European Journal of Paediatric Neurology 42 (2023) 34–41

Contents lists available at ScienceDirect

European Journal of Paediatric Neurology

journal homepage: www.journals.elsevier.com/european-journal-of-paediatric-neurology

Inflammatory markers in cerebrospinal fluid of paediatric spinal muscular atrophy patients receiving nusinersen treatment

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ARTICLE INFO

Keywords:
Spinal muscular atrophy
Gene therapy
Biomarker
Multiplex immunoassay
Inflammation
MCP1/CCL2

ABSTRACT

Spinal muscular atrophy (SMA) is a progressive motor neuron disease with onset during infancy or early childhood. Recent therapeutic advances targeting the genetic defect that underlies SMA improved survival in patients with infantile onset SMA (type 1) and improved motor function in SMA type 1–3. The most commonly used therapy for SMA, the antisense oligonucleotide nusinersen, is delivered by repeated intrathecal injections. The long-term safety effects of this procedure, however, have not yet been investigated in detail. We here present case reports of three children with SMA in which routine laboratory investigation revealed increased leukocyte counts in cerebrospinal fluid (CSF) collected during the course of nusinersen treatment. To further characterize this observation, we used a multiplex method to analyse a broad spectrum of inflammatory markers in the CSF of these patients. We found that interleukin-10 (IL10) was consistently elevated in CSF with increased leukocyte counts, but other inflammatory markers were not. Based on this analysis we selected 7 markers for further analysis in a cohort of 38 children with SMA and determined their expression during the course of nusinersen therapy. No consistent association was found between levels of inflammatory markers and the duration of nusinersen therapy in individual patients. However, monocyte chemoactive protein 1 (MCP1/CCL2) -a neuroprotective protein secreted by astrocytes and previously associated with SMA- levels increased over the course of nusinersen treatment, indicating a possible neuroprotective mechanism associated with nusinersen therapy. In summary, our findings confirm that repeated intrathecal injections are safe and do not trigger unwanted immune responses.

1. Introduction

Hereditary proximal spinal muscular atrophy (SMA) is characterized by stalled early gross motor development and deterioration of muscle strength throughout life, including of respiratory and bulbar muscles [1]. SMA is caused by deficiency of the survival motor neuron (SMN) protein due to the homozygous loss-of-function of the *survival motor neuron 1* gene (*SMN1*) [2]. A point mutation in exon 7 of the highly homologous *SMN2* causes exclusion of exon 7 in the majority of *SMN2* mRNA, leading to a truncated protein with limited functionality [3,4]. A minority of *SMN2* mRNA transcripts, however, includes exon 7 and leads to the production of full-length SMN protein. As such, SMA severity is inversely correlated with *SMN2* copy number, although this correlation is incomplete and up to 40% of clinical variability cannot be explained by variation in *SMN2* copy number alone [5].

Understanding the mechanisms underlying exon 7 splicing in *SMN2* has led to the development of two *SMN2* splicing modifiers that have now been approved for the treatment of SMA. One of these drugs, nusinersen (Spinraza), is an antisense oligonucleotide (ASO) that increases *SMN2* exon 7 inclusion by binding to a sequence (ISS-N1) upstream of exon 7 [6]. Although the long-term efficacy of nusinersen therapy will need to be further established in the coming years, an increasing number of post-marketing studies amongst multiple populations, age groups and SMA types now all indicate that nusinersen

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https://doi.org/10.1016/j.ejpn.2022.12.003
Received 21 July 2022; Received in revised form 1 November 2022; Accepted 5 December 2022
Available online 7 December 2022

therapy leads to disease stabilisation or meaningful clinical improvements in the vast majority of SMA patients, as determined by motor function and development (see for example refs. [7–10]).

Nusinersen is an ASO that cannot pass the blood-brain barrier and therefore needs to be administered by repeated intrathecal injections. The need for repeated intrathecal injections at the scale and frequency required for nusinersen treatment represents a relatively novel concept in (paediatric) neurology. It is therefore important to implement routine, long-term monitoring of any adverse events that may be related to the procedure. The clinical trials that led to the approval indicated that the procedure was safe and well-tolerated [7,8,11,12], and did not report side-effects other than those commonly associated with lumbar puncture (e.g. post-puncture headache). However, since the approval of nusinersen, specific, other adverse events have been reported, including communicating hydrocephalus [13] and aseptic meningitis [14]. Although the incidence of these adverse reactions is unknown, they may indicate an inflammatory process of the meninges after nusinersen administration. Moreover, other groups have described so-called nusinophages, macrophages with granules containing nusinersen [15–17], similarly pointing towards an ongoing immune response. Despite these observations, a systematic investigation of the presence and levels of inflammatory markers over the course of nusinersen therapy has not yet been reported.

