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Multiple metal exposures associate with higher amyotrophic lateral sclerosis risk and mortality independent of genetic risk and correlate to self-reported exposures: a case-control study

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Abstract

Background: The pathogenesis of amyotrophic lateral sclerosis (ALS) involves both genetic and environmental factors. This study investigates associations between metal measures in plasma and urine, ALS risk and survival, and exposure sources.

Methods: Participants with and without ALS from Michigan provided plasma and urine samples for metal measurement via inductively coupled plasma mass spectrometry. Odds and hazard ratios for each metal were computed using risk and survival models. Environmental risk scores (ERS) were created to evaluate the association between exposure mixtures and ALS risk and survival and

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Contributorship

DGJ, JD, LZ, KMB, BM, SAB, ELF, and SAG conceived and designed the study. ST and SAG contributed to the acquisition of the data. DGJ, JD, and KMB performed the statistical analyses. DGJ, EJK, and SAG interpreted the data, drafted the text, and prepared the figures. All authors critically reviewed and approved the final version of the manuscript. SAG is the guarantor of the study.

Competing Interests

DGJ: None.

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exposure source. ALS (ALS-PGS) and metal (metal-PGS) polygenic risk scores were constructed from an independent genome-wide association study and relevant literature-selected SNPs.

Results: Plasma and urine samples from 454 ALS and 294 control participants were analyzed. Elevated levels of individual metals, including copper, selenium, and zinc, significantly associated with ALS risk and survival. ERS representing metal mixtures strongly associated with ALS risk (plasma, OR=2.95, CI=2.38–3.62, $p<0.001$; urine, OR=3.10, CI=2.43–3.97, $p<0.001$) and poorer ALS survival (plasma, HR=1.37, CI=1.20–1.58, $p<0.001$; urine, HR=1.44, CI=1.23–1.67, $p<0.001$). Addition of the ALS-PGS or metal-PGS did not alter the significance of metals with ALS risk and survival. Occupations with high potential of metal exposure associated with elevated ERS. Additionally, occupational and non-occupational metal exposures associated with measured plasma and urine metals.

Conclusion: Metals in plasma and urine associated with increased ALS risk and reduced survival, independent of genetic risk, and correlated with occupational and non-occupational metal exposures. These data underscore the significance of metal exposure in ALS risk and progression.

Keywords

Amyotrophic lateral sclerosis (ALS); metals; polygenic risk score; environmental risk score

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) causes cranial, trunk, limb, and respiratory weakness.¹ Pathogenesis is multifaceted and the gene-environment hypothesis proposes that ALS results from environmental exposures acting upon a genetically susceptible background.¹ We previously reported that polygenic risk contributes to the development of ALS² and environmental risk scores (ERS), representing persistent organic pollutants (POPs) mixtures, associate with ALS risk and survival.^{3–5} Thus, both genetic and environmental factors contribute to ALS in our Michigan ALS cohort.

Several studies using diverse biosamples, such as blood,⁶ cerebrospinal fluid (CSF),^{7, 8} and teeth,⁹ link metal exposures to ALS.^{10, 11} While significant associations between metal exposures and ALS vary across studies, selenium, lead, copper, aluminum, cadmium, and zinc have been identified as significant risk factors for ALS in multiple studies, enhancing the statistical evidence behind these associations.¹¹ However, these studies are limited by relatively small sample sizes,¹⁰ restricting the generalizability of findings and the ability to detect subtle associations. While recent studies suggest the potential for gene-metal interactions to influence ALS outcomes,^{12, 13} no studies have yet to comprehensively examine this interaction.

To address these gaps and build upon existing research, we conducted a large cohort study to investigate the association between metal exposures and ALS, as well as the potential influence of gene-metal interactions on ALS. We investigated the impact of metal levels in plasma and urine, individually and as a mixture modeled by ERS, on ALS risk and survival. This approach captured a comprehensive picture of metal exposures from diverse sources, as well as the biotransformation within and elimination processes from the body.¹⁴

We also examined the relationship between genetic background and metal exposures in ALS risk and determined if measured metal mixtures associated with self-reported occupational exposures. These findings provide valuable insight into the role of metals in underlying disease mechanisms and identify potentially modifiable ALS risk factors.

METHODS

Participants

Participant recruitment details are previously published.^{2–5, 11, 15–19} Briefly, all ALS patients meeting Gold Coast criteria seen in the University of Michigan Pranger ALS clinic are approached for enrollment and depositing of blood and urine samples into the University of Michigan ALS Biorepository, provided they are able to consent in English. Control participants are identified via population outreach using a University of Michigan research database, random address mailing, and Facebook advertisements. These population outreach efforts target individuals falling with the same sex and age distribution of the ALS participants. All participants provided informed consent for this University of Michigan IRB approved study (HUM28826) and demographic information, including age, race, education, military service history, medical history, and supplements/vitamins usage. Blood and/or urine samples collected at the time of recruitment were stored in the Michigan ALS Biorepository. Plasma samples were collected between February 26, 2012 - December 7, 2022 and urine samples were collected between September 23, 2015 - September 9, 2022. Additional information abstracted from medical records included ALS disease characteristics, including onset segment, age of symptom onset, age of diagnosis, ALS family history, El Escorial Criteria, and revised ALS functional rating scale (ALSFRS-R) score. The El Escorial Criteria and ALSFRS-R obtained upon initial presentation to the Pranger ALS clinic are utilized for analysis. *C9orf72* expansion status was obtained from medical records and previously published data.²

Biosamples

Detailed methods are reported in online Supplemental File 1. Briefly, plasma and urine samples were analyzed by inductively coupled mass spectrometry (ICP-MS) at the Dartmouth Trace Element Analysis Core. The Dartmouth Trace Element Analysis Core did not provide normal reference ranges.

Polygenic scores

Two polygenic risk scores (PGS) were utilized. The first, an ALS-PGS, was constructed as previously reported² but updated with weights from a recent large-scale ALS genome-wide association study (GWAS, Supplemental Table 1).²⁰ PRSice-2 software was used for clumping and pruning SNPs using default settings, followed by weight application from the parent GWAS. No cutoff for SNPs (p-value threshold of 1.0) was used. Notably, the ALS-PGS was exclusively constructed for participants not included in the large ALS GWAS. A metal-PGS was constructed to represent metal metabolism (metal-PGS), utilizing relevant SNPs from literature related with higher body burden of metals, or poorer outcomes from xenobiotic exposures (Supplemental Table 2). As the selected SNPs originated from various

studies with diverse outcomes, weights were not applied based on effect estimates; instead, the metal-PGS was constructed based on the count of risk alleles.

Genetic samples

Online Supplemental File 1 shows genotyping analysis of sample batches. QC filtering strategies at the SNP level are detailed in Supplemental Figure 1, with sample-level filtering strategies illustrated in Supplemental Figure 2.

Missing data and multiple imputation

Measurements of metals in plasma and urine are subject to missingness due to detection limits. Five metals detected below the limit of detection (LOD) in over 40% of samples were excluded from ERS construction. Certain metals were manually excluded from ERS construction because the biofluid source was unsuitable for accurate and representative measurement.¹⁴ For plasma ERS, arsenic,¹⁴ cadmium,¹⁴ and silver were excluded.²¹ For urine ERS, manganese,¹⁴ silver,²¹ and strontium were excluded.²² Metal concentrations below LOD were imputed by $LOD/\sqrt{2}$.

Certain adjustment covariates exhibited missing data. In the risk model, these covariates included military service (n=50 for plasma, and n=37 for urine). In the survival analyses, these covariates included family history of ALS (n=12 for plasma, and n=8 for urine), onset segment (n=2 for both plasma and urine), El Escorial criteria (n=17 for plasma, and n=20 for urine), ALSFRS-R (n=2 for plasma, and n=1 for urine), and time between symptom onset and diagnosis (n=5 for plasma, and n=4 for urine). Multiple imputation with chained equations was used for missing adjustment covariates and stratified by case-control status for each sample source. The control imputation model included metals (log-transformed), age at sample collection, sex, and military service. The case imputation model additionally included age at diagnosis, onset segment, ALS family history, El Escorial criteria, ALSFRS-R, smoking status, education, and cumulative hazard rate (Nelson-Aalen estimator) from diagnosis to last follow-up. Twenty multiple imputation datasets were generated using a predictive mean matching model.

Risk

For ALS risk models, single metal logistic regression models adjusted for age at sample collection, sex, and military service were constructed. Models were fit independently across 20 imputation datasets and results across models were pooled using Rubin's rule. *P*-values were adjusted for multiple comparisons by Benjamini-Hochberg correction. To assess the potential confounding effects of education and smoking, sensitivity analyses were conducted by including education or smoking as additional covariates, respectively.

