS = familial ALS; NND = nonneurodegenerative controls (patient number [*n*] for serum/CSF).



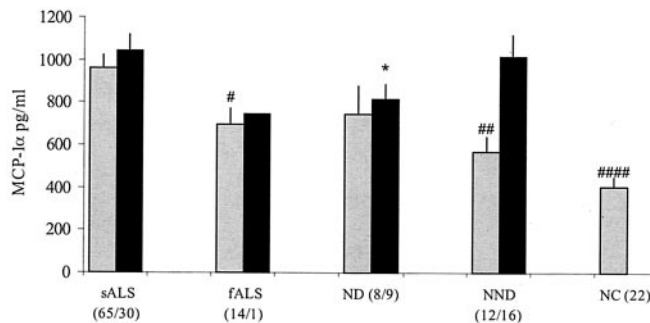
**Figure 2.** Average serum 4-hydroxy-2,3-nonenal (HNE) levels grouped according to clinical disability: early, mid, and late stages. Early stage vs mid, late stage sporadic ALS (sALS) populations ( $n = 42$ ), neurology disease controls (DC;  $n = 43$ ), and normal controls (NC;  $n = 16$ ), Student  $t$ -test: # $p < 0.05$ , #### $p < 0.00001$  (serum samples per population [ $n$ ]).

sALS patient population, HNE levels were elevated at all stages of disease compared with NC and all neurology disease control subjects (fALS, NND, ND) ( $p \leq 0.01$ ). The population means of serum sALS HNE levels at different clinical stages were statistically different by one-way ANOVA ( $p = 0.03$ ), with HNE levels measured at mid and late stages of disease significantly above those measured at early stage by two-tailed Student  $t$ -test (early vs mid stage,  $p = 0.006$ ; early vs late stage,  $p = 0.008$ ). There was no difference in levels measured at mid and late stages of disease in sALS patient sera (figure 2).

Serum HNE levels measured from 42 sALS patients collected at multiple time points throughout their disease courses positively correlated with advancing disease ( $R = 0.172$ ,  $p = 0.02852$ ) (figure 3); however, there was no relationship between change in HNE levels and rate of progression (points/month;  $r = 0.10488$ ,  $p = 0.242$ ). In regard to immunomodulatory therapy, 79% were receiving therapy at any one time during their disease course. Of these, 33% were receiving immunomodulatory therapy at all time points analyzed. There was no difference in averaged HNE



**Figure 3.** Serum 4-hydroxy-2,3-nonenal (HNE) levels measured at multiple time points positively correlate with progressive clinical disability (Appell score) in sporadic ALS patients ( $n = 42$ ; 162 samples).

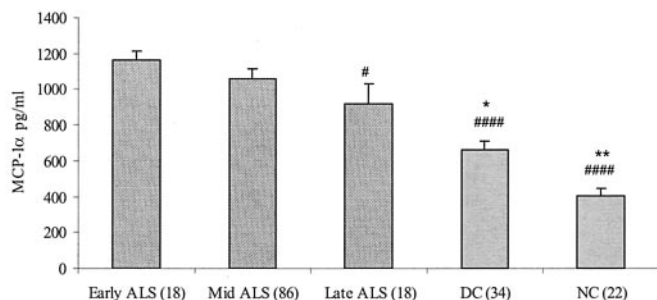


**Figure 4.** Average monocyte chemoattractant protein-1 $\alpha$  (MCP-1 $\alpha$ ) levels in serum (gray columns) and CSF (black columns) of patients and normal control subjects (NC) measured at baseline clinical visit and random sampling, respectively. Serum MCP-1 $\alpha$  levels from sporadic ALS (sALS) patients vs controls, Student  $t$ -test: # $p < 0.05$ , ## $p < 0.001$ , #### $p < 0.00001$ ; CSF MCP-1 $\alpha$  levels from sALS patients vs controls, Student  $t$ -test: \* $p < 0.05$ . fALS = familial ALS; ND = neurodegenerative controls; NND = nonneurodegenerative controls (patient number [ $n$ ] for serum/CSF).

levels measured in those patients maintained on immunomodulatory therapy ( $641.8 \pm 66.4$  pg/mL;  $n = 14$ ) compared with those who never received such therapy ( $663.1 \pm 52.8$  pg/mL;  $n = 12$ ) ( $p = 0.82$ ).

