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Diagnostic and Prognostic Value of Plasma NfL and Creatinine in a Korean Cohort of Amyotrophic Lateral Sclerosis and Spinal and Bulbar Muscular Atrophy: A Cross-Sectional Study

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ABSTRACT

Objective: Biomarkers that can distinguish motor neuron disease spectrum conditions, such as spinal and bulbar muscular atrophy (SBMA) and amyotrophic lateral sclerosis (ALS), could facilitate early-stage diagnosis. This study used a multibiomarker approach to improve the differential diagnosis between SBMA and ALS.

Methods: In this study, plasma samples were collected from patients with SBMA ($n = 44$), male patients with ALS ($n = 26$), and healthy controls ($n = 20$). Plasma levels of neurofilament light chain (NfL), creatine (Cr), and Cr kinase (CK) were measured. Disease severity was assessed using the Revised ALS Functional Rating Scale scores and its subscores in both patient groups. Additionally, SBMA-specific functional measures, including SBMA Functional Rating Scale (SBMAFRS) scores and the 6-Min Walk Test (6MWT), were evaluated in patients with SBMA.

Results: NfL levels were significantly higher in patients with ALS than in patients with SBMA and healthy controls, and were significantly influenced by age. When the three biomarkers were entered into logistic regression models, NfL levels alone could be used to differentiate ALS from SBMA almost perfectly, and the inclusion of Cr or CK levels did not improve this differentiation. Within the SBMA group, however, Cr levels were correlated with the lower-limb subscore of the SBMAFRS and with 6MWT results, which points to a role in tracking motor function rather than in diagnosis.

Conclusions: Our results indicated that plasma NfL levels could be used to reliably distinguish ALS from SBMA, whereas plasma Cr levels were better suited as a marker of muscle function and disease severity in SBMA. Measuring both of these may therefore aid diagnosis as well as prognostic assessment in patients with suspected motor neuron disease.

1 | Introduction

Motor neuron diseases (MNDs) are a heterogeneous group of disorders that involve motor neuron degeneration, resulting

primarily in progressive motor weakness. They encompass several conditions, including amyotrophic lateral sclerosis (ALS), spinal and bulbar muscular atrophy (SBMA), progressive bulbar palsy, primary lateral sclerosis, and progressive

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muscular atrophy. ALS is characterized by degeneration of both upper and lower motor neurons and is attributed to multifactorial mechanisms, whereas SBMA predominantly affects lower motor neurons and is associated with a trinucleotide repeat expansion in the androgen receptor gene [1]. Nevertheless, these two diseases share common clinical features, such as progressive limb weakness and bulbar symptoms [2]. Clinical differentiation between patients with lower motor neuron-predominant ALS and those with SBMA can be challenging, especially in the early stages, and can sometimes lead to misdiagnosis. Currently, time-consuming neurologic examinations and electrophysiological studies serve as key primary diagnostic tools and can be supplemented by cerebrospinal fluid studies, genetic testing, and neuroimaging. However, some of these studies can be painful and can impose financial burdens on patients [3, 4]. Furthermore, these diseases are characterized by the absence of effective disease-modifying therapies, and reliable prognostic biomarkers complicate both disease monitoring and the objective evaluation of emerging therapeutic interventions.

Neurofilament light chain (NfL) has emerged as the most extensively studied blood biomarker for ALS [3, 5–7]. NfL is regarded as a sensitive marker of neuroaxonal degeneration [8]. Thus, blood NfL levels are elevated in various neurological conditions, including Alzheimer's disease, traumatic brain injury, multiple sclerosis, and frontotemporal lobar degeneration [9]. Several studies have consistently demonstrated that serum NfL levels are significantly higher in patients with ALS than in healthy controls and patients with SBMA [3, 6, 7, 10]. In contrast, levels of myopathy-associated biomarkers such as creatine kinase (CK) and creatinine (Cr) are altered in patients with SBMA. A general indicator of muscle damage is the blood concentration of CK [11, 12], and CK is among the proteins most distinctly identified as higher in the plasma of patients with SBMA than of those with ALS [3, 13–15]. However, blood CK levels in SBMA are not significantly correlated with SBMA Functional Rating Scale (SBMAFRS) scores, disease duration, or the number of CAG repeats [3, 16]. Serum Cr levels, commonly used as an indicator of muscle mass, are another potential biomarker for SBMA [13, 16, 17]. They generally decline during both the presymptomatic and symptomatic phases of the disease and exhibit a strong correlation with scores of symptomatic scales such as the SBMAFRS and 6-Min Walk Test (6MWT) results [3, 18, 19]. In the present study, we evaluated three potential blood biomarkers previously implicated in MND, namely NfL, Cr, and CK, in patients with SBMA and ALS. Correlations between levels of these biomarkers and disease-related functional impairment scale scores were investigated, along with their utility in the differential diagnosis of these two diseases, which can be challenging to diagnose in the early stages, thereby prompting the application of genetic testing in SBMA.

