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Longitudinal Metabolomics in Amyotrophic Lateral Sclerosis Implicates Impaired Lipid Metabolism

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Abstract

Objective: Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by altered metabolome and energy homeostasis, manifesting with body mass index changes and hypermetabolism—both prognostic of disease progression and survival. The cross-sectional ALS metabolome has been characterized, but longitudinal correlations to functional decline are lacking.

Methods: We longitudinally evaluated metabolomes from ALS plasma and terminal *postmortem* spinal cord and brain motor cortex tissue. We constructed three plasma models. A linear mixed effects model correlated all metabolite levels across all time points to their corresponding functional scores. An interaction model predicted longitudinal change in function from baseline metabolites, while a progression model identified metabolites linked to a 20 or 50% drop in function. In *postmortem* samples, differential metabolites in onset versus second spinal cord segments served as a surrogate of disease progression. Mendelian randomization assessed potential causality from metabolites.

Results: In plasma, all models primarily selected lipid metabolites and sub-pathways, in addition to amino acids, xenobiotics, and various less frequently selected pathways. Among lipids, fatty acids and sphingomyelins were predominant, along with plasmalogens, phosphatidylcholines, and lysophospholipids. Sex interaction findings were nominal. In spinal cord, sphingomyelin and long-

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Author Contributions

SAG and ELF contributed to the conception and design of the study; KG, MGS, D-GJ, SGT, LZ, JH, SAG, and ELF contributed to the acquisition and analysis of data; MGS, SAG, and ELF contributed to drafting the text or preparing the figures.

Potential Conflicts of Interest

The authors report no competing interests relevant to this publication.

Supplementary information

The online version contains supplementary material.

chain saturated and monounsaturated fatty acids were more abundant in the onset segment tissue, whereas phosphatidylcholines and phosphatidylethanolamines were less abundant. Mendelian randomization suggested that impaired carnitine and short chain acylcarnitine metabolism may be genetically determined in ALS, along with various antioxidant derivatives.

Interpretation: Our findings suggest metabolomic changes primarily involving different lipid classes and carnitine metabolism may underscore ALS severity and progression.

Keywords

fatty acid; human; motor cortex; motor neuron disease; spinal cord; sphingomyelin

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease with death from respiratory failure within 2 to 4 years of symptom onset.^{1,2} While the primary pathology in ALS occurs in the central nervous system,³ changes in the metabolome,^{4–6} energy homeostasis,⁷ and body mass index (BMI)^{8,9} are an integral part of the disease process.

Altered metabolism in ALS is complex and multifaceted; ALS patients experience global hypermetabolism¹⁰ with elevated resting energy expenditure.¹¹ Metabolic changes occur across various tissues, including brain, spinal cord, and muscle, and result from alterations in both glucose and lipid metabolism as well as impaired mitochondrial function with inefficient tricarboxylic acid cycle and oxidative phosphorylation activity.⁹ This impaired metabolic function in ALS manifests by altered levels in energy substrates and intermediates; poor energy production in ALS could also impact downstream processes, inducing broad changes to the metabolome overall, including to bioactive metabolites and lipids.⁹ In cross-sectional analysis, specific metabolites significantly correlate with disease status, based on the revised ALS functional rating scale (ALSFRS-R),⁵ suggestive that metabolic status may reflect functional status.

Moreover, hypermetabolism¹² and steeper BMI changes¹³ associate with faster disease progression and poorer survival, indicating that metabolome changes may occur over the disease course longitudinally. However, despite well-established associations between the metabolome and ALS,^{4,5,9,14} most previous studies were cross-sectional. Our goal in this study was to overcome limitations of cross-sectional analyses and assess longitudinal changes in the ALS metabolome throughout the disease course, including the terminal time point. We hypothesized that plasma metabolite changes would track with disease progression, serving as potential biomarkers and offering new insights into ALS pathophysiology.

We performed untargeted plasma metabolomics early in the disease course and longitudinally 6 and 12 months later. In tandem, *postmortem* spinal cord and brain motor cortex were also analyzed in a participant subset, the first analysis, to our knowledge, of various central nervous system tissues in human ALS. In plasma, we generated models that associated metabolite abundance to ALS functional status by ALSFRS-R across the disease course. An interaction model leveraged baseline metabolites to predict change in ALSFRS-R

and progression model identified metabolites linked to a 20 and 50% drop in ALSFRS-R relative to baseline score. We also performed sex interaction analyses since sex impact metabolism¹⁵ and ALS,^{16,17} including metabolites.⁵ In tissue, mirroring disease progression, we assessed differential metabolites in the onset versus opposing spinal cord segment to identify species potentially linked to temporal changes in disease.

By leveraging longitudinal metabolomic profiles from a prospective cohort of individuals with ALS, our analysis identified several plasma lipid metabolites and lipid sub-pathways that correlated with disease course, along with select amino acids and xenobiotics. In spinal cord, select sphingomyelin and long-chain saturated and monounsaturated fatty acid species were nominally more abundant in the onset tissue, whereas select phosphatidylcholines and phosphatidylethanolamines were less abundant. Mendelian randomization identified impaired carnitine and short chain acylcarnitine metabolism as possible genetic determinants in ALS, along with various antioxidant derivatives. Our findings reveal longitudinal changes in the ALS metabolome that associate with disease progression, yielding potential new ALS biomarkers and therapeutic targets.

Materials and Methods

Participants and Biosamples

Protocols were previously published and details are provided in the SI.^{4,5,18} Briefly, all ALS patients seen at the University of Michigan Pranger ALS Clinic older than 18 years and able to communicate in English were invited to participate and provided oral and written informed consent. ALS patients were diagnosed using the original/revised El Escorial criteria, and all were included meeting the criteria of definite, probable, probable-lab supported, possible (revised criteria),¹⁹ or suspected (original criteria).²⁰ All ALS patients also met the Gold Coast criteria.²¹ Patients provided non-fasting plasma samples during their regular clinic visit, which were collected at baseline (T1) and approximate 6-month intervals (T2 at 6 months, T3 at 12 months; Fig 1A). Brain and spinal cord samples were from patients who consented to donate tissue following death. The University of Michigan institutional review board approved the study (HUM00028826).

Metabolomic Profiling

Metabolon (Durham, North Carolina) performed untargeted metabolomics of plasma and central nervous tissue samples by ultrahigh-performance liquid chromatography-tandem mass spectroscopy; details are provided in the SI. Metabolomics was performed in two batches. Internal standards accounted for instrument variability and a well-characterized human plasma technical replicate accounted for process variability.

Statistical and Data Analysis

Statistical and data analysis details are provided in the SI.

Participant Demographics. Participant demographics were summarized by median (25th-75th percentile) for continuous variables and counts (percentages) for categorical variables.

Plasma Metabolite to ALSFRS-R Models. The longitudinal plasma metabolomics dataset contained a total of 692 metabolites measured from 548 samples from 317 participants. Three plasma models were constructed: Model 1 (linear mixed effects model), Model 2 (longitudinal interaction model), Model 3 (progression model) (Fig 1B). Model 1 employed all metabolite abundances measured at all time points, *i.e.*, from baseline up to 12 months, and their corresponding ALSFRS-R scores at the time of sample collection. Model 2 employed baseline metabolite abundance to predict longitudinal change in ALSFRS-R, from baseline to 6 months, to examine disease progression, assuming a constant rate over time. There were 15 missing values for ALSFRS-R score out of 387 total scores, or 3.9%. Missing ALSFRS-R scores were imputed with the average values between the data points preceding and following the time point of the missing value. Models 1 and 2 adjusted for age, sex, BMI at study entry, and onset segment. Benjamini-Hochberg (BH)-adjusted p-values<0.05 were computed for Models 1 and 2 (Supplementary Tables S1, S2, S4, S5). Model 1 results were reported using a threshold of BH-adjusted p-value<0.05. Model 2 results were reported using a nominal p-value<0.05 due to the more exploratory nature of this analysis, with lower power to detect due to the added variable of time versus Model 1.

