

# Glial Fibrillary Acidic Protein in Cerebrospinal Fluid of Nusinersen-Treated Patients with Spinal Muscular Atrophy

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# Abstract

## Background

Activated astroglia is involved in the pathophysiology of neurodegenerative diseases and has also been described in animal models of spinal muscular atrophy (SMA). Given the urgent need of biomarkers for treatment monitoring of new RNA-modifying and gene replacement therapies in SMA, we examined glial fibrillary acidic protein (GFAP) concentrations as a marker of astrogliosis in the cerebrospinal fluid (CSF) of children and adult patients with SMA before and during treatment with nusinersen.

## Methods

58 adult patients and 21 children with genetically confirmed 5q-associated SMA from 4 German motor neuron disease specialist care centers and 30 age- and sex-matched controls were prospectively included in this study. GFAP concentration was measured in CSF and motor performance and disease severity were assessed.

## Results

CSF GFAP concentrations did not differ from controls but showed higher levels in more severely affected patients after adjustment for patients' age. Within 14 months of nusinersen treatment, CSF GFAP concentrations did not change significantly.

## Conclusions

GFAP concentration in CSF of patients with long-standing SMA is not useful to assess disease severity or predict treatment response, but might support the hypothesis that glial activation is involved in SMA pathology.

## Background

5q-associated spinal muscular atrophy (SMA) is a lower motor neuron disease based on a lack of survival of motor neuron (SMN) protein caused by a loss-of-function mutation of the *Survival of motor neuron 1* gene (*SMN1*) (1). The deficiency of SMN protein primarily leads to death of motor neurons. However, selective depletion of SMN protein within motor neurons of mice only generates a very mild, late-onset SMA-like phenotype (2) which contrasts the severe phenotype resulting from ubiquitously low expression of SMN protein (3). These findings suggest that SMA pathophysiology is more complex and involves non-neuronal tissue (e.g. astrocytes) apart from motor neurons alone (2). Astrocytes are crucial for neuronal homeostasis, trophic support of neurons and synaptic plasticity. Thus, the involvement of astrocytes is widely accepted in other motor neuron diseases like amyotrophic lateral sclerosis (4, 5). Glial fibrillary acidic protein (GFAP) is the principal intermediate filament in mature astrocytes, a key element of their cytoskeleton and increased expression of GFAP is an indication of reactive gliosis, a process which has been shown to be highly related to brain damage and aging (6). Elevated GFAP

concentrations in tissue, serum or cerebrospinal fluid (CSF) already have been reported for several neurodegenerative and neuroinflammatory diseases, stroke and traumatic brain injuries (6–11), and were found to have prognostic as well as predictive value. Astrogliosis, visualized by GFAP staining, has been described in the spinal cord of SMA mice (*SMN $\Delta$ 7*) and patients with SMA, especially located in the gray matter of the ventral horn and astrocytic processes form glial bundles along the ventral roots (3, 12–15). Likewise, recent research has increasingly focused on the involvement of glial cells in the development and maintenance of SMA and on inferring new strategies for therapeutic approaches (16, 17). In SMA models, viral-based restoration of SMN protein levels in astrocytes attenuated disease progression and improved neuromuscular integrity (3). Astrocytes, derived from induced pluripotent stem cells from SMA mice, showed morphological and functional changes as signs of activation and astrocyte-motor neuron co-cultures presented impaired synaptic formation and interaction (18).

The aim of this study was to evaluate GFAP concentration in CSF as biomarker for disease severity and treatment monitoring in patients with SMA and to further investigate the non-neuronal involvement in the pathophysiology of SMA.

## Methods

### Standard Protocol Approvals, Registrations, and Patient Consents

58 adult patients and 21 children with genetically confirmed 5q-associated SMA from 4 German motor neuron disease specialist care centers (Departments of Neurology in Dresden, Ulm, Hannover and Göttingen) and 30 age- and sex-matched controls were prospectively included in this study between 2017 and 2020. The local ethics committees of all participating sites approved the study and all patients signed written informed consent.

