Biochimica et Biophysica Acta 1842 (2014) 79–87

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Amino Acids as biomarkers in the SOD1^{G93A} mouse model of ALS

Monica Bame, Robert E. Grier [1], Richard Needleman, William S.A. Brusilow *

Department of Biochemistry and Molecular Biology, Wayne State University School of Medicine, 540 E. Canfield Street, Detroit, MI 48230, USA

ARTICLE INFO

Article history:
Received 22 August 2013
Received in revised form 26 September 2013
Accepted 7 October 2013
Available online 12 October 2013

Keywords:
ALS
Plasma amino acid
Biomarker
Methylation

ABSTRACT

The development of therapies for Amyotrophic Lateral Sclerosis (ALS) has been hindered by the lack of biomarkers for both identifying early disease and for monitoring the effectiveness of drugs. The identification of ALS biomarkers in presymptomatic individuals might also provide clues to the earliest biochemical correlates of the disease. Previous attempts to use plasma metabolites as biomarkers have led to contradictory results, presumably because of heterogeneity in both the underlying genetics and the disease stage in the clinical population. To eliminate these two sources of heterogeneity we have characterized plasma amino acids and other metabolites in the SOD1^{G93A} transgenic mouse model for ALS. Presymptomatic SOD1^{G93A} mice have significant differences in concentrations of several plasma metabolites compared to wild type animals, most notably in the concentrations of aspartate, cystine/cysteine, and phosphoethanolamine, and in changes indicative of methylation defects. There are significant changes in amino acid compositions between 50 and 70 days of age in both the SOD1^{G93A} and wild type mice, and several of the age-related and disease-related differences in metabolite concentration were also gender-specific. Many of the SOD1^{G93A}-related differences could be altered by treatment of mice with methionine sulfoximine, which extends the lifespan of this mouse, inhibits glutamine synthetase, and modifies brain methylation reactions. These studies show that assaying plasma metabolites can effectively distinguish transgenic mice from wild type, suggesting that one or more plasma metabolites might be useful biomarkers for the disease in humans, especially if genetic and longitudinal analysis is used to reduce population heterogeneity.

1. Introduction

The intense efforts made in unraveling the genetic and neurological bases of amyotrophic lateral sclerosis (ALS) have not led to the discovery of drugs that can substantially change the progress of the disease: fasciculations, muscle weakness and atrophy, and death from respiratory failure within 3–5 years of diagnosis. No effective therapy exists, and a definitive diagnosis can only be made after significant neuronal damage has already occurred [1].

Drug development has relied heavily on a mouse model in which genomic insertion of multiple copies of the human mutant SOD1 gene, most often SOD1^{G93A}, causes a disease very similar to ALS. Most of the drugs found to be useful in increasing survival in the ALS mouse model have failed clinical trials [2]. Many reasons can be postulated for this failure: obvious differences between the disease in mice and humans (e.g., cause of death), the difficulty of conducting dose response studies in a sufficiently large population of patients having a relatively rare disease, and most importantly the absence of biological markers of disease progression that would increase the statistical power of drug trials. In addition, ALS clinical studies rely on the enrollment of patients at different disease stages and whose underlying genetics may reflect different biochemical abnormalities. ALS patients with SOD1 mutations represent only about 5% of familiar cases and would thus be underrepresented in clinical trials [1,3].

Successful drug discovery is greatly facilitated by the identification of appropriate biomarkers, defined as an 'objective measurement that acts as an indicator of normal biological processes, pathogenic processes, or pharmacological response to therapeutic intervention' [4]. In addition to serving as a surrogate marker in drug studies, such biomarkers can establish the underlying biochemistry of ALS, and lead to early diagnosis and the identification of drug targets. The identification of biomarkers in ALS has been a very active area of investigation, employing transcriptional studies, protein profiling in blood and CSF, imaging, and electrophysiological techniques [5]. While these techniques have identified some potential ALS biomarkers, so far none have proven to be clinically useful.

The ideal biomarker for drug development is one for which there is an inexpensive and minimally invasive assay that can be done in the usual hospital setting. Plasma amino acid concentrations satisfy these criteria. Although the proximate cause of death in ALS is the destruction of motor neurons, ALS is a multi-organ disease that also affects the liver and skeletal muscle, two organs which are central participants in determining plasma amino acid concentrations [6,7]. In several studies

* Corresponding author.
E-mail address: wbrusilo@med.wayne.edu (W.S.A. Brusilow).
[1] Department of Pathology, Wayne State University School of Medicine and Harper University Hospital.

0925-4439/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbadis.2013.10.004

on human patients, however, plasma amino acid levels have not proved to be of clinical value in either disease diagnosis or as a measure of disease progression. While statistically significant differences between unaffected and ALS patients have been found, the amino acid biomarkers identified have varied from study to study and have produced no consistent pattern of disease-related differences. In one study tyrosine, valine, methionine, leucine, and isoleucine were found to be reduced in ALS and increases in glutamine and serine; another study showed increases in threonine, glutamate and cystine, and reductions in phenylalanine and histidine; a third report found only decreases in alanine, isoleucine, methionine, leucine and tyrosine [8–10].

We reevaluate here the use of amino acids as biomarkers in the $SOD1^{G93A}$ mouse, thus eliminating several sources of heterogeneity found in clinical studies. In this mouse model the disease proceeds in a highly uniform way with motor symptoms appearing at around 70 days and death occurring at around 125 days. As a result, in the asymptomatic $SOD1^{G93A}$ mouse we are able to identify amino acid biomarkers both of ALS and of disease progression.

Methionine sulfoximine (MSO), a drug which inhibits a central enzyme in amino acid metabolism, glutamine synthetase, and which we have found to increase survival (especially in females), also normalizes some of the amino acid abnormalities, and does so in a gender-specific manner [11]. Furthermore, some of the differences seen in amino acid profiles of $SOD1^{G93A}$ mice are gender-specific, with males and females showing different metabolic profiles; the profiles also differ by the disease stage.

Our results suggest that plasma analysis of certain amino acids may prove to be an effective tool in clinical studies on humans, if such biomarkers can also be identified in patients. Previous studies present results from analysis of a single plasma sample, but repeatedly sampling patients during the disease process will significantly reduce biological variability, allowing the identification of a "biochemical or metabolic" classification of changing plasma amino acid profiles during the normal disease process. Used in conjunction with the identification of the particular gene affected in each FALS (Familial Amyotrophic Lateral Sclerosis) patient, this approach may allow the use of plasma amino acid analysis as a biomarker for ALS.

2. Materials and methods

2.1. Animal breeding and care

All animal experiments were approved by Wayne State University's Animal Investigation Committee. $SOD1^{G93A}$ breeding mice were obtained from Jackson Laboratories. Female C57BL/6*SJL mice were crossed with B6SJL males over expressing the human SOD1 transgene containing the G93A mutation ($SOD1^{G93A}$).

2.2. Experimental groups

The 50 day group consisted of untreated wild-type and $SOD1^{G93A}$ mice from which liver, central nervous system tissue and plasma were collected at 50 ± 2 days of age. The 70 day group consisted of wild-type and $SOD1^{G93A}$ mice that were given weekly i.p. injections of saline or 20 mg/kg body weight MSO (Sigma Chemical Company, no. 5379) starting at 50 ± 2 days of age and were euthanized at 70 ± 2 days of age. Mice were euthanized and plasma collected for analysis within 1–3 days after the final injection.

2.3. Plasma and tissue collection

Mice were anesthetized with an i.p. injection of Avertin (0.4–0.75 mg/g body weight) and blood was collected via cardiac puncture. Blood was placed in BD Microtainer tubes containing lithium heparin and centrifuged at 16,000 ×*g* for 3 min in a Sorvall Biofuge pico desktop centrifuge to obtain plasma. For all animals, 100 μL aliquots of plasma were taken for plasma ammonia analysis and the remaining plasma was immediately frozen on dry ice for later use. All tissue samples were placed in centrifuge tubes and immediately frozen on dry ice and thawed prior to homogenization.

2.4. Plasma ammonia determination

Plasma ammonia levels were determined using the Berthelot indophenol reaction as described by Brusilow [12]. Ammonia levels were determined by comparing sample absorbances to those of an ammonium sulfate standard curve and values were expressed as μmol of ammonia per liter of plasma.

2.5. Plasma amino acid analysis

Plasma samples that were not used for ammonia determination were immediately frozen on dry ice. They were analyzed for plasma metabolites using a BioChrom 30+ Amino Acid Analyzer in the Biochemical Genetics Laboratory in the Detroit Medical Center. The plasma samples were deproteinized with 5-sulfosalicylic acid (SSA). 12 μg SSA was added to 350 μl of plasma, and after 5 min on ice, the precipitate was spun down, and the supernatant fraction was filtered and treated with 200 mM DTT for an hour at 37 °C, which converts all the cystine in the plasma to cysteine for analysis. Reported levels of cysteine therefore represent plasma cystine plus cysteine.

