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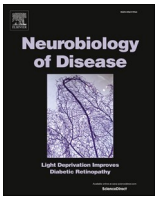

Nusinersen corrects L-arginine deficiency in the cerebrospinal fluid of patients with severe spinal muscular atrophy

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ABSTRACT

Spinal Muscular Atrophy (SMA) is a progressive neuromuscular disorder caused by homozygous loss of the *survival motor neuron 1* (*SMN1*) gene, leading to reduced SMN protein expression. Increasing evidence implicates neurotransmission deficits in the pathophysiology of SMA. In particular, alterations in neuroactive amino acids involved in glutamatergic neurotransmission have recently been identified in both the cerebrospinal fluid (CSF) of SMApatients and the spinal cord of SMNΔ7 mouse models. L-arginine, a precursor of nitric oxide, plays a critical role in glutamatergic receptor signalling, influencing neurotransmitter release, synaptic plasticity, and neuroprotection. However, it remains unclear whether SMN deficiency affects L-arginine metabolism in SMA. To address this, we used high-performance liquid chromatography to investigate whether SMN deficiency alters L-arginine homeostasis in the central nervous system of SMNΔ7 mice and in the CSF of SMA patients with varying disease severity, both before and after treatment with the SMN-inducing therapy Nusinersen. Notably, we observed significantly reduced L-arginine levels in the brainstem and spinal cord of symptomatic SMA mice compared to age-matched wild-type littermates. Consistent with these findings, we revealed lower L-arginine levels in severe SMA1 patients compared to milder SMA2 and SMA3 patients and healthy controls, enhancing the translational strength of our findings. Importantly, Nusinersen-mediated SMN upregulation fully restored L-arginine homeostasis in the CSF of severe SMA1 patients. In conclusion, our results demonstrate a dysregulation of L-arginine in SMA and highlight a role for SMN-enhancing therapies in restoring neurochemical alterations observed in patients with this neurodegenerative disease.

Abbreviations: ANCOVA, analysis of covariance; ANOVA, analysis of variance; BMI, body mass index; CHOP-INTEND, Children's Hospital of Philadelphia infant test of neuromuscular disorders; CNS, central nervous system; CSF, cerebrospinal fluid; L-Arg, L-arginine; HFMSE, Hammersmith functional motor scale-expanded; HPLC, high-performance liquid chromatography; IQR, interquartile range; NIV, non-invasive ventilation; P, postnatal day; SMA, spinal muscular atrophy; SMN, survival motor neuron; TCA, trichloroacetic acid; WT, wild-type.

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1. Introduction

Spinal Muscular Atrophy (SMA) is a rare progressive neuromuscular disease caused by the homozygous inactivation of the *survival motor neuron 1* (*SMN1*) gene, leading to motor neuron degeneration and skeletal muscle atrophy (Tisdale and Pellizzoni, 2015). The *SMN2* gene, a paralog of *SMN1*, produces approximately 10 % of functional SMN protein and its varying copy number is a key determinant of disease severity in patients with SMA (Mercuri et al., 2022; Wirth et al., 2020; Wirth, 2021). Accordingly, SMA is categorized into distinct severity types (type 0–4) based on the age at onset and the highest motor milestone achieved, with lower classification levels indicating greater disease severity (Grice and Liu, 2023; Lu et al., 2024; Mercuri et al., 2022; Wirth, 2021).

Three gene-targeting therapies that increase SMN protein expression have been approved for SMA, including Nusinersen, Risdiplam, and Onasemnogene-abeparvovec (Chaytow et al., 2021; Haque and Yokota, 2024; Mercuri et al., 2020; Tizzano and Finkel, 2017). On one hand, while current SMA treatments have demonstrated efficacy in enhancing motor function across a broad patient population —particularly when administered early— they do not provide a definitive cure for SMA (Al-Taie and Köseoğlu, 2023; Chaytow et al., 2018; Dalangin et al., 2020; Kong et al., 2021; Li et al., 2023; Łusakowska et al., 2023; Mercuri et al., 2020; Mercuri et al., 2022; Pascual-Morena et al., 2024; Ravi et al., 2021; Wirth et al., 2020; Wirth, 2021). On the other, the lack of validated targets for enhancing the effects of SMN-inducing drugs through combinatorial therapy continues to pose a significant challenge (Chaytow et al., 2018; Reilly et al., 2023).

Previous studies in animal models have shown that cell-autonomous mechanisms in motor neurons alone cannot fully account for the SMA phenotype (Fletcher et al., 2017; Imlach et al., 2012; Mentis et al., 2011; Simon et al., 2019). Accordingly, dysregulation of afferent monoaminergic and glutamatergic neurotransmission on motor neurons has emerged as key downstream consequence of SMN deficiency likely contributing to the broader pathophysiology of SMA (Delestrée et al., 2023; Fletcher et al., 2017; Hassan et al., 2025; Imlach et al., 2012; Lotti et al., 2012; Mentis et al., 2011; Pagiazitis et al., 2025; Prat-Ortega et al., 2025; Simon et al., 2019; Valsecchi et al., 2023). Consistent with glutamatergic neurotransmission abnormalities in SMA, neuroanatomical studies have revealed a loss of glutamatergic synapses between proprioceptive and descending fibres that connect to motor neurons (Fletcher et al., 2017; Ling et al., 2010; Mentis et al., 2011; Simon et al., 2025; Simon et al., 2019). Moreover, we have recently shown dysregulation of L-glutamate, L-glutamine and L-serine metabolism in the cerebrospinal fluid (CSF) of patients with severe SMA1 compared to both milder SMA cases and healthy controls (Hassan et al., 2025). Collectively, these synaptic and neurochemical deficits may contribute to alter motor neuron firing and impair skeletal muscle contraction (Fletcher et al., 2017, Ling et al., 2010, Mentis et al., 2011, Simon et al., 2025, Simon et al., 2019). Consistent with this, it has been shown that the upregulation of excitatory neurotransmission through distinct mechanisms can improve motor function in SMA models (Biondi et al., 2010; Biondi et al., 2008; Biondi et al., 2012; Branchu et al., 2013; Fletcher et al., 2017; Grondard et al., 2005; Hassan et al., 2025; Prat-Ortega et al., 2025; Simon et al., 2019).

Besides the well-known excitatory amino acids L-glutamate and L-aspartate, L-arginine (L-Arg) is the sole precursor of nitric oxide (NO). Specifically, Ca^{2+} influx through NMDARs triggers the activation of nitric oxide synthase (NOS), which catalyses the conversion of L-Arg into NO (Snyder, 1992). This gaseous neurotransmitter, modulates synaptic plasticity and excitatory neurotransmission (Rickard and Gibbs, 2003), forming a feedback loop where NO induces NMDAR S-nitrosylation and activates cGMP-dependent signalling pathways (Francis et al., 2010; Jaffrey et al., 2001). NO influences neurotransmitter release, receptor signalling, astrocytic glutamate reuptake, glutamate-glutamine cycle (Contestabile, 2000; Myslivecek et al., 1996; Virarkar et al., 2013), as well as angiogenesis (Ziche and Morbidelli, 2000), and neurovascular coupling processes that are increasingly recognized as disrupted in SMA (Delestrée et al., 2023; Fletcher et al., 2017).

