Cerebrospinal fluid proteomic profiling in nusinersen-treated patients with spinal muscular atrophy

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Abstract
Promising results from recent clinical trials on the approved antisense oligonucleotide nusinersen in pediatric patients with 5q-linked spinal muscular atrophy (SMA) still have to be confirmed in adult patients but are hindered by a lack of sensitive biomarkers that indicate an early therapeutic response. Changes in the overall neurochemical composition of cerebrospinal fluid (CSF) under therapy may yield additive diagnostic and predictive information. With this prospective proof-of-concept and feasibility study, we evaluated non-targeted CSF proteomic profiles by mass spectrometry along with basic CSF parameters of 10 adult patients with SMA types 2 or 3 before and after 10 months of nusinersen therapy, in comparison with 10 age- and gender-matched controls. These data were analyzed by bioinformatics and correlated with clinical outcomes assessed by the Hammersmith Functional Rating Scale Expanded (HFMSE). CSF proteomic profiles of SMA patients differed from controls. Two groups of SMA patients were identified based on unsupervised clustering. These groups differed in age and expression of proteins related to neurodegeneration and neuroregeneration. Intraindividual CSF changes and may therefore be suitable for diagnostic and predictive analyses.

KEYWORDS
antisense oligonucleotide, cerebrospinal fluid, mass spectrometry, nusinersen, proteomics, spinal muscular atrophy

Abbreviations: ABC, ammonium bicarbonate; ACN, acetonitrile; ALSFRS-R, amyotrophic lateral sclerosis functional rating scale–revised; APC, absolute protein cluster; ASO, antisense oligonucleotide; CDH18, cadherin 18; COL6A1, collagen type VI alpha 1; CPE, carboxypeptidase E; CSF, cerebrospinal fluid; DDA, data-dependent acquisition; DPC, differential protein changes and may therefore be suitable for diagnostic and predictive analyses.

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1 | INTRODUCTION

Spinal muscular atrophy (SMA) is an autosomal-recessive neuromuscular disease characterized by degeneration of alpha motor neurons and progressive muscle wasting thus leading to a continuous loss of movement ability. The underlying genetic causes are homozygous deletions or loss-of-function mutations in the survival motor neuron (SMN) 1 gene with retained function of at least one copy of the paralogous gene SMN2, both located on chromosome 5q13 (Lunn & Wang, 2008). The SMN protein is ubiquitously expressed, whereas motor neurons are most vulnerable to its deficiency (Singh, Howell, Ottesen, & Singh, 2017). Classically, four different clinical phenotypes of SMA are distinguished (SMA 1–4) based on the age at onset and achieved motor milestones: type 1 (“non-sitters”), type 2 (“sitters”), type 3 (“walkers” with onset of first symptoms before (3a) or after (3b) the age of 3 years), and type 4 (adult onset); and an additional type 0 for the severest form with a neonatal onset (Lunn & Wang, 2008).

Recently, the orphan drug antisense oligonucleotide (ASO) nusinersen was clinically approved by the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) as the first disease-modifying treatment for all types of 5q-linked SMA. Nusinersen consists of 18 nucleotides with high specificity for the intron downstream of exon 7 in the SMN2 pre-mRNA, a region that is normally occupied by heterogeneous nuclear ribonucleoproteins A1/A2 and referred to as intron splicing silencer N1, thus promoting the inclusion of exon 7 in the SMN2 mRNA transcript. This splice modification of the SMN2 pre-mRNA increases the amount of full-length SMN protein and thus compensates for the genetic defect in the SMN1 gene (Bennett, Baker, Pham, Swayne, & Geary, 2017; Wurster & Ludolph, 2018). Because of its inability to cross the blood–brain barrier, nusinersen requires intrathecal administration (Stolte, Totzeck, & Kizina, 2018; Wurster, Winter, et al., 2019b). This treatment has shown encouraging results in children with infantile-onset (SMA 1) and later-onset (SMA 2) SMA (Darras, Chiriboga, et al., 2019a; Finkel et al., 2017; Mercuri, Darras, & Chiriboga, 2018; Michelson et al., 2018; Pane et al., 2019), but efficacy data for adult patients are pending, while current treatment costs are high (Editorial, The Lancet Neurology, 2017).

As plasma phosphorylated neurofilament heavy chain (pNfH) is emerging as a potential marker of disease activity and treatment response in infants with SMA 1 (Darras, Crawford, et al., 2019b), biomarkers indicating a potential early therapeutic response in adolescent and adult patients are lacking to date. For biomarker screenings in SMA, cerebrospinal fluid (CSF) is a promising bioanalyte because (1) it is in direct contact with the diseased spinal cord and lower brain stem and (2) nusinersen is directly administered to the CSF compartment. In addition, identifying nusinersen-dependent deregulations of the CSF molecular composition might give rise to novel insights into underlying disease mechanisms. Routine CSF analyses would also disclose potential treatment-related changes in basic CSF parameters which might be used as safety readout.