Model 3 was a Cox regression model that correlated baseline metabolite abundance to progression to an event (PTE), defined as a 20% (PTE20) or 50% (PTE50) decline in ALSFRS-R relative to diagnostic ALSFRS-R score.²² PTE20 was defined as the first longitudinal ALSFRS-R score that was $\leq 0.8 \times$ initial ALSFRS-R or death. PTE50 was defined as the first longitudinal ALSFRS-R score that was $\leq 0.5 \times$ initial ALSFRS-R or death. Participants who did not experience PTE20 or PTE50 or were lost to follow-up were censored. The model was adjusted for age at initial sample, sex, BMI at entry, onset segment (bulbar versus non-bulbar, *i.e.*, cervical, lumbar), diagnostic ALSFRS-R, time between symptom onset and diagnosis, and definite versus non-definite (*i.e.*, probable, probable-laboratory supported, possible, suspected) diagnostic El Escorial criteria. BH-adjusted p-values<0.05 were computed for Model 3 (Supplementary Tables S6–S7), but results were reported using a nominal p-value<0.05 due to the more exploratory nature of this analysis, with lower power to detect due to the right-censored data.

Plasma metabolomics pathway analysis. Unsupervised gene set variation analysis assessed pathway activity in each participant sample based on the abundance of all metabolites within each pathway for Models 1, 2, and 3.

Plasma metabolomics sex interaction analysis. Sex interaction analysis was performed for Models 1 and 2 by adding the interaction effects between sex and pathway as model covariates. Models were adjusted for age, BMI, and onset segment. BH-adjusted p-values<0.05 were computed for the sex interaction of Models 1 and 2 (Supplementary Tables S3), but results were reported using a nominal p-value<0.05 due to the more exploratory nature of this analysis, with lower power to detect due to the added variable of sex versus Models 1 and 2.

Spinal cord and brain metabolomics analyses. Analysis first examined unique and overlapping metabolites across lumbar spinal cord, cervical spinal cord, and brain motor cortex samples, summarized by sub-pathways and super-pathways. Next, analysis of

differential metabolites in the onset versus a second spinal cord segment was used as a surrogate of disease progression. Pairwise Wilcoxon test identified significant metabolites and pathways; BH-adjusted p-values<0.05 were computed (Supplementary Table S9), but results were reported using nominal p-value<0.05 due to the small sample size.

Mendelian randomization. Mendelian randomization (MR) was performed using single-nucleotide polymorphisms from published genome wide association studies of ALS²³ and plasma metabolites²⁴ in participants of European ancestry since participants in this study were 97% White. Significance was based on inverse variance weighted (IVW) p-value<0.05.

Results

ALS Participant Characteristics of Plasma Cohort

From 317 ALS participants, we had available 548 plasma samples, 317 at baseline (previously published),⁵ 142 at T2 (*i.e.*, 6 months), 87 at T3 (*i.e.*, 12 months) (Table 1). The cohort was typical of ALS, with baseline median age of 64.0 years, within peak ALS prevalence, greater proportion of males, and a distribution of bulbar versus spinal (cervical, lumbar) onset within anticipated ranges,² which remained relatively constant longitudinally. As expected with time, participants grew older (p-value<0.0001, T1 to T3) and disease progressed, based on an ALSFRS-R decline (p-value<0.0001, T1 to T3). Median BMI for the ALS cohort did not change significantly with time.

Plasma Metabolomics Linear Mixed Effects Model 1

Model 1 investigated the association of metabolite abundance to corresponding ALSFRS-R score at the time of sample collection across all time points, from baseline to 12 months. This model is an extension of our previously published purely cross-sectional baseline analysis⁵ by further correlating metabolite levels to ALSFRS-R across the continuum of disease. Model 1 selected 262 significant metabolites (adjusted p-value<0.05), of which 80 contributed significantly to the model ($|\beta|>0.2$; Fig 2A, Supplementary Fig S1A, Supplementary Table S3). Lipids were prominently featured (54%, primarily positively correlated to ALSFRS-R), followed by xenobiotics (19%, exclusively positively correlated to ALSFRS-R), amino acids (19%, two-thirds positively correlated to ALSFRS-R), and a few percent of various additional metabolites. The lipid class constituted fatty acids (26%), sphingomyelins (23%), plasmalogens (16%), lysoplasmalogens and lysophospholipids (12%), phosphatidylcholines (9%), and a few percent of various additional lipids. The most significant metabolite was the plasmalogen 1-(1-enyl-palmitoyl)-2-linoleoyl-GPC (P-16:0/18:2) (Padj=5.29E-28), which also contributed the most to Model 1 ($\beta=0.495$).

Next, Model 1 assessed the aggregated levels of sub-pathway metabolite members to identify sub-pathways significantly associated with ALSFRS-R scores across all time points. There were 56 significant sub-pathways (adjusted p-value<0.05), of which 18 correlated positively and 38 negatively with ALSFRS-R scores (Fig 2B, Supplementary Fig S1B, Supplementary Table S2). Lipid sub-pathways continued to be predominant, at 39% (of which approx. 60% correlated negatively with ALSFRS-R), followed by xenobiotics (16%) and amino acids (14%), with fewer sub-pathways from nucleotides (7%),

cofactors and vitamins (7%), peptides (7%), carbohydrates (5%), and energy (3.6%). Of lipid sub-pathways, most were related to fatty acids (41%); however, sub-pathways were selected, *e.g.*, primary and secondary bile metabolism, that were not represented when we examined individual metabolites. Evaluating sub-pathway level significance also identified candidates not selected by examining individual metabolites, *e.g.*, sub-pathways belonging to carbohydrate super-pathway.

Sex interaction in Model 1 was nominal, with 6 sub-pathways belonging to amino acid, lipid, carbohydrate, and xenobiotic super-pathways (Supplementary Fig S1C, Supplementary Table S3).

Plasma Metabolomics Longitudinal Interaction Model 2

We next assessed whether baseline metabolite abundances could predict ALSFRS-R at 6 months, *i.e.*, predict net change in functional status 6 months from baseline. Given the interaction analysis, we focused on the 52 nominally significant metabolites with a time interaction, of which 31 correlated positively with ALSFRS-R change, *i.e.*, with functional decline, and 21 correlated negatively with disease progression (Fig 3, Supplementary Fig S2A, Supplementary Table S4). Most were lipids, comprising 52% (~56% positively correlated to ALSFRS-R change), followed by amino acids (25%, approximately equally positively or negatively correlated to ALSFRS-R change), xenobiotics (12%, all positively correlated to ALSFRS-R change), and various others (12%). Of the lipids, fatty acids (26%), diacylglycerols (22%), phosphatidylcholines (22%), and other phosphatidyl species (15%) were represented, with only a single sphingomyelin and single hexosylceramide species.

Summing baseline metabolites identified seven nominally significant sub-pathways linked to net ALSFRS-R change, three that were lipid sub-pathways, with diacylglycerol as the most significant (negatively linked to ALSFRS-R), along with fatty acid, amino (positively linked to ALSFRS-R), and sphingosines (negatively linked to ALSFRS-R) (Supplementary Fig S2B, Supplementary Table S5). Additional pathways were food component/plant (xenobiotics super-pathway), vitamin A metabolism (cofactors and vitamins super-pathway), glutathione metabolism (amino acid super-pathway), and drug – gastrointestinal (xenobiotics super-pathway). Model 2 selected some sub-pathways that Model 1 selected, *i.e.*, vitamin A metabolism, food component plant, drug (gastrointestinal), and fatty acid (amino), but also selected unique sub-pathways, including glutathione metabolism, sphingosines, and diacylglycerol (Fig 4), possibly indicative of sub-pathways that may predict functional status.