**Cytokines and chemokines.** To determine whether indexes of immune activation are widespread in ALS patients analogously to the HNE levels, serum levels of several cytokines and chemokines were measured from samples collected at first clinical visit from all ALS patients (familial and sporadic), ND and NND control subjects, and random samples from NC subjects. There were no statistical differences in serum concentrations of either the IL-6 or INF $\gamma$  cytokines between the ALS, neurologic disease control, and NC subjects (see table 2). TNF $\alpha$  and GMCSF levels were undetectable for most ALS patients and control subjects; however, one of six of the sALS and one of six of the fALS samples contained assayable levels of GMCSF. With respect to chemokines, IP-10 and GRO $\alpha$  detectable levels were not significantly different between the ALS, neurologic disease control, and NC patients (see table 2). MIP-1 $\alpha$  levels were below detectable limits in all samples assayed. MCP-1 $\alpha$  serum levels were statistically elevated in sALS patients above most control populations.

**MCP-1 $\alpha$ .** MCP-1 $\alpha$  levels in sera were analyzed from the sampled populations and found to be statistically different by one-way ANOVA ( $p < 0.0001$ ). CSF $\alpha$  MCP-1 $\alpha$  population means were not statistically different by one-way ANOVA ( $p = 0.25$ ) (figure 4). Post-hoc analysis using a two-paired Student  $t$ -test for serum MCP-1 $\alpha$  revealed that levels measured from sALS patients were elevated above those of fALS ( $p = 0.0097$ ), ND ( $p = 0.0012$ ), and NC ( $p < 0.00001$ ) subjects. Serum levels were not different between sALS patients and ND control subjects ( $p = 0.150$ ). Serum levels from fALS, ND, and NND control subjects were significantly elevated above those of NC subjects ( $p \leq 0.05$ ). Averaged MCP-1 $\alpha$  levels were unchanged for patients receiving immunomodulatory therapy ( $869.9 \pm 86.6$  pg/mL;  $n = 36$ ) or not ( $1,086.8 \pm 93.8$  pg/mL;  $n = 29$ )

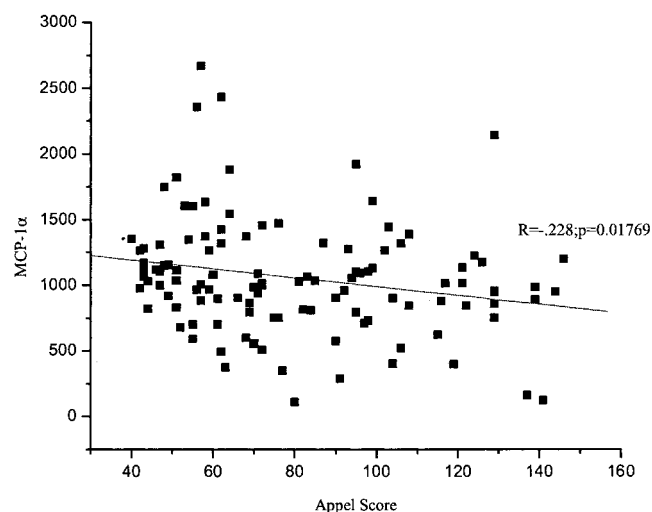


**Figure 5.** Average serum monocyte chemoattractant protein-1α (MCP-1α) levels grouped according to clinical disability: early, mid, and late stages. Early stage vs mid- and late-stage ALS populations ( $n = 34$ ), neurology disease controls (DC;  $n = 34$ ), and normal controls (NC;  $n = 22$ ), Student *t*-test: # $p < 0.05$ , #### $p < 0.00001$ ; late-stage ALS vs DC and NC, Student *t*-test: \* $p < 0.05$ , \*\* $p < 0.001$  (serum samples per population [ $n$ ]).

at time of baseline clinic visit, although there was a trend toward significance for lower levels in patients on immunomodulatory therapy ( $p = 0.09$ ).

Average MCP-1α levels were grouped according clinical categories of disability and found to be elevated above neurology disease (disease control: fALS, ND, NND) and NC subjects at all stages of disease ( $p < 0.0001$ ). The population means of serum sALS MCP-1α levels at different clinical stages were not statistically different by one-way ANOVA ( $p = 0.57$ ), but post-hoc analysis using two-tailed Student *t*-test showed a decline in MCP-1α levels measured at late stage compared with early stage of disease ( $p = 0.04$ ) (figure 5).