2 | Materials and Methods

2.1 | Participants (Study Subjects)

This study included 90 participants. Rather than being recruited from a single screened population, participants were assigned into three predefined, independent cohorts: 26 patients with

ALS, 44 patients with SBMA, and 20 healthy controls. Patients with SBMA were eligible if they had genetically confirmed expansion of more than 38 CAG repeats in the first exon of the androgen receptor gene [20]. Sex-matched male patients with ALS who met the probable or clinically definite ALS criteria according to the revised El Escorial criteria were also enrolled. Twenty age-matched healthy male controls with no known history of neurological disorders were also recruited as controls. Written informed consent was obtained from all participants prior to enrollment. This study was reviewed and approved by the Institutional Review Board of Kyungpook National University Chilgok Hospital (approval number: KNUMC-2023-01-036-001). Blood samples were collected from all participants. Three patients with ALS without Cr or CK level measurements were excluded from the analyses. The clinical and genetic characteristics of the study participants are summarized in Table 1.

2.2 | Clinical Assessment

Clinical and demographic characteristics were obtained from patients with ALS and with SBMA, including age and disease duration. For ALS, we further analyzed onset subtypes, categorized as bulbar-onset or limb-onset. The CAG repeat length was obtained from genetic testing in patients with SBMA. Revised ALS Functional Rating Scale (ALSFRS-R) scores and subscores were assessed in both patients with ALS and patients with SBMA. Further, we assessed SBMA-specific functional scores using the Korean version of the SBMAFRS (K-SBMAFRS) total score and subscores for patients with SBMA [21, 22]. Cr and CK levels were obtained from routine laboratory data within 3 months of plasma NfL measurement. Three patients with ALS who lacked Cr or CK measurements within this interval were excluded from analyses involving these biomarkers.

2.3 | Plasma Collection

Overall, 10 mL of blood was collected from each participant in the morning into ethylenediaminetetraacetic acid tube. After collection, the tubes were generally inverted and incubated at room temperature for 30 min, and then centrifuged at 3000 rotations per min for 10 min. Plasma was aliquoted into new 1.5-mL tubes and stored at -80°C . Routine blood sample data were used to assess Cr levels.

2.4 | NfL Measurements

Human plasma NfL concentrations were quantified using the NF-light V2 Advantage Kit (Item 104073, Quanterix) on a single molecule array (Simoa) HD-X analyzer, following previously described protocols [23]. Briefly, plasma samples were diluted 1:4 according to the manufacturer's recommendations. The assay was performed using a fully automated two-step protocol. In the first step, samples were incubated with paramagnetic beads coated with capture antibodies, together with a biotinylated detector antibody. After washing, streptavidin- β -galactosidase was added to bind the detector antibody, enabling enzymatic labeling of the captured targets. Following a final wash, the beads were resuspended in

TABLE 1 | Clinical and genetic profile of participants in the study.

	HC (<i>n</i> = 20)	ALS (<i>n</i> = 26)	SBMA (<i>n</i> = 44)	<i>p</i> ALS vs. SBMA
Mean age (years)	63.15 ± 8.33	66.15 ± 8.42	58.27 ± 11.38	0.002 ^a
Disease duration (months)		13.23 ± 9.85	117.6 ± 65.40	< 0.001 ^b
ALS subtype (bulbar/limb)		9 (34.62%)/17 (65.38%)		
ALSFRS-R		33.65 ± 10.64	36.86 ± 5.37	0.519 ^b
ALSFRS bulbar subscore		8.35 ± 3.54	9.55 ± 1.39	0.474 ^b
ALSFRS fine motor subscore		7.85 ± 3.00	9.52 ± 1.76	0.030 ^b
ALSFRS gross motor subscore		7.31 ± 3.43	6.98 ± 2.11	0.422 ^b
ALSFRS respiratory subscore		10.15 ± 2.41	10.82 ± 1.26	0.552 ^b
CAG repeat			46.30 ± 4.50	
K-SBMAFRS			35.70 ± 8.43	
SBMAFRS bulbar subscore			13.66 ± 2.55	
SBMAFRS upper limb subscore			5.57 ± 1.66	
SBMAFRS trunk subscore			9.61 ± 3.04	
SBMAFRS lower limb subscore			3.68 ± 1.89	
SBMAFRS respiratory subscore			3.18 ± 0.87	
6-min walk test			292.8 ± 124.9	
Creatinine (mg/dL)		0.65 ± 0.22	0.55 ± 0.13	0.052 ^a
Creatine kinase (IU/L)		281.7 ± 275.7	654.8 ± 414.6	< 0.001 ^b
Neurofilament light chain (pg/mL)	19.33 ± 7.92	123.6 ± 97.2	11.96 ± 7.86	< 0.001 ^b

Note: Data are presented as mean ± SD or number (%) unless otherwise indicated.

