ORIGINAL ARTICLE

Serum asymmetric dimethylarginine level correlates with the progression and prognosis of amyotrophic lateral sclerosis

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Abstract

Background and purpose: The aim was to investigate the association between serum asymmetric dimethylarginine (ADMA) levels and the progression and prognosis of amyotrophic lateral sclerosis (ALS), and to compare cerebrospinal fluid (CSF) and serum ADMA levels with other biomarkers of ALS.

Methods: Serum ADMA levels of sporadic ALS patients ($n = 68$), disease control patients ($n = 54$) and healthy controls ($n = 20$) were measured using liquid chromatography tandem mass spectrometry. Correlations of the ADMA level and other markers (nitric oxide and neurofilament light chain levels) were analyzed. Changes in the ALS Functional Rating Scale Revised (ALSFRS-R) score from the onset of disease (ALSFRS-R pre-slope) was used to assess disease progression. Survival was evaluated using the Cox proportional hazards model and Kaplan–Meier analysis.

Results: The serum ADMA level was substantially higher in patients with ALS than in healthy controls and disease controls. Serum ADMA level correlated with CSF ADMA level ($r = 0.591$, $p < 0.0001$) and was independently associated with the ALSFRS-R pre-slope ($r = 0.505$, $p < 0.0001$). Patients with higher serum ADMA levels had less favorable prognoses. CSF ADMA level significantly correlated with CSF neurofilament light chain level ($r = 0.456$, $p = 0.0002$) but not with nitric oxide level ($r = 0.194$, $p = 0.219$).

Conclusion: Serum ADMA level is an independent biomarker of ALS disease progression and prognosis and reflects the degree of motor neuron degeneration.

KEYWORDS
amyotrophic lateral sclerosis, biomarker, dimethylarginine

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by the selective loss of upper and lower motor neurons. Despite decades of research into the molecular mechanisms underlying the pathogenesis of ALS and the development of molecular targeting therapy, to date only a few drugs have been shown to be effective for ALS. One of the difficulties in performing clinical trials for ALS patients is the large variation in the clinical courses of the patients [1,2], as evaluated by survival time or the revised ALS Functional Rating Scale (ALSFRS-R). To obtain better patient stratification and more reliable measures for monitoring the therapeutic effects of potential treatments, it is imperative to develop biomarkers that accurately reflect the pathophysiology and predict the progression and prognosis of ALS.

It was recently reported that protein arginine dimethylation is upregulated in the spinal cord of patients with ALS and that cerebrospinal fluid (CSF) levels of asymmetric dimethylarginine (ADMA) can be a biomarker of ALS disease progression and prognosis [3]. It is hence hypothesized that increased CSF ADMA levels might reflect the hypermethylation of RNA-binding proteins in the spinal motor neurons and surrounding glial cells of ALS disease patients. Interestingly, recent reports have shown that RNA-binding proteins that are targets of arginine dimethylation demonstrate abnormal aggregation or mislocalization in the motor neurons of sporadic ALS patients [4–7], indicating the involvement of abnormal arginine dimethylation in the pathogenesis of ALS.

In this study, the changes in serum ADMA levels in ALS patients and the correlation of ADMA level with neurofilament light chain (NfL) level were analyzed, to understand how ADMA is associated with the pathology of ALS. The correlation between ADMA and nitric oxide (NO) levels in the CSF was also analyzed to determine whether increased ADMA affects ALS pathology through the suppression of NO synthesis. Moreover, towards clinical application, the usefulness of serum ADMA level for predicting disease progression and prognosis was analyzed.