In this non-targeted proof-of-concept and feasibility study, we used high accuracy mass spectrometry (MS)-based CSF proteomic analysis (Bereman, Beri, Enders, & Nash, 2018; Coscia et al., 2018; Zhang et al., 2015) in a restricted number of adult patients with later-onset SMA (SMA 2 and 3). We (i) evaluated SMA-specific CSF proteomes in comparison with healthy controls, (ii) screened for candidate proteins specifically deregulated in response to the first 10 months of nusinersen treatment in correlation with changes in motor function, and (iii) analyzed basic CSF parameters before and during therapy in comparison with healthy controls.

2 | METHODS

2.1 | Standard protocol approvals, registration, and patient consents

This prospective exploratory monocentric case–control study was approved by the institutional ethics board (University of Heidelberg; S-554/2018), and all participants gave written informed consent. The study conforms with The Code of Ethics (Declaration of Helsinki; World Medical Association 2013).

2.2 | Study design

2.2.1 | Participants and sampling

A total of 10 non-pediatric patients with genetically confirmed 5q-linked SMA 2 or 3 were enrolled at the Department of Neurology of Heidelberg University Hospital from December 2017 to April 2018. The first 10 patients who were started on treatment with nusinersen at our department were recruited to the study. Patient characteristics are given in Table 1. The study was not pre-registered. SMA 2 or 3 was clinically classified based on the age at symptom onset and achievement of motor milestones (Lunn & Wang, 2008). Patients with SMA 1 or 4 were not available at our department during the accrual period. Each clinical indication to start treatment on nusinersen was provided by neurologists with longstanding experience in neuromuscular diseases. Exclusion criteria were age < 18, and pregnancy. No randomization, blinding, or sample calculation was performed in this study. A detailed medical history was taken in all patients, and comprehensive neurologic examinations were performed including assessments for the Amyotrophic Lateral Sclerosis Functional Rating Scale–Revised (ALSFRS-R) score (Abdulla et al., 2013).

Severity of clinical symptoms was assessed by the Hammersmith Functional Motor Scale–Expanded (HFMSE), a rating scale for the evaluation of gross motor functions validated for SMA 2 and 3 patients (Glanzman et al., 2011; O’Hagen, Glanzman, & McDermott,
Clinical and genetic characteristics of SMA patients

| Patient No. | SMA type | Age [years] | Gender [m/f] | Duration of symptoms [years] | SMN1 mutation | SMN2 copies [n] | ALSFRS-R score [0–48] T0 | HFMSE score [0–66] T0 | HFMSE score [0–66] T10 | HFMSE score ΔT0–T10 |
|-------------|----------|-------------|--------------|------------------------------|---------------|------------------|----------------|----------------|----------------|----------------|----------------|
| 1           | 3b       | 18.5        | m            | 6                            | Het. Δ7/8, c.283G > C | 2               | 38             | 44             | 47             | 3              |
| 2           | 3a       | 24.2        | f            | 20                           | Het. Δ7/8, c.3’ + 6T>G | 2               | 37             | 36             | 38             | 2              |
| 3           | 3b       | 27.3        | m            | 19                           | Homozygous Δ7/8   | 4               | 38             | 26             | 35             | 9              |
| 4           | 3b       | 38.8        | m            | 23                           | Homozygous Δ7/8   | 3               | 41             | 41             | 48             | 7              |
| 5           | 3a       | 33.8        | f            | 23                           | Homozygous Δ7/8   | 2               | 30             | 10             | 10             | 0              |
| 6           | 3a       | 48.5        | f            | 46                           | Homozygous Δ7/8   | 4               | 29             | 15             | 27             | 12             |
| 7           | 3b       | 50.0        | m            | 34                           | Homozygous Δ7/8   | 4               | 41             | 56             | 56             | 0              |
| 8           | 3b       | 50.7        | m            | 46                           | Homozygous Δ7/8   | 4               | 28             | 7              | 7              | 0              |
| 9           | 3b       | 46.3        | f            | 37                           | Het. Δ7/8, c.821C > T | 3               | 33             | 6              | 6              | 0              |
| 10          | 2        | 29.8        | f            | 29                           | Homozygous Δ7/8   | 4               | 25             | 0              | 0              | 0              |

