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Exploring Cerebrospinal Fluid IgG *N*-Glycosylation as Potential Biomarker for Amyotrophic Lateral Sclerosis

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Abstract

Amyotrophic lateral sclerosis (ALS) is a fatal motor neuron disease for which the existing candidate biomarkers (neurofilaments) have low specificity. Changes in blood IgG *N*-glycosylation have been observed in several diseases, including ALS, whereas cerebrospinal fluid (CSF) IgG has been less studied. Here, we characterized *N*-glycans of CSF IgG from ALS patients in comparison with a control group of other neurological diseases. Cerebrospinal fluid was collected from patients with ALS ($n = 26$) and other neurological diseases ($n = 10$). *N*-Glycans were released from CSF purified IgG with peptide *N*-glycosidase F, labeled with 2-aminobenzamide and analyzed by NP-HPLC chromatography in combination with exoglycosidase digestion and MALDI-TOF mass spectrometry. The *N*-glycosylation profile of ALS CSF IgG consisted of diantennary *N*-glycans predominantly with proximal fucose and some bisecting GlcNAc; agalacto-, mono-, and digalactosylated as well as α2,6-sialylated structures were detected. Differences between ALS and control patients were observed; most relevant was the increase in ALS CSF IgG of the level of galactosylated structures defined here as Gal-index (median 46.87 and 40.50% for ALS and controls, respectively; $p = 0.006$). The predictive value of the Gal-index (AUC = 0.792, $p = 0.007$) considering ROC analysis had potential utility as a diagnostic test for ALS and was comparable to that of phosphoneurofilament heavy chain (AUC = 0.777, $p = 0.011$), which was used as benchmark marker for our group of patients. The results provide the basis to further explore the potential of IgG *N*-glycan galactosylation as biomarker for ALS by using larger cohorts of patients and controls.

Keywords Amyotrophic lateral sclerosis · Biomarker · Cerebrospinal fluid · Glycoproteins · Immunoglobulin G · *N*-Glycosylation

Introduction

Amyotrophic lateral sclerosis (ALS) is the most frequent motor neuron disease, with an incidence of 2.6/100,000 and a prevalence of 7–9/100,000 in Europe with a mean life expectancy of 30 months from the first symptoms [1]. Although the disease can affect any adult and there are juvenile forms, typically, onset occurs between 50 and 70 years [2]. About 5–10% of the patients have a positive family history for the disease, but most cases (90–95%) are sporadic. The only available drug used worldwide for this disease is Riluzole, but its effectiveness in increasing survival is modest [3].

There has been a strong effort towards the identification of molecular markers for supporting diagnosis and prognosis, aiming its use in future clinical trials [4–6]. At present, neurofilaments appear as the most promising candidate biomarkers for ALS. Results from different groups including multicenter studies showed that phosphoneurofilament heavy chain (pNFH) and neurofilament light chain from CSF and

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blood [7–10] are increased in ALS patients, particularly in fast progressors. Unfortunately, neurofilaments are also increased in other neurological diseases but still constitute a valuable benchmark for ALS.

Other potential markers have been proposed including other cytoskeletal proteins, markers of oxidative stress, aggregated proteins, growth factors, neuroinflammation-associated molecules, microRNA, protein, and metabolic signatures [6]. Changes in post-translational modifications were also observed in ALS; for example, concerning protein glycosylation, changes were observed for serum [11, 12] and CSF glycoproteins [8]. Particularly, for serum IgG, the increase of the *N*-glycan A2BG2 has been proposed as potential ALS biomarker [11, 12].

IgG is an abundant glycoprotein from human serum (10–15 mg/mL) [13] and from CSF (typical concentration 22 mg/L) [14]. In healthy individuals, CSF IgG predominantly originates from the blood. Although there is much information concerning serum IgG glycosylation and its alteration in disease [13, 15], studies describing CSF IgG glycosylation are scarce. Only recently, some reports appeared which showed that specific *N*-glycans are differentially represented in serum versus CSF IgG [16, 17].

In this work, with the aim of discovering novel biomarkers for ALS, we elucidate the *N*-glycan structures from CSF IgG of ALS patients using high-resolution NP-HPLC chromatography in combination with an *N*-glycan reference library, exoglycosidase digestion, and MALDI-TOF mass spectrometry; pNFH is presented as biomarker benchmark for ALS. Variations of specific *N*-glycans comparative with a neurological disease-control group are presented.

Materials and Methods

ALS Patients and Controls

All patients included were regularly followed at the Neuromuscular Unit of the Department of Neurosciences (Hospital de Santa Maria, Lisbon). ALS patients had probable or definite disease and showed disease progression, according to the revised El Escorial criteria [18]. As inclusion criteria, we established age between 18 and 80 years and informed consent. Patients with other medical conditions on gastrostomy, taking supplements other than vitamins, and symptoms of respiratory distress or cognitive changes were excluded. At the time of CSF sampling, the patients were observed and the disease severity was scored by applying ALSFRS (Amyotrophic Lateral Sclerosis Functional Rating Scale) [19]. We included a group of 26 patients with ALS and 10 controls with other neurological diseases (Table 1). The control group included patients with normal pressure hydrocephalus, degenerative disease other than ALS (multiple system atrophy), axonal peripheral neuropathy (vasculitis), demyelinating spinal cord lesion, idiopathic brachial plexopathy, and chronic inflammatory demyelinating neuropathy (CIDP). Except for the single patient with normal pressure hydrocephalus, the clinical presentation of the remaining patients could be considered as ALS-mimicking disorders.

In all patients, CSF was collected as part of the diagnostic work-up. In all included patients, serology for *Borrelia burgdorferi* and *Treponema pallidum* (CSF) and retrovirus (blood) were negative. CSF was collected by lumbar puncture into polypropylene tubes without additives and immediately stored at − 80 °C. Patients signed permission for biobank storage, and further studies were agreed by the local Ethic's committee. Research was done in accordance with the Helsinki Declaration as revised in 2013 (www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects).