The neuromodulatory activity of L-Arg is essential for brain development and its biosynthesis within the central nervous system (CNS) is a limiting factor during early life (Contestabile et al., 2003; Cormack et al., 2019; Erens et al., 2022; Kalhan and Bier, 2008).

In addition, L-Arg contributes to mitochondrial integrity and creatine biosynthesis linking cellular energy metabolism and motor neuron resilience (Kazak and Cohen, 2020; Ostojic, 2021; Wyss and Kaddurah-Daouk, 2000; Zilio et al., 2022). Importantly, dysregulation of L-Arg metabolism has been previously implicated in amyotrophic lateral sclerosis (ALS) (Arnold et al., 2024; Flores et al., 2012; Urushitani and Shimohama, 2001) and spinal cord injury, where excessive NO production exacerbates neuronal damage.

Despite growing evidence of neurotransmission abnormalities in SMA, little is known about how SMN deficiency alters amino acid metabolism in the CNS. In particular, no studies have systematically investigated L-Arg as a potential metabolic signature of disease severity. Moreover, reliable CSF biomarkers that reflect the extent of neurometabolic restoration following SMN-enhancing therapies are still lacking. Addressing these gaps may offer both mechanistic insight and clinical utility in monitoring therapeutic efficacy and guiding combinatorial interventions.

Based on prior evidence of glutamatergic signalling disruptions (Alves et al., 2020; Deutsch et al., 2021; Errico et al., 2022; Saffari et al., 2021), we hypothesize that SMN deficiency may lead to a measurable deficit in L-Arg levels in the CNS and CSF, and that this metabolic disturbance represent a tractable target for intervention through SMN-enhancing therapies.

To investigate the involvement of L-Arg metabolism in SMA pathophysiology, we designed a translational study with the following aims: (*i*) To profile L-Arg levels in CNS tissues of a severe SMA mouse model and in the CSF of SMA patients with varying disease severity; (*ii*) To assess whether treatment with the SMN-enhancing drug Nusinersen restores L-Arg homeostasis; (*iii*) To explore correlations between L-Arg levels and clinical/demographic parameters such as motor function —assessed using the Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders (CHOP INTEND) and Hammersmith Functional Motor Scale – Expanded (HFSME)— and age. To ensure the clinical robustness and generalizability of our results, we conducted a bicentric study involving two nationally recognized paediatric neuromuscular reference centres: the Bambino Gesù Children's Hospital in Rome and the Giannina Gaslini Institute in Genoa.

Importantly, this integrated preclinical/clinical approach enabled us to reveal both L-Arg disruptions in the developing CNS and validation of this neurometabolic signatures in patients, thereby enhancing the translational robustness of our findings. Moreover, we propose SMNΔ7 as a suitable mouse model to further investigate the mechanistic link between SMN deficiency and L-Arg perturbation.

2. Results

2.1. Selective decrease of L-arginine levels in the brainstem and spinal cord of SMA mice

We investigated the effects of SMN deficiency on L-Arg homeostasis in specific brain regions of the preclinical mouse model SMNΔ7 at different stages of symptomatology. Specifically, we quantified L-Arg levels in the cortex, cerebellum, brainstem, and spinal cord of SMNΔ7 mice and their wild-type (WT) littermates across disease progression from early (postnatal day (P)1 and P5) to late (P12) symptomatic stages (Fig. 1; Suppl. Table 1; Suppl. Raw Data).

HPLC analysis revealed a significant downregulation of L-Arg in the brainstem of SMA mice compared with WT littermates at both P5 ($p = 0.0379$, Mann-Whitney test) and P12 ($p = 0.0175$) (Fig. 1; Suppl.