MATERIALS AND METHODS

Patient registry and follow-up

Patients who were diagnosed as having ALS or followed up for ALS at Osaka University Hospital between July 2016 and August 2020 were prospectively enrolled. The data were collected from patients who agreed to participate in the Osaka University Longitudinal Biomarker Study for Neuromuscular Diseases. In total, 68 ALS patients with definite, probable, probable laboratory-supported or possible ALS according to the revised El Escorial criteria were included. Although genetic testing was not performed, all of the 68 patients were confirmed to have no family history of ALS. The patients were registered in the Osaka University Longitudinal Biomarker Study for Neuromuscular Diseases with written informed consent. The clinical scores listed below were obtained when specimens were collected from the patients. CSF was collected from ALS patients ($n = 58$) by lumbar puncture in the morning after fasting overnight. The CSF samples were centrifuged at 400g for 10 min at 4°C, and aliquots were stored at −80°C. Muscle strength was manually tested and scored using the scale of the Medical Research Council (manual muscle testing, MMT). Disease onset was defined as when the patients became initially aware of muscle weakness or the impairment of swallowing, speech or respiration. The Japanese version of the ALSFRS-R [2] was used as a scale to evaluate activities of daily living. The reliability of the Japanese version of the ALSFRS-R has been confirmed previously [8]. To evaluate the functional decline in the ALSFRS-R, the slope defined as (decrease in the value within a duration)/duration was calculated. The pre-slope was used to evaluate the decline in the ALSFRS-R from the time of onset to diagnosis (registration) and was calculated as ALSFRS-R pre-slope = (ALSFRS-R at registration − 48)/(duration from onset to diagnosis). The primary end-point was defined as either the introduction of tracheostomy positive pressure ventilation (TPPV) or death of the patient, and the time a patient reached the primary end-point was determined by telephone follow-up. TPPV-free survival was defined as survival in the TPPV cases.

Clinical data of the ALS patients, disease control patients, and healthy controls

The average age at venipuncture (ALS, 63.5 [47.5–79.5] years; disease controls, 63.0 [47.75–78.25] years; healthy controls, 59.0 [45.0–74.0] years) and the sex ratio (male:female, ALS, 41:27; disease controls, 32:22; and healthy controls, 12:8) were not significantly different between the three groups. The average duration from onset to registration (months) in patients with ALS was 21.1 (5.12–37.1), the average ALSFRS-R at registration was 38.7 (32.5–44.9) and the average forced vital capacity (FVC) at registration was 84.9% (63.0%–106.8%), which are consistent with previous studies [2,9–11]. At the end of this study, 33 patients had reached the primary end-point, and the average duration from registration to the primary end-point was 23.64 ± 14.33 months. The remaining 35

patients were alive at the end of the study, and the average duration of their observation was 26.27 ± 14.72 months. Regarding disease form (initial symptoms), 13 patients showed the bulbar form and 55 patients showed the spinal form. The disease controls included 54 patients with Parkinson's disease ($n = 10$), Parkinson's syndrome ($n = 9$), polyneuropathy ($n = 7$), multiple sclerosis ($n = 6$), myositis ($n = 6$), brain infarction ($n = 5$), dystonia ($n = 3$) and other disorders ($n = 8$). Furthermore, 20 volunteers were recruited to be included as age-matched and sex-matched healthy controls without neurological disorders or illnesses affecting neuromuscular or renal function in the healthy control group.

Measurement of arginine analogs

Arginine, N(omega) and N(omega)-dimethyl-L-arginine (ADMA) were used to construct standard curves (Enzo Life Sciences). ADMA-d6 was prepared according to the method of Kennedy et al. [12]. ADMA was measured using a high-performance liquid chromatography tandem mass spectrometry system (Quattro Premier XE Mass Spectrometer; Waters Corporation). A 5-μl sample solution that was deproteinated by methanol was injected into an Intrada Amino Acid column (2×50 mm; Imtakt) at 40°C. Chromatography was performed at a flow rate of 0.6 ml/min using a step gradient alternating between a mixture of acetonitrile:tetrahydrofuran 25 mmol/l aqueous ammonium formate:formic acid (9:25:16:0.3) and a mixture of 100 mmol/l aqueous ammonium formate:acetonitrile (80:20). ADMA was analyzed using the multiple reaction monitoring mode of tandem mass spectrometry in positive ion mode. The cone voltage was 22–25 V, collision energy was 13–22 and transitions were m/z $203 \rightarrow 46$ for ADMA.

Quantification of plasma and CSF NfL concentrations

The concentrations of plasma and CSF NfL were quantified as previously described [13,14], using Simoa NF-light Advantage Kit and a Simoa HD-1 analyzer according to the manufacturer's protocol (Quanterix). All samples were analyzed in duplicate.

