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SERIES XXXIV

EXPLORATORY BIOMARKERS IN RARE NEUROLOGICAL DISORDERS

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Summary

Abstract

Spinal Muscular Atrophy (SMA) is an autosomal recessive disorder due to progressive degeneration of lower motor neurons (LMN) resulting in muscle atrophy and possible respiratory and bulbar involvement. Pathogenic hallmark is the loss of function of Survival Motor Neuron Protein (SMN), encoded by mutated or deleted *SMN1* gene. *SMN2*, the centromeric paralogous gene of *SMN1*, produces only a small amount of functional SMN (Levin, 2019). Nusinersen is the first disease modifying therapy approved for adult SMA patients. Neurofilaments light chain (NfL) are intermediate filaments exclusively expressed in neurons. As NfL levels raise following axonal damage, they might be promising diagnostic and prognostic biomarkers in motor neuron diseases (Gaetani, et al., 2019). A different intermediate filament, peripherin (PRPH) assembles with neurofilaments mostly in peripheral neurons and LMN (Zhao & Liem, 2016). Human Profilin-1 (PFN-1) is a small actin-binding protein that promotes actin polymerization and has a role in regulating cytoskeletal architecture and dynamics of neurons (Witke, 2004). This study aims to investigate the role of NfL, PRPH and PFN-1 as disease and/or treatment-response biomarker in a cohort of adult SMA type 2 and 3 patients.

33 SMA type 2 and 3 patients were recruited at the Neuromuscular Center of University-Hospital of Padova, where nusinersen treatment was administered from February 2018 to September 2021 in a loading phase (L1 baseline, L2 day 14, L3 day 28, and L4 day 63) and a maintenance phase (M1-Mx, every four months). Cerebrospinal fluid (CSF) samples were collected at each administration; NfL was tested at each time point, additional neurodegeneration biomarkers total tau (t-Tau) and phosphorylated tau (p-Tau) proteins were tested at time points L1 and L3. PFN-1 was tested at each time point as an exploratory muscular biomarker. Further testing of PRPH was conducted at baseline on 20 type 3 patients. NfL concentration was determined with a commercial enzyme-linked immunosorbent assay (ELISA) kit (UmanDiagnostics, Umea, Sweden). T-Tau and pTau were measured with an automated Chemiluminescent Enzyme Immunoassay (CLEIA) analyzer (LUMIPULSE G600 II by Fujirebio, Japan); PFN-1 was measured in serum with a commercial ELISA kit (Cusabio, China); PRPH was tested in serum with a commercial ELISA kit (Abbexa, UK). Both PFN-1 and PRPH results were compared each to a control group of healthy subjects (HC). Neuromuscular outcomes were tested at L1, L4, M1, M2 and M3 with appropriated validated motor scales.

Baseline CSF NfL, t-Tau and p-Tau levels were overall included in the reference ranges for healthy donors. Mean NfL was 211.97 ± 180.9 ng/L in SMA patients, compared to 809.53 ± 1,065.26 ng/L in controls. Correlation was found between baseline log[NfL] and age both in SMA patients and control group. Also log[t-Tau] and log[p-Tau] correlated with log[NfL] at L1, but not at L3, although a slight significant increase was found in t-Tau and p-Tau at L3. NfL significantly increased in loading phase until L3 (mean increase 285.45 ng/L). From L4 NfL started to decrease and no significant difference was found with baseline at M1, M2 and M3. PFN-1 at baseline was higher in SMA than in healthy controls (mean 989 vs 608 ng/L, p=0.0018). PFN-1 showed a complex dynamic during loading phase, with a significant reduction at L4. PRPH was significantly higher compared to HC (median 5.653 µg/L vs 3.168 µg/L, p=0.02). No correlation was found between NfL and motor scores at each time point.

Our study partially reinforces recently published results in similar patients (Wurster, et al., 2019) (Wurster, et al., 2020) (Faravelli, et al., 2020), adding insights on NfL dynamic during the first month of treatment. Neurodegenerative biomarkers might inadequately relate to long disease duration. Serum PFN-1 and PRPH might be more sensitive to reveal LMN damage, therefore subsequent studies are needed to clarify their potential prognostic value.

Neurofilaments as a possible biomarker in adult SMA type 2 and 3 patients undergoing Nusinersen treatment

Introduction

Spinal Muscular Atrophy (SMA) is an autosomal recessive disorder due to progressive degeneration of lower motor neurons resulting in muscle atrophy, proximal limbs and axial muscle weakness and possible respiratory and bulbar involvement. It is a severe disease, leading cause of inherited infant death, whose incidence is 1/11000 of live births, though onset can occur at more adult age; prognosis depends on clinical phenotypes with most serious forms fatal by the age of 2 years old (Waldrop & Kolb, 2019).

Pathogenic hallmark is the loss of function of Survival Motor Neuron Protein (SMN), encoded by mutated or deleted survival motor neuron 1 (*SMN1*) gene located in 5q11.2-q13.3 (Lefebvre, et al., 1995). *SMN2*, the centromeric paralogous gene of *SMN1*, produces only a small amount of functional SMN due to the exclusion of exon 7 in mRNA transcript (Levin, 2019).

Molecular genetic testing is the fundament of SMA diagnosis following clinical suspicion: homozygous deletion of exon 7 of *SMN1* gene (often coexisting with exon 8 deletion) has a sensitivity of 95% and a specificity of almost 100% (Mercuri, et al., 2012). *SMN2* gene can be present with various copy number, ranging from 0 to 4 per chromosome 5 in the general population, and SMA patient usually have at least 1 copy; molecular quantification of *SMN2* copy numbers should be routinely performed because of its prognostic implications: less severe types 3 and 4 have generally a higher copy number, although exceptions might be encountered at individual level (Mercuri, et al., 2018).

SMA is clinically classified based on the maximum achieved motor function and on the age of onset in five phenotypes (type 0 to type 4) (Mercuri, et al., 2012). Type 3 SMA patients are also divided into *sitters* and *walkers*, depending on the current maximum motor function (Mercuri, et al., 2018).

Consensus statements on the standard of supportive care have contributed in the last decades to an improvement in natural history of every SMA phenotype (Mercuri, et al., 2018). Nevertheless, Nusinersen, a *SMN2* splicing modifier able to promote the inclusion of exon 7 in the *SMN2* transcript and to increase the production of functional SMN protein, is the first

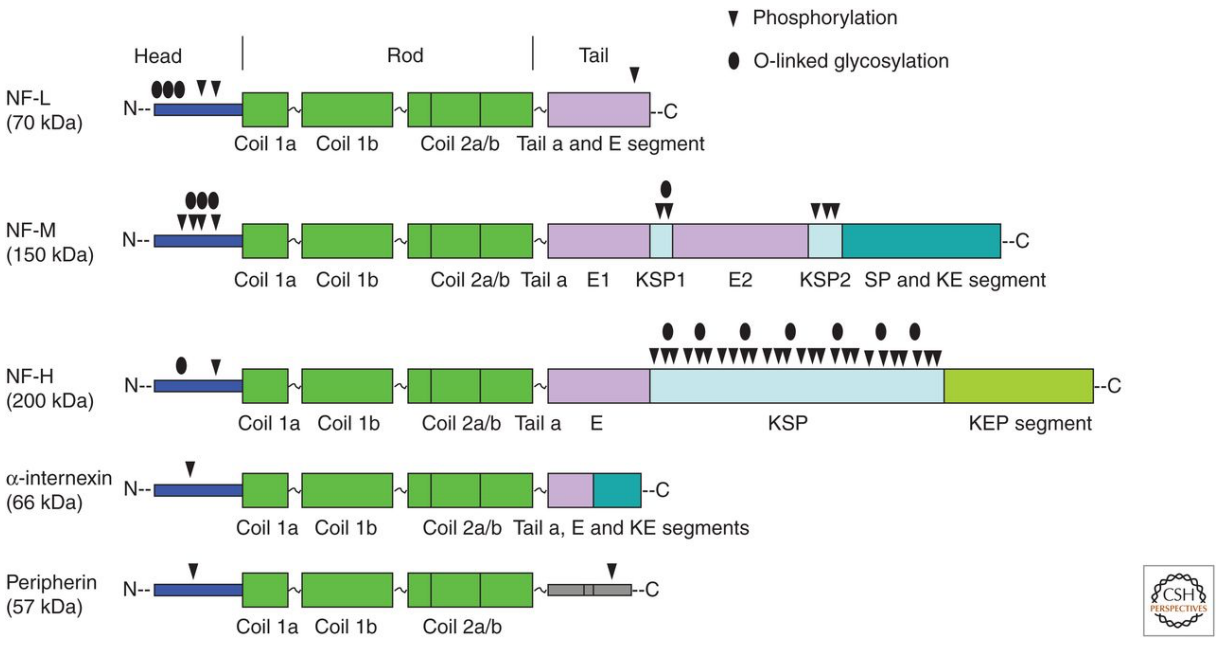
disease modifying therapy approved for adult SMA patients, following the results of clinical trials (Finkel, et al., 2017) (Mercuri, et al., 2018). Nusinersen is an antisense oligonucleotide (ASO) that must be administered intrathecally because it does not penetrate the blood-brain barrier; once in the CNS it distributes in the CSF and is cleared in the systemic circulation, with reported half-life of 135-177 days in the CSF (Neil & Bisaccia, 2019).

This and other novel emerging therapies will deeply change SMA natural history and stress the need for reliable outcome measures to capture clinical changes and to assess therapy response, alongside to validated clinical assessment scores.

Neurofilaments

Neurofilaments (NFs) are class IV intermediate filaments exclusively expressed in neurons, where they contribute to maintaining stability and growth of axons and the impulse conduction (Yuan, et al., 2017). Based on their molecular weight on SDS-polyacrylamide gel electrophoresis (Julien & Mushynski, 1983), NFs are classified in light chain (NfL), middle chain (NfM) heavy chain (NfH), α-internexin and peripherin (*Figure 1*) (Yuan, et al., 2017). NfL is the main core component of neurofilaments in central nervous system (CNS), together with α-internexin, where peripherin is the major associated component in the peripheral nervous system (PNS); NfM and NfH localize more peripherally (Yuan, et al., 2017).

Figure 1 Domain structure and posttranslational modifications of neurofilament (NF) subunits (Yuan, et al., 2017)



Almost twenty years ago a first highly sensitive and specific monoclonal immunoassay for the detection and quantification of NFL in cerebrospinal fluid (CSF) was described (Norgren, et al., 2003); in this Enzyme-Linked Immunosorbent Assay (ELISA) with a large measuring range of 60-64000 ng/L, NFL concentration was up to 600 folds higher in patients with cerebral infarction, amyotrophic lateral sclerosis (ALS) and Multiple Sclerosis (MS) than in healthy controls. Subsequent international efforts were soon undertaken to validate the laboratory assay, pointing that under controlled conditions the inter-laboratory agreement might be acceptable and that inter-laboratory coefficient of variation (CV) <15% should be a target (Petzold, et al., 2010). Growing interest for NfL as biomarkers of neuronal injury further led to assess whether its concentration in the peripheral circulation (i.e. serum or plasma), yet more accessible specimens for the long-term follow-up of chronic diseases or disabling neurological events, might reflect intrathecal setting. In a study involving MS patients undergoing intrathecal administration of rituximab through a surgical inserted ventricular catheter, Bergman et al. found a robust correlation between CSF and serum NfL concentrations measured with the same immunoassay transferred on a single-molecule array (Simoa) platform (Bergman, et al., 2016), with CSF containing more than 20-folds greater NfL quantity than in blood. As already suggested, though, the pathophysiological context should be carefully considered before assuming a permanent correlation between CSF and peripheral blood, since Nfs might originate also from PNS (Gafson, et al., 2020).