Sex interaction analysis of Model 2 yielded no significant sub-pathways.

Plasma Metabolomics Progression Model 3

Finally, Model 3 was progression to an event (PTE), defined as a drop of 20% (PTE20) or 50% (PTE50) in function relative to baseline ALSFRS-R. This model then correlated baseline metabolite abundance to time between diagnosis to this decrease, of 20 or 50%, in baseline ALSFRS-R score. For participants earlier in their disease course with higher ALSFRS-R scores, a drop of 20 or 50% translates to a relatively larger ALSFRS-R change compared to participants later in their disease course with lower ALSFRS-R scores (Fig 1B).

Therefore, this model indirectly accounts for disease severity of participants. The choice of event was based on a survey of ALS clinicians and researchers, where 90% of respondents considered a 20% drop in ALSFRS-R as “somewhat clinically significant”, and a drop of 50% as “very clinically meaningful”.²⁵ PTE has been used previously to evaluate ALS progression.²²

PTE20 and PTE50 selected 55 and 61 nominally significant metabolites, respectively, which overlapped to a significant extent (46 shared metabolites) (Supplementary Table S6). Most PTE20-selected metabolites were lipids (55%), followed by amino acids (25%) and xenobiotics (13%), mirrored, as expected, by PTE50-selected metabolites, which were 49% lipids, 26% amino acids, and 15% xenobiotics. For the PTE20 model, within lipid super-pathways, there were 27% sphingomyelins, 13% lysophospholipids, 10% monoacylglycerols, and 27% of various fatty acid species. The breakdown by lipids for the PTE50 model was similar, as anticipated, with 39% sphingomyelins, 13% lysophospholipids, 10% monoacylglycerols, 19% of various fatty acid species, and, additionally, 10% hexosylceramides. We next performed pathway enrichment analysis of all selected metabolites to identify sub-pathways especially relevant to ALS progression (Fig 5). Both sphingomyelins and monoacylglycerols were significantly enriched for PTE20 and PTE50, suggesting these lipid sub-pathways are relevant to ALS progression, across the spectrum of disease severity. None of the amino acid or xenobiotic sub-pathways enriched.

When we summed sub-pathway metabolites, new significant sub-pathways emerged for PTE20 and PTE50, including oxidative phosphorylation and dipeptides (Supplementary Table S7), in addition to sub-pathways that represented singly-selected metabolites, e.g., monoacylglycerols, fatty acids. Interestingly, PTE20 and PTE50 sub-pathway examination did not select sphingomyelins, as opposed to examination by single metabolites, which selected several sphingomyelins. This may again suggest dysregulation of specific sphingomyelin species in ALS as opposed to general impairment in the entire sub-pathway.

Lastly, there was some overlap between Model 3 PTE50 with both Model 1 and Model 2, although there were also uniquely selected metabolites, such as hexosylceramides and medium chain fatty acids (Fig 4).

ALS Participant and Sample Characteristics of Spinal Cord and Brain Cohort

As a terminal time point, we also metabolically profiled 94 cervical and 81 lumbar spinal cord and 87 brain motor cortex tissue samples from 103 ALS cases (Table 2). Ten (9.7%) participants provided one sample, 27 (26.2%) provided two samples, and 66 (64.1%) provided all three samples (Table 2, Supplementary Fig S3A). Among cervical tissue donors, median age at diagnosis was 64.2 years and median age at autopsy was 65.5 years with 37.2% bulbar, 33.0% cervical, and 25.5% lumbar onset ALS. Findings were similar among lumbar and motor cortex sample donors except they varied slightly in the distribution of onset segment.

ALS Cervical and Lumbar Spinal Cord and Motor Cortex Metabolites

We detected 703, 665, and 701 metabolites in cervical, lumbar, and motor cortex samples, respectively, that were annotated and with <20% missingness (Supplementary Fig S3B).

Regarding metabolite overlap, 564 were shared by all three tissues, 638 were shared by cervical and lumbar, and 593 and 595 were shared by cervical and lumbar, respectively, with motor cortex (Supplementary Fig S3B, Supplementary Table S8). There were 36, 36, and 41 metabolites unique to cervical, lumbar, and cortex, respectively. As anticipated for myelin-rich nervous tissue, 256 (45%) of the 564 metabolites shared by all tissue types belonged to the lipid super-pathway. Second most abundant were 146 metabolites (26%) of the amino acid super-pathway, followed by nucleotide, carbohydrate, cofactors and vitamins, peptide, xenobiotics, energy, and partially characterized molecules. These 564 metabolites represented 97 sub-pathways, top among them the lysophospholipid, methionine, cysteine, S-adenosylmethionine and taurine metabolism, and sphingomyelin sub-pathways with 24, 22, and 21 members, respectively (Supplementary Table S8). In sum, fatty acid acylcarnitines, spanning saturated long-chain, saturated medium-chain, short-chain, monounsaturated, and polyunsaturated species, had 30 metabolite members.

Differential Metabolites in Onset Versus Second Spinal Cord Segment in ALS

We did not have control autopsy tissue; therefore, to identify metabolites linked to ALS progression, we assessed differential species between the onset tissue versus the second segment as a surrogate. We compared lumbar to cervical metabolites in the 20 participants with lumbar onset ALS that provided both lumbar and cervical samples, and cervical to lumbar metabolites in the 18 participants with cervical onset that provided both samples. No metabolites significantly differed between onset versus second segment although 33 were nominally significant (Fig 6, Supplementary Table S9). Most were lipids (69%), especially sphingomyelins (15% of total) and long-chain saturated and monounsaturated fatty acids (12% of total), that were more abundant in the onset tissue. In contrast, phosphatidylcholines (9% of total) and phosphatidylethanolamines (6% of total) were more abundant in the progression tissue. Other significantly differential metabolites included amino acids (3 species, 9% of total), cofactors and vitamins (3 species, 9% of total), energy metabolites (2 species, 6% of total), and very few others. Only sphingomyelins and lipids enriched (Supplementary Fig S4). Finally, progression was the strongest determinant of metabolite abundance compared to sex or onset segment.

Mendelian Randomization and Possible Causal Relation of Metabolites to ALS

We performed Mendelian randomization (MR) using a large genome wide association study of ALS patients (n=27,205) and controls (n=110,881) of European ancestry,²³ as well as one of 8,299 participants of European ancestry linked to 1,702 plasma metabolites.²⁴ Datasets of European ancestry were selected since participants in this study were 97% White. MR identified 114 significant metabolites (IVW p-value<0.05), of which 98 were annotated (Supplementary Table S10). Heterogeneity and pleiotropy testing yielded no significant metabolites. As might be expected from Models 1, 2, and 3, which consistently selected lipid, amino acid, and xenobiotic super-pathways, MR metabolites were, in large part, lipids (25%), amino acids (20%), and xenobiotics (16%), with a smaller proportion of energy, carbohydrate, nucleotide, peptide, cofactor and vitamin, and partially characterized molecules. Among lipid sub-pathways, sphingomyelin and diverse fatty acid and carnitine metabolism sub-pathways were more prominently featured, which contained short- and

medium-chain fatty acid acylcarnitine metabolites, along with a few various additional lipid sub-pathways.

MR metabolites overlapped with metabolites both significantly and nominally selected by each of the Models, 1, 2, and 3, and the CNS analysis (Supplementary Table S10). Most of the overlapping metabolites were lipids and amino acids. One highly recurrent candidate was sphingomyelin (d18:2/24:1, d18:1/24:2), which may have genetically determined elevated levels in ALS via 32 single-nucleotide polymorphisms. In Model 1, sphingomyelin (d18:2/24:1, d18:1/24:2) levels correlated negatively with ALSFRS-R, i.e., higher levels with lower functional status, and it favored the time to event for a 20 or 50% drop in ALSFRS-R in Model 3. Finally, in the CNS model, sphingomyelin (d18:2/24:1, d18:1/24:2) was more abundant in the onset than in the second segment, a reflection of more progressive disease.