Measurement of NO concentration

The nitrite concentration in the CSF was measured using a nitrite assay kit following the manufacturer's instructions (BioVision). Absorbance at 540 nm was measured using a microplate reader.

Statistical analysis

A one-way ANOVA was performed to compare the age between groups. The chi-squared test was performed to compare the sex ratio between groups. Pearson's correlation analysis was performed to analyze the correlation between factors. Survival time was defined as the time from disease onset to death or the introduction of TPPV. The Kaplan–Meier method was used to estimate survival curves, and the survival curves of the two groups were compared using the log rank test. The Cox proportional hazards model, which included age, the ALSFRS-R slope at registration, FVC, phenotype at onset (bulbar or non-bulbar) and serum ADMA level (ng/ml), was applied to analyze the effects of these variables on survival time. The hazard ratio (HR) and 95% confidence interval (CI) were estimated. To determine an optimal cut-off value of serum ADMA to predict the prognosis of ALS, all the cut-off values were tested by Cox regression analysis, and the best value was selected. Multivariate regression analyses with stepwise variable selection (alpha = 0.05 for inclusion and alpha = 0.10 for exclusion) was also performed to analyze the effect of ADMA on ALSFRS-R. The Statistical Package for the Social Sciences 23.0J software (IBM Japan) was used to perform statistical analyses.

Ethics statement

This study was conducted in accordance with the Declaration of Helsinki and the Ethical Guidelines for Medical and Health Research involving Human Subjects endorsed by the Japanese government. The Ethics Committee of Osaka University Graduate School of Medicine approved the study (approval number 19089-3). All the patients, including disease controls, were informed about the study and written consent was obtained.

RESULTS

Serum ADMA level is increased in ALS patients and correlates with disease progression

Serum ADMA level was found to be significantly higher in ALS patients than in healthy controls and disease controls (Figure 1; ALS vs. healthy controls, $p < 0.0001$, and ALS vs. disease controls, $p = 0.006$). ADMA levels in the disease controls were slightly higher than in the healthy controls, but the difference was not statistically significant ($p = 0.051$). Subgroup analysis of disease controls showed that atypical parkinsonian disorders (aPD) and neuropathy groups had significantly higher serum ADMA levels than healthy controls (healthy controls vs. aPD, $p < 0.0001$, and healthy controls vs. polyneuropathy, $p = 0.007$) (Figure S1). ADMA levels were then compared in the serum and CSF and it was found that serum ADMA levels significantly correlated with CSF levels (Figure 2a, $r = 0.591$, $p < 0.032$). On the other hand, there was no correlation in L-arginine level between serum and CSF (Figure 2b, $r = 0.591$, $p < 0.032$), probably because serum L-arginine levels are affected by diet and several other factors.

Serum ADMA level and several clinical parameters were then compared and it was found that serum ADMA level strongly

correlated with disease progression (ALSFRS-R pre-slope) (Figure 2c, $r = 0.505$. $p < 0.0001$) but not with disease severity at each time-point (ALSFRS-R) (Figure 2d, $r = -0.261$, $p = 0.032$), age (Figure S1a, $r = 0.138$, $p = 0.241$) or disease duration at venipuncture (Figure S2b, $r = -0.286$, $p = 0.017$). Contrary to our previous study of the CSF, in the present study the ADMA/L-arginine ratio did not reflect disease progression (Figure S3a, $r = 0.256$, $p = 0.033$), also probably owing to the variability of serum L-arginine level. Importantly, multivariate linear regression analysis demonstrated that amongst serum ADMA, age, sex, MMT, FVC, and ALSFRS-R, only serum ADMA was an independent factor that was associated with disease progression (Table 1).