The demographic and clinical data of patients were collected including age, sex, disease onset, baseline weight and height, clinical subtype, number of *SMN2* copies if available and ambulatory status.

Patients received nusinersen treatment according to the prescribing information for up to 14 months (Visit 7 = V7). CSF was obtained by lumbar puncture (LP), which was performed for intrathecal administration of nusinersen.

The samples designated for GFAP assay were stored at -80°C within 2 hours after centrifugation (5 min; 800 g; RT). In total, 214 CSF samples were analyzed for GFAP concentration at three time points (V1 = baseline, V5 = 6.2 ± 0.6 months, V7 = 14.2 ± 0.9 months) using ELISA kits (BioVendor, Brno, Czech Republic) at 1:3 dilution according to the instructions of the manufacturer. For quality control, a CSF pool was measured in triplicates per plate additionally to duplicates of the control samples included in the GFAP ELISA kits. The mean intra-assay and inter-assay coefficients of variation were < 15% for both the

kit controls and the CSF pool. One patient was excluded from the analysis since the CSF sample of that patient was insufficient for GFAP determination at baseline.

To monitor motor and functional outcome, established motor scores (Hammersmith Functional Motor Scale Expanded – HFMSE (19), Revised Upper Limb Module – RULM (20), Children’s Hospital of Philadelphia Infant Test of Neuromuscular Disorders - CHOP INTEND (21)) as well as the revised ALS-Functional Rating Scale (ALSFRS-R) (22) were assessed concurrently at each visit. Motor scores comprise several items rating different motor skills with higher scores indicating better function. Ratings were performed according to the respective manuals.

## Statistical analysis

Statistical analysis and data visualization were performed using SPSS Statistics 27 (IBM, Chicago (IL), USA) and GraphPad Prism 5 (GraphPad Software Inc., San Diego (CA), USA). Unless otherwise stated, GFAP data and the assessed scores are presented as median  $\pm$  interquartile range (IQR). GFAP data were not normally distributed as tested by Shapiro-Wilk test ( $p < 0.001$ ). We therefore applied rank-based, non-parametric tests for the baseline analysis. To estimate the comparability of study group and control group, we used Pearson's Chi-squared test for equal distribution regarding sex and Mann-Whitney U test concerning conformity of age. To investigate the association between GFAP values and disease severity, we correlated GFAP baseline values with demographic features and clinical assessments using Spearman's rank correlation coefficient ( $\rho$ ). Due to the significant association with age, we considered it a confounding factor and controlled for baseline age by partial correlation. A correlation coefficient of  $\rho < 0.3$  was considered as a weak,  $\rho = 0.3 - 0.59$  as a moderate, and  $\rho > 0.6$  as a strong correlation (modified from (23)). We used Mann-Whitney U test or one-way analysis of covariance (ANCOVA) with post-hoc Bonferroni adjustment for comparison of GFAP (dependent variable) between different patient subgroups considering age as covariate. To meet the assumptions of ANCOVA, we applied square root transformation to GFAP data. For longitudinal analysis under nusinersen treatment, we performed Wilcoxon signed-rank test to include all available data ( $N = 58$ ) for the comparison between baseline (V1) and 14-month follow-up (V7, representing third maintenance dose). Data sets with missing values were excluded pairwise for cross-sectional and longitudinal analysis. Statistical significance threshold was set to  $< 0.05$ .

## Results

58 adult patients and 21 children with SMA type 1 ( $N = 7$ ), type 2 ( $N = 33$ ) or type 3 ( $N = 39$ ) were included in the analysis. Median age was 31 years (IQR 17 – 43), 52% were female. The control group was age- and sex-matched and comprised 23 adults and 7 children without neurodegenerative or neuroinflammatory disease (healthy controls:  $N = 23$ , normal pressure hydrocephalus:  $N = 3$ , idiopathic Bell’s palsy:  $N = 4$ ). In the control group, median age was 30 years (IQR 17 - 44), 60% were female. The distribution of sex or age did not differ significantly between the groups. Details of study group characteristics and study profile are presented in Table 1 and Figure 1.