Nevertheless, further comparative works evaluated CSF and serum NfL with laboratory methods holding different analytical sensitivity: in a seminal paper, Kuhle et al. demonstrated strong CSF-serum correlation in paired samples on ELISA, electrochemiluminescence immunoassay (ECL) and Simoa (Kuhle, et al., 2016); moreover the three platforms showed a high agreement on CSF, while serum levels were more comparable between Simoa and ECL, due to lower analytical sensitivity of ELISA (78 ng/L, versus 0.62 ng/L and 15.6 ng/L of Simoa and ECL respectively).

Nfs levels raise as a result of axonal damage (Khalil, et al., 2018) (Gaetani, et al., 2019), NfL in particular has proven to be a sensible biomarker for axonal white matter injury (Bergman, et al., 2016), and seem to be promising diagnostic and prognostic biomarkers also in motor neuron diseases, especially ALS, where motor neurons with long axons are damaged, releasing high amount of axonal proteins (Gaiani, et al., 2017) (Benatar, et al., 2018) (Lu, et al., 2015) (Steinacker, et al., 2016).

Therefore, the need for robust clinical laboratory validation of this and other blood-based biomarkers holding the potential for dramatically improving the management of rare neurological disorders has already been remarked (Musso & Plebani, 2019).

More recently, Nfs have been investigated also in SMA patients, with controversial results. Indeed, while SMA type 1 patients showed elevated CSF and serum NfL and phosphorylated NfH (pNfH) levels at baseline, with a decline during nusinersen treatment inversely related to CHOP-INTEND score values (Finkel, et al., 2017) (Darras, et al., 2019) (Winter, et al., 2019) (Olsson, et al., 2019), limited evidence concerning type 2 and 3 patients is currently available. Wurster and colleagues (Wurster, et al., 2019) did not detect elevated NfL and pNfH CSF levels in adolescent and adult patients compared to controls, neither before nor during therapy with nusinersen.

Initial longitudinal analysis of serum NfL during nusinersen treatment (baseline, 6 months, 10 months, 14 months) revealed almost stable levels (Wurster, et al., 2020) and a first study concerning loading phase only (Totzeck A., 2019) analyzed CSF and serum NfH in 11 type 3 SMA patients and found broadly stable values within the normal range at baseline, 14 days, 28 days and 2 months.

Interestingly, concentration of total tau protein (t-Tau), currently a steady biomarker of cortical degeneration in dementias, in CSF of SMA type 1 children were found also high at baseline and decreasing along with NfL following ASO therapy (Olsson, et al., 2019) (Winter, et al., 2019).

<u>Exploratory biomarkers: Profilin 1 (PFN-1) and Peripherin (PRPH)</u>

PFN-1 is a small actin-binding protein that promotes actin polymerization; it is required in both the presynaptic and the postsynaptic compartment and has a role in regulating cytoskeletal architecture and dynamics of neurons (Witke, 2004). Cytoskeletal defects have been identified in several motor neuron diseases and mutations in *PNF-1* gene were recently reported in 1-2% of familial ALS (Wu, et al., 2012). As PFN-1 directly interacts with SMN and a colocalization of PFN-1 and SMN has been demonstrated in cultured cells and in mouse motoneurons (Giesemann, et al., 1999), PFN-1 showed as a candidate exploratory pathogenic biomarker for SMA patients.

PRPH is an intermediate filament which assembles with NFs mostly in neurons of the peripheral nervous system (PNS) (Yuan, et al., 2017) and in neurons of the central nervous system (CNS) with projections to peripheral structures, such as lower motor neurons (LMNs) (Zhao & Liem, 2016). PRPH function is incompletely understood though a role in neurite growth and stability has been suggested, therefore it might be considered as a potential candidate biomarker in motor neuron diseases (MND).

Aim of the study

This study aims to investigate the role of NfL as disease and treatment-response biomarker in a cohort of adult SMA type 2 and type 3 patients during loading and maintenance nusinersen dosing, exploring its correlation to validated neurodegeneration biomarkers as total tau (t-Tau) and phosphorylated tau (p-Tau) proteins and to Human Profilin-1 (PFN-1) and Human Peripherin (PRPH) as exploratory biomarkers.

Materials and methods

Study population.

In our study we recruited 3 SMA type 2 and 30 SMA type 3 patients who referred to the Neuromuscular Center of University-Hospital of Padova for nusinersen treatment from February 2018 to September 2021. All the patients had a molecularly defined 5q SMA diagnosis and they were divided into two subgroups, *sitters* and *walkers*, based on actual motor function status. All patients underwent nusinersen intrathecal therapy (12 mg each dose) according to the administration protocol approved by AIFA, which requires:

- loading phase, composed by four administrations: *loading dose 1 (L1)* at the baseline; *loading dose 2 (L2)* after 14 days; *loading dose 3 (L3)*, at the 28th day from baseline, and *loading dose 4 (L4)*, at the 63rd day from baseline.
- maintenance phase in which nusinersen was administrated every four months (M_n).

Data from the CSF analysis of NfL were compared to a control group of 44 patients recruited for a previous study conducted at the Neurological Department of Padova (Gaiani A, 2017). This group includes patients who underwent lumbar puncture for mononeuropathies, primary headache and patients sampled to exclude meningitis or inflammatory CNS diseases and whose diagnostic workup excluded neurological disorders.

Results on safety and efficacy of nusinersen treatment in our patients have been recently published together with a large Italian cohort of adult SMA (Maggi, et al., 2020).

Neuromuscular evaluation

Neuromuscular evaluation was conducted in all patients before the beginning of the therapy (L1), at the end of the loading phase (L4), and thereafter every 4 months at each maintenance dose (M1, M2 and M3). The neuromuscular assessment protocol included validated motor scales as Hammersmith Functional Rating Scale Expanded (HFMSE) (Glanzman, et al., 2011), Revised Upper Limb Module (RULM) (Mazzone E.M., 2017) and the Six-Minutes Walk test (6MWT) for walkers patients (Dunaway Young S., 2016). We additionally performed Medical Research Council (MRC) in all the patients (as average score calculated from evaluation of head extensor and flexor, shoulder abductors and flexors, brachial biceps and triceps, brachioradialis, external and internal rotators of upper limbs, extensors and flexors of carpi, flexor digitorum superficialis and profundus, extensor digitorum, thumb abductor, thigh

extensors, thigh adductors and abductors, leg flexors, quadriceps, anterior and posterior tibialis, sural triceps, peroneal muscles) and North Star Ambulatory Assessment (NSAA), considered appropriate evaluation tool for ambulatory patients (Tiziano, et al., 2013) (Mercuri, et al., 2008) (Werlauff, et al., 2012).

Cerebrospinal fluid (CSF) and serum biomarkers analysis.

CSF samples were collected within therapy administration procedure. Just before drug administration (12 mg, 5 mL) an equal CSF volume was collected. Patients with severe scoliosis or history of vertebral arthrodesis underwent a CT scan-guided procedure in Neuroradiology Unit of University-Hospital of Padova.

CSF samples were collected at each therapy infusion during loading dose phase and maintenance phase, until the sixth administration (M6); paired serum samples were collected at each time point, when available.

CSF samples were centrifuged, aliquoted and then stored in polypropylene vials at -80°C.

NfL quantitative determination has been performed at each time point from L1 to M3 and then at M6 as last follow up with NF-light® enzyme-linked immunosorbent assay (ELISA) in vitro diagnostic kit (UmanDiagnostics, Sweden) according to manufacturer instructions. Briefly: lyophilized bovine NfL standard was reconstituted with aqueous buffered solution with detergent to obtain a concentration of 10000 ng/L, a standard dilution series was then prepared at 5000, 2500, 1000, 500, 100 and 0 ng/L; CSF samples were diluted 1:1 also with aqueous buffered solution with detergent, then 100 µl each of standards and samples were added in the plate wells previously washed three times with wash buffer and incubated for 1 hour at room temperature (RT) on a plate shaker; after three washing cycles, 100 µl of freshly diluted biotin labelled anti NF-L monoclonal antibody was added to each well and the plate was incubated for 45 minutes at RT with agitation; following three more washing cycles, 100 µl of freshly diluted Streptavidine Horseradish peroxidase conjugate was added to each well and the plate was incubated for 30 minutes at RT with agitation; after three more washing cycles and the addition of 100 µl of tetramethylbenzidine substrate, the plate was incubated for 15 minutes at RT with agitation, then the immunoreaction was stopped with the addition of 50 µl of H_2SO_4 solution and the absorbance of each well was read with an automated reader at the wavelength of 450 nm; inter-assay CV calculated with repeated samples from the control group as internal quality control was <15%.

T-Tau and pTau were measured at time points L1 and L3 with a fully automated Chemiluminescent Enzyme Immunoassay (CLEIA) analyzer (LUMIPULSE G600 II by Fujirebio, Japan).

PFN-1 was measured with a commercial manual ELISA kit (Cusabio, China) in CSF samples of 6 patients (3 sitters 3 walkers) at time points L1, L3 and M2 and in every available serum sample from time point L1 to time point M6. PFN-1 was also tested in 19 serum samples of healthy donors as a control group, samples were anonymized and classified as diagnostic leftovers.

PRPH was measured with a commercial manual ELISA kit (Abbexa, UK) in CSF and serum samples of time point L1 from 20 SMA type 3 patients; patients with other neurological disorders (ALS, Spinal-Bulbar Muscular Atrophy, Alzheimer Dementia, Peripheral Neuropathies) and healthy controls were tested as control groups. Results of PRPH in biofluids of patients with motor neuron diseases have been recently published (Sabbatini, et al., 2021).

Statistical analysis.

Variables were summarized as mean, standard deviation (SD) or median and range as appropriate. Distributions of quantitative and ordinal variables between groups were compared with the Wilcoxon-Mann-Whitney test or Student's t test as appropriate. Correlation between quantitative and/or ordinal variables was tested with the Spearman method. Concurrent effects of age, SMA diagnosis and tau protein levels on log-transformed concentration of neurofilaments were tested in multivariate ANOVA/ANCOVA models as appropriated. The distribution of outcome measures and CSF and serum biomarkers at different time points was compared to baseline with the Wilcoxon test for paired data. Statistical significance was set at $p < 0.05$. Analyses were performed with R v.3.5.3.

Results

Population study.

As resumed in *Table 1*, in our study we recruited 3 SMA type 2 and 30 SMA type 3, 19 males and 11 females, with average age of 35.5 years old (range 14-68). Mean disease duration was 29.6 years; among SMA type 3 14 patients lost the ambulation, while 16 patients were walkers at the time of the study. All the patients had molecularly confirmed SMA diagnosis, 24 presented *SMN1* homozygous deletion of exon 7-8, 3 homozygous deletion of exon 7. Three patients were compound heterozygous for an exon 7 deletion on one allele and a point mutation on the other (c.734C>T - p.Pro245Leu: SMA 3b, walker; c.305G>A - p.Trp102Ter: SMA 3a, walker with assistance; c.469C>T - p.Gln157Ter: SMA 3a, walker with assistance). All these mutations are already described in literature (Brichta L., 2008; https://databases.lovd.nl/shared/variants/SMN1/unique). Of our type 3 SMA patients, 18 had 4 *SMN2* copies, 11 had 3 *SMN2* copies and only one had 2 *SMN2* copies, according to literature data (Calucho M., 2018).

Motor scales values at baseline (L1) are shown in *Table 2*; of our 16 ambulatory patients one refused to perform NSAA scale and 6MWT, and 3 refused to do both motor tests.