In our cohort of children with SMA patients receiving nusinersen therapy, we observed three patients with elevated leukocyte counts in CSF during treatment, without clear clinical signs of inflammation. This finding led us to systematically investigate the expression of a range of inflammatory markers in patient cerebrospinal fluid (CSF) in these patients using multiplex immunoassays. Based on an initial screen to determine which inflammatory markers could reliably be measured, we subsequently performed a larger-scale experiment in a cohort of 38 paediatric SMA patients to identify possible inflammatory markers associated with nusinersen therapy and, additionally, investigate their suitability as candidate biomarkers.

2. Materials and methods

2.1. Patients

All patients had been included previously in a prospective population-based cohort study on SMA in the Netherlands [18–20]. Patient characteristics were collected during interviews with parents using standardized questionnaires and physical examination as described before [18–22]. We distinguished patients as SMA types 1–3 based on age at disease onset and best of two acquired WHO motor milestones (i.e. sitting and walking independently), and further classified patients into SMA subtypes [1,18,20]. In short, patients with type 1c achieve the ability to roll or lift their head in prone position, patients with type 2a learn to sit independently, while those with type 2b also learn to stand or take a few steps with support. Patients with type 3 learn to walk independently and have a disease onset before (type 3a) or after (type 3b) the age of three years [1,18,20]. A genetic diagnosis of SMA and *SMN2* copy number was confirmed using multiplex ligation-dependent probe amplification (SALSA MLPA kit P021–B1, MRC-Holland) as described in detail previously [5].

2.2. CSF collection and multiplex immunoassay

Five millilitres of CSF was collected at each injection according at the time points indicated in the standard nusinersen treatment protocol, starting with loading doses at baseline (day zero, T0), day 14 (T1), day 28 and day 63, and followed by intrathecal injections every four months (T5 in this study was at 10 months after the start of therapy) [23]. At each injection, 3 mL of CSF was sent for routine clinical laboratory analysis that included determination of blood cell counts, protein and glucose levels. Moreover, 2 mL of CSF was centrifuged at 1,000×*g* at 4 °C for 10 min, aliquoted and stored at −80 °C until analysis. Prior to multiplex immunoassay on the Luminex platform, samples were passed through a 0.22 μm filter to remove debris. Multiplex analysis of 16 proteins was performed as described previously [24]. Briefly, color-coded magnetic beads (Luminex) were conjugated to antibodies that had previously been validated to specifically detect the protein of interest. After incubating our CSF samples with color-coded, antibody-coated beads, a mix of biotinylated antibodies was used for detection of the analytes and fluorescence intensity was determined on a Flexmap 3D system (Luminex).

2.3. Experimental design

In this study we used a two-stage experimental design. In the first stage, as it was unclear which proteins could reliably be measured in CSF, we analysed CSF samples from patients with increased leukocyte counts and patients without increased leukocyte counts (matched for sex, age, SMA type, disease duration and remaining motor function) for a broad range of analytes. All analytes we included had previously been validated on our multiplexing platform [24] and that could thus be reliably and reproducibly quantified. We included proteins of which the expression is thought to be linked to the activity or regulation of key parts of the immune system including interleukin-1 beta (IL1B), IL3, IL6, IL10, IL12, IL13, IL17, IL18, and interferon gamma (IFNG) [25,26]. Also, we included proteins that had been previously linked to SMA, motor neuron function or the neuromuscular system including brain-derived neurotrophic factor (BDNF), FAS, vascular endothelial growth factor (VEGF), tumor necrosis factor alpha (TNFA), angiopoietin-1 (ANG1), complement C5 alpha chain (C5a) and monocyte chemoattractant protein 1 (MCP1; also called C-C motif chemokine 2, CCL2) [27–33]. For the second stage of our analysis, we selected 7 of the above proteins based on the results of the first stage experiment. We analysed these proteins at three different time points during the course of nusinersen treatment (T0, T1 and T5) in 38 patients for whom we had samples from these time points available. Graphs were generated in Rstudio (version 1.3.959) using ggplot 2. Statistical tests were performed in Graphpad Prism (version 9.3), by comparing the means of multiple groups with a one-way ANOVA followed by post hoc Tukey *t*-test with correction for multiple testing.

2.4. Ethical approval

Patients included in this study participate in an ongoing prospective population-based cohort study on SMA in the Netherlands, approved by the UMC Utrecht Medical Ethical Committee (No. 09–307/NL29692.041.09). We obtained written and oral informed consent from both parents of each patient.