Table 1  
Study group characteristics

		<b>SMA</b>	<b>Controls</b>
		<b>(N = 79)</b>	<b>(N = 30)</b>
Age [yr],		31	30
median (IQR)		(17 - 43)	(17 - 44)
Age of onset [yr],		1	
median (IQR)		(0 - 3)	
Disease duration [yr],		28	
median (IQR)		(15 - 37)	
Sex,	female	41 (52)	18 (60)
N (%)	male	38 (48)	12 (40)
SMA type,	1	7 (9)	
N (%)	2	33 (42)	
	3	39 (49)	
<i>SMN2</i> copy number,	2	9 (16)	
N (%)	3	31 (53)	
	4+	18 (31)	
	unknown	21	
Weight [kg],		50	
median (IQR)		(33 - 65)	
Height [cm],		158	
median (IQR)		(145 - 170)	
BMI [kg/m <sup>2</sup> ],		20.5	
median (IQR)		(16.1 - 23.4)	
Scoliosis,	present	50 (63)	
N (%)	not present	29 (37)	
Spondylodesis,	present	24 (30)	
N (%)	not present	55 (70)	

IQR, interquartile range; BMI, body mass index; *SMN2*, *Survival of motor neuron 2* gene

		SMA (N = 79)	Controls (N = 30)
Wheelchair-use, N (%)	never	9 (11)	
	occasionally	6 (8)	
	permanently	64 (81)	
Mobility, N (%)	never able to walk	40 (51)	
	lost ability to walk	24 (30)	
	still able to walk	15 (19)	
IQR, interquartile range; BMI, body mass index; <i>SMN2</i> , <i>Survival of motor neuron 2</i> gene			

### CSF GFAP concentrations in treatment-naïve patients did not differ from age- and sex-matched controls, but indicated disease severity

GFAP concentrations in the CSF of treatment-naïve SMA patients did not differ from the control group ( $F(1, 105) = 0.024$ ,  $p = 0.877$ ; Figure 2A, Table 2). GFAP concentrations correlated with patients' ( $\rho = 0.405$ ,  $p < 0.001$ ,  $N = 79$ ; Figure 2B) and controls' age ( $\rho = 0.544$ ,  $p = 0.002$ ,  $N = 30$ ; Figure 2B), but were not associated with age of disease onset, height, weight or *SMN2* copy number (see Table 3). Higher GFAP levels were associated with lower motor function (Table 4) in treatment-naïve patients. Furthermore, GFAP concentrations did not correlate with Chitotriosidase 1 concentrations which was measured within the same cohort at the same time ( $\rho = 0.081$ ,  $p = 0.483$ ,  $N = 79$ ; calculated by partial rank correlation controlling for patients' age and height; Figure S1 displays scatter dot plot of raw data) (24). GFAP concentrations were higher in patients with SMA type 2 compared to type 3 ( $F(2, 74) = 3.673$ ,  $p < 0.05$ , partial  $\eta^2 = 0.090$ ) after adjustment for patients' age, but did not differ between SMA type 1 vs. 2 or type 1 vs. 3 or compared to controls or regarding *SMN2* copy number ( $F(4, 51) = 0.333$ ,  $p = 0.855$ ). Moreover, patients who were able to walk had higher GFAP concentrations than patients who were not ( $F(1, 75) = 4.813$ ,  $p < 0.05$ , partial  $\eta^2 = 0.060$ ).