Associations between serum ADMA levels and other biomarkers

Moreover, plasma NfL levels were measured to analyze the degree of neurodegeneration in patients and it was found that ADMA level significantly correlated with NfL level (Figure 3a, $r = 0.430$, $p = 0.002$). Interestingly, ADMA level did not correlate with NO level (Figure 3b, $r = 0.270$, $p = 0.080$), indicating that the increase in ADMA level was independent of NO dysregulation. Multivariate linear regression analysis demonstrated that, amongst serum ADMA, creatinine, albumin, NO and plasma NfL level, serum ADMA level was independently associated with disease progression (Table 2).

Serum ADMA is a prognostic marker for ALS

Next, a multivariate Cox regression analysis of survival time was performed, and it was found that serum ADMA level can predict the survival of patients independent of age, ALSFRS-R pre-slope, FVC, and onset symptom (bulbar or non-bulbar) (Table 3).

The optimal cut-off score of the ADMA level that produced the highest HR for predicting the prognosis of ALS was then analyzed. When a cut-off of ADMA >118.83 ng/ml was used, the HR
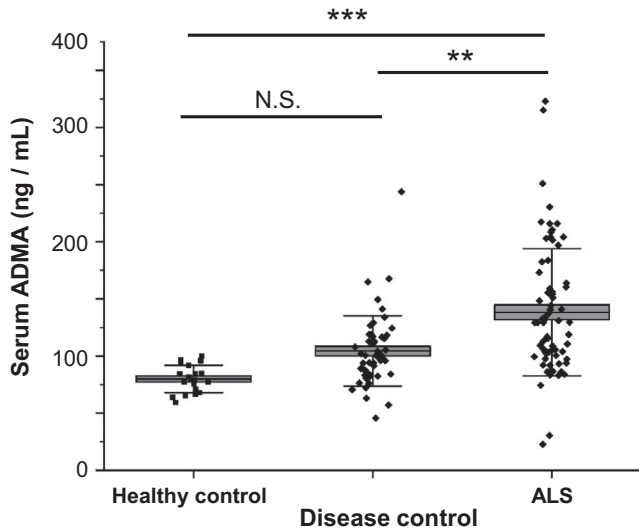
FIGURE 1 Increased serum ADMA level in ALS patients. Serum ADMA concentrations of healthy controls ($n = 20$), disease controls ($n = 54$) and ALS patients ($n = 68$). **$p < 0.01$, ***$p < 0.0001$, one-way ANOVA with the Bonferroni test
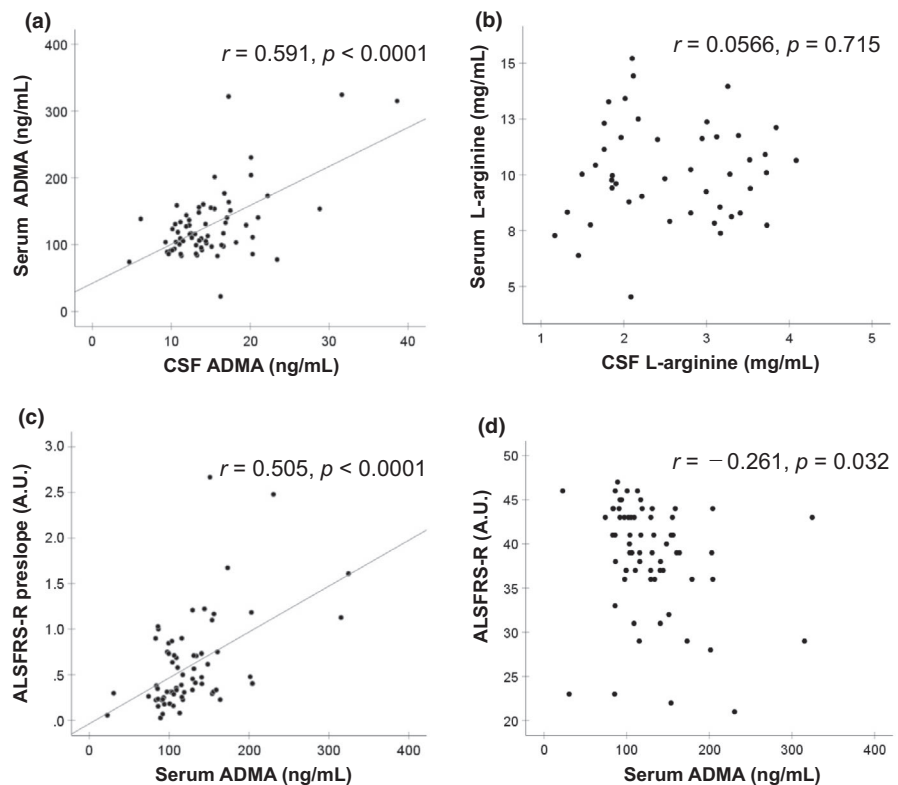
FIGURE 2 Correlation between serum ADMA level and disease progression. (a) Correlation between CSF and serum ADMA levels ($n = 58$). (b) Correlation between CSF and serum L-arginine levels ($n = 58$). (c), (d) Correlation of serum ADMA concentrations and disease progression scores (ALSFRS-R pre-slope) or disease severity score (ALSFRS-R) ($n = 68$ for both analyses). Pearson's correlation analysis was performed for statistical analysis