Mean N/A 36.8 N/A 28.3 N/A 3.2 34.0 24.1 27.4 3.3

SEM N/A 3.7 N/A 4.0 N/A 0.3 1.8 6.1 6.4 1.4

Note: All results are presented as mean values ± SEM.
Abbreviations: ALSFRS-R, Amyotrophic Lateral Sclerosis Functional Rating Scale–Revised; Δ7/8, deletion of exons 7/8; f, female; HFMSE, Hammersmith Functional Motor Scale–Expanded for SMA; Het., heterozygous; m, male; N/A, not applicable; SEM, standard error of the mean; SMA, 5q-linked spinal muscular atrophy; SMN1, survival motor neuron gene 1; SMN2, survival motor neuron gene 2; T0, first nusinersen injection at baseline; T10, sixth nusinersen injection after 10 months.

2.3 | Routine CSF analysis

CSF was collected in polypropylene tubes (Falcon 15 ml Conical Centrifuge Tubes; Thermo Fisher Scientific, catalogue number: 14–959-53A), and was immediately analyzed for routine CSF parameters between 8 a.m. and 5 p.m., that is, cell count, cytology, glucose, lactate, total protein, CSF-serum quotients of albumin (Q Alb), immunoglobulin (Ig)G (Q IgG), IgA (Q IgA), and IgM (Q IgM), and oligoclonal IgG bands, within 1 hr after lumbar puncture (Jesse et al., 2011). Briefly, cells in the CSF were manually counted using a Fuchs-Rosenthal counting chamber (Brand, Wertheim, Germany, catalogue number: 719805) and a CSF cell count of > 4 cells/μL was considered as elevated. Five minutes pre-centrifugation at 700 g of 5–10 ml CSF in a Hettich Rotina 35 (Hettich, Tuttinglen, Germany, catalogue number 17005) was performed to enrich the cells. For mass spectrometry proteomic analysis, cell-free supernatant was decanted carefully to avoid contamination by proteins from CSF cells, frozen and stored at −80°C until analysis. For cytology, cell sediment was resuspended in 0.2 ml of cell culture medium and cytospins were prepared in a Shandon Cytospin 3 (Thermo Shandon Limited, Cheshire, UK, catalogue number: 74000201) at 72 g for 10 min. After air drying, CSF specimens were stained using a panoptic Pappenheim stain by performing
May-Grünwald (Merck, Darmstadt, Germany, catalogue number: 1014240500) followed by Giemsa (Merck, Darmstadt, Germany, catalogue number: 1014240500) staining. Quantitative assessment of leukocyte subpopulations was performed by an expert with longstanding experience in CSF cytology (B.W.). A total of 20 CSF/serum pairs derived from 10 SMA patients at T₀ and T₁₀, and controls. CSF, cerebrospinal fluid; MS, mass spectrometry; n.s., not significant; PCA, principal component analysis; T₀, first nusinersen injection at baseline; T₁₀, sixth nusinersen injection after 10 months

2.4 | Mass spectrometry CSF proteomic analysis

2.4.1 | Preparation of CSF specimens

An appropriate volume of CSF from patients or controls at each time point containing 100 µg of protein was processed for proteomic analysis. The volumes of all CSF specimens in a single experiment were equalized by adding 100 mM ammonium bicarbonate (Sigma-Aldrich, München, Germany, catalogue number: 09830-500G). For protein reduction, dithiotreitol (Sigma-Aldrich, catalogue number: D9779-1G) was added to a final concentration of 1.4 mM, and samples were incubated at 600 rounds per minute (rpm) and 45°C for 30 min on a ThermoMixer (Eppendorf, Hamburg, Germany, catalogue number: 5382000015). Cysteine residues were alkylated by adding iodoacetamide (Sigma-Aldrich, catalogue number: I1149-5G) to a final concentration of 2.75 mM and incubating the samples in the dark at 600 rpm and 25°C for 30 min. Thereafter, protein was precipitated to remove the solvent as described (Wessel & Flugge, 1984). After precipitation, pellets were resuspended in 142 µl ammonium bicarbonate and incubated at 600 rpm and 25°C for 60 min. Trypsin (Promega, Madison, WI, USA, catalogue number: V5280) was added at a ratio of 1:25 to protein concentration and samples were incubated at 600 rpm and 37°C overnight. Enzymatic activity was stopped by adding 7.5 µl 20% trifluoroacetic acid (Sigma-Aldrich, catalogue number: 302031-100Ml). Samples were centrifuged at 16,200 g for 2 min and the peptides-containing supernatant was kept and stored at −20°C until MS analysis. Peptide concentration was measured using the Pierce™ Quantitative Colorimetric Peptide Assay (Life Technologies GmbH, Darmstadt, Germany, catalogue number: 23275). Four µg of peptides was diluted to 20 µl with 0.1% trifluoroacetic acid and 500 ng was loaded on a cartridge trap column, packed with Acclaim PepMap300 C18, 5 µm, and 300 Å wide pore (Thermo Scientific, Waltham, USA, catalogue number: 069690). Peptides were subsequently eluted over a 120 min gradient ranging from 2% to 95% acetonitrile (Fisher Scientific GmbH, Schwerte, Germany, catalogue number: A955-1) applying a flow rate of 300 nI/min on a nanoEase MZ Peptide analytical column. Peptides were analyzed on a QExactive-HF-X mass spectrometer (Thermo Scientific, Waltham, USA, catalogue number: 0726042) using a data-dependent acquisition strategy. Precursor scans were acquired at a resolution of 120,000 followed by fragment scans of the 15 most abundant ions at a resolution of 30,000. Acquired fragment ions were dynamically excluded for 60 s.