Environmental risk scores (ERS) were constructed to assess multiple metals concurrently. As metal levels differ between plasma and urine samples, separate ERS scores for plasma (ERS^{PR}; plasma risk ERS) and urine (ERS^{UR}; urine risk ERS) were developed. An adaptive elastic net approach applied to the combined 20 imputed datasets using the R-package *miselect*²³ determined ERS weights. To optimize performance of the adaptive elastic net,

hyperparameters were carefully tuned through a 5-fold cross-validation process to minimize deviance, based on the log likelihood for a logistic regression model.

To test for effect modification between genetic predisposition to ALS and elevated metal levels, logistic regression models evaluated the interaction between plasma/urine levels and ALS-PGS. To test for effect modification between altered metals metabolism genetics and elevated metal levels, logistic regression models evaluated the interaction between plasma/urine levels and metal-PGS. Adjustment covariates for both interaction models included age at sample collection, sex, military service, and the first five genetic principal components.² Of note, the risk model did not adjust for *C9orf72* gene positivity status as there were no *C9orf72* positive control cases.²

Survival

Cox proportional hazards models adjusted for age at diagnosis, sex, family history of ALS, bulbar vs non-bulbar onset, diagnostic El Escorial criteria, ALSFRS-R, and time between symptom onset and diagnosis were fit for each metal model post-diagnosis survival with subsequent Benjamini-Hochberg correction. Sensitivity analyses, in which smoking was included as an additional covariate, were conducted to assess the potential confounding effect of smoking on the results. To ensure that the definition of survival time did not impact the results, a sensitivity analysis was conducted by defining survival time from the date of sample collection to the event of interest.

Survival ERS was constructed via application of a Cox regression with ridge penalty to the combined twenty imputation datasets, following published methods.^{3, 4} Again, separate ERS were developed for plasma (ERS^{PS}; plasma survival ERS) and urine (ERS^{US}; urine survival ERS). Hyperparameters of the ridge penalty were tuned through a 5-fold cross-validation process with the aim of minimizing the deviance based on the log partial likelihood for a Cox model. Kaplan-Meier survival curves depicted the survival functions of quartiles based on ERS. Survival curves were adjusted for covariates via the inverse probability weights method. Propensity scores were computed using multinomial logistic regression models, with ERS quartiles as outcome variables and adjustment covariates (as stated above) as predictors.

Cox survival models assessed interactions between genetic factors (ALS-/metal-PGS) and plasma/urine metal levels. Models were adjusted for age at diagnosis, sex, family history of ALS, bulbar vs. non-bulbar onset, initial El-Escorial criteria, ALSFRS-R, time between symptom onset and diagnosis, the first five genetic principal components, and *C9orf72* gene positivity.

Occupational and non-occupational exposures

Occupational¹¹ and non-occupational²⁴ data were generated as previously described. Briefly, participants provided a list of prior job titles and responsibilities that were sorted into standard occupational classification (SOC) codes (Supplemental Table 3) and answered questions regarding known metal exposures in their most recent occupation and non-occupational settings. SOC codes were separated into high- and low-metal exposure groups based on previously published results.¹¹ Distributions of ERS^{PR} and ERS^{UR} by high-/

low-metal exposure groups were compared using Wilcoxon tests. Associations between occupational/non-occupational metal exposures and urine/plasma metals were assessed using the partial least squares method feature in the R-package “mixOmics”²⁵ and estimated associations were visualized in heatmap plots.

Associations between metal concentrations and metal-PGS

An analysis was conducted to assess the associations between the metal-PGS and log concentrations of metals in plasma and urine. The associations were examined using linear regression models adjusted for sex and two genetic ancestry principal components.

RESULTS

Participants and metals analysis

The full population cohort included 454 ALS and 295 control participants, with plasma samples from 387 ALS and 281 control participants (Table 1, Figure 1), and urine samples from 322 ALS and 194 control participants (Table 2, Figure 1). In both the plasma (Table 1) and urine (Table 2) cohorts, ALS individuals were older, more frequently male, and had lower educational attainment than control participants. ALS characteristics were consistent between plasma and urine cohorts with 93% and 91% of participants presenting with classical ALS, 31% and 27% meeting El Escorial Definite ALS criteria, and 26% and 27% having bulbar onset, respectively (Tables 1 and 2). Both plasma and urine samples were available for 255 ALS and 181 control participants (Supplemental Table 4).

Among the 42 familial ALS cases in our plasma sample cohort, 9 patients had a *C9orf72* mutation, 7 patients had other mutations, 15 patients had no mutations identified, and 11 patients had no available test results (Supplemental Table 5A). In the 30 familial ALS cases in our urine sample cohort, 7 patients had a *C9orf72* mutation, 5 patients had other mutations 12 patients had no mutations identified, and 6 patients had no available test results (Supplemental Table 5B).

Concentrations of the metals detected in plasma and urine samples are listed in Supplemental Tables 6 and 7, respectively. Metals found below the LOD in over 40% of plasma samples included aluminum, beryllium, mercury, molybdenum, and uranium, while in urine samples, these were beryllium, chromium, manganese, mercury, and silver. Among plasma metals, levels of cadmium, copper, lead, selenium, vanadium, and zinc were significantly ($p_{adjusted} < 0.05$) higher in ALS versus control participants, while antimony, arsenic, barium, and strontium levels were lower (Supplemental Table 6). In urine, concentrations of aluminum, barium, cadmium, copper, iron, molybdenum, selenium, strontium, tin, uranium, vanadium, and zinc were significantly higher in ALS versus control participants (Supplemental Table 7). Correlations among metals were more pronounced in urine samples than plasma, and few metals showed significant interclass correlation between plasma and urine measures (Supplemental Figure 3).

ALS risk

Plasma. Odds ratios (ORs) for metals ranged from 0.58 for antimony to 1.58 for selenium (Figure 2). Metals significantly associated with elevated ALS risk included copper, lead, selenium, and zinc. Conversely, antimony, barium, nickel, and strontium were linked with reduced ALS risk. Metals included in the ERS^{PR}, to represent the metal mixture, are detailed in Supplemental Table 8. Analysis revealed a significant association between the ERS^{PR} and ALS risk (Supplemental Figure 4A), with a one standard deviation (SD) increase in ERS^{PR} based on the control population corresponding to an OR=2.95 ($p<0.001$, Figure 2).

The model was then adjusted for ALS-PGS to account for the cumulative burden of genetic predisposition and environmental exposure. Following adjustment for ALS-PGS (Supplemental Figure 5; Supplemental Table 9A), copper, selenium, and zinc associated with increased ALS risk, while antimony and strontium were linked to decreased risk. Notably, the ERS^{PR} for metal mixtures remained significantly elevated with OR=2.71 ($p<0.0001$). Next, models with metals and PGS interaction terms explored the interplay between ALS polygenic risk, metal exposure, and disease susceptibility. Only chromium exhibited a statistically significant interaction with the ALS-PGS ($p<0.01$).

We next incorporated a PGS constructed with genes related to metal metabolism (metal-PGS) into our risk model to account for metal metabolism variability. Adjustment for metal-PGS reaffirmed associations with copper, zinc, and selenium to increased ALS risk, and antimony, nickel, and strontium with decreased ALS risk (Supplemental Figure 6; Supplemental Table 9B). Again, the ERS^{PR} remained significantly elevated with an OR=2.93 ($p<0.0001$). Copper and molybdenum showed significant associations with the metal-PGS.

Urine. Analysis of urine samples revealed associations between barium, cadmium, copper, molybdenum, selenium, tin, uranium, vanadium, and zinc with increased ALS risk (Figure 3); no metals associated with decreased risk. Consistent with plasma models, the ERS^{UR} exhibited a significant association with ALS risk (OR=3.10, $p<0.001$; Supplemental Table 8; Supplemental Figure 4B). These findings remained consistent after adjusting for ALS-PGS (Supplemental Figure 7; Supplemental Table 9C) and metal-PGS (Supplemental Figure 8; Supplemental Table 9D). Significant metal and gene interactions were observed between molybdenum and ALS-PGS, as well as arsenic, barium, nickel, and vanadium with metal-PGS.

ALS survival

Plasma. No metals exhibited a significant impact on ALS survival (Figure 4). As expected, the ERS^{PS} representing the cumulative effects of multiple metals (Supplemental Table 8) associated with poorer survival (HR=1.37, $p<0.001$; Supplemental Figure 9A). ALS participants were stratified into quartiles according to their ERS^{PS}, revealing a striking difference in survival times between quartile 4 (1.59 years; highest exposure) and quartile 1 (3.75 years; lowest exposure; Figure 5A). Upon adjustment for ALS-PGS, cadmium, manganese, lead, and tin negatively impacted ALS survival, with interactions detected between ALS-PGS and molybdenum, selenium, and strontium (Supplemental Figure 10).

Following adjustment for metal-PGS, no significant associations were found between metal main effects and ALS survival. However, significant interactions were identified between metal-PGS and iron, manganese, thallium, and the ERS^{PS} (Supplemental Figure 11).