was 3.58 (95% CI 1.700–7.570, p = 0.001). The registered patients were divided into two categories using this cut-off score. Figure 4 shows the Kaplan–Meier curves for the primary end-point of patients in the two categories. The difference between the curves was statistically significant by the log rank test (p = 0.004). In addition, to investigate the additive value of ADMA level on the benchmark biomarker NfL, Kaplan–Meier curves were drawn for four groups of patients according to their ADMA and NfL levels: those with high levels of both ADMA and NfL had the worst prognosis. The results of the log rank test between the groups showed that only the group with high values of both ADMA and NfL was significantly different from the group with low values of ADMA and NfL and the group with high values of ADMA and low values of NfL (Figure S4). It was also confirmed that serum ADMA can predict the survival of patients independently of plasma NfL levels (Table 4).

Finally, the longitudinal change of serum ADMA levels during the disease progression was investigated. The second blood examination could only be obtained from eight patients after a mean duration of 15.75 ± 3.06 months. Interestingly, serum ADMA levels did

not change significantly over time (Figure S5), indicating that serum ADMA is not the consequence of disease progression but rather reflects the upstream of the disease.

DISCUSSION

In this study, it has been shown that serum ADMA level is well correlated with CSF ADMA level and is useful for evaluating ALS disease progression and prognosis, similarly to what was previously reported for CSF ADMA [15]. Interestingly, ADMA level correlates more strongly with the ALSFRS-R slope than the ALSFRS-R. This suggests that, unlike the decrease in creatinine level, which simply reflects decreased muscle volume and is hence just a consequence of ALS symptoms, the increase in ADMA level more accurately reflects the disease state of the patients. Indeed, it was shown that serum ADMA level correlates with plasma NfL level, which reflects axonal degeneration and is a pathological biomarker for ALS [13,14,16–20].

Here it has been shown that serum ADMA level is increased in ALS patients and is associated with disease progression score (ALSFRS-R slope) independently of several clinical scores and blood biomarkers, including plasma NfL. Serum ADMA level also predicts disease prognosis independently of the ALSFRS-R slope, FVC, age and onset symptom (bulbar or non-bulbar).

Our previous study mainly focused on the ADMA/l-arginine ratio, considering that ADMA competes with NO synthase for binding to l-arginine and to inhibit NO production. In the present study, serum ADMA levels were used instead of its ratio with l-arginine as a target marker because it was found that serum and CSF l-arginine levels did not correlate with each other, although serum ADMA levels correlated with CSF ADMA levels. It is considered that serum ADMA level reflects brain pathology; however, l-arginine is more affected by other factors, such as diet, and hence the ratio of serum ADMA and l-arginine level was not as useful as that of CSF ADMA and l-arginine levels. Moreover, serum ADMA level did not correlate with NO level, indicating that a high ADMA level might not indicate the insufficient production of NO in the central nervous system, but

TABLE 1 Multivariate linear regression analysis with stepwise variable selection; comparison between serum ADMA and clinical ALS parameters