IgG Purification and *N*-Glycans Release

IgG was purified from 300 μl CSF by affinity chromatography using Protein G HP SpinTrap from GE Healthcare following the supplier's instructions. CSF was added to cartridges pre-equilibrated in binding buffer (20 mM sodium phosphate pH 7.0), which were incubated with gentle mixing, 4 min, for IgG binding. The cartridges were then centrifuged 30 s, 100×*g*, and washed twice with binding buffer. IgG elution was done with 0.1 M glycine-HCl, pH 2.7, and the recovered fractions (eluate 1 and eluate 2) were neutralized immediately after collection with 1 M Tris-HCl, pH 9.0. All fractions (12 μl) were analyzed by SDS-PAGE (Fig. S1). CSF volumes of 300, 450, and 600 μl were tested and the lower amount provided enough material for the analytical procedures.

IgG containing-eluates were six- to 12-fold concentrated in the Speed Vac and IgG was precipitated with five volumes of ice-cold acetone, for 2 h at − 20 °C and collected from the pellet after centrifugation at 21000×*g*, 5 min, 6 °C. The pellets

Table 1 Demographic and clinical features of the patients included. Median and interquartile range is shown

	Gender (M/F)	Age (years)	Disease duration (years)	ALSFRS	Onset site (bulbar/spinal)	Total protein (mg/ml)	pNFH (pg/ml)
Controls	(6/4)	64.6 (55.7–72.3)	–	–	–	0.48 (0.37–0.71)	378 (236–809)
ALS	(18/8)	57.0 (49.1–64.3)	0.9 (0.6–1.8)	37 (34–38)	(1/25)	0.49 (0.37–0.57)	1672 (536–3092)

were dried in the Speed Vac and solubilized in 20 μl 3.3 mM Tris-HCl pH 7.0 containing 6 M urea; then, 3 μl 0.5 M 2-mercaptoethanol, 2.5% SDS in peptide *N*-glycosidase F (PNGase F; EC 3.5.1.52) digestion buffer (100 mM sodium phosphate, 0.1% sodium azide, pH 7.5) and 2 μl PNGase F digestion buffer were added. Samples were then incubated at 65 °C for 30 min and Tween-20 was added to 0.5% in a total volume of 150 μl. IgG was deglycosylated with 15 mU PNGase F at 37 °C overnight. Deglycosylation was checked from the IgG heavy chain molecular mass shift in SDS-PAGE. Samples were adsorbed onto Hypercarb cartridges (25 mg; Thermo Hypersil-Keystone) and after cartridge washing with water; the *N*-glycans were eluted with 150 μl of 40% acetonitrile, 0.1% TFA, and neutralized with 1.6 μl of 2.5% NH_4OH. Samples were dried and stored at − 20 °C until subjected to 2-AB-labeling and NP-HPLC analysis. As control, 5-μg human immunoglobulin (molecular size) BRP (European Pharmacopoeia (EP) Reference Standard Y0000488) were processed in parallel.

2-Aminobenzamide Labeling and Separation of *N*-Glycans by NP-HPLC

N-Glycans were labeled with 2-aminobenzamide (2-AB) using a GMP validated derivatization procedure based on the previously published method of Bigge [20] with reagents from Sigma-Aldrich. After derivatization, the 2-AB-labeled glycan mixtures were purified from excess labeling reagents by gel filtration using PD MiniTrap G-10 size exclusion columns from GE Healthcare. The size exclusion columns were conditioned with 8-ml ultrapure water prior to sample application. The columns were washed with 700-μl ultrapure water followed by elution of 2-AB-labeled *N*-glycans with 500-μl ultrapure water. Control for sialic acid stability during 2-AB-labeling was performed using tri- and tetrasialylated (α2,6-sialylated) *N*-glycans from bovine fetuin.

Mapping of 2-AB-labeled glycans was done with an ACQUITY UPLC BEH glycan column from Waters Corporation (Milford, MA, USA). An HPLC system from Dionex (ThermoFisher Scientific) with an Ultimate 3000 RS Fluorescence detector FLD-3400RS was used. Mobile phase A was 85% (*v*/*v*) acetonitrile and mobile phase B was 250 mM ammonium formate pH 4.4, at a flow rate of 0.40 ml/min and at a column temperature of 60 °C. Detection was at excitation wavelength = 347 nm and emission wavelength = 420 nm. The gradient was as follows: starting conditions 88% A and 12% B; a gradient from 1 to 25 min to 82% A; gradient for 20 min to 70% A; gradient for 10 min to 20% A; and an increase to 88.0% A and re-equilibration of the column isocratically for 10 min. NP-HPLC analysis was done under GMP conditions using validated SOP. The equivalent amounts to 75–150 μl CSF and typically 1.25 μg IgG BRP were applied per run. 2-AB-labeled reference oligosaccharide standards of known structure were run within each analytical sample sequence (Table S1). These standard *N*-glycan structures have been characterized according to GMP qualification tests and are from a glycan library of the company.