3. Results

3.1. Rare observations of elevated leukocyte counts in paediatric SMA patients

The cohort of SMA patients receiving nusinersen therapy in the Netherlands was initially limited to children up to the age of 9.5 years and consisted of 72 patients. From this cohort, we noticed elevated leukocyte counts in the CSF of three children during routine laboratory monitoring. Other variables that were monitored included CSF protein, glucose and erythrocyte levels and were generally all within the normal range. The first child started treatment presymptomatically (four days after birth). This child has two *SMN2* copies and a baseline CHOP INTEND score of 43. Elevated leukocyte counts were observed in the first three CSF samples, and elevated protein levels in the first four CSF samples (sample taken prior to injection 1: leukoctyes 7 × 10^6/L, 70% monocytes, 25% lymphocytes; protein 1.91 g/L; injection 2: leukocytes 9 × 10^6/L, 77% lymphocytes, 16% monocytes; protein 1.52 g/L;

injection 3: leukocytes 6 × 10ˆ6/L, 53% lymphocytes -within range-, 23% monocytes, 9% macrophages; protein 1.05 g/L; injection 4: leukocytes 2 × 10ˆ6/L -within range-; protein 0.77 g/L). There were no clinical symptoms accompanying these laboratory findings. After the fourth injection, no abnormalities were found in the CSF of this patient again. Later during treatment, the parents have occasionally reported increased body temperature after nusinersen injection, unrelated to elevated leukocyte counts. The second child started treatment at the age of 30 months, has SMA type 2a with three *SMN2* copies and a baseline HFMSE score of 4. In total this child has now received 12 injections without complications. Elevated CSF leukocyte counts (20 × 10ˆ6/L; 75% lymphocytes, 19% monocytes) was observed once, prior to the fifth injection. The CSF was colourless. Other variables were within normal range. The third child started treatment at the age of 50 months, has SMA type 1c with three *SMN2* copies and a baseline CHOP INTEND of 42. In total this child has had 13 injections, without complications. We observed elevated leukocyte levels in the CSF once (6 × 10ˆ6/L; 86% lymphocytes, 13% monocytes), prior to the sixth injection. The CSF was colourless and erythrocyte and protein levels were within the normal range (protein 0.22 g/L).

Other than mildly elevated body temperature in the first child, no clinical signs associated with inflammation or immune response were reported for these patients. Also, no chronic leucocytosis was observed in these patients (increased leukocytes were present during the first 4 weeks of nusinersen therapy in patient 1). Although it is therefore likely that the clinical effect of increased leukocytes in CSF during nusinersen therapy is limited, we decided to study in more detail if this phenomenon was associated with the activation of specific parts of the immune system as this has not been systematically characterised before.

3.2. Increased interleukin-10 (IL10) expression is associated with increased leukocyte count

As generally protein makes up only a small proportion of CSF (on average 0.2 g per litre of CSF) and reference values available for CSF cytokines have mainly been reported based on adult cohorts, we first asked what proteins could be reliably detected in the CSF of children with SMA. For this, we used a multiplex assay based on the Luminex platform, as this allowed us to make efficient use of scarce patient material while reliably detecting proteins at a high sensitivity using reproducible assays that have been validated previously (see materials and methods). The human immune response is regulated through a complex interplay of many (extracellular) elements, including chemokines, cytokines, growth factors, peptides and complement factors. We here tried to cover a broad range of the human immune response by selecting analytes that are known to be markers or regulators of key elements of the immune system. Moreover, we included proteins that were part of pathways that had previously been linked to SMA or motor neuron function. We analysed CSF samples from time points at which elevated leukocytes counts were observed and compared those to samples taken from the same patients at baseline and after leukocyte counts were back within normal range. Moreover, we analysed CSF samples from age-matched patients from the same time points but without CSF anomalies. The results from this initial screen are presented in Table 1. First, we looked for associations between the expression of inflammatory markers and the of elevated CSF leukocytes. Interleukin-10 (IL10) was consistently elevated in each sample in which increased leukocyte levels were found but could not be detected in any of the other samples. Although patient 2 showed higher IL6 and IFNG levels when elevated leukocyte counts were reported, we did not observe a further consistent

Table 1
Screen to determine detectable cytokines and other proteins in SMA patient CSF.

Patient	Description	IL10	IL1B	IL6	MCP1	C5A	ANG1
1	Elevated leukocytes (after 1st injection)	3.26	7.72	ND	1314.75	468.21	41.38
2	Elevated leukocytes (after 4th injection)	34.06	7.49	327.91	764.58	175.48	56.7
3	Elevated leukocytes (after 5th injection)	5.83	7.07	4.87	192.73	39.06	69.91
Average baseline	Normal leukocytes (baseline)	ND	5.90	2.60	1227.47	221.90	100.27
(total n = 3)	Range (min - max)	–	4.56–7.39	–	369.95–2909.38	9.05–644.53	82.71–121.53
	ND (not detected) in *n* samples	3	–	2	–	–	–
Average control	Normal leukocytes (after 4–8 injections)	ND	6.48	4.74	538.10	26.68	90.03
(total n = 8)	Range (min - max)	–	4.56–10.92	2.53–6.95	372.7–856.38	6.41–99.15	52.04–138.92
	ND (not detected) in *n* samples	8	–	6	–	–	–