Abbreviations: ALS, amyotrophic lateral sclerosis; ALSFRS-R, ALS functional rating scale-revised; HC, healthy control; SBMA, spinal and bulbar muscle atrophy; SBMAFRS, SBMA functional rating scale.

^aWelch's *t*-test.

^bMann-Whitney *U* test.

resorufin β-D-galactopyranoside substrate solution and transferred to a Simoa disc for signal detection. Enzyme activity was quantified as the average number of enzymes per bead, which is proportional to the NfL level in the sample. All samples were measured in duplicate, and the mean of the two measurements was used for subsequent analyses.

2.5 | Assessment of Symptom Scores in Patients With ALS and With SBMA

Overall functional scale was evaluated using the ALSFRS-R and the K-SBMAFRS in patients with ALS and SBMA, respectively. The ALSFRS-R comprises four domains: bulbar, fine motor, gross motor, and respiratory function. The K-SBMAFRS is an SBMA-specific functional scale consisting of five subscores: bulbar, upper limb, trunk, lower limb, and breathing. Higher total scores on both the ALSFRS-R and K-SBMAFRS reflect better motor function. The 6MWT, which reflects overall cardiopulmonary and motor function, was also assessed in all ambulatory patients with SBMA. The mean, standard deviation, minimum, maximum, and results of normality tests for these scores are summarized in Table 1.

2.6 | Statistical Analysis

Descriptive statistics, including means and standard deviations, were calculated for each patient group. Group comparisons were performed using Welch's *t*-test for normally distributed variables and the Mann-Whitney *U* test for nonparametric data. Linear models comparing biomarker levels between the ALS and SBMA groups in cross-sectional analyses were adjusted for age. Biomarker levels (NfL, Cr, and CK) were natural log-transformed to better approximate normality and improve variance homogeneity, as required for the analyses. Multivariable logistic regression, with ALS/SBMA as the response, was used to evaluate predictive models, and receiver-operating characteristic (ROC) curves were constructed from the logistic scores. Areas under the curve (AUC) for different ROC curves were compared using the DeLong method, and optimal cutoff points were estimated using Youden's index. Improvements in model fit were assessed using the *p* values from AUC comparisons and the Akaike Information Criterion (AIC), based on the reference model including only ln (NfL). Spearman's rank correlation was used to assess the association between age-adjusted biomarkers and symptomatic measurements. All analyses and data visualization were carried out using R (V4.4.1).

3 | Results

3.1 | Participant Demographics and NfL Level Distribution

The study population did not originate from a single screened general cohort; rather, it comprised three predefined cohorts that were recruited independently. The final analysis included 26 patients with ALS, 44 patients with SBMA, and 20 healthy controls, totaling 90 participants. The baseline demographic and clinical characteristics of each independently enrolled group are summarized in Table 1. Age ranged from 38 to 82 years (mean, 61.6 years). NfL levels exhibited a wide distribution, from 4.88 to 421.79 pg/mL. Among the participants, 26 (28.9%) were diagnosed with ALS; they were aged 47–82 years (mean, 66.2 years) and had NfL levels ranging from 21.94 to 421.79 pg/mL. The SBMA group comprised 44 patients (48.9%) aged 38–80 years (mean, 58.3 years), with NfL levels between 4.88 and 42.40 pg/mL. The remaining 20 participants (22.2%), who served as controls, were aged 52–77 years (mean, 63.2 years) and had NfL levels ranging from 9.24 to 36.20 pg/mL. Participant demographics are presented in Table 1.

3.2 | Effects of Age on NfL, Cr, and CK Levels

Because NfL levels are sensitive markers of acute axonal damage and show age-related variability in blood plasma, assessing how they vary with age is essential [24]. A *t*-test revealed a statistically significant age difference between patients with ALS and those with SBMA ($p < 0.005$), implying a difference in the age of onset between the two diseases. Cr and CK levels were measured only in patients with ALS and SBMA, whereas NfL levels were also measured in healthy controls. Given the well-documented sex differences in plasma Cr and CK levels, and considering that SBMA exclusively affects men, this study was restricted to male patients with ALS [25, 26].