Table 2  
CSF GFAP levels in treatment-naïve patients with SMA and controls

	<b>SMA</b> <b>(N = 79)</b>	<b>Controls</b> <b>(N = 30)</b>
CSF GFAP [ng/mL], median	0.743	0.578
(IQR)	(0.479 – 0.959)	(0.512 – 0.887)
range	0.091 – 2.295	0.305 – 2.843
GFAP, Glial fibrillary acidic protein concentration; CSF, cerebrospinal fluid; IQR, interquartile range		

Table 3

Correlation between CSF GFAP concentration and characteristics of treatment-naïve patients with SMA

<b>Spearman rho</b>	<b>CSF GFAP [ng/ml]</b>
Age [yr]	rho = 0.405 <b>p &lt; 0.001</b> N = 79
Partial correlation controlled for age	
Height [cm]	rho = 0.050 p = 0.663 N = 79
Weight [kg]	rho = 0.070 p = 0.543 N = 79
<i>SMN2</i> copy number	rho = -0.080 p = 0.554 N = 58
Disease onset [yr]	rho = -0.216 p = 0.061 N = 77
Disease duration [yr]	rho = 0.173 p = 0.136 N = 77
GFAP, Glial fibrillary acidic protein concentration; CSF, cerebrospinal fluid; rho, (partial) rank correlation coefficient	

Table 4

Correlation between CSF GFAP concentration and disease severity scores in treatment-naïve patients with SMA

Controlled for age	CSF GFAP [ng/ml]
HFMSE	rho = -0.381 p = <b>0.002</b> N = 63
RULM	rho = -0.294 p = <b>0.019</b> N = 64
ALSFRS-R	rho = -0.330 p = <b>0.007</b> N = 66
CHOP INTEND	rho = -0.193 p = 0.619 N = 10
GFAP, Glial fibrillary acidic protein concentration; CSF, cerebrospinal fluid; HFMSE, Hammersmith Functional Motor Scale Expanded; RULM, Revised Upper Limb Module; ALSFRS-R, revised ALS Functional Rating Scale; CHOP INTEND, Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders; rho, partial rank correlation coefficient	

## CSF GFAP concentrations decreased in SMA patients with motor improvement following nusinersen treatment

After 14 months of nusinersen treatment, GFAP concentrations did not differ significantly from baseline levels ( $p = 0.158$ ; Figure 3; raw data displayed in Figure S2). However, lower GFAP concentrations were observed in two thirds of individuals and the decrease was significant in patients with motor improvement as indicated by increased HFMSE scores after 14 months of nusinersen treatment (median change -11.6%,  $p < 0.05$ ; Table 5). For responders and non-responders, however, the median change of GFAP concentrations within the observation period was similar ( $U = 50$ ,  $z = -0.758$ ,  $p = 0.476$ ). In order to compare our results with those of Olsson et al.(25), we screened our study cohort for patients who met the inclusion criteria of Olsson et al.. We identified two children with SMA type 1 carrying 2 *SMN2* copies who were treated within their first year of life (#57, #74). Similar to Olsson et al., GFAP concentrations declined during nusinersen treatment. In fact, GFAP concentrations decreased by 70% in these two type 1 patients with recent onset and notably, CHOP INTEND scores improved coincidentally (Figure S3).

**Table 5** Dynamics in CSF GFAP concentration during 14 months of nusinersen treatment

	14-month analysis				p value
	N	median (IQR)	difference versus baseline		
			median (IQR)	(%)	
<b>CSF GFAP</b> [ng/mL]	58	0.5838 (0.4144 – 0.8982)	-0.0488 (-0.1199 – 0.1049)	-7.4	0.158
<b>HFMSE increase</b>	21	0.4725 (0.4104 – 0.7337)	-0.0700 (-0.2275 – -0.0017)	-11.6	<b>0.018</b>
<b>HFMSE decrease</b>	6	0.8858 (0.7337 – 1.2268)	-0.0371 (-0.2468 – 0.5370)	-4.0	0.753

GFAP, Glial fibrillary acidic protein concentration; CSF, cerebrospinal fluid; HFMSE, Hammersmith Functional Motor Scale Expanded; IQR, interquartile range; p value calculated by Wilcoxon signed-rank test

