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Original Contribution

Blood Lead, Bone Turnover, and Survival in Amyotrophic Lateral Sclerosis

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Blood lead and bone turnover may be associated with the risk of amyotrophic lateral sclerosis (ALS). We aimed to assess whether these factors were also associated with time from ALS diagnosis to death through a survival analysis of 145 ALS patients enrolled during 2007 in the National Registry of Veterans with ALS. Associations of survival time with blood lead and plasma biomarkers of bone resorption (C-terminal telopeptides of type I collagen (CTX)) and bone formation (procollagen type I amino-terminal peptide (PINP)) were estimated using Cox models adjusted for age at diagnosis, diagnostic certainty, diagnostic delay, site of onset, and score on the Revised ALS Functional Rating Scale. Hazard ratios were calculated for each doubling of biomarker concentration. Blood lead, plasma CTX, and plasma PINP were mutually adjusted for one another. Increased lead (hazard ratio (HR) = 1.38; 95% confidence interval (CI): 1.03, 1.84) and CTX (HR = 2.03; 95% CI: 1.42, 2.89) were both associated with shorter survival, whereas higher PINP was associated with longer survival (HR = 0.59; 95% CI: 0.42, 0.83), after ALS diagnosis. No interactions were observed between lead or bone turnover and other prognostic indicators. Lead toxicity and bone metabolism may be involved in ALS pathophysiology.

amyotrophic lateral sclerosis; blood lead; bone turnover; survival; veterans

Abbreviations: ALS, amyotrophic lateral sclerosis; ALSFRS-R, Revised ALS Functional Rating Scale; CI, confidence interval; CTX, C-terminal telopeptides of type I collagen; HR, hazard ratio; MMP, matrix metalloproteinase; PINP, procollagen type I amino-terminal peptide; VA, Veterans Affairs.

Although most patients with amyotrophic lateral sclerosis (ALS) succumb quickly, about 10% survive more than 10 years (1). Explanations for the heterogeneous survival profile of ALS remain to be discovered. Older age at diagnosis, bulbar onset, lower forced vital capacity, and shorter diagnostic delay (i.e., time interval between first symptom onset and ALS diagnosis) are all consistently related to a worse prognosis (1–4). Female sex, higher diagnostic certainty, lower score on the ALS Functional Rating Scale, and greater rate of disease progression after diagnosis may be associated with a worse prognosis, but the associations are less consistent (5–8). More recently, rate of decline of ALS functional rating score before diagnosis and executive dysfunction have also been discussed as predictors of poor prognosis for ALS (9).

Lead exposure has long been hypothesized to be a culprit in the development of ALS. Higher concentrations of lead in both blood and bone have been observed among ALS patients compared with controls (10, 11). Blood lead level is generally considered to reflect contemporaneous environmental exposure to lead, but it may also reflect mobilization of bone lead to blood (12, 13). The dynamics of lead distribution between blood and bone are related to bone-turnover status, and a higher level of bone demineralization leads to increased release of lead from bone to blood (13). Lead released during demineralization may affect bone cells directly and promote increased bone turnover (14–16), so that a positive feedback cycle is created. Nevertheless, the association of ALS risk with blood lead is independent of bone turnover, as shown in a previous study that reported a 2.6-fold increase in ALS risk for each doubling of blood lead concentration after adjustment for bone formation and resorption (11). Interestingly, that study also found that ALS patients had higher levels of bone

resorption than controls, although bone formation levels were similar (11).

ALS is increasingly recognized as a disease involving whole body physiology and multiple nonneural cell populations (17, 18). Little research, however, has specifically addressed bone metabolism in ALS etiology or survival. Similarly, the association between blood lead and ALS survival has rarely been investigated (19). We hypothesized that blood lead and bone turnover would both be associated with length of survival after ALS diagnosis, independent of other known prognostic indicators. We used data from a cohort of 145 US veterans with ALS to address this hypothesis.