	ALSFRS-R pre-slope	
	Coefficient (95% CI)	p value
Serum ADMA	0.004 (0.000–0.006)	**<0.0001**
Age	−0.001 (−0.009–0.007)	0.775
Sex	0.043 (−0.171–0.256)	0.692
MMT	0.014 (−0.003–0.032)	0.102
FVC	−0.005 (−0.010–0.001)	0.110
ALSFRS-R	−0.033 (−0.058–0.009)	**0.009**

Abbreviations: ADMA, asymmetric dimethylarginine; ALS, amyotrophic lateral sclerosis; ALSFRS-R, ALS Functional Rating Scale Revised; CI, confidence interval; FVC, forced vital capacity; MMT, manual muscle testing. Bold values are significant.
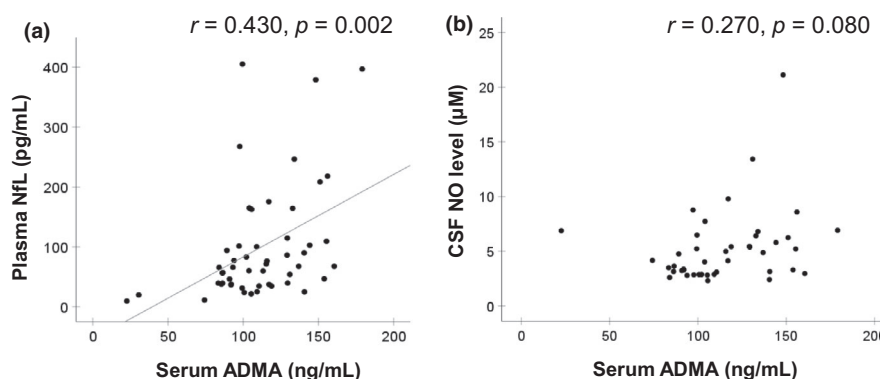
FIGURE 3 Comparison between serum ADMA level and plasma NfL or CSF NO level. (a) Correlation between serum ADMA concentration and plasma NfL level (n = 51). (b) Correlation between serum ADMA level and plasma NfL level (n = 43). Pearson's correlation analysis was performed for statistical analysis

TABLE 2 Multivariate linear regression analysis with stepwise variable selection; comparison amongst serum ADMA and other biomarkers

	ALSFRS-R pre-slope	
	Coefficient (95% CI)	p value
Serum ADMA	0.006 (0.001–0.011)	**0.024**
Creatinine	−0.536 (−1.255–0.183)	0.139
Albumin	−0.30 (−0.429–3.69)	0.880
Nitric oxide	−0.003 (−0.048–0.041)	0.878
Plasma NfL	0.002 (0.000–0.004)	**0.045**

Abbreviations: ADMA, asymmetric dimethylarginine; ALS, amyotrophic lateral sclerosis; ALSFRS-R, ALS Functional Rating Scale Revised; CI, confidence interval; NfL, neurofilament light chain. Bold values are significant.

TABLE 3 Multivariate Cox regression analysis of the survival of ALS patients, with adjustments of covariates

	Primary end-point	
	HR (95% CI)	p value
Serum ADMA	1.008 (1.002–1.017)	**0.014**
Age	1.063 (1.022–1.111)	**0.003**
ALSFRS-R pre-slope	7.268 (2.658–19.872)	**<0.0001**
FVC	1.007 (0.986–1.024)	0.608
Onset (bulbar or non-bulbar)	2.541 (1.006–6.421)	**0.049**
n = 68 (33 subjects reached the primary end-point and 35 subjects were censored)		

Abbreviations: ADMA, asymmetric dimethylarginine; ALS, amyotrophic lateral sclerosis; ALSFRS-R, ALS Functional Rating Scale Revised; CI, confidence interval; FVC, forced vital capacity; HR, hazard ratio. Bold values are significant.

TABLE 4 Multivariate Cox regression analysis of the survival of ALS patients between serum ADMA and NfL

	Primary end-point	
	HR (95% CI)	p value
Serum ADMA	1.024 (1.004–1.043)	**0.017**
Plasma NfL	1.004 (0.999–1.010)	0.143
n = 45 (15 subjects reached the primary end-point and 30 subjects were censored)		

Abbreviations: ADMA, asymmetric dimethylarginine; ALS, amyotrophic lateral sclerosis; CI, confidence interval; HR, hazard ratio; NfL, neurofilament light chain. Bold value is significant.

might indicate the importance of the hypermethylation of arginines within RNA-binding proteins in the pathogenesis of sporadic ALS.