2.6. Glutamine synthetase activity assays

Brain preparations for GS activity were carried out as described by Meister [13]. Liver sample preparation was carried out using a modified version of Pierson's protocol for preparation of samples for CPSI activity assays [14]. GS activity was determined using a modified version of the gamma-glutamyltransferase reaction described by Meister [13]. The reaction was carried out for 10 min at 37 °C in the presence of 100 mM L-glutamine. Sample absorbances were read at a wavelength of 535 nm, and the extinction coefficient of the product, glutamyl hydroxamate, is reported to be .850 M^{-1} cm^{-1} [15]. *Ovariectomization* was performed on 30–40 day-old females, before the animals had reached sexual maturity, and were carried out as described previously [16].

GS activity assays were performed on brain and liver homogenates from ovariectomized and intact 70-day female mice. Absorbance values were converted to micromoles of product formed by comparing them to an L-γ-glutamyl hydroxamate standard curve and specific activities were determined accordingly.

2.7. Glutaminase activity assays

Glutaminase activity assays were carried out on the same brain homogenates used for GS activity assays. Brain glutaminase activity was determined by monitoring the production of ammonia using a modified version of Sigma's protocol for the enzymatic assay of glutaminase from *E. coli*. Test tubes containing 0.9 mL of glutaminase reaction buffer (100 mM sodium phosphate, and 20 mM L-glutamine, pH 8.0) were equilibrated to 37 °C in a water bath and 100 μL of brain homogenate was added. The reaction was allowed to proceed for 15 min at 37 °C before being quenched with 9 mL of cold deionized water on ice. A 100 μL aliquot from the quenched reaction mixture was added to a tube containing 200 μL of deionized water and 50 μL of the prepared ion-exchange resin used for determining ammonia levels. The ammonia assay was then carried out from this point to determine the amount of ammonia produced during the reaction. Ammonia produced during the reaction was quantified by reading absorbances of samples at 630 nm and comparing them to absorbance values from an ammonium sulfate standard curve. Glutaminase activity assays

were performed on 5–8 brain samples per treatment group for each gender at every time point.

2.8. Carbamoyl phosphate synthase I activity assays

Sample preparation and carbamoyl phosphate synthetase I enzymatic activity assays were carried out according to a modified version of a protocol by Pierson [14]

3. Results

Death occurs in SOD1^{G93A} mice at around 125 days of age with muscle weakness appearing at about 90 days. We collected plasma and measured amino acid levels in asymptomatic SOD1^{G93A} and congenic wild type mice at 50 and 70 days of age.

3.1. Plasma amino acid profiles differ between asymptomatic *SOD1^{G93A}* mice and congenic wild type mice

Table 1 shows those plasma amino acids and other metabolites that differ statistically ($p<0.05$) between asymptomatic SOD1^{G93A} mice and congenic wild type mice, pooling the data for both 50 and 70-day time points. In cases where the distribution was severely non-normal as shown by the Shapiro–Wilk test, the nonparametric Mann–Whitney test for significance was used, as indicated.

The cutoff two-sided p value was $p<0.05$ for both tests, but since low p values do not reflect the magnitude of statistical differences, we also used a standard measure of effect size, Cohen's d statistic, to identify the most important differences [17]. The test provides a measure of the overlap of the distribution in standard deviation units of amino acid concentrations between wild type and SOD1^{G93A} mice. For example, if $d = 0.8$ then the mean for SOD1^{G93A} mice is at the 79th percentile of the value obtained for the wild type mice; if $d = 1.0$, the 84th percentile; if $d = 2.0$, the 97.7th percentile. The standard interpretation of effect sizes is to identify $d > 0.8$ as a 'large effect' and $0.5 < d < 0.8$ as a 'medium effect'. Large effect sizes are found for phosphoserine (Phser), phosphoethanolamine (Pea), aspartate (Asp), citrulline (Citr), cysteine (Cys), and arginine (Arg); moderate effect sizes are found for glutamine (Gln), glutamate (Glu), ornithine (Orn), sarcosine (N-methylglycine) (Sarc), and methionine (Met). We include serine (Ser), which approaches our $p<0.05$ cutoff, since it is relevant to our later discussion of the phosphoserine pathway. The 95% confidence intervals on d are large, so a conservative measure is to use the minimum value to identify the most reliable major differences: cysteine (1.4), aspartate (0.7), and phosphoserine (0.6). Of course, this restriction does not exclude the other amino acids with smaller d values as being useful diagnostic markers.

3.2. The amino acid profile can identify presymptomatic *SOD1^{G93A}* mice

If a disease-related difference has a large Cohen effect size, it implies that there is low overlap between the amino acid concentrations in the two populations; in other words, the larger the d, the more likely that the plasma concentration of that amino acid can correctly assign a mouse to the correct group. We therefore further analyzed the concentrations of cysteine, aspartate, phosphoserine, citrulline, and phosphoethanolamine to attempt to predict the mouse genotype.

A logistic regression analysis using cysteine, aspartate, phosphoserine, citrulline and phosphoethanolamine correctly classifies 26 of 31 (84%) wild type and 26 of 27 (96%) SOD1^{G93A}mice, essentially the same as using only cysteine (81% wild type and 96% SOD1^{G93A}). Conducting the same regression analysis using just aspartate correctly classifies 71% of the wild type and 82% of the SOD1^{G93A} mice; regression with just phosphoethanolamine correctly classifies 74% of the wild type and 70% of the SOD1^{G93A} mice.

A Receiver Operating Characteristic (ROC) analysis is commonly used to evaluate the tradeoffs between test sensitivity (true positives) and test specificity (false positives). The larger the area under the curve (AUC), the better the diagnostic strength of the test, with the maximum area possible equal to 1. The AUC for cysteine, phosphoethanolamine, and aspartate is 0.9, 0.8, and 0.8 respectively. So these amino acids can be confidently used for a robust identification of presymptomatic SOD1^{G93A} mice.

3.3. The amino acid profile changes between 50 and 70 days of age in both wild type and *SOD1^{G93A}* mice

The amino acid profiles of both wild type and *SOD1^{G93A}* mice change between the ages of 50 and 70 days (Tables 2A and 2B). Wild-type mice show significant increases in average plasma levels of alanine, asparagine, valine, isoleucine, leucine, phenylalanine, and proline while *SOD1^{G93A}* mice show significant increases in average plasma levels of urea and sarcosine with age. Changes common to both genotypes are

Table 1
Comparison of the average metabolite levels of all untreated and saline-treated wild-type mice to those of all untreated and saline-treated SOD1^{G93A} mice, irrespective of age. All values are expressed as averages in μmol/L (SEM). For the plasma ammonia analysis n = 57 wild-type (WT) mice and n = 55 SOD1^{G93A} mice (*). For the remainder of the plasma metabolites n = 31 wild-type mice and n = 27 SOD1^{G93A} mice. In cases where data were non-normally distributed the Mann–Whitney test for significance was used to determine p values (†). Normal three-letter abbreviations for amino acids were used, in addition to Citr (citrulline), Pea (phosphoethanolamine), Phser (Phosphoserine), and Sarc (sarcosine).

Metabolite	WT Mean (SEM)	SOD1^{G93A} Mean (SEM)	*p*	*d* (95% CI)
Ammonia *	45 (4)	58 (5)	0.05	0.4 (0.0–0.7)
Arg	117 (6)	94 (5)	0.01	0.8 (0.2–1.3)
Asp	16.1 (1.1)	10.2 (0.5)	0.00	1.2 (0.7–1.8)
Citr	103 (4)	84 (3)	0.00	1.0 (0.4–1.5)
Cys	22 (3)	0.2 (0.9)	0.00 †	2.0 (1.4–2.6)
Gln	570 (13)	634 (23)	0.02	0.7 (0.1–1.2)
Glu	27 (4)	18 (2.6)	0.04	0.6 (0.0–1.1)
Met	92 (6)	74 (5)	0.03	0.6 (0.08–1.1)
Orn	90 (6)	73 (5)	0.04	0.6 (0.05–1.1)
Pea	9.4 (1.1)	3.2 (1.1)	0.00	1.0 (0.5–1.6)
Phser	12.6 (1.2)	6.4 (0.8)	0.00	1.1 (0.6–1.7)
Sarc	9.3 (3.1)	23 (4)	0.01 †	0.7 (0.2–1.2)
Ser	155 (5)	142 (4)	0.06	0.5 (0.0–1.0)

Table 2A
Age-related changes in average plasma metabolite levels of wild-type mice. All values are expressed as averages in μmol/L (SEM). For the plasma ammonia analysis n = 31 50-day old wild-type (WT) mice and n = 27 70-day old wild-type mice (*). For the remainder of the plasma metabolites n = 19 50-day old wild-type mice and n = 12 70-day old wild-type mice. Normal three-letter abbreviations for amino acids were used, in addition to AAAA (α-aminoadipic acid), Citr (citrulline), Pea (phosphoethanolamine), Phser (Phosphoserine), and Taur (Taurine).