Table 1: Demographic and clinical data of the study population

	SMA type II	**SMA type 3**			**Total**
		Sitter	**Walker**	**Total**	
n =	3	14	16	30	33
Male / Female	3 / 0	12 / 2	7 / 9	19 / 11	22 / 11
Age (range, y)	20.5 y (14 – 22)	34.5 y (20 – 57)	37.18 y (17 – 68)	36.5 y (17 – 68)	35.5 y (14 – 68)
Age of onset (range, SD)	11.16 months (9.6 – 12, ±1.32)	3.43 y (1-7, ± 2.43)	11.75 y (0.25-28, ± 6.5)	7.9 y (1-28, ± 6.53)	7.12 y (0.25 – 28, ±6.53)
Disease duration (y)	19 y (15 – 23)	33 y (14 – 52)	28.8 y (10 – 51)	30.8 y (10 – 52)	29.6 y (10 – 52)
Age at loss of ambulation (y, range, SD)	Not acquired	18.7 y (4-47) ±13.3)	-	-	-
SMN 1					
- Deletion exon 7-8	3	13	11	24	27
- Deletion exon 7	-	-	3	3	3
- Compound heterozygosity	-	-	3	3	3
SMN2 copies					
- 2	0	0	1	1	1
- 3	2	6	5	11	13
- 4	1	8	10	18	19
NIV					
- None	1	13	15	28	29 (88%)
- Intermittent	2	1	1	2	4 (12%)
- Permanent	0	0	0	0	0
Arthrodesis	3	4	1	5	8 (24.2%)
Salbutamol	0	2	2	4	4 (12%)
Physical therapy	2	12	11	23	25 (74%)

Table 2: Baseline (L1) functional data

	Group	N	Mean	SD	Median	Min	Max
HFMSE	**SMA II**	3	2	2	2	0	4
	SMA III sitters	14	6.79	7.84	3.5	0	27
	SMA III walkers	16	47.12	12.6	48.5	27	62
	Tot.	33	25.9	23.19	27	0	62
RULM	**SMA II**	3	14	8.72	18	4	20
	SMA III sitters	14	16.36	9.68	17	0	31
	SMA III walkers	16	34.44	3.74	37	25	37
	Tot.	33	24.9	11.76	29	0	37
MRC	**SMA II**	3	2.13	0.46	2.19	1.64	2.56
	SMA III sitters	14	2.5	0.64	2.57	1.17	3.35
	SMA III walkers	16	3.91	0.45	3.88	3.3	4.59
	Tot.	33	3.14	0.92	3.32	1.17	4.59
NSAA	**SMA III walkers**	15	17.6	10.11	15	3	30
6MWT (m)	**SMA III walkers**	13	367.23	119.16	345	121	588

Baseline CSF NfL, t-Tau and p-Tau.

Baseline CSF NfL, t-Tau and p-Tau (**Table 3**) levels were overall included in the reference ranges for healthy donors provided by testing assay manufacturers (NfL < 290 ng/L age < 30 yr, < 380 ng/L < 40 yr, < 830 ng/L < yr 60; t-Tau 146-410 ng/L, p-Tau 21,5-59 ng/L). In one patient aged 68 years old NfL measure was minimally above reference, this sample was pre-diluted due to small volume and intra-assay CV was >20%. Mean NfL was 211.97 ± 180.9 ng/L in SMA patients, compared to 809.53 ± 1,065.26 ng/L in controls. No significant difference was found between *sitters* group and walkers group. Baseline NfL values were also analyzed considering SMA phenotype and SMN2 copy number *(Table 4*), and no differences were found.

Correlation was found between baseline log[NfL] and age (F*igure 2*) both in SMA patients ($r=0.69$, $p<0.0001$) and control group ($r=0.75$, $p<0.0001$). ANCOVA on the entire population showed independent effects of age and SMA versus controls ($p<0.0001$ and $p<0.001$). Also log[t-Tau] and log[p-Tau] correlated to log[NfL] ($r=0.48$, $p=0.005$ and $r=0.53$, $p=0.002$ respectively) (*Figure 3*).

Table 3 CSF biomarker at baseline (ng/L)

Group		NfL	t-Tau	p-Tau 181
SMA II	**Mean**	149.33	239	27.2
	SD	48.8	236.54	14.29
	Median	129	133	29.5
	Min	114	74	11.9
	Max	215	510	40.2
SMA III Sitter	**Mean**	219.07	210.57	31
	SD	120.44	82.52	13.3
	Median	181.5	179	29.3
	Min	111	106	14.5
	Max	502	418	70.2
SMA III Walker	**Mean**	217.5	146.93	23.1
	SD	236.75	72.8	11
	Median	162	115	20.7
	Min	41	75	10
	Max	1068	298	44.3
Total	**Mean**	211.97	182.30	26.8
	SD	180.9	100.11	1250
	Median	168	160	23.3
	Min	41	74	10
	Max	1068	510	70.2

Table 4: Baseline CSF [NfL] values of SMA patients in relation to clinical phenotype and SMN2 copy number

	[NfL] baseline (ng/L)					
	N	**Mean**	**SD**	**Median**	**Min**	**Max**
SMA II	3	149.33	48.8	129	114	215
SMA III Sitter	14	219.07	120.44	181.5	111	502
SMA III Walker	16	217.5	236.7	162	41	1068
***SMN2* = 2**	1	268	-	-	-	-
***SMN2* = 3**	13	155.62	36.78	147	111	231
***SMN2* = 4**	19	247.58	231.22	183	41	1068
TOT	33	211.97	180.9	168	41	1068
Control group	44	809.53	1065.26	395.53	75.75	5927.88

Figure 2: Correlation between log[Nfl] and age.

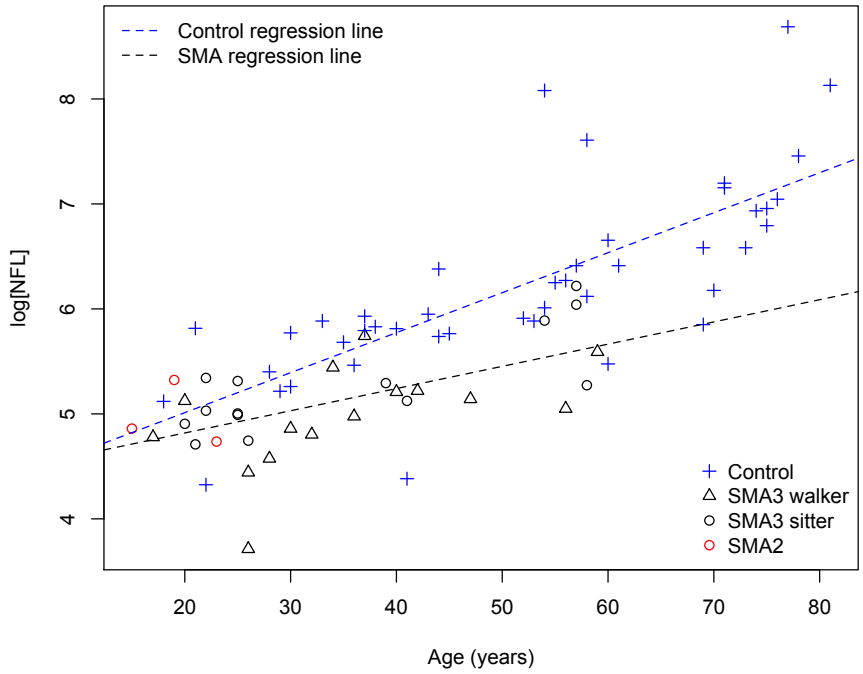
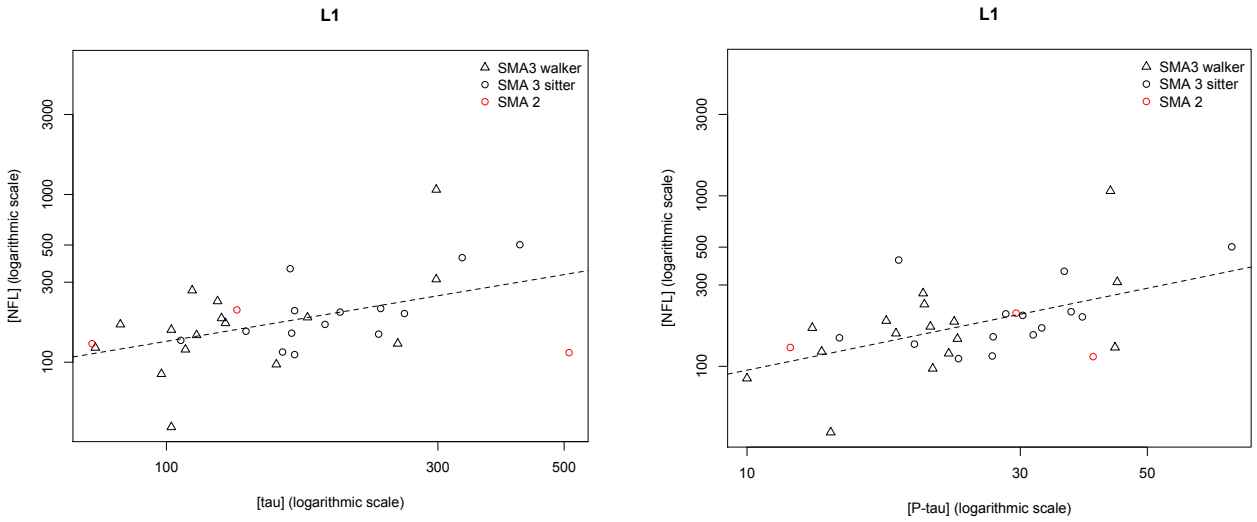


Figure 3: Correlation between CSF log[NfL] and [t-Tau] and [p-Tau 181] at baseline



Longitudinal analysis of CSF biomarkers.

CSF NfL was analyzed at baseline L1, L2, L3, L4, M1, M2 and M3 (*Table 5*).

A significant increase in mean NfL concentrations was measured during loading phase until L3 (*Figure 4*), with a mean increase of 285.4 ng/L from the baseline. From L4 NfL started to decrease and no difference was found with baseline at M1, M2 and M3; at 26 months follow-up (M6) concentration was slightly higher than baseline (p = 0.0022), but not significantly different from M3.

No correlation was found between NfL values and HFMSE, RULM, 6WMT and NSAA scores at each time point (data not shown).

A slight significant increase was found in t-Tau (p = 0.022) and p-Tau (p = 0.004) concentrations at time point L3 (*table 6*, *figure 5*). At this timepoint value of CSF log[t-Tau] and log[p-Tau] was not correlated with CSF log[NfL] (*figure 6*).