Digestion of 2-AB-Labeled *N*-Glycans with Exoglycosidases

For structure elucidation/confirmation, total 2-AB-labeled *N*-glycans (corresponding to 75 μl CSF) were digested in 10–20 μl of 0.1 M ammonium acetate buffer pH 5.0 with the following exoglycosidases: α(2-3,6)-sialidase (*Arthrobacter ureafaciens* EC 3.2.1.18, Roche); α-L-(1-2,3,4,6)-fucosidase (bovine kidney EC 3.2.1.51, Sigma); and β(1-3,4)-galactosidase (bovine testes, Prozyme). The digested *N*-glycan incubation mixtures were diluted with 50-μl water and were applied onto a pipette tip filled with 20–25 μl of porous graphitic carbon (Hypercarb) which had previously been conditioned with 100 μl 80% acetonitrile containing 0.1% TFA and 150-μl water. After washing with 3 × 150-μl water, *N*-glycans were eluted with 150 μl 25% acetonitrile, dried in a Speed Vac concentrator, and stored at − 20 °C. For NP-HPLC glycan mapping, *N*-glycans were solubilized in 75% acetonitrile/20% water and 2–10 pmol were applied per run.

Analysis of 2-AB-Labeled *N*-Glycans by MALDI-TOF MS

2-AB-labeled *N*-glycans were analyzed with a Bruker ULTRAFLEX time-of-flight (TOF/TOF) instrument as previously described [21]. Mass spectra were recorded in the reflector and positive ion mode. Samples of 1 μl at an approximate concentration of 5 pmol/μl were mixed with equal amounts of the matrix 2,5-dihydroxybenzoic acid. The mixture was spotted onto a stainless steel target and dried at room temperature before analysis. Data were acquired using FlexControl 3.4 software (Bruker, Germany) averaging up to 1000 individual spectra for optimum signal quality. Peak identification was done manually with Compass DataAnalysis version 4.2 Bruker from unprocessed spectra. Compatible structures were assigned to various *N*-glycans on the basis of their observed *m/z* values using the GlycoWorkbench 2 software [22], considering 2-AB-labeling at the reducing end, 1-Da accuracy and searched in the databases CFG, Carbbank, GlycomeDB, or Glycosciences.

Phosphoneurofilament Heavy Chain Quantification

pNFH from the CSF was quantified using the ELISA kit from BioVendor Research and Diagnostic Products (Czech Republic, no. RD191138300R), as previously described [8, 23].

Peak Quantification and Statistical Analysis

Quantification of peaks from the NP-HPLC chromatograms was done using the Chromeleon 6.8 software. First, all peaks eluting between 10 and 45 min were selected manually and their relative percentages were calculated; 41 peaks were detected. Then, only peaks above 1% relative percentage (and peak 6) were considered for final quantification (15 peaks). Two samples were processed and analyzed independently, and reproducibility was high with coefficient of variation of individual peaks typically below 5%; the remaining samples were processed and analyzed once.

Statistical analysis was performed with Graph-Pad Prism 6. Normality was checked by the D'Agostino and Pearson omnibus normality test and most sample distributions were not normal. Therefore, peak percentages were presented as median and the interquartile range (IQR, 25–75% percentiles) and statistical comparison was done using the non-parametric Mann-Whitney test. Spearman non-parametric correlation analysis was used. Values of $p < 0.05$ were considered as statistically significant. Receptor operator characteristic (ROC) curve analysis was performed and area under the curve (AUC) was calculated.

Results

We included 26 ALS patients (18 male, 8 female) with a median age of 57.0 and 10 patients with other neurological diseases, predominantly ALS-mimicking disorders (6 male, 4 female) with median age 64.6 (Table 1); age was comparable between groups ($p = 0.103$). ALS patients had median disease duration of 0.9 years. Their clinical severity score according to the Amyotrophic Lateral Sclerosis-Function Rating Scale was ALSFRS 37. The median protein concentration was similar for ALS patients and controls (0.49 and 0.48 mg/mL, respectively).

IgG was purified by affinity chromatography using Protein G HP SpinTrap that binds IgG1–4 subclasses (Fig. S1). *N*-Glycans were released enzymatically from the purified IgG with PNGase F and fluorescently labeled with 2-AB for increased sensitivity and quantitative analysis. 2-AB-labeled glycans from control and ALS patients and standard human serum IgG BRP were separated by NP-HPLC (Fig. 1). Fifteen peaks eluting between 10 and 45 min were identified based on identical migration to a reference library of 2-AB-labeled *N*-glycan standards (Table S1). Structure assignment was further corroborated by sequential digestions of *N*-glycans from IgG BRP (Fig. S2), CSF IgG of control patients (Fig. S3), and ALS