2.5 | Data processing

Raw data files from mass spectrometer were analyzed with MaxQuant (version 1.6.6.0, RRID:SCR_014485) using standard conditions. Label-free quantification intensity values from MaxQuant analysis were used for further data preparation with Perseus (version 1.6.2.2, RRID:SCR_015753). Label-free quantification intensity value
matrix was filtered for possible contaminants and log transformed. Missing values were imputed with a normal distribution with a downshift accounting for likely low abundance missing values. The resulting matrix was normalized, and a z-score calculated. MS proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org, RRID:SCR_004055) via the PRIDE partner repository (Vizcaino, Cote, & Csordas, 2013, RRID:SCR_003411) with the dataset identifier PXD016757.

2.5.1 | Differential protein analysis

Resulting matrix files were further analyzed with R (www.r-project.org, version 3.6.0, RRID:SCR_001905) using customized scripts. For differential expression the r-based package “limma” (version 3.40.2) was used and a p-value < 0.05 adjusted with the Benjamini-Hochberg procedure for multiple testing was considered significant. Proteins with a significant p-value and a log₂ fold-change > 1 or < -1 were used in the Ingenuity Pathway Analysis (IPA, QIAGEN Inc., RRID:SCR_008653).

2.5.2 | Dimensionality reduction, clustering, and correlation network analysis

Principle component analysis (PCA) is a common method for linear dimensionality reduction that can visualize high-dimensional data in a two-dimensional diagram using principle components (Jolliffe & Cadima, 2016). We implanted PCA in R with the function “prcomp”, and visualization was done with the package “ggplot2” (version 3.2.1). Unsupervised clustering was performed using the package “ConsensusClusterPlus” (version 1.48.0) in R. K-means algorithms try to find different subgroups where the data points of the members have a high similarity. K-means distance clustering was considered most suitable for the present dataset and clusters ranging from k = 2 to k = 4 were evaluated. A weighted protein correlation network analysis (WPCNA) can identify clusters of proteins that show high-intensity correlation and therefore similar concurrent regulation (Horvath & Dong, 2008). Subsequent association with clinical variables identifies correlations between protein clusters and variables. We implemented the WPCNA with the R package “WGCNA” (version 1.68) with minor adaptations for the use in protein analysis. Soft threshold was automatically picked, and the network type was defined as signed hybrid. Associations with cluster assignments were calculated using the package “globaltest” (version 5.38.0). All R scripts are customized implementations of above-mentioned packages, and the source code can be made available upon reasonable request.

2.6 | Statistical analysis

Statistical analysis was performed in R (version 3.6.0, RRID:SCR_001905) and Microsoft Excel 2016 (Microsoft Corporation, RRID:SCR_016137). Normal distributions of age, HFMSE score, and CSF parameters were assessed with the Shapiro-Wilk test before parametric testing. For comparisons between different groups of samples the two-sided unpaired Student’s t-test was used, whereas for comparisons between T₀ and T₅₀ samples the two-sided paired Student’s t-test was applied. No outliers have been excluded. All results are reported as mean values ± standard error of the mean (SEM) or median and interquartile range (IQR), and ranges as appropriate. Statistical tests were all two tailed and the level of significance was defined at p < .05.