This study has some limitations and biases. First, the number of patients enrolled was small and validation in a larger cohort is
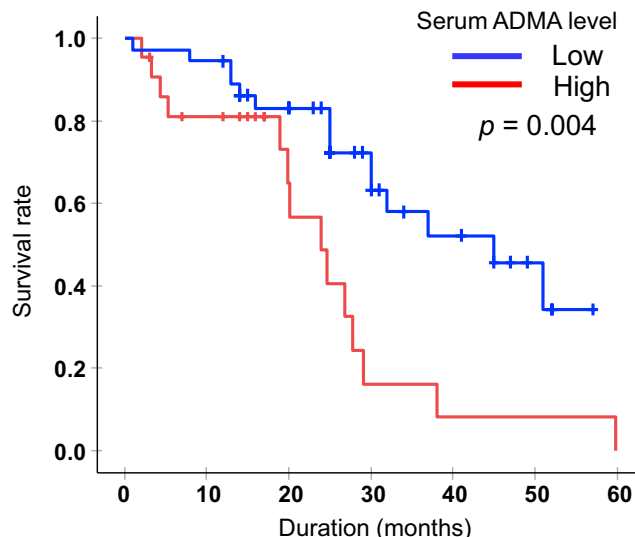
FIGURE 4 Serum ADMA level predicts the prognosis of ALS patients. Kaplan–Meier curve according to serum ADMA level (low, <118.83 ng/ml, blue line; high, >118.83, red line). Kaplan–Meier curves for the primary end-point were compared by the log rank test. There was a statistically significant difference between the curves ($p = 0.004$) [Colour figure can be viewed at wileyonlinelibrary.com]

required in the future. Also, more detailed longitudinal analysis of the ADMA levels is required in the future study. Secondly, as the ADMA level was slightly higher in disease controls than in healthy controls, alterations in ADMA levels should be evaluated more widely in other neurological disorders, to conclude the specificity of this biomarker in ALS. Finally, the pathomechanism by which an increased ADMA level affects disease progression remains unclear. Further studies are required to confirm that arginine hypermethylation is directly involved in the pathogenesis of ALS, and to determine whether ADMA can be a useful biomarker for clinical applications.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

Kensuke Ikenaka: Conceptualization (equal); data curation (equal); formal analysis (equal); funding acquisition (equal); investigation (equal); project administration (equal); resources (equal); writing—original draft (equal); writing—review and editing (equal). Yasuhiro Maeda: Formal analysis (lead). Yuji Hotta: Formal analysis (equal). Seiichi Nagano: Conceptualization (supporting); resources (equal); supervision (supporting); writing—original draft (supporting). Shinichiro Yamada: Resources (equal). Daisuke Ito: Formal analysis (supporting); resources (equal); writing—original draft (supporting). Ryota Hattori: Data curation (equal); investigation (equal); resources (equal). Keita Kakuda: Resources (equal); writing—original draft (supporting). Harutsugu Tatebe: Formal analysis (equal). Naoki Atsuta: Conceptualization (supporting); formal analysis (supporting); supervision (equal). César Aguirre: Methodology (equal); supervision (equal); writing—original draft (equal). Yasuyoshi Kimura: Resources

(equal); supervision (equal); writing—original draft (supporting). Kousuke Baba: Resources (equal); supervision (supporting); writing—original draft (supporting). Takahiko Tokuda: Formal analysis (equal). Masahisa Katsuno: Conceptualization (equal); resources (equal); supervision (equal); writing—original draft (supporting). Kaunori Kimura: Formal analysis (equal). Gen Sobue: Conceptualization (equal); funding acquisition (equal); project administration (equal); supervision (equal). Hideki Mochizuki: Funding acquisition (equal); resources (equal); supervision (equal).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

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SUPPORTING INFORMATION

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