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1 **Dysregulated balance of D- and L-amino acids modulating glutamatergic neurotransmission**
2 **in severe spinal muscular atrophy**
3

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35

36 **Abstract**

37 Spinal muscular atrophy (SMA) is a neuromuscular disorder caused by reduced expression of the
38 survival motor neuron (SMN) protein. In addition to motor neuron survival, SMN deficiency affects
39 the integrity and function of afferent synapses that provide glutamatergic excitatory drive essential for
40 motor neuron firing and muscle contraction. However, it is unknown whether deficits in the metabolism
41 of excitatory amino acids and their precursors contribute to neuronal dysfunction in SMA. To address
42 this issue, we measured the levels of the main neuroactive D- and L-amino acids acting on
43 glutamatergic receptors in the central nervous system of SMNΔ7 mice as well as the cerebrospinal
44 fluid (CSF) of SMA patients of varying severity before and after treatment with the SMN-inducing
45 drug Nusinersen. Our findings reveal that SMN deficiency disrupts glutamate and serine metabolism
46 in the CSF of severe SMA patients, including decreased concentration of L-glutamate, which is
47 partially corrected by Nusinersen therapy. Moreover, we identify dysregulated L-glutamine to L-
48 glutamate conversion as a shared neurochemical signature of altered glutamatergic synapse metabolism
49 that implicates astrocyte dysfunction in both severe SMA patients and mouse models. Lastly, consistent
50 with a correlation of higher CSF levels of D-serine with better motor function in severe SMA patients,
51 we show that daily supplementation with the NMDA receptor co-agonist D-serine improves
52 neurological deficits in SMNΔ7 mice. Altogether, these findings provide direct evidence for
53 dysregulation of D- and L-amino acid metabolism linked to glutamatergic neurotransmission in severe
54 SMA and have potential implications for treating this neurological disorder.

55

56

57

58 **Keywords**

59 Spinal muscular atrophy, cerebrospinal fluid, Nusinersen, central nervous system, glutamatergic
60 neurotransmission, NMDA receptors, D-serine.

61

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62 **Introduction**

63 Spinal muscular atrophy (SMA) is the most common genetic cause of infant mortality and is
64 characterized by the progressive degeneration of spinal motor neurons and skeletal muscle atrophy (1-
65 3), with increasing evidence of multiorgan pathology (3, 4). SMA is caused by homozygous mutations
66 in the *Survival Motor Neuron 1* (*SMN1)* gene (5), while the paralogue gene *SMN2* - which is present
67 in variable copy numbers - can only partially compensate for *SMN1* loss due to a single nucleotide
68 change affecting its pre-mRNA splicing (6). SMA patients are classified into three main clinical groups
69 (Types 1, 2 and 3) based on the age of onset and severity of the disease, which inversely correlate with
70 the number of *SMN2* copies and the SMN protein levels (1).
71
72 Three different therapeutic approaches aimed at increasing SMN expression through splicing
73 modulation or viral-mediated gene replacement have been approved for the treatment of SMA (4).
74 Among these, Nusinersen (Spinraza) - the first FDA-approved drug for the treatment of SMA (7) - is
75 an antisense oligonucleotide administered intrathecally that corrects the splicing of *SMN2* pre-mRNA,
76 increasing the concentration of functional SMN protein (4, 6, 8). Clinical data show that Nusinersen
77 induces remarkable improvement of motor function in SMA patients, especially when treated pre-
78 symptomatically (9). Despite significant advances in SMA therapy, however, the current consensus is
79 that neither Nusinersen nor other treatments can cure the disease (4, 8, 10). In particular, there are still
80 unmet needs to address the incomplete correction of disease symptoms and the variability in clinical
81 response to treatment. One of the major limitations is the absence of validated targets whose
82 modulation could increase the clinical benefit of SMN-inducing drugs through combinatorial therapy
83 (11, 12). In this regard, the identification of neurometabolic markers linked to disease severity and
84 correlated with functional outcomes after treatment would be crucial for explaining differences in
85 clinical responses and guiding discovery of new therapies, including nutritional support. However, our
86 current understanding of the biochemical and neurochemical abnormalities associated with SMA
87 pathology and their specific response to SMN-inducing therapies is very limited.
88
89 Studies in model organisms revealed that cell-autonomous deficits in motor neurons alone cannot
90 account for the disease phenotype and implicated dysfunction of excitatory neuronal networks that
91 control motor neuron output as important players of SMA pathophysiology (13-17). The best
92 characterized example is the dysfunction and loss of glutamatergic synapses from proprioceptive
93 sensory neurons to motor neurons, which have emerged as some of the earliest manifestations of the
94 disease in SMA mice (13, 14, 17, 18). The resulting reduction in afferent glutamatergic
95 neurotransmission causes downregulation of Kv2.1 channels and decreased firing of SMA motor

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96 neurons, contributing to impaired muscle contraction and motor dysfunction (17). Consistent with
97 SMA motor neurons suffering from reduced excitatory drive, increasing neuronal activity and
98 glutamate signaling on motor neurons improve motor function in animal models of SMA (13, 15, 17,
99 19). Importantly, recent studies have expanded the detrimental effects of SMN deficiency in neuronal
100 networks beyond hypoglutamatergic signaling to include noradrenergic and serotonergic
101 neurotransmission (20, 21). However, it is unknown whether deficits in the metabolism of excitatory
102 amino acids contribute to synaptic dysfunction in SMA sensory-motor circuits.
103
104 L-glutamate (L-Glu) is the most important excitatory amino acid in the central nervous system (CNS)
105 (22). It plays a pivotal role in orchestrating neurodevelopmental processes, synaptic transmission, and
106 plasticity within the brain and spinal cord through stimulation of ionotropic (NMDA, AMPA, and
107 kainate) and metabotropic (mGlu) receptors (23-28). Besides neurotransmission, L-Glu controls
108 fundamental cellular pathways, including non-essential amino acid synthesis and energy metabolism
109 by directly regulating α-ketoglutarate levels and, in turn, the Krebs cycle (29). The closely related
110 dicarboxylic amino acid L-aspartate (L-Asp) and its D-enantiomer derivative D-Asp are also known to
111 act as primary agonists of NMDARs (26, 30-32) and mGluR5 (33), while D-serine (D-Ser) functions
112 in glutamatergic signaling by acting as an NMDAR co-agonist (34, 35). Lastly, L-glutamine (L-Gln)
113 and L-Ser play a primary role in modulating the synthesis of L-Glu and D-Ser and, together with L-
114 Asp, are involved in essential cellular processes including energy homeostasis, ammonium recycling,
115 redox balance and in the biosynthesis of amino acids, nucleotides, and membrane lipids (36).
116
117 Despite their critical roles for neuronal function, possible alterations in the physiological levels of
118 neuroactive amino acids in the CNS of either SMA patients or animal models have not been
119 investigated. Here, we addressed this question by performing a comprehensive neurotransmitter
120 profiling in the spinal cord and brain of severe SMA mice as well as in the cerebrospinal fluid (CSF)
121 of SMA patients across the spectrum of disease severity. We also investigated the impact of Nusinersen
122 therapy on the CSF levels of excitatory amino acids and their precursors implicated in glutamatergic
123 neurotransmission. Overall, our findings provide direct evidence for dysregulation of amino acid
124 metabolism linked to glutamatergic neurotransmission that may contribute to motor dysfunction in
125 severe SMA.
126