## Discussion

After initially assuming that the SMA phenotype was based on a motor neuron-specific pathology, recent research elucidated a more systemic disorder (16, 26). Astrocytes play a dual role in this non-neuronal involvement. As in many other neurodegenerative diseases, reactive astrogliosis was reported in the spinal cord of SMA type 2 (15) and 3 (14) patients including glial bundles in anterior roots (12) (for review (6, 16)). Additionally, astrocytes are crucial in the non-cell-autonomous pathophysiology of motor neuron diseases. For SMA, Rindt et al. revealed the importance of astrocytes in SMA pathology since they observed improved life span and motor function after restoration of SMN protein levels specifically in astrocytes (3). Interestingly, when restoring SMN protein levels in motor neurons only, improvements were only minor (27, 28), underlining the importance of the non-cell-autonomous effects. Thus, astrocytes might not only be activated secondarily to form a reactive gliosis, e.g. as a consequence of motor neuron degeneration or triggered by activated microglia, but also might be induced intrinsically by the SMN protein deficiency itself. Since, on the one hand, nusinersen treatment prevents motor neuron degeneration and consequently formation of astrogliosis and on the other hand, nusinersen treatment might restore SMN protein levels also directly in non-neuronal tissue such as astrocytes, one could postulate that GFAP concentrations are elevated in the CSF of SMA patients and decrease in response to nusinersen treatment, proposing GFAP as a candidate biomarker in SMA.

In our study cohort, GFAP concentrations in SMA patients did not differ significantly from age- and sex-matched controls before the start of treatment. In contrast, Olsson et al. reported higher GFAP

concentrations in children with SMA type 1 and 2 copies of *SMN2* gene (aged 0.5 – 4 months; controlled for age and sex) (25). SMA type 1 is characterized by a fast disease progression and a rapid destruction of motor neurons. Our cohort mainly comprised SMA type 2 and 3, who might be characterized by a less extended astroglial activation and subsequently lower GFAP concentrations due to milder disease activity. Hence, the comparability of the two studies is limited.

Higher GFAP concentration was associated with more impaired motor function and patients with SMA type 2 and non-ambulatory patients had a higher GFAP concentration than patients with SMA type 3 and patients who were still able to walk, respectively. This still supports the hypothesis of astrocyte involvement in SMA pathogenesis. However, despite the correlation of GFAP concentration with disease severity, its limited applicability to distinguish SMA samples from control samples does not advocate for a diagnostic or prognostic use, at least in adult patients with SMA.

Olsson et al. reported decreasing GFAP concentrations during nusinersen treatment in severely affected children with SMA and short treatment delay (25). Fitting to this observation, two of our patients with similar characteristics presented a remarkable decrease associated with motoric improvement. Overall however, GFAP concentrations did not significantly change during nusinersen treatment in our cohort, which could be due to the diverse composition of our cohort and the very small proportion of patients with early disease onset and short treatment delay compared to Olsson et al.. Still, two thirds of all patients showed a decline and the median GFAP concentration after 14 months approximated the median GFAP concentration of the control group. In addition, patients with improving HFMSE scores during treatment presented a significant reduction of GFAP concentrations. We therefore hypothesize that there might be an attenuation of astrogliosis during nusinersen treatment also in our cohort.

Our study has some limitations. Statistical analysis could be compromised by the small proportion of patients with SMA type 1 compared to type 2 and 3 within our study cohort. Further studies are needed to investigate the role of astroglia in the pathophysiology of SMA and the suitability of GFAP concentrations in CSF as a marker of astroglial activation.

## Conclusion

GFAP concentration in CSF is not useful for assessing disease severity or predicting treatment response in patients with long-standing SMA, but might support the hypothesis that glial activation is involved in SMA pathology and may be modulated by nusinersen treatment.

## Declarations

**Ethics approval and consent to participate** The local ethics committees of all participating sites approved the study and all patients and controls signed written informed consent.

**Consent for publication** Not applicable.

**Availability of data and materials** The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Competing interests** MF reports non-financial support from Biogen outside the submitted work.

PS reports no disclosures.