METHODS

Study participants

Enrollment of veterans with ALS in the US National Registry of Veterans with ALS (Veterans Affairs (VA) Registry) began in April 2003 and ended in September 2007 (20). Active recruitment involved searches of VA inpatient and outpatient databases for veterans with ALS and other motor neuron diseases (20, 21) Passive recruitment involved multiple nationwide publicity efforts, aiming to enroll other veterans with ALS not treated within the VA healthcare system. Neurologists with expertise in ALS reviewed all relevant medical records for potentially eligible veterans and assigned diagnoses using an algorithm based on the revised El Escorial Criteria (22). Information was also collected on clinical characteristics of the ALS patients, including site of onset, diagnostic delay, and the Revised ALS Functional Rating Scale (ALSFRS-R) score (range, 0–48, with lower score indicating worse function).

A total of 200 neurologist-confirmed patients with motor neuron diseases had blood samples collected during January–September 2007. Because we were interested primarily in ALS, we excluded 30 cases of progressive muscular atrophy and 7 cases of primary lateral sclerosis, and we included patients with clinically definite, clinically probable–lab supported, clinically probable, and clinically possible ALS in our analysis ($n = 163$). Further, because the majority of the cases were white men, 3 female cases and 9 nonwhite male cases were excluded to reduce sample heterogeneity. An additional 6 cases with missing covariate data (see below) were excluded, leaving 145 cases in the final analyses.

Institutional review boards at the National Institute of Environmental Health Sciences, the Durham VA Medical Center, Duke University, and Copernicus Group approved the present study.

Measurements of lead and bone turnover

The VA Registry conducted home visits with enrollees, during which up to 4 tubes of blood were collected, including a whole blood sample in a metal-free tube. Detailed procedures for sample collection and laboratory assays have been described previously (11). Briefly, inductively coupled plasma mass spectrometry was used for measurement of blood lead, accompanied by careful prevention of metal contamination during analysis and multiple quality-control efforts. Biomarkers for bone formation and resorption were measured in plasma. Bone formation was assessed using procollagen type I amino-terminal peptide (PINP) and bone resorption using plasma C-terminal telopeptides of type I collagen (CTX). PINP and CTX are both specific to bone compared with other connective tissues, and were stable under field conditions (11).

Follow-up

All participants were followed from the diagnosis date until the date of death or July 25, 2013, whichever came first. Information on death was obtained from the Austin Vital Status File (23). Using the National Death Index (NDI) (24) as the gold standard, a validation study of Austin Vital Status File found 98.3% sensitivity and 99.8% specificity for identifying deaths among veterans (23). We considered only death, not beginning of patient ventilation, as the endpoint, following a published recommendation (25).

Enrollees of the VA Registry were contacted by telephone at approximately 6-month intervals to monitor their health, including performance on the ALSFRS-R. For the present study, we used the ALSFRS-R score closest to the time of sample collection.

Statistical analysis

We described the concentrations of blood lead and plasma CTX and PINP according to different clinical characteristics of the ALS patients (age at diagnosis, diagnostic certainty, site of onset, diagnostic delay, and ALSFRS-R score). Correlations among the 3 biomarkers and between the biomarkers and clinical characteristics that were treated as continuous variables (age at diagnosis, diagnostic delay, and ALSFRS-R score) were assessed using Spearman's rank correlation coefficient. We used analysis of variance methods or t tests to compare levels of the 3 biomarkers across categories of diagnostic certainty and site of onset.

For our main analyses, we estimated associations between individual biomarkers and survival using hazard ratios and their 95% confidence intervals derived from Cox proportional hazard models, with time since ALS diagnosis as the underlying timescale. Because patients were enrolled at various lag times after diagnosis and because only patients who survived until collection of blood samples were enrolled in the present study, all survival models were fitted with delayed entry at the actual time of sample collection (i.e., days since diagnosis) to avoid potential immortal time bias (26). Using directed acyclic graphs (27), an adjustment for age at diagnosis, site of onset, diagnostic certainty, diagnostic delay, and ALSFRS-R score was deemed sufficient to estimate the association of different biomarkers with survival after ALS diagnosis. Blood lead, plasma CTX, and plasma PINP were used in the Cox models as $\log_2$-transformed continuous variables. In addition, we created the "bone resorption-to-formation ratio"—calculated as CTX/PINP—to assess the importance of unbalanced bone resorption and formation on ALS (28); it was also used as a $\log_2$-transformed continuous variable. To assess the contribution of bone turnover to the association of blood lead and ALS survival, and vice versa, the 3 biomarker variables were mutually adjusted in models with each as separate variables. We also examined models that replaced the 2 separate bone

turnover variables with the CTX/PINP ratio as a $\log_2$-transformed continuous variable.