Patient	TNFA	IL12	IL13	IL18	VEGF	FAS	IL3	IL17	IFNG	BDNF
1	ND	ND	2.62	18.31	9.92	86.04	73.49	ND	ND	ND
2	1.79	11.95	3.16	1.91	4.41	61.18	65.71	ND	2.89	ND
3	2.11	15.32	2.76	1.56	4.81	65.93	103.69	ND	ND	ND
Average baseline	1.69	13.64	2.87	25.25	8.65	70.83	96.27	0.94	ND	ND
(total n = 3)	1.42–2.07	10.43–16.06	1.08–4.79	–	3.78–17.5	37.96–115.52	46.72–145.82	–	–	–
	–	–	–	2	–	–	1	2	3	3
Average control	1.54	10.75	3.45	2.73	3.59	52.80	55.28	2.86	–	–
(total n = 8)	1.26–1.99	5.74–13.05	0.27–6.05	2.56–3.42	2.62–6.95	32.16–74.52	29.6–85.51	–	–	–
	1	3	1	5	–	–	3	7	–	8

CSF samples from patients with increased leukocyte counts (patients 1, 2 and 3) were analysed for 16 cytokines and proteins to determine what samples could reliably be measured. For each patient, baseline control samples and age-matched controls were included in the analysis. Because not all analytes could be measured in all samples, the table indicates the number of samples for which this was not possible (ND; not detected). The average values for each analytes and the range (min – max) are presented.
IL: interleukin (1B, 3, 6, 10, 12, 13, 17, 18); MCP1: monocyte chemoattractant protein 1 (also known as C-C motif chemokine 2 or CCL2); C5A: complement C5 alpha chain; ANG-1: angiopoietin-1; TNF: tumor necrosis factor; VEGF: vascular endothelial growth factor; FAS: Fas cell surface death receptor; BDNF: brain derived neurotrophic factor; IFNG: interferon gamma.

association between increased leukocyte levels and other proteins included in our analysis. Second, because protein levels in CSF are low, we found that most other proteins included in our analysis were below the detection limit of the Luminex platform or could only be detected in a limited number of samples. However, MCP1 (CCL2), C5A and ANG1 could reliably and consistently be detected in almost all samples and were therefore selected for further analysis in a larger cohort of SMA patients.

3.3. MCP1 (CCL2) expression increases during nusinersen treatment

We included all children with SMA who started treatment with nusinersen between May 2017 and July 2019 and for whom CSF samples were available at baseline (T0), two weeks after the start of therapy (T1) and 10 months after starting treatment (T5), providing a total cohort of 38 patients for these analyses. We chose these timepoints to capture key phases of nusinersen: T0 samples may possibly contain SMA-dependent, treatment-unrelated immunological markers; whereas T1 and T5, respectively, contain acute or chronic immunological responses to nusinersen treatment or repeated intrathecal injections. The characteristics of the patients included in this analysis are summarized in Table 2. We again used a multiplex assay based on the Luminex platform to determine proteins levels of MCP1, C5A and ANG1 in 114 CSF samples. We also included IL1B, IL6, IL10 and TNFA as a representative sample of inflammatory markers we were able to measure in our initial screen. We included analysis of the protein RANTES as a quality control for possible cell contamination in our samples, in particular contamination with erythrocytes. Based on the presence of detectable levels of RANTES in 5 samples, we excluded those from further analysis, leaving 109 results from CSF samples for further analysis. The results from this experiment are presented in Table 3. Further association between inflammatory markers (IL1B, IL6, IL10, TNFA) and nusinersen therapy was also absent in this larger cohort.

The expression of MCP1, ANG1 and C5A for each of the time points included in our analysis is shown in Fig. 1. For ANG1, levels were highly variable, and we did not observe an association between ANG1 levels and treatment timepoints or SMA types (Fig. 1A). For C5A and MCP1 (Fig. 1B and C), we noticed particularly high protein levels in the youngest patients included in our cohort, that decreased quickly after therapy start. Because we had limited availability of baseline CSF samples for type 1 patients in our centre, we were not able to analyse this observation in more detail. It seems to represent a similar pattern of high CSF protein levels shortly after birth, as has been previously observed in studies of CSF from SMA patients (see e.g. Ref. [34]). Because we were able to include more type 2 (n = 22) and type 3 (n = 12) patients in our study, we analysed the pattern in which the expression of MCP1 and C5A changed over time with treatment in more detail (Fig. 2). When normalising the expression of these proteins to baseline levels, to control for variability in expression between patients at the start of therapy, we observed that MCP1 expression showed an increased trend over time with treatment (Fig. 2A and B). For patients with type 3, this increase was significant compared to baseline levels (Fig. 2B; T0: 699.3 pg/mL, T5: 941.9 pg/mL, $P = 0.0014$). Compared to MCP1, C5A levels remained stable over time with treatment, and we did not observe significant changes (Fig. 2C and D). Finally, we investigated if increased MCP1 levels over the course of nusinersen treatment correlated with other clinical characteristics of the patients included in our analysis. However, we did not identify a correlation between MCP1 levels and age at disease onset, delay of therapy start, HFMSE score or *SMN2* copy number.