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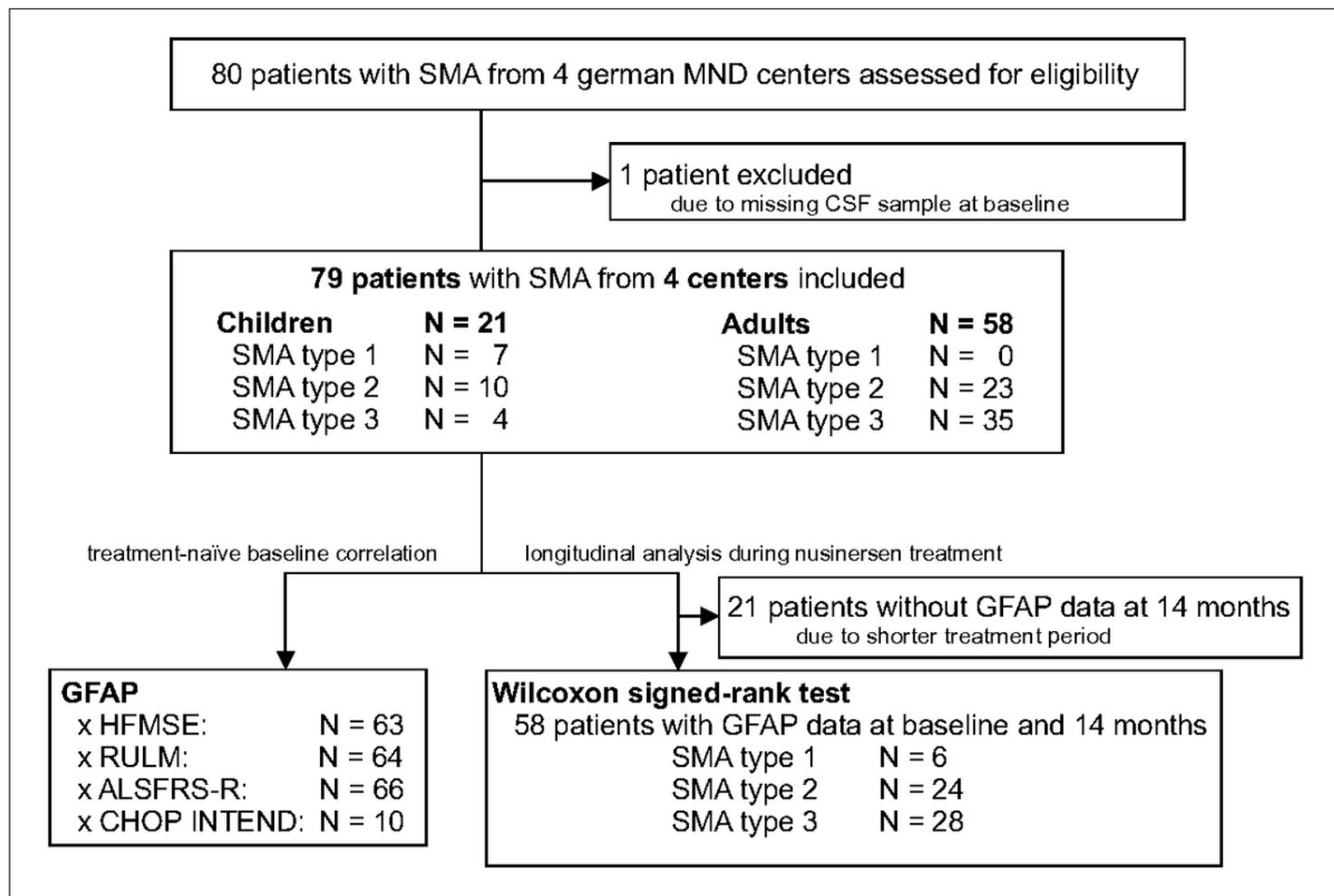
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