Figure 4: Longitudinal analysis of CSF log[NfL] during treatment course (p compared to baseline)

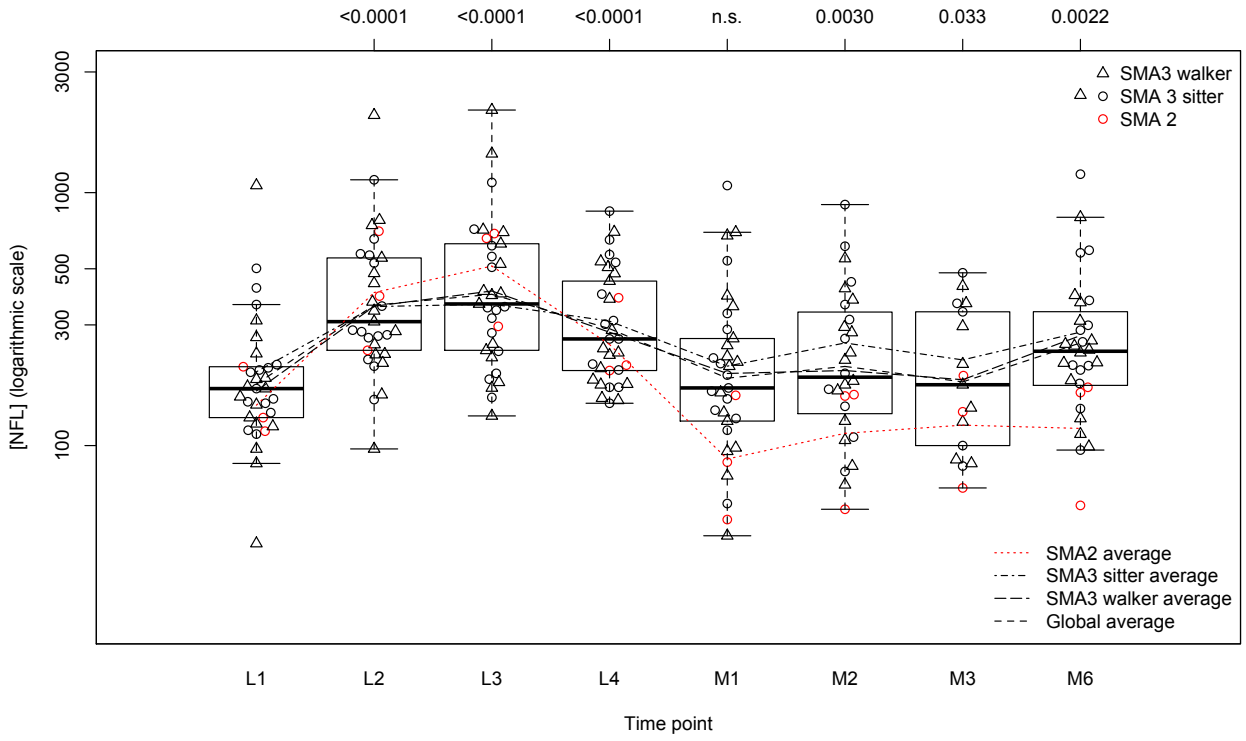


Table 5: CSF NFL ng/L descriptive statistics at each time point

	Group	N	Mean	SD	Median	Min	Max
	Control group	44	809.53	1065.26	395.53	75.75	5927.88
L1	**SMA II**	3	149.33	48.78	129	114	205
	SMA III sitters	14	219.07	120.44	181.5	111	502
	SMA III walkers	16	217.5	236.75	162	41	1068
	total	33	211.97	180.89	168	41	1068
L2	**SMA II**	3	444	237.64	390	238	704
	SMA III sitters	14	411.36	259.07	284	152	1124
	SMA III walkers	16	468.62	458.22	325	97	2025
	total	33	442.09	360.53	309	97	2025
L3	**SMA II**	3	548.33	219	660	296	689
	SMA III sitters	14	422.14	258.56	347	155	1097
	SMA III walkers	16	553.75	525.56	396	131	2121
	total	33	497.42	404.90	363	131	2121
L4	**SMA II**	3	263.33	104.61	208	198	384
	SMA III sitters	14	359.29	211.94	283	147	845
	SMA III walkers	16	317.06	168.12	237.5	151	699
	total	33	330.09	181.82	264	147	845
M1	**SMA II**	3	98.33	54.55	86	51	158
	SMA III sitters	14	269.64	257.92	179.5	59	1067
	SMA III walkers	16	250.81	195.40	210	44	697
	total	33	244.94	217.73	169	44	1067
M2	**SMA II**	3	124	58.89	157	56	159
	SMA III sitters	11	326	247.58	265	79	897
	SMA III walkers	14	233.64	138.40	199	70	549
	total	28	258.18	190.70	186.5	56	897
M3	**SMA II**	3	131	60.65	136	68	189
	SMA III sitters	5	273.6	174.93	338	83	482
	SMA III walkers	9	212.22	123.71	174	85	427
	total	17	215.94	134.76	174	68	482
M6	**SMA II**	3	130	62.48	162	58	170
	SMA III sitters	13	353.54	291.91	257	96	1183
	SMA III walkers	16	404.81	564.12	243.5	99	2430
	total	32	358.22	439.76	236	58	2430

Table 6: Longitudinal [t-Tau] (on top) and [p-Tau 181] (table below)

Group	Time	Mean	SD	Median	Min	Max
SMA II	**L1**	181.6	121.1	156	74	510
	L3	211	100.3	177	138	396
SMA III Sitters	**L1**	210.6	82.5	179	106	418
	L3	254.1	150.2	179.5	84	635
SMA III Walkers	**L1**	146.9	72.8	118	75	298
	L3	184.8	67.7	162	103	322
Total	**L1**	182.30	100.11	160	74	510
	L3	219.00	116.97	177	84	635

Group	Time	Mean	SD	Median	Min	Max
SMA II	**L1**	26.6	11.5	29.5	11.9	40.2
	L3	31.4	10.1	30.4	24.8	33.2
SMA III Sitters	**L1**	31	13.3	29.3	14.5	70.2
	L3	35.3	14.1	30.4	15.2	59.4
SMA III Walkers	**L1**	23.1	11	20.7	10	44.3
	L3	29	10	25.8	17.5	53.5
Total	**L1**	26.8	12.5	23.3	10	70.2
	L3	31.83	11.82	29.9	15.2	59.4

Figure 5: Analysis of CSF log[t-Tau] and log[p-Tau 181] at L1 and L3

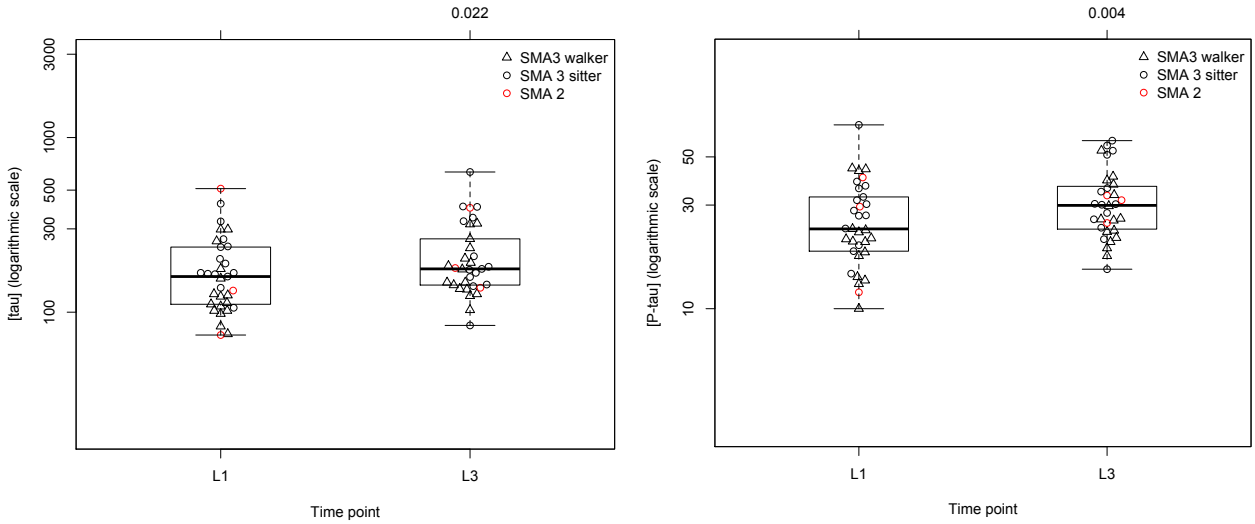
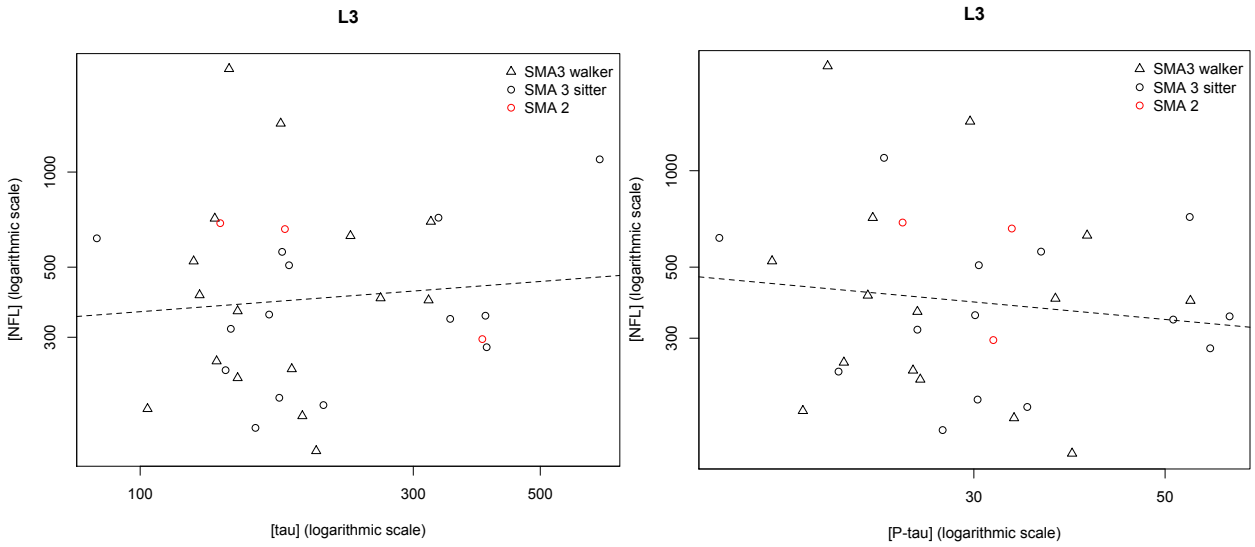


Figure 6: Correlation between CSF log[NfL] and [t-Tau] and [p-Tau 181] at L3 timepoint



Preliminary CSF results of PFN-1 and PRPH

Preliminary testing of PFN-1 in all 18 CSF samples (6 patients at 3 different time points) found concentrations below the lower limit of quantification (<31.25 ng/L). A spike and recovery experiment with assay standard (table 7), adapted from Andreasson et al. with modifications (Andreasson, et al., 2015), was then set up to partially validate CSF as a matrix for the specific assay kit, resulting in acceptable percentage of recovery (75-94%). Recovery was calculated as *%Rec = Measured OD / Expected value OD * 100*. Therefore, CSF concentrations were believed undetectable due to the analytical sensitivity of the assay and further testing were conducted in serum only, as prescribed by manufacturer.

Table7: spike and recovery test for PFN-1 in CSF

Sample	Spike specimen	Spike neat concentration ng/L	OD Spike	Spike dilution	Measured OD	Measured concentration ng/L	Expected value OD	%Rec
CSF 1					0,063	< 31.25		
CSF 1 +st250	assay standard	250	0,503	0.5 (1:2)	0,211	66.40	0,283	75
CSF 1 +st500	assay standard	500	0,892	0.5 (1:2)	0,448	204.51	0,4775	94

We also conducted spike and recovery test for PRPH in two CSF samples with two different spikes: assay standard solution at neat concentration of 20 µg/L and human serum at neat concentration of 9,66 µg/L and obtained results in table 8.

Table 8: spike and recovery test for PRPH in CSF

Sample	Sample neat concentration µg/L	Spike specimen	Spike neat concentration µg/L	Spike dilution	Measured concentration µg/L	%Rec
CSF A	0,456	assay standard	20	1:5	6,973	165
		assay standard		1:10	4,417	200
		assay standard		1:20	1,717	128
		serum sample	9,660	1:5	1,880	78
		serum sample		1:10	1,179	80
		serumsample		1:20	0,787	73
CSF B	0,434	assay standard	20	1:5	6,286	148
		assay standard		1:10	3,705	166
		assay standard		1:20	2,087	167
		serum sample	9,660	1:5	1,926	82
		serum sample		1:10	1,265	91
		serum sample		1:20	0,948	111

Recovery was calculated as *%Rec = (Measured concentration - Calculated initial concentration of neat sample)/Theoretical Spiked concentration*100*

Given an acceptance range of 80-120%, we noted a better recovery with human serum as spike specimen at each concentration, probably due to an optimized assay reaction with serum proteins rather than standard solution. However, we added spike and recovery test on serum sample and obtained comparable results with spiked assay standard, pointing that a negative matrix effect for CSF versus serum might be excluded. As results for CSF samples of SMA patients were all below the lower limit of quantification (< 0.312 µg/L), further analysis was conducted for serum only.