Urine. In contrast to plasma findings, multiple urine metals associated with shorter ALS survival, including cadmium, copper, nickel, tin, and zinc (Figure 6). However, consistent with plasma results, the ERS^{US} (Supplemental Table 8) correlated with shorter ALS survival (HR=1.44, $p<0.001$) (Supplemental Figure 9B). Survival curves based on ERS^{US} are depicted in Figure 5B, with estimated median survival times of 3.24 years in quartile 1 and 1.79 years in quartile 4, translating to a 1.45-year survival difference. After adjusting for ALS-PGS, copper, nickel, tin, and zinc continued to correlate with poorer survival, while cadmium did not (Supplemental Figure 12). Significant interactions were detected between antimony, barium, beryllium, molybdenum, nickel, selenium, uranium, zinc, and the ERS^{US} and the ALS-PGS ($p<0.1$). No associations were found between metals and ALS survival following adjustment for metal-PGS, although the ERS^{US} remained correlated with shorter survival (Supplemental Figure 13). Interactions between metals and metal-PGS were observed for beryllium, copper, thallium, tin, vanadium, zinc, and the ERS^{US}.

Metal concentrations and metal-PGS

The associations between metal concentrations and metal-PGS are summarized in Supplemental Table 10. Notably, an association was observed with beryllium in plasma, as well as inverse associations between aluminum and mercury in urine, with metal-PGS scores ($p<0.05$).

Occupational and non-occupational metal exposures

Plasma. ERS^{PR} scores for individuals grouped into the high-metal exposure group (job titles of “Building and Grounds Cleaning and Maintenance,” “Construction and Extraction,” “Installation, Maintenance, and Repair,” and “Production Occupations”) were significantly higher than ERS^{PR} scores for those grouped into the low-metal exposure group (Supplemental Figure 14A). Furthermore, occupational metal exposures positively associated with antimony, iron, and thallium levels (Supplemental Figure 15). Finally, reported exposures to metals outside the occupational setting positively associated with increased aluminum, cadmium, chromium, lead, manganese, uranium, vanadium, and zinc levels (Supplemental Figure 16).

Urine. Again, individuals in the high-metal exposure group based on SOC codes had higher ERS^{UR} scores than those in the low-metal exposure group (Supplemental Figure 14B). Self-reported occupational metal exposures positively associated with mercury and thallium levels (Supplemental Figure 17). Additionally, self-reported metal exposure in non-occupational activities demonstrated positive associations with arsenic, cadmium, copper, iron, lead, mercury, molybdenum, nickel, selenium, strontium, thallium, tin, uranium, and zinc (Supplemental Figure 18).

Supplement/vitamin usage

Recognizing the potential impact of supplement usage on metal levels, we compared metal levels characterized by participants' supplement/vitamin usage. In plasma samples, significant differences in median metal concentrations between individuals using supplements/vitamins and those abstaining from them were only detected for selenium and vanadium (Supplemental Table 11). No significant differences were observed for urine measures (Supplemental Table 12). Overall, supplement/vitamin usage does not appear to substantially impact outcomes.

Sensitivity Analyses

To address potential residual confounding related to education, a sensitivity analysis was conducted by including education as an additional covariate in the logistic regression models. The results were consistent with our original findings in both plasma samples (Supplemental Table 13) and urine samples (Supplemental Table 14). This suggests that the observed associations are robust and not significantly influenced by differences in education levels.

Additionally, to address the potential impact of smoking behavior on our findings, we included smoking status (Former Smoker, Smoker, Non-Smoker) as an additional covariate in both logistic regression and survival analyses. The findings continued to align closely with the original analyses for both plasma and urine samples, as detailed in Supplemental Table 15 (risk) and Supplemental Table 16 (survival) for plasma, as well as Supplemental Table 17 (risk) and Supplemental Table 18 (survival) for urine. Notably, the sensitivity analyses demonstrated significant associations between elevated levels of aluminum and antimony in urine with increased ALS risk, and higher selenium in urine with shorter ALS survival, which were not observed in the original analyses ($p_{adjusted} < 0.05$). Despite these changes, the ORs and HRs for these metals remained consistent between the original and sensitivity analyses, indicating that the overall strength and direction of the associations did not significantly change.

A sensitivity analysis using survival time defined as the time from the date of sample collection was conducted to address potential bias from the time gap between ALS diagnosis and sample collection. The results were consistent with our original findings in both plasma (Supplemental Table 19) and urine (Supplemental Table 20) samples, reinforcing the robustness of our initial analyses.

DISCUSSION

ALS is influenced by genetic and environmental factors, including metals.¹⁰ By measuring plasma and urine metal levels, we confirmed that individual metals, such as copper, selenium, and zinc, associated with greater ALS risk and shorter survival (Figure 7). Notably, the most robust associations with both risk and survival were observed with cumulative ERS, representing a mixture of multiple metals. Despite its influence on ALS susceptibility, genetic background did not significantly alter the relationship between metal exposure and ALS risk or survival. Finally, engagement in occupations with heightened

metal exposure associated with increased ERS. Overall, our report supports previous research and underscores the pivotal role of metals in ALS pathophysiology within this Michigan-based cohort.

The associations between copper, selenium, and zinc and ALS are consistent with previous clinical studies.^{6–9, 26} Exposure to these metals can occur through diet, drinking water, supplements, and environmental pollutants. In preclinical models, accumulation of these metals induces oxidative stress, disrupts mitochondrial function, and impairs protein folding.^{8, 27, 28} all mechanisms implicated in ALS.¹ For selenium, excess levels promote SOD1 accumulation in the mitochondria of human neuroblastoma cells,⁸ a feature also observed in neurons during ALS pathogenesis.⁸ In regards to zinc, the superoxide dismutase 1 (SOD1) enzyme protects cells from oxidative stress and zinc ions are essential for SOD1 stability and enzymatic activity. Mutations in SOD1 are associated with ALS¹ and alterations in zinc levels cause SOD1 misfolding and aggregation.²⁹ Indeed, chronic oral administration of zinc sulfate to transgenic mice overexpressing the human mutated form of SOD1 (G93A) decreases survival.³⁰ While the exact mechanisms linking these metals to ALS are not fully understood, accumulating evidence suggest that their altered levels contribute to ALS pathophysiology.

In the current study, antimony, nickel, and strontium associated with decreased ALS risk. Research investigating the effects of antimony on human health are limited. However, antimony associates with neurobehavioral changes and neurotoxicity in preclinical models.³¹ Regarding nickel, a population-based case-control study in Denmark did not detect an association between occupational exposure to nickel and ALS.³² It is surprising that nickel would be protective against ALS, considering it influences oxidative stress, increases DNA damage, and induces apoptosis.³² Similarly, the protective role of strontium conflicts with our previous study which analyzed human permanent teeth and found that strontium during late childhood and adolescence correlated with ALS.⁹ This discrepancy could stem from the biosample analyzed, as strontium uptake in teeth occurs during tooth formation, reflecting exposure levels during that specific developmental stage.⁹ Thus, the potential protective effects of antimony, nickel, and strontium against ALS warrant further confirmation and investigation.

The use of plasma and urine samples to measure metal exposures offers several advantages, e.g., urine sampling is relatively easy and non-invasive, and both reflect integrated exposure. However, measurements must be interpreted cautiously. Levels in plasma and urine may fluctuate due to diet, hydration status, and circadian rhythms, potentially leading to variability in measurements;¹⁴ though, it is worth noting that supplement or vitamin use did not impact metal levels in the current study. Additionally, blood and urine often represent recent, rather than chronic, exposures. Moreover, some metals may not be well-represented in plasma or urine. Zinc is distributed intracellularly in the musculoskeletal system and plasma zinc does not represent intracellular accumulation.¹⁴ Urinary zinc is an accurate biomarker but again represents recent exposures.¹⁴ Biomarkers such as hair, teeth, and nails can provide a historical record of metal accumulation over time and have been used to study chronic metal exposure and ALS.^{9, 33, 34} However, challenges remain regarding biomarker

suitability, variability in sample quality, and growth rate.¹⁴ Thus, selecting the appropriate biomarker is crucial for accurately detecting metal levels and assessing exposure.

Most prior epidemiological investigations involving metals and ALS associations centered on individual metals as opposed to mixtures. However, a mixtures approach using the ERS better reflects reality. Here, we found that metal mixtures, represented by ERS, had a greater effect on ALS risk and survival than single metal exposures. ERS derived from the quantification of metal levels in biofluids have been used to explore the relationship between metal exposure and disease across various health domains, including obesity and its comorbidities,³⁵ glucose homeostasis,³⁶ and heart rate regulation.³⁷ Thus, ERS are a valuable tool for studying multi-pollutant exposures and their impact on disease.