To assess the crude associations of blood lead and bone turnover with ALS survival, we adjusted only for age at diagnosis using 4 categories with cutpoints at the quartiles. To assess the adjusted association in accordance with the directed acyclic graphs, we further included site of onset (spinal, bulbar), diagnostic certainty (definite, probable, possible), diagnostic delay (≤344 and >344 days), and ALSFRS-R score (1–29 and 30–48). Diagnostic delay and ALSFRS-R score were dichotomized at the medians among all patients.

We examined the adequacy of the Cox models in several ways. We assessed the proportional hazards assumption for all variables in all models using methods based on Schoenfeld partial residuals and found little indication of violation of the assumption. We assessed the linearity of each continuous variable in fully adjusting models using likelihood ratio tests, comparing a model where the variable entered as a linear term only with a larger model where that variable entered as a natural cubic spline with knots at the quintiles of its distribution. We assessed the overall fit of the Cox models using a score test (29) and provided Akaike information criterion (AIC) as a model comparison index. Only $\log_2$(CTX/PINP) showed evidence of slight nonlinearity, and the corresponding spline fit showed a strong, essentially monotone exposure-response relationship. The overall goodness-of-fit of the fully adjusting model of $\log_2$(CTX/PINP) was also questionable, likely due to the nonlinearity (the corresponding spline model showed no such lack of fit). Nevertheless, we chose to present the results of the linear parameterization for $\log_2$(CTX/PINP) to provide a single summary hazard ratio, assessing a trend across the monotone relationship. Finally, we used influence diagnostics to check for influential observations.

We also conducted several exploratory analyses. We evaluated potential changes in the associations of lead and bone turnover with ALS survival by 4 clinical characteristics: diagnostic certainty, diagnostic delay, site of onset, and ALSFRS-R score. Because results from the main analyses were largely similar whether CTX and PINP were used as individual variables or as a ratio (CTX/PINP), we used the latter for these additional analyses. Specifically, we added a term for interaction between each of the 4 clinical characteristics and lead or CTX/PINP in separate models and used Wald χ^2 tests of the interaction terms and $\alpha = 0.1$ to identify statistically different associations. To address the potential bias created by including study patients at various lag times after diagnosis, we conducted an analysis restricted to cases with a sample collection delay shorter than the median of the entire cohort (days from diagnosis to sample collection ≤392). To assess potential residual confounding due to adjusting for diagnostic delay as a dichotomized variable, in 2 additional analyses, we further adjusted for diagnostic delay as a continuous variable, using either a single linear term or using both linear and quadratic terms. In all additional analyses, we adjusted for age at diagnosis, diagnostic certainty, site of onset, diagnostic delay, and ALSFRS-R score.

Analyses were conducted using SAS, version 9.3 (SAS Institute, Inc., Cary, North Carolina). For interaction analysis, we calculated stratum-specific hazard ratios and their 95% confidence intervals using the HAZARDRATIO statement in PROC PHREG.

RESULTS

The mean age at ALS diagnosis was 62.8 years (range, 34–83). Most patients had a diagnosis of clinically probable ALS and spinal onset (Table 1).

Plasma CTX varied by diagnostic certainty ($P = 0.003$) and site of onset ($P = 0.02$), whereas blood lead and plasma PINP were similar across these categories (all $P > 0.05$). Blood lead was statistically significantly correlated with plasma CTX but not plasma PINP, although plasma CTX and plasma PINP were correlated with each other (Table 2). Blood lead was positively correlated with age at diagnosis and negatively correlated with ALSFRS-R score. Plasma CTX was negatively correlated with diagnostic delay and ALSFRS-R score, whereas plasma PINP was not correlated with any of these variables (Table 2).