Serum MCP-1α levels measured from 34 sALS patients with samples collected at multiple time points throughout their disease courses negatively correlated with advancing disease ( $R = -0.228$ ,  $p = 0.01769$ ) (figure 6). There was no relationship between change in MCP-1α levels and rate of progression (points/month;  $r = 0.18364$ ,  $p = 0.194$ ). In regard to immunomodulatory therapy, 80% of these pa-



**Figure 6.** Serum monocyte chemoattractant protein-1α (MCP-1α) levels measured at multiple time points negatively correlate with progressive clinical disability (Appel score) in sporadic ALS patients ( $n = 34$ ; 133 samples).

tients were receiving therapy at any one time during their disease course. Of these, 52% were on immunomodulatory therapy at all time points analyzed. There was no difference in averaged MCP-1α levels measured in patients maintained on immunomodulatory therapy ( $944.2 \pm 83.1$  pg/mL;  $n = 15$ ) compared with those who never received such therapy ( $1,122.91 \pm 152.6$  pg/mL;  $n = 9$ ) ( $p = 0.30$ ).

**MCP-1α and HNE levels.** Because of an observed increase in HNE levels and decrease of MCP-1α levels with advancing disease in sALS patients, we investigated the potential relationship between HNE and MCP-1α by measuring MCP-1α released from cultured human macrophages exposed to varying HNE concentrations (0.1 pM to 10 μM). Secretion of MCP-1α from cultured macrophages exposed to low levels of HNE (0.1 pM to 1.0 nM) was increased at 0.1 pM HNE ( $p = 0.029$ ) and maximally increased at 1.0 nM HNE ( $p = 0.0037$ ). However, MCP-1α levels were suppressed with further increases of HNE compared with 1.0 nM (100 mM,  $p = 0.039$ ; 1.0 μM,  $p = 0.00032$ ; and 10 μM,  $p = 0.00023$ ) (figure 7). However, there was no correlation between MCP-1α and HNE levels in the sera of sALS patients ( $n = 34$ ) with samples collected at multiple time points during their disease course ( $R = 0.005$ ,  $p = 0.95$ ) (figure 8).

**Discussion.** We sought to determine whether HNE is present systemically in ALS patients and correlates with disease progression, stage, and drug therapy. HNE is a reactive and cytotoxic by-product of aldehydic oxidation generated from the degradation of membrane ω3-polyunsaturated fatty acids. It can rapidly diffuse through lipid bilayers to modify extracellular or intracellular membrane proteins and enzymes necessary for normal cellular function, and it is implicated in several disease processes including neurodegeneration.<sup>1,12</sup> Similar to other markers of oxidative damage found to be elevated in ALS tissues,<sup>32,33</sup> we previously documented increased levels of HNE in the CSF and spinal cord tissue of ALS patients,<sup>1,15</sup> which supports a role for oxidative stress in ALS. Likewise, immune activation has been documented in affected areas of postmortem CNS tissue, in addition to elevated levels of pro-inflammatory cytokines in the CSF and sera of living ALS patients. However, it is unclear at what stage in the disease these pathways are activated and how their interaction is relevant to disease pathogenesis.

In this study, serum and CSF HNE level were significantly elevated in ALS patients compared with all controls. When grouped according to clinical stage of disease, serum levels were consistently elevated above control values even at early stages of disease. Further, HNE levels from sALS serum and CSF samples were significantly elevated above those collected from fALS patients, suggesting that the familial and sporadic forms are qualitatively different in regard to oxidative stress. This may reflect the presence of distinct mechanisms involved in the pathogenesis of either form of ALS.

HNE levels measured during advanced stages of disease were significantly elevated above those collected at earlier or moderate stages of disease and

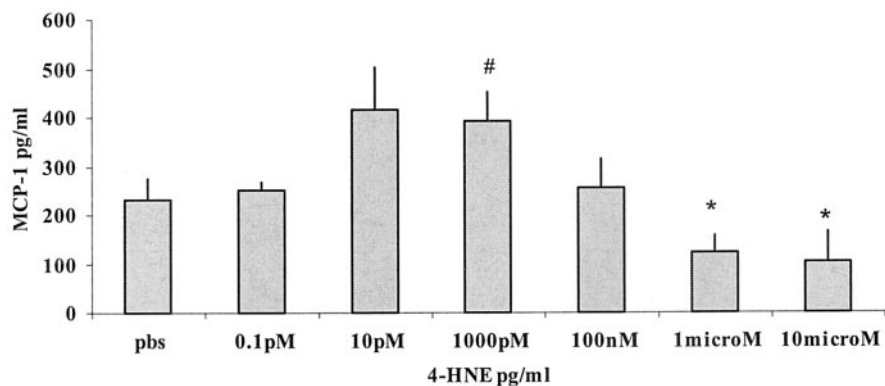


Figure 7. Monocyte chemoattractant protein-1 $\alpha$  (MCP-1 $\alpha$ ) release from cultured human macrophages upon 1-hour exposure to 4-hydroxy-2,3-nonenal (4-HNE) at varying concentrations. MCP-1 $\alpha$  release increased from baseline (phosphate-buffered solution control) with exposure to 1,000 pM HNE, Student t-test: #p < 0.05. These levels declined with increasing HNE concentration (1 and 10  $\mu$ M), Student t-test: \*p < 0.05 (four experiments, three to four wells per treatment).

positively correlated with stage of disease, implicating HNE as a possible early indicator of oxidative stress and marker of disease burden. HNE levels were not altered by immunomodulatory drug therapy, nor did they correlate with rate of progression.