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127 ## Results

128 **SMN deficiency perturbs glutamate and serine metabolism in the CSF of severe SMA patients.**
129 We performed a real-world, retrospective study to determine the effects of SMN deficiency on the
130 concentration of neuroactive amino acids and their precursors in the CSF of untreated SMA patients
131 across the disease-severity spectrum using HPLC (Fig.1A). This analysis included CSF from SMA1
132 (*n* = 34), SMA2 (*n* = 22), and SMA3 (*n* = 17) patients as well as age-matched control subjects (*n* = 7)
133 whose clinical and demographic features are presented in Table 1.
134
135 Using the non-parametric Kruskal-Wallis test, we found significant L-Glu and L-Gln changes in the
136 CSF of SMA patients (L-Glu, *P* = 0.007; L-Gln, *P* = 0.023) (Fig. 1B,C; Suppl. Table 1). Importantly,
137 further *post-hoc* comparisons highlighted a significant reduction of L-Glu in the CSF of SMA1 patients
138 compared to controls (Fig. 1B; Suppl. Table 1). The L-Gln to L-Glu ratio was also significantly affected
139 in SMA patients (*P* = 0.005; Kruskal-Wallis) (Fig. 1D; Suppl. Table 1). Accordingly, we found
140 increased L-Gln/L-Glu ratio in both SMA1 and SMA2 patients compared to controls (respectively, *P*
141 = 0.006 and *P* = 0.036; Mann-Whitney with Bonferroni correction) (Fig. 1D; Suppl. Table 1). In
142 addition, the D-Ser to total Ser ratio – which is a reliable index of D-Ser metabolism (37) – showed a
143 significant difference among clinical conditions (*P* < 0.001; Kruskal-Wallis) (Fig. 1H and Suppl. Table
144 1). The following *post-hoc* analysis revealed an increased D-Ser/total Ser ratio in SMA1 patients
145 compared to those with milder forms of SMA (*P* < 0.001; Fig 1H; Suppl. Table 1). The Kruskal-Wallis
146 test showed significant changes in L-Asp levels in SMA patients (*P* = 0.037) (Fig. 1E; Suppl. Table 1),
147 and the CSF levels of D-Asp were below the detection limit of our HPLC settings (0.01 pmol). Lastly,
148 ANCOVA analysis performed on natural log-transformed data to evaluate the potential confounding
149 effects of age and sex indicated that only variations in the levels of L-Glu (*P* = 0.006) and the L-Gln/L-
150 Glu ratio (*P* = 0.003) were significantly associated with the clinical condition. These findings show
151 that SMN deficiency leads to significantly decreased concentrations of L-Glu in the CSF of severe
152 SMA1 patients and to altered L-Gln to L-Glu conversion in the CSF of both SMA1 and SMA2 patients.
153
154 We next investigated whether the CSF concentrations of amino acids within each SMA patient type
155 were associated with age or motor function assessed by CHOP-INTEND (SMA1) or HFMSE (SMA2
156 and SMA3) clinical assays. Non-parametric Spearman's correlation analysis revealed that the levels of
157 L-Glu and L-Gln as well as the L-Gln/L-Glu ratio showed no significant correlation with age or clinical
158 parameters in SMA1 patients (Table 2). In contrast, D-Ser levels and the D-Ser/total Ser ratio were
159 negatively correlated with age (Table 2; Suppl Fig. 1) and positively correlated with CHOP-INTEND
160 (Table 2; Suppl. Fig. 2). Since we also found a negative correlation between age and CHOP-INTEND

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161 (Table 2; Suppl. Fig. 3), we performed multivariate linear regression analysis considering age as a
162 confounding factor. This analysis did not confirm the significance of the association between D-Ser or
163 D-Ser/total Ser ratio with CHOP-INTEND (respectively, $P = 0.074$ and $P = 0.203$), highlighting the
164 putative influence of age on this correlation. In SMA2 patients, statistical analysis showed a significant
165 negative correlation between D-Ser levels and the D-Ser/total Ser ratio with age (Table 2; Suppl. Fig.
166 1). There were also positive correlations of D-Ser levels and the D-Ser/total Ser ratio with the HFMSE
167 score (Table 2; Suppl. Fig. 2), which failed to reach statistical significance probably due to the small
168 sample size. Similar to SMA1 patients, age and motor function were negatively correlated in SMA2
169 individuals (Table 2; Suppl Fig. 3). In SMA3 patients, we found a negative correlation of the D-
170 Ser/total Ser ratio with age but not with the HFMSE score (Table 2; Suppl Fig. 1 and 2). There was
171 also no correlation between age and the HFMSE score (Table 2; Suppl. Fig. 3). Interestingly, no
172 significant associations occurred between either D-Ser levels or the D-Ser/total Ser ratio and age in
173 control subjects (Table 2; Suppl Fig. 1), consistent with the possibility that age-dependent decrease in
174 D-Ser levels is specific to the disease state. However, the limited sample size of controls might affect
175 this result. The confounding effects of age notwithstanding, these results highlight a potential
176 correlation between greater D-Ser levels and the D-Ser/total Ser ratio and better motor function, which
177 is especially apparent in severe SMA patients.
178
179 **Nusinersen modulates glutamate, glutamine, and serine levels in the CSF of severe SMA patients.**
180 We next sought to determine the effects of Nusinersen treatment on the CSF levels of neuroactive
181 amino acids and whether it could normalize the alterations observed in untreated, early-onset SMA
182 patients. For this longitudinal analysis, we used a subgroup of SMA patients ($n = 18$ SMA1, $n = 17$
183 SMA2, and $n = 14$ SMA3) for whom CSF samples were available both prior to (T0) and 302 days after
184 (T302) initiation of treatment, corresponding to the maintenance phase of Nusinersen therapy (Figure
185 2A; Table 3).
186
187 The non-parametric Wilcoxon matched-pairs test revealed that Nusinersen therapy significantly
188 increased the levels of L-Glu ($P = 0.035$) and L-Gln ($P = 0.025$) in the CSF of SMA1 patients relative
189 to the corresponding drug-free baseline (Fig. 2B, C; Suppl. Table 2). Moreover, Nusinersen
190 administration significantly increased L-Ser levels ($P = 0.004$) and decreased the D-Ser/total Ser ratio
191 in the CSF of SMA1 patients ($P = 0.007$) (Fig. 2F,H; Suppl. Table 2). In SMA2 patients, we did not
192 observe significant changes in the concentration of neuroactive amino acids after Nusinersen treatment
193 (Suppl. Fig. 4; Suppl. Table 2). In SMA3 patients, Nusinersen treatment decreased the CSF levels of
194 several amino acids, which differs from its effects in both SMA1 and SMA2 patients. Accordingly, we