We observed 113 deaths during follow-up. After adjustment for age, a 1-unit increment of $\log_2$-transformed blood lead (i.e., a doubling of blood lead concentration) was associated with shorter survival after ALS diagnosis (Table 3). A doubling of plasma CTX was also associated with shorter survival, whereas a doubling of plasma PINP was associated with longer survival. The associations for blood lead and plasma CTX diminished slightly after additional mutual adjustment of blood lead, plasma CTX, and plasma PINP, and the comparison of Akaike information criterion results suggests that the mutual adjustment is desirable, but the association of plasma PINP with ALS was not affected by adjustment for blood lead (Table 3). Further adjustment for other known prognostic indicators did not change these associations substantively (Table 3). Similarly, a 1-unit increment of $\log_2$-transformed CTX/PINP was associated with shorter survival, regardless of mutual adjustment of blood lead or further adjustments for other prognostic indicators (Table 3).

No statistically significant interactions were detected between either blood lead or plasma CTX/PINP and other prognostic indicators, except for a stronger association for CTX/PINP among patients with a long diagnostic delay than among those with a shorter delay ($P_{\text{interaction}} = 0.02$; Table 4).

Including only patients with a sample collection delay of ≤392 days ($n = 73$; deaths = 60) did not change greatly the association for blood lead (hazard ratio (HR) = 1.58; 95% confidence interval (CI): 1.05, 2.38), and slightly weakened the association for plasma CTX/PINP (HR = 1.72; 95% CI: 1.18, 2.50), compared with the main analyses (Table 3). Adjusting for diagnostic delay as a continuous variable, either using only a linear term or using both linear and quadratic terms, yielded largely similar results (in the fully adjusting models, blood lead HR = 1.42; 95% CI: 1.06, 1.89 when diagnostic delay was adjusted for linearly; HR = 1.42; 95% CI: 1.07, 1.90 when diagnostic delay was adjusted for using both linear and quadratic terms). Further adjustment for other known prognostic indicators gave similar results in these sensitivity analyses (data not shown).

DISCUSSION

We found that both higher levels of blood lead and increased bone resorption predicted shorter survival after diagnosis among patients with ALS. The associations of survival with blood lead and bone turnover were independent of each other and of other prognostic indicators. Together with the previously reported associations of blood lead and bone turnover with ALS risk (11),

Table 1. Presence of Lead and Biomarkers of Bone Formation and Resorption According to Clinical Characteristics, Among 145 US Military Veterans With Amyotrophic Lateral Sclerosis, 2007–2013

Variable	No. of Cases	%	Lead, μg/dL, Mean (SD)	CTX, ng/mL, Mean (SD)	PINP, ng/mL, Mean (SD)
All participants	145	100	2.35 (1.28)	0.60 (0.33)	36.5 (17.5)
Age at diagnosis, years					
34–55	28	19	1.72 (0.92)	0.66 (0.29)	40.3 (25.2)
56–60	32	22	2.19 (1.08)	0.57 (0.28)	37.6 (14.9)
61–70	42	29	2.60 (1.32)	0.51 (0.31)	32.6 (11.0)
71–83	43	30	2.62 (1.45)	0.68 (0.39)	37.0 (18.1)
Diagnostic certainty					
Clinically definite ALS	27	19	2.30 (0.94)	0.74 (0.34)	43.3 (25.3)
Clinically probable ALS	97	67	2.30 (1.33)	0.53 (0.29)	34.6 (14.8)
Clinically possible ALS	21	14	2.63 (1.45)	0.73 (0.40)	36.4 (15.0)
Site of onset					
Spinal	113	78	2.23 (1.10)	0.57 (0.33)	36.3 (16.9)
Bulbar	32	22	2.74 (1.75)	0.69 (0.30)	37.3 (19.7)
Diagnostic delay, days					
48–208	38	26	2.21 (1.14)	0.64 (0.33)	39.5 (22.9)
209–341	35	24	2.16 (0.98)	0.65 (0.33)	34.4 (12.1)
342–634	36	25	2.23 (1.18)	0.60 (0.36)	38.4 (14.2)
635–5,974	36	25	2.79 (1.67)	0.51 (0.30)	33.5 (18.1)
ALSFRS-R score					
1–21	39	27	2.85 (1.54)	0.86 (0.39)	38.2 (22.2)
22–30	35	24	2.23 (1.11)	0.59 (0.29)	33.6 (15.8)
31–37	37	26	2.28 (1.15)	0.45 (0.19)	37.5 (18.4)
38–45	34	23	1.95 (1.10)	0.48 (0.22)	36.4 (11.0)