Figure 1 illustrates NfL levels among patients with ALS and with SBMA and healthy controls. As displayed in Figure 1A, patients with ALS exhibited significantly higher NfL levels than patients with SBMA and healthy controls. However, a difference was observed between the SBMA and healthy control groups, suggesting that age may contribute to NfL level variability. Following adjustment for age using linear regression (Figure 1B), the NfL difference between healthy controls and the SBMA group was markedly attenuated and no longer significant. This finding emphasizes the critical importance of accounting for age when assessing NfL levels as a biomarker. These results may suggest the importance of including age as a covariate in logistic regression when using biomarkers to distinguish between ALS and SBMA. Nonetheless, the mean age difference between ALS and SBMA (Table 1) complicated the use of age as a covariate in the logistic regression, leading to unexpected results in which patients clustered primarily by age. This indicates that using age-adjusted biomarker levels, obtained through linear regression, in a logistic regression model without including age as a covariate would be more reasonable than performing multivariate logistic regression with age as a covariate.

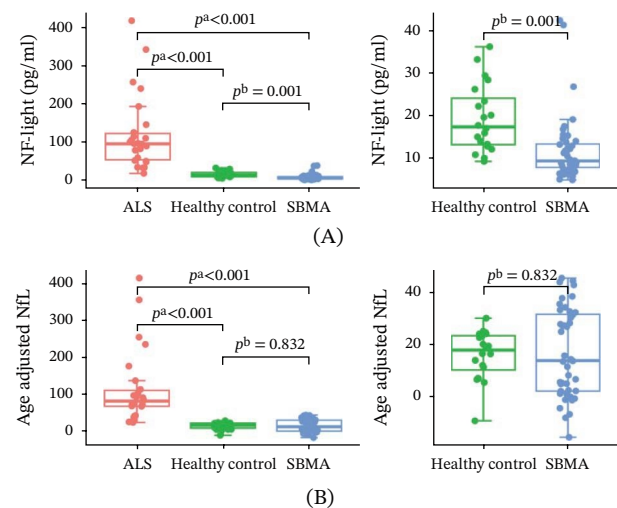


FIGURE 1 | The Importance of age adjustment in NfL levels. Box plots with Mann–Whitney *U* test (p^a) and Welch's *t*-test (p^b) results for NfL levels across ALS, SBMA, and healthy control groups are presented. Although the NfL levels in the ALS patient group are significantly elevated compared to SBMA and control groups in both (A) raw and (B) age-adjusted levels, the significant difference in NfL levels between the control and SBMA patients in raw data diminishes after age adjustment. The right figures provide a zoomed-in view of the Healthy Control and SBMA groups, which are difficult to distinguish in the left original figures.

3.3 | Improved Normality Through Log-Transformation

Figure 2 presents box plots illustrating the distributions of the levels of the three biomarkers (NfL, Cr, and CK) across the ALS and SBMA groups. To assess normality and homogeneity of variance, both the raw and logarithmically transformed data were analyzed using the Shapiro–Wilk test. The logarithmic transformation changed the distributions of NfL and CK levels, increasing the normality of their distribution and improving variance homogeneity. For Cr levels, logarithmic transformation slightly increased normality only in the SBMA group. As shown in Figure 2D, variance homogeneity was significantly increased for log-transformed NfL levels. These results suggest that logarithmically transformed NfL, Cr, and CK levels were more appropriate for subsequent analyses. Therefore, log-transformed data were used, adjusted for age using linear regression, resulting in age-adjusted, log-transformed biomarker levels in the subsequent analyses.

3.4 | Logistic Regression Analysis for Classifying Two Groups (ALS and SBMA) Based on NfL, Cr, and CK

We performed logistic regression analysis to classify patients as having ALS or SBMA. The analysis evaluated three biomarker-level variables (NfL, Cr, and CK), both individually and in combination. Figure 3 presents ROC curves for several predictive models using age-adjusted, log-transformed NfL, Cr, and CK levels to classify patients into the respective groups. In Figure 3A, the ROC curve compares healthy controls with the ALS and SBMA groups to evaluate the effect