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195 found lower levels of L-Glu (*P* = 0.006) resulting in a small increase in the L-Gln/L-Glu ratio (*P* =
196 0.019) as L-Gln levels were comparable between groups (*P* = 0.064) (Suppl. Fig. 5A-C; Suppl. Table
197 2). There were also reduced levels of L-Ser (*P* = 0.019), D-Ser (*P* = 0.016), and L-Asp (*P* = 0.038) in
198 Nusinersen-treated SMA3 patients relative to baseline (Suppl. Fig. 5D-F; Suppl. Table 2). Overall,
199 these results reveal that Nusinersen-dependent upregulation of SMN modulates the metabolism of L-
200 Glu, L-Gln, and L-Ser while decreasing the D-Ser/total Ser ratio in the CSF of SMA1 patients, partially
201 counteracting the dysregulation of amino acids associated with the severe form of the disease prior to
202 treatment.
203
204 We next investigated the association of changes in amino acid levels with demographic and clinical
205 parameters of SMA patients following 302 days of Nusinersen treatment. Non-parametric Spearman's
206 correlation analysis highlighted a positive correlation between the D-Ser/total Ser ratio and CHOP-
207 INTEND but the lack of significant correlation with age in Nusinersen-treated SMA1 patients (Suppl.
208 Fig. 6; Table 4). However, when controlling for age by multivariate linear regression, the association
209 between D-Ser/total Ser and CHOP-INTEND was lost (*P* = 0.136). As expected for the early-onset
210 severe form of the disease, we found a negative correlation between age and CHOP-INTEND in SMA1
211 patients (Table 4). In Nusinersen-treated SMA2 patients, we found a positive correlation of L-Ser and
212 D-Ser levels with HFMSE (Suppl. Figure 7-8; Table 4). Specifically, L-Ser and D-Ser levels were
213 negatively correlated with age, which was also negatively correlated with HFMSE **(**Table 4**)**. However,
214 only age but not L-Ser or D-Ser remained significantly associated with HFMSE after multivariate linear
215 regression analysis (L-Ser: *P* = 0.308; D-Ser: *P* = 0.809).
216
217 Lastly, statistical analysis showed only a negative correlation of the D-Ser/total Ser ratio with age in
218 Nusinersen-treated SMA3 patients (Table 4). These results show a positive correlation between D-
219 Ser/total Ser and CHOP-INTEND in SMA1, and between L-Ser or D-Ser and HFMSE in SMA2.
220 However, the influence of age on motor function in early-onset SMA patients complicates the
221 interpretation of the observed amino acid variations in relationship to motor improvement.
222

223 **Dysregulation of glutamate-glutamine metabolism in the brain and spinal cord of SMA mice**

224 To expand on our investigation of the effects of SMN deficiency on the levels of neuroactive amino
225 acids, we conducted HPLC analysis (Fig.3B) in brain and spinal cord tissues isolated from SMA mice
226 at early (postnatal day 3, P3) and late (P11) symptomatic stages of the disease. SMN depletion did not
227 change the levels of any amino acid tested in the brain or spinal cord from SMNΔ7 mice compared
228 with WT at P3 (Fig. 3C,D; Suppl. Fig. 8; Suppl. Table 3). In contrast, we found a significant increase

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229 in the concentration of L-Gln and L-Gln/L-Glu ratio in both the brain and spinal cord of SMNΔ7 mice
230 compared to WT at P11 (Fig 3E,F; Suppl. Fig. 9; Suppl. Table 3). Furthermore, SMNΔ7 mice displayed
231 an increased D-Asp/total Asp ratio in the spinal cord at P11 (Suppl. Fig. 9; Suppl. Table 3).
232
233 We then used two-way ANOVA followed by Tukey's *post-hoc* comparisons to further analyze the
234 neurochemical variations during postnatal CNS development in WT and SMNΔ7 mice. This
235 highlighted a physiological, age-dependent drop of the L-Gln/L-Glu ratio in the brain of WT mice that
236 does not occur in SMNΔ7 mice, resulting in a higher L-Gln/L-Glu ratio in SMNΔ7 relative to WT mice
237 at P11 (Fig. 3G; Suppl. Table 4). Similarly, we found that the L-Gln/L-Glu ratio in the spinal cord of
238 SMNΔ7 mice significantly increased relative to WT littermates at P11 (Fig. 3H; Suppl. Table 4).
239 Furthermore, despite significant age-dependent changes between WT and SMNΔ7 mice were found
240 for the D-Ser/total Ser ratio in the brain and the D-Asp/total Asp ratio in the spinal cord, no differences
241 were highlighted between genotypes at each single time point using the Tukey's multiple comparisons
242 *post-hoc* analysis (Suppl. Fig. 10, Suppl. Table 4).
243
244 These findings indicate that SMN deficiency significantly impacts the metabolism of neuroactive
245 amino acids involved in glutamatergic neurotransmission in the brain and spinal cord of SMA mice at
246 the disease end stage. Importantly, the increase in the L-Gln/L-Glu ratio emerges as a conserved
247 signature of neurochemical dysregulation in the CSF of severe SMA patients and the CNS of mouse
248 models.
249
250 **D-serine supplementation moderately improves motor function in SMA mice.**
251 The results of our neurochemical profiling highlighted the dysregulation of amino acid metabolism
252 acting on glutamatergic neurotransmission as well as a potential correlation between higher D-Ser/total
253 Ser ratio in the CSF and better motor function in severe SMA patients. Since D-Ser is a major co-
254 agonist of NMDARs (34, 35), we sought to investigate the phenotypic effects of increasing D-Ser
255 levels in the CNS of SMA mice.
256
257 First, to validate that intraperitoneal (IP) administration of D-Ser increases its levels in the mouse spinal
258 cord, we performed a single injection of vehicle or D-Ser at a dosage of 500 mg/kg in WT mice at P3
259 followed by HPLC analysis 1 h post-injection (Fig. 4A). As expected (38), we found that the
260 concentration of D-Ser (median [IQR] of nmol/mg of protein, D-Ser = 28.97 [25.29-58.61] vs vehicle
261 = 2.81 [2.71-3.09], $P = 0.0286$, Mann-Whitney test) and the D-Ser/total Ser ratio (D-Ser = 51.90

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262 [47.54-67.22] vs vehicle = 11.02 [10.34-11.68], $P = 0.0286$) were strongly increased in the spinal cord
263 of D-Ser-injected mice relative to control mice injected with vehicle (Fig. 4A-C).
264
265 Next, we analyzed the effects of daily supplementation of D-Ser (500mg/kg) starting at birth on SMN
266 expression and the phenotype of SMA mice, which included daily measures of weight gain, motor
267 function, and survival. Western blot analysis of spinal cord tissue isolated at P11 from WT and SMA
268 mice either with or without amino acid supplementation demonstrated that D-Ser does not increase
269 SMN protein levels (Fig. 4D,E). Interestingly, phenotypic analysis showed a moderate improvement
270 of motor function assessed by the righting reflex in D-Ser-treated relative to untreated SMA mice (Fig.
271 4F). This motor benefit appeared in the second postnatal week and was not associated with an increase
272 in weight gain, which was similar for D-Ser-treated and untreated SMA mice (Fig. 4G). Lastly, despite
273 fewer early deaths and some slightly longer-lived mice being noted, treatment with D-Ser did not
274 increase the median survival of SMA mice (Fig. 4H). Overall, these results are consistent with the
275 possibility that increased CNS levels of D-Ser may partially improve motor function in a mouse model
276 of SMA by acting on glutamatergic neurotransmission.
277
278
279

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280 **Discussion**

281 Selective degeneration of motor neurons and skeletal muscle atrophy are hallmarks of SMA in both
282 patients and mouse models (1, 6). However, cell-autonomous deficits in motor neurons alone cannot
283 account for the SMA phenotype and dysfunction of neuronal networks that control motor output has
284 been implicated in disease etiology (6). Accordingly, several studies have shown that reduced
285 excitatory drive to motor neurons (13-17) and metabolomic dysfunction in fundamental intracellular
286 pathways (39) may play a role in SMA pathophysiology. Nevertheless, while a variety of synaptic
287 deficits in motor neuron afferents have been documented (13, 14, 17, 21, 40), whether SMN deficiency
288 directly alters the content of neurotransmitters in the CNS of animal models and SMA patients is
289 unknown. Here, we addressed this issue by investigating changes in the levels of the most abundant
290 excitatory D- and L-amino acids that drive glutamate receptor signaling or act as their immediate
291 precursors in the CNS of SMNΔ7 mice and in the CSF of SMA patients of differing severity before
292 and after Nusinersen therapy.
293