Abbreviations: ALS, amyotrophic lateral sclerosis; ALSFRS-R, Revised ALS Functional Rating Scale; CTX, plasma C-terminal telopeptides of type I collagen; PINP, plasma procollagen type I amino-terminal peptide; SD, standard deviation.

our present findings shed light on the potential involvement of lead toxicity and bone metabolism in ALS etiology and survival.

In a previous case-control study based in 2 tertiary ALS care centers in New England (United States), we found a positive association for lead in both blood and bone with ALS risk (10). The association was not explained by a decrease in physical activity among ALS patients and was not modified by a gene affecting lead toxicokinetics, δ-aminolevulinic acid dehydratase (*ALAD*) (10, 30). In the same study, we found that elevated bone lead was associated with longer survival after ALS diagnosis (for patella lead, HR = 0.5 (95% CI: 0.2, 1.0), and, for tibia lead, HR = 0.3 (95% CI: 0.1, 0.7)), but the association was less clear for blood lead (HR = 0.9; 95% CI: 0.8, 1.0) (19). Our more recent case-control study in US military veterans further substantiated the positive relationship of blood lead to ALS risk by directly controlling for bone turnover status in the analyses; this study also found that the association of blood lead with ALS risk was not modified by *ALAD* (11).

It is difficult to directly compare the results of the survival analyses from the New England study with those of the present study because the former had no data on bone turnover, whereas the present study lacked bone lead measurements. Given the similar associations of blood lead with ALS risk noted in both studies, the contrasting results of blood lead in relation to ALS survival are puzzling. Increased bone resorption leads to elevated blood lead (13, 31), and possibly results in decreased bone lead. Thus the association of blood lead with shorter survival after ALS diagnosis in the present study might be consistent with an association between bone lead and longer survival after ALS diagnosis. The relationship to ALS survival of both blood and bone lead warrants further study.

Biological plausibility of lead involvement in ALS etiology has been discussed extensively. Mechanisms involved in lead neurotoxicity—including increased oxidative stress, mitochondrial dysfunction, and excitotoxicity—are also involved in ALS pathogenesis (32). Previous studies reporting an association of ALS with a higher level of lead exposure, whether through self-report or measured through biomarkers, are mostly cross-sectional and consequently not able to establish a temporal relationship. In the present prospective study we showed that lead exposure, namely higher blood lead, was associated not only with a higher ALS risk (11) but also with a shorter survival after diagnosis. It is important to keep in mind, however, that all the measures of association are calculated per 1 doubling of blood lead concentration, which represents a small or a

Table 2. Spearman Correlations Between Continuous Study Variables, Among 145 US Military Veterans With Amyotrophic Lateral Sclerosis, 2007–2013

Variable	Lead	CTX	PINP
Lead	1.000		
CTX	0.237[a]	1.000	
PINP	0.059	0.194[b]	1.000
Age at diagnosis, years	0.281[c]	0.011	−0.056
Diagnostic delay, days	0.118	−0.169[b]	−0.041
ALSFRS-R score	−0.325[c]	−0.466[c]	0.043

Abbreviations: ALSFRS-R, Revised Amyotrophic Lateral Sclerosis Functional Rating Scale; CTX, plasma C-terminal telopeptides of type I collagen; PINP, plasma procollagen type I amino-terminal peptide.
[a] $P < 0.01$.
[b] $P < 0.05$.
[c] $P < 0.001$.

large change in absolute lead level depending on the starting point.