3 | RESULTS

3.1 | Characteristics of SMA patients and controls

Of the 10 SMA patients included in this study, 1 patient was classified as SMA 2 and 9 as SMA 3 (3 patients with 3a and 6 with 3b). Mean age was 36.8 ± 3.7 years (range: 18–50 years; 5 female, 5 male patients). Mean duration of clinical symptoms at study entry was 28.3 ± 4.0 years. The homozygous deletion of exons 7 and 8 of the SMN1 gene was found in 7 of 10 patients (70%). Three patients (30%) were diagnosed with a compound heterozygous mutation of the SMN1 gene (1 patient with SMA 3a (c.‘3 + 6T>G) (Wirth et al., 1999) and 2 patients with SMA 3b (c.283G > C; c.821C > T)). At study entry, the mean ALSFRS-R score was 34.0 ± 1.8, and the mean HFMSE score was 24.1 ± 6.1. After achievement of CSF maintenance concentrations after 10 months of nusinersen treatment at T₁₀ (i.e., after five completed injections), 5 of 10 patients showed an increase in the HFMSE score, whereas the other patients remained clinically stable. Of the five patients with unaltered HFMSE scores during treatment, four patients had high disease severity (Table 1). Overall changes in the HFMSE score were significantly different in SMA patients when compared with baseline at T₀ (p = .04). Nota bene, this study was not intended to evaluate the efficacy of nusinersen in these patients. Patients’ clinical and genetic characteristics are summarized in detail in Table 1. Mean age of the 10 selected age- and gender-matched controls was 35.7 ± 3.3 years (range: 18–48 years; 5 female, 5 male patients). There was no difference in age between SMA patients and controls (p = .84).

3.2 | Basic CSF parameters in SMA patients and controls in response to nusinersen treatment

Detailed CSF characteristics of SMA patients and controls are given in Table 2. Briefly, basic CSF parameters in SMA patients at baseline did not differ from controls in mean white cell count (p = .33), lactate (p = .75), total protein (p = .50), and Qₐₕ values (p = .58). Only one SMA patient showed immunoglobulin G (IgG) synthesis with identical oligoclonal immunoglobulin G (IgG) bands in CSF and serum, indicating systemic but not autochthonous immune activation in the central nervous system (CNS). None of the other SMA patients and none of the 6 of 10 available controls revealed IgG synthesis in CSF or serum. Basic CSF parameters of the SMA patients did not differ between T₀ and
and T10 in mean white cell count \( (p = .21) \) and lactate \( (p = .07) \). Total protein concentrations were increased \( (0.441 \text{ vs. } 0.372 \text{ g/L}; p < .01) \) in the SMA patient group after 10 months of nusinersen treatment compared with the pre-treatment condition (Table 2; Figure S1a). There was also a trend toward an increased Q_{alb} after nusinersen treatment as reported recently (Wurster, Günther, et al., 2019a). However, this increase did not reach the level of significance in our patient cohort \( (p = .07) \). A recent study detected reactive immune cells in the CSF of nusinersen-treated patients (Gingele et al., 2019). In our cohort, 2/10 SMA patients had few activated lymphocytes in the CSF at T0 and 6/10 patients had mononuclear and/or phagocytic cells at T10.

### 3.3 CSF proteomic profiles in unselected SMA patients at baseline and in response to nusinersen treatment

MS-based CSF proteomic analysis of SMA CSF specimens and controls identified a total of 822 different proteins. A median of 519 different proteins was identified in each SMA specimen (IQR: 466–575; range: 404–591; Figure S1b, S1c). A PCA using all identified proteins separated control from SMA specimens (Figure 1b). Of the 822 proteins identified, 42 (5.1%) were differentially expressed in SMA CSF specimens and controls at baseline \( (T_0) \) (Figure 1c; Table S1). Sixteen \((38\%)\) of these 42 proteins were also differentially found in SMA CSF specimens after 10 months of nusinersen treatment \( (T_{10}) \) when compared with controls. Additional eight proteins were differentially expressed in SMA CSF specimens in comparison with controls only at T10 (Figure 1c; Table S2), suggesting a high overlap of differentially regulated proteins in pre-treatment and post-treatment specimens.

Notably, throughout the entire unselected SMA cohort, not a single CSF protein was differentially expressed in response to 10 months of nusinersen treatment compared with T0 (Figure 1c). Among the top deregulated proteins, Parkinson’s disease protein 7 (PARK7), also known as protein deglycase DJ-1, was identified to be the most up-regulated protein in SMA CSF specimens at baseline \( (T_0) \) compared with controls (Table S1). PARK7/DJ-1 acts as a mediator of neuroprotection in a variety of neurodegenerative disorders (Antipova & Bandopadhyay, 2017).

### 3.4 Age-related clustering of CSF proteomes in SMA patients associated with distinct molecular signaling networks

A k-means distance unsupervised clustering of the protein profiles identified two stable clusters of CSF proteomes within the SMA
patients with high accuracy (Figure S2). The two clusters mainly separate on principle component 1 in a PCA analysis with SMA patients only (variance: 26%; Figure 2a) and are thereafter referred to as clusters APC1 (APC, absolute protein cluster; green; Figure 2a) and APC2 (blue; Figure 2a). Of note, each SMA CSF specimen remained within its assigned cluster, irrespective of the nusinersen treatment (Figure 2a). Moreover, hierarchical clustering of each SMA CSF proteome detected a close relationship between SMA CSF proteomes pre-treatment and post-treatment, highlighting the fingerprint of an individual CSF over changes related to nusinersen treatment (Figure 2b).