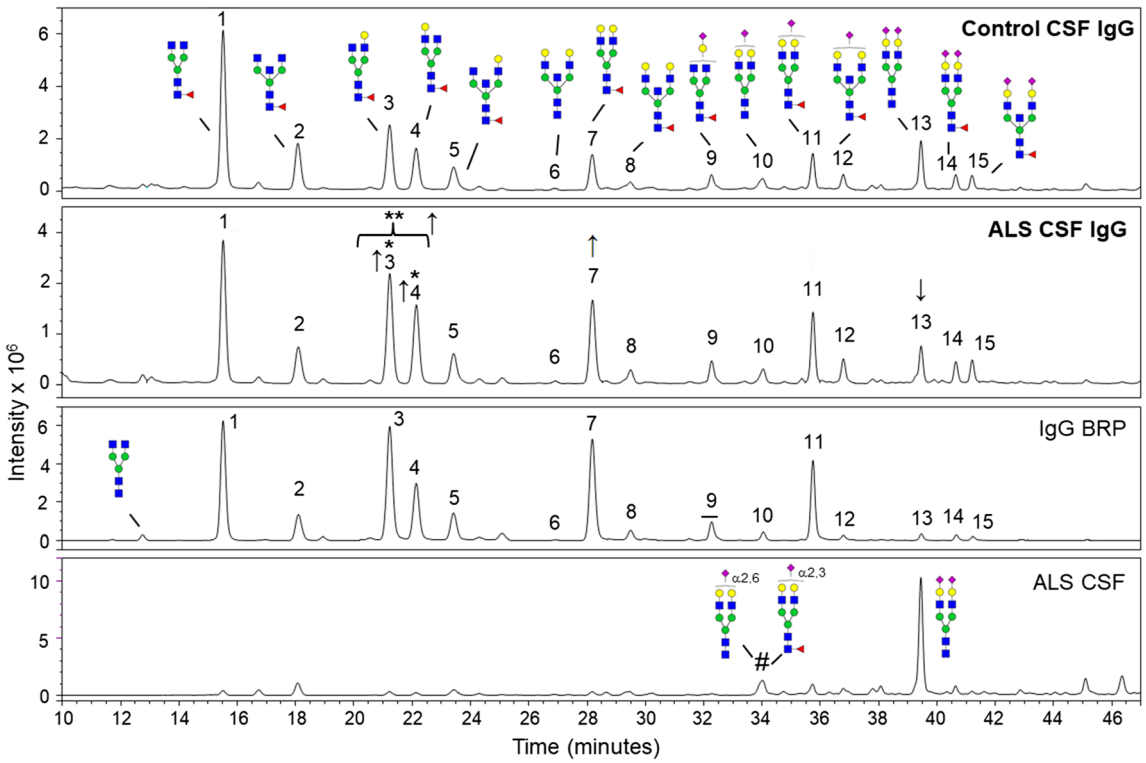

Fig. 1 NP-HPLC profiles of 2-AB-labeled *N*-glycans of CSF IgG from representative control and ALS patients. Major increases (upwards arrow) and decreases (downwards arrow) as well as statistically significant differences are indicated. The corresponding structures are represented according to the Consortium for Functional Glycomics. Median values from peaks 1–15 of control and ALS patients are presented in Table 2. The *N*-glycan profiles from human serum IgG BRP as standard, and from CSF of an ALS patient for comparison are shown. Number sign indicates the peak that was previously found increased in CSF *N*-glycans (8)

patients (Fig. S4), with exoglycosidases α(2-3,6)-sialidase from *A. ureafaciens*, α-L-(1-2,3,4,6)-fucosidase from bovine kidney, and β(1-3,4)-galactosidase from bovine testes. Furthermore, the assigned peaks were collected from NP-HPLC preparative runs of control as well as ALS patients and analyzed individually by MALDI-TOF MS (Fig. S5). The identified *m/z* values were compatible with the proposed structures (Table 2).

Total 2-AB-labeled *N*-glycans from CSF IgG of control and ALS patients were also analyzed by MALDI-TOF MS and compared with the profile from IgG BRP (Fig. 2). The *m/z* values observed were coherent with the NP-HPLC profiles.

The structures detected consisted of diantennary glycans predominantly with proximal fucose (peaks 1–5, 7–9, 11, 12, 14, 15) with (peaks 2, 5, 6, 8, 12, 15) or without bisecting GlcNAc (peaks 1, 3, 4, 7, 9, 10, 11, 13, 14). Mono- and disialylated structures with α2,6-linked Neu5Ac (peaks 9–15) were present. On the other hand, structures lacking galactose in one or two branches (peaks 1–5) were largely represented.

Peaks 1–15 were quantified as relative percentages (Table 2). The following differences were observed in ALS: significant increases in monogalactosylated diantennary glycans with proximal Fuc (peaks 3 and 4); trend towards an increase in proximally fucosylated diantennary glycans (peak 7); and trend towards a decrease for disialylated afucosylated diantennary glycan (peak 13) (Fig. 1).

When peaks were grouped based on structural features, a significant increase of total mono- and digalactosylated glycans (defined here as Gal-index) (1.16-fold, $p = 0.006$) (Fig. 3a) was observed in ALS patients concomitant to a trend towards a decrease of agalactosylated structures (0.85-fold; $p = 0.087$) (Table 2). In agreement, there was a negative correlation between Gal-index levels and agalactosylated glycan levels (Spearman $r = -0.784$, $p < 0.0001$) for all subjects. There were no differences in the levels of sialylated, proximally fucosylated, nor bisecting GlcNAc–containing glycans between ALS patients and controls. There was a significant negative correlation ($r = -0.749$; $p = 0.0001$) between sialylated glycans and disease duration (in a subgroup with disease duration below 2 years).

For comparison, peaks 1–15 from serum IgG BRP were also quantified (Table S2). A lower proportion of Gal-index and fucosylated structures together with increased levels of sialylated as well as bisecting GlcNAc-containing structures was observed in the cohorts of ALS and control patients relative to human serum IgG.

pNFH was used as a benchmark. pNFH levels were significantly higher in ALS patients (1672 pg/ml) than in controls (378 pg/ml, $p = 0.010$) (Table 1, Fig. 3a) as previously reported [8, 23]. There was a weak but significant correlation between pNFH and Gal-index considering all subjects ($r = 0.333$, $p = 0.047$).

ROC analysis was performed to evaluate Gal-index diagnostic accuracy; AUC was 0.792 ($p = 0.007$) (Fig. 3b). ROC analysis of pNFH levels from the same subjects gave an AUC of 0.777 ($p = 0.011$) (Fig. 3b). Therefore, the predictive value of the Gal-index is comparable with that of pNFH for the patients analyzed here.

Both Gal-index and pNFH levels showed a significant negative correlation with disease duration ($r = -577$, $p = 0.003$; $r = -0.656$, $p = 0.0004$). In agreement, Gal-index and pNFH were significantly lower in ALS patients with a disease duration longer than 1 year ($p = 0.004$ and $p = 0.021$, respectively) (Fig. 3c). On the other hand, Gal-index did not show correlation with the rate of functional decline (calculated as (40 - ALSFRS)/disease duration (months)) ($r = 0.268$, $p = 0.195$) contrary to pNFH ($r = 0.741$, $p < 0.0001$). Moreover, Gal-index correlated negatively with age at sampling ($r = -594$, $p = 0.001$) in ALS but not in controls ($r = -0.430$, $p = 0.218$); pNFH did not correlate with age at sampling in ALS ($r = -0.164$, $p = 0.424$). No significant correlation was detected between Gal-index or pNFH and ALSFRS or the corresponding respiratory subscore.