294 Our findings reveal that SMN deficiency strongly affects the levels of D- and L-amino acids related to
295 glutamatergic neurotransmission in the CSF of severe SMA patients as well as in the brain and spinal
296 cord of SMNΔ7 mice. Accordingly, we found lower levels of L-Glu in SMA1 patients and a trend
297 toward reduction in SMA2 and SMA3 patients compared to controls. L-Glu reduction also results in a
298 significant increase of the L-Gln/L-Glu ratio in both SMA1 and SMA2 patients compared to control
299 subjects. Interestingly, a prominent increase in the L-Gln/L-Glu ratio also occurs in the brain and spinal
300 cord of SMNΔ7 mice at late symptomatic stages. Thus, deregulated L-Gln to L-Glu conversion
301 emerges as a neurochemical signature of altered glutamatergic metabolism shared by severe SMA
302 patients and animal models that may contribute to impaired excitatory neurotransmission in the disease
303 state.
304

305 The SMA-related changes in the L-Gln/L-Glu ratio are suggestive of abnormalities in the glutamate-
306 glutamine cycle, an imbalance of which has been associated with various neurological and psychiatric
307 disorders (41). This cycle involves the functional interaction between neurons and astrocytes in the
308 tripartite glutamatergic synapse where pre- and post-synaptic nerve terminals and perisynaptic
309 astrocytic processes recycle L-Glu for neurotransmission (Fig. 3A). Following synaptic release, L-Glu
310 binds to ionotropic and metabotropic glutamate receptors located post-synaptically and is then taken
311 up by astrocytes via excitatory amino acid transporters to prevent excitotoxicity caused by excessive
312 activation of glutamate receptors (42). In astrocytes, the enzyme glutamine synthetase converts the
313 main part of L-Glu into L-Gln, which is shuttled to the neuron via sodium-coupled neutral amino acid

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314 transporters. L-Gln is then deaminated to L-Glu by glutaminase in the mitochondria of neurons and
315 either transferred by vesicular glutamate transporters into synaptic vesicles for the further rounds of
316 neurotransmission or converted in α-ketoglutarate for Krebs cycle activity. Thus, in addition to
317 perturbing glutamatergic neurotransmission, SMN deficiency may also trigger an abnormally increased
318 conversion of L-Glu in α-ketoglutarate into the Krebs cycle as a compensatory mechanism to
319 counteract the pervasive energy failure due to mitochondrial abnormalities associated with SMA (39,
320 43). Importantly, previous *in vitro* and *in vivo* studies have highlighted several deficits induced by
321 SMN deficiency in SMA astrocytes that may contribute to the dysfunction of the tripartite synapse and
322 the neurochemical alterations observed here (44-47).
323
324 Notably, our neurochemical profiling revealed an upregulation of the D-Ser/total Ser ratio in the CSF
325 of SMA1 patients compared to individuals with SMA2 and SMA3, reflecting opposite trends toward
326 decrease or increase of L-Ser and D-Ser levels, respectively. Based on the pharmacological properties
327 of D-Ser as a potent endogenous co-agonist of NMDARs (34, 35), these findings further extend the
328 link between SMN deficiency and dysregulation of amino acid metabolism acting on glutamatergic
329 neurotransmission in severe SMA. As is the case for the glutamate-glutamine cycle described above,
330 an increase in the D-Ser/total Ser ratio points to dysfunction of astrocyte metabolism in SMA1 patients.
331 Accordingly, *de novo* synthesis of L-Ser in the CNS occurs exclusively in astrocytes through the
332 phosphorylated pathway, which employs 3-phosphoglycerate generated by glycolysis; L-Ser is then
333 shuttled to neurons for the biosynthesis of D-Ser catalyzed by serine racemase (SR) (48, 49) (Fig. 3A).
334 Interestingly, our clinical data highlight a positive correlation between D-Ser levels or the D-Ser/total
335 Ser ratio and motor function assessed by CHOP-INTEND in SMA1 patients and a similar trend in
336 SMA2 patients assessed by HFSME. However, the correlation between D-Ser metabolism and motor
337 function should be interpreted cautiously due to the potentially confounding effect of age. Accordingly,
338 age was negatively correlated with D-Ser levels, the D-Ser/total Ser ratio, and motor function in SMA1
339 and SMA2 patients; and age-adjusted partial correlation and multiple regression analyses did not
340 confirm the association between D-Ser metabolism and clinical scores. The inverse correlation of D-
341 Ser levels or the D-Ser/total Ser ratio with age observed in the CSF of SMA patients and controls is in
342 agreement with previous observation in a large cohort of pediatric individuals (50).
343
344 Our longitudinal analysis of the neurochemical composition of the CSF in SMA patients before and
345 after treatment with Nusinersen further supports the role of SMN in regulating the concentration of D-
346 and L-amino acids that modulate glutamatergic neurotransmission, especially in patients affected by
347 the most severe form of the disease. Accordingly, we found that Nusinersen induces a significant

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348 increase in the levels of L-Glu and L-Gln as well as upregulation of L-Ser with consequent reduction
349 of the D-Ser/total Ser ratio in the CSF of treated SMA1 patients relative to their drug-free baseline.
350 These findings are consistent with previous untargeted metabolomic studies highlighting the effect of
351 Nusinersen on glutamate metabolism in SMA1 patients (39), which was accompanied by modulation
352 of energy-related Krebs cycle and glutathione metabolism that depend on direct L-Glu supply.
353 Although the mechanisms driving the observed neurochemical variations remain unclear, these results
354 link Nusinersen-dependent SMN upregulation to an improved astrocyte-dependent metabolism of
355 amino acids related to glutamatergic neurotransmission and energy metabolism, which are strongly
356 affected in severe SMA (51, 52). Interestingly, and in contrast to SMA1 patients, Nusinersen did not
357 affect the levels of neuroactive amino acids in the CSF of SMA2 patients and slightly decreased the
358 concentration of L-Glu, L-Asp, L-Ser, D-Ser as well as the L-Gln/L-Glu ratio in SMA3 patients. The
359 opposite effects of Nusinersen on the CSF concentrations of L-Glu and L-Ser in SMA1 and SMA3
360 patients confirm that the impact of SMN upregulation on amino acid metabolism follows distinct
361 biochemical trajectories depending on SMA severity. Lastly, we found a positive correlation of motor
362 function with the D-Ser/total Ser ratio in SMA1 patients and with the levels of L-Ser or D-Ser in SMA2
363 patients after 302 days of Nusinersen treatment. As is the case for the analysis in naïve SMA patients,
364 however, it remains unclear whether these neurochemical differences are directly associated with
365 motor improvement because age is a significant confounding factor in these correlations. Nevertheless,
366 it is important to highlight that L-Ser supplementation is currently in use for the therapy of the Neu-
367 Laxova syndrome, characterized by severe peripheral malformations and microcephaly (53) and in
368 Phase I clinical trials for the treatment of an inherited form of peripheral neuropathy (54) and ALS
369 (55). Future longitudinal studies in larger cohorts of SMA patients and age-matched healthy individuals
370 will help address the issue.
371
372 Given the difficulty in conclusively establishing cause-effect relationships between changes in the CSF
373 levels of neuroactive amino acids and clinical outcomes in SMA patients, we sought to initially address
374 this issue in a preclinical *in vivo* setting by studying the phenotypic effects of D-Ser metabolism
375 modulation in a mouse model of SMA. The focus on D-Ser was prompted by our observations that the
376 D-Ser/total Ser ratio in the CSF of SMA1 patients i) is higher relative to that of SMA2 and SMA3
377 patients, ii) positively correlates with motor function, and iii) decreases after Nusinersen therapy. If
378 changes in the D-Ser/total Ser ratio are biologically relevant to SMA pathology, two main scenarios
379 can be envisioned that take into consideration the role of D-Ser as a potent agonist of NMDAR (34,
380 35). On one hand, increased levels of D-Ser may be beneficial and reflect a homeostatic attempt to
381 counteract deficits in glutamatergic neurotransmission at NMDARs through enhanced L-Ser to D-Ser