We then analyzed differentially regulated proteins between the two clusters, APC1 and APC2. 268 proteins were significantly up-regulated in APC1 and 109 proteins in APC2 (Figure 2b, 2c; Table S3). A subsequent Ingenuity Pathway Analysis (IPA) revealed molecular networks related to “outgrowth”, “sprouting”, and “shape change of neurites” to be associated with cluster APC1, whereas the networks “neuronal degeneration” and “ataxia” were up-regulated in cluster APC2. Potential transcription factors involved in this regulation are PLA2R1, NFE2L2, TP73, and IL15 (Figure 2d). Correlation with clinical data revealed an age dependency in the two clusters with a younger mean age at study entry in APC1 versus APC2 clusters (28.2 ± 4.6 vs. 43.6 ± 3.4 years, \( p = .03 \); Figure 3a). However, despite a proteomic profile associated with increased neuronal loss in APC2 cluster patients, the mean HFMSE score at baseline (T\(_0\)) was similar in both clusters (22.4 ± 8.5 vs. 25.8 ± 9.7, \( p = .80 \); Figure 3b). WPCNA identified four protein clusters in SMA CSF specimens (Figure 3c). Gene ontology (GO) analyses revealed that two of these clusters were composed of proteins associated with neuronal development, whereas the third

\[\text{FIGURE 2}\]

MS proteome profiling in the CSF of \( n = 10 \) adult SMA patients identifies two distinct clusters of molecular signaling networks. (a) PCA of CSF proteomic profiles of SMA patients identifies two different clusters (APC1 and APC2) with a km-distance consensus clustering. (b) Hierarchically clustered heatmap showing z-scores of 377 CSF proteins differentially regulated between the two clusters. (c) Volcano plot with proteins differentially regulated between clusters APC1 and APC2. Proteins with a fold change > 2 are highlighted in red, and proteins above the dashed line are regarded significant based on an adjusted \( p \)-value < 0.05. Protein names in black identify the top 10 up- and down-regulated proteins, whereas protein names in purple represent the proteins identified in the network in (d). (d) Ingenuity pathway analysis (IPA) of differential molecular networks between APC1 and APC2 identifies a network associated with neuroregeneration (APC1) and neurodegeneration (APC2). Orange boxes predict up-regulation and blue down-regulation in APC1. APC, absolute protein cluster; CSF, cerebrospinal fluid; MS, mass spectrometry; PCA, principal component analysis; SMA, spinal muscular atrophy; \( T_0 \), first nusinersen injection at baseline; \( T_{10} \), sixth nusinersen injection after 10 months.
cluster comprised immune-related proteins and the fourth cluster a mix of various pathways. The two neuronal development clusters were associated with the APC1 cluster, whereas the immune-related cluster was associated with the APC2 cluster and the fourth mix cluster did not show association with any specific group (Figure 3c).

3.5 Changes in CSF proteomic profiles in response to nusinersen treatment

The intraindividual proteomic changes in the CSF were analyzed in response to treatment with nusinersen. Comparing T_{10} with T_{0}, CSF total protein concentrations increased in all 10 patients during nusinersen treatment (p < .01), whereas relevant changes in the CSF white cell count were not observed (Fig. S1a, Table 1). We subsequently defined a difference z-score matrix that is composed of the intraindividual abundance differences of each identified protein before and after nusinersen treatment (T_{0}-T_{10}). A k-means distance clustering now including the changes in each protein abundance identified two proteome clusters (Fig. S3), separating in a principle component analysis. Of note, these differential clusters are distinct from the previous APC1 and APC2 clusters (see Figures 2 and 3) and therefore referred to as DPC1 and DPC2 clusters (DPC, differential protein cluster). The patients of cluster DPC2 showed a relative up-regulation of the proteins neuronal pentraxin-1 (NPTX1), semaphorin 7A (SEMA7A), carboxypeptidase E (CPE), and collagen type VI alpha 1 (COL6A1), and down-regulation of cadherin 18 (CDH18), in comparison with DPC1 patients (Figure 4b; Table S4). The three patients in cluster DPC2 did not improve in their HFMSE scores between T_{0} and T_{10}, whereas five of the seven patients in cluster DPC1 did (ΔHFMSE score between T_{0} and T_{10}: 4.7 in DPC1 vs. 0.0 in DPC2, p = .04; Figure 4c). Constrictively, the overlap of APC and DPC clusters was not complete, hindering predictions for a patients’ DPC cluster assignment from baseline CSF analyses.