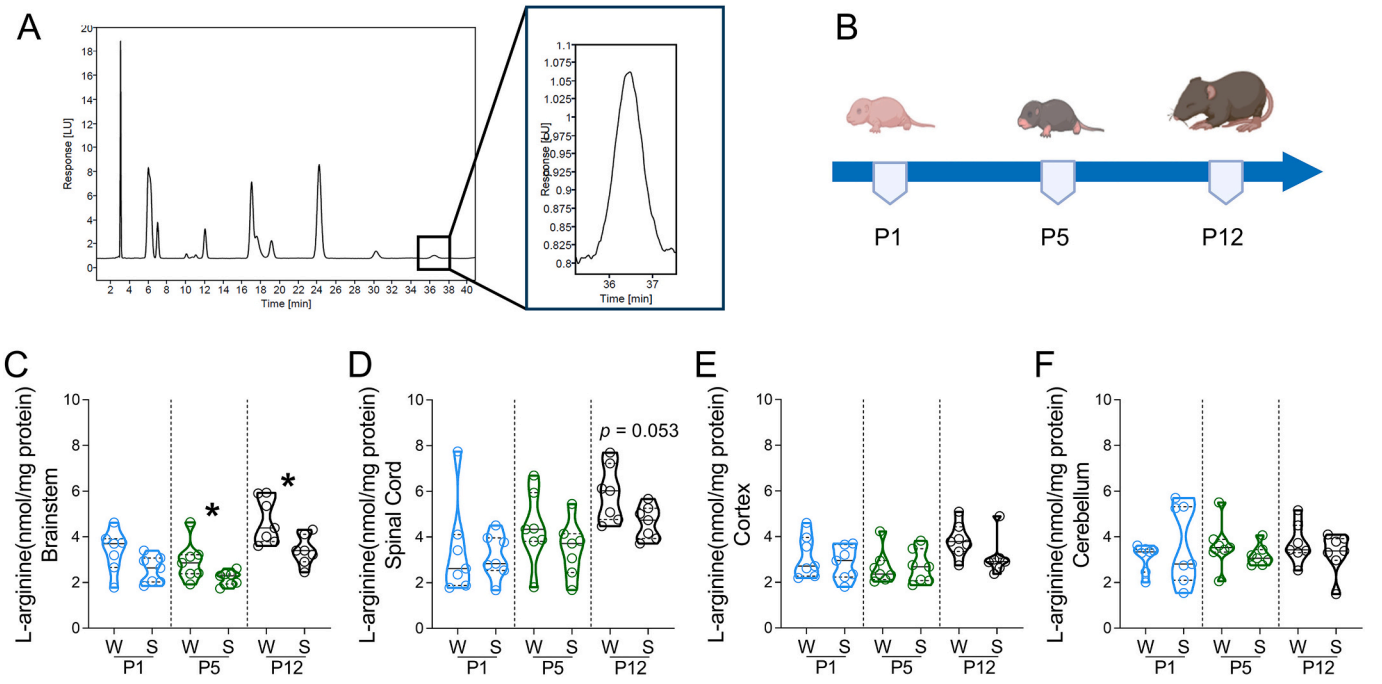

Fig. 1. Decreased L-arginine levels in the brainstem and spinal cord of SMA mice at overt-symptomatic stage of the disease. (a) Representative chromatogram obtained from spinal cord of a wild-type (W) mouse at post-natal day (P) 12 with L-arginine peak magnification. (b) Schematic representation of time points (P1, P5, and P12) corresponding to key phases in early postnatal development and disease progression in SMNΔ7 mice at which mice have been sacrificed in the present study (created with Biorender.com). Levels of L-arginine in brainstem (c), spinal cord (d), cortex (e) and cerebellum (f) in W and SMA (S) mice at P1, P5, and P12. Data are expressed as nmol/mg of protein and are shown as violin plots representing the median with interquartile range. *$p < 0.05$ (Mann-Whitney test). Dots represent values from each mouse analyzed, $n = 7$/group.

Table 1). A strong trend toward reduction very close to significance was observed in the spinal cord at P12 ($p = 0.0530$) (Fig. 1; Suppl. Table 1). In contrast, no significant alterations in L-Arg levels were detected in the cortex and cerebellum at all ages (Fig. 1; Suppl. Table 1). These findings suggest that L-Arg deficiency in the brainstem and spinal cord represents a neurochemical signature of SMN deficiency in SMNΔ7 during symptomatic stages of disease.

2.2. *SMN depletion induces L-arginine deficiency in the CSF of severe SMA1 patients*

We investigated whether the SMN deficiency-induced reduction in L-Arg observed in the CNS of SMNΔ7 mice is reflected in the CSF of untreated SMA patients across the disease severity spectrum. Specifically, we analyzed L-Arg content in naïve SMA1 ($n = 29$), SMA2 ($n = 19$), and SMA3 ($n = 13$) patients, as well as in age-matched control subjects ($n = 7$), whose clinical and demographic features are detailed in Table 1. The analysis of L-Arg levels was performed using ANCOVA on natural log-transformed data, considering age and sex as confounding factors. The statistical analysis evidenced that L-Arg concentration in the CSF is significantly associated with the diagnosis ($p = 0.008$) (Fig. 2, Suppl. Table 2). The following Fisher's post-hoc analysis revealed that L-Arg levels are significantly reduced in SMA1 patients compared with control subjects ($p = 0.010$) and the less severe SMA2 ($p = 0.005$) and SMA3 patients ($p = 0.011$) (Fig. 2 and Suppl. Table 2).

Next, we assessed whether clinical interventions targeting nutritional or respiratory deficits in SMA patients —such as gastrostomy, non-invasive ventilation (NIV) and tracheostomy— have an impact on L-Arg levels. Statistical analysis in SMA1 patients revealed that none of these medical supports could perturb L-Arg levels (Suppl. Table 3). Due to the small sample size and uneven distribution of patients receiving these interventions, we could not conduct the same analysis for SMA2 and SMA3 patients. Similarly, ANCOVA analysis including age, sex, and diagnosis as confounding factors suggests that *SMN2* copy number does not affect L-Arg concentration in our cohort (Suppl. Table 4).

Overall, our neurochemical analysis highlights a selective down-regulation of L-Arg levels in the CSF of the most severe SMA1 patients, compared to SMA2 and SMA3 patients, and control subjects.

Table 1
Demographic and clinical characteristics of patients with SMA and control subjects enrolled in the study.

Variable	Controls (n = 7)	SMA1 (n = 29)	SMA2 (n = 19)	SMA3 (n = 13)
Sex (M:F:N/A)	3:4:0	13:15:1	6:13:0	6:6:0
Age, median of years [IQR]; N/A	7 [4–12]; 0	2.9 [0.8–4.70]; 1	5.3 [1.6–10.80]; 1	14.3 [3.3–17]; 3
SMN Copy number (2:3:4:N/A)		(25:2:0:2)	(2:16:0:1)	(3:6:1:3)
BMI, median [IQR]; N/A		13 [13–16]; 9	18 [16–20]; 6	19 [16–22]; 6
CHOP-INTEND, median [IQR]; N/A		14 [3–17]; 2		
HFSME, median [IQR]; N/A			12 [8–15]; 2	44 [31–55]; 3
Gastrostomy (Yes: No:N/A)		17:10:2	0:18:1	0:9:4
NIV (Yes:No:N/A)		12:15:2	6:12:1	1:8:4
Tracheostomia (Yes:No:N/A)		17:10:2	0:18:1	0:9:4

Data are expressed as absolute frequencies or median with interquartile range (IQR). Abbreviations: BMI = Body Max Index; CHOP-INTEND = Children's Hospital Of Philadelphia Infant Test Of Neuromuscular Disorders; HFMSE = Hammersmith Functional Motor Scale Expanded; NIV = Non-invasive ventilation; N/A = count of missing data.

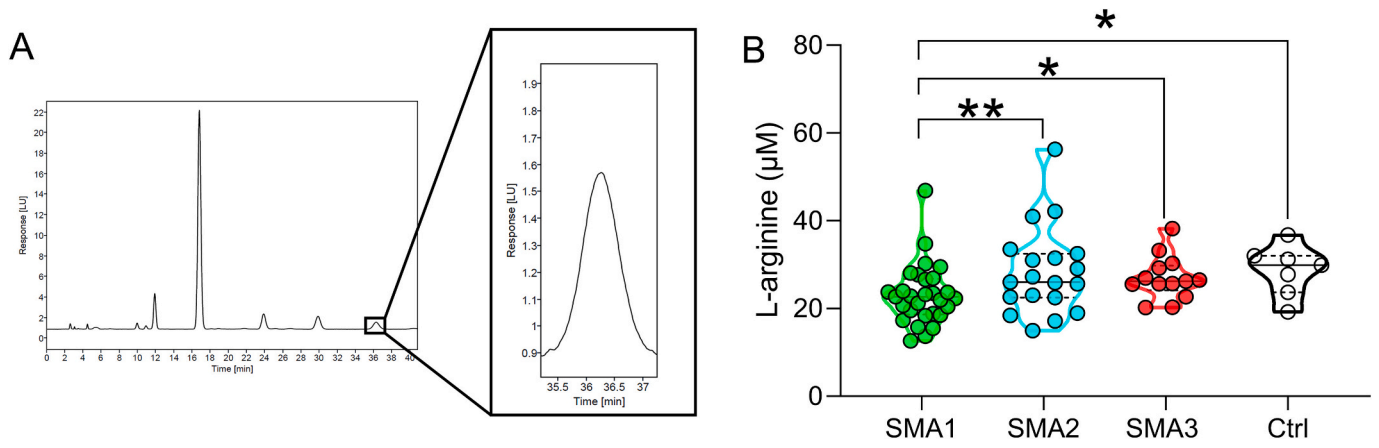

Fig. 2. The concentration of L-arginine in cerebrospinal fluid is influenced by the disease severity of spinal muscular atrophy patients. (a) Representative chromatogram obtained from the cerebrospinal fluid (CSF) of a 1-year-old patient showing the peak of L-arginine; (b) Levels of L-arginine (µM) in the indicated cohorts of drug-free SMA1 ($n = 29$), SMA2 ($n = 19$) and SMA3 ($n = 13$) patients as well as control individuals (n = 7). Data are shown as violin plots representing the median with interquartile range (IQR). For statistical analysis data were natural log transformed and ANCOVA considering age and sex as confounding factors was performed, $^{*}p < 0.05$, $^{**}p < 0.01$.