Pathway enrichment yielded carnitine and fatty acid metabolism (BCAA metabolism), containing two short-chain fatty acid acylcarnitine species (butyrylcarnitine, propionylcarnitine (Supplementary Fig S5). However, the most significantly enriched sub-pathways were hemoglobin and porphyrin metabolism and partially characterized molecules, both containing bilirubin and biliverdin, and various related degradation products. Glutathione metabolism was also highlighted, comprising antioxidant derivatives and oxidized byproducts thereof.

Discussion

ALS pathophysiology is characterized by changes to the metabolome^{4–6} and energy homeostasis,^{7,9} manifesting clinically by correlation of BMI^{8,13,26} and hypermetabolism¹² to disease progression and survival. Low BMI at diagnosis or BMI loss in the years leading up to diagnosis may presage rapid ALS progression.^{8,26} Similarly, hypermetabolism in ALS adversely affects survival,^{12,27} with elevated resting energy expenditure.²⁸ Thus, overall, metabolic and metabolomic changes may underscore ALS progression. Indeed, we^{5,8} and others¹⁴ demonstrated that specific plasma and cerebrospinal fluid (CSF) metabolites, lipids, and/or metabolic modules correlate with cross-sectional ALS functional status^{5,14} and BMI trajectories.⁸ Specifically, we found that ALS plasma was characterized by altered lipid, amino acid, and xenobiotic sub-pathways, with several lipid species, including sphingomyelins and long-chain acylcarnitines, correlated to cross-sectional ALSFRS-R.

Herein, we hypothesized that ALS progression would be similarly marked by longitudinal changes in metabolome. We examined individual metabolites as well as sub-pathways since the sum of their collective member metabolites contributes to biological processes. We extend our prior findings in this new longitudinal dataset comprising granular metabolomic and functional data. Across the continuum of disease, individual lipid species and/or lipid sub-pathways continue to remain prominent in all Models 1, 2, and 3, including sphingomyelins, fatty acids, plasmalogens, phosphatidylcholines, and lysophospholipids. Interestingly, fatty acid, but not sphingomyelin, sub-pathways remained prominent in sub-pathway analysis, possibly suggesting broadly impaired fatty acid metabolism in ALS, linked to functional status, but only alterations in specific sphingomyelin species. The two

models that integrated changes to ALSFRS-R, Models 2 and 3, selected several fatty acid sub-pathways, especially long-chain saturated acylcarnitine species, which may suggest that impaired baseline fatty acid metabolism may be prognostic of disease progression, aligned with hypermetabolism in ALS.^{12,27}

We also build on prior work by analysis of differential metabolite species between onset versus second spinal cord segment, as a surrogate of ALS progression and as a terminal time point. This metabolomic analysis is, to our knowledge, the largest to date, in terms of sample size and the types of central nervous system tissue assessed.²⁹ We found that most differential metabolites were lipids; among them, a few sphingomyelin and long-chain saturated and monounsaturated fatty acid species were nominally more abundant in the onset segment, whereas phosphatidylcholines and phosphatidylethanolamines were nominally less abundant.

Although impaired metabolism in ALS, especially of lipids,^{4,5,30} is relatively well-established, few prior studies were longitudinal.^{31–34} A relatively small analysis of ALS CSF identified altered glycolytic metabolite abundance at 2-year follow-up by proton nuclear magnetic resonance, a method of limited resolution, so the study lacked granular metabolomic information.³² A mass spectrometry analysis of serum found ALS cases (n=40) were uniquely perturbed in sphingolipids and glycerophospholipids at 2-year follow-up versus controls, as herein, but the study lacked correlation to functional status. Finally, a relatively small mass spectrometry lipidomics analysis of serum from ALS (n=28) versus control (n=22) participants identified 14 lipid classes that changed with time in ALS, especially diacylglycerols,³¹ which we noted as a differential class herein. Although the investigators did not examine correlation of lipid abundance to ALSFRS-R, they found that diacylglycerol (18:1/18:1) correlated to neurofilament light chain levels,³¹ a marker of neuronal damage and progression in ALS. Collectively, the reported literature, albeit using small sample sizes, supports our current findings.

Besides tracking with disease progression, and potentially serving as biomarkers, metabolomic changes can also shed insight into ALS pathophysiology and potential treatment avenues. We previously reported altered β -oxidation metabolites in cross-sectional ALS plasma analysis, evidenced by partially metabolized acylcarnitine intermediates.⁵ Here, we noted the presence of long-chain acylcarnitines linked to ALSFRS-R in plasma and spinal cord tissue, now also supporting aberrant β -oxidation in ALS progression.^{35,36} Evidence from mutant *SOD1* mouse models also uphold altered β -oxidation and fatty acid metabolism, including in spinal cord.^{37,38} Enhancing β -oxidation capacity by administering L-carnitine to *SOD1*^{G93A} mice slows motor function deterioration and extends life.³⁹ However, although the precursor acetyl-L-carnitine has been considered as a potential ALS treatment,⁴⁰ an idea supported by the current findings, it is not approved clinically. Moreover, our prior⁴¹ and current MR analysis indicates possible genetically determined causality from specific fatty acid, acylcarnitine, and carnitine species, further bolstering dysfunctional β -oxidation in ALS. Enrichment analysis persisted in selecting carnitine and fatty acid metabolism, underscoring the centrality of this metabolic pathway in ALS, along with sub-pathways comprising antioxidant derivatives and oxidized byproducts, possibly linked to oxidative stress, another ALS hallmark.³

We also previously reported differential plasma sphingomyelins in ALS versus control participants, linked to cross-sectional ALSFRS-R.⁵ We expand these findings here by showing that sphingomyelins further correlate with disease progression longitudinally in plasma and in onset versus second spinal cord segment, adding to the growing literature on this lipid class in ALS.⁴² Cross-sectional studies have noted altered sphingolipids, including sphingomyelins, in ALS plasma⁴³ and spinal cord,^{44–46} corroborated by longitudinal studies in early⁴⁵ and symptomatic stages.^{33,37} Modifying activity of enzymes involved in specific sphingolipid species biosynthesis, *e.g.*, glucocerebrosides, alters ALS progression rate in mouse models.^{45,46} Moreover, the occurrence of monogenic childhood-onset ALS with mutant serine palmitoyltransferase subunit 1⁴⁷ further supports a causative or early role for sphingolipid metabolism in ALS. Indeed, our MR analysis identified a sphingolipid, sphingomyelin (d18:2/24:1, d18:1/24:2), with potentially genetically determined elevated levels in ALS, which was shared with metabolites selected by Models 1 and 3, as well as the CNS analysis. The specific role of this sphingomyelin (d18:2/24:1, d18:1/24:2) in ALS remains unclear, but warrants further investigation.

In addition to fatty acids and sphingomyelins, we found plasma plasmalogens, phosphatidylcholines, lysoplasmalogens, lysophospholipids, and diacylglycerols and/or sub-pathways correlated with ALSFRS-R over time. In spinal cord, phosphatidylcholine and phosphatidylethanolamine metabolites were more abundant in the second segment. ALS changes in plasmalogens, phosphatidylcholines, lysophosphatidylcholines, and/or phosphatidylethanolamines have been observed in cross-sectional⁴⁴ and longitudinal assessments.^{31,33,37} Plasmalogens,⁴⁸ phosphatidylcholines, and phosphatidylethanolamines⁴⁹ are cell membrane components that regulate various biological functions, *e.g.*, mitochondrial function, membrane fusion, proliferation, survival, and cholesterol efflux. Phosphatidylcholines and phosphatidylethanolamines are synthesized from diacylglycerols through the Kennedy pathway,⁵⁰ rendering the pool of these bioactive lipids interconnected.