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382 conversion, which is tuned down after Nusinersen treatment enhances L-Glu levels. On the other hand,
383 increased levels of endogenous D-Ser could be harmful to neurons via excitotoxicity – a possibility
384 consistent with previous studies of the motor neuron disease amyotrophic lateral sclerosis (ALS) (56-
385 58). Our results show that systemic administration of D-Ser by IP injection strongly increases D-Ser
386 levels and the D-Ser/total Ser ratio in the mouse CNS as expected from previous studies (38, 59).
387 Interestingly, daily supplementation of D-Ser in severe SMA mice ameliorates motor function while
388 having no effects on weight gain and survival. Furthermore, the motor improvement is independent
389 from SMN whose low expression levels in the spinal cord are unaffected by D-Ser. These findings
390 suggest the possibility that elevated D-Ser levels do not have deleterious but rather beneficial effects
391 on the severe motor phenotype of SMA mice by enhancing glutamatergic neurotransmission at the
392 GluN1 subunit of NMDARs. This interpretation is in agreement with previous studies showing that
393 physical and pharmacological approaches aimed at increasing neuronal activity and NMDAR signaling
394 improve motor function in animal models of SMA (15, 17, 19, 60-62).
395
396 In conclusion, our study shows that SMN deficiency disrupts the physiological balance of neuroactive
397 D- and L-amino acids linked to glutamatergic receptors signaling in the CNS of SMA mice and the
398 CSF of severe SMA patients. The resulting defects may compound the deleterious effects associated
399 with the loss of excitatory synapses on motor neurons in spinal sensory-motor circuits as well as
400 interfere more broadly with glutamatergic neuronal networks in the brain of severe SMA patients,
401 including cognitive deficits. Moreover, our findings identify modulation of glutamate and serine
402 metabolism as downstream targets of Nusinersen treatment in SMA patients and support further
403 investigation of pharmacological approaches, such as D-Ser supplementation, aimed at improving
404 glutamatergic neurotransmission deficits for use in combination therapies with SMN-inducing drugs.

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405 ## Materials and Methods

406 ### Patients' characteristics

407 This is a two-center study (Bambino Gesù Hospital, Rome, Italy; Giannina Gaslini Institute, Genoa,
408 Italy) conducted on seventy-three patients affected by SMA1 (n = 34), SMA2 (n = 22) and SMA3 (n
409 = 17) who received intrathecal treatment with Nusinersen (12 mg) (Table 1). Additionally, seven non-
410 neurological pediatric control subjects aged 2.5-14 years were included in the study (Table 1). The
411 study was approved by the local Ethics Committees of the two Hospitals (2395_OPBG_2021). All
412 participants and/or their legal guardians signed a written informed consent. CSF samples were collected
413 at day 0 (T0; baseline) and day 302 (T302; after 5 Nusinersen injections) and used for detection of
414 amino acids. For SMA1 patients, we collected n = 34 CSF samples at T0, and n = 18 at T302. For
415 SMA2 patients, we collected n = 22 CSF samples at T0, and n = 17 at T302. For SMA3 patients, we
416 collected CSF samples from 17 patients at T0, and 14 CSF samples at T302 (Table 3). For longitudinal
417 analysis, we considered the subgroup of SMA patients (n = 18 SMA1, n = 17 SMA2, and n = 14 SMA3)
418 for whom CSF samples were available both prior to (T0) and 302 days after (T302) initiation of
419 treatment, corresponding to the maintenance phase of Nusinersen therapy (Figure 2A; Table 3). All
420 patients were clinically diagnosed and genetically confirmed, and the *SMN2* copy number was also
421 determined. All SMA1 patients, irrespective of age and disease severity, were part of the Expanded
422 Access Programme (EAP) for compassionate use to patients with the infantile form only, which
423 occurred in Italy between November 2016 and November 2017. The overall clinical response of these
424 patients to Nusinersen treatment has previously been reported as part of the full Italian cohort and
425 showed that therapeutic efficacy is related to age and clinical severity at baseline (63, 64). The SMA2
426 and SMA3 patients have also been reported previously (65).

427

428 ### Clinical evaluation

429 Assessment of patients was performed at T0 and T302. At each visit, extensive clinical examination
430 was performed by experienced child neurologists or pediatricians with expertise in SMA, and
431 anthropometric measurements and vital parameters were collected. Patients' feeding status (oral
432 nutrition, nasogastric tube (NG) or percutaneous gastrostomy), nutritional status postulated by Body
433 Mass Index (BMI), and respiratory function (spontaneous breathing, non-invasive ventilation (NIV) or
434 tracheostomy) were recorded.

435 For SMA1 patients, five children were younger than 5 months, while all the others were older than 5
436 months at the beginning of treatment with ages ranging from 6 months to 10 years. Eleven patients had
437 tracheostomy and thirteen were under NIV for <16h/day. Nineteen patients had gastrostomy, and the
438 BMI fell into the underweight range (< 18.5) in all patients. The age of the SMA2 patients included in

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439 this study ranged from 9 months to 13.6 years at baseline. Seven of these patients were under NIV, and
440 fourteen patients were in spontaneous breathing. None had tracheostomy or gastrostomy, and the BMI
441 fell below 18 in eight patients. Regarding the SMA3 patients, one was under NIV for < 16h/day and
442 none had gastrostomy. At T0 and T302, all patients were assessed using standardized motor function
443 tests chosen according to their age and motor function. Functional assessments were performed by
444 expert physiotherapists trained with standardized procedure manuals (66) and reliability sessions.
445 SMA1 patients were assessed with the CHOP-INTEND (67, 68), a functional scale including 16 items
446 aimed at assessing motor function in weak infants. Each item is scored from 0 to 4 (0 being no response
447 and 4 being the complete level of response), with a total score ranging from 0 to 64. SMA2 and SMA3
448 patients were evaluated with the HFMSE (68, 69), a scale of 33 items investigating the child's ability
449 to perform different activities. The total score can range from zero, if all the activities are failed, to 66,
450 indicating better motor function. All patients were not wearing spinal jackets or orthoses during the
451 evaluations.
452 SMA1, SMA2 and SMA3 patients significantly differed in age (SMA1 *vs* SMA2, $P = 0.006$; SMA1
453 *vs* SMA3, $P < 0.0001$; SMA2 *vs* SMA3, $P = 0.004$; Mann-Whitney test). BMI was lower in SMA1
454 compared to SMA2 and SMA3 patients ($P = 0.0001$ and $P = 0.002$, respectively; Mann-Whitney test)
455 while sex was not different among SMA groups ($\chi^2 = 0.045$, $P = 0.978$).
456