Table 2
Patient characteristics.

	All patients (n = 38)	SMA type 1 (n = 4)	SMA type 2 (n = 22)	SMA type 3 (n = 12)
Male sex n (%)	22 (58)	4 (100)	11 (50)	7 (58)
Age disease onset	12 (0–36)	3.5 (0–13)	9 (2.5–18)	19 (12–36)
Disease duration start therapy	47 (2–108)	24 (2–101)	47.5 (12–108)	47 (9–95)
Age at first dose	59 (2–115)	27.5 (2–114)	55.5 (21–115)	68 (39–113)
SMN2 copy number n (%)				
2	3 (8)	3 (50)	0	0
3	27 (71)	1 (50)	22 (100)	4 (33)
4	8 (19)	0 (0)	0	8 (67)
CHOP INTEND at baseline	33 (20–46)	33 (20–46)	n.a.	n.a.
HFMSE at baseline	19.5 (2–56)	n.a.	13 (2–38)	47.5 (34–56)

A cohort of 38 patients for which baseline, T1 and T5 CSF samples were available were included for further analyses. N = number; n.a. = not applicable; *SMN2* = survival motor neuron 2; CHOP INTEND= Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders; HFMSE= Hammersmith Functional Motor Scale Expanded. All ages and disease duration are given in months, unless otherwise stated. All outcomes are given in median (range) unless otherwise stated.

4. Discussion

Our knowledge about the long-term effect and safety of repeated intrathecal injections to treat neurological diseases is still limited. At the same time, the number of genetic therapies for neurological diseases in advanced stages of clinical development and requiring intrathecal delivery is rapidly increasing [35]. Because SMA is at the forefront of these developments, and we occasionally noticed elevated CSF leukocytes in the cohort of children with SMA receiving nusinersen, we decided to focus on characterising expression profiles of inflammatory markers in response to repeated intrathecal injections. Our results further confirm that repeated intrathecal delivery of gene-targeting therapies is safe, also when focusing on markers of inflammation.

IL10 was the only cytokine included in our analyses that was consistently upregulated in response to increased leukocyte counts. IL10 is a cytokine with an anti-inflammatory function and is expressed by different cells from the adaptive and innate immune system [36,37]. Previous work suggests that IL10 is required to prevent host damage after infection or to inhibit an immune response [36]. Its exact *in vivo* role remains incompletely understood, but it has been suggested that IL10 downregulates the production of other, pro-inflammatory cytokines [36,37] and can protect against tissue damage caused by inflammatory diseases of the CNS [37]. The anti-inflammatory function of IL10 is a possible explanation of the association between elevated leukocytes counts in the CSF and increased IL10 that we observe in our current study. As such, it may contribute to our observation that increased leukocyte counts did not lead to infection or inflammation. Of note, the increased leukocyte counts observed in our studies seemed to be mainly lymphoid, although the youngest patient displayed a shift from monocytic to a lymphoid reaction. Unfortunately, we did not have access to a larger number of CSF samples for this patient to further detail the cytokines that may be regulating this change in CSF leukocyte counts.

In the publication reporting the results of the phase I clinical trial of nusinersen, the analysis of cytokine levels in CSF (IL6, TNFA, MCP1) was mentioned [11]. This study was performed in small numbers of SMA patients receiving lower doses of nusinersen and found no abnormalities for these cytokines at day 7 and 28 post-dose, although no further details were provided [11]. Importantly, changes in e.g. MCP1 expression in our studies were only measurable after longer treatment durations, which illustrates that it may be important to measure molecular markers for longer follow-up intervals. Furthermore, several other studies have previously reported typical laboratory values (e.g. protein levels, erythrocyte counts, glucose levels) for CSF samples from adult and adolescent patients with SMA [38–41]. These studies reported an overall increase in protein levels over the course of nusinersen therapy, which we did not generally observe in our study. However, in the youngest patients included in our analysis (SMA type 1) we noticed higher overall

Table 3
Protein expression in CSF per timepoint and per SMA type.