Model 1 also selected bile acid lipid sub-pathways, that may be indicative of impaired lipid absorption and metabolism in the gut; indeed, ALS patients may have an altered microbiome⁵¹ and some microbiome-derived metabolites in the current study, *e.g.*, indolelactate, correlated with ALSFRS-R. In our previous gut microbiome-plasma metabolome correlation, gut microbial structure at the phylum and genera level differed in ALS versus control participants.⁴¹ In the integrated analyses, several microbiota genera/species strongly correlated to multiple metabolites, primarily lipids, including bile acids, which associated to functional ALS status and differentiated cases from controls.

Besides lipids, amino acid metabolism and xenobiotics were recurrent in all three plasma models but to a lesser extent in spinal cord tissue. Altered amino acid metabolism in ALS has also been reported by cross-sectional^{52–54} and longitudinal^{52,55} human and animal studies, including of histidine, arginine, and proline,⁵² as well as creatine metabolism.⁴³ The latter, creatine, however, is likely a byproduct of muscle wasting. Selected xenobiotic metabolites in baseline and longitudinal analysis belonged mostly to xanthine and benzoate metabolism and food component/plant sub-pathways, as previously seen in cross-sectional studies.⁵ Sub-pathway analysis further selected various drug sub-pathways. Notably, “drug,

gastrointestinal” and “drug, respiratory” abundance increased with disease progression, likely reflecting symptom management of ALS patients.

Our study has some limitations. First, a limitation intrinsic to most human studies, is the tissue issue, since our longitudinal analysis was restricted to accessible blood samples, which may not necessarily reflect the disease process in nervous tissue or may instead reflect downstream processes. However, there is no method to longitudinally capture brain and spinal cord tissue, which was only represented at the terminal time point by the spinal cord and brain cohort. Nevertheless, metabolomics analysis of plasma, though non-organ specific, can still reflect complex biochemical processes occurring throughout the body, including impaired systemic metabolism in ALS. Second, we had some cohort attrition in our longitudinal analysis, frequent in ALS studies,⁵⁶ which may have biased our findings. Third, our study involved ALS participants that had not fasted prior to providing blood collection since it was deemed unethical to request it. However, although diet influences circulating lipid levels, studies have shown that a plasma lipidomics signature of disease can occur independent of diet and/or fasting state.^{57,58} Moreover, although we collected blood from ALS patients at the same time intervals, samples may have been collected at different time points in their disease course. Also, we assumed a linear rate of disease progression to impute missing ALSFRS-R scores, which is often, but not always, the case.⁵⁹ However, ALSFRS-R scores had to be imputed for only 3.9% of the data. A fourth limitation was the time window between a patient’s death and the time of autopsy, which may have allowed for *postmortem* changes in the metabolome. Additionally, the spinal cord and brain cohort was relatively small and did not include any tissues from healthy controls to facilitate direct comparisons to ALS, which instead relied on comparisons of the initial to the second affected spinal segment as a surrogate of disease progression. Finally, since this was an exploratory study that encompassed all detected metabolites, we lost power to detect significant correlations of specific metabolites and pathways to ALSFRS-R scores and relied on nominal p-values for Models 2 and 3, sex interaction analysis, and CNS metabolites. Future larger studies that analyze select target metabolites and pathways are needed to confirm our more exploratory findings.

Nevertheless, our study also had many strengths, including a longitudinal design using an agnostic metabolome-wide analysis platform that detects and identifies an extensive number of metabolites in plasma from a relatively large cohort of ALS participants. Additionally, the metabolomic platform included multiple standards to assure low day-to-day variability and ability to mitigate batch effects. In our data analysis pipeline, we performed a sex interaction analysis for the longitudinal plasma data, which is relevant to ALS, including differences to metabolites and lipids by male versus female patients;⁵ however, the spinal cord and brain cohort was too small to examine the impact of sex on central nervous tissue metabolomes. Finally, we also assessed both metabolites individually and cumulatively within sub-pathways. The former method identified robust single metabolites, whereas the second measured the sum of metabolites within sub-pathways, which considers minor but cumulative contributions from multiple metabolites, an appropriate approach for a heterogeneous disease like ALS.

In summary, we show for the first time, to our knowledge, that differences especially in plasma lipid species and sub-pathways, amino acids, xenobiotics, and various additional less frequently selected pathways, track with ALS progression, specifically with functional status by ALSFRS-R. Examination by lipid class pinpointed fatty acids and sphingomyelins, along with plasmalogens, phosphatidylcholines, and lysophospholipids. In spinal cord, sphingomyelin and long-chain saturated and monounsaturated fatty acid species were nominally more abundant in onset tissue, while phosphatidylcholines and phosphatidylethanolamines were less abundant. Overall, our findings suggest that metabolic and metabolomic changes may underscore disease severity and progression in ALS and offer new opportunities for both biomarker and therapeutic development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data Availability

Anonymized data will be shared upon request from any qualified investigator.

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Summary for Social Media

1. If you and/or a co-author has a Twitter handle that you would like to be tagged, please enter it here.

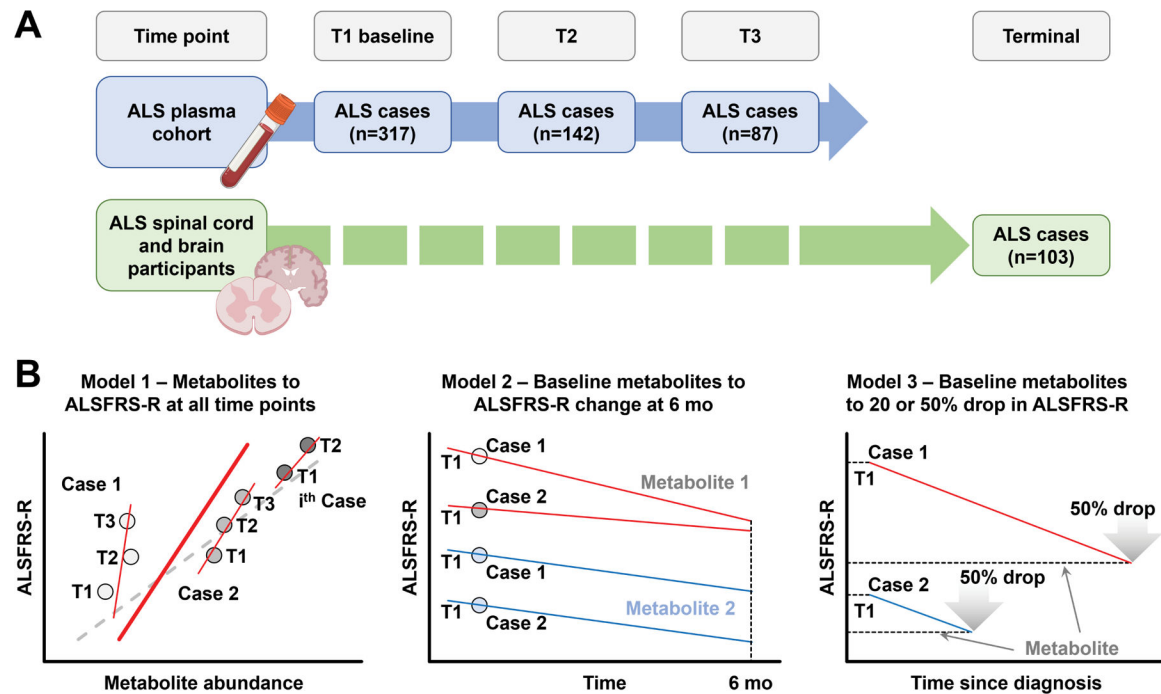
@EvaFeldmanMDPhD
2. What is the current knowledge on the topic?