457 **Intrathecal treatment with Nusinersen**

458 Intrathecal administration of 12 mg of Nusinersen was performed in a hospital environment. Fasting
459 less than 4 h was planned for the procedure in SMA1 patients, while the time between the last meal
460 and the lumbar puncture was 6-8 h in SMA2 and SMA3 patients. In SMA1 the procedure was carried
461 out without sedation, whereas for SMA2 and SMA3 patients a sedation with midazolam was applied.
462 No severe adverse events were reported. After the infusion, all patients were recommended to lie for 2
463 h to avoid any possible post-lumbar puncture symptoms.
464

465 **CSF sample collection**

466 CSF samples were collected at the time of intrathecal administration of Nusinersen in polypropylene
467 tubes and stored at -80°C until further analysis. Amino acid levels were measured in the CSF sample
468 of each patient. Exclusion criteria included the presence of symptoms or changes in blood biochemical
469 and haematological parameters suggestive of a systemic inflammatory state, and/or
470 immunosuppressive treatments ongoing in the last 6 months before inclusion.
471

472 **Animals**

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473 Experiments in mice were performed according to the international guidelines for animal research and
474 approved by the Animal Care Committee of "Federico II" University of Naples, Italy and the Ministry
475 of Health, Italy. Heterozygous SMNΔ7 carrier mice ($Smn^{+/-}$; $SMN2^{+/+}$; $SMN\Delta7^{+/+}$) were purchased
476 from Jackson Laboratory (stock number 005025) and bred to obtain $Smn^{+/+}$ (WT) animals and $Smn^{-/-}$
477 (SMA) animals. Mice were housed with 12 h light/dark cycle and were given free access to food and
478 water. All efforts were made to minimize animal suffering and to reduce the number of animals used.
479 The colony was maintained by interbreeding carrier mice, and the offspring were genotyped by PCR
480 assays on tail DNA according to the protocols provided by Jackson Laboratory as previously reported
481 (70). Data were obtained from brain and spinal cord tissue of WT and SMNΔ7 mice isolated at P3 and
482 P11, considering P0 as the day of birth.
483

484 **HPLC detection**

485 CSF samples (100 µl) were mixed in a 1:10 dilution with HPLC-grade methanol (900 µl) and
486 centrifuged at 13,000 × g for 10 min; supernatants were dried and then suspended in 0.2 M TCA.
487 Mouse brain and spinal cord frozen samples were homogenized in 1:10 (w/v) 0.2 M TCA, sonicated
488 (4 cycles, 10 s each), and centrifuged at 13,000 × g for 20 min. TCA supernatants from mice and human
489 samples were then neutralized with NaOH and subjected to pre-column derivatization with o-
490 phthaldialdehyde (OPA)/N-acetyl-L-cysteine (NAC). Diasteroisomer derivatives were resolved on a
491 UHPLC Agilent 1290 Infinity (Agilent Technologies, Santa Clara, CA, USA) using a ZORBAX
492 Eclipse Plus C8, 4.6 × 150 mm, 5 µm (Agilent Technologies, Santa Clara, CA, USA) under isocratic
493 conditions (0.1 M sodium acetate buffer, pH 6.2, 1% tetrahydrofuran, and 1.5 mL/min flow rate). A
494 washing step in 0.1 M sodium acetate buffer, 3% tetrahydrofuran, and 47% acetonitrile was performed
495 after every single run. Identification and quantification of D-Asp, L-Asp, L-Glu, D-Ser, L-Ser and L-
496 Gln were based on retention times and peak areas, compared with those associated with external
497 standards. All the precipitated protein pellets from mice samples were solubilized in 1% SDS solution
498 and quantified by bicinchoninic acid (BCA) assay method (Pierce™ BCA Protein Assay Kits,
499 Thermofisher scientific, Rockford, IL, USA). The concentration of amino acids in tissue homogenates
500 was normalized to the total protein content and expressed as nmol/mg protein. Amino acid levels in
501 the CSF were expressed as micromolar (µM).
502

503 **Drug treatment and behavioral assays in SMA mice**

504 For studies of D-Ser supplementation in SMA mice, all procedures were performed on postnatal mice
505 in accordance with the NIH guidelines and approved by the Institutional Laboratory Animal Care and
506 Use Committee of Columbia University. FVB.Cg-$Grm7^{Tg(SMN2)89Ahmb}$ $Smn1^{tm1Msd}$

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507 Tg(SMN2*delta7)4299Ahmb/J (JAX Strain # 005025) mice were interbred to obtain SMA mutant
508 mice (71). Mice were housed in a 12h/12h light/dark cycle with access to food and water *ad libitum*.
509 Mice from all experimental groups were monitored daily for weight, motor function, and survival from
510 birth to 21 days of age. The righting reflex was assessed by placing the mouse on its back and
511 measuring the time it took to turn upright on its four paws (righting time). The cut-off test time was 60
512 s. For each testing session, the test was repeated three times, and the mean of the recorded times was
513 calculated. D-Ser (Sigma #S4250) was dissolved in water, filter sterilized and delivered daily at a dose
514 of 500 mg/kg by intraperitoneal injections starting from P0. Approximately equal proportions of mice
515 of both sexes were used, and aggregated data were presented because gender-specific differences were
516 not found.
517
518 **Protein analysis**
519 For Western blot analysis, mice were euthanized and spinal cord collection was performed in a
520 dissection chamber under continuous oxygenation (95%O_2/5%CO_2) in the presence of cold (~12°C)
521 artificial cerebrospinal fluid (aCSF) containing 128.35mM NaCl, 4mM KCl, 0.58mM NaH_2PO_4,
522 21mM $NaHCO_3$, 30mM D-Glucose, 1.5mM $CaCl_2$, and 1mM $MgSO_4$. Total protein extracts were
523 generated by homogenization of spinal cords in SDS sample buffer (2% SDS, 10% glycerol, 5% ß-
524 mercaptoethanol, 60mM Tris-HCl pH 6.8, and bromophenol blue), followed by brief sonication and
525 boiling. Proteins were quantified using the *RC DC*™ Protein Assay (Bio-Rad) and 25µg of protein
526 extract was analyzed by SDS/PAGE on 12% polyacrylamide gels followed by Western blotting as
527 previously described (72). Anti-SMN mouse monoclonal antibody (BD Transd Lab, clone 8, #610646;
528 1:10,000), anti-GAPDH mouse monoclonal antibody (Sigma, clone 6C5, #MAB374, 1:50,000), and
529 HRP conjugated goat anti-mouse secondary antibody (Jackson #115-035-044; 1:10,000) were used.
530 The signal was detected using an iBrigth CL1500 Imaging System (Thermo Fisher Scientific) and
531 image quantification was processed with the iBright Analysis Software (version 5.1.0).
532
533 **Statistical analysis**
534 Statistical analyses were performed using SPSS software v.27 (SPSS Inc., Chicago, IL, USA) and R
535 Language v.4.3.2 (R Foundation for Statistical Computing, Vienna, Austria). Normality distribution
536 was assessed by q-q plot and Shapiro–Wilk test. Quantitative variables were expressed by the median
537 and interquartile range (IQR), while qualitative variables were by absolute or relative frequency. The
538 correlation was evaluated by non-parametric Spearman's rho. The effect of confounders on correlation
539 was evaluated by partial correlation and multivariate linear regression on natural log-transformed data.
540 Differences between independent groups were studied by the non-parametric Kruskal-Wallis test