<u>Serum PFN-1</u>

Comprehensive results for time points L1-M3 are summarized in *table 9*; of note serum samples from SMA 2 patients were not available for each time point. As shown in *figure 7*, PFN-1 at baseline was higher in SMA group than in healthy controls (mean 989 vs 608 ng/L, p=0.0018 Student's t test). PFN-1 showed a complex dynamic during loading phase, with a significant reduction at L4 compared to baseline, different from NfL evolution both in sitters

and in walkers patients (*Figure 8*). Results of 32 samples from time point M6 were not directly compared to previous points because of a relatively high variance measured between different lot numbers of the ELISA kit, whose standardization will need to be further evaluated. A correction was applied on M6 concentrations for a mean bias of 198% calculated on 4 samples from the control group tested in each assay plate, resulting in the following descriptive statistics: mean 949, SD 391, median 936, min 180, max 1833 ng/L.

No correlation was found with NfL and motor scores at each time point.

Figure 7: serum PFN-1 at baseline compared to control groups

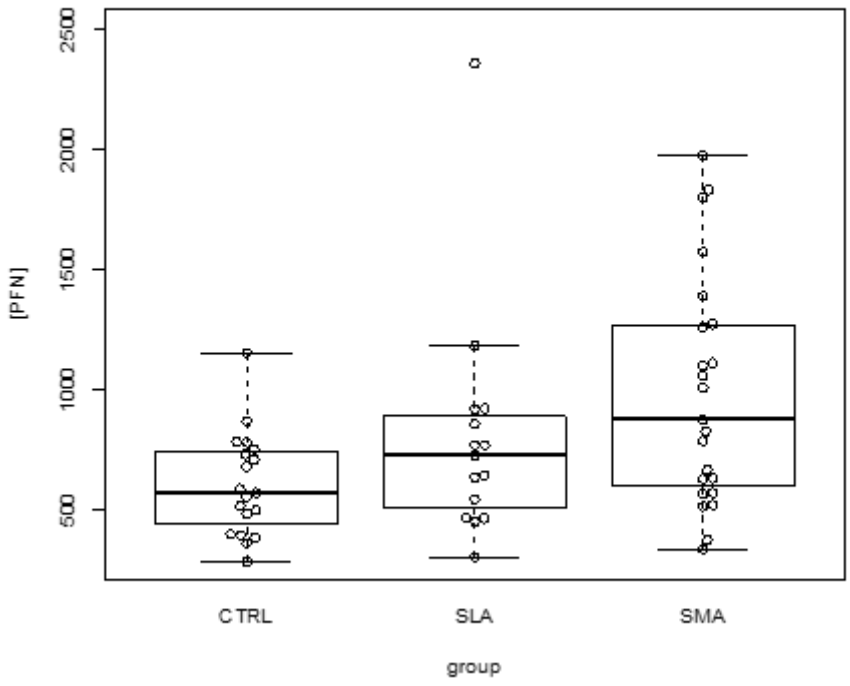
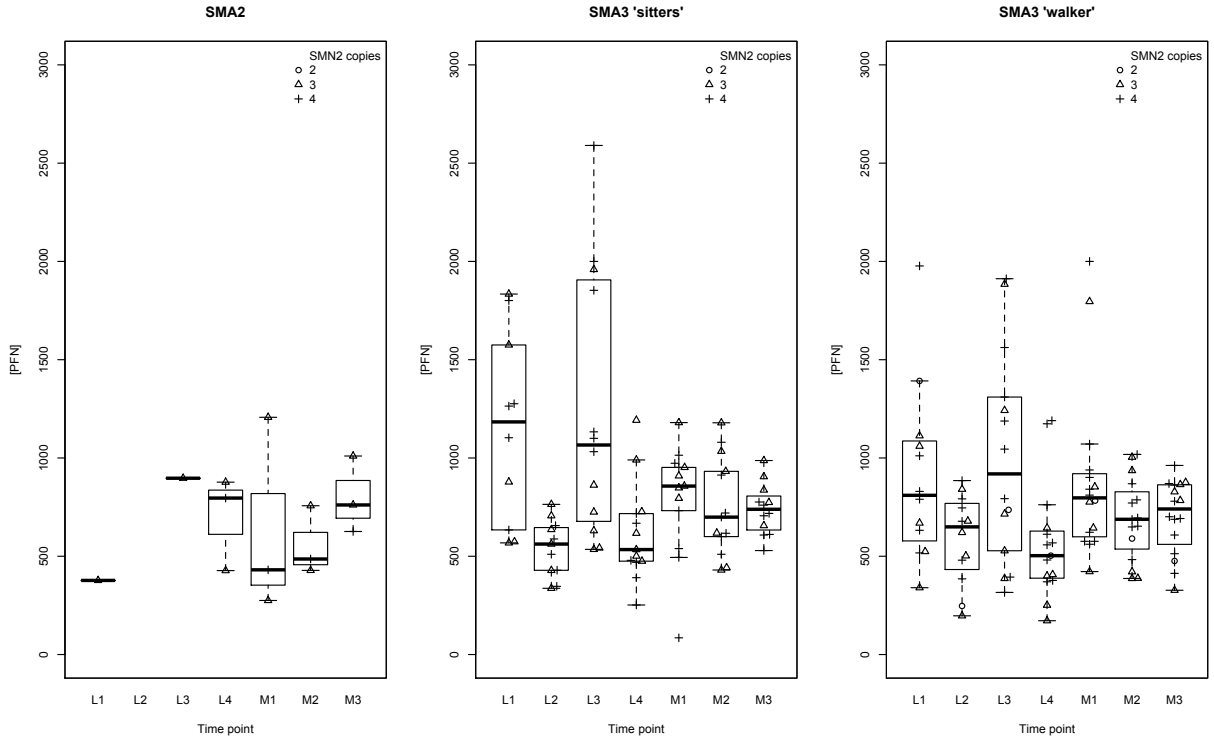


Table 9: serum PFN-1 ng/L descriptive statistics at each time point

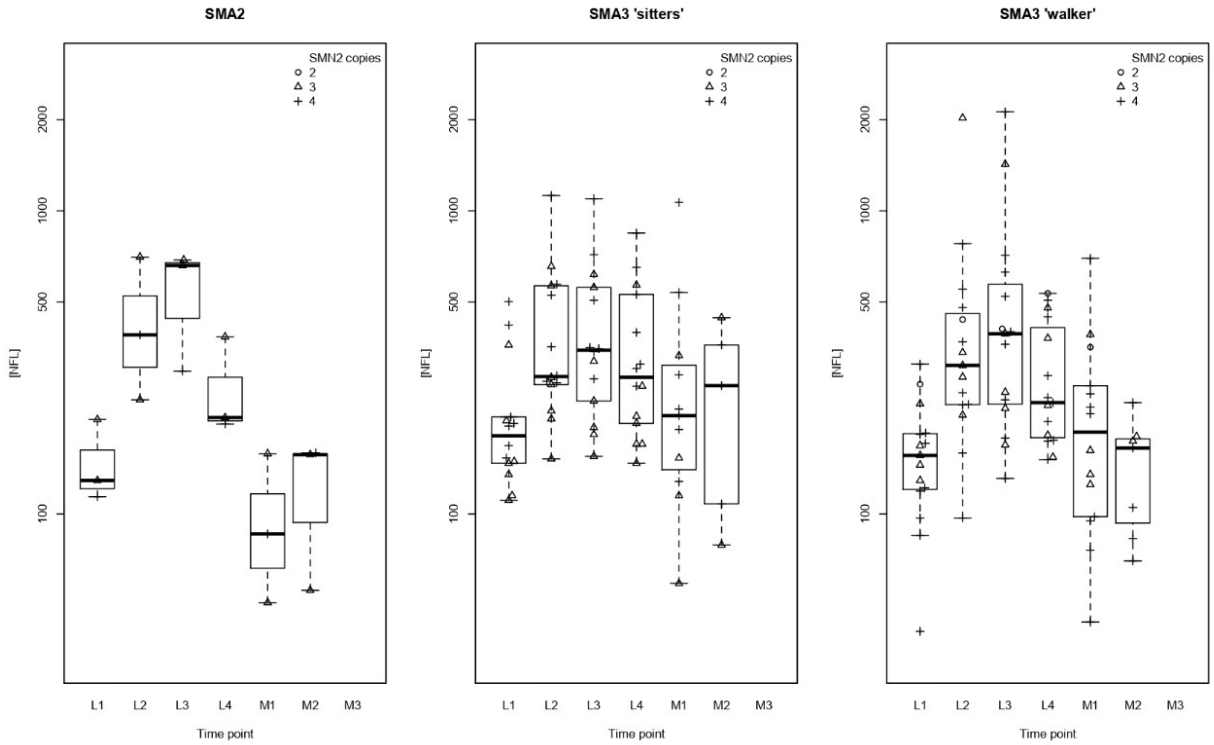
	Group	N	Mean	SD	Median	Min	Max
L1	**SMA II**	1	377	-	-	-	-
	SMA III sitters	10	1151	485	1183.5	568	1834
	SMA III walkers	12	905	450	810	340	1977
	total	23	989	480	878	340	1977
L2	**SMA II**	-	-	-	-	-	-
	SMA III sitters	11	542	144	562	337	764
	SMA III walkers	12	588	227	649.5	197	885
	total	23	566	189	588	197	885
L3	**SMA II**	1	897	-	-	-	-
	SMA III sitters	12	1247	683	1066	535	2590
	SMA III walkers	14	1001	537	919	316	1912
	total	27	1106	599	1032	316	2590
L4	**SMA II**	3	700	240	796	427	877
	SMA III sitters	13	616	252	534	252	1192
	SMA III walkers	15	565	293	503	172	1190
	total	31	599	266	534	172	1192
M1	**SMA II**	3	638	499	431	275	1207
	SMA III sitters	13	795	283	857	85	1180
	SMA III walkers	16	886	430	797	422	2000
	total	32	825	377	826	85	2000
M2	**SMA II**	3	557	176	486	428	757
	SMA III sitters	13	752	250	699	430	1179
	SMA III walkers	15	689	211	688	387	1018
	total	31	703	226	688	387	1179
M3	**SMA II**	3	799	195	761	626	1010
	SMA III sitters	12	739	131	739	529	987
	SMA III walkers	16	703	187	740.5	327	962
	total	31	726	165	760	327	1010

Figure 8: Longitudinal analysis of serum [PFN] A and CSF [NfL] B

A



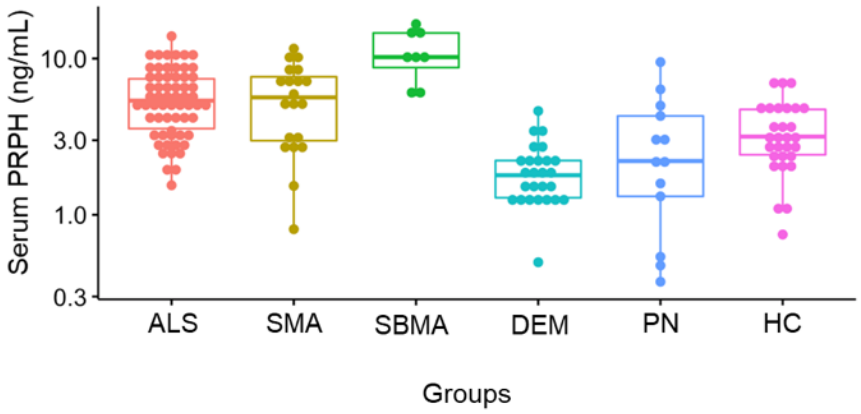
B



Serum PRPH

PRPH levels were significantly higher in SMA patients (median 5.653 µg/L; IQR 2.982-7.654 µg/L) compared to healthy controls (HC, median 3.168 µg/L; IQR 2.422-4.726 µg/L, p=0.02), dementia (DEM, median 1.793 µg/L; IQR, 1.287-2.226 µg/L) and Peripheral Neuropathies (PN, median 2.21 µg/L; IQR 1.313-4.293 µg/L); other MND groups, ALS and Spinal-Bulbar Muscular Atrophy (SBMA), also showed significantly higher concentrations (median 5.376 µg/L and 10.224 µg/L for ALS and SBMA respectively). Gender and age did not influence PRPH levels in any of the groups tested individually.