ALS is characterized by hypermetabolism and changes in body mass index (BMI) along with altered plasma and brain and spinal cord metabolome.
3. What question did this study address?

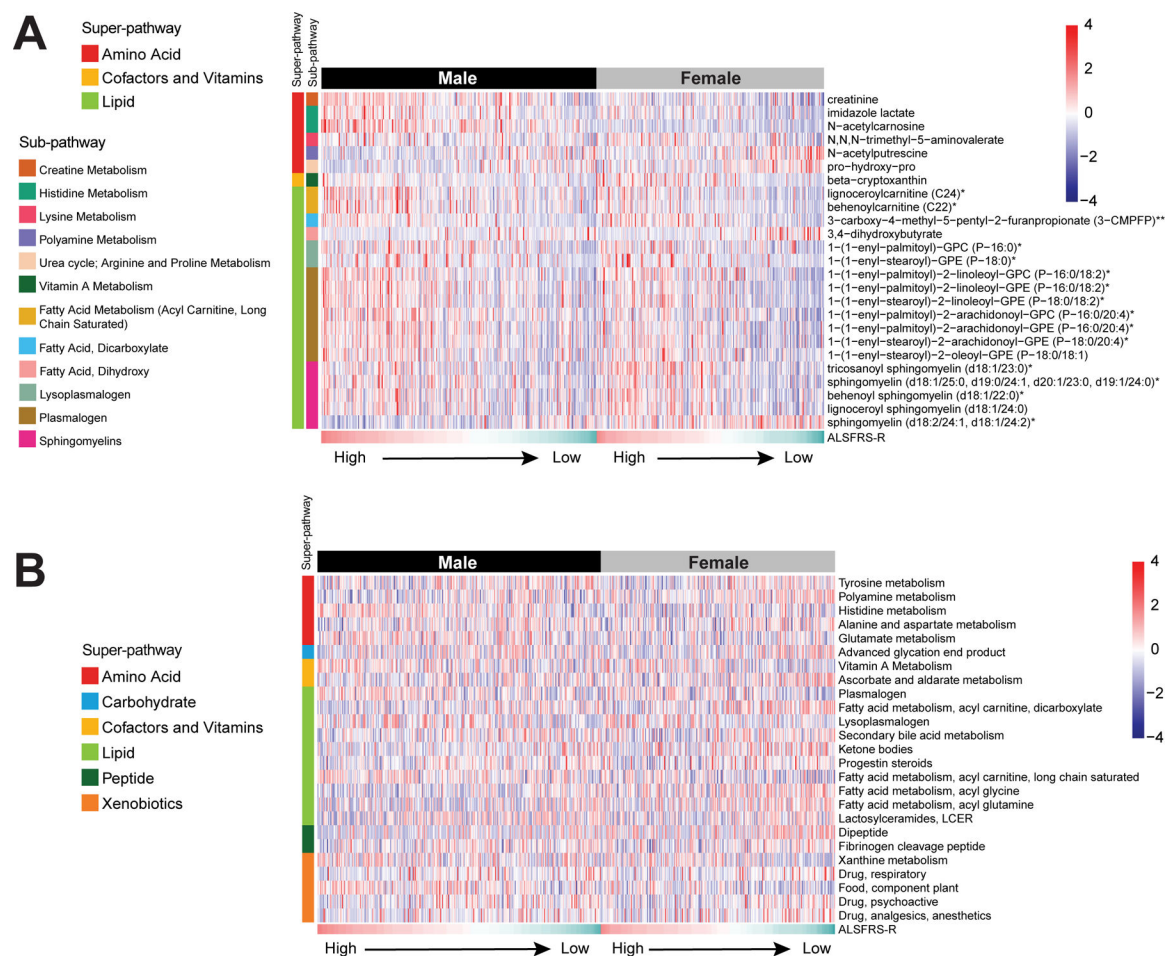
Although studies have shown that hypermetabolism and BMI changes are associated with faster disease progression and poorer survival, most studies of the metabolome to date have been cross-sectional. This study assessed whether changes in the plasma metabolome also tracked with ALS disease progression.
4. What does this study add to our knowledge?

This study profiled plasma metabolome and functional status longitudinally in ALS patients early in the disease course and longitudinally 6 and 12 months later along with postmortem spinal cord as a terminal time point. Our analysis identified several plasma lipid metabolites and lipid sub-pathways that correlated with disease course, along with select amino acids and xenobiotics, and differences in select lipid species in onset versus second spinal cord segment.
5. How might this potentially impact on the practice of neurology?

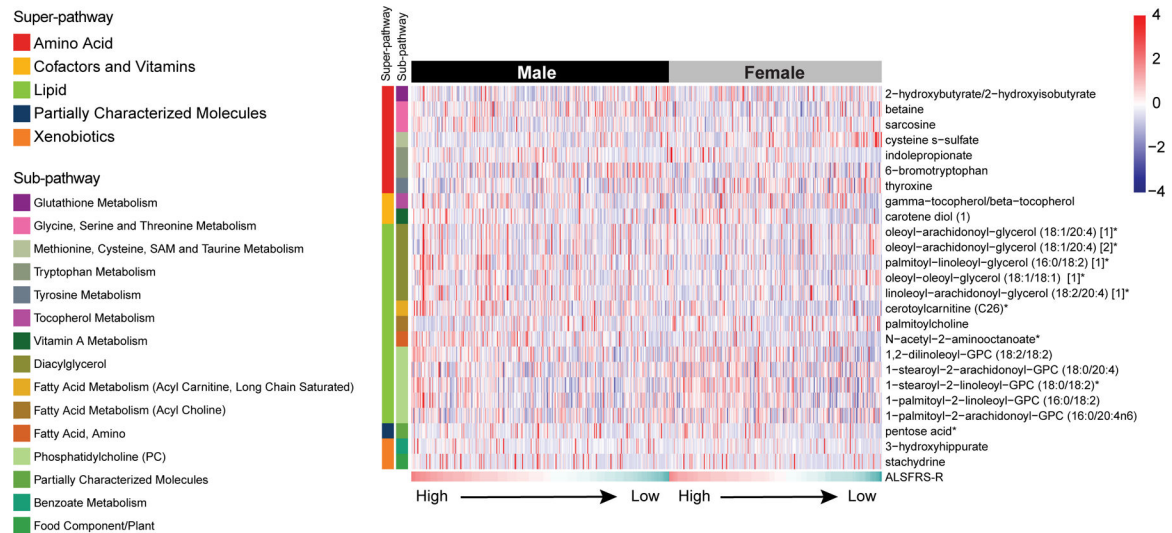
Potentially, metabolites that track with disease progression could serve as biomarkers and metabolomic changes can also shed insight into ALS pathophysiology and potential treatment avenues.

**FIGURE 1:**

Overview of study design and models. (A) Number of ALS participants at each time point in the plasma cohort (blue) and spinal cord and brain cohort (green). (B) Graphic representation of the models for plasma metabolomics analysis. **Linear mixed effects Model 1** correlated metabolite abundances to ALSFRS-R scores across all time points (T1, T2, T3) for all participants (case 1, case 2, ..., ith case). Linear regressions for each participant as fine red lines. Linear regression accounting for the entire metabolite and ALSFRS-R dataset across all time points shown as a bold red line. **Longitudinal interaction Model 2** correlated baseline metabolite abundances (T1 time point only) to predict ALSFRS-R change at 6 months for all participants (only case 1 and case 2 shown). For metabolite 1, differing baseline metabolite abundances predicted differing ALSFRS-R change at 6 months (different slopes) indicating a time interaction. In this illustration, higher metabolite 1 levels predict faster decline. Thus, metabolite 1 predicts ALS progression. For metabolite 2, differing baseline metabolite abundances correlate to the same ALSFRS-R change at 6 months (same slopes) indicating no time interaction. Thus, metabolite 2 does not predict ALS progression. **Progression Model 3** correlated baseline metabolites to 20 or 50% drop in ALSFRS-R. For case 1 with less severe disease, 50% drop in ALSFRS-R shown as an illustration. For case 2 with more severe disease, 50% drop in ALSFRS-R shown. The same percent drop in ALSFRS-R translates to differing net changes in ALSFRS-R over potentially different time scales based on the initial disease severity. Figure created in part in [Biorender.com](https://www.biorender.com).