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541 followed, if statistically significant, by post-hoc tests performed by the Mann-Whitney test with
542 Bonferroni's correction. The effect of confounders was evaluated by ANCOVA on natural log-
543 transformed variables. Differences between dependent groups were studied by non-parametric
544 Friedman test followed, if statistically significant, by post-hoc tests performed by Wilcoxon Signed
545 Ranks Test with Bonferroni's correction. Amino acid concentrations in the CNS of SMA and WT mice
546 were compared using Mann-Whitney test and two-way ANOVA followed by Tukey's *post-hoc* using
547 Prism 8 version 8.0.2. For studies of D-Ser supplementation in SMA mice, statistical analysis of SMN
548 protein levels was performed by one-way ANOVA with Šídák's multiple comparisons test. Differences
549 in weight gain and motor function were analyzed by two-way ANOVA with Tukey's multiple
550 comparison test. A comparison of survival curves was performed using the Log-rank (Mantel-Cox)
551 test. Prism 10 for macOS version 10.3.1 was used for these statistical analyses.
552

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553 **Author Contributions**

554 A.H. and R.d.V. conducted HPLC experiments and acquired data; T.N. acquired data and prepared
555 figures; T.N. and M.V. analyzed data; M.J.C., S.Y. and H.Y. conducted in vivo experiments on SMA
556 mice, acquired and analyzed data; A.D.A., C.P., C.B. and E.B. provided CSF samples of patients; X.K.,
557 V.V. and G.P. provided brain and spinal cord samples of SMA mice; F.E., L.P. and A.U. wrote the
558 manuscript; A.U. designed research studies.

559

560 **Declaration of Competing Interest**

561 C.B. received advisory board honoraria from Avexis, Biogen, Novartis and Roche. The other authors
562 declare no competing interests.

563

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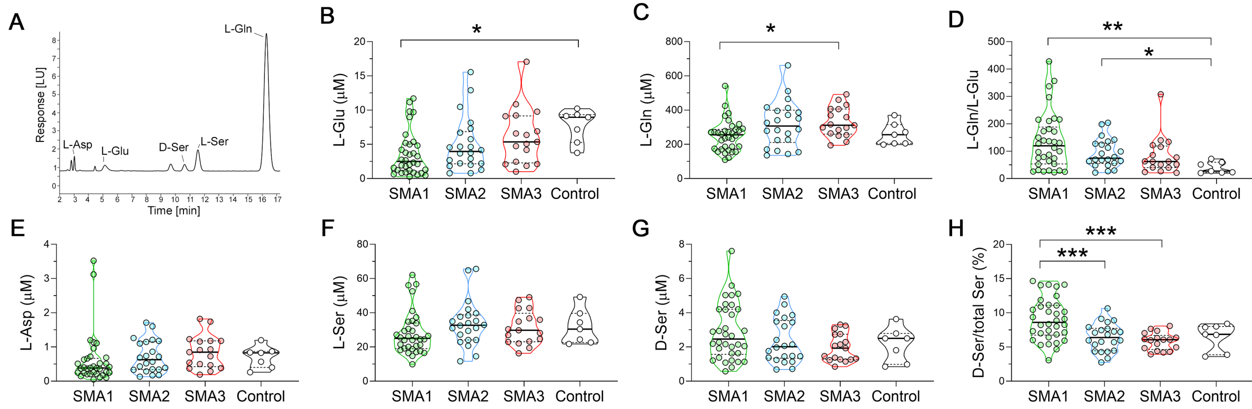

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759 **Figure 1. Levels of neuroactive amino acids in the CSF of SMA patients and control individuals.**
760 **(A)** Representative chromatogram showing the peaks of L-aspartate (L-Asp), L-glutamate (L-Glu), D-
761 serine (D-Ser), L-serine (L-Ser), and L-glutamine (L-Gln) in CSF of SMA1 patients. **(B-H)** Levels of
762 L-Glu **(B)**, L-Gln **(C)**, L-Gln/L-Glu ratio **(D)**, L-Asp **(E)**, L-Ser **(F)**, D-Ser **(G)** and D-Ser/total Ser
763 percentage ratio **(H)** in the indicated cohorts of SMA1, SMA2 and SMA3 patients as well as control
764 individuals. Data are shown as violin plots representing the median with interquartile range (IQR). **P*
765 < 0.05, ***P* < 0.01(Mann-Whitney test with Bonferroni's correction). Dots represent values from each
766 individual analyzed.
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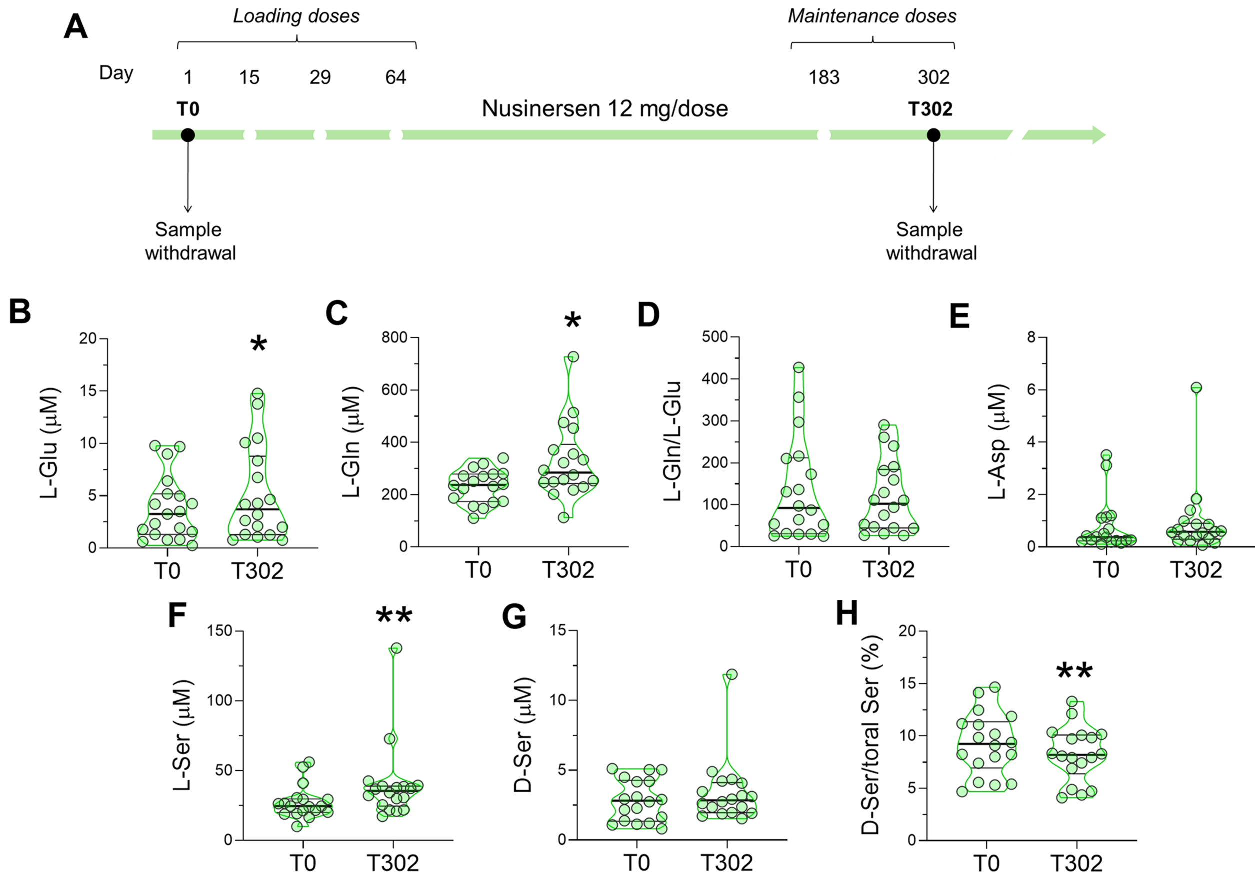