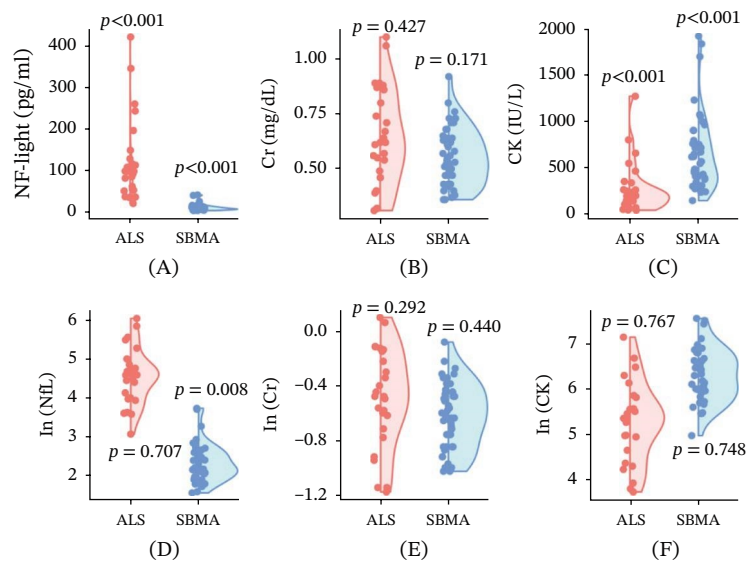


FIGURE 2 | Normality assessment for NfL, Cr, and CK levels in raw and log-transformed data. p values were calculated using the Shapiro–Wilk test for normality, where $p = 1.0$ indicates perfect normality and $p = 0.0$ indicates complete nonnormality. (A–C) The raw data for the three biomarkers show deviations from normality, particularly for NfL and CK. (D–F) After natural log transformation, the data demonstrate improved normality and variance homogeneity, meeting the assumptions required for statistical analyses.

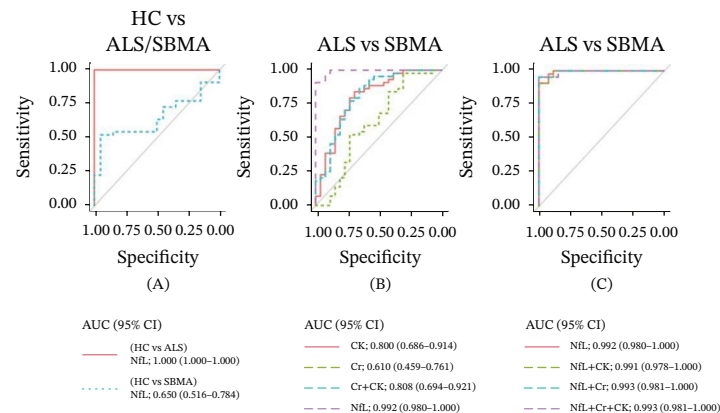


FIGURE 3 | Receiver-operating characteristic (ROC) curves for the classification of healthy controls (HC), ALS, and SBMA patients. (A) ROC curves comparing HC versus ALS and SBMA patients using logistic regression models with NfL. The cutoff values adjusted for age 60 are as follows: HC versus ALS = 22.97 pg / mL, HC versus SBMA = 11.34 pg / mL. (B and C) ROC curves comparing SBMA versus ALS patients using logistic regression models with NfL, Cr, CK, and their combinations. The cutoff values for each biomarker, adjusted for age 60, are as follows: NfL = 22.67 pg / mL, Cr = 0.78 mg / dL, CK = 299.5 IU / L. All three biomarker levels were natural log-transformed and age-adjusted using linear regression. Confidence intervals (CI) were calculated using DeLong's method.

of NfL in distinguishing these disease groups from healthy controls. The NfL model achieved perfect discrimination for healthy controls versus patients with ALS (AUC = 1.000). For healthy controls versus patients with SBMA, the same model yielded an AUC of 0.650. Thus, we found that NfL levels were highly effective for distinguishing healthy controls from patients with ALS but not from patients with SBMA.

We next evaluated the biomarkers that differentiate SBMA from ALS. For the ROC curves of the three biomarkers shown in Figure 3B, the model including NfL levels exhibited excellent discrimination, with an AUC of 0.992 (95% confidence interval: 0.980–1.000; cutoff value = 22.67 pg / mL at age 60). The regression coefficients are presented in Table 2. Although CK shows a difference between the SBMA and ALS groups (Figure 2), the CK-based model, with an AUC of 0.800, did not

perform as well as the NfL-based model. The CK + Cr model yielded results similar to those of the CK model, whereas the multivariate logistic regression models that added Cr or CK to NfL (Figure 3C) showed no significant improvements, as indicated by the p values for AUC comparisons and AIC values (Table 2). Because NfL levels alone distinguished ALS from SBMA with excellent accuracy (AUC = 0.992), adding Cr or CK did not significantly improve model performance. Neither the AUC comparisons nor the AIC values changed significantly when Cr or CK levels were added (Table 2). Thus, in the present cohorts, Cr and CK levels did not appear to improve distinguishing between the two diseases.

To evaluate diagnostic performance during the clinically challenging early stage of disease, we repeated the classification

TABLE 2 | Multivariate logistic regression coefficients for Figure 3.