	Baseline (detected in *n* samples)				After 1st injection (detected in *n* samples)				After 5th injection (detected in *n* samples)			
	All SMA (37)	Per SMA type (*n*)			All SMA (35)	Per SMA type (*n*)			All SMA (37)	Per SMA type (*n*)		
		1 (3)	2 (22)	3 (12)		1 (3)	2 (21)	3 (11)		1 (4)	2 (21)	3 (12)
IL1B (95% CI)	1.51 (1.31–1.71) (8)	2.06 (1)	1.51 (2)	1.41 (5)	1.62 (0.94–2.29) (3)	1.53 (1)	1.92 (1)	1.4 (1)	1.58 (1.28–1.88) (11)	1.33 (3)	1.66 (7)	1.75 (1)
IL6	6.34 (1)	– –	6.34 (1)	– –	– (0)	– –	– –	– –	11.51 (1)	– –	11.51 (1)	– –
IL10	2.55 (1)	– –	– –	2.55 (1)	– (0)	– –	– –	– –	– (0)	– –	– –	– –
TNFA	2.75 (−1.58–7.09) (4)	4.05 (2)	1.46 (2)	– –	2.07 (−0.80–4.95) (3)	2.34 (2)	– –	1.55 (1)	1.35 (1.24–1.46) (9)	1.26 (1)	1.34 (5)	1.38 (3)
MCP1	756.6 (668.2–845.1) (37)	1123.08 (3)	737.93 (22)	699.33 (12)	904.5 (705.9–1103) (35)	2078.58 (3)	829.88 (21)	726.86 (11)	1001 (887.4–1115) (37)	1176.84 (4)	1001.28 (21)	941.94 (12)
ANG1	33.17 (27.52–38.81) (35)	26.67 (3)	40.32 (21)	21.28 (11)	33.66 (27.16–40.17) (31)	12.98 (1)	38.98 (21)	23.55 (9)	26.81 (22.08–31.54) (33)	36.75 (3)	30.04 (21)	15.93 (19)
C5A	44.64 (30.08–59.19) (37)	123.78 (3)	32.33 (24)	47.41 (12)	41.83 (26.96–56.71) (35)	146.21 (3)	31.30 (21)	33.47 (11)	32.24 (27.13–37.36) (37)	51.54 (4)	27.55 (21)	34.01 (12)

Overview of protein expression levels in 38 SMA patients. Initially, 114 CSF samples were analysed, after quality control 5 were excluded from further analysis. The average (mean) for all SMA patients at each time point is presented with the 95% confidence interval. As not all analytes reached detectable levels in each sample, the number of samples on which each average value is based is presented below the average between brackets. IL: interleukin (IL1B, IL6, IL10); MCP1: monocyte chemoattractant protein 1; C5A: complement C5 alpha chain; ANG1: angiopoietin-1; TNF: tumor necrosis factor.