This is the first study, to our knowledge, to assess the potential role of bone turnover biomarkers in ALS survival. It is possible that altered bone turnover status, especially increased bone resorption, is secondary to decreased physical activity in ALS patients, because CTX was inversely correlated with both diagnostic delay and ALSFRS-R score. This could also result in higher blood lead concentration. However, adjustment for diagnostic delay and ALSFRS-R score attenuated but did not eliminate the association of bone turnover with ALS survival.

It is also possible that changes in bone turnover have pathophysiological significance in and of themselves. Although data relevant to this issue are scarce, factors related to poor bone health, including hyperparathyroidism (33–35) and vitamin D deficiency (33, 34, 36, 37), may be related to ALS. Correction of hyperparathyroidism did not alter disease course in one study of 5 patients (35), but supplementation with vitamin D delayed the decline in the ALSFRS-R score in some although not all studies (36–38) and improved motor function in *G93A SOD1* transgenic mice (39). A molecular link between bone turnover and ALS may be provided by matrix metalloproteinases (MMPs) and their inhibitors (40). For example, MMP-9 activity increased, whereas MMP-2 activity decreased, in ALS animal models and patients (40). MMPs, especially MMP-9, have been associated with rapid disease progression and poorer survival in ALS (40). MMP-9 is primarily expressed by osteoclasts, which resorb bone tissue, whereas MMP-2 is expressed by osteoblasts, which synthesize bone. Therefore, the relationship of bone turnover to ALS can be explained, at least partially, by activities of bone cells and their expressed MMPs. The strength of the associations of ALS with bone turnover biomarkers in the present study, and the suggested potential of mesenchymal stem cells as a therapy for ALS (41), underscore the importance of further study of this hypothesis.

Factors linking blood lead, bone turnover, and ALS might also explain the present findings. Homocysteine might be one such factor. A role of homocysteine in both risk and prognosis of ALS has been proposed, based on both experimental and clinical studies (42). The proposed pathologic mechanisms of homocysteine also overlap with the proposed mechanisms of ALS (43). Homocysteine promotes the activity of osteoclasts and suppresses the activity of osteoblasts, as well as decreasing bone blood flow and directly reducing bone strength (44). Blood lead, although not tibia lead, might be related to increased homocysteine (45). Further studies that include measurement of homocysteine may help disentangle these relationships.

Table 3. Associations of Blood Lead Concentration and Bone Turnover Status With Survival After Diagnosis, Among 145 US Military Veterans With Amyotrophic Lateral Sclerosis, 2007–2013

Model	AIC	Variable[a]							
		Lead		CTX		PINP		CTX/PINP	
		HR	95% CI	HR	95% CI	HR	95% CI	HR	95% CI
1[b]	877.56	1.53	1.16, 2.01						
2[b]	852.17			2.40	1.79, 3.22	0.55	0.39, 0.77		
3[b]	852.21							2.15	1.67, 2.76
4[b]	849.92	1.36	1.02, 1.82	2.26	1.67, 3.05	0.55	0.39, 0.76		
5[c]	852.43	1.38	1.03, 1.84	2.03	1.42, 2.89	0.59	0.42, 0.83		
6[b]	849.16	1.39	1.05, 1.85					2.06	1.60, 2.65
7[c]	851.11	1.40	1.06, 1.87					1.85	1.40, 2.45

Abbreviations: AIC, Akaike information criterion; CI, confidence interval; CTX, plasma C-terminal telopeptides of type I collagen; HR, Hazard ratio; PINP, plasma procollagen type I amino-terminal peptide.
[a] Biomarker variables were $\log_2$-transformed and modeled as continuous; original units: lead, μg/dL; CTX and PINP, ng/mL. Blank entries indicate that the variable was not included in that model.
[b] The model adjusted for age at diagnosis (categorical using quartile cutpoints).
[c] The model adjusted for age at diagnosis (categorical using quartile cutpoints), diagnostic certainty (definite, probable, possible), site of onset (bulbar, spinal), diagnostic delay (dichotomized at the median of 344 days), and Revised Amyotrophic Lateral Sclerosis Functional Rating Scale score (dichotomized at the median of 30).