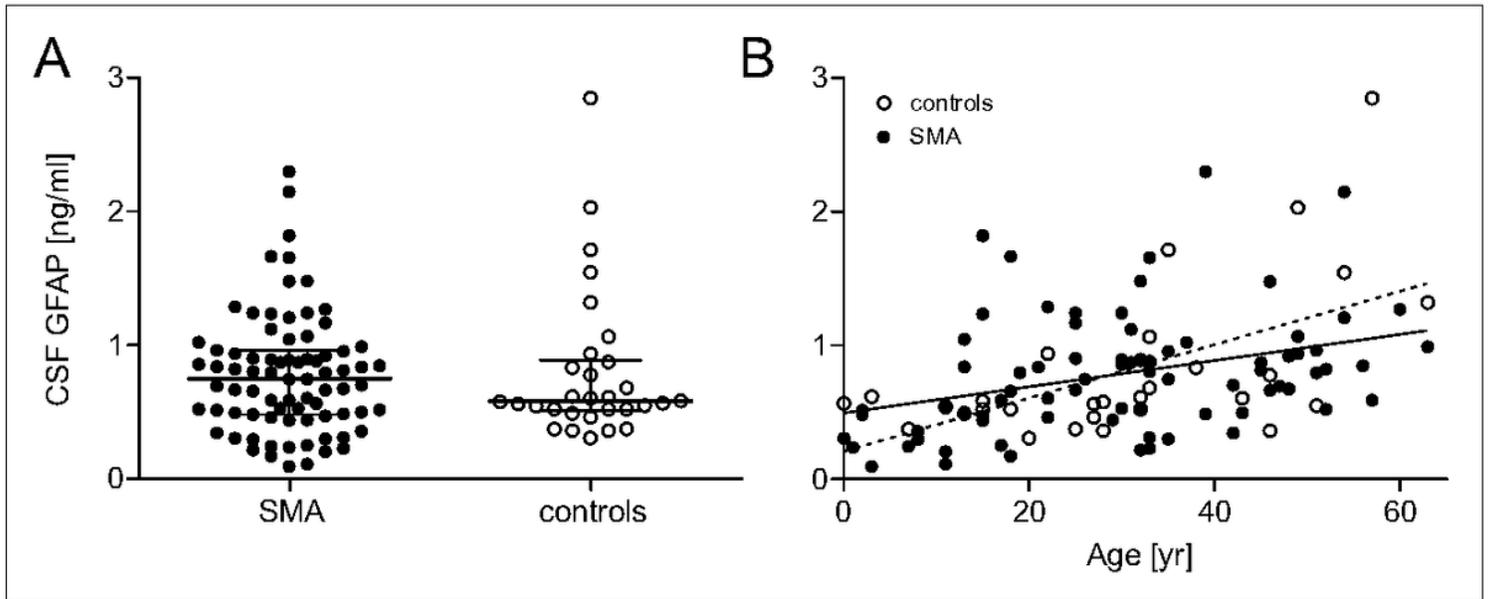
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## Figures