Owing to the ability of HNE to form adducts with a variety of cellular proteins necessary for normal physiologic function,<sup>8,9,12,13</sup> lipid peroxidation in ALS could also contribute to neuronal death. Indexes of increased oxidative stress have been reported in the sera or plasma of ALS patients, in addition to elevated levels in urine, which correlate with rate of progression.<sup>33</sup> Although we did not find any correlation with HNE serum levels and rate of progression, we did find a positive correlation with disease burden, thus supporting a proximate role for lipid peroxidation in ALS progression.

The systemic presence of increased HNE in the serum of ALS patients may reflect either the presence of lipid peroxidation in the CNS with diffusion to the peripheral circulation or the activation of additional pathways originating outside the CNS, leading to the formation of HNE and its related adducts. HNE is a lipophilic molecule that is able to diffuse

across lipid membranes. Therefore, it is possible that serum levels reflect diffusion of HNE from the CNS to the periphery. The levels of CSF HNE were 2.3 times higher than that measured in the sera of our sALS patients, supporting the generation of HNE within the CNS and its diffusion to the periphery. Likewise, in Alzheimer and Parkinson disease patients, levels of CSF HNE have been reported as elevated above those measured in the plasma, possibly owing to diffusion of HNE to the peripheral circulation in these populations.<sup>34,35</sup> In accord, products of oxidative stress that are not lipophilic may not be significantly elevated in the serum or plasma of patients with primary neurodegenerative disease, such as what has been reported for 8-hydroxyguanosine, a hydrophilic marker of DNA oxidative damage, in Alzheimer and Parkinson disease patients.<sup>36,37</sup> However, 8-hydroxyguanosine levels are elevated in the plasma of ALS patients compared with levels measured in the spinal fluid,<sup>35</sup> implicating a peripheral source of oxidative stress that is specific to sALS. Currently, there is no documentation of increased oxidative stress in peripheral organs or tissues of ALS patients. However, pathologic alterations are evident in peripheral tissues of ALS patients,<sup>38-40</sup> and these may reflect changes induced by lipid peroxidation originating outside the CNS.

Initiation of lipid peroxidation and other forms of oxidative damage can be induced by several mechanisms related to the disease process.<sup>16-18</sup> Immune activation is one such mechanism that can serve as a significant source of oxidative stress through the release of free radicals, nitric oxide, and pro-inflammatory molecules.<sup>19,20,41</sup> Similar to the presence of increased HNE and HNE-modified proteins in ALS, immune activation has also been documented in ALS patients. Activated microglia, T lymphocytes, and immunoglobulins are present in postmortem ALS spinal cord,<sup>4,42,43</sup> in addition to activated peripheral blood T lymphocytes and elevated levels of TNF $\alpha$  and IL-6 in the serum or CSF.<sup>5,6,23</sup> We therefore measured the concentration of several cytokines and chemokines in the sera of ALS patients compared with control subjects to determine if a possible relationship may exist between HNE and immune activation. Of the cytokines and chemokines

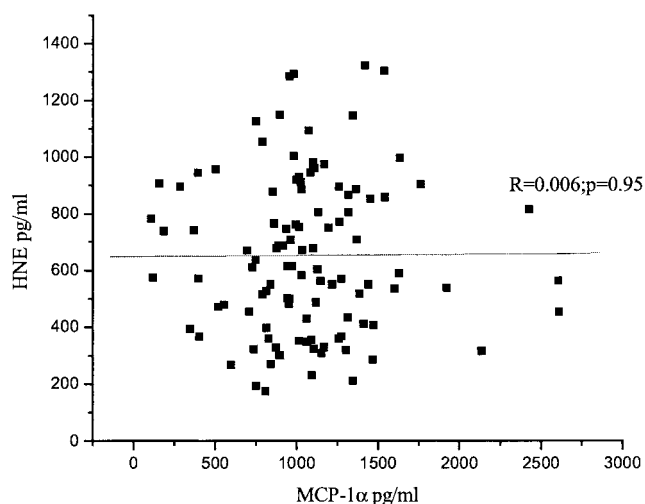


Figure 8. Serum 4-hydroxy-2,3-nonenal (HNE) levels and serum monocyte chemoattractant protein-1 $\alpha$  (MCP-1 $\alpha$ ) levels measured at multiple time points do not correlate (n = 34; 108 samples).

measured, only MCP-1 $\alpha$  was significantly elevated in the sera of ALS patients compared with both disease and NC subjects, although less than that observed for HNE in the sampled populations.