Table 4. Associations of Blood Lead Concentration and Bone Turnover Status With Mortality Rate, Stratified by Diagnostic Certainty, Diagnostic Delay, Site Of Onset, and Functional Rating Score, Among 145 United US Military Veterans With Amyotrophic Lateral Sclerosis, 2007–2013[a]

Stratum	No. of Cases	Lead			CTX/PINP		
		HR	95% CI	$P_{interaction}$	HR	95% CI	$P_{interaction}$
Diagnostic certainty				0.30			0.68
Definite	27	1.84	0.82, 4.16		1.43	0.71, 2.89	
Probable	97	1.51	1.08, 2.12		1.88	1.35, 2.63	
Possible	21	0.89	0.46, 1.73		2.14	1.16, 3.94	
Diagnostic delay				0.44			0.02
Long (345–5,974 days)	71	1.55	1.06, 2.27		2.42	1.70, 3.45	
Short (48–344 days)	74	1.25	0.82, 1.89		1.36	0.93, 1.99	
Site of onset				0.64			0.88
Spinal	113	1.47	1.04, 2.08		1.87	1.35, 2.59	
Bulbar	32	1.28	0.79, 2.07		1.80	1.11, 2.90	
ALSFRS-R score				0.63			0.25
High (30–48)	73	1.30	0.85, 1.98		1.54	1.01, 2.34	
Low (1–29)	72	1.50	1.01, 2.24		2.11	1.48, 3.00	

Abbreviations: ALSFRS-R, Revised Amyotrophic Lateral Sclerosis Functional Rating Scale; CI, confidence interval; CTX, plasma C-terminal telopeptides of type I collagen; HR, Hazard ratio; Pb, lead; PINP, plasma procollagen type I amino-terminal peptide.

[a] All models included age, $\log_2$Pb, $\log_2$(CTX/PINP), diagnostic certainty, diagnostic delay, site of onset, and ALSFRS-R score. Models also included appropriate interaction terms to evaluate effect modification.

The present study has several limitations. Some stem from the limited number of deaths that we observed, given the number of variables in some of our models. We sought to honor a rule of thumb that about 10 deaths per parameter is a reasonable number when relying on large sample inferences (46–48). Models with interactions and some subgroup analyses fall below this guideline and should be interpreted cautiously. To honor this guideline, we dichotomized some potential confounders instead of using additional categories, leaving residual confounding as a possible concern. Similarly, we reported a model for linear trend in $\log_2$(CTX/PINP) rather than the more complex nonlinear model. Although we took into account multiple possible confounders in the present analyses, unknown and unmeasured confounding remains a concern. Caution should therefore be exercised when interpreting these findings.

Another limitation is that half of the ALS patients were diagnosed 392 days before sample collection, representing a selected group of patients with longer survival or different levels of blood lead and bone turnover compared with other ALS patients. The fact that higher blood lead and bone turnover were associated with shorter survival argues against the possibility of spurious positive associations due to survival bias, because including long survivors should only have underestimated the real associations. Further, sensitivity analyses including only patients with blood sampled within 392 days of diagnosis gave similar results.

Because the study was based on US military veterans, these findings may not be generalizable to other ALS patients. However, this does not compromise the internal validity of our findings regarding the biological relationships among lead, bone turnover, and ALS survival.

The present study has several key strengths, including the simultaneous consideration of biomarkers for both lead and bone turnover, relatively large sample size, and the availability of information on important covariates and clinical features, including known prognostic factors. We used a highly sensitive assay to measure blood lead, which enabled us to detect small differences in a population with low absolute levels. Controlling for bone turnover was another main strength, allowing us to infer that the association between blood lead and ALS survival was not due entirely to changes in bone turnover in patients with lowered activity levels.

In summary, our study suggests that higher blood lead, higher bone resorption, and lower bone formation are all associated with shorter survival after ALS diagnosis. If these findings are verified in independent populations, given the strength of their associations with ALS survival, bone turnover biomarkers may prove useful in predicting prognosis for ALS patients.

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