The gene-environment hypothesis suggests that interactions between genetic factors and environmental exposures influence an individual's risk for developing a particular disease.¹ Several studies suggest that environmental factors, such as metals, interact with genetic variants to influence ALS onset, progression, and severity.^{12, 13} Additionally, previous studies successfully integrated environmental and genetic factors into risk prediction models for cancer.^{38, 39} However, we found that accounting for ALS risk genes or genes involved in metal metabolism did not influence the relationship between metal exposure and ALS. Relationships between genes, environmental exposures, and disease risk are complex and adjusting for underlying genetic heterogeneity may not fully capture the complicated nature of these interactions. Additionally, our current knowledge of the genetic basis of ALS and metal metabolism may be incomplete and other genetic factors or pathways involved in ALS risk or metal metabolism may be unaccounted for in our current PGS. Construction of our metal-PGS was limited by the lack of available relevant GWAS of metal levels and is, in turn, a weak genetic instrument for assessing any potential interaction with ALS-PGS. Its association with only a few metals further confirms the need for a more robust future metal-PGS prior to drawing meaningful conclusions. We also do not know how well the metal-PGS reflects metals metabolism. Factors such as efficiency of elimination and excretion of metals from the body may explain inverse associations observed with urine metals. Future studies require a newly constructed metal-PGS in the setting of well-characterized exposures and samples measured in a relevant period to determine true interaction with metal exposures.

Finally, we previously showed significant associations between workplace metal exposure and ALS risk and identified SOC codes that were likely to report occupational exposure to metals.¹¹ Here, ALS participants categorized into a high-metal exposure group exhibited higher ERSs compared to those in the low-metal exposure groups. These results support the finding that occupations linked to metal exposures associate with elevated ALS risk. Furthermore, occupational and non-occupational metal exposures²⁴ correlated with metal levels in plasma and urine. This association underscores the potential impact of both activities on overall metal exposure in individuals, emphasizing the necessity of accounting for occupational and environmental sources when evaluating overall exposure risk.

Recognizing these exposures as modifiable risk factors may open avenues for targeted prevention. By avoiding high-risk activities associated with occupational and non-

occupational metal exposures, individuals might lower their overall exposure levels, potentially mitigating ALS risk. However, the strength of these associations varied, with some being weak or even negative. This variability highlights the complex nature of metal exposures and their measurements. Given this variability, further research is needed to better understand these associations and their implications for ALS pathogenesis.

This study benefits from a large sample size and biomarker-based exposure measurements, reducing recall bias. However, limitations do exist. First, the study represents a cross-sectional analysis and may miss potential fluctuations in metal levels over time. Consequently, this snapshot may not accurately reflect lifetime exposures as metal levels are subject to sampling variation and metabolic pathways, potentially altering their concentrations over extended periods. Second, sampling after disease onset may confound results, blurring the distinction between disease progression effects and true baseline measures, and future longitudinal prospective studies will be crucial. Third, while our findings suggest an association between metals and ALS, they do not establish causation. Fourth, unlike the ALS-PGS, the metal-PGS does not have a large GWAS from which to draw weights and its construction is more ad hoc and less robust than standard methods used for the ALS-PGS. Large genetic studies of xenobiotic metabolism would be critical to further understand gene-environment interactions. Fifth, there is a potential limitation related to the time gap between diagnosis and sample collection. This variability introduces a form of left truncation, as patients must survive until the time of sample collection to be included in the study, potentially biasing our findings by excluding patients with shorter survival times who may have different exposure profiles. Future studies with more uniform timing of sample collection relative to diagnosis are necessary to validate our findings and mitigate this potential source of bias. Sixth, although we adjusted for age, sex, and military service, other potential confounders, such as socioeconomic status and lifestyle factors, may not be fully accounted for. Sensitivity analyses indicated that education and smoking did not significantly impact our findings, but we recognize the possibility of unidentified confounding factors that could influence the results. Lastly, given the study used a Michigan-based cohort, validation across different cohorts is critical for broader generalizability.

To conclude, we demonstrate that metal mixtures, as represented by cumulative ERS, associate with increased ALS risk and reduced ALS survival, regardless of genetic predisposition. These associations correlate with self-reported occupational metal exposure. Utilizing quantitative assessments of metal levels in biofluids like plasma or urine could offer a pathway to predict future ALS risk, providing a promising avenue for early identification and intervention. Overall, these results offer valuable insights into the complexities of ALS pathogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data Availability

Sharing of non-identifiable data will be considered at the reasonable request of a qualified investigator.

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What is already known on this topic:

Metal exposure is linked to amyotrophic lateral sclerosis (ALS) risk and survival, however information regarding the impact of metal mixtures and the influence of underlying genetic heterogeneity on the relationship between metals and ALS is lacking.

What this study adds:

This study demonstrates that mixtures of metals significantly associate with increased risk of ALS and reduced survival, independent of genetic risk. Additionally, occupations linked to increased metal exposure correlate with higher environmental risk scores, supporting the relationship between occupational metal exposure and increased ALS risk.

How this study might affect research, practice or policy:

This study reinforces the relationship between metal exposure and ALS pathophysiology and underscores the importance of further research into the underlying mechanisms driving this association to inform targeted risk reduction strategies.

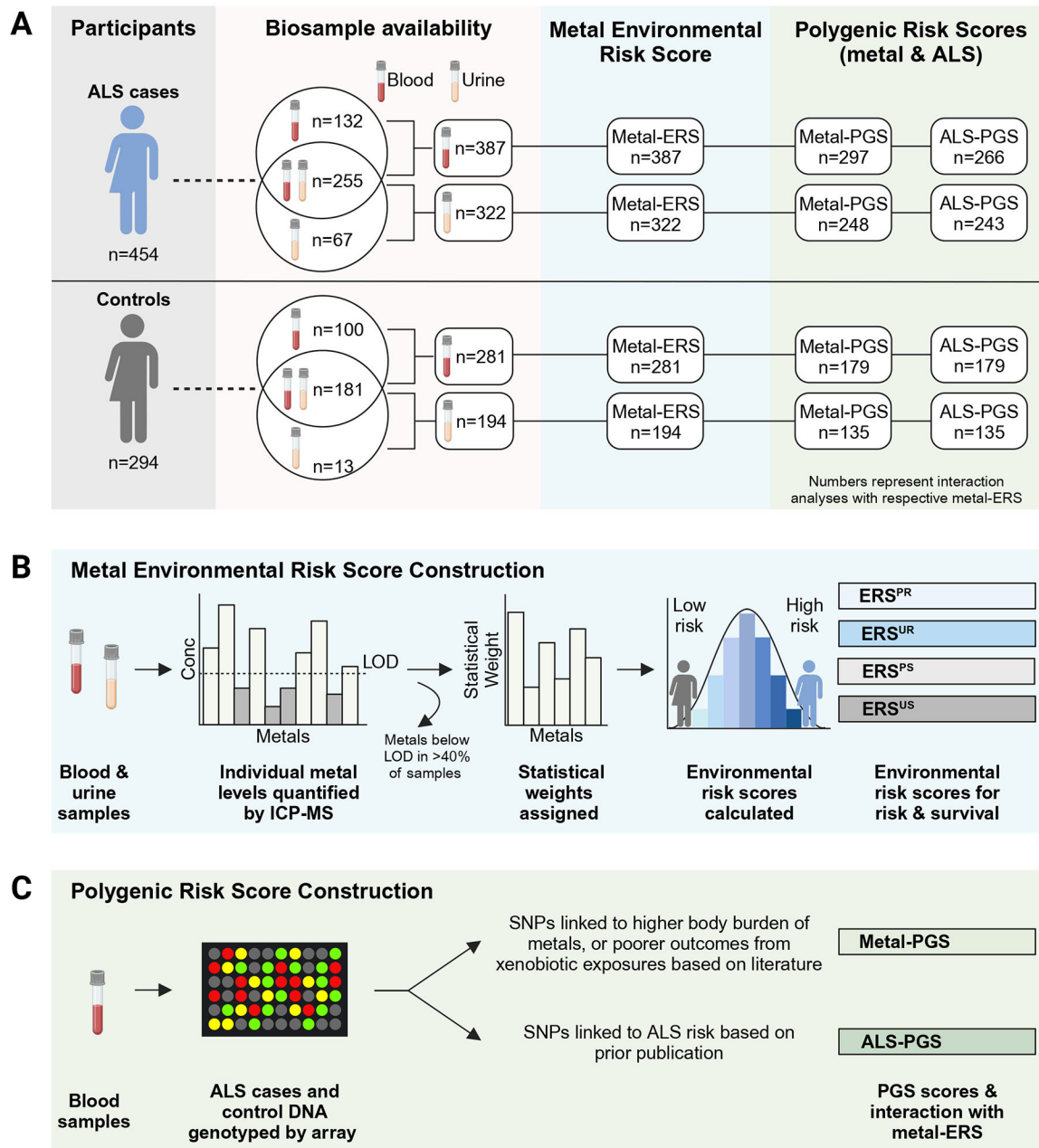


Figure 1. Study overview.