4 DISCUSSION

Controlled clinical trials conducted in pediatric patients with infantile-onset and later-onset SMA demonstrated significant improvement of motor functions in patients treated with the ASO nusinersen compared with sham procedure (Darras, Chiriboga, et al., 2019a; Finkel et al., 2017; Mercuri et al., 2018; Pane et al., 2019). However, it still remains unclear whether adult SMA patients with first onset of symptoms during childhood or adolescence also benefit from the cost-intensive therapy with nusinersen, as clinical data or reliable biomarkers that indicate an advantageous disease-modifying effect in this patient population are lacking to date (Michelson et al., 2018).

CSF is in direct contact with the CNS including the spinal cord and lower brain stem that are affected in SMA, and thus is a potential source of neurochemical biomarkers. Furthermore, CSF serves as a medium of molecular signaling and contains a multitude of regulatory proteins and peptides relevant for physiologic and pathologic CNS functions. However, a CSF proteome study in SMA has not been reported so far, neither in pediatric nor in adult patients.

Here, we present a non-targeted, control-matched CSF proteomic analysis in adult patients with later-onset SMA in correlation with clinical outcome, immediately before (T_{0}) and 10 months after initiation of nusinersen treatment (T_{10}), along with changes in basic CSF parameters (Figure 1a). Concordant with recent findings from another group (Gingele et al., 2019), we observed a higher number of reactive mononuclear cells in CSF of nusinersen-treated patients compared with baseline and controls (Table 2). We also found a consistent increase in CSF total protein (Table 2; Fig. S1a) and a trend of rising Q_{abs} values (Table 2) during nusinersen therapy indicating a slight dysfunction in CSF flow. Whether this can be solely attributed to a specific pharmacologic effect of the ASO nusinersen or, alternatively, might be a consequence of the repetitive lumbar punctures or the disease itself remains unclear and requires further investigation. Therefore, we recommend...
to routinely determine basic CSF parameters including cytology, the total protein concentration, and the CSF/serum albumin ratio (Qa) in patients undergoing intrathecal treatments as a safety readout.

Plasma pNfH was recently described as a potential marker of disease activity and treatment response in infants with SMA 1 (Darras, Crawford, et al., 2019b). In contrast, another recent study provided evidence that CSF concentrations of neurofilament light chain (NFL) and pNfH are neither elevated in adolescent or adult SMA 2 and SMA 3 patients compared with controls nor influenced by the loading dosing of nusinersen (treatment day 63) (Wurster, Günther, et al., 2019a). Hence, the authors of the latter work concluded that CSF neurofilament concentrations would not be useful as diagnostic or monitoring markers in adolescent/adult SMA 2 or 3 (Wurster, Günther, et al., 2019a).

In our study, we chose a longer evaluation period (10 months) to assure stable steady-state concentrations of nusinersen in the CSF, that is, after completion of the loading dosing on days 0, 14, 28, and 63, plus one maintenance dose after another 4 months (Figures 1a and 2a). Based on MS analysis, PCA of a total of 822 CSF proteins allowed a clear-cut delineation of two distinct SMA clusters, plus the control cluster (Figure 1b). Nusinersen treatment led to decreasing differences between SMA and control CSF proteomes (Figure 1c), but not to a switch of cluster assignments (Figure 2a). Notably, not a single CSF protein was consistently found to be differentially expressed in response to nusinersen treatment (Figure 1c). The reasons for this finding are speculative: The slow disease progression in SMA might hinder the detection of changes to the CSF neurochemical composition during the relatively short evaluation period. Moreover, this result might be caused by the robustness of an intrathalamic CSF proteome fingerprint toward treatment-related influences or simply an effect of the limited patient number investigated in this study, or both.

The two identified CSF proteome clusters in SMA patients differed in that 268 proteins were relatively up-regulated in cluster APC1 while down-regulated in APC2. Conversely, another 109 proteins were relatively down-regulated in cluster APC1 and up-regulated in cluster APC2 (Figure 2b and c). Both CSF proteome clusters were associated with activation of distinct molecular signaling networks (Figure 2d). Additional correlation with clinical data at study entry revealed that both clusters were related to age (Figure 3a) but not to motor function (Figure 3b). Of note, the CSF proteome cluster related to younger patient age (APC1; 28.2 ± 4.6 years) was associated with molecular networks linked to neuroregeneration, whereas in the cluster related to older age (APC2; 43.6 ± 3.4 years) signaling networks associated with neurodegeneration were up-regulated (Figure 2d).