Figure 9 Serum PRPH concentrations in different disease groups (Sabbatini, et al., 2021)



Discussion

We evaluated CSF neurodegenerative biomarkers as possible outcome measures for adult SMA patients treated with recent approved antisense oligonucleotide (ASO) nusinersen. In our cohort of 33 SMA 2 and 3 (14 sitters and 16 walkers) patients we found no increase in the concentrations of NfL, t-Tau and p-Tau at baseline and after 14 months of treatment compared to accepted reference ranges for healthy donors; baseline NfL values did not differ significantly between clinical phenotypes nor between patients with different *SMN2* copy number.

We found a direct correlation between baseline CSF NfL and age, both in SMA and in control group. Analysis of covariance (ANCOVA) on the entire population showed independent effects of age on SMA versus controls, with SMA patients presenting CSF NfL values, when age corrected, lower than control group. Literature data show a direct correlation between CSF NfL and age (Vågberg, et al., 2015), with a 2.5-fold increase in NfL levels between the age of 20 years and 50 years (Gaetani L, 2019; Khalil M, 2018) and a reported exponential yearly increase (Yilmaz, et al., 2017). The effect of aging on NfL release has been recently reinforced by the extensive results of a prospective study on serum NfL of a community-based neurologically healthy population (Khalil, et al., 2020) that also excluded gender-related differences; the study measured a nonlinear yearly median increase of NfL concentration that was different per age group, moreover NfL concentration was a strong predictor of future brain volume loss.

Conversely, studies on early onset SMA patients reported an inverse correlation with age at SMA diagnosis for CSF pNfH (Darras BT, 2019) and no correlation between NfL and age (Olsson B, 2019).

Longitudinal CSF NfL analysis in our cohort during treatment showed a statistically significant increase during the loading phase, with a progressive mild decrease during the maintenance phase. These results are in contrast with the results observed in type 1 SMA (Winter B, 2019) (Darras BT, 2019) (Olsson B, 2019) but showed similar baseline values to other type 2 and 3 patients (Wurster CD, Neurofilament light chain in serum of adolescent and adult SMA patients under treatment with nusinersen, 2020) (Faravelli, et al., 2020). The reasons for these differences between early versus late onset SMA are not completely clear. High Nfs levels

might reflect neuronal plasticity developing early in CNS maturation or disease and Nfs level might tend to normalize later on. Indeed, as all type 1 SMA were recruited in the first year of life compared to much later age in adult, disease duration at time of CSF sampling might play a pivotal role. As Kariyawasam et al. have recently pointed out in a comprehensive review, plasma pNfH also declined in untreated patients at advancing age, questioning its utility as biomarker for treatment response and hypothesizing a reduction in motor neuron pool or in disease activity (Kariyawasam, 2019).

Supporting the recent considerations of Wurster et al. (Wurster CD, Neurofilament light chain in serum of adolescent and adult SMA patients under treatment with nusinersen, 2020), Lombardi et al. (Lombardi, 2019) found low NfL levels in serum also in patients with spinal and bulbar muscular atrophy (SBMA), in which the prominent LMN involvement seem to be better related to muscular rather than neurodegenerative biomarkers.

Confirming these results t-Tau and p-Tau levels in our SMA group were also within reference range, although longitudinal analysis of CSF t-Tau and p-Tau over L1-L3 timepoints showed a statistically significant increase.

Notably there are currently no data on NfL dynamic during the first month of the loading phase, as the previously published results (Wurster CD, Neurochemical markers in CSF of adolescent and adult SMA patients undergoing nusinersen treatment, 2019) (Wurster CD, Neurofilament light chain in serum of adolescent and adult SMA patients under treatment with nusinersen, 2020) (Faravelli, et al., 2020) encompassed time points at two and six months of administration.

In a cohort of progressive MS patients undergoing administration of rituximab through a permanent intrathecal catheter, NfL concentrations significantly increased of 5 folds over baseline at 1-month follow up after the first dose and then declined after 3 months and returned to baseline at 6; in this study an NfL peak after a neurosurgical procedure was an incidental finding that was presumed to be related to the main white matter lesion during catheter insertion (Bergman, et al., 2016). Interestingly, the frequent CSF sampling through lumbar punctures for an extended follow-up period was not considered responsible for this peculiar dynamic.

In our cohort NfL were not significantly correlated to clinical motor scores, while the latter, particularly HFMSE, revealed a statistically significant improvement in the functional condition due to therapy (Maggi, et al., 2020). This imbalance between clinical and biochemical status

seems to point out that Nfs might not be reliable prognostic biomarker in treated adult SMA patients.

Moreover, PFN-1 as an exploratory cytoskeletal biomarker changed significantly during the first two months of treatment, supporting the assumption of an increased neuronal remodeling during the loading phase. To our knowledge this is the first report of PFN-1 determination in serum of SMA patients.

PRPH was also first reported in body fluids of neurological patients, revealing higher concentrations in MND in general and in SMA patients. As already suggested, that might be ascribed to the more prominent LMN injury in the PNS occurring in these disorders (Sabbatini, et al., 2021).

Conclusions

Our study reinforces the differences observed in adult SMA patients compared to type 1, having the latter a better correlation to NfL levels as a surrogate treatment biomarker. Baseline NfL resulted within proposed reference range and did not correlate to clinical phenotype, SMN2 copy number and functional status. In wider terms, all putative biomarkers studied did not reveal a univocal trend, therefore implying that neurodegenerative biomarkers might inadequately relate to a degenerative disease marked by a long duration. NfL with confirmatory t-Tau and p-Tau testing at baseline did not highlight an ongoing central axonal degeneration process, while serum PFN-1 and even more PRPH might be more sensitive to LMN injury.

Some insights were indeed revealed about the possible biological effects of nusinersen treatment during the first month of treatment. Results of shorter time points in our cohort might suggest a putative limited axonal remodelling after ASO injection, although a transient side effect of lumbar puncture might not be excluded.

Additional studies are needed to explore novel candidate biomarkers that might help predict patients prognosis, monitor treatment response and optimize new therapeutic options and that might intercept a potential remodeling involving axonal or muscular compartments, or even neuromuscular junction. PRPH and exploratory synaptic biomarkers N-CAM, Agrin, FGF-21 and GDF-15 will be further tested.

Validation of a GAD65 antibodies chemiluminescence immunoassay for CSF in neurological syndromes

Introduction

Antibodies against glutamic acid decarboxylase isoform 65 (GAD65) have been found in different severe neurological conditions associated to altered synthesis of γ-aminobutyric acid (GABA), particularly stiff person syndrome (SPS) and its variants (including paraneoplastic), cerebellar ataxia (CA), epilepsy and limbic encephalitis (LE). SPS, first described in 1956 (Moersch & Woltman, 1956), is a rare disorder with a prevalence of 1-2 cases per million, mostly adults, and is more frequent in women; it has an insidious onset of axial muscle stiffness, progressively involving proximal and distal limbs, and within a chronic course pain, dysautonomic and psychiatric symptoms can occur (Baizabal-Carvallo, 2019). GAD-abs levels 100-folds higher than those found in Diabetes mellitus 1 (DM1) are reported up to 80% of SPS patients (Saiz, et al., 2008) and intrathecal synthesis in cerebrospinal fluid (CSF) is frequently described (Gresa-Arribas, et al., 2015) and support the correlation of autoantibodies with the neurological signs, though a direct pathogenic role is yet to be confirmed (Baizabal-Carvallo, 2019). Nevertheless, an international agreement on decisional level for GAD-abs in neurological diseases is still lacking.

GAD-abs are commonly tested in clinical laboratories with enzyme-linked immunosorbent assays (ELISA) specifically developed for serum to support DM1 diagnosis, so that results above the upper limit of detection (2000 KIU/L) are usually not quantified.

Chemiluminescence immunoassays (CLIA) for GAD-abs have recently proved good analytical performances compared to ELISA and have been validated for clinical practice in automated test systems (Cosma, et al., 2019), with linearity up to 280 KIU/L, due to the intended endocrinological target.

This study aims to validate CSF testing on a CLIA platform for neurological disorders and to determine a clinical cut-off or decisional level of GAD-abs in CSF for typical neurological GAD-related diseases.

Materials & Methods

CSF samples from neurological patients previously classified with GAD-related syndromes in preliminary work collected at Department of Neurology, Ospedale S. Antonio, Padova and Department of Neurology, Ospedale Ca' Foncello, Treviso (Zoccarato, unpublished) and CSF leftovers samples from diagnostic routine workup of Department of Laboratory Medicine, University-Hospital of Padova, from patients with other neurological conditions previously anonymized as control group were retrospectively tested on an automated CLIA system (MAGLUMI 2000 Plus by Snibe, China). CSF samples were stored at -20 °C after routine testing and thawed immediately before GAD-ab analysis. This commercial sandwich immunoassay is validated for in vitro diagnostic use for serum only; manufacturer declares a measuring range of 1.0-280.0 KIU/L with a limit of blank of 1 KIU/L, it has a traceable calibrator for GAD65 (WHO 1st Reference Reagent 97/550).

CSF samples were also tested on an ELISA commercial kit (RSR Limited, UK or Euroimmun, Germany) with an automated system (DSX by Technogenetics, Italy or Triturus by Grifols, Spain) and results were compared to CLIA assay.

Precision evaluation

CSF samples with low GAD-ab concentration were pooled and divided into three aliquots, calibrator 1 (13.7 KIU/L) and calibrator 2 (169.3 KIU/L) were added to an aliquot each; precision was estimated with triple measurements of each aliquot for three consecutive days, following a modified Clinical and Laboratory Standards Institute (CLSI) EP15-A3 protocol, and compared to manufacturer declared results appropriately interpolated using a linear function.

Linearity assessment

Two samples (Sample 53 measured GAD-ab level of 212.5 KIU/L, sample 130 88.8 KIU/L) were diluted with kit buffer (phosphate buffered saline containing bovine serum and NaN_3 <0.1%, previously tested for GAD-ab); different linear dilutions were prepared with a fixed final volume and tested in duplicate, average values were compared to expected GAD-ab concentrations as explained in the CLSI EP06 A:2003 guideline.

Statistical analyses

Clinical threshold was calculated with receiver-operator characteristic (ROC) analysis and Youden index cutoff with confidence (CI) intervals 95%. Proportional and constant bias between CLIA and ELISA were estimated with Passing-Bablok regression and Bland-Altman analysis. Analysis were performed using Stata v 16.1 (StataCorp, Lakeway Drive, TX, USA).

Results

CSF samples of 42 patients from Neurology Unit O.S.A. of University-Hospital of Padova were collected at the Department of Laboratory Medicine. 11 patients had clinical features possibly related to GAD-Ab (SPS, CA, Temporal epilepsy, Encephalitis), the other underwent lumbar puncture for the diagnostic workup of dementia, other phenotypes of epilepsy, infectious diseases, cerebrovascular disorders, headache, NMDAR encephalitis, oncologic diseases.