(A) Participants with (n=454) or without (n=294) amyotrophic lateral sclerosis (ALS) provided blood and/or urine samples. Environmental risk scores (ERS) of multiple metals associated with ALS risk and survival were calculated for both blood and urine samples. Polygenic risk scores (PGS) for ALS risk or genes related to metal metabolism were available for a subset of participants. (B) Blood and urine samples were collected from study participants and analyzed by inductively coupled plasma mass spectrometry (ICP-MS) to measure the concentrations of various metals. After removing metals in which over 40% of the samples fell under the limit of detection (LOD), the statistical weight of each metal was determined, and overall ERSs were calculated. Individual ERS for plasma and urine samples

were developed for risk (plasma, ERS^{PR}; urine, ERS^{UR}) and survival (plasma, ERS^{PS}; urine, ERS^{US}). These scores represent the cumulative exposure to various metals and serve as a comprehensive indicator of environmental risk. Image created using [BioRender.com](https://www.biorender.com). (C) DNA samples from ALS and control participants were genotyped to obtain single-nucleotide polymorphisms (SNP) data. ALS-PGS was computed based on statistical weights from an independent genome-wide association study (GWAS). The metal-PGS was constructed using unweighted SNPs linked to higher body burden of metals or poorer outcomes from xenobiotic exposures based on a review of multiple publications from the literature.

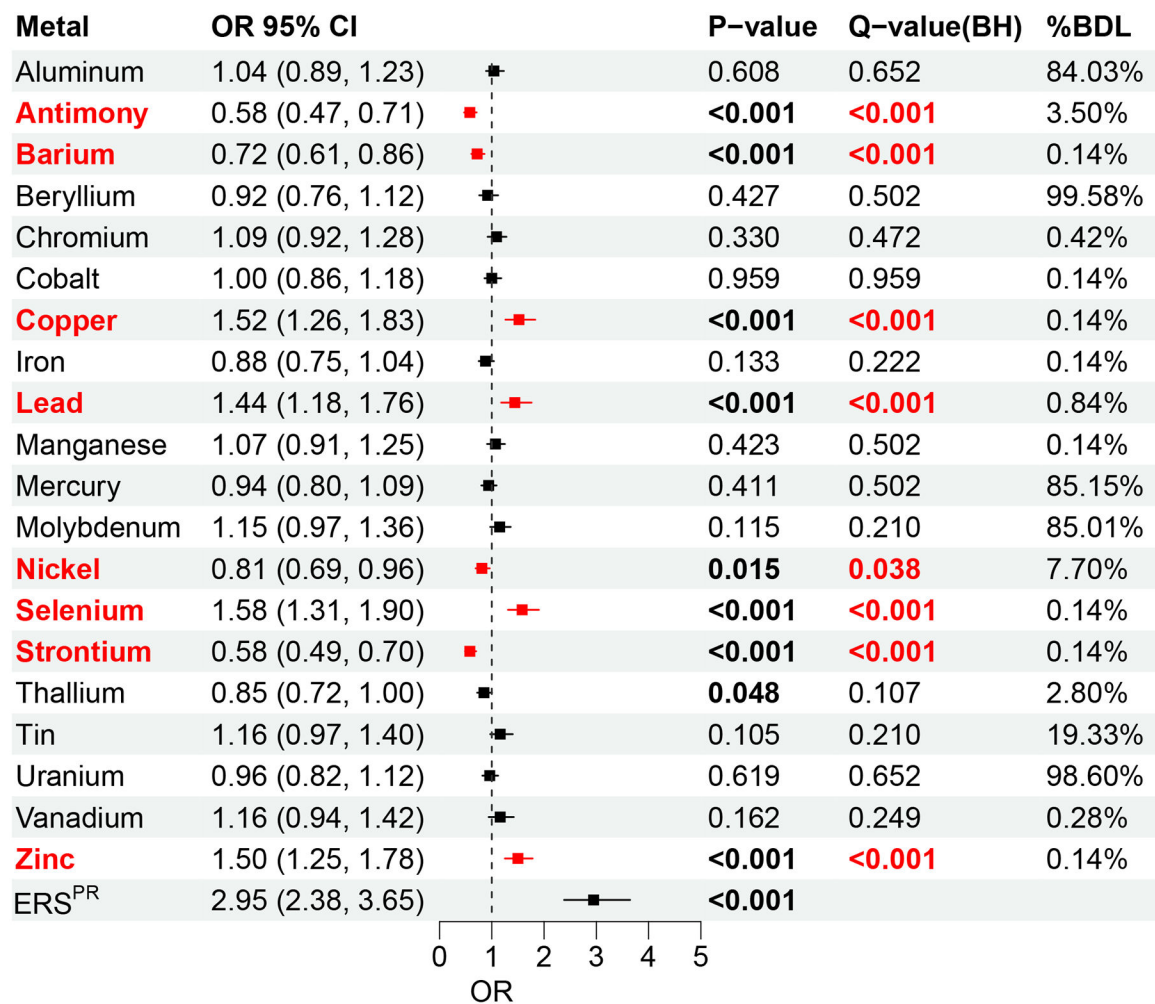


Figure 2. Plasma single metal and mixture associations with ALS risk.

Single metal logistic regression models based on plasma samples from ALS (n=387) and control (n=281). The outcome is case/control status, the predictors are log-transformed, standardized metal levels, and the covariates are continuous age at sample collection, sex, and military service. Mixture metal ERS^{PR} (plasma risk environmental risk score). %BDL, percentage of samples below detection limit; ALS, amyotrophic lateral sclerosis; BH, Benjamini-Hochberg, red font are significant correlations; CI, confidence interval; OR, odds ratio corresponding to one standard deviation increase in log-transformed metals.

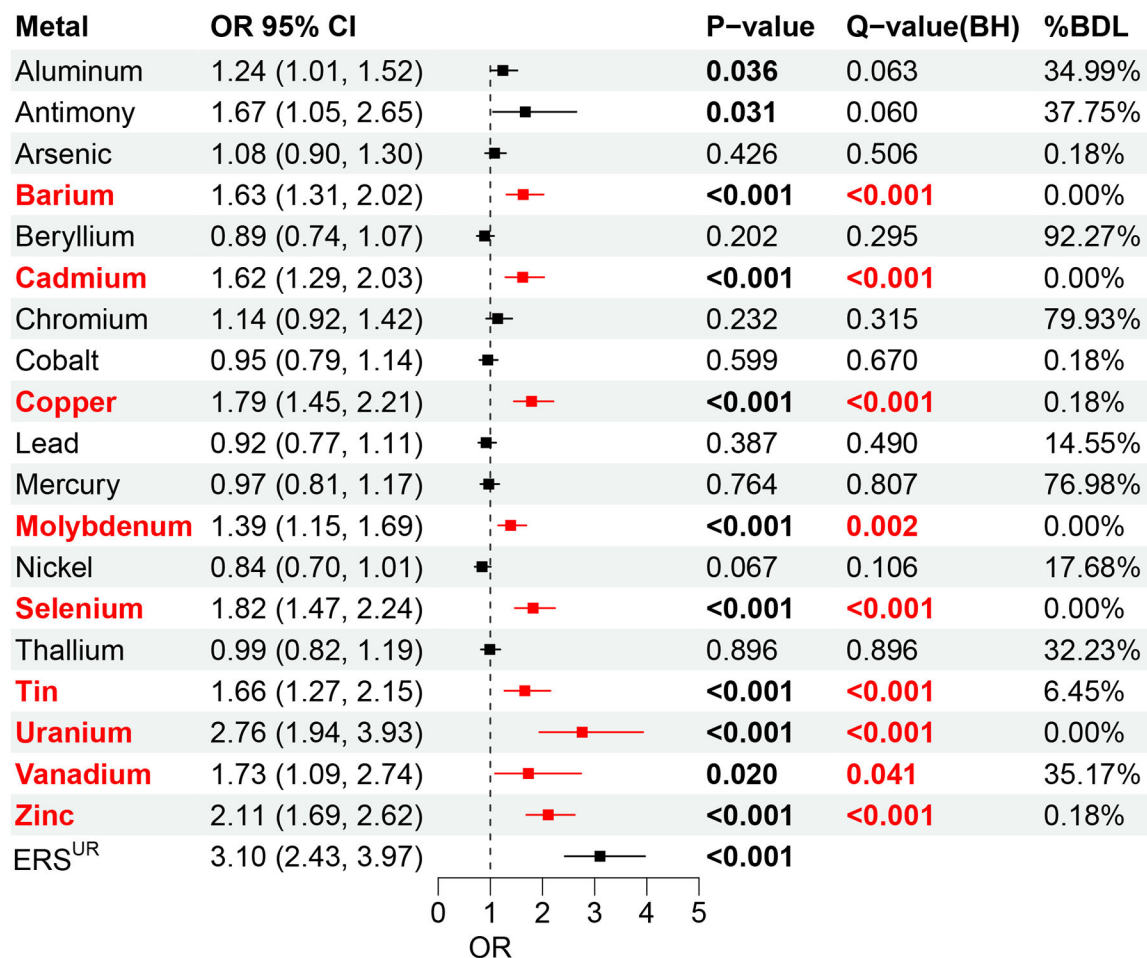


Figure 3. Urine single metal and mixture associations with ALS risk.