Metabolite	50 day WT Mean (SEM)	70 day WT Mean (SEM)	*p*	*d* (95% CI)
Ammonia	52 (5)	27 (3)	0.00	1.1(0.5–1.7)
AAAA	26 (3)	14.7 (2.8)	0.02	0.9 (0.2–1.7)
Ala	578 (24)	675(29)	0.02	1.0 (0.2–1.7)
Asn	39 (2)	50 (5)	0.03	0.9 (0.1–1.6)
Asp	18.0 (1.5)	13.1 (1.4)	0.03	0.8 (0.1–1.6)
Citr	109 (5)	92 (7)	0.04	0.8 (0.2–1.5)
Cys	28 (2)	13.3 (6)	0.01	1.0 (0.2–1.7)
Glu	35 (4)	14.9 (3.6)	0.00	1.2 (0.5–2.0)
Ile	87 (4)	110 (9)	0.01	1.0 (0.3–1.8)
Leu	145 (5)	186 (15)	0.01	1.2 (0.4–2.0)
Pea	11.5 (1.3)	6.1 (1.7)	0.02	0.9 (0.2–1.7)
Phe	60 (2)	73 (5)	0.01	1.1 (0.4–1.9)
Phser	14.4 (0.8)	9.6 (2.7)	0.05	0.8 (0.03–1.5)
Pro	89 (4)	117 (10)	0.01	1.1 (0.4–1.9)
Taur	464 (33)	328 (20)	0.01	1.2 (0.4–1.9)
Val	247 (8)	312 (25)	0.01	1.1 (0.4–1.9)

Table 2B
Age-related changes in average plasma metabolite levels of SOD1^{G93A} mice. All values are expressed as averages in units of μmol/L (SEM). For the plasma ammonia analysis n = 31 50-day old SOD1^{G93A} mice and n = 34 70-day old SOD1^{G93A} mice (*). For the remainder of the plasma metabolites n = 13 50-day old SOD1^{G93A} mice and n = 14 70-day old SOD1^{G93A} mice. Normal three-letter abbreviations for amino acids were used, in addition to AAAA (α-aminoadipic acid), Pea (phosphoethanolamine), Taur (Taurine), and Sarc (sarcosine).

Metabolite	50 day SOD1^{G93A} Mean (SEM)	70 day SOD1^{G93A} Mean (SEM)	*p*	*d* (95% CI)
Ammonia	78(7.5)	35(3)	0.00	1.4(0.9–2.0)
AAAA	21 (4)	10.6 (1.4)	0.01	1.1 (0.3–1.9)
Asp	11.3 (0.9)	9.1 (0.3)	0.03	1.0 (0.2–1.8)
Glu	26 (3)	10.3 (3.2)	0.00	1.5 (0.7–2.4)
Pea	5.0 (2.1)	0(0)	0.03	1.0 (0.2–1.8)
Sarc	11.8 (5.3)	32 (5)	0.01	1.1 (0.3–1.9)
Taur	429 (37)	320 (26)	0.02	1.0 (0.2–1.8)
Urea	8089 (697)	10527 (433)	0.01	1.2 (0.4–2.0)

decreases in taurine, phosphoethanolamine, aspartate, alpha-amino adipic acid (AAAA), and glutamate.

Wild type and *SOD1^{G93A}* mice therefore undergo different metabolic programs as reflected in their plasma amino acid profiles during this asymptomatic period.

3.4. At both 50 and 70 days wild type and *SOD1^{G93A}* mice have different plasma amino acid profiles

Table 3 compares amino acid profiles of wild-type and *SOD1^{G93A}* mice at 50 and 70 days of age. 50 day old *SOD1^{G93A}* mice have higher plasma ammonia levels and urea levels than wild type mice of the same age. In addition, *SOD1^{G93A}* mice have higher levels of glutamine (the precursor of glutamate and ammonia), and lower levels of several intermediates of the urea cycle – aspartate, citrulline, and arginine. Remarkably, cysteine levels are extremely low in *SOD1^{G93A}* mice when compared to the wild type, with cysteine undetectable in the plasma of all but one *SOD1^{G93A}* mouse.

There are fewer significant differences between saline-treated *SOD1^{G93A}* and wild-type mice at 70 days of age than at 50 days of age. Ammonia is higher (because of a significant difference in males although not in females (gender data shown below in Table 7), phosphoethanolamine and aspartate are significantly lower, and cysteine was low or undetectable at both ages of *SOD1^{G93A}* mice. Lower glycine and higher sarcosine in *SOD1^{G93A}* mice only become apparent at 70 days.

The combined 50 day and 70 day results (Table 1) identified 12 metabolites that are different in wild type and *SOD1^{G93A}* animals: phosphoserine, phosphoethanolamine, ammonia, aspartate, glutamine, glutamate, sarcosine, citrulline, cysteine, ornithine, methionine, and arginine. Only three of these, phosphoethanolamine, aspartate, and cysteine, remain significantly different between wild type and *SOD1^{G93A}* mice at 70 days of age (Table 3). But the older mice also show differences in sarcosine and glycine that are absent in younger mice.

Table 3
Average concentrations of plasma metabolites of 50 and 70 day old wild-type and SOD1^{G93A} mice. All values are expressed as averages in μmol/L (SEM), and 70-day old animals had been injected with saline once per week starting at 50 days of age. For the plasma ammonia analysis of 50-day old animals n = 31 wild-type (WT) and 31 SOD1^{G93A} untreated mice, and for 70-day old animals n = 27 wild-type and 34 SOD1^{G93A} saline treated mice. For the remainder of the plasma metabolites n = 19 wild-type and 13 SOD1^{G93A} 50-day old untreated mice and n = 12 wild-type and 14 SOD1^{G93A} 70-day old saline treated mice. In cases where the data were non-normally distributed the Mann–Whitney test for significance was used to determine p values (†). Abbreviations are as described in Tables 1–2B.

Metabolite	50 day-old mice			70 day-old mice		
	WT Mean (SEM)	SOD1^{G93A} Mean (SEM)	*p*	WT, saline Mean (SEM)	SOD1^{G93A}, saline Mean (SEM)	*p*
Ammonia	52 (5)	78 (8)	0.02	27 (3)	35 (3)	0.07
Ala	578 (24)	673 (44)	0.04	675(29)	594 (33)	0.08
Arg	122 (8)	87 (8)	0.01	109 (8)	101 (8)	0.5
Asn	39 (2)	48 (4)	0.04	51 (5)	43 (3)	0.2
Asp	18.0 (1.5)	11.3 (0.9)	0.00	13.1 (1.4)	9.2 (0.4)	0.01
Citr	109 (5)	87 (6)	0.01	92 (7)	81 (4)	0.2
Cys	28 (2)	0.4 (0.4)	0.00 †	13.3 (5.5)	0.0 (0.0)	0.03 †
Gln	560 (13)	677 (37)	0.00	585 (27)	594 (24)	0.8
Gly	299 (7)	285 (13)	0.3	314 (12)	266(12)	0.00
Lys	320 (13)	274 (16)	0.03	290 (18)	280 (19)	0.7
Met	87 (6)	65 (7)	0.02	98 (12)	82 (7)	0.2
Pea	11.5 (1.3)	5.0 (2.0)	0.01	4.9(3.3)	0(0)	0.03 †
Phser	14.4 (0.8)	6.8 (1.2)	0.00	9.6 (2.7)	6.0 (1.0)	0.2
Sarc	11.9 (4.4)	11.8 (5.3)	1.0	4.9(3.3)	32 (5)	0.00
Urea	9867 (358)	8089 (697)	0.02	10640 (460)	10530 (430)	0.9

3.5. Ovariectomization alters the plasma amino acid profile and enzymes involved in glutamine metabolism in *SOD1^{G93A}* mice

Significant gender differences in metabolites and clinical features have been observed both in patients and in animal studies of ALS [18–20]. We have previously shown that female SOD1^{G93A} mice have delayed neuromuscular degeneration compared to male SOD1^{G93A} mice [21]. Methionine sulfoximine (MSO), a potent inhibitor of a central enzyme of nitrogen metabolism, glutamine synthetase, improves the survival of both males and females, but with a much larger effect on females [11,16]. Ovariectomization or castration eliminates the effect of MSO on both neuromuscular degeneration and survival, consistent with the view that estrogen is protective in ALS [16,22], and one aspect of that protection involves an MSO-sensitive pathway. Because of these results with MSO, and the disease-dependent alterations in ammonia and metabolites involved in the urea cycle (arginine, ornithine, citrulline, aspartate), we studied the effects of ovariectomization on metabolites and enzymes involved in central processes of nitrogen metabolism, in order to better define estrogen-dependent pathways that might be involved in the disease process.

We ovariectomized mice between 30 and 40 days of age, before maturity, then, at 70 days of age, we measured the activity of three enzymes involved in nitrogen metabolism: glutamine synthetase levels in the liver and brain, glutaminase in the brain, and carbamoyl phosphate synthetase I (CPSI), the rate limiting enzyme of the urea cycle, in liver at 70 days of age in intact and ovariectomized SOD1^{G93A} mice (Table 4). Ovariectomized SOD1^{G93A} females have significantly lower levels of brain and liver glutamine synthetase ($p = 0.04$ and $p = 0.003$ respectively), lower liver CPSI ($p = 0.05$), and elevated brain glutaminase activity ($p = 0.002$). Thus the specific activities of two enzymes involved in incorporation of ammonia into organic molecules are lowered by ovariectomization, and an enzyme involved in the production of ammonia is elevated by ovariectomization, demonstrating an effect of sex hormones on ammonia metabolism in these mice.