Precision

Repeatability and intermediate precision of CLIA assay in a 3-days analysis are reported in Table 10, intra-assay and total CV% were both ≤ 11%.

Table 10 Precision evaluation obtained with pools of CSF samples and calibrators

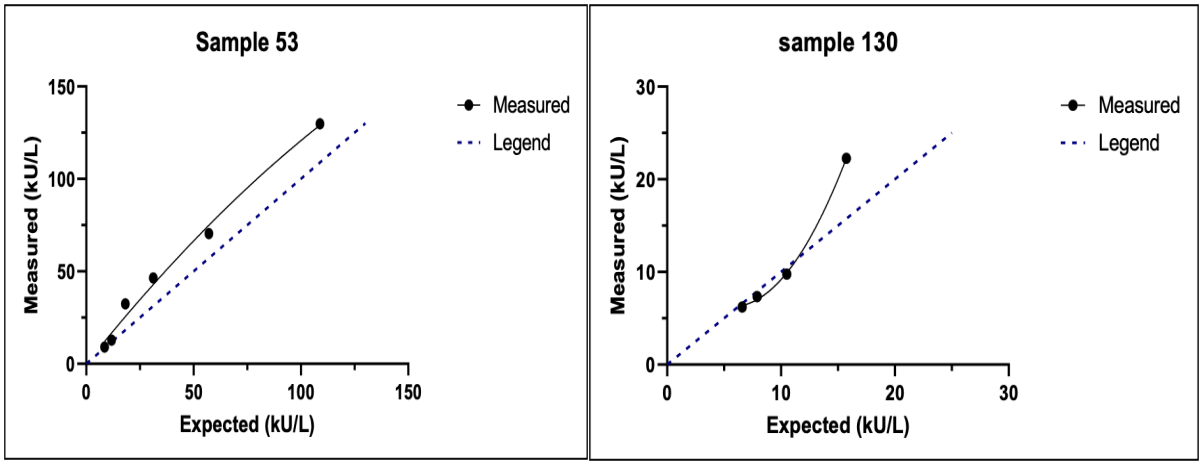
Design	Level KIU/L	Measured repeatability CV%	Measured intermediate precision CV%	Manufacturer obtained repeatability CV%	Manufacturer obtained intermediate precision CV%
3x3 CLSI EP15-A3	5	≤ 11.0	10.18	5.33	8.26
	9	≤ 4.4	3.1	5.18	7.86
	102	≤ 1.9	1.06	2.17	2.5

Linearity

Dilution linearity for two samples is reported in Figure 10.

Kit buffer, phosphate-buffered saline (PBS) and a pool of GAD-Ab negative CSF samples were previously tested as dilution means (data not shown) and Passing-Bablok regression and Bland-Altman analysis showed no constant bias between the three specimens.

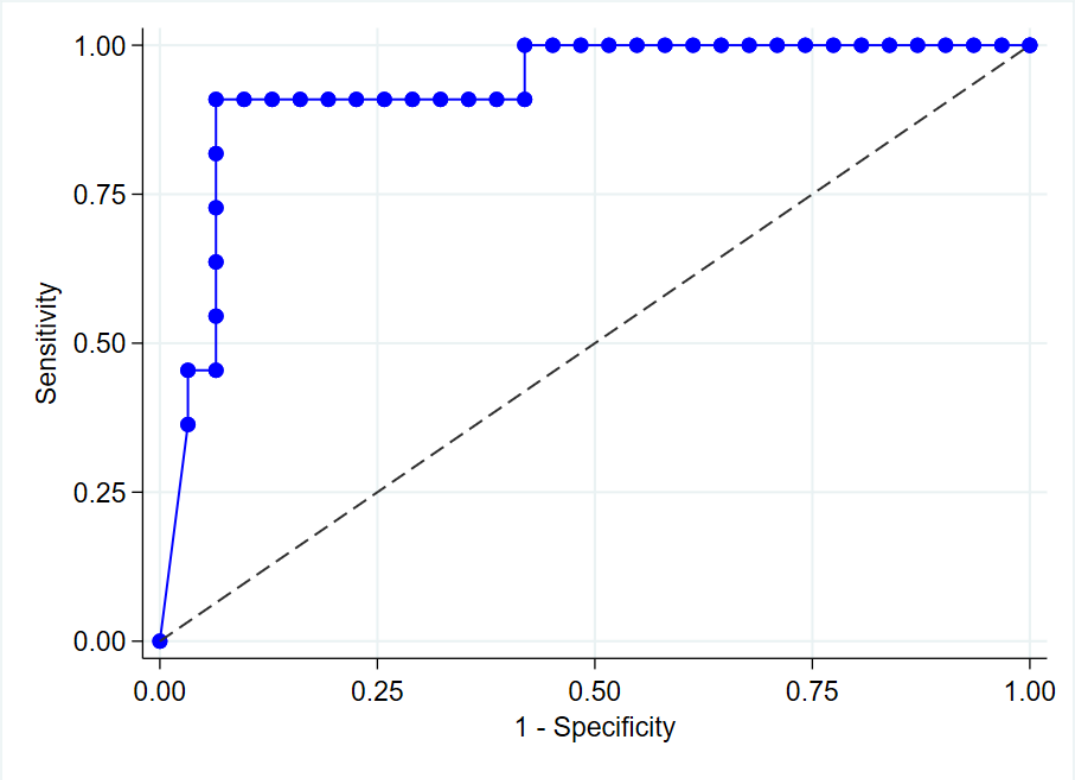
Figure 10: Linearity assessment



Clinical cut-off/decisional level

ROC curve for outcome (GAD-related syndromes: SPS, CA, Epilepsy and LE, PNS versus non-GAD pathology) had AUC=0.9238 (95%CI:0.832-1.000)(Figure 11); with Best cut-off 18 kIU/L sensitivity was 90.9% % (95%CI: 58.7% to 99.8%), specificity 93.5% (95%CI: 78.6% to 99.2%); positive likelihood ratio 14.09 (95%CI: 3.64-54.54), negative likelihood ratio 0.10 (95%CI: 0.01-0.63).

Figure 11: ROC curve



Conclusions

Validation and standardization of GAD-abs testing for CSF are long-awaited goals for neurological clinical practice, due to the crucial impact of intrathecal synthesis on therapeutic management and long-term follow-up.

CLIA systems are expected to increase in clinical laboratories due to their performance advantages and their flexibility, agreement analysis and establishment of specific decisional levels should be pursued to facilitate clinical management of complex patients.

Cross Sectional Analysis of Antibody-Associated Encephalitis Testing

Introduction

In the last years, several autoantibodies against surface and intracellular neuronal antigens have been identified in complex neurological syndromes including encephalitis; though some of their associated syndromes might be similar, the topographic distinction results in different pathogenic mechanisms and outcomes (Dalmau & Graus, 2018).

Antibody-mediated encephalitis is a group of inflammatory brain diseases in which neuropsychiatric symptoms, abnormal movements, dysautonomia and a decreased level of consciousness are associated with antibodies directed against neuronal cell-surface proteins (NSA): N-methyl-D-aspartate receptor (NMDAR), leucine-rich glioma-inactivated 1 (LG1), contactin-associated protein-like 2 (CASPR2), GABA type B receptor (GABABR), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) being the most frequent (Dalmau & Graus, 2018). Paraneoplastic neurological syndromes (PNS) are a heterogeneous group of disorders of central and peripheral nervous system, including subacute cerebellar degeneration and subacute sensory neuronopathy (Zoccarato, et al., 2017), occurring in association with cancer, where autoantibodies against intracellular antigens (IA) i.e., anti-Hu, anti-Yo, anti-Ri, anti-amphiphysin, anti-Ma2/Ta, react with both nervous system and cancer antigens mimicking neuronal antigens (Darnell & Posner, 2003).

Although the pathophysiological aspect of these autoantibodies is still not completely understood, neuronal surface antibodies are supposed to directly mediate the central nervous system (CNS) damage, while intracellular antigens antibodies are rather considered an epiphenomenon of a T-cells mediated paraneoplastic process (Ricken, et al., 2018).

Exact epidemiologic data of antibody-associated encephalitis are lacking: annual incidence of all types encephalitis is estimated in 5-8/100,000 but in about 40% of cases the underlying cause cannot be established (Granerod, et al., 2010), while PNS are supposed to affect 0.01% of patients with cancer (Darnell & Posner, 2003). In the last decade clinical laboratories benefited of the development of commercial methods (Zoccarato, et al., 2017) – immunoblotting for IA and indirect immunofluorescence cell-based assays for NSA (Ricken, et al., 2018)–leading to an increased number of studies on larger populations.

Extensive epidemiological studies of gender-related mechanisms in autoimmune diseases have led to highlight a degree of sexual dimorphism in the immune system, with increased

antibody production and cell-mediated response in women, who have a higher CD4+ T cell count and Th1 cytokine production (Moroni, et al., 2012). However, sex hormones profiles alone were not sufficient to explain the higher prevalence of autoimmunity in female, suggesting that other factors, particularly differences in gene expression, might influence disease presentation and clinical course (Nussinovitch & Shoenfeld, 2012). Gender-related differences have been also described in central nervous system (CNS) structure, mainly due to sex hormones regulating neurotransmission and neuronal development and conferring a different risk for neurodegenerative diseases (Nussinovitch & Shoenfeld, 2012). While for Multiple Sclerosis (MS) and Myasthenia Gravis (MG) experimental studies strengthened the hypothesis on the physiopathological mechanisms determining a gender-based different incidence (mainly related to sex hormones production and estrogen receptors expression) (Nussinovitch & Shoenfeld, 2012) (Altintas, et al., 2020), for antibody-associated encephalitis data on prevalence for sex and age groups have been so far mostly related to the different incidence of associated tumors (Altintas, et al., 2020).

Materials & Methods

Cross sectional analysis of neurological antibody tests collected in a centralized diagnostic clinical laboratory (Department of Laboratory Medicine, University-Hospital of Padova, Italy) of a tertiary care Academic Institution serving a large territory of Veneto region from 2009 to March 2021. Testing for NSA and IA was conducted on commercial assays; antibodies against LG1, CASPR2, GABAR, AMPAR1,AMPAR2 and NMDAR were detected in serum and CSF on indirect immunofluorescence cell based assay (CBA) using human embryonic kidney 293 fixed cells transfected with different antigens for each biochip (Autoimmune Encephalitis Mosaic 1, Euroimmun, Lubeck, Germany); antibodies against Amphyphisin, CV2, Hu, Ma2/Ta, Ri and Yo were tested on linear immunoblotting (Neuronal Antigens Profile 2, Euroimmun Lubeck, Germany).

Test requests and results were retrospectively extracted from laboratory database and demographic and clinical data were collected when available in the hospital electronic database of University-Hospital of Padova.

Statistical analyses

Statistical analyses were performed using Stata v 16.1 (StataCorp, Lakeway Drive, TX, USA). Mean and standard deviation were used to describe data. T-test was used to evaluate Age differences between groups, while Fisher's exact test was used to compare proportions. Logistic regression was used to estimate the Odds ratio between studied variables and antibody positivity (exact logistic regression was performed when required).

Results

Data on 3497 patients (55% male, 45% female) tested from January 2009 to March 2021 for IA were collected through laboratory database; mean age (and SD) was not significantly different for males and females (59.9 ± 18.9 yrs vs 59.6± 19.9 yrs, p = 0.681). We collected a total of 125 positive results, 64 male and 61 female with median age 64 yrs. The most frequent antibodies detected were anti-Yo (36.8%), and anti-Hu (16%). Four patients had more than one antibody positivity. Number of patients for each antibody, median age and tumor association are summarized in Table 11.