Single metal logistic regression models based on urine samples from ALS (n=322) and controls (n=194). The outcome is case/control status, the predictors are log-transformed, standardized metal levels, and the covariates are continuous age at sample, sex, military service. Mixture metal ERS^{UR} (urine risk environmental risk score). %BDL, the percentage of samples below detection limit; CI, confidence interval; ALS, amyotrophic lateral sclerosis; BH, Benjamini-Hochberg, red font are significant correlations; OR, odds ratio corresponding to one standard deviation increase in log-transformed metals.

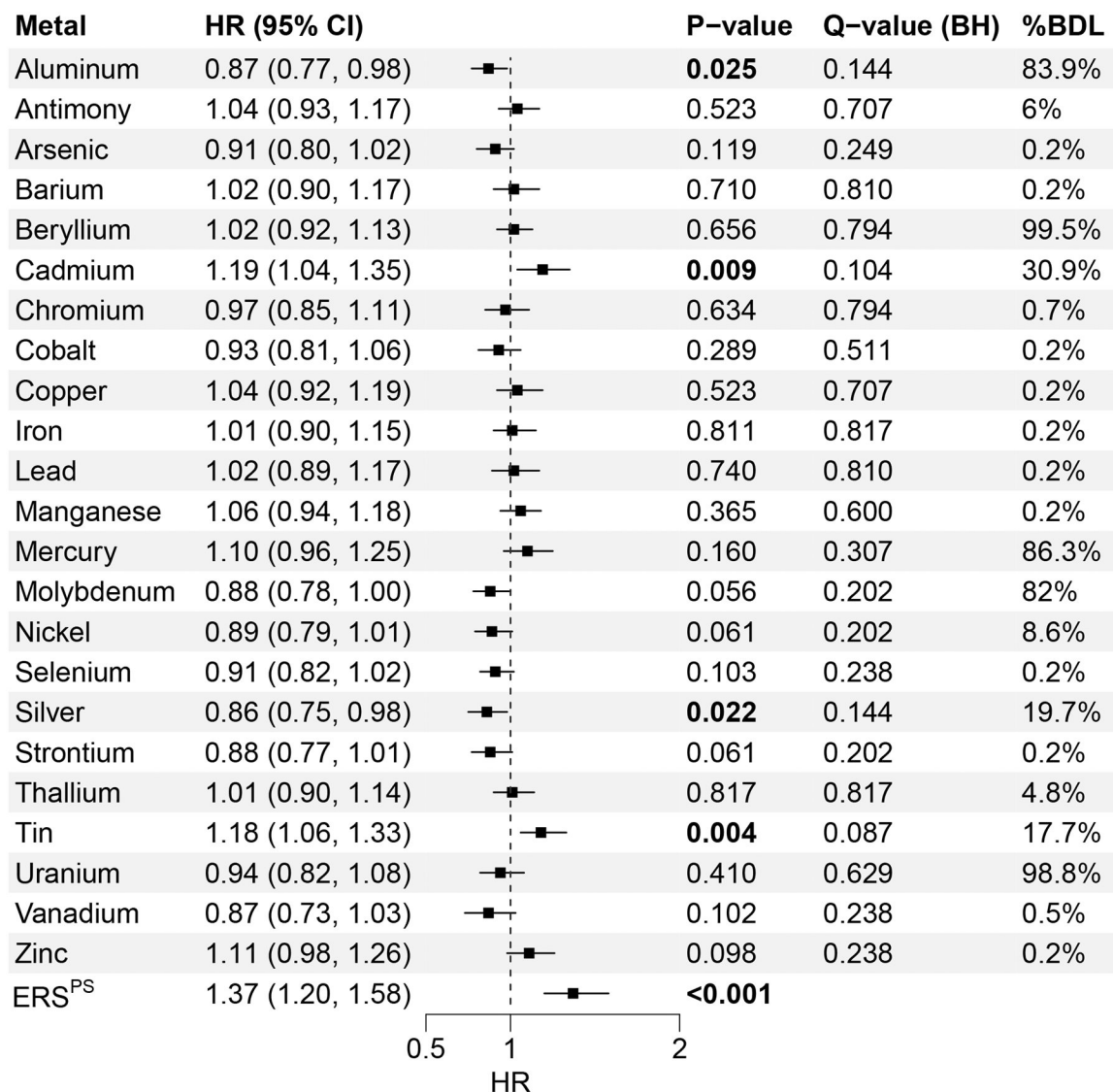
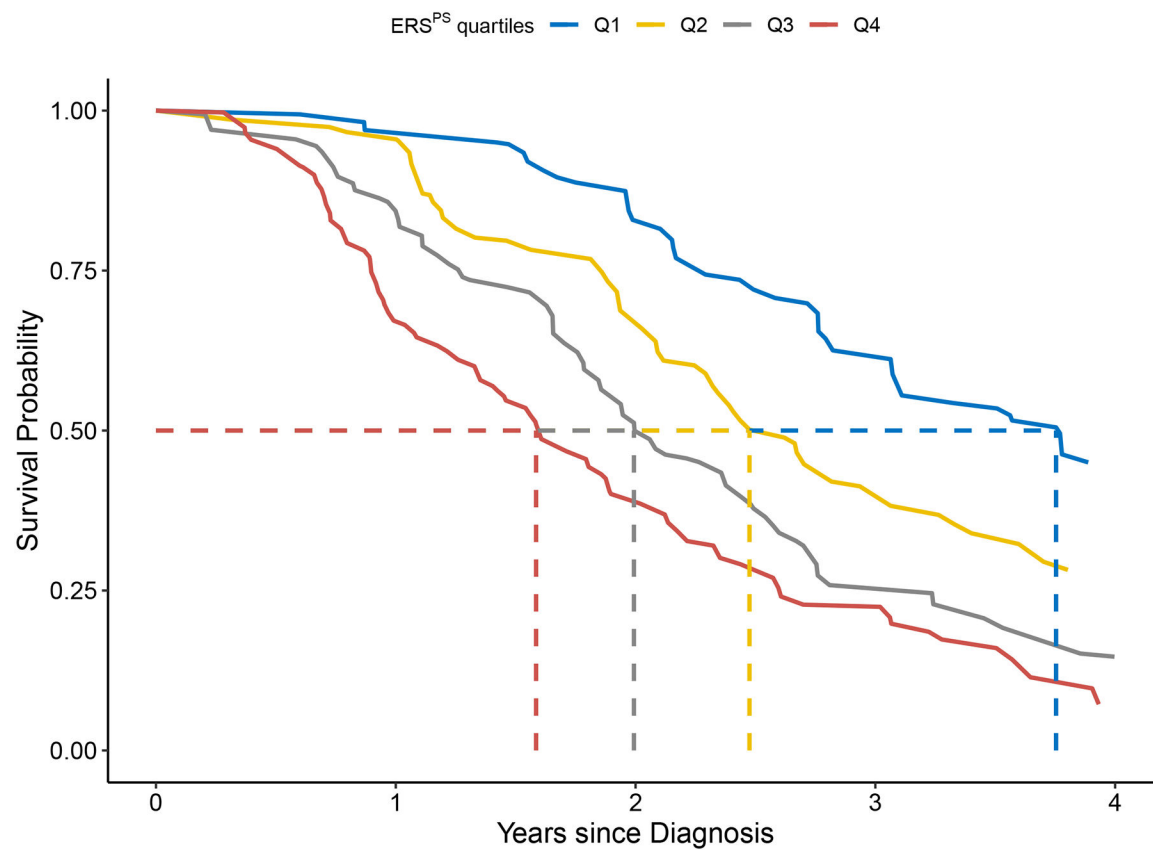


Figure 4. Cohort survival analysis based on plasma samples.

Cox proportional hazards survival models from plasma samples from ALS cases (n=387). The outcome is survival time since diagnosis (in years), the predictors are log-transformed, standardized metal levels, and adjustment covariates are age at diagnosis, sex, family history of ALS, onset segment, El Escorial criteria, ALSFRS-R, and time between symptom onset and diagnosis. %BDL, the percentage of samples below detection limit; BH, Benjamini-Hochberg, red font are significant correlations; CI, confidence interval; ERS^{PS}, plasma survival environmental risk score; HR, hazards ratio corresponding to one standard deviation increase in log-transformed metals.