2.3. Correlation analysis of L-arginine CSF levels with demographic and clinical characteristics of SMA patients

We investigated whether L-Arg CSF levels correlated with age or motor function within each SMA type. Motor function was assessed by CHOP-INTEND for SMA1 patients and HFMSE for SMA2 and SMA3 patients (Table 2). Non-parametric Spearman's correlation analysis in SMA1 patients revealed no significant relationship between L-Arg levels and either age or motor activity (Table 2; Fig. 3). In contrast, SMA2 patients showed a significant negative correlation between L-Arg levels and age ($r = -0.544$, $p = 0.019$, Spearman's correlation) (Table 2; Fig. 3), and a positive correlation with HFMSE scores ($r = 0.568$; $p = 0.017$) (Table 2; Fig. 3). However, since age and HFSME were negatively correlated in this group ($r = -0.693$; $p = 0.002$), we performed a partial correlation analysis between L-Arg and HFSME while controlling for age. This analysis did not confirm a significant correlation ($r = -0.019$, p-value = 0.945), suggesting that the apparent association between L-Arg levels and motor function may be confounded by age. Finally, no significant correlations between L-Arg levels and either age or motor performance were observed in SMA3 patients (Table 2; Fig. 3). Taken together, these findings indicate that while no direct correlation was found between L-Arg levels and motor functions across SMA types, a significant age-dependent variation in L-Arg levels was selectively identified in SMA2 patients.

Table 2
Correlation between L-Arginine and clinical or demographic variables in drug-free (T0) patients with SMA and control subjects.

		CHOP-INTEND /HFMSE	L-arginine
SMA1	Age	$r = -0.430$ $p = \mathbf{0.025}$* $N = 27$	$r = -0.118$ $p = 0.541$ N = 29
	CHOP-INTEND		$r = 0.120$ $p = 0.550$ N = 27
SMA2	Age	r = −0.693 p = **0.002** $N = 17$	r = −0.544 p = **0.019** N = 18
	HFMSE		r = 0.568 p = **0.017** N = 17
SMA3	Age	$r = 0.182$ $p = 0.614$ N = 10	$r = 0.067$ $p = 0.855$ N = 10
	HFMSE		$r = -0.261$ $p = 0.466$ $N = 10$
Controls	Age		$r = -0.321$ $p = 0.482$ N = 7

Non-parametric Spearman's rho coefficients (ρ) and *P*-values (*p*) are indicated. Significant *p*-values are shown in bold with hysteric (*). Abbreviations: CHOP-INTEND = Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders; HFMSE = Hammersmith Functional Motor Scale Expanded.

2.4. Nusinersen therapy restores L-arginine levels in the CSF of severe SMA1 patients

To determine whether Nusinersen-mediated SMN upregulation influences the CSF levels of L-Arg and counteracts the reduction observed in SMA1 patients, we analyzed the subset of SMA1 ($n = 14$), SMA2 (n = 14), and SMA3 ($n = 10$) patients with available CSF samples at both T0 (baseline) and T302 (maintenance phase of Nusinersen therapy) (Table 3). Compared to their relative baselines before treatment, we found that Nusinersen therapy selectively increased the CSF levels of L-Arg in SMA1 patients ($p = 0.035$, paired Wilcoxon test) but neither in SMA2 nor in SMA3 patients (Fig. 4; Suppl. Table 5; Suppl. Raw Data). Thus, Nusinersen can correct L-Arg deficiency in the CSF of SMA1 patients.

2.5. Correlation analysis of CSF L-arginine levels with demographic and clinical characteristics of Nusinersen-treated SMA patients

We then examined the correlation between L-Arg levels and the clinical and demographic characteristics of SMA patients following Nusinersen treatment (Table 4). Non-parametric Spearman's correlation analysis showed that Nusinersen treatment does not alter the negative correlation between age and motor outcome observed in untreated SMA1 (T0: $r = -0.554$, $p = 0.040$; T302: $r = -0.635$, $p = 0.020$) and SMA2 (T0: $r = -0.551$, $p = 0.041$; T302: $r = -0.847$, $p < 0.0001$) patients (Table 4). Additionally, the negative correlation between L-Arg levels and age that is specific for SMA2 patients persisted following Nusinersen treatment (T0: $r = -0.626$, $p = 0.017$; T302: $r = -0.824$, $p < 0.0001$) (Table 4), indicating that Nusinersen does not counteract the progressive reduction in L-Arg levels in these patients.

3. Discussion

Dysfunction of glutamatergic and monoaminergic neurotransmission

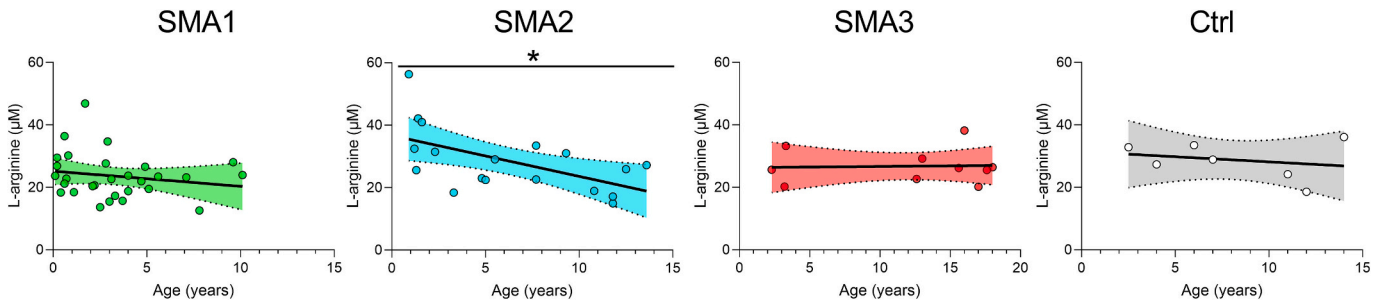

Fig. 3. L-arginine displays an age-dependent reduction in the CSF of SMA2 patients. Correlation between cerebrospinal fluid (CSF) concentrations of L-arginine (μM) and age (years) in SMA1 ($n = 29$), SMA2 ($n = 18$), SMA3 ($n = 10$) patients, and age-matched controls (CTRL) ($n = 7$). Each dot represents an individual subject. Lines and shadows represent the best fit line and its 95 % confidence interval, respectively. Non-parametric Spearman correlation: $^*p < 0.05$.