**FIGURE 2:**

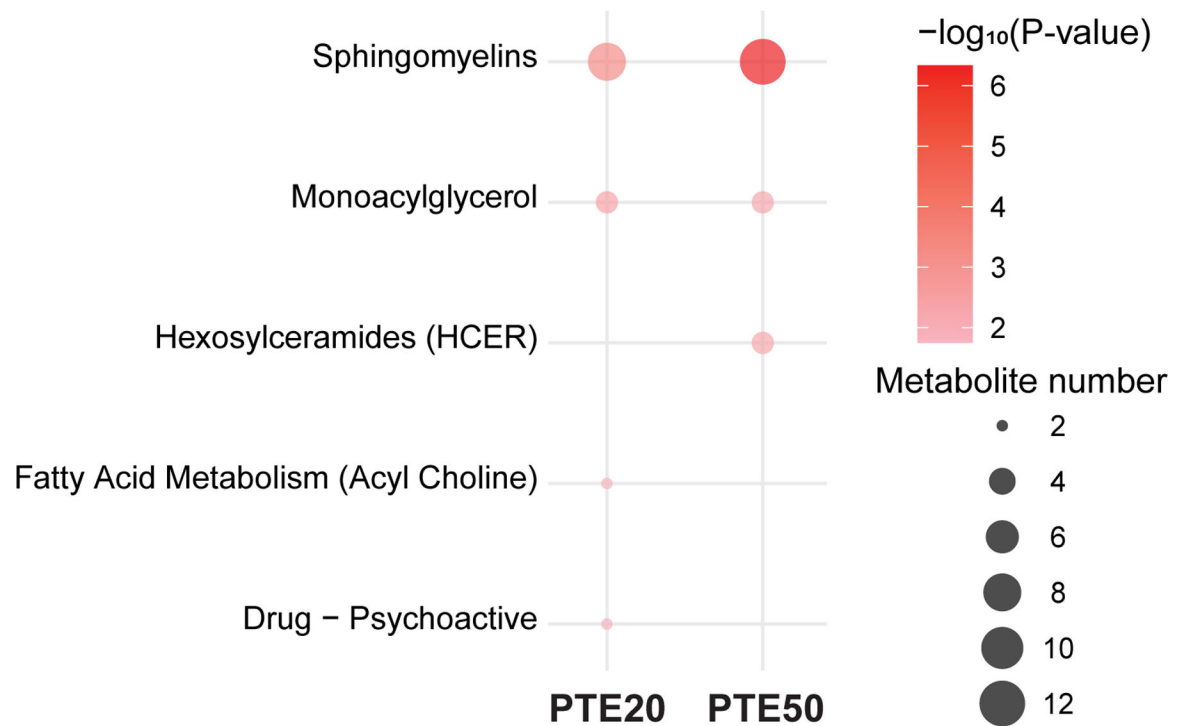
Plasma metabolomics linear mixed effects Model 1: Metabolite abundance to ALSFRS-R across all time points. (A) Heatmap visualization of the relative abundance in the top 25 metabolites out of 80 (Supplementary Table S1) with adjusted p-value (P_{adj})<0.05 and $|\beta|>0.2$ to ALSFRS-R. (B) Heatmap visualization of the relative abundance of the top 25 enriched sub-pathways with P_{adj} <0.05 to ALSFRS-R (Supplementary Table S2). Heatmaps grouped by male (black) and female (grey) and sorted by ALSFRS-R, from high (red; better functional status) to low score (turquoise; poorer functional status). Relative abundance scaled from higher (red) to lower (blue) according to the legend. Single asterisk * indicates a compound that has not been confirmed based on a standard, but that the analytical platform is confident in its identity. Double asterisk ** indicates a compound for which a standard is not available, but that the analytical platform is reasonably confident in its identity, or the information provided. ADMA, asymmetric dimethylarginine; GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine; SDMA symmetric dimethylarginine. Legends ordered according to metabolite/sub-pathway order in heatmaps.

**FIGURE 3:**

Plasma metabolomics longitudinal interaction Model 2: Baseline metabolite abundance to predict ALSFRS-R change at 6 months. Heatmap visualization of the relative abundance of the top 25 baseline metabolites (Supplementary Table S4) with nominal p-value < 0.05 to change in ALSFRS-R. Heatmaps grouped by male (black) and female (grey) and sorted by ALSFRS-R, from high (red; better functional status) to low score (turquoise; poorer functional status). Relative abundance scaled from higher (red) to lower (blue) according to the legend. Single asterisk * indicates a compound that has not been confirmed based on a standard, but that the analytical platform is confident in its identity. GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine. Legend ordered according to metabolite order in heatmap.



FIGURE 4: Sub-pathways selected across all three Models, linear mixed effects, longitudinal interaction, and progression models. Dot plot of sub-pathways selected by longitudinal interaction (Model 1, only top 25 sub-pathways shown), longitudinal interaction (Model 2), and progression (Model 3, progression to event for 50% drop in initial ALSFRS-R) models. Larger dot size represents higher sub-pathway significance level. For Model 1, dot color represents estimate that correlates positively (red) or negatively (blue) with ALSFRS-R. For Model 2, dot color represents estimate that correlates positively (red) or negatively (blue) with change in ALSFRS-R at 6 months. For Model 3, dot color represents estimate that correlates positively (red) or negatively (blue) with progression to 50% drop in initial ALSFRS-R.

**FIGURE 5:**

Plasma metabolomics progression Model 3: Baseline metabolite abundance to progression to event. The events were defined as a 20% (PTE20) and 50% (PTE50) drop in diagnostic ALSFRS-R score. Dot plot of enrichment analysis of PTE20 and PTE50 metabolites selected by Model 3 (Supplementary Table S6). Dot color represents least (light pink) to most (red) significant enriched sub-pathways; dot size represents number of metabolite members in sub-pathways.