Ovariectomization also altered the amino acid profile in SOD1^{G93A} mice (Table 5). Aspartate, proline, and α-aminoadipic acid levels are

Table 4
The effects of ovariectomization on enzyme activity in female SOD1^{G93A} mice. All specific activities are expressed as averages of nmol product/minute/mg protein (SEM). n = 8 non-ovariectomized (OVR) and n = 7 ovariectomized female SOD1^{G93A} mice (‡). n = 10 non-ovariectomized and n = 9 ovariectomized female SOD1^{G93A} mice (†). Abbreviations: GS (Glutamine Synthetase), CPSI (Carbamoyl Phosphate Synthetase I).

Enzyme (Tissue)	Non-OVR Specific activity Mean (SEM)	OVR Specific activity Mean (SEM)	*p*
GS (brain) ‡	1345 (97)	1099 (29)	0.04
Glutaminase (brain) ‡	56 (7)	99 (8)	0.002
GS (liver) †	814 (26)	695 (21)	0.003
CPSI (liver) †	252 (11)	213 (14)	0.05

Table 5
The effects of ovariectomization on average plasma metabolite levels of 70-day old female SOD1^{G93A} mice. All values are expressed as averages in μmol/L (SEM). For the plasma metabolite analysis n = 12 wild-type females, n = 12 non-ovariectomized SOD1^{G93A} females, and n = 10 ovariectomized SOD1^{G93A} females. For the remainder of the plasma metabolites n = 6 wild-type females, n = 7 non-ovariectomized SOD1^{G93A} females, and n = 11 ovariectomized SOD1^{G93A} females. p values were obtained from the analysis comparing non-ovariectomized SOD1^{G93A} female mice to ovariectomized SOD1^{G93A} female mice. Abbreviations are as described in Tables 1–2B.

Metabolite	WT females Non-OVR Mean (SEM)	SOD1^{G93A} females		p
		Non-OVR Mean (SEM)	OVR Mean (SEM)	
Ammonia	33 (5)	33 (4)	41 (4)	0.2
AAAA	16.1 (5.4)	11.2 (32.7)	19.6 (1.4)	0.006
Asp	13.3 (2.4)	9.2 (0.6)	4.6 (1.7)	0.04
His	82 (3)	82 (7)	63 (3)	0.01
Lys	318 (33)	318 (18)	250 (16)	0.01
Pro	121 (20)	70 (19)	113 (7)	0.02
Sarc	8.9 (5.6)	38 (5)	0.0 (0.0)	0.00
Ser	174 (19)	152 (6)	121 (6)	0.003
Thr	204 (30)	190 (11)	154 (11)	0.06

elevated in plasma from ovariectomized SOD1^{G93A} females, while threonine, serine, sarcosine, lysine, and histidine are all significantly lower in ovariectomized SOD1^{G93A} females.

3.6. Methionine Sulfoximine (MSO), an inhibitor of glutamine synthetase affects the plasma amino acid profile of SOD1^{G93A} mice

Methionine sulfoximine (MSO), a mechanism-based inhibitor of glutamine synthetase extends the survival of the SOD1^{G93A} mouse and is more effective in females than in males. Wild type and SOD1^{G93A} mice were treated once a week with i.p. injections of either saline or 20 mg/kg MSO starting at 50 days of age. This dosage has been shown to be well-tolerated by mice, even when injected three times per week [23] and is the dosage and timing we have used in our previous studies [11,16,26]. 50 days was chosen as the starting point in order to treat mice that were adults but presymptomatic. Table 6 shows the plasma metabolites that differ in SOD1^{G93A} mice after MSO treatment. SOD1^{G93A} mice treated with MSO show several changes: phosphoethanolamine, α-aminoadipic acid, and ornithine levels increase, while average sarcosine levels decrease. Asparagine, alanine, arginine, and cysteine levels increase significantly with MSO treatment, while – not surprisingly – average glutamine levels decrease. Cysteine, which is undetectable in saline-treated SOD1^{G93A} mice, becomes detectable after MSO treatment.

Table 6
The effects of MSO treatment on average plasma metabolite levels in SOD1^{G93A} mice. All values are expressed as averages in μmol/L (SEM). For the plasma ammonia analysis n = 34 mice/treatment group. For the remainder of the plasma metabolites n = 14 for saline treated mice and n = 16 for MSO treated mice. Abbreviations are as described in Tables 1–2B.

Metabolite	Saline Mean (SEM)	20 mg/kg MSO Mean (SEM)	p
Ammonia	35 (3)	52 (5)	0.00
AAAA	10.6 (1.4)	17.6 (1.7)	0.00
Ala	594 (33)	743 (55)	0.00
Arg	101 (8)	137 (11)	0.01
Asn	43 (3)	57 (4)	0.01
Cys	0.0 (0.0)	5.3 (2.1)	0.03
Gln	594 (24)	449 (29)	0.00
Lys	280 (19)	358 (25)	0.02
Orn	74 (6)	96 (8)	0.02
Pea	0(0)	4.7 (1.2)	0.01
Sarc	32 (5)	18.3 (3.4)	0.03

3.7. The effect of MSO on amino acid levels is gender specific

Since MSO extends the survival of the SOD1^{G93A} mouse in a gender specific manner, with females living longer than males, we might expect that it would also affect plasma amino acid profiles in a gender-dependent fashion [11,16].

The plasma metabolites that are altered by MSO can be seen in Table 6 and the gender specific changes in Table 7. MSO treatment has a minimal effect on wild type female mice, only slightly elevating plasma ammonia, but does affect SOD1^{G93A} female mice, elevating plasma ammonia and cysteine levels and significantly decreasing plasma levels of glutamine and sarcosine.

Male wild type mice treated with MSO show significant elevations in average plasma levels of ammonia, phosphoserine, phosphoethanolamine, glutamate, ornithine, and lysine. Male SOD1^{G93A} mice treated with MSO also show moderate elevations in average plasma ammonia levels, and much like wild type males, show elevations in average levels of phosphoethanolamine. These mice also show significant increases in average plasma levels of α-aminoadipic acid, alanine, and tryptophan, changes that are not observed in any other group.

It is interesting to note that in 70-day old mice, SOD1^{G93A} females show elevated levels of plasma sarcosine when compared to their wild-type counterparts (Table 3), and that while MSO treatment lowers plasma sarcosine levels in these mice, ovariectomized female SOD1^{G93A} mice have undetectable levels of sarcosine in their plasma (Table 5). As discussed below, these differences point to an alteration of methylation/demethylation reactions in the SOD1^{G93A} mice.

Table 7
The effects of MSO treatment according to genotype and gender. All values are expressed as averages in μmol/L (SEM). For the plasma ammonia analysis n = 12 mice/gender/treatment. For the remainder of the plasma metabolites n values are as follows: 6 wild-type males and 6 wild-type females treated with saline, 7 SOD1^{G93A} males and 7 SOD1^{G93A} females treated with saline, 8 SOD1^{G93A} males and 8 SOD1^{G93A} females treated with MSO, and 5 wild-type males and 5 wild-type females treated with MSO. p values highlighted in bold face font were those that approached significance, while those marked with ND indicate that p values were not determined due to non-normally distributed data.

Group	Metabolite	Saline Mean (SEM)	20 mg/kg MSO Mean (SEM)	p
WT females	Ammonia	33 (5)	48 (4)	**0.02**
	Arg	119 (14)	118 (10)	0.9
	Cys	12.4 (7.9)	27 (8)	0.2
	Gln	639 (35)	587 (68)	0.5
	Sarc	8.9 (5.7)	12.2 (8.1)	0.7
SOD1^{G93A} Females	Ammonia	33 (4)	53 (6)	**0.01**
	Arg	115 (8)	152 (14)	**0.04**
	Cys	0.0 (0.0)	3.6 (2.9)	ND
	Gln	647 (26)	465 (45)	**0.005**
	Sarc	38 (5)	20 (5)	**0.02**
WT males	Ammonia	22 (4)	34 (2)	**0.02**
	AAAA	13.2 (2.3)	18.2 (4.7)	0.4
	Ala	674 (28)	792 (102)	0.3
	Cys	14.2 (8.3)	27 (7)	0.3
	Glu	8.3 (3.8)	29 (6)	**0.01**
	Lys	262 (9)	323 (21)	**0.02**
	Orn	71 (11)	102 (7)	**0.04**
	Pea	1.0 (1.0)	9.0 (3.1)	**0.03**
	Phser	6.5 (1.1)	13.5 (2.1)	**0.01**
	Trp	50 (5)	30 (12.6)	0.1
SOD1^{G93A} males	Ammonia	32 (5.2)	50 (7)	**0.05**
	AAAA	10.1 (0.8)	16.8 (2.3)	**0.02**
	Ala	521 (43)	710 (54)	ND
	Cys	0.0 (0.0)	7.0 (3.1)	**0.02**
	Glu	11.1 (5.5)	23 (4)	0.1
	Orn	73 (10)	97 (18)	0.09
	Pea	0.6 (0.6)	5.7 (1.7)	**0.02**
	Phser	6.6 (1.5)	7.9 (2.9)	0.7
	Trp	43 (7)	66 (4)	**0.01**

4. Discussion

Progress in ameliorating the suffering of ALS patients has been significantly hindered by the lack of useful biomarkers for early disease diagnosis and for monitoring the efficacy of drug treatment. Many biochemical, neurological, and radiological correlates of ALS have been reported but none have proved to have the requisite sensitivity or specificity.