Table 3
Demographic and clinical Information of sub-cohort of patients treated with Spinraza.

Variable	SMA1 (n = 14)	SMA2 (n = 14)	SMA3 (n = 10)
Sex (M:F:N/A)	7:7:0	2:12:0	4:5:1
Age, median of years [IQR]; N/A	2.8 [0.7–4.9]; 0	5.3 [2.3–9.3]; 0	13 [3.3–16]; 1
SMN Copy number (2:3:4:N/A)	(14:0:0:0)	(1:13:0:0)	(3:5:1:1)
BMI, median [IQR]; N/A	13 [12–14]; 5	18 [16–20]; 3	19 [16–22]; 3
CHOP-INTEND, median [IQR]; N/A	14 [3–17]; 0		
HFSME, median [IQR]; N/A		11 [8–14]; 0	39 [31–55]; 1
Gastrostomy (Yes:No:N/A)	10:4:0	0:14:0	0:8:2
NIV (Yes:No:N/A)	6:8:0	5:9:0	1:7:2
Tracheostomia (Yes:No:N/A)	5:9:0	0:14:0	0:8:2

Data are expressed as absolute frequencies or median with interquartile range (IQR). Abbreviations: BMI = Body Max Index; CHOP-INTEND = Children's Hospital Of Philadelphia Infant Test Of Neuromuscular Disorders; HFMSE = Hammersmith Functional Motor Scale Expanded; NIV = Non-invasive ventilation; N/A = count of missing data.

in the spinal cord are emerging as key pathological features of SMA (Delestrée et al., 2023; Fletcher et al., 2017; Hassan et al., 2025; Imlach et al., 2012; Lotti et al., 2012; Mentis et al., 2011; Pagiazitis et al., 2025; Simon et al., 2019; Valsecchi et al., 2023). These alterations likely contribute to both neuromuscular and autonomic deficits characteristic of the disorder (Biondi et al., 2010; Biondi et al., 2008; Biondi et al., 2012; Branchu et al., 2013; Chaytow et al., 2018; Fletcher et al., 2017; Grondard et al., 2005; Leo et al., 2022; Liu et al., 2015; Mercuri et al., 2022; Quinlan et al., 2019; Simon et al., 2025; Simon et al., 2019; Sun and Harrington, 2019; Tharaneetharan et al., 2021; Tisdale and Pellizzoni, 2015; Wirth, 2021; Wishart et al., 2010). Consistent with recent findings linking SMN deficiency to disrupted homeostasis of neuroactive amino acids involved in neurotransmission in *drosophila*, mice, and humans (Errico et al., 2022; Hassan et al., 2025; Scatolini et al., 2025), here we found significantly lower L-Arg occurrence in the CSF of naive SMA1 patients compared to milder SMA2 and SMA3 types and healthy controls. In SMA mice at P5 and P12, L-Arg reduction is selectively observed in the brainstem and spinal cord, but not in higher brain structures like the cortex and cerebellum. These findings localize the perturbance of L-Arg levels in motor neuron-rich areas that are highly vulnerable in SMA (Murray et al., 2008; Woschitz et al., 2022). However, further investigations are needed to better characterize the L-Arg occurrence within distinct cortical and cerebellar subregions, which were not assessed in the present study.

L-Arg plays a crucial role in CNS neurotransmission as precursor of NO, which regulates astrocytic glutamate uptake and stimulates glutamine synthetase activity, thereby influencing the glutamate-glutamine cycle, which is essential for maintaining synaptic glutamate levels and sustaining excitatory neurotransmission (Grima et al., 2001). Given the observed L-Arg reduction and reported glutamatergic dysfunction in SMA (Errico et al., 2022; Hassan et al., 2025), we hypothesize that an alteration of NO signalling, particularly in SMA1 patients, may contribute to impaired astrocytic glutamate recycling, leading to excessive extracellular glutamate accumulation (Dhurandhar et al.,

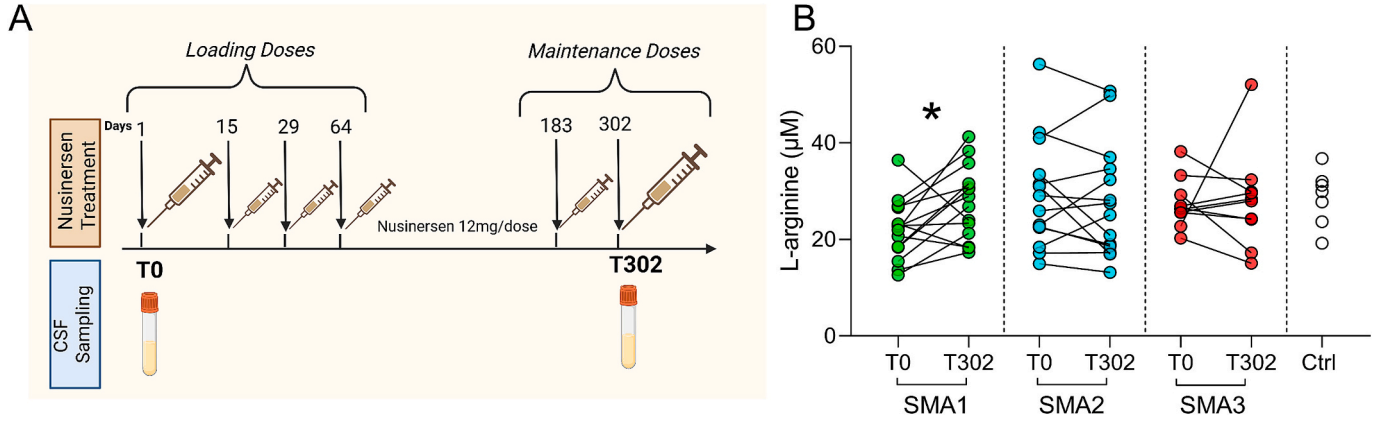

Fig. 4. Nusinersen selectively increases L-arginine concentrations in the cerebrospinal fluid of patients with SMA1. (a) Schematic representation of the timeline of intrathecal Nusinersen administration and cerebrospinal fluid (CSF) collection in SMA patients including loading and maintenance dose phases with sample collection points at T0 and T302 (created with Biorender.com); (b) Spaghetti plots represent L-arginine concentrations (μM) in CSF of SMA1 ($n = 14$), SMA2 (n = 14), SMA3 (n = 10) patients at baseline (T0) and after 302 days of Nusinersen treatment (T302). L-arginine concentrations (μM) in CSF of controls (Ctrl) is also reported. Each dot indicates an individual subject. Paired Wilcoxon test, $^*p < 0.05$.