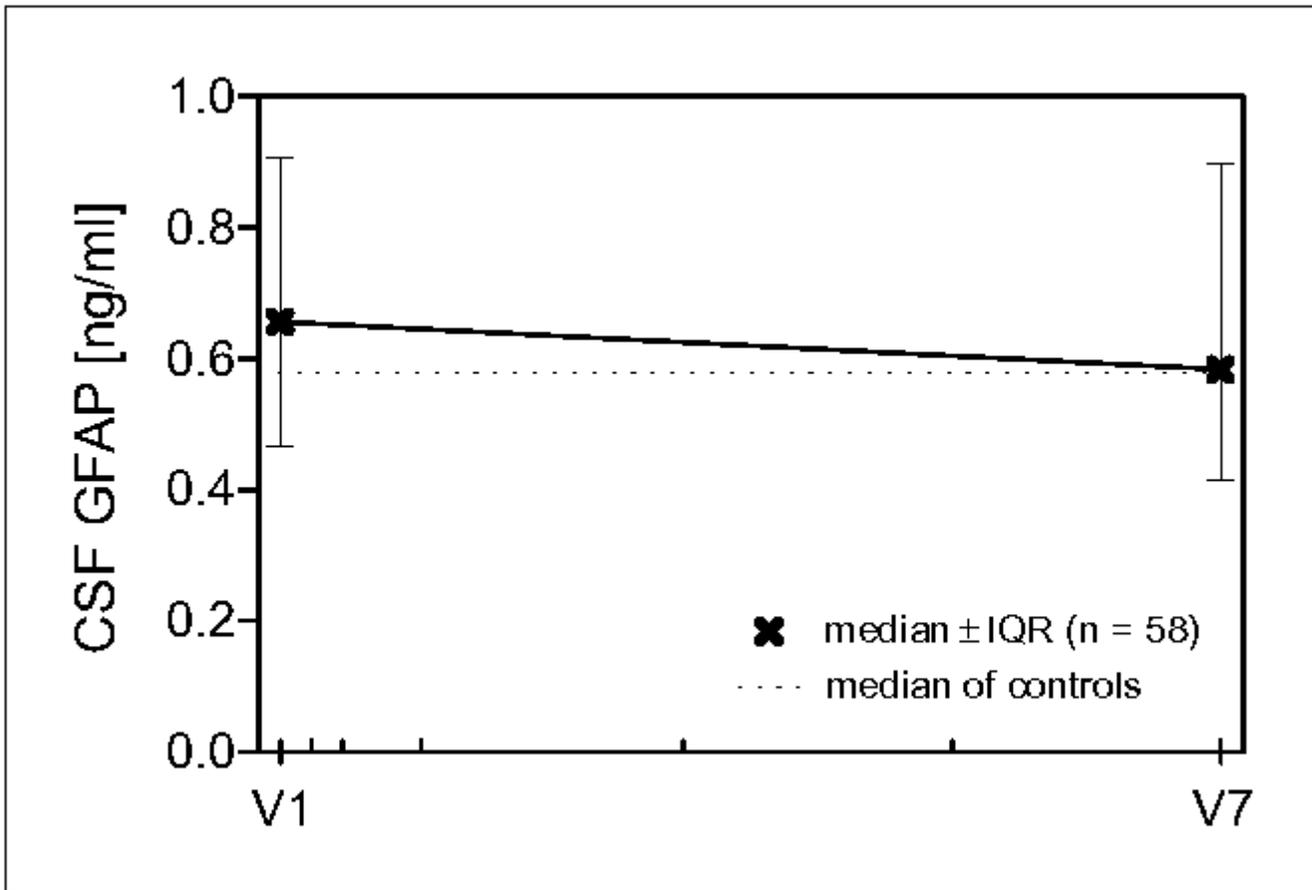
**Figure 1**

Study profile. GFAP, Glial fibrillary acidic protein concentration; CSF, cerebrospinal fluid; HFMSE, Hammersmith Functional Motor Scale Expanded; RULM, Revised Upper Limb Module; ALSFRS-R, revised ALS Functional Rating Scale; CHOP INTEND, Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders



**Figure 2**

CSF GFAP concentrations before treatment initiation. (A) Baseline CSF GFAP concentrations comparing diseased individuals (closed circles; N = 79) to controls (open circles; N = 30). Horizontal line shows median, whiskers illustrate interquartile range (0.25 - 0.75), each icon represents an individual patient. (b) Correlation between age and CSF GFAP concentration before treatment initiation; each icon represents an individual person; closed circles illustrate patients with SMA (N = 79), open circles display controls (N = 30). Solid line shows regression line of patients with SMA, dashed line shows regression line of controls. CSF, cerebrospinal fluid; GFAP, glial fibrillary acidic protein



**Figure 3**

Median change of CSF GFAP concentrations during nusinersen treatment. Median change of CSF GFAP concentrations from baseline (V1) to 14 months (V7), x marks median, whiskers illustrate interquartile range (0.25 – 0.75), dotted line indicates median GFAP concentration of control individuals. Each upward tick on the x-axis indicates the time of nusinersen administration. N = 58 CSF, cerebrospinal fluid; GFAP, glial fibrillary acidic protein

## Supplementary Files

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