The search for useful biomarkers has applied diverse methods: transcriptional analysis, high sensitivity identification of proteins and metabolites by mass spectroscopy and high resolution NMR, and diffusion tensor radiography [5,24]. The ideal biomarker would be measured following a minimally invasive procedure that is inexpensive and rapid to assay, and would use clinical protocols currently available in all hospitals; sophisticated analytic tools, while valuable in identifying the underlying biochemical processes in ALS are less suitable in clinical settings.

Amino acid analysis of blood plasma satisfies all these criteria, and several studies have in fact shown abnormalities of plasma amino acid profiles in ALS patients, but different amino acids have been identified as abnormal in different studies. Iwasaki and colleagues found elevated levels of aspartate, glutamate, and glycine; Perry and coworkers found increased glutamate, glutamine, and cysteine, while threonine, methionine, phenylalanine, and histidine decreased; Ilzecka and colleagues found higher glutamine and serine, and lower tyrosine valine, methionine, leucine and isoleucine [8,9,25]. More sophisticated and larger metabolomic screens have also identified plasma amino acids or their breakdown products that differ in ALS patients [4,26].

The failure to find a consistent clinical amino acid profile for ALS is not surprising since the patient populations are highly heterogeneous, both in their underlying genetics and in their disease stage. The use of small patient populations and single instead of longitudinal analysis of samples also decreased the statistical power of these studies. In addition, the primary causes of ALS – still unknown – might well involve different metabolic pathways, with all leading to a common endpoint of muscle atrophy and respiratory failure [27–29].

In this report we reinvestigate the possibility of using plasma amino acids as biomarkers for the disease in the SOD1^{G93A} mouse model of ALS. Our studies take advantage of the synchronized disease progression and genetic uniformity of the SOD1^{G93A} model, and we have confined our analysis to 'asymptomatic' mice, trying to identify early biochemical changes; however pathological changes in SOD1^{G93A} mice have also been observed 13 days before our first plasma amino acid analysis at 50 days [30]. Compared to our mouse model, the heterogenous disease presentation in patients means that a metabolomic 'snapshot' at one time point in a genetically diverse population is unlikely to reveal useful biomarkers for disease.

We have addressed two questions: First, can we identify plasma amino acid biomarkers using this model in which genotype and disease stage is precisely defined? Second, do the plasma amino acids profiles provide information about the biochemical basis of ALS?

4.1. Plasma amino acid profiles in wild type and SOD1^{G93A} mice

ALS affects not only motor neurons, but is a multi-organ disease affecting liver and muscle, the major organs involved in amino acid metabolism. In later stages of the disease where muscle wasting is prominent, levels of amino acids reflecting muscle breakdown should increase [7,31–33]. In addition, changes in the amino acid profile might be expected due to general acidosis in the SOD1^{G93A} mouse [34].

Three changes in the amino acid profile might be expected, each reflecting a current hypothesis about ALS: mitochondrial dysfunction, oxidative stress, and excitotoxicity. Mitochondrial dysfunction might affect amino acid intermediates of the urea and TCA cycles; oxidative stress may be reflected in changes in the methionine/cysteine/glutathione pathway and the arginine/NO pathway; excitotoxiciy may be reflected in levels of glutamate, glycine, aspartate, glutamine, and serine.

We observed all three types of predicted changes, with significant changes in amino acids involved in the urea cycle, neuron excitation, and methylation and the related transsulfuration pathway: cysteine, methionine, glycine, sarcosine, phosphoserine, and phosphoethanolamine. These amino acids participate in GSH synthesis, methylation of DNA, RNA, and lipids, and lipid synthesis. The observed changes suggest aberrant methylation, a condition that is seen not only in ALS but also in other neurodegenerative diseases [35–38].

4.2. Alterations in mitochondrial processes and metabolites

In plasma from 50 day old mice, there are significant differences between SOD1^{G93A} and wild type in metabolites associated with mitochondrial processes. Ammonia levels are higher in the SOD1^{G93A} mice, and urea cycle intermediates arginine, ornithine, and citrulline, are all lower. Glutamate, aspartate, and urea are lower in SOD1^{G93A} animals, and glutamine is higher. At 70 days, only the differences in ammonia and aspartate persist. Both the 50 day differences and the 70 day differences indicate an effect of the SOD1^{G93A} mutation and/or the disease process on the ammonia-handling characteristics of mitochondrial metabolism. Besides the potentially pathogenic effects of altered metabolic pathways in mitochondria, ammonia itself is extremely toxic and is normally maintained at very low levels. Elevated ammonia might contribute to cell death properties seen in neurodegeneration. Ovariectomization of SOD1^{G93A} females lowers levels of glutamine synthetase activity in the brain and liver, lowers levels of CPSI activity in the liver, and raises levels of glutaminase activity in the brain. Reduced estrogen therefore is correlated to lowered activity of two enzymes that incorporate ammonia into other molecules, and increased activity of an ammonia producing enzyme.

4.3. Changes in oxidative metabolism as biomarkers in ALS

Cysteine and cystine play multiple biological roles, including immune cell function, synthesis of glutathione (GSH), the major antioxidant of the brain, and cell signaling through its Cys/CySS redox ratio [39,40]. Cystine is exchanged for cellular glutamate through the cystine/glutamate antiporter followed by its reduction to cysteine; this exchange plays an important role in glutamateric signaling [41]. Our amino acid analysis does not detect cystine, homocysteine, or glutathione.

Our measurements show that cysteine levels are extremely low in SOD1^{G93A} mice, with cysteine completely undetectable in all but one SOD1^{G93A} mouse (Table 1). Cysteine concentrations are so abnormal that they alone can be used to distinguish between wild type and SOD1^{G93A} asymptomatic mice. A logistic regression using only cysteine correctly classifies 25 of 31 wild type mice (81%) and 26 of 27 SOD1^{G93A} mice (96%); a ROC curve from this classification has an area of 0.9. Low cysteine is seen in many disease states: HIV infection, cancer, sepsis, and intestinal disorders [42,43]. These conditions are often associated with low natural killer (NKT) activity and muscle wasting.

Cysteine concentrations are independent of gender and age, but are increased by MSO treatment in SOD1^{G93A} mice ($p = 0.04$, Mann–Whitney for 70 day old mice; $p = 0.00$ combining 50 and 70 day old mice). Oxidative damage has been implicated in the progression of ALS, with mutant SOD1^{G93A} mice engineered to have defect in γ-glutamylcysteine ligase (the first and rate limiting enzyme in glutathione synthesis) showing a 55% reduction in average lifespan [44]. Since cysteine is rate-limiting, low cysteine may lead to reduced GSH synthesis and sensitivity to ROS. When SOD1^{G93A} expression is confined to skeletal muscle ROS increases and extensive muscle damage occurs. Motor neurons expressing mutant SOD1^{G93A} in cell culture also have lower GSH and GSH levels in the brain of the SOD1^{G93A} are also altered [11,32,45].

4.4. Metabolites involved in methylation and related pathways as biomarkers in ALS

The disease-related changes observed in these studies suggest aberrant methylation, a condition that is seen not only in ALS but also in other neurodegenerative diseases [35–38]. Defects in DNA methylation have been linked to neurodegenerative disease including ALS ([36,46,47]. In NSC34 cultured cells increases in the DNA methyltransferase enzyme (Dnmt), Dnmt3a [13]causes neurodegeneration, and this enzyme is also expressed in the motor cortex and spinal cord motor neurons of SALS (Sporadic Amyotrophic Lateral Sclerosis) patients. In addition, SALS patients show large scale changes in brain methylation patterns [38]. A possible biomarker for this pathway is homocysteine, a central intermediate in the methylation pathway, which is elevated in ALS patients [48].