Table 4
Correlation between amino acids and clinical or demographic variables in drug-free (T0) and treated (T302) patients with SMA and control subjects.

		T0		T302	
		CHOP-INTEND /HFMSE	L-Arginine	CHOP-INTEND /HFMSE	L-Arginine
SMA1	Age	**r = −0.554 p = 0.040* N = 14**	*r* = −0.055 *p* = 0.852 *N* = 14	**r = −0.635 p = 0.020* N = 13**	*r* = 0.077 *p* = 0.794 *N* = 14
	CHOP-INTEND		r = 0.243 p = 0.403 N = 14		r = 0.443 p = 0.130 N = 13
SMA2	Age	**r = −0.551 p = 0.041* N = 14**	**r = −0.626 p = 0.017* N = 14**	ρ = **−0.847 *p* = 0.000*** N = 14**	**r = −0.824 p = 0.000** N = 14**
	HFMSE		r = 0.560 p = 0.037* N = 14		r = 0.695 *p* = 0.006** N = 14
SMA3	Age	*r* = 0.151 *p* = 0.699 N = 9	*r* = 0.317 *p* = 0.406 *N* = 9	*r* = 0.075 *p* = 0.847 *N* = 9	*r* = −0.067 *p* = 0.865 N = 9
	HFMSE		*r* = −0.301 *p* = 0.431 N = 9		*r* = 0.569 *p* = 0.110 N = 9

Non-parametric Spearman's rho coefficients (ρ) and related *P*-values (*p*) are indicated. Significant *p*-values are shown in bold. Abbreviations: CHOP-INTEND = Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders; HFMSE = Hammersmith Functional Motor Scale Expanded.

2024). This imbalance may be particularly detrimental to motor neurons, which are highly susceptible to glutamate-induced excitotoxicity (Dhurandhar et al., 2025; Schmitt et al., 2023; Wiesinger, 2001). Hence, alterations of L-Arg could interfere with neurodevelopmental trajectories and contribute to motor coordination impairments and autonomic dysfunctions characteristic of SMA (Ando et al., 2020; Le et al., 2005). On the other hand, the pronounced L-Arg deficiency observed at T0 in SMA1 patients may reflect its increased consumption mediated by inducible NOS (iNOS) activity in the context of heightened neuroinflammation, as supported by the elevated cytokine levels we previously reported in this population (Nuzzo et al., 2023). These alterations suggest that disrupted NO signalling may contribute to SMA pathogenesis. While this model is biologically plausible and aligns with our clinical observations, it remains speculative and requires direct experimental confirmation. Future studies are needed to validate whether L-Arg/NO pathway disruption plays a causal role in SMA pathogenesis and whether CSF L-Arg could serve as a reliable biomarker for therapeutic monitoring.

Besides its role in neurotransmission, L-Arg is a key intermediate in the urea cycle, a metabolic pathway responsible for converting toxic ammonia into the less harmful urea. Although initially identified in the liver, a recent study demonstrated that under normal conditions, enzymes involved in the urea cycle are expressed in astrocytes, leading to a non-cyclic pathway in these cells (Ju et al., 2022). Notably, the urea cycle operates between the cytoplasm and mitochondria. Given that mitochondrial function is impaired in SMA (Boyd et al., 2017; Watson et al., 2021; Yeo and Darras, 2020; Zilio et al., 2022), we hypothesize that L-Arg deficiency may reflect or further exacerbate mitochondrial integrity by disrupting nitrogen metabolism and energy homeostasis in the brainstem and spinal cord.

A previous untargeted NMR-based metabolomic study revealed reduced CSF creatine and creatinine levels in severe SMA1 patients compared to milder cases (Errico et al., 2022). This finding aligns with serum and urine findings suggesting that creatinine, a breakdown product of creatine, could serve as a valuable biomarker for disease severity and progression (Alves et al., 2020; Deutsch et al., 2021; Zhao et al., 2024). Creatine is synthesized primarily in the liver and kidney from L-Arg and glycine via the action of arginine:glycine amidinotransferase, followed by methylation through guanidinoacetate *N*-methyltransferase (Kazak and Cohen, 2020; Wyss and Kaddurah-Daouk, 2000). Afterward, creatine is transported to energy-demanding tissues, such as muscle and CNS, where it is phosphorylated for energy storage. Over time, creatine is non-enzymatically converted into creatinine, which is excreted in urine. In this context, the observed reduction in L-Arg may therefore contribute to impaired creatine synthesis and subsequent lower creatinine levels, as noted in both CSF and peripheral biofluids (Alves et al., 2020; Deutsch et al., 2021; Errico et al., 2022; Zhao et al., 2024). Further biochemical studies assessing L-Arg and creatine levels in both the CNS and peripheral organs of SMA mouse models are warranted. Nonetheless, the present findings highlight a potential metabolic impact of dysregulation within the L-Arg–creatine pathway in infants with severe SMA1 (Errico et al., 2022; Freigang et al., 2021; Meneri et al., 2023; Saffari et al., 2024).

Importantly, The restoration of L-Arg levels in SMA1 patients following Nusinersen therapy may reflect a broader normalization of amino acid metabolism and neurochemical homeostasis driven by SMN upregulation (Errico et al., 2022; Zhuang et al., 2024). We propose that the upregulation of L-Arg may represent a SMN-dependent metabolic rescue contributing to the restoration of glutamatergic neurotransmission, neurovascular function and cellular homeostasis through pathways downstream of NO signalling (Kuznetsova et al., 2023; Tarabal et al., 2014; Zhou et al., 2022; Zilio et al., 2022). Conversely, the absence of a Nusinersen-related effect on L-Arg concentrations in treated SMA2 and SMA3 patients supports previous findings indicating that the metabolic and immune response to treatment varies according to SMA disease severity (Errico et al., 2022; Hassan et al., 2025; Nuzzo et al., 2023; Valsecchi et al., 2023). Our findings provide additional support for the emerging view that SMA patients exhibit disease severity-specific therapeutic responses to SMN-enhancing therapies, reinforcing the need for personalized or stratified therapeutic approaches.

While current clinical assessments primarily rely on motor function scales, our results suggest that biochemical correction such as L-Arg normalization may represent an additional indicator of treatment efficacy. This aligns with recent evidence showing that SMN-enhancing therapies can modulate broader cellular pathways, including metabolism, RNA processing, and mitochondrial function (Errico et al., 2022; Kuznetsova et al., 2023; Tarabal et al., 2014; Zhou et al., 2022; Zhuang et al., 2024; Zilio et al., 2022). Thus, tracking CSF-based metabolic markers may offer complementary insight into patient response, especially in cases where motor outcomes plateau or diverge from biochemical recovery.