Since IgG galactosylation is decreased with age [24], to exclude any effect of age on the observed Gal-index difference, we compared subgroups of control ($n = 9$) and ALS ($n = 15$) patients with more closely matching median ages of 64.1 (IQR 55.5–69.0) and 63.0 (IQR 59.7–67.7), respectively. Gal-index was still higher for ALS 46.57 (41.39–47.45)% than for controls 39.62 (36.02–45.33)% ($p = 0.035$). On the other hand, since decreases in IgG galactosylation were observed by others for serum IgG in CIDP [25], and considering that in ALS IgG from CSF mostly derives from transfer from the blood, to exclude any possible effect of CIDP on the observed CSF, Gal-index difference here was used as control subgroup without CIDP ($n = 8$). Median and interquartile range was 39.90 (35.88–45.54)%; Gal-index was still 1.17-fold higher in ALS than in control subgroup ($p = 0.017$). Therefore, age and CIDP from controls did not affect the difference in Gal-index observed between controls and ALS patients in this study.

Previously, we identified the increase of a peak composed predominantly of A2G2S(6)1 in *N*-glycans from total CSF glycoproteins of ALS patients [8]. That peak comigrated with peak 10 from CSF IgG (Fig. 1, fourth panel), which was non-significantly decreased in ALS (0.91-fold, $p = 0.374$) (Table 2).

We have screened for the A2BG2 structure (peak 6) in CSF IgG, since it has been found by other groups to be increased in human serum IgG in ALS [11, 12] and SOD1G93A mouse model [12]. We identified this structure in CSF IgG, since the corresponding peak comigrated with the A2BG2 peak from IgG BRP and presented the expected *m/z* value in MALDI-TOF MS analysis (Fig. S5). A2BG2 was weakly represented in CSF IgG (median (IQR) 0.25 (0.19–0.30) and 0.26 (0.21–0.36), in controls and ALS, respectively) and only a small non-significant increase (1.04-fold, $p = 0.323$) was observed for the ALS patients in the present study (Table 2).

Table 2 Comparison of the relative percentages of *N*-glycan fractions between controls and ALS patients

NP-HPLC fraction	Structures (CFG)	Structures	*m/z*	Controls (%) Median (IQR)	ALS (%) Median (IQR)	p	ALS/ Controls	AUC (p)
1		FA2	1605.6	23.66 (19.93-27.67)	20.95 (16.65-23.03)	0.166	0.89	-
2		FA2B	1808.7	10.09 (7.15-11.16)	8.57 (6.68-10.25)	0.111	0.85	-
3		FA2[6]G1	1767.7	13.58 (12.01-16.35)	16.05 (14.68-16.94)	0.018 *	1.18	0.756 (0.019)
4		FA2[3]G1	1767.7	8.29 (6.84- 8.93)	9.33 (8.23-10.54)	0.044 *	1.13	0.719 (0.044)
5		FA2B[6]G1	1970.7	6.16 (5.58- 8.65)	6.46 (5.58-7.19)	0.883	1.05	-
6		A2BG2	1986.7	0.25 (0.19-0.30)	0.26 (0.21-0.36)	0.323	1.04	-
7		FA2G2	1929.7	8.72 (6.78-10.77)	11.38 (8.65-12.66)	0.052	1.31	-
8		FA2BG2	2132.8	2.23 (1.83- 3.21)	2.65 (2.04-2.93)	0.481	1.19	-
9		FA2G1S(6)1	2058.8 2080.8($2Na^+$-H^+)	2.41 (2.20- 2.84)	2.55 (2.42-2.99)	0.438	1.06	-
10		A2G2S(6)1	2074.7 2096.7($2Na^+$-H^+)	2.49 (2.11- 2.71)	2.26 (1.88-2.76)	0.374	0.91	-
11		FA2G2S(6)1	2220.8 2242.8($2Na^+$-H^+)	6.76 (4.94- 9.32)	7.52 (6.29-8.58)	0.475	1.11	-
12		FA2BG2S(6)1	2423.9 2445.9($2Na^+$-H^+)	3.08 (2.49- 3.66)	3.03 (2.75-3.33)	0.788	0.98	-
13		A2G2S(6)2	2365.8 2387.8($2Na^+$-H^+) 2409.8($3Na^+$-2H+)	5.43 (4.15- 8.06)	4.28 (3.49-6.73)	0.067	0.79	-
14		FA2G2S(6)2	2511.9	1.91 (1.60- 2.23)	1.85 (1.65-2.17)	0.855	0.97	-
15		FA2BG2S(6)2	2715.0 2737.0($2Na^+$-H^+) 2759.0($3Na^+$-2H+)	2.17 (1.97-2.77)	2.24 (2.01-2.49)	0.735	1.03	-
Agalactosylated (1,2)	-	-	-	33.81 (27.08-36.89)	28.84 (25.70-32.37)	0.087	0.85	-
Monogalactosylated w/o bisecting GlcNAc (3,4)	-	-	-	22.40 (20.31- 24.05)	25.41 (23.48-26.71)	0.006 **	1.13	0.792 (0.007)
Mono+digalactosylated w/o bisecting GlcNAc (3,4,7)	-	-	-	30.43 (26.81-34.70)	37.18 (32.84-39.08)	0.009 **	1.22	0.781 (0.010)
Monogalactosylated (3-5)	-	-	-	29.67 (25.54-31.22)	31.96 (30.50-32.83)	0.004 **	1.08	0.804 (0.005)
Digalactosylated (6-8)	-	-	-	11.75 (9.75-13.31)	14.22 (11.71-16.13)	0.041 *	1.21	0.723 (0.041)
Gal-index (3-8)	-	-	-	40.50 (36.16- 47.96)	46.87 (41.44-48.30)	0.006 **	1.16	0.792 (0.007)
Sialylated (9-15)	-	-	-	25.45 (22.35-27.72)	25.03 (22.42-26.24)	0.684	0.98	-
Bisecting GlcNAc-containing (2,5,6,8,12,15)	-	-	-	23.63 (20.32-28.70)	23.01 (20.49-26.03)	0.471	0.97	-
Fucosylated (1-5,7-9, 11,12,14,15)	-	-	-	92.03 (89.07-93.18)	93.16 (90.35-94.37)	0.100	1.01	-