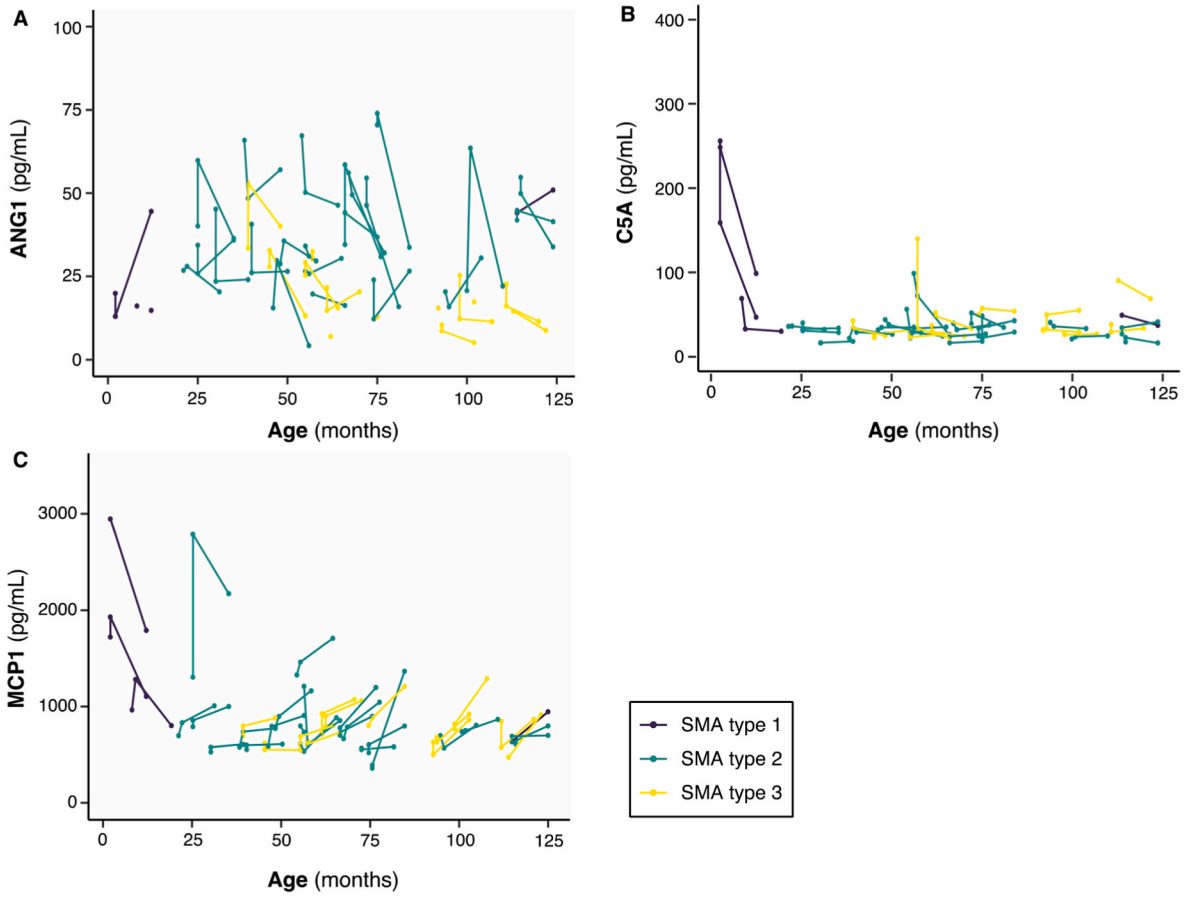

Fig. 1. MCP1, ANG1 and C5A in CSF in individual patients treated with nusinersen. ANG1 (A), C5A (B) and MCP1 (C) expression levels were measured using multiplex immunoassays in patients with SMA type 1 (n = 4, dark blue), type 2 (n = 22, green) and type 3 (n = 12, yellow). For each patient, three time points were included in our analysis (baseline, 14 days after the first injection, and after 10 months of therapy). Although protein levels were measured at 3 time points for each patient, after quality control 109 samples were included for final analysis and therefore for some patients for some proteins only 1 or 2 data points could be included in this figure. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