Although Nusinersen treatment modulates central L-Arg levels in SMA1 patients, no correlation was found between L-Arg levels and motor functions, as measured by the CHOP-INTEND score. In contrast, a positive correlation emerged between L-Arg levels and the HFSME in SMA2 patients. While this association may suggest a link between L-Arg and motor function, we cannot exclude the possibility that age is a confounding factor in this relationship. Interestingly, a significant age-dependent decline in L-Arg levels was observed only in SMA2 patients and not in other SMA subtypes or controls. This decline persisted even after Nusinersen treatment, raising the possibility that it reflects either an intrinsic metabolic feature of the SMA2 subtype or progressive disease worsening. However, further studies in age-matched cohorts are needed to disentangle the effects of age from disease progression.

While this study is observational and does not directly address mechanistic pathways, our findings contribute to a better understanding of SMA pathophysiology from both molecular and clinical perspectives. At the molecular level, the observed disruption of L-Arg homeostasis in motor neuron–rich regions of the CNS, may be associated with alterations in NO signalling, glutamate handling, and synaptic function,

which have been implicated in motor neuron vulnerability. Clinically, the selective restoration of L-Arg levels by Nusinersen in SMA1 patients points to its utility as a pharmacodynamic biomarker, offering a complementary tool for treatment monitoring beyond motor function assessments. This dual perspective strengthens the relevance of our results for both basic neuroscience and translational medicine.

Additionally, methodological strength of the current study include its multicentre design involving two Italian hospitals and the use of a longitudinal paired-sample approach, which enabled the monitoring of CSF L-Arg levels in clinically well-characterized SMA1, SMA2 and SMA3 patients treated with Nusinersen. This design minimizes inter-individual variability and highlights the value of longitudinal CSF biomarker monitoring in rare paediatric neurological disorders, where patient numbers are often limited. Our study is further strengthened by the use of validated analytical techniques, including HPLC for metabolite quantification, and the implementation of statistical analyses such as ANCOVA, which accounted for age and sex as potential confounders. These strategies improve the robustness and interpretability of our findings, particularly in a heterogeneous clinical population.

However, some limitations should be acknowledged. First, while our study suggests a potential link between L-Arg dysregulation and SMN levels in SMA pathology, the underlying mechanisms remain poorly understood. Lastly, one important limitation of this study is the relatively small sample size, particularly for the control group and SMA3 patients. This may reduce statistical power and limit our ability to detect subtle differences, particularly in milder SMA phenotypes. As such, the findings should be interpreted with caution, and future studies with larger cohorts will be important to validate and extend these observations.

In conclusion, our study highlights L-Arg deficiency in the CSF of SMA1 and in the brainstem and spinal cord of SMA mouse models during symptomatic stage, underscoring the relevance of amino acid metabolic disruption in SMA pathophysiology. However, further mechanistic studies investigating L-Arg biosynthesis and its metabolic pathways —particularly L-Arg/NO and L-Arg/creatine— are needed to clarify the causal link between SMN deficiency and reduced L-Arg levels as well as L-Arg homeostasis disruption consequences in SMA pathophysiology. This study, for the best of our knowledge, represents the first integrated preclinical and clinical evidence of L-Arg dysregulation in SMA. Notably, the restoration of L-Arg homeostasis following Nusinersen in SMA1 patients suggests that L-Arg may serve as a downstream target for SMN-inducing therapies and as a candidate pharmacodynamic biomarker of therapeutic response. Our findings highlight the clinical relevance of amino acid metabolism in SMA and emphasize the need to explore complementary therapeutic strategies, including amino acid supplementation, to overcome SMA-associated metabolic dysfunction and improve treatment efficacy.

4. Methods

4.1. Animal model

SMNΔ7$^{+/+}$; SMN2$^{+/+}$; *Smn*$^{+/-}$ mice (stock number 005025; Jackson Laboratory, Bar Harbor, Maine, U.S.) were interbred to generate *Smn*$^{-/-}$ and *Smn*$^{+/+}$ offspring, which respectively served as models for severe Spinal Muscular Atrophy (SMA) and healthy controls (WT). The pups were kept with their mother until the day of sacrifice, under standard conditions (12/12-h light/dark cycle and ad libitum access to food and water. Efforts aimed to minimize both the number of animals used and their distress. Both males and females were included in the experiments. All procedures complied with institutional guidelines, national regulations (D.L. N.26, 04/03/2014), and international directives (Directive 2010/63/EU). The study was approved by the Italian Ministry of Health (protocol #160/2020-PR) and the Ethics Committee of the University of Turin. A total of 42 animals were used (21 WT and 21 SMA mice), obtained from 28 different litters, randomly assigned to the experimental groups. Specifically, $n = 7$ animals were included for each time point and genotype. Researchers were not blinded during group assignment, as no substantial differences between litters were expected based on our previous experience. For each mouse, L-Arg was quantified in spinal cord, brainstem, cortex, and cerebellum. No animals were excluded from the analyses as outliers.

4.2. Mice genotyping

To identify WT and SMA mice, tail snips were collected at P0 for genotyping, following the PCR protocol provided by Jackson Laboratory. Briefly, DNA was extracted from mouse tail incubating a small portion of tail in 60 μL of NaOH (50 mM, PanReac AppliChem, ITW Reagents, Monza, Italy, ref. 181,691.1211) and 6 μL of 1 M TRIS-HCl at 95 °C for 10 min under gentle shaking. The absence of the survival motor neuron gene (*Smn*) was determined by PCR analysis using primers that amplify a fragment of the *Smn* gene, yielding a 420 bp product for the wild-type (WT) allele and a 150 bp product for the knockout (SMA) allele. The primers used were: *Smn* fwd 5'-TTTTCTCCCTCTTCAGAGTGAT-3′, *Smn* wt rev 5'-CTGTTTCAAGGGAGTTGTGGC-3′, and *Smn* tg rev 5'-GGTAACGCCAGGGTTTTCC-3′, as recommended by the supplier (Jackson Laboratory).

4.3. Sample collection

WT and SMA mice were sacrificed through cervical dislocation at P1, P5 and P12. Fresh cortex, cerebellum, brainstem, and spinal cord were collected on dry ice and immediately stored at −80 °C, before HPLC analyses.

4.4. Patients' characteristics and enrolment

This bicentric study was conducted in collaboration between two Italian paediatric neuromuscular reference centres: the Bambino Gesù Children's Hospital in Rome and the Giannina Gaslini Institute in Genoa, Italy. This multicentre enrolment enhances the representativeness of the patient cohort and strengthens the external validity of our findings. A total of 61 SMA patients diagnosed with SMA1 ($n = 29$), SMA2 ($n = 19$), and SMA3 ($n = 13$), and $n = 7$ age-matched control subjects participated in the study (Table 1).