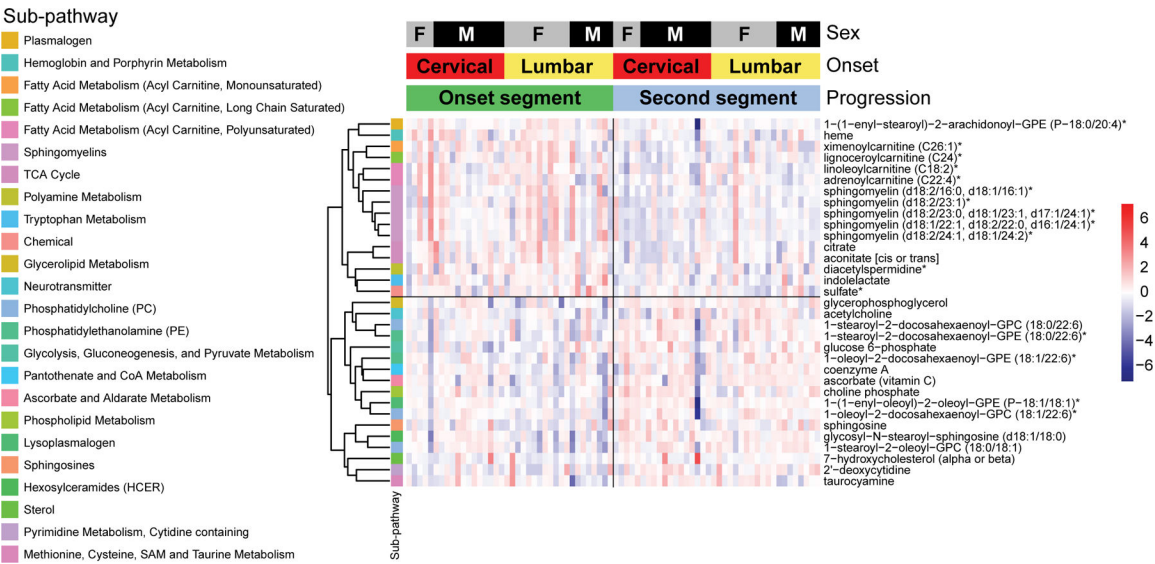


FIGURE 6: Differential metabolites in onset versus second segment spinal cord tissue in ALS. Heatmap visualization of the relative abundance of the 33 metabolites (Supplementary Table S9) with p-value<0.05 that differed in onset versus second segment. Heatmap grouped by onset (green) and second (blue) segment, cervical (red) and lumbar (yellow) onset, and male (black; M) and female (grey; F). Relative abundance scaled from higher (red) to lower (blue) according to the legend. Single asterisk * indicates a compound that has not been confirmed based on a standard, but that the analytical platform is confident in its identity. GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine.

Table 1.
Demographics of ALS participants of plasma cohort

	T1 (baseline) (n=317)	T2 (6 months) (n=142)	T3 (12 months) (n=87)	P-value
Age at plasma collection (years)	64.0 (56.0–71.0)	64.2 (55.3–70.4)	65.0 (58.1–70.7)	<0.0001
Sex				
Female	145 (45.7%)	62 (43.7%)	40 (46.0%)	
Male	172 (54.3%)	80 (56.3%)	47 (54.0%)	
BMI at study entry (kg/m ²)	26.1 (23.2–29.8)	26.6 (23.4–29.8)	27.1 (23.4–29.5)	0.98
Race/ethnicity				
Asian	2 (0.6%)	0	0	
Hispanic	1 (0.3%)	0	0	
Black or African American	7 (2.2%)	4 (2.8%)	2 (2.3%)	
White	307 (96.8%)	138 (97.2%)	85 (97.7%)	
Family History of ALS				
No	302 (95.3%)	136 (95.8%)	83 (95.4%)	
Yes	15 (4.7%)	6 (4.2%)	4 (4.6%)	
<i>C9orf72</i> repeat expansion status				
Positive	21 (6.6%)			
Intermediate	2 (0.6%)			
Negative	230 (72.6%)			
Unknown/missing	64 (20.2%)			
Diagnostic El Escorial criteria				
Definite	85 (26.8%)	32 (22.5%)	20 (23.0%)	
Probable	94 (29.7%)	45 (31.7)	28 (32.2%)	
Probable, lab supported	77 (24.3%)	33 (23.2%)	17 (19.5%)	
Possible	43 (13.6%)	21 (14.8%)	16 (18.4%)	
Suspected	18 (5.7%)	11 (7.7%)	6 (6.9%)	
Missing	0 (0%)	0 (0%)	0 (0%)	
Age at symptom onset (years)	61.7 (53.0–68.2)	61.1 (52.3–67.2)	61.6 (53.7–67.2)	
Age at diagnosis (years)	63.2 (54.5–69.7)	62.2 (53.6–68.7)	62.6 (54.9–69.0)	
Time from symptom onset to diagnosis (years)	1.0 (0.6–1.7)	1.1 (0.9–1.6)	1.6 (1.4–1.9)	
Time from diagnosis to time of sample collection (years)	0.6 (0.4–1.0)	1.2 (0.7–1.9)	1.2 (0.7–1.9)	
ALSFERS-R at plasma collection	32 (26–37)	29 (23–35)	27 (19–33)	<0.0001
Onset Segment				
Bulbar	90 (28.4%)	33 (23.2%)	21 (24.1%)	
Cervical	109 (34.4%)	56 (39.4%)	32 (36.8%)	
Lumbar	118 (37.2%)	53 (37.3%)	34 (39.1%)	

Continuous variables, age at plasma collection (years), BMI at study entry (kg/m²), and ALSFRS-R at plasma collection, represented as median (25th – 75th percentile).

Categorical variables, sex, race/ethnicity, family history of ALS, and onset segment, represented as N (%; rounded to the first decimal place).

Significance with time assessed by a linear mixed model.

ALSFRS-R, ALS functional rating scale – revised; BMI, body mass index.

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Table 2.
Demographics of ALS participants of spinal cord and brain cohort by sample type

	Cervical samples (n=94)	Lumbar samples (n=81)	Motor cortex samples (n=87)
Age at diagnosis (years)	64.2 (57.5–71.8)	64.0 (57.7–71.1)	64.3 (57.6–71.7)
Age at autopsy (years)	65.5 (59.8–73.3)	65.4 (59.8–73.3)	65.5 (59.9–73.1)
Sex			
Female	47 (50%)	41 (50.6%)	46 (52.9%)
Male	47 (50%)	40 (49.4%)	41 (47.1%)
Race/ethnicity			
Asian	0 (0%)	0 (0%)	0 (0%)
Hispanic	1 (1.1%)	0 (0%)	1 (1.1%)
Black or African American	0 (0%)	0 (0%)	0 (0%)
White	93 (98.9%)	81 (100%)	86 (98.9%)
Family History of ALS			
No	74 (78.7%)	63 (77.8%)	65 (74.7%)
Yes	10 (10.6%)	9 (11.1%)	11 (12.6%)
Unknown	10 (10.6%)	9 (11.1%)	11 (12.6%)
Diagnostic El Escorial criteria			
Definite	30 (31.9%)	25 (30.9%)	27 (31.0%)
Probable	27 (28.7%)	20 (24.7%)	25 (28.7%)
Probable, lab supported	17 (18.1%)	15 (18.5%)	17 (19.5%)
Possible	11 (11.7%)	12 (14.8%)	11 (12.6%)
Suspected	4 (4.3%)	4 (4.9%)	2 (2.3%)
Missing	5 (5.3%)	5 (6.2%)	5 (5.7%)
Age at symptom onset (years)	62.1 (54.6–70.2)	61.8 (55.9–70.2)	62.3 (54.7–70.2)
Age at diagnosis (years)	64.2 (57.5–71.8)	64.0 (57.7–71.1)	64.3 (57.6–71.7)
Time from symptom onset to diagnosis (years)	1.1 (0.6–2.0)	1.1 (0.6–2.0)	1.1 (0.7–1.9)
Time from diagnosis to time of autopsy (years)	1.9 (1.3–2.9)	1.9 (1.3–2.9)	1.8 (1.2–2.9)
Final recorded ALSFRS-R	36 (32–39)	36 (32–40)	36 (31.25–39)
Onset Segment			
Bulbar	35 (37.2%)	28 (34.6%)	30 (34.5%)
Cervical	31 (33.0%)	28 (34.6%)	27 (31.0%)
Lumbar	24 (25.5%)	21 (25.9%)	26 (29.9%)
Unknown	4 (4.3%)	4 (4.9%)	4 (4.6%)

Continuous variables, age at plasma collection (years), BMI at study entry (kg/m^2), and ALSFRS-R at plasma collection, represented as median (25th – 75th percentile).

Categorical variables, sex, race/ethnicity, family history of ALS, and onset segment, represented as N (%; rounded to the first decimal place).

Significance with time assessed by a linear mixed model.

ALSFRS-R, ALS functional rating scale – revised.