NP-HPLC fractions from preparative runs of pools of control as well as ALS patients were analyzed by MALDI-TOF MS (Fig. S5), and *m/z* values (monoisotopic masses $[M + Na]^+$) of major peaks are shown; adducts with 2 or 3 Na^+ are indicated in the table. Gal-index, mono-/digalactosylated structures. *w/o*, without

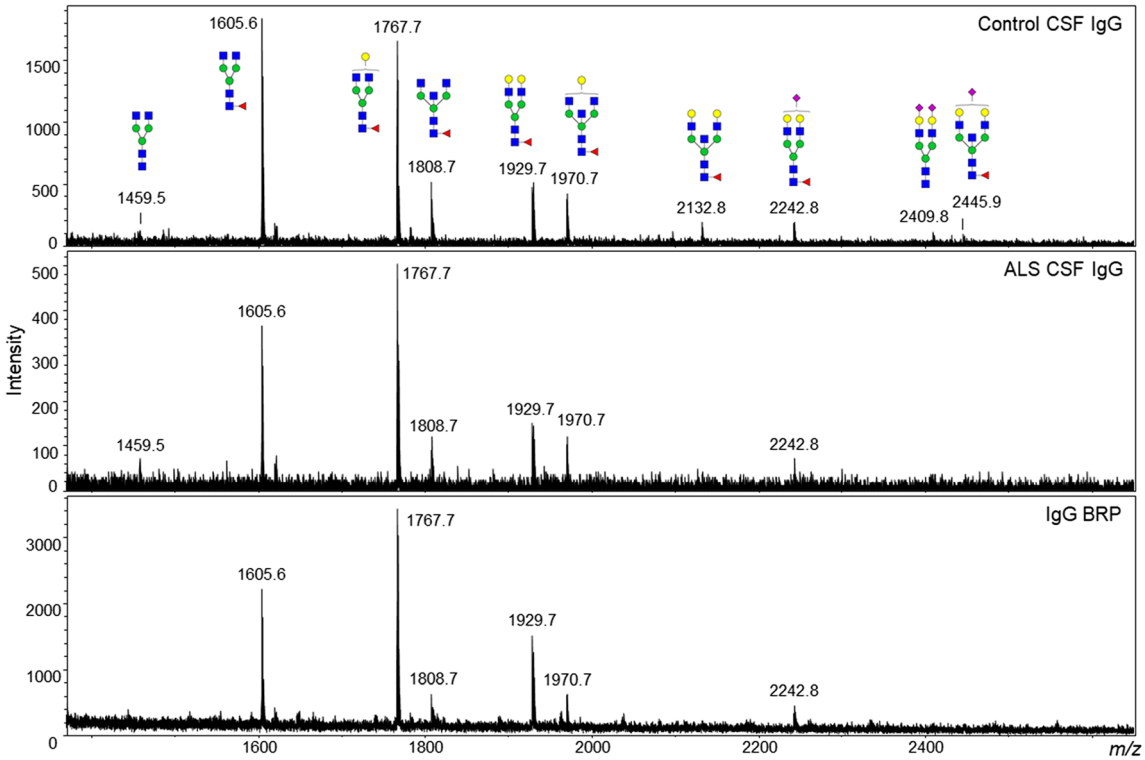

Fig. 2 MALDI-TOF MS analysis of 2-AB-labeled *N*-glycans of CSF IgG from control and ALS patients. The profile of 2-AB-labeled *N*-glycans from human serum IgG BRP standard is shown. Complete list of *m/z* values with compatible structures is presented in Table 2

Discussion

Whereas studies on glycosylation of human serum IgG [26] as well as recombinant therapeutic monoclonal antibodies [27] are abundant, CSF IgG glycosylation has been less studied. Particularly in ALS, to our knowledge, this is the first study that presents the *N*-glycan profile and detailed structure analysis of CSF IgG. Major structures were of the diantennary type predominantly with proximal fucose and some bisecting GlcNAc; the profile consisted of agalacto-, mono-, and digalactosylated as well as α2,6-sialylated structures.

The *N*-glycan composition of CSF IgG was similar to that of standard human serum IgG BRP but the relative amounts were distinct. CSF IgG from our control and ALS groups contained lower amounts of galactosylated and fucosylated structures than IgG BRP, which is in agreement with reports where CSF versus serum IgG1 from the same individuals (the group containing healthy controls and non-inflammatory neurological diseases) were compared [16]. Concerning bisecting GlcNAc-containing structures, we found higher levels in CSF IgG in both our control and ALS groups than in IgG BRP. Other groups also reported increases in bisecting GlcNAc for CSF versus serum IgG1 in multiple sclerosis (MS) patients but no significant changes for controls (healthy and non-inflammatory neurological diseases) [16]. On the other hand, decreases in bisecting GlcNAc for all IgG subclasses (IgG1, IgG2/3, IgG4) in CSF compared to serum were observed for patients with mild cognitive impairment [17]. Concerning sialylation, we observed that CSF IgG had more sialic acid than IgG BRP in contrast to that found for MS patients [16]. The differences observed may be due to several factors that include distinct compositions of the control groups, stage of disease, number of samples, or presence of inflammation (see discussion below). In addition to the comparison with IgG BRP, since in ALS CSF IgG mainly originates from the blood through transport via the blood-brain barrier (intrathecal oligoclonal IgG synthesis has been reported only in few ALS cases from 1.9% [28] to 3.5% [29]), it would be relevant to study glycan profiles from CSF and serum IgG for the same ALS individuals at the same time to understand the transfer of individual IgG glycoforms through the blood-brain-barrier.