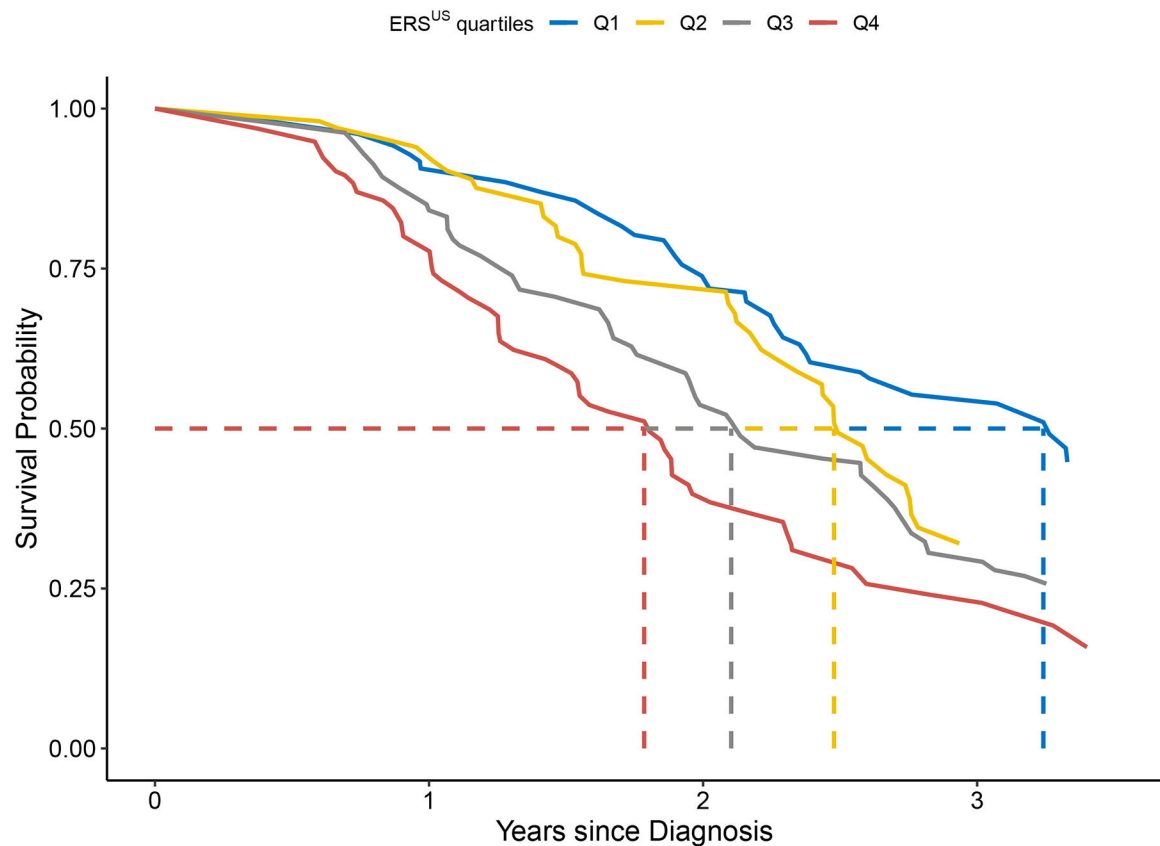


Figure 5. Adjusted survival curves stratified by environmental risk score (ERS) quartile.

Kaplan-Meier survival curves of ERS quartiles adjusted for covariates with the inverse probability weights method for (A) plasma (ERS^{PS}) and (B) urine (ERS^{US}) sample sets. Adjusted covariates are age at sample collection, sex, military service, and family history of ALS. Dashed lines indicate the median survival in each ERS strata. The estimated adjusted median survival times for plasma (A) are 3.75 years for Quartile 1 (Q1), 2.48 years for Quartile 2 (Q2), 1.99 years for Quartile 3 (Q3), and 1.59 years for Quartile 4 (Q4). Estimated adjusted median survival times for urine (B) are 3.24 years for Q1, 2.48 years for Q2, 2.10 years for Q3, and 1.79 years for Q4. Adjusted survival curves and adjusted median survival time estimates are pooled across all 20 imputed datasets.

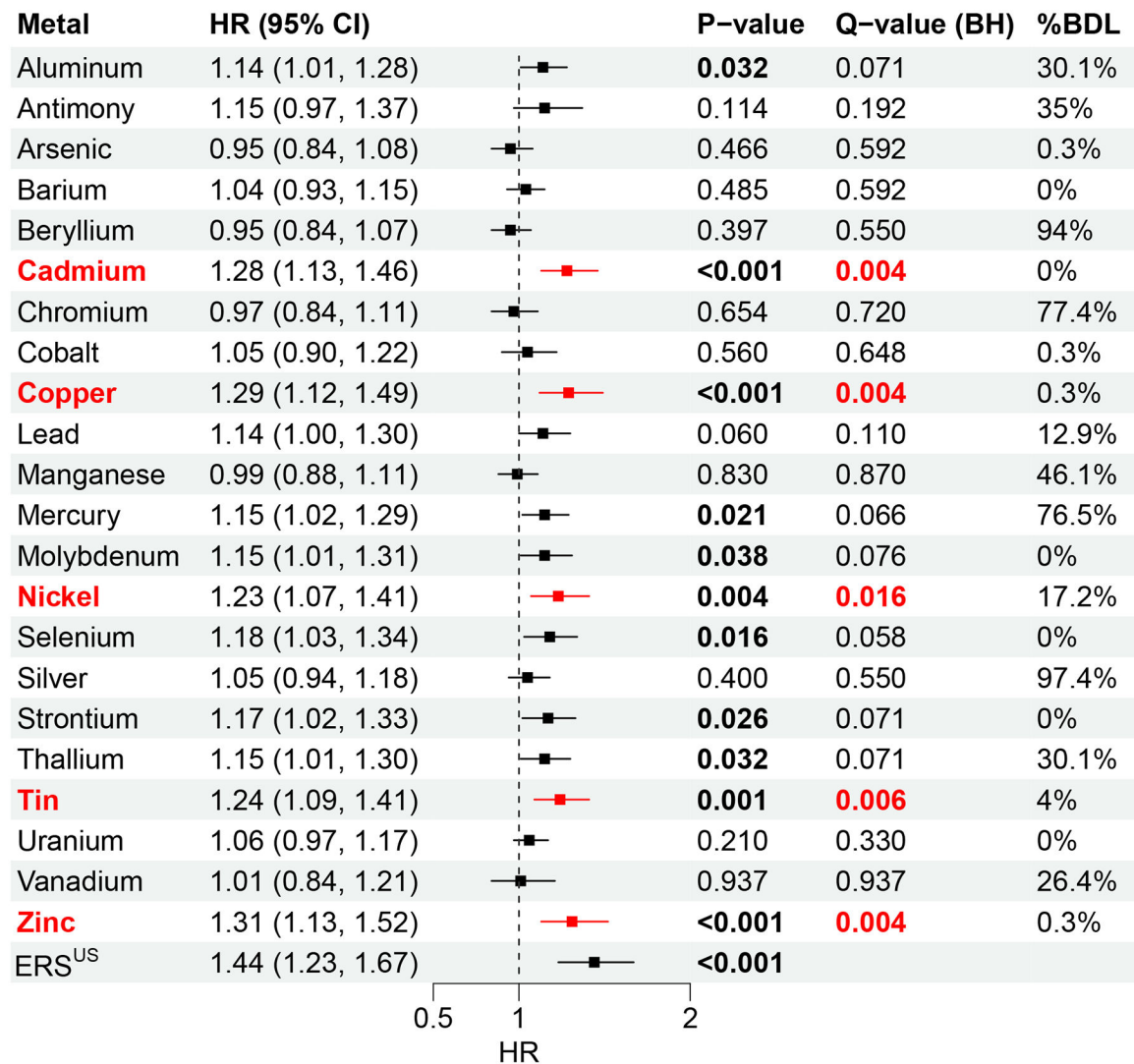
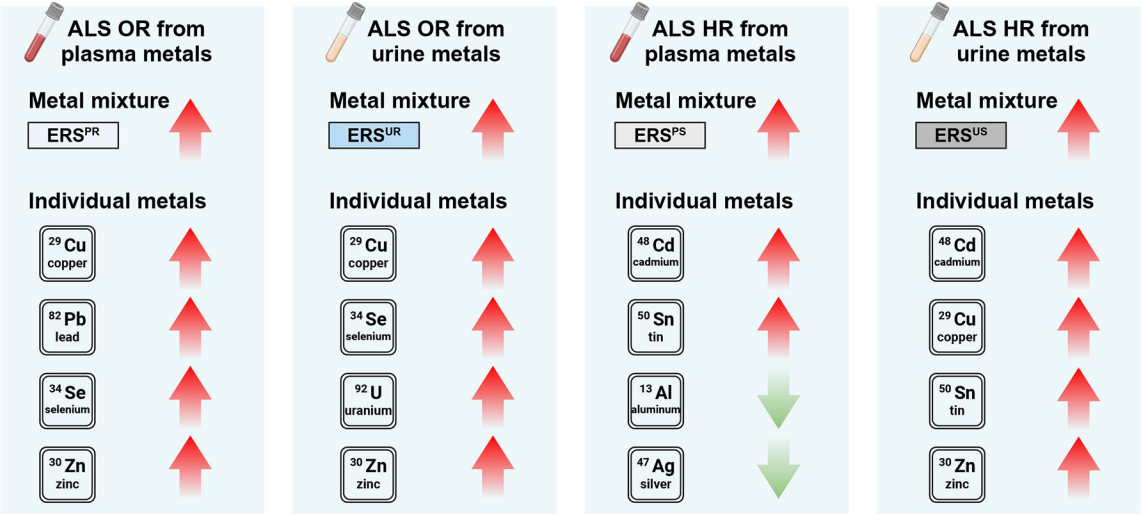


Figure 6. Cohort survival analysis based on urine samples.