MCP-1 $\alpha$  is a member of the CC-chemokine family and is responsible for recruitment of mononuclear cells including monocytes, microglia, activated T cells, and dendritic cells to sites of inflammation and injury through interaction with its receptor, CCR2.<sup>44</sup> MCP-1 $\alpha$  has been documented to recruit mononuclear cells to the CNS in animal models, resulting in significant immunopathology.<sup>45,46</sup> In our sALS population, MCP-1 $\alpha$  levels were significantly elevated during early stage of disease and significantly declined by late stages when grouped according to stage of disease or advancing disability.

For samples assayed at multiple time points for individual patients, levels did not correlate with rate of progression and were not different in patients maintained on immunomodulatory therapy vs untreated patients. There was a negative correlation between MCP-1 $\alpha$  levels and advancing disease. The importance of this is unclear but may reflect a decrease in immune activation and recruitment due to the progressive loss of motor neurons with disease progression.

Similar to HNE levels in the sera, the systemic presence of MCP-1 $\alpha$  may reflect a CNS or peripheral source of activation. Although CSF and serum MCP-1 $\alpha$  levels in our ALS patient population were not different, others have a reported significant increase of CSF MCP-1 $\alpha$  levels compared with serum levels in sALS patients, suggesting that MCP-1 $\alpha$  is a marker predominantly for neuroinflammation.<sup>47</sup> Similar to MCP-1 $\alpha$  levels measured in the serum and CSF of our ALS patients, CSF MCP-1 $\alpha$  levels were not statistically different from serum levels measured within disease control populations or with CSF levels measured between these populations, with the exception of our ND control subjects. A recent report from others in our group of increased CSF MCP-1 $\alpha$  in sALS over NND controls<sup>48</sup> is likely a reflection of differences in sample size and patient selection between the two studies. Nevertheless, our findings suggest that immune activation involves the activation and recruitment of immune cells from the CNS and peripheral sites in ALS. In accord, we have found a significant infiltration of myeloid dendritic cells in addition to elevated levels of MCP-1 $\alpha$  in the spinal cord of sALS patients compared with disease controls. These cells are likely originating from peripheral sites and are being recruited to the CNS through their major chemokine, MCP-1.<sup>48</sup>

Immune infiltration such as is present in ALS CNS tissue is associated with a significant generation of free radicals sufficient to initiate pathways leading to lipid peroxidation and oxidative stress. Likewise, oxidative damage from lipid peroxidation may be important in the activation and recruitment of immune cells to sites of injury, thus creating a pathologic loop of cellular damage and death. Sup-

port for this is based on recent studies showing that oxidized proteins induce chemokine expression in affected tissues,<sup>49</sup> are chemotactic for mononuclear cells,<sup>50</sup> and activate and induce proliferation of mononuclear cells.<sup>51</sup> In addition, MCP-1 $\alpha$  has been shown to directly activate monocytes, leading to an increased generation of free radicals.<sup>52</sup>

Although in our study we did not find a correlation between HNE and MCP-1 $\alpha$  levels in sALS patients during the course of their disease, HNE levels positively correlated and MCP-1 $\alpha$  levels negatively correlated with advancing disease. Further, previous studies have documented enhanced release of MCP-1 $\alpha$  in a variety of cells and tissues in response to oxidative stress,<sup>53,54</sup> implicating a relationship between oxidative and immune activation and recruitment in a variety of disease processes. To better understand this relationship, we measured the release of MCP-1 $\alpha$  from cultured human macrophages in response to varying concentrations of HNE. Macrophages released increasing amounts of MCP-1 $\alpha$  in a dose-dependent manner to increasing concentrations of HNE. With further increases in HNE, comparable with those measured in our patient population, MCP-1 $\alpha$  release was suppressed in the absence of significant cell loss or injury. This biphasic response has been observed with other cell populations upon exposure to HNE and may reflect activation and subsequent inhibition of pathways relevant to protein transport, release and possibly synthesis.<sup>55,56</sup> Thus, it is possible that lipid peroxidation may be a dominant pathway in the modulation of immune activation and recruitment,<sup>57</sup> and the immune/inflammatory response may be the consequence of increased oxidative stress rather than its cause. Furthermore, HNE is implicated as a possible biomarker of the extent of disease.

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