Model	Odds ratio (p)				p ^b vs. ln(NfL)	AIC (ΔAIC) vs. ln(NfL)	Accuracy	LOOCV AUC mean ± SE ^c	LOOCV accuracy mean ± SE ^c
	ln(NfL)	ln(Cr)	ln(CK)	AUC (95% CI) ^a					
ln(NfL), ln(Cr), ln(CK)	−12.796 (0.050)	3.919 (0.155)	0.148 (0.817)	0.993 (0.981–1.000)	0.866	20.647 (0.529)	0.971	0.993 ± 0.008	0.971 ± 0.025
ln(NfL), ln(Cr)	−13.067 (0.043)	4.165 (0.105)	—	0.993 (0.981–1.000)	0.866	18.701 (−1.417)	0.971	0.993 ± 0.008	0.971 ± 0.025
ln(NfL), ln(CK)	−8.586 (0.012)	—	0.578 (0.342)	0.991 (0.978–1.000)	0.727	21.147 (1.029)	0.943	0.991 ± 0.008	0.945 ± 0.069
ln(Cr), ln(CK)	—	−1.608 (0.129)	1.669 (<0.001)	0.808 (0.694–0.921)	0.001	76.320 (56.202)	0.786	0.808 ± 0.070	0.787 ± 0.124
ln(NfL)	−8.602 (0.007)	—	—	0.992 (0.980–1.000)	Ref	20.118 (Ref)	0.943	0.992 ± 0.006	0.944 ± 0.055
ln(Cr)	—	−1.209 (0.172)	—	0.610 (0.459–0.761)	<0.001	94.425 (74.307)	0.729	0.610 ± 0.078	0.729 ± 0.054
ln(CK)	—	—	1.640 (<0.001)	0.800 (0.686–0.914)	0.001	76.717 (56.599)	0.786	0.800 ± 0.059	0.786 ± 0.050

Note: ln(NfL), ln(Cr), and ln(CK) are adjusted by age (using linear regression).
Abbreviations: AIC, Akaike Information Criterion; AUC, area under the curve; CI, confidence interval; CK, Creatine kinase; Cr, creatinine; LOOCV, leave-one-out cross validation; NfL, neurofilament light chain; Ref, reference.
^aConfidence intervals for AUCs are estimated by DeLong's method.
^bp values are for comparisons of AUCs (using DeLong test) between the ln(NfL) alone and other models.
^cThe jackknife standard error estimate.

analysis in patients with early-stage SBMA, defined by symptom duration rather than time from diagnosis, since diagnostic delays are common in this slowly progressive disease. Patients within 10 years of symptom onset were classified as having early-stage ($n = 28$; mean disease duration 76.7 ± 26.2 months, mean K-SBMAFRS score 38.6 ± 7.6 , mean 6MWT result 345.5 ± 88.0 m). All of whom were ambulatory with relatively preserved motor function (Table S1). In this subset, ln(NfL) continued to distinguish ALS from SBMA well, with an AUC of 0.962, whereas adding ln(Cr) or ln(CK) did not significantly improve classification (DeLong test; Figure S2).

3.5 | Correlation Between Plasma Biomarkers and Clinical Features of Patients With ALS and With SBMA

We conducted correlation analyses between levels of three biomarkers and symptom measures, including ALSFRS-R (analyzed separately in the ALS and SBMA groups) and K-SBMAFRS (SBMA group) scores. The correlation coefficients and corresponding p values for each measure are presented in Figure 4. Plasma NfL and CK levels were useful for differential diagnosis. Nonetheless, neither exhibited an association with symptom-related clinical indicators in ALS or SBMA. Conversely, Cr levels exhibited a significant correlation with K-SBMAFRS lower limb subscores and 6MWT results in patients with SBMA. Scatter plots of the two significant SBMA symptom measures versus Cr levels are presented in Figure 5. The SBMAFRS lower limb subscore refers to items related to the lower limbs and assessing walking and climbing stairs [21].

4 | Discussion

Consistent with the findings of prior studies, plasma NfL levels emerged as the most effective biomarker for differentiating ALS from SBMA. Blood NfL levels tend to increase with age [24]. Age-adjusted NfL concentrations alone demonstrated superior differential diagnostic performance compared with combined analyses that incorporated other biomarkers, such as Cr and CK (Figure 3). In addition, age-adjusted plasma NfL levels were nearly identical between patients with SBMA and healthy controls, consistent with findings from prior studies [3, 10, 16].