When the ALS group of patients was compared with a group of controls with other neurological diseases, significant higher levels of the mono- and digalactosylated structures (Gal-index) were observed in ALS; concomitantly, a trend towards a decrease of agalactosylated structures was detected; there were no significant differences for total sialylated *N*-glycans, proximally fucosylated nor bisecting GlcNAc-containing structures. The monosialylated glycan A2G2S(6)1 was decreased here for ALS CSF IgG, which indicated that its previous increase from total CSF glycoproteins [8] originated from glycoprotein(s) other than IgG that is still to be identified.

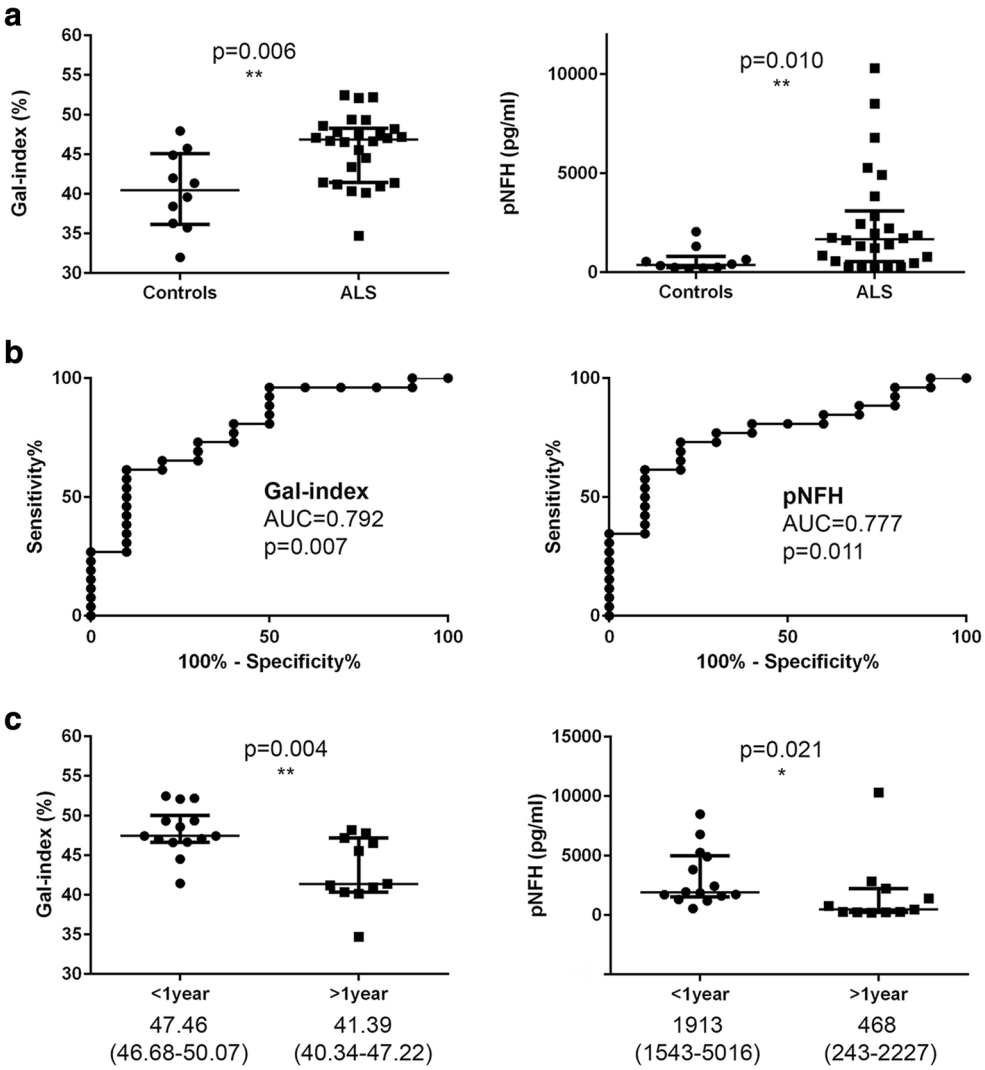

Fig. 3 Comparison of Gal-index and pNFH levels in control and ALS patients. **a** Gal-index and pNFH levels. **b** ROC curve analysis of the Gal-index and pNFH. **c** Gal-index and pNFH levels for patients with disease duration below and above 1 year

In our study, we have used as benchmark biomarker pNFH, which has unequivocally been shown by different groups and in multicenter studies to be increased in ALS CSF [7–10]. Here, pNFH levels were also found elevated in our ALS group (4.4-fold) with AUC = 0.777 ($p = 0.011$) using ROC curve analysis. In comparison, the Gal-index was also increased for the same ALS patients (1.2-fold) with AUC = 0.792 ($p = 0.007$). The predictive values from ROC curves were comparable and with potential utility as a diagnostic test for ALS. In view of these results, it will be crucial to investigate Gal-index in larger groups of controls and ALS patients for further validation. On the other hand, in contrast with pNFH, Gal-index did not correlate with the rate of functional decline, indicating that it is not useful as an indicator of disease progression.