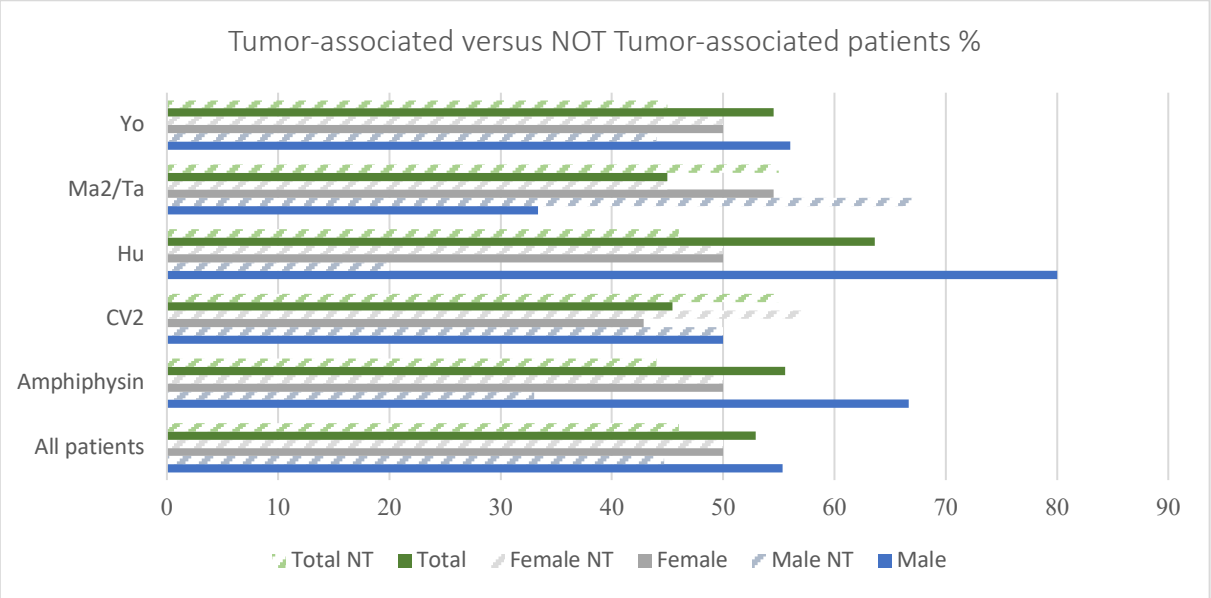
Table 11: Antibodies against intracellular antigens positive patients

	Male	**Female**	**Total**
Positive patients n.	64	61	125
Median age (range)	63 years (1-83)	65 years (5-86)	64 years (1-86)
Tumor-associated % (n. unknown)	55% (17)	50% (23)	53% (40)
Amphiphysin n.	6	8	14
Median age Amphiphysin	70 years	54 years	59 years
Tumor-associated % (n. unknown)	67% (3)	50% (2)	56% (5)
CV2 n.	6	9	15
Median age CV2	57 years	68 years	62 years
Tumor-associated % (n. unknown)	50% (2)	43% (2)	45% (4)
Hu n.	9	11	20
Median age Hu	57 years	71 years	66 years
Tumor-associated % (n. unknown)	80% (4)	50% (5)	64% (9)
Ma2/Ta n.	13	14	27
Median age Ma2/Ta	57 years	61 years	58 years
Tumor-associated % (n. unknown)	33% (4)	55% (3)	45% (7)
Ri n.	2	1	3
Median age Ri	67 years	73 years	70 years
Tumor-associated % (n. unknown)	100% (1)	na	100% (2)
Yo n.	28	18	46
Median age Yo	63 years	66 years	65 years
Tumor-associated % (n. unknown)	56% (3)	50% (10)	55% (13)

Antibody positivity was not different between males and females in the entire cohort (Fisher's exact p=0.411), neither was in the anti-Yo (Fisher's exact p=0.137), anti-Ma2/Ta (Fisher's exact p=0.920), anti-Hu (Fisher's exact p=0.629), anti-Amphiphysin (Fisher's exact p=0.578) and anti-CV2 (Fisher's exact p=0.416) subset of patients.

In 85 of 125 positive patients, tumor association could be identified through electronic database (Figure 12 shows percentages of males and females tumor- and not-tumor associated). Tumor was not gender related in the entire cohort (Fisher's exact p=0.667) nor in single antibody subset (data not shown). Seven out of 11 anti-Hu positive patients with clinical records had cancer: 4 small cells lung carcinoma (SCLC), 1 lung neuroendocrine tumor (NET) and 2 tumors of CNS.

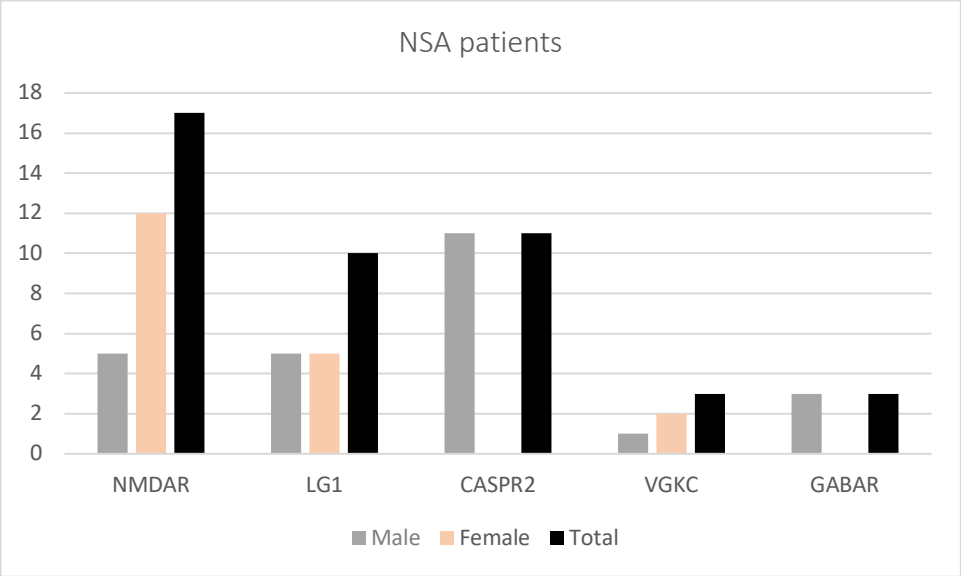
Figure 12: Positive patients for antibodies anti intracellular antigens Tumor- and NOT Tumor-associated %



From December 2013 to December 2020, 1108 patients (53% male, 47% female), mean age 47.9±26.4 yrs were tested for NSA, 44 were positive. Age did not differ between positive and negative subjects (t=0.41, p= 0.681, mean 46.3±26.9 and 48±26.4 yrs respectively). The most frequent antibody was anti-NMDAR (38.6%), followed by anti-CASPR2 (25%) and anti-LG1 (22.7%); 3 patients had both anti-CASPR2 and anti-LG1. Gender was not associated to antibody positivity in the NSA cohort (Fisher's exact p=0.648). However, in the CASPR2 and NMDAR positive subset a gender specific association was identified (Fisher's exact p=0.001

and Fisher's exact p=0.005, respectively) (Figure 13). The logistic regressions were performed, adjusting results for age, since mean age differed for specific antibody positivity: CASPR2 65.7±10.3 yrs, GABABR49.3±21.9 yrs, LG1 62.9±15.6 yrs, VGKC (CASPR+LG1) 21.8±26.1 yrs, NMDAR 27.9±26.1 yrs. Female gender underlined a minor susceptibility to CASPR2 positivity (OR 0.739, 95%CI: 0.001 to 0.542), while it enhanced risk for NMDAR positivity (OR 6.182, 95% CI: 1.256 to 30.436).Tumor association was not calculated for NSA due to few clinical data available, however no anti-NMDAR patient had a solid tumor before or after the diagnosis.

Figure 13: Gender distribution of patients positive for antibody anti neuronal surface antigens



Discussion

Antibody-mediated encephalitis and PNS are heterogenous disorders affecting both males and females of all age groups with peculiar clinical characteristics of specific antibody positivity. In this cross-sectional analysis of 12-years laboratory requests of IA testing, we found 125 positive results, of which 46 were anti-Yo antibodies (36.8%), a percentage similar to what was previously reported by an epidemiological study on PNS recently conducted in Northeastern Italy (30%) (Vogrig, et al., 2020). Unlike other large records on IA (Graus, et al., 2004) (Seluk, et al., 2019), anti-Hu antibodies were not the most frequent finding in suspected PNS: 16% in our report versus 41% in 34 Israelian patients (Seluk, et al., 2019).

We found no association between gender and antibodies against intracellular antigens, neither considering all antibodies nor for single antibody group, though a slight prevalence of

male patients with anti-Yo antibodies was emerging (ratio male/female 1.6:1). The second most frequent antibody positivity was anti-Hu, with a negligible female majority (11 vs. 9). A recent review of gender issues in autoimmune encephalitis (Altintas, et al., 2020) reports a male/female ratio of 3:1 for anti-Hu (Graus, et al., 2001), although the authors themselves argued that the frequency of women in the study was much lower than in previous series (Dalmau, et al., 1992) (Lucchinetti, et al., 1998), and considered this a possible effect of the different incidence among women of specific associated lung cancer between different countries.

No statistical association appeared between gender and tumor association; however, male with anti-Hu were the subgroup most affected by cancer (80%). The most common tumor associated to anti-Hu patients was SCLC, as stated in previous comprehensive reports (Graus, et al., 2004); accordingly, anti-Hu is also the most frequent antibody detected in SCLC patients affected with neurological disease (Zekeridou, et al., 2019), with the exception of a more prevalent anti-SOX2 antibodies reported in a single-region prospective study (Gozzard, et al., 2015).

During a 7-years observational period, 44 positive NSA patients were reported, the most frequent antibody being anti-NMDAR (38.6%), according with larger International (Dalmau & Graus, 2018) and Italian (Gastaldi, et al., 2020) cohorts. Mean age differed in each antibody subgroup, being anti-NMDAR patients younger than anti-CASPR2 and anti-LG1 subjects, as recently reaffirmed in a comprehensive review (Dalmau & Graus, 2018).

Gender-association was found in the subgroups anti-NMDAR, where female gender had an OR 6.182, and anti-CASPR2 where OR for female was 0.739, as every subject was male. Anti-NMDAR encephalitis affects women with a high female/male ratio, with or without the compresence of cancer (Altintas, et al., 2020). In fact, none of our patients with anti-NMDAR harbored a solid tumor, even though a convincing oncologic association mostly with ovarian teratoma has been long accepted (Dalmau & Graus, 2018) (Graus, et al., 2010) (Titulauer, et al., 2013), probably due to the relative smaller number of patients in the present study. Initial studies on estrogen regulation of NMDA receptors in rats unveiled a complex interaction depending on receptor subunits, age and ovarian status, moreover they indicated a stimulating role of NMDAR in neurotransmission of GnRH neurons in rats of reproductive age (Gore, 2001). An exhaustive explanation of molecular mechanisms underlying the strong gender distribution is still lacking even for anti-CASPR2, for which a substantiated genetic

predisposition due to specific HLA haplotypes has been reported (Binks, et al., 2018), as it has been for anti-LG1; interestingly gender-related differences were not highlighted for this genetic association (Mueller, et al., 2018) (Altintas, et al., 2020).

Conclusions

Gender-related differences, already widely accepted in autoimmune diseases, appear in the distribution of NSA autoimmune encephalitis, while paraneoplastic diseases associated to IA seem to correlate to the specific cancer incidence. Experimental studies are needed to clarify the role of sex hormones and their receptors in the developing of these rare conditions.

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