The sex distribution was balanced across the groups ($\chi^2 = 1.675$, $p = 0.643$). The age in control subjects was comparable with SMA1, SMA2 and SMA3 groups. SMA1 patients were significantly younger than SMA3 ($p < 0.001$, Kruskall-Wallis test; controls vs SMA1, $p = 0.070$; controls vs SMA2, $p > 0.999$; controls vs SMA3, $p > 0.999$; SMA1 vs SMA2, $p = 0.233$; SMA1 vs SMA3, $p = 0.001$; SMA2 vs SMA3, $p = 0.240$; Dunn's test with Bonferroni correction) (Table 1). The percentages of patients requiring clinical procedures such as gastrostomy, NIV, or tracheostomy are reported in Table 1.

SMA patients with a confirmed genetic diagnosis and available SMN2 copy number were enrolled in the study. Individuals were excluded if they had prior exposure to SMN-modulating therapies or comorbidities potentially affecting central amino acid metabolism. Control CSF samples were obtained from age- and sex-matched paediatric patients undergoing lumbar puncture for Idiopathic intracranial hypertension or suspected meningitis at Bambino Gesù Children's Hospital. The study received approval from the respective local Ethics Committees of the two hospitals (2395_OPBG_2021). Written informed consent was obtained from the legal guardians of all participants. CSF samples were collected on day 0 (T0; baseline), at the time of the first Nusinersen administration, and day 302, which corresponds to the maintenance phase of Nusinersen therapy (T302). All patients underwent clinical diagnosis and genetic confirmation, and the SMN2 copy number was also assessed. All SMA1 patients, irrespective of age or disease severity, were enrolled in the Expanded Access Programme (EAP) for compassionate use, which was exclusively available to patients with the

infantile form and took place in Italy between November 2016 and November 2017. The overall clinical response of these patients to Nusinersen treatment has been well-documented in previous literature (Coratti et al., 2021; Pane et al., 2019; Pane et al., 2018). Details regarding the clinical assessment of patients at T0 and T302, as well as Nusinersen intrathecal injection, have been previously reported (Errico et al., 2022; Hassan et al., 2025; Nuzzo et al., 2023; Valsecchi et al., 2023).

4.5. HPLC detection

CSF samples and brain structures were analyzed in accordance with previously established protocols (Hassan et al., 2025). In summary, 100 µL of CSF was combined with 900 µL of HPLC-grade methanol in a 1:10 dilution and subsequently centrifuged at 13,000 $\times g$ for 10 min. The resulting supernatants were dried, and the resultant pellet was resuspended in 0.2 M trichloroacetic acid (TCA) before undergoing centrifugation at 13,000 $\times g$ for additional 10 min. Mouse tissues were subjected to sonication in TCA followed by centrifugation at 13,000 $\times g$ for 20 min. The TCA supernatants were then neutralized with sodium hydroxide (NaOH) and subjected to pre-column derivatization utilizing o-phthalaldehyde (OPA) in conjunction with *N*-acetyl-L-cysteine (NAC). Diastereoisomer derivatives were resolved using a UHPLC Agilent 1290 II Infinity (Agilent Technologies, Santa Clara, CA, USA) equipped with a ZORBAX Eclipse Plus C8 column (4.6 × 150 mm, 5 µm) (Agilent Technologies, Santa Clara, CA, USA), under isocratic conditions utilizing a mobile phase comprising 0.1 M sodium acetate buffer at pH 6.2, 1 % tetrahydrofuran, with a flow rate of 1.5 mL/min. A washing procedure involving 0.1 M sodium acetate buffer, 3 % tetrahydrofuran, and 47 % acetonitrile was performed following each analytical run.

For each HPLC run, mice tissue samples were randomised according to age and genotype to minimize batch effect. For each tissue, samples were divided in two different baches ($n = 21$ samples/each). Similarly, for CSF samples, the same percentage of controls, SMA 1, SMA 2, and SMA 3 were used in each batch. Samples obtained from the same patient at T0 and T302 were run in the same batch. The chromatogram analysis was carried out in blind.

The identification and quantification of L-Arg were conducted based on retention times and peak areas, which were compared against those of external and internal standards. Calibration curve was set up using increasing concentrations of external standards prior to the experiment. The limit of detection (LOD) for L-Arg was 0.73 pmol while limit of quantification (LOQ) was 1.5 pmol. For each experimental batch, L-Arg internal standard was used as quality control. Intra- and inter-assay coefficients of variation were assessed within the whole experiment and are, respectively, 0.013 pmol ± 0.0005 pmol and 0.035 pmol ± 0.021 pmol.

The concentration of L-Arg in the CSF is reported in micromolar (µM). Furthermore, the protein pellet derived from mouse tissues was solubilized in a 1 % SDS solution and quantified using the bicinchoninic acid (BCA) assay method (Pierce™ BCA Protein Assay Kits, Thermo Fisher Scientific, Rockford, IL, USA). The concentration of L-Arg in tissue homogenates was normalized to the total protein content and expressed as nanomoles per milligram of protein (nmol/mg protein).

4.6. Statistical analysis

Statistical analyses were performed using SPSS software version 27 (SPSS Inc., Chicago, IL, USA) and Prism 8, version 8.0.2. Normality distribution was assessed using the Kolmogorov-Smirnov and Shapiro–Wilk tests. Quantitative variables were expressed by the median and interquartile range (IQR), while qualitative variables were by absolute frequency. The correlation was evaluated using non-parametric Spearman test. The effect of confounders on correlation was evaluated by partial correlation. Due to the non-normal data distribution, differences between independent groups were studied by the non-parametric Kruskal-Wallis test followed by post-hoc Dunn's test with Bonferroni's correction. Differences in L-Arg levels between patients and control groups were explored using ANCOVA model, adjusted for age and sex, on natural log-transformed variables, followed by Fisher's post hoc multiple comparisons. Similarly, ANCOVA model adjusted for age, sex, and diagnosis was used to compare L-Arg levels among SMA patients with different SMN2 copy number. The Wilcoxon Signed-Ranks Test was used to study differences between dependent groups. For mice data, differences between independent groups were studied by Mann-Whitney test. Groups were considered significantly different when $p \leq 0.05$. Missing data were automatically excluded from the calculation.

CRediT authorship contribution statement

Amber Hassan: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Raffaella di Vito:** Writing – original draft, Investigation, Formal analysis, Data curation. **Anna Caretto:** Resources. **Tommaso Nuzzo:** Writing – review & editing, Formal analysis. **Adele D'Amico:** Resources. **Chiara Panicucci:** Resources. **Claudio Bruno:** Resources. **Enrico Bertini:** Resources. **Alessandro Vercelli:** Resources. **Marina Boido:** Resources. **Francesco Errico:** Writing – review & editing, Writing – original draft, Supervision. **Livio Pellizzoni:** Writing – review & editing, Writing – original draft. **Alessandro Usiello:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

E.B. received advisory board honoraria from Roche, Biogen, PTC, Red Nucleus. The other authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2025.107046.

Data availability

The authors confirm that the data supporting the findings of this study are available within the supplementary materials.

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