Unlike plasma NfL levels, which exhibit a strong correlation with the extent of axonal damage and neurodegeneration, Cr and CK levels are associated with muscle damage in various myopathies and are more closely related to the pathophysiology of SBMA. In line with the findings of prior research, our results indicated that plasma CK levels were significantly higher in patients with SBMA than in those with ALS. Previous studies have suggested that blood Cr levels could be used to effectively differentiate between ALS and SBMA [5, 27]. However, in this study, although plasma Cr levels were lower in patients with SBMA, Cr levels were not effective in differentiating the two diseases. Several factors may account for this finding, including differences in analytical adjustments, the make-up and disease stage of the cohorts, and factors we could not measure directly, such as muscle mass and renal function. However, the

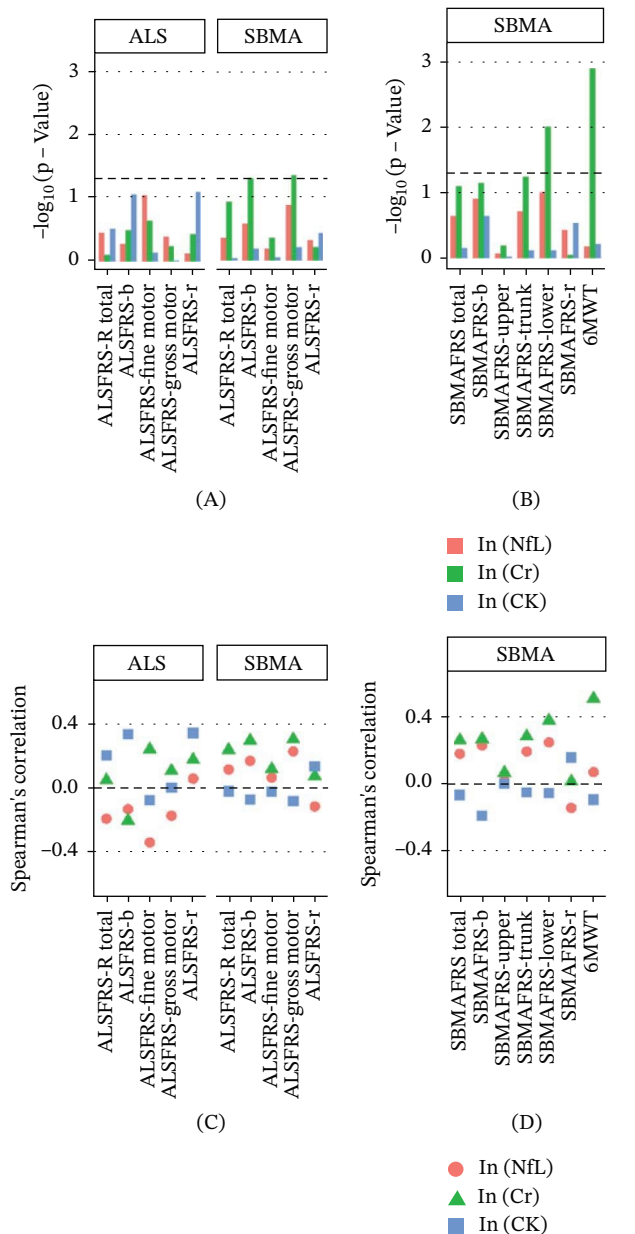


FIGURE 4 | Correlations between biomarkers and symptomatic measurements in each patient groups. (A and B) p values and (C and D) Spearman's correlation coefficients are plotted for all pairwise comparisons among the three age-adjusted biomarkers (NfL, Cr, CK) and measured symptoms. The dashed line in A and B indicates a p value of 0.05.

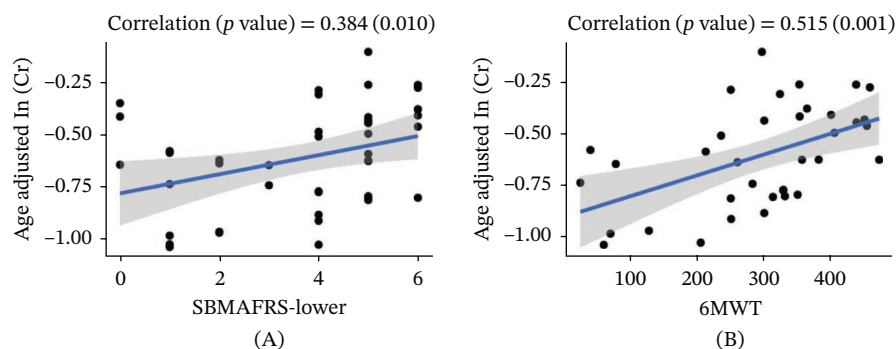


FIGURE 5 | Scatter plots display selected symptom measurements versus Cr levels in SBMA patients. They are chosen for their weak significance in Spearman's correlations, as depicted in Figure 4. Natural log transformed Cr levels show a positive correlation with SBMAFRS-lower and 6MWT scores.

sensitivity analysis showed that adjusting for these additional variables only slightly changed Cr values. Thus, the limited incremental diagnostic value of Cr may be attributable to the strong diagnostic performance of NfL alone.