We have found striking changes in metabolites in the linked methylation and transsulfuration pathways in SOD1^{G93A} mice. Serine, phosphoserine, sarcosine, phosphoethanolamine, methionine, glycine, cysteine, are metabolites in closely integrated metabolic pathways, and all show changes in SOD1^{G93A} mice. Briefly, the methionine cycle regenerates methionine from homocysteine in a vitamin B-12 dependent methylation with 5-methyltetrahydrofolate as the donor. Methionine can then be converted to S-adenosylmethionine (SAM) which is the primary donor for the methylation of DNA, RNA, and proteins. After the transfer of the methyl group, SAM is recycled to homocysteine. Alternatively, homocysteine can react with betaine to form methionine and dimethylglycine, the later is then converted to sarcosine (N-methylglycine). Sarcosine is rapidly degraded to glycine, a precursor to both serine and ethanolamine. Homocysteine is also a precursor of cysteine and hence glutathione, and these reactions comprising the transsulfuration pathway. SAM can also methylate phosphatidylethanolamine to phosphatidylcholine [49].

SOD1^{G93A} mice show a remarkable elevation in sarcosine at 70 days of age ($p = 0.00$, $d = 1.8$, 0.8–2.7) (Tables 2A and 2B). Sarcosine (N-methylglycine) is an intermediate in the choline-to-glycine pathway and is metabolized to glycine, serine, and ethanolamine; it is also an inhibitor of glycine transporter 1 [50]. Sarcosine in SOD1^{G93A} mice is elevated in both males and females at this age, and MSO reduces it in both genders. Since elevations are not seen at 50 days, sarcosine is potentially a biomarker of progression. In contrast to sarcosine, glycine becomes lower in 70 day SOD1^{G93A} mice ($p = 0.00$, $d = 1.5$, 0.6–2.4). Sarcosine can be made from dimethylglycine in the homocysteine to methionine reaction or directly from glycine by glycine-N-methyltransferase; conversely sarcosine can be converted to glycine by sarcosine dehydrogenase. These reactions are of importance to neurological function and it has been postulated that they control the important SAM/SAH ratio [51,52]. Sarcosine inhibits glycine transport into astrocytes by blocking the Gly1T1 transporter, which would be expected to activate the NMDA receptor by occluding the D-serine/glycine site.

Phosphoserine concentrations are lower in SOD1^{G93A} mice than in wild type. Lowered phosphoserine has also been found in the CSF of ALS patients [53]. Phosphoserine is converted to serine by phosphoserine phosphatase. Alterations in phosphoserine phosphatase may play a role in glutamate neurotoxicity in Alzheimer's disease, and phosphoserine is elevated in ecstasy (MDMA) users [54,55]. Serine is synthesized from glucose through 3-phosphoglycerate with this dephosphorylation as the final reaction. Serine may also be derived from glycine produced from the degradation of proteins or phospholipids. D-serine, produced from L-serine by L-serine racemase enhances glutamate toxicity in ALS [56,57]. Enzymes involved in D/L serine metabolism have altered transcription in asymptomatic SOD1^{G37R} mice [58].

Phosphoethanolamine is present in high concentrations in the brain and is a precursor to both acetylcholine and the high-energy donor CDP-ethanolamine. Decreased levels of phosphoethanolamine are found in Alzheimer's and Huntington's disease [59]. In our studies, phosphoethanolamine levels vary with genotype, gender, and age. We measured decreases with age in both wild type and *SOD1^{G93A}* mice, but *SOD1^{G93A}* mice have significantly lower levels at both ages. Wild type females have the same concentration at both ages, but males have significantly lower levels at 70 days of age. In contrast female *SOD1^{G93A}* mice show a large decrease with age.

4.5. Effects of age and gender on plasma amino acids

The results reported here imply that to be useful in ALS clinical practice, amino acid profiles must control for gender and disease stage. Both wild type and SOD1^{G93A} mice exhibited significant changes in plasma amino acid composition between 50 and 70 days of age. While it may seem surprising that we found amino acid changes between the seemingly short, 50-70 day time span in wild type mice, in humans this is roughly equivalent to early human childhood where children from 3 months to 4 years of age also show age and gender related changes in metabolites [60]. These changes then may be part of the maturation process that differs between wild type and SOD1^{G93A} animals. We also found that ovariectomization changes the plasma amino acid profile and alters the levels of brain and liver glutamine synthetase, liver glutaminase, and liver CPSI of SOD1^{G93A} mice. CPSI is dependent upon gender in humans, and sarcosine, the metabolite most strongly affected by ovariectomization, is also under androgen control in humans. Estrogens have been postulated as being protective in ALS [22,61,62].

4.6. Use of plasma amino acid profiles to identify the underlying biochemical defects in ALS

Amino acid profiles reflect a complex interaction between diet, genetics, pharmacological compartment kinetics, and metabolic states. It is difficult to identify the underlying biochemical defects from these changing profiles because while we understand to some extent the network of amino acid synthesis and degradation pathways and the kinetic parameters (at least in vitro) of the enzymes involved we lack information about the correlations of amino acid concentrations and the exchange kinetics between the organ compartments involved in amino acid metabolism. Indeed, the underlying metabolic defect(s) may change over the course of the disease, as seen by the fact that our SOD1^{G93A} mice have distinctly different profiles at 50 and 70 days (Table 3).

Nevertheless the changes we observe lend support to theories about the role of mitochondria and oxidative stress in ALS, and in addition suggests that the recent striking observation of a methylation defect in ALS may be reflected in plasma amino acids and metabolites such as sarcosine and thereby provide a practical method for following disease progression in a clinical setting.

The appropriate statistical tools for approaching this task have been developed primarily from research on transcription networks in the last decade. These tools include both standard (principal component analysis, partial least squares) and newer methods for network analysis [5]. We have not applied these more sophisticated tools to our analysis of amino acid profiles because it would require an analysis of many hundred mice and would have little clinical relevance. The appropriate next study would involve measuring plasma amino acids, over time in ALS patients that had been grouped by disease stage, gender, and type of ALS (e.g. sporadic, familial), compared to matched control groups.

4.7. Useful biomarkers for ALS

The mouse model closely mimics many of the features of the human disease. It seems likely that all ALS patients have both common biochemical changes due to motor neuron death and muscle atrophy and unique changes dependent upon the underlying genetic defect. Our observation of cysteine as a biomarker for the SOD1^{G93A} mouse

has not been observed in the previous studies of plasma amino acids, possibly due to the mixed patient population studied and may be an example of a change unique to the SOD1^{G93A} allele.

Given the relatively small number of control mice (31 wild type vs 27 SOD1^{G93A}) identifying the key amino acids in a logistic regression model is difficult. The large number of amino acids measured relative to the mouse population studied means that a good statistical fit can be derived from many different combinations of amino acids. However, aspartate, cysteine, and phosphoethanolamine are abnormal in SOD1^{G93A} mice at both 50 and 70 days of age. Using all three metabolites allows the correct identification of 80% (24/30) of wild type and 96% (22/23) of SOD1^{G93A} mice with a ROC of 0.92(SE = 0.04). Using only aspartate and phosphoethanolamine we identify the same number of wild type mice correctly, but only 18/23 SOD1^{G93A} with ROC = 0.86(SE = 0.06). Therefore, those metabolites are useful disease biomarkers in mice.

4.8. Usefulness of plasma amino acid analysis to study effects of drugs

Our studies on the effects of MSO suggest that plasma amino acid analysis might be used to screen potentially useful ALS drugs. From our previous measurements of grip strength and survival we know that MSO affects both males and female SOD1^{G93A} mice, but with a larger positive effect on female survival [11,16]. At 70 days, the diagnostic metabolites for the SOD1^{G93A} mice are phosphoethanolamine, aspartate, sarcosine, glycine, and cysteine (Table 3); we omit discussion of NH_4^+ since this is directly dependent upon glutamine synthetase. Of these only aspartate is closely related to an amino acid pathway expected to be influenced by MSO, which is a site-specific inhibitor of glutamine synthetase. Nonetheless, MSO affects the level of phosphoethanolamine, sarcosine, and cysteine (Table 6) in SOD1^{G93A} mice, in all cases significantly changing the levels in the direction of the wild type values. It has been shown that MSO also increases glycogen deposit in the brain [63], a possible compensatory mechanism for reducing acidosis recently observed in the SOD1^{G93A} mouse [34]. Acidosis by itself can modulate the levels of brain and plasma amino acids [64,65]. When mice are treated with MSO, phosphoethanolamine is increased in both wild type and SOD1^{G93A} mice, and sarcosine decreases but only in female mice. Cysteine is not changed in wild type mice but levels become non-zero in SOD1^{G93A} mice; the statistical significance is difficult to calculate due to the severely non-normal distribution. MSO has no effect on plasma amino acids in wild type female mice. It is interesting to note that in addition to inhibiting glutamine synthetase, MSO also has major effects on levels of brain S-adenosylmethionine and methylation reactions, consistent with the disturbances in methylation-related metabolites that we have found [66–68].

Given these observations, even in the absence of any information of MSO on disease progress, it is clear that MSO would be flagged as a potentially useful drug once the amino acid profile of treated and untreated SOD1^{G93A} mice had been established and compared to wild type mice. Similar analysis of the effects of drugs on plasma metabolites might therefore be useful in identifying therapeutically useful compounds.