Our results on increased levels of monogalactosylated glycans are in contrast with those previously reported for serum IgG *N*-glycans in ALS patients where decreases had been observed [11]. In addition, we identified the structure A2BG2 previously reported to be 2.1-fold significantly increased in human serum IgG [11] and also in mSOD1G93A mouse serum, particularly, at late-stage disease [12] when it has been suggested to enhance antibody-dependent cell-mediated cytotoxicity (ADCC) [11, 12]. In our study, we have found only low levels of A2BG2 in human CSF and although there was a 1.04-fold increase for the ALS patients, the difference was not significant. The difference in IgG source (CSF or serum), the cohorts analyzed, and the very low abundance of the A2BG2 structure in human CSF and serum could be the source of the discrepancies observed. Concerning sialylated glycans, we found a significant negative correlation with the disease duration (below 2 years) in human CSF IgG *N*-glycans, which agreed with the report for serum IgG from the mSOD1G93A mouse model where sialylated glycans were consistently downregulated with increasing days with the disease [12].

IgG glycosylation has been found to be highly relevant in inflammatory and autoimmune diseases. IgG binds the antigens via the Fab region and Fc gamma receptors

(FcγRs) on immune cells via the Fc region. This causes inflammatory effector functions of IgG, including ADCC. Glycosylation at Asn-297 *N*-glycosylation site from Fc affects IgG effector functions. On the other hand, increased levels of IgG sialylation are anti-inflammatory requiring DC-SIGN, STAT6 signaling, and FcγRIIB [30]. In fact, a common therapeutic approach for inflammatory and autoimmune diseases consists of intravenous immunoglobulin (IVIG) application where IgG Fc glycosylation, particularly sialylation, is of relevance for the immunomodulatory activity. Furthermore, increased levels of IgG1 Fc galactosylation were also reported to have anti-inflammatory activity by promoting association between FcγRIIB and dectin-1 [31]. For therapeutic antibodies, afucosylation leads to the enhancement of ADCC via increased affinity for the FcγRIIIA from immune cells, and galactosylation may enhance ADCC [27]. Increased levels of agalactosylated structures are usually associated with inflammatory and autoimmune diseases [26]. For example, in serum IgG, decreased galactosylation and sialylation concomitant to the increased levels of agalactosylated structures was found in rheumatoid arthritis [13, 32]; hypogalactosylation of serum IgG Fc but not Fab regions was detected in antineutrophil cytoplasmic antibodies (ANCA)–associated systemic vasculitis [33]. *N*-Glycosylation of Fab has been less studied but appears to contribute to the modulation of immunity [34].

In neurological diseases, lower levels of galactosylation in blood IgG were observed in Lambert-Eaton myasthenic syndrome and myasthenia gravis [35] and decreased sialylation and galactosylation were detected in serum IgG in CIDP [25]. Concerning CSF IgG, the results are scarce, but significant results have recently appeared in the literature concerning MS, which is a typical autoimmune disease of the central nervous system with frank impairment of blood-brain barrier and IgG production from clonal B cells in the CSF [16, 36]. In MS, decreased galactosylation was observed for CSF IgG1 Fc [16] in agreement with increased agalactosylation for CSF IgG [36]. In addition, IgG1 Fc afucosylation was found to be reduced, and bisecting GlcNAc elevated [16].

Neuroinflammation in the context of neurodegenerative diseases is a particularly complex topic. In comprehensive reviews [37, 38], neuroinflammation in ALS, Alzheimer's and Parkinson's diseases, has been proposed to appear as a consequence of the pathology affecting neurons, whereas in MS, a primary defect in the immune system targeting components of the myelin sheath results in subsequent neurodegeneration. In this context, even if there was clear evidence for the presence of neuroinflammation [39] and indications of autoimmunity associated with ALS [40], the administration of immunosuppressive drugs as well as plasmapheresis or IVIGs did not affect disease progression [40]. This supported the notion that inflammation and autoimmunity are not primary events in ALS. Accordingly, in this work, we have found increased CSF *N*-glycan IgG galactosylation (Gal-index) in ALS compared with the disease-control group in contrast to what is known in MS [16, 36]. Also, in Parkinson's disease, increases of mono- and digalactosylated structures concomitant to a decrease of agalactosylated glycans have recently been found in serum IgG relative to a control group composed of healthy individuals and some patients with mild to chronic diseases other than dementia [41].

To fully understand the relevance of IgG galactosylation in ALS, further studies need to be performed with larger study groups: ALS groups and several control groups including ALS-mimicking disorders as in this study and also other neurodegenerative diseases sorted by inflammatory status as well as healthy individuals. CSF is a very useful source for disease biomarkers since it is in contact with the central nervous system; however, its collection is invasive, which is a drawback concerning the practicality of a potential diagnostic test and also limits the number of samples available for investigation, particularly, for healthy controls. Therefore, considering that CSF IgG in ALS predominantly originates from the blood (see discussion above), a very useful approach will be to screen for the changes in IgG galactosylation associated with ALS in the blood (serum or plasma) of ALS patients in comparison with the controls.

In conclusion, in this study, we presented the *N*-glycosylation profile of IgG from the CSF of ALS patients and showed that levels of galactosylated *N*-glycans (here defined as Gal-index) were higher in ALS patients than in controls with other neurological diseases with a predictive value comparable to the benchmark pNFH and with potential utility as diagnostic test for ALS. Therefore, the results open novel perspectives to further explore Gal-index as a potential ALS biomarker by analyzing IgG from larger cohorts of controls and ALS patients. The Gal-index also is a promising parameter to be investigated in other neurodegenerative diseases.

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Compliance with ethical standards

Patients signed permission for biobank storage, and further studies were agreed by the local Ethic's committee. The research was done in accordance with the Helsinki Declaration as revised in 2013 (www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects).

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