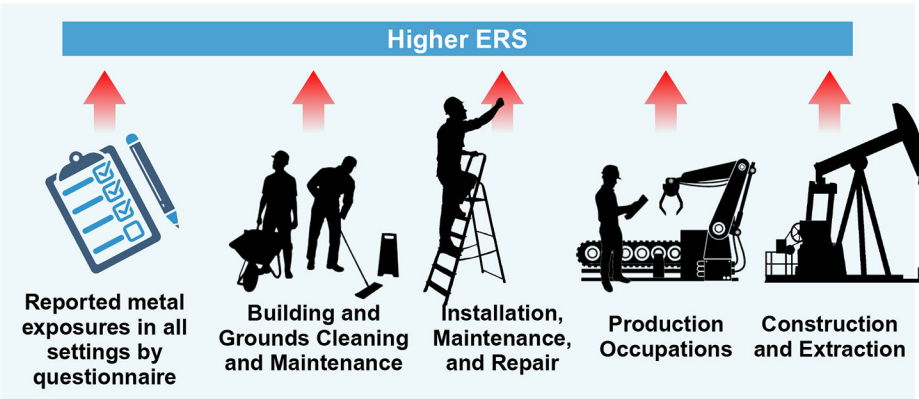
Cox proportional hazards survival models from urine samples from ALS cases (n=322).

The outcome is survival time since diagnosis (in years), the predictors are log-transformed, standardized metal levels, and adjustment covariates are age at diagnosis, sex, family history of amyotrophic lateral sclerosis (ALS), onset segment, El Escorial criteria, ALSFRS-R, and time between symptom onset and diagnosis. %BDL, the percentage of samples below detection limit; BH, Benjamini-Hochberg, red font are significant correlations; CI, confidence interval; ERS^{US}, urine survival environmental risk score; HR, hazards ratio corresponding to one standard deviation increase in log-transformed metals.

A Correlation of individual metals and metal mixtures on ALS risk and survival



B Correlation of metal mixture ERS to occupations



C ERS and PGS interaction

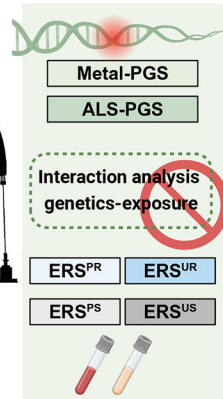


Figure 7. Study summary.

(A) Associations of individual metals and environmental risk scores (ERS) with ALS risk and survival. Variables with odds ratios (OR) >1 or hazard ratios (HR) >1 are indicated as red upward arrows, while those with OR<1 or HR<1 are indicated as green downward arrows. **(B)** Associations between ERSs and occupational metal exposures. Occupations and occupational metal exposures are reported by questionnaire. Occupational groups are defined by Standard Occupational Classification (SOC) codes. Four occupational groups identified in the literature as exhibiting high metal exposure levels: “Building and Grounds Cleaning and Maintenance” (SOC 37), “Construction and Extraction” (SOC 47), “Installation, Maintenance, and Repair” (SOC 49), and “Production Occupations” (SOC 51) had higher ERS^{PR} and ERS^{UR}. **(C)** We did not observe evidence of interaction between PGS and ERS measures on ALS risk and survival models.

Table 1.
ALS and control participant demographics: Plasma

Covariate	ALS, N = 387 ¹	Control, N = 281 ¹	p-value ²
Age at Sample Collection	65 (58–73)	62 (55–68)	<0.001
Sex			0.038
Female	171 (44%)	147 (52%)	
Male	216 (56%)	134 (48%)	
Ethnicity			0.036
Hispanic or Latino	4 (1.0%)	9 (3.4%)	
Not Hispanic or Latino	378 (99%)	255 (97%)	
Missing	5	17	
Race			0.2
American Indian and Alaska native	2 (0.5%)	0 (0%)	
Asian	1 (0.3%)	4 (1.4%)	
Black or African American	13 (3.4%)	10 (3.6%)	
White or Caucasian	370 (96%)	267 (95%)	
Missing	1	0	
Military Service	46 (12%)	22 (8.9%)	0.2
Missing	15	35	
Education			<0.001
High school or less	109 (29%)	13 (5.3%)	
Bachelor's degree	75 (20%)	93 (38%)	
Some postsecondary	125 (34%)	51 (21%)	
Graduate degree	63 (17%)	90 (36%)	
Missing	15	34	
Family History of ALS	42 (11%)	0 (0%)	<0.001
Missing	12		
C9orf72 Expansion			0.005
Positive	20 (7.9%)	0 (0%)	
Negative	233 (92%)	95 (100%)	
Missing	134	186	
Age at Diagnosis	65 (57–72)		
Missing	2		
Diagnosis			
ALS	358 (93%)		
ALS/Frontotemporal dementia	17 (4.4%)		
Brachial amyotrophic diplegia	6 (1.6%)		
Flail leg	2 (0.5%)		
Progressive muscular atrophy	4 (1.0%)		
El Escorial Criteria			
Definite	114 (31%)		
Probable	114 (31%)		

Covariate	ALS, N = 387 ¹	Control, N = 281 ¹	p-value ²
Probable, lab supported	100 (27%)		
Possible	42 (11%)		
Suspected	0 (0%)		
Missing	17		
Onset Segment			
Bulbar	99 (26%)		
Cervical	126 (33%)		
Lumbar	152 (39%)		
Respiratory	4 (1.0%)		
Thoracic	4 (1.0%)		
Missing	2		
ALSFRS-R	37 (33–41)		
Missing	2		
Time Between Symptom Onset and Diagnosis (Years)	1.04 (0.64–1.77)		
Missing	5		
Time Between Diagnosis and Sample (Years)	0.58 (0.27–1.13)		
Missing	2		

¹Median (25%–75%); n (%)

²Wilcoxon rank sum test; Pearson's Chi-squared test; Fisher's exact test

Abbreviations: ALS, amyotrophic lateral sclerosis; ALSFRS-R, Revised ALS Functional Rating Scale

Table 2.
ALS and control participant demographics: Urine

Covariate	ALS, N = 322 ¹	Control, N = 194 ¹	p-value ²
Age at Sample Collection	65 (58–72)	61 (55–68)	<0.001
Sex			<0.001
Female	122 (38%)	107 (55%)	
Male	200 (62%)	87 (45%)	
Ethnicity			0.022
Hispanic or Latino	3 (0.9%)	8 (4.3%)	
Not Hispanic or Latino	316 (99%)	179 (96%)	
Missing	3	7	
Race			0.3
American Indian and Alaska native	1 (0.3%)	0 (0%)	
Asian	2 (0.6%)	2 (1.0%)	
Black or African American	8 (2.5%)	10 (5.2%)	
White or Caucasian	310 (97%)	182 (94%)	
Missing	1	0	
Military Service	37 (12%)	16 (9.6%)	0.4
Missing	10	27	
Education			<0.001
High school or less	78 (25%)	8 (4.8%)	
Bachelor's degree	68 (22%)	66 (39%)	
Some postsecondary	110 (35%)	30 (18%)	
Graduate degree	56 (18%)	64 (38%)	
Missing	10	26	
Family History of ALS	30 (9.6%)	0 (0%)	<0.001
Missing	8		
C9orf72 Expansion			0.009
Positive	18 (8.4%)	0 (0%)	
Negative	196 (92%)	72 (100%)	
Missing	108	122	
Age at Diagnosis	65 (57–72)		
Missing	2		
Diagnosis			
ALS	292 (91%)		
ALS/Frontotemporal dementia	16 (5.0%)		
Brachial amyotrophic diplegia	10 (3.1%)		
Flail leg	1 (0.3%)		
Progressive muscular atrophy	3 (0.9%)		
El Escorial Criteria			
Definite	86 (28%)		
Probable	95 (31%)		

Covariate	ALS, N = 322 ¹	Control, N = 194 ¹	p-value ²
Probable, lab supported	84 (28%)		
Possible	37 (12%)		
Suspected	0 (0%)		
Missing	20		
Onset Segment			
Bulbar	85 (27%)		
Cervical	116 (36%)		
Lumbar	112 (35%)		
Respiratory	4 (1.3%)		
Thoracic	3 (0.9%)		
Missing	2		
ALSFRS-R	38 (34–41)		
Missing	1		
Time Between Symptom Onset and Diagnosis (Years)	1.05 (0.63–1.74)		
Missing	4		
Time Between Diagnosis and Sample (Years)	0.48 (0.25–0.88)		
Missing	2		

¹Median (25%–75%); n (%)

²Wilcoxon rank sum test; Pearson's Chi-squared test; Fisher's exact test

Abbreviations: ALS, amyotrophic lateral sclerosis; ALSFRS-R, Revised ALS Functional Rating Scale