4.9. Summary

The SOD1^{G93A} mutation that causes an ALS-like disease in mice produces significant changes in plasma amino acids and other metabolites, most significantly those concentrated in the methylation and related pathways affecting levels of cysteine, serine, phosphoserine, taurine, phosphoethanolamine, sarcosine, glycine, methionine, and aspartate. The fact that these changes are observed in presymptomatic animals suggests that the analysis of plasma metabolites might be useful in monitoring the progression of ALS in humans, if similar amino acid analyses can be done on well-defined groups of ALS patients over time.

Acknowledgements

We acknowledge the technical assistance of Weiping Mattingly.

References

[1] R. Dengler, El Escorial or Awaji criteria in ALS diagnosis, what should we take? Rom. J. Neurol. Psychiatry 123 (2012) 217–218.
[2] N. Nirmalananthan, L. Greensmith, Amyotrophic lateral sclerosis: recent advances and future therapies, Curr. Opin. Neurol. 18 (2005) 712–719.
[3] A. Al-Chalabi, A. Jones, C. Troakes, A. King, S. Al-Sarraj, L.H. den Berg, The genetics and neuropathology of amyotrophic lateral sclerosis, Acta Neuropathol. 124 (2012) 339–352.
[4] M.R. Turner, M.C. Kiernan, P.N. Leigh, K. Talbot, Biomarkers in amyotrophic lateral sclerosis, Lancet Neurol. 8 (2009) 94–109.
[5] P.-F. Pradat, M. Dib, Biomarkers in amyotrophic lateral sclerosis: facts and future horizons, Mol. Diagn. Ther. 13 (2009) 115–125.
[6] Y. Nakano, K. Hirayama, K. Terao, Hepatic ultrastructural changes and liver dysfunction in amyotrophic lateral sclerosis, Arch. Neurol. 44 (1987) 103–106.
[7] S. Abmayr, P. Weydt, Skeletal muscle in amyotrophic lateral sclerosis: emerging concepts and therapeutic implications, Phys. Med. Rehabil. Clin. N. Am. 16 (2005) 1091–1097(xi–xii).
[8] J. Iłzecka, Z. Stelmasiak, J. Solski, S. Wawrzycki, M. Szpetnar, Plasma amino acids percentages in amyotrophic lateral sclerosis patients, 25 (2003) 69–73.
[9] T.L. Perry, C. Krieger, S. Hansen, A. Eisen, Amyotrophic lateral sclerosis: amino acid levels in plasma and cerebrospinal fluid, Ann. Neurol. 28 (1990) 12–17.
[10] W. Camu, M. Billiard, M. Baldy-Moulinier, Fasting plasma and CSF amino acid levels in amyotrophic lateral sclerosis: a subtype analysis, Acta Neurol. Scand. 88 (1993) 51–55.
[11] F. Ghoddoussi, M.P. Galloway, A. Jambekar, M. Bame, R. Needleman, W.S.A. Brusilow, Methionine sulfoximine, an inhibitor of glutamine synthetase, lowers brain glutamine and glutamate in a mouse model of ALS, J. Neurosci. 290 (2010) 41–47.
[12] S.W. Brusilow, Phenylacetylglutamine may replace urea as a vehicle for waste nitrogen excretion, 29 (1991) 147–150.
[13] A. Meister, Glutamine synthetase from mammalian tissues, Methods Enzymol. 113 (1985) 185–199.
[14] D. Pierson, A rapid colorimetric assay for carbamyl synthetase I, J. Biochem. Biophys. Methods 3 (1980) 31–37.
[15] R.A. Ronzio, A. Meister, Phosphorylation of methionine sulfoximine by glutamine synthetase, Proc. Natl. Acad. Sci. U. S. A. 59 (1968) 164–170.
[16] M. Bame, P. Pentiak, R. Needleman, W. Brusiow, Effect of sex on lifespan, disease progression, and the response to methionine sulfoximine in the SOD1 G93A mouse model for ALS, Gend. Med. 9 (2012) 524–535.
[17] J. Cohen, A power primer, Psychol. Bull. 112 (1992) 155–159.
[18] P. McCombe, R. Henderson, Effects of gender in amyotrophic lateral sclerosis, Gend. Med. 7 (2010) 557–570.
[19] K. Frutiger, T.J. Lukas, G. Gorrie, S. Ajroud-Driss, T. Siddique, Gender difference in levels of Cu/Zn superoxide dismutase (SOD1) in cerebrospinal fluid of patients with amyotrophic lateral sclerosis, Amyotroph. Lateral Scler. 9 (2008) 184–187.
[20] S. Hadano, A. Otomo, R. Kunita, K. Suzuki-Utsunomiya, A. Akatsuka, M. Koike, M. Aoki, Y. Uchiyama, Y. Itoyama, J.-E. Ikeda, Loss of ALS2/Alsin exacerbates motor dysfunction in a SOD1-expressing mouse ALS model by disturbing endolysosomal trafficking, PLoS One 5 (2010) e9805.
[21] M. Bame, Understanding the gender-based mechanism of MSO in ALS mice: a metabolic characterization of the SOD1G93A mouse model, (Ph.D. Thesis) Wayne State University School of Medicine, 2012.
[22] H. Blasco, A.-M. Guennoc, C. Veyrat-Durebex, P.H. Gordon, C.R. Andres, W. Camu, P. Corcia, Amyotrophic lateral sclerosis: a hormonal condition? Amyotroph. Lateral Scler. 13 (2012) 585–588.
[23] M. Blin, W.E. Crusio, T. Hévor, J.-F. Cloix, Chronic inhibition of glutamine synthetase is not associated with impairment of learning and memory in mice, Brain Res. Bull. 57 (2002) 11–15.
[24] K.A. Lawton, M.E. Cudkowicz, M.V. Brown, D. Alexander, R. Caffrey, J.E. Wulff, R. Bowser, R. Lawson, M. Jaffa, M.V. Milburn, J.A. Ryals, J.D. Berry, Biochemical alterations associated with ALS, Amyotroph. Lateral Scler. 13 (2012) 110–118.
[25] Y. Iwasaki, K. Ikeda, M. Kinoshita, Plasma amino acid levels in patients with amyotrophic lateral sclerosis, J. Neurosci. 107 (1992) 219–222.
[26] K.A. Lawton, A. Berger, M. Mitchell, K.E. Milgram, A.M. Evans, L. Guo, R.W. Hanson, S.C. Kalhan, J.A. Ryals, M.V. Milburn, Analysis of the adult human plasma metabolome, Pharmacogenomics 9 (2008) 383–397.
[27] A. Serio, B. Bilican, S.J. Barmada, Astrocyte pathology and the absence of non-cell autonomy in an induced pluripotent stem cell model of TDP-43 proteinopathy, Proc. Natl. Acad. Sci. 110 (2013) 4697–4702.
[28] H.P. Phatnani, P. Guarnieri, B.A. Friedman, M.A. Carrasco, M. Muratet, S. O'Keeffe, C. Nwakeze, F. Pauli-Behn, K.M. Newberry, S.K. Meadows, J.C. Tapia, R.M. Myers, T. Maniatis, Intricate interplay between astrocytes and motor neurons in ALS, Proc. Natl. Acad. Sci. 110 (2013) E756–E765.
[29] A. Wuolikainen, P.M. Andersen, T. Moritz, S.L. Marklund, H. Antti, ALS patients with mutations in the SOD1 gene have an unique metabolomic profile in the cerebrospinal fluid compared with ALS patients without mutations, Mol. Genet. Metab. 105 (2012) 472–478.
[30] A.Y. Chiu, P. Zhai, M.C. Dal Canto, T.M. Peters, Y.W. Kwon, S.M. Prattis, M.E. Gurney, Age-dependent penetrance of disease in a transgenic mouse model of familial amyotrophic lateral sclerosis, Mol. Cell 6 (1995) 349–362.
[31] L. Dupuis, L. Dupuis, H. Oudart, H. Oudart, F. René, F. René, J.-L. Gonzalez de Aguilar, J.-L. Gonzalez de Aguilar, J.-P. Loeffler, J.-P. Loeffler, Evidence for defective energy