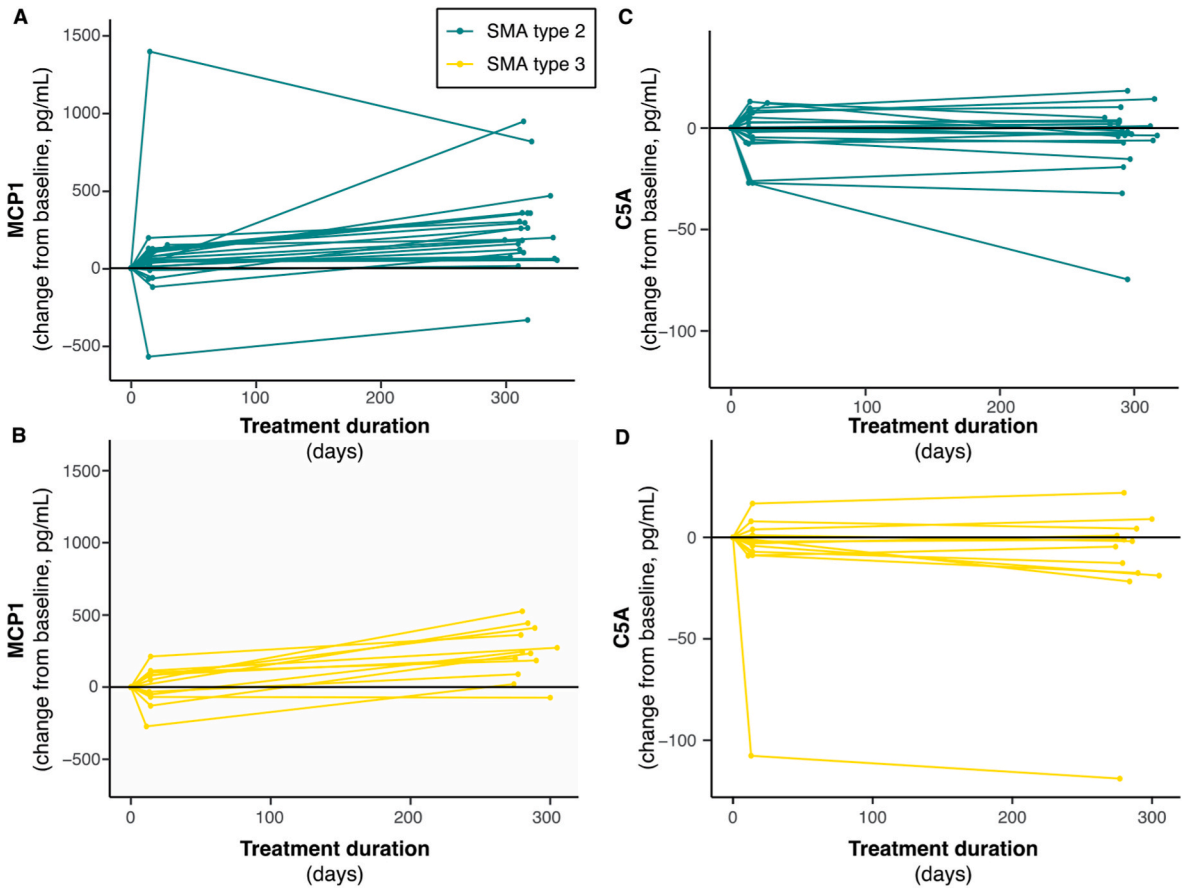

Fig. 2. MCP1 and C5a expression changes compared to baseline in CSF of patients treated with nusinersen. To illustrate the development of MCP1 (A, B) and C5A (C, D) expression profiles over time, protein expression values were plotted as change compared to baseline measurements. This illustrates that MCP1 expression in type 3 patients (B) increased over time compared to baseline in all but one patient. A similar trend is visible in type 2 patients for MCP1 (A), but is absent in C5A (C, D).

CSF protein, and also higher levels of specific proteins (e.g. MCP1 and C5A) levels at the start of treatment, that decreased over time. Interestingly, protein levels in CSF have previously been found to be increased in young infants (up to 6 months) compared to older children and adolescents, independent of underlying pathology [42]. Possible explanations for increased protein levels in young infants are birth trauma, an immature blood-brain barrier or different CSF flow rate. This observation is an example of our limited knowledge about reference values for CSF of (very) young children. In addition to basic reference values such as leukocyte counts and protein levels, reference values for specific proteins (including C5A, MCP1 and ANG1) have not been reported for the age groups investigated in our current study. This warrants care in interpreting results, as many studies, including our current study, reported results on CSF analyses of SMA patients only, as dedicated control groups are not often available.

Other than for diagnostic purposes, CSF samples were previously not regularly obtained and collected from children. With the arrival of genetic therapies that need intrathecal delivery also comes the possibility to readily obtain CSF without extra invasive procedures to the children receiving nusinersen therapy. This now highlights CSF as a source for determining longitudinal molecular markers of disease progression and therapy efficacy. This is especially relevant for nusinersen treatment, as the long-term clinical efficacy, safety and cost-effectiveness remain unclear. Indeed, a growing number of previous studies have reported findings on both specific and unbiased approaches to determine and identify CSF-derived biomarkers for monitoring treatment efficacy. Many groups have focused on various forms of neurofilament as a biomarker for SMA severity and treatment response (e.g. Refs. [34,39,43–47]). Especially children with SMA type 1 show very high neurofilament levels, that sharply decrease with treatment [39,43,47]. It should be noted, however, that neurofilament levels also decrease with age and that differences in neurofilament levels between treated and untreated patients in older SMA patients (e.g. type 2 and type 3) is less pronounced. Moreover, recent studies have used unbiased approaches to determine on a large scale what protein [38], extracellular RNAs and micro RNAs (miRNAs) could be measured in CSF obtained from SMA patients [48]. These approaches highlighted e.g. miR-146a, a known miRNA associated with astrocyte function in SMA. Interestingly, in our current paper, we identified MCP1 as a promising candidate for monitoring nusinersen efficacy, as it increased significantly over time in nusinersen-treated type 2 and type 3 patients. MCP1 levels did not correlate with clinical outcomes, including motor scale improvement in these patients, but we cannot exclude that this is partially due to subtle phenotypic improvements that may currently not be adequately captured by existing motor scales. MCP1 has been linked to SMA previously, when it was shown to be secreted by astrocytes and thereby protect against degeneration of motor neurons [32], possibly by interacting with an axonal isoform of the SMN protein [33]. Our results may therefore reflect a nusinersen-induced improvement of astrocyte function in SMA, which may prove an interesting insight both for biomarker research and further understanding of the role of astrocytes in SMA pathogenesis [49].

5. Conclusions

We confirm that repeated intrathecal injections of nusinersen to treat children with SMA are well-tolerated and safe, also when investigating markers of inflammation. In rare patients with elevated leukocyte

numbers, we were able to measure IL10, suggesting that IL10 may be a promising candidate to monitor immune response to intrathecal injections in patients for which this is relevant. MCP1 (CCL2) showed a modest but consistent increase with treatment over time, especially in type 3 children, and may therefore be an interesting biomarker candidate for future research.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We would like to thank our colleagues at the Multiplex Core Facility UMC Utrecht for their support with the processing of the CSF samples in this study. We also acknowledge the support of our colleagues involved in the administration of nusinersen, allowing for standardized collection of CSF samples. This study was supported by grants from Stichting Spieren voor Spieren (to WLvdP), the European Union's Horizon 2020 Research and Innovation Program under the Marie Skłodowska-Curie Grant Agreement No. 956185 (SMABEYOND ITN, to WLvdP) and Prinses Beatrix Spierfonds (W.OB21-01, to EJNG).

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