It should be noted that, in our cohort, NfL levels alone could be used to distinguish ALS from SBMA almost perfectly, and adding Cr or CK levels did not improve this differentiation. The value of measuring Cr and CK levels, if any, seems to lie elsewhere chiefly in reflecting muscle involvement in SBMA rather than in differential diagnosis. Nevertheless, Cr levels were the only biomarker to correlate with some symptom-related measures, including lower-limb subscores of the K-SBMAFRS and 6MWT results. This suggests that Cr levels primarily reflect skeletal muscle function and disease severity in SBMA rather than serving as a differential diagnostic biomarker, in line with earlier work linking serum Cr levels to functional measures and disease progression.

In addition to NfL levels as used in this study, another marker of neurodegeneration, plasma neurofilament heavy chain, has been reported to increase only in ALS and to remain unchanged in SBMA [6]. These findings support a hypothesis, proposed in SBMA animal model studies and pathological investigations [28, 29], stating that under-recognized muscle damage possibly contributes to the pathogenesis of SBMA. However, central nervous system-related pathological changes, such as tremors, mild cognitive impairment, and white matter abnormalities, which are not readily explained by skeletal muscle impairment, are also common in patients with SBMA [30, 31]. Therefore, further research is warranted to identify plasma biomarkers associated with the neuropathology of SBMA.

This study had some limitations. First, the cohort comprised mainly patients with early-stage ALS and ambulatory SBMA; therefore, our findings may not be generalizable to patients with more advanced disease. Most of the patients with SBMA had relatively preserved functional status, representing early clinical stages, defined by symptom duration and functional severity. Nevertheless, one of the primary objectives of our study was to distinguish between ALS and SBMA in their early stages, which may make our results potentially more reliable for this purpose. Second, the sample size was relatively small. Further studies with larger cohorts from multiple countries and ethnic backgrounds are needed to validate and consolidate these findings. Finally, this was a single-center study, which is an inherent

limitation. However, the single-center design enabled consistent functional scoring by a single experienced evaluator and ensured uniform data acquisition and analysis using the same SiMoA method. These factors contributed to the reliability and consistency of our results.

Two further caveats concern the biomarker adjustment itself. Cr and CK levels depend on more than age and are also affected by muscle mass, muscle wasting, weight loss tied to disease, nutritional state, and renal function. We corrected for age and used a sensitivity analysis to check whether adding body mass index, disease duration, and functional severity data changed the results. The Cr and CK values changed only slightly (Figure S1). However, measures of muscle mass or renal function were not consistently available across the cohort, so we could not rule out residual confounding, and the limited diagnostic value seen for Cr and CK levels should be interpreted cautiously.

The second caveat is age matching. Because ALS and SBMA typically begin at different ages, our two groups were not age-matched. Plasma NfL levels increase with age, so rather than entering age as a covariate—which pushed the groups apart largely along the age axis—we removed the effects of age using linear regression before fitting the logistic models. This narrowed the gap between patients with SBMA and controls, but regression cannot be assumed to remove the effects of age entirely because the groups differed in participant age, and a degree of residual confounding is possible. Confirming these results will require larger cohorts that are matched for age.

Our findings align with those of previous studies, which suggest that age-adjusted plasma NfL levels are a sensitive and promising biomarker for distinguishing ALS from SBMA in early diagnostic stages. Plasma Cr levels exhibited minimal discriminatory potential for differentiating between ALS and SBMA but correlated with motor function parameters in patients with SBMA, including lower-limb subscores of the K-SBMAFRS and 6MWT results, suggesting their potential utility as a surrogate biomarker of SBMA. Further studies with larger and more diverse cohorts are warranted to substantiate and extend our findings.

Author Contributions

Shinrye Lee and Youngtae Choi have contributed to the work equally and should be regarded as cofirst authors.

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Ethics Statement

Ethical approval for the study was granted by the Institutional Review Boards of the Kyungpook National University Chilgok Hospital, Korea (KNUMC-2023-01-036-001).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

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

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Supporting Information.** Figure S1: Assessment of potential clinical covariates influencing Cr and CK levels. Figure S2: ROC curve analysis restricted to early-stage SBMA patients. Table S1: Clinical and genetic profile of SBMA participants in the study.