homeostasis in amyotrophic lateral sclerosis: benefit of a high-energy diet in a transgenic mouse model, Proc. Natl. Acad. Sci. 101 (2004) 11159–11164.
[32] G. Dobrowolny, M. Aucello, E. Rizzuto, S. Beccafico, C. Mammucari, S. Bonconpagni, S. Belia, F. Wannenes, C. Nicoletti, Z. Del Prete, N. Rosenthal, M. Molinaro, F. Protasi, G. Fanò, M. Sandri, A. Musarò, Skeletal muscle is a primary target of SOD1G93A-mediated toxicity, Cell Metab. 8 (2008) 425–436.
[33] A. Finkelstein, G. Kunis, A. Seksenyan, A. Ronen, T. Berkutzki, D. Azoulay, M. Koronyo-Hamaoui, M. Schwartz, Abnormal changes in NKT cells, the IGF-1 axis, and liver pathology in an animal model of ALS, PLoS One 6 (2011) e22374.
[34] J.C. Dodge, C.M. Treleaven, J.A. Fidler, T.J. Tamsett, C. Bao, M. Searles, T.V. Taksir, K. Misra, R.L. Sidman, S.H. Cheng, L.S. Shihabuddin, Metabolic signatures of amyotrophic lateral sclerosis reveal insights into disease pathogenesis, Proc. Natl. Acad. Sci. U. S. A. 108 (2013) 10812–10817.
[35] A. Fuso, The 'golden age' of DNA methylation in neurodegenerative diseases, Neurobiol. Aging (2012) 1–12.
[36] Z. Xu, X. Li, DNA methylation in neurodegenerative disorders, Curr. Transl. Geriatr. Gerontol. Rep. 1 (2012) 199–205.
[37] Z. Xu, H. Li, P. Jin, Epigenetics-based therapeutics for neurodegenerative disorders, Curr. Transl. Geriatr. Gerontol. Rep. 1 (2012) 229–236.
[38] J.M. Morahan, B. Yu, R.J. Trent, R. Pamphlett, A genome-wide analysis of brain DNA methylation identifies new candidate genes for sporadic amyotrophic lateral sclerosis, Amyotroph. Lateral Scler. 10 (2009) 418–429.
[39] J.W. Zhu, J.F. Yuan, H.M. Yang, S.T. Wang, C.G. Zhang, L.L. Sun, H. Yang, H. Zhang, Extracellular cysteine (Cys)/cystine (CySS) redox regulates metabotropic glutamate receptor 5 activity, Biochimie 94 (2012) 617–627.
[40] T.B. Levring, A.K. Hansen, B.L. Nielsen, M. Kongsbak, M.R. von Essen, A. Woetmann, N. Odum, C.M. Bonefeld, C. Geisler, Activated human CD4 T cells express transporters for both cysteine and cystine, Sci. Rep. 2 (2012) 266.
[41] R. Bridges, V. Lutgen, D. Lobner, D.A. Baker, Thinking outside the cleft to understand synaptic activity: contribution of the cystine-glutamate antiporter (System xc-) to normal and pathological glutamatergic signaling, Pharmacol. Rev. 64 (2012) 780–802.
[42] W. Dröge, A. Gross, V. Hack, R. Kinscherf, M. Schykowski, M. Bockstette, S. Mihm, D. Galter, Role of cysteine and glutathione in HIV infection and cancer cachexia: therapeutic intervention with N-acetylcysteine, Adv. Pharmacol. 38 (1997) 581–600.
[43] V. Hack, D. Schmid, R. Breitkreutz, C. Stahl-Henning, P. Drings, R. Kinscherf, F. Taut, E. Holm, W. Dröge, Cystine levels, cystine flux, and protein catabolism in cancer cachexia, HIV/SIV infection, and senescence, FASEB J. 11 (1997) 84–92.
[44] M.R. Vargas, D.A. Johnson, J.A. Johnson, Decreased glutathione accelerates neurological deficit and mitochondrial pathology in familial ALS-linked hSOD1G93A mice model, Neurobiol. Dis. 43 (2011) 543–551.
[45] G. D'Alessandro, E. Calcagno, S. Tartari, M. Rizzardini, R.W. Invernizzi, L. Cantoni, Glutamate and glutathione interplay in a motor neuronal model of amyotrophic lateral sclerosis reveals altered energy metabolism, Neurobiol. Dis. 43 (2011) 346–355.
[46] B.A. Chestnut, Q. Chang, A. Price, C. Lesuisse, M. Wong, L.J. Martin, Epigenetic regulation of motor neuron cell death through DNA methylation, J. Neurosci. 31 (2011) 16619–16636.
[47] K.D. Robertson, DNA methylation and human disease, Nat. Rev. Genet. 6 (2005) 597–610.
[48] S. Zoccolella, I.L. Simone, P. Lamberti, V. Samarelli, R. Tortelli, L. Serlenga, G. Logroscino, Elevated plasma homocysteine levels in patients with amyotrophic lateral sclerosis, 70 (2008) 222–225.
[49] S. Mudd, J. Bronson, M. Bronson, R. Jacobs, S. Stabler, R. Allen, D. Vance, C. Wagner, Methyl balance and transmethylation fluxes in humans, Am. J. Clin. Nutr. 85 (2007) 19–25.
[50] K. Socała, D. Nieoczym, C. Rundfeldt, P. Wlaź, Effects of sarcosine, a glycine transporter type 1 inhibitor, in two mouse seizure models, Pharmacol. Rep. 62 (2010) 392–397.
[51] J.T. Dever, A.A. Elfarra, The biochemical and toxicological significance of hypermethionemia: new insights and clinical relevance, Expert Opin. Drug Metab. Toxicol. 6 (2010) 1333–1346.
[52] J.M. Mato, M.L. Martínez-Chantar, S.C. Lu, Methionine metabolism and liver disease, Nutrition 28 (2008) 273–293.
[53] B.M. Patten, Y. Harati, L. Acosta, S.S. Jung, M.T. Felmus, Free amino acid levels in amyotrophic lateral sclerosis, Ann. Neurol. 3 (1978) 305–309.
[54] H.J. Stuerenburg, K. Petersen, C. Buhmann, M. Rosenkranz, T. Baeumer, R. Thomasius, Plasma amino acids in ecstasy users, 24 (2003) 348–349.
[55] W.E. Klunk, R.J. McClure, J.W. Pettegrew, Possible roles of l-phosphoserine in the pathogenesis of Alzheimer's disease, Mol. Chem. Neuropathol. 15 (1991) 51–73.
[56] J. Sasabe, S. Aiso, Aberrant control of motoneuronal excitability in amyotrophic lateral sclerosis: excitatory glutamate/D-serine vs. inhibitory glycine/gamma-aminobutanoic acid (GABA), Chem. Biodivers. 7 (2010) 1479–1490.
[57] J. Sasabe, T. Chiba, M. Yamada, K. Okamoto, I. Nishimoto, M. Matsuoka, S. Aiso, D-Serine is a key determinant of glutamate toxicity in amyotrophic lateral sclerosis, EMBO J. 26 (2007) 4149–4159.
[58] C.S. Lobsiger, S. Boillée, D.W. Cleveland, Toxicity from different SOD1 mutants dysregulates the complement system and the neuronal regenerative response in ALS motor neurons, Proc. Natl. Acad. Sci. 104 (2007) 7319–7326.
[59] D.W. Ellison, M.F. Beal, J.B. Martin, Phosphoethanolamine and ethanolamine are decreased in Alzheimer's disease and Huntington's disease, Brain Res. 417 (1987) 389–392.
[60] J. Nikkilä, M. Sysi-Aho, A. Ermolov, T. Seppänen-Laakso, O. Simell, S. Kaski, M. Oresic, Gender-dependent progression of systemic metabolic states in early childhood, Mol. Syst. Biol. 4 (2008) 197.
[61] K. Mittelstrass, J.S. Ried, Z. Yu, J. Krumsiek, C. Gieger, C. Prehn, W. Roemisch-Margl, A. Polonikov, A. Peters, F.J. Theis, Discovery of sexual dimorphisms in metabolic and genetic biomarkers, PLoS Genet. 7 (2011) e1002215.
[62] A. Sreekumar, L.M. Poisson, T.M. Rajendiran, A.P. Khan, Q. Cao, J. Yu, B. Laxman, R. Mehra, R.J. Lonigro, Y. Li, M.K. Nyati, A. Ahsan, S. Kalyana-Sundaram, B. Han, X. Cao, J. Byun, G.S. Omenn, D. Ghosh, S. Pennathur, D.C. Alexander, A. Berger, J.R. Shuster, J.T. Wei, S. Varambally, C. Beecher, A.M. Chinnaiyan, Metabolomic profiles delineate potential role for sarcosine in prostate cancer progression, Nature 457 (2009) 910–914.
[63] J. Folbergrová, J.V. Passonneau, O.H. Lowry, D.W. Schulz, Glycogen, ammonia and related metabolities in the brain during seizures evoked by methionine sulphoximine, J. Neurochem. 16 (1969) 191–203.
[64] M. Yudkoff, Y. Daikhin, I. Nissim, I. Nissim, Acidosis and astrocyte amino acid metabolism, Neurochem. Int. 36 (2000) 329–339.
[65] R. Šafránek, M. Holeček, J. Kadlčíková, L. Šprongl, C. Mišlanová, M. Kukan, J. Chládek, Effect of acute acidosis on protein and amino acid metabolism in rats, Clin. Nutr. 22 (2003) 437–443.
[66] O.Z. Sellinger, R.A. Schatz, P. Gregor, Cerebral methylations in epileptogenesis, Adv. Neurol. 44 (1986) 465–473.
[67] R.A. Schatz, O.Z. Sellinger, Effect of methionine and methionine sulphoximine on rat brain S-adenosyl methionine levels, J. Neurochem. 24 (1975) 63–66.
[68] R.A. Schatz, O.Z. Sellinger, The elevation of cerebral histamine-N-and catechol-O-methyl transferase activities by L-methionine-dl-sulfoximine, J. Neurochem. 25 (1975) 73–78.