Assessment of intrathalamic abundance differences of each identified protein before and after nusinersen treatment (T0–T10) based on a difference z-score matrix identified two clusters distinct from the previous ones (DPC1 and DPC2: Figure 4a) that only showed a partial overlap with the two clusters APC1 and APC2 (Figure 4b) from the unsupervised analysis. Those CSF clusters, DPC1 and DPC2, featured differential expression of five proteins (Figure 4b), and correlated with the HFMSE score (Figure 4c): The three patients of cluster DPC2 who remained on a stable HFMSE score under nusinersen treatment showed a relative up-regulation of NPTX1, SEMA7A, and CPE, also known as neurotrophic factor-α1 (CPE-NFα1), and COL6A1, and down-regulation of CDH18, whereas five of seven patients in cluster DPC1 improved clinically in response to treatment (Figure 4b and c; Table S4). Of note, all of these proteins are associated with either neuronal or muscular processes: NPTX1 is involved in coordinating synaptic strength (Schaukowitch et al., 2017), SEMA7A fulfills functions in promoting axonal outgrowth (Pasterkamp, Kolk, Hellemans, &
Koledkin, 2007), CPE is a neurotrophic factor with neuroprotective functions during stress and neurodevelopment (Xiao, Yang, & Loh, 2019), mutated COL6A1 underlies collagen type VI myopathy (Bushby, Collins, & Hicks, 2014), and the cell–cell adhesion molecule CDH18 plays a pivotal role in the development of the neural circuitry and in mature synaptic function (Redies, Hertel, & Hubner, 2012). Albeit clear associations of these candidate proteins with mostly neuroregenerative functions are evident, it still needs to be validated to what extent they are relevant in SMA patients and their individual response to nusinersen treatment, and might then be developed as potential biomarkers.

One important insight from our data is that future longitudinal intradividual analyses of CSF proteomes should integrate changes in single CSF protein abundances in each patient. Reactive and/or phagocytic cells that were observed in post-treatment CSF may influence the proteomic analysis. As the proteomic analysis was performed from CSF supernatant after early centrifugation of cells, a large bias derived from phagocytosis is yet unlikely.

The authors are aware that a major drawback of this study is the low number of cases. The patient cohort at hand only included one patient with SMA 2 while all other patients had SMA 3a or 3b. Furthermore, control subjects received only one lumbar puncture each, and their CSF, unlike in SMA patients, was not reanalyzed after 10 months. Nevertheless, proteomic analyses from protein-poor bioanalytes such as CSF are technically demanding, while the detection of CSF biomarkers in genetic disorders, for which a rising number of ASO- and RNA interference (RNAi)–based therapies will be available in the near future, is still an unmet need (Roovers, Jonghe, & Weckhuysen, 2018; Rossor, Reilly, & Sleigh, 2018; Wurster & Ludolph, 2018). Hence, we provide here first proof-of-concept data showing that CSF proteomic analyses are feasible in this slowly progressive neurodegenerative disease. Herewith they permit clustering of patient groups according to distinct molecular signaling networks in correlation with clinical outcome parameters, and thus might give rise to the identification of relevant, potentially predictive biomarkers. Future studies are needed to apply this high-throughput method and confirm findings in larger multicenter patient cohorts.

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AUTHOR CONTRIBUTIONS

Tobias Kessler: acquisition of data, analysis and interpretation of data, statistical analysis, study coordination, and writing the manuscript; Pauline Latzer: acquisition of data, analysis and interpretation of data, and critical revision of manuscript for intellectual content; Dominic Schmid: acquisition of data, analysis and interpretation of data, and critical revision of manuscript for intellectual content; Markus Möhlenbruch: acquisition of data, analysis and interpretation of data, and critical revision of manuscript for intellectual content; Afshin Saffari: analysis and interpretation of data, and critical revision of manuscript for intellectual content; Jennifer Kollmer: analysis and interpretation of data, and critical revision of manuscript for intellectual content; Markus Weiler: acquisition of data, analysis and interpretation of data, and critical revision of manuscript for intellectual content; Wolfgang Wick: analysis and interpretation of data, and critical revision of manuscript for intellectual content; Christian Ulfert: acquisition of data, analysis and interpretation of data, and critical revision of manuscript for intellectual content; Christian Herweh: acquisition of data, analysis and interpretation of data, and critical revision of manuscript for intellectual content; Brigitte Wildemann: acquisition of data, analysis and interpretation of data, and critical revision of manuscript for intellectual content; Markus Weiler: study concept and design, acquisition of data, analysis and interpretation of data, statistical analysis, study supervision and coordination, and writing the manuscript.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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