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772 **Figure 2. Effect of Nusinersen on the levels of neuroactive amino acids in the CSF of SMA1**
773 **patients. (A)** Schematic representation of the timeline of intrathecal Nusinersen administration and
774 CSF collection in SMA patients. **(B-H)** Levels of L-glutamate (L-Glu) **(B)**, L-glutamine (L-Gln) **(C)**,
775 L-glutamine/L-glutamate (L-Gln/L-Glu) ratio **(D)**, L-aspartate (L-Asp) **(E)**, L-serine (L-Ser) **(F)**, D-
776 serine (D-Ser) **(G)** and D-serine/total serine (D-Ser/total Ser) percentage ratio **(H)** in the CSF of SMA1
777 patients before treatment (T0, n=18) and at the time of the sixth (T302, n=18) injection of Nusinersen.
778 $^{*}P < 0.05$, $^{**}P < 0.01$, compared to T0 (Wilcoxon matched-pairs signed ranks test). Data are shown
779 as violin plots representing the median with interquartile range (IQR). Dots represent values from
780 individual SMA1 patients.
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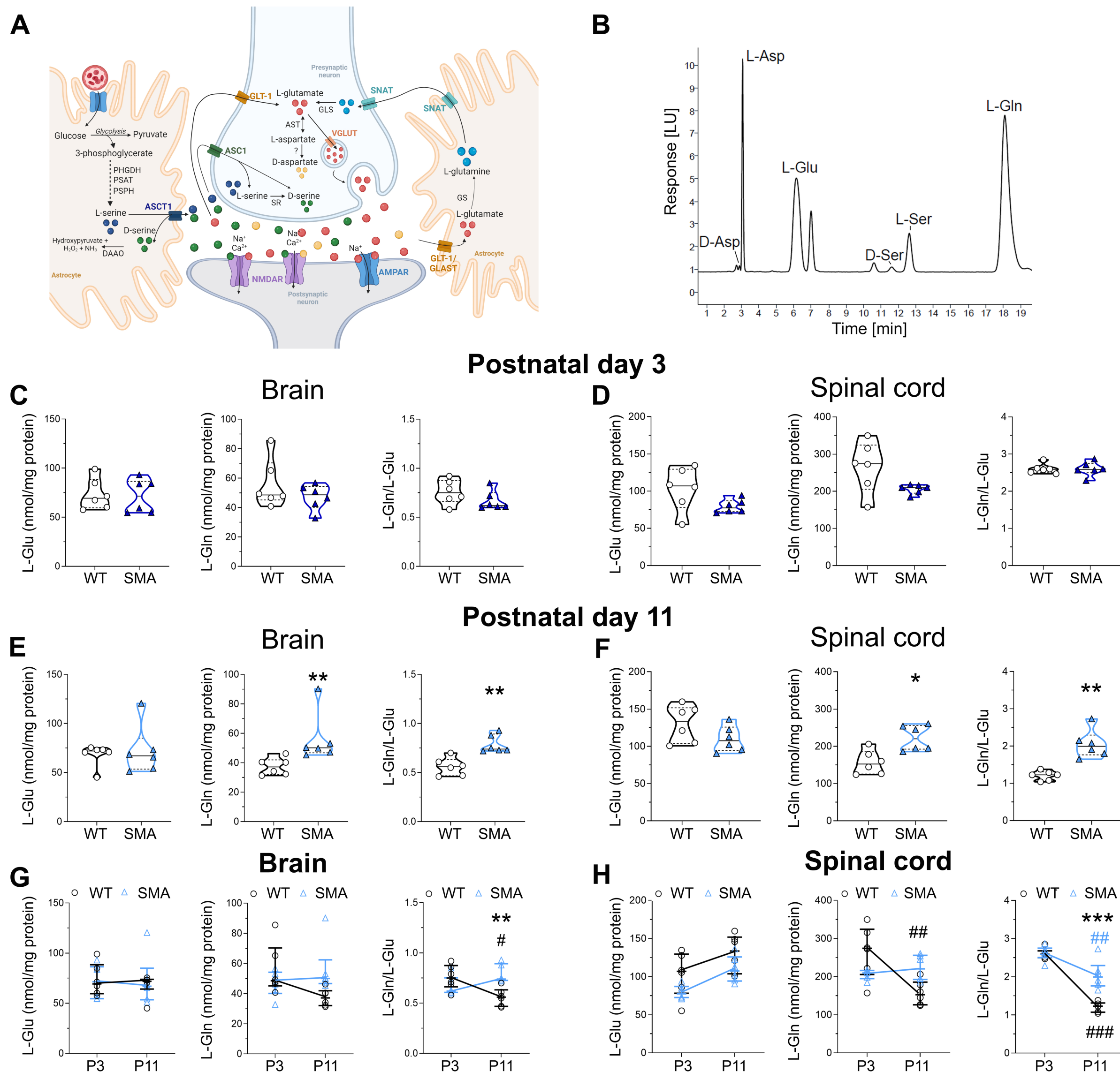

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783 **Figure 3. Analysis of neuroactive amino acid levels in the brain and spinal cord of SMNΔ7 mice**
784 **at early and late symptomatic stage of the disease. A)** Schematic model of the tripartite
785 glutamatergic synapse showing the main localization of the amino acids analyzed in this study. Image
786 created with BioRender.com (www.biorender.com). Abbreviations: PHGDH: phosphoglycerate
787 dehydrogenase; PSAT: phosphoserine aminotransferase; PSPH: phosphoserine phosphatase; DAAO:
788 D-amino acid oxidase; SR: serine racemase; GLS: glutaminase; GS: glutamine synthetase; ASCT1:
789 alanine, serine, cysteine transporter 1; ASC1: alanine, serine, cysteine transporter 1; GLT-1: glutamate
790 transporter 1; SNAT: sodium-coupled neutral amino acid transporter; GLAST: glutamate aspartate
791 transporter; NMDAR: N-methyl-D-aspartate receptor; AMPAR: alpha-amino-3-hydroxy-5-methyl-4-
792 isoxazolepropionic acid receptor; VGLUT: vesicular glutamate transporter. **B)** Representative
793 chromatogram showing the peaks of L-aspartate (L-Asp), L-glutamate (L-Glu), D-serine (D-Ser), L-
794 serine (L-Ser), and L-glutamine (L-Gln) in the brain homogenate of SMNΔ7 mice. **(C-F)** Levels of L-
795 Glu, L-Gln and L-Gln/L-Glu ratio in the brain and spinal cord of wild type (WT) and SMNΔ7 mice at
796 postnatal day 3 (P3), and P11. The average amounts of amino acids detected were normalized for mg
797 of total proteins. Dots represent values from individual mice. Amino acid levels are expressed as violin
798 plots representing median with interquartile range (IQR) and analyzed by Mann-Whitney test (**P* <
799 0.05, ***P* < 0.01, compared to age-matched WT mice). **(G,H)** Amino acid levels were also analyzed
800 as two-way ANOVA, followed by Tukey's multiple comparisons test (***P* < 0.01, ***P* < 0.0001,
801 compared to age-matched WT mice; ##*P* < 0.01, ###*P* < 0.0001, compared to genotype-matched P3

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802 mice). Amino acid levels were shown as scatter dot plots representing median with interquartile range
803 (IQR) while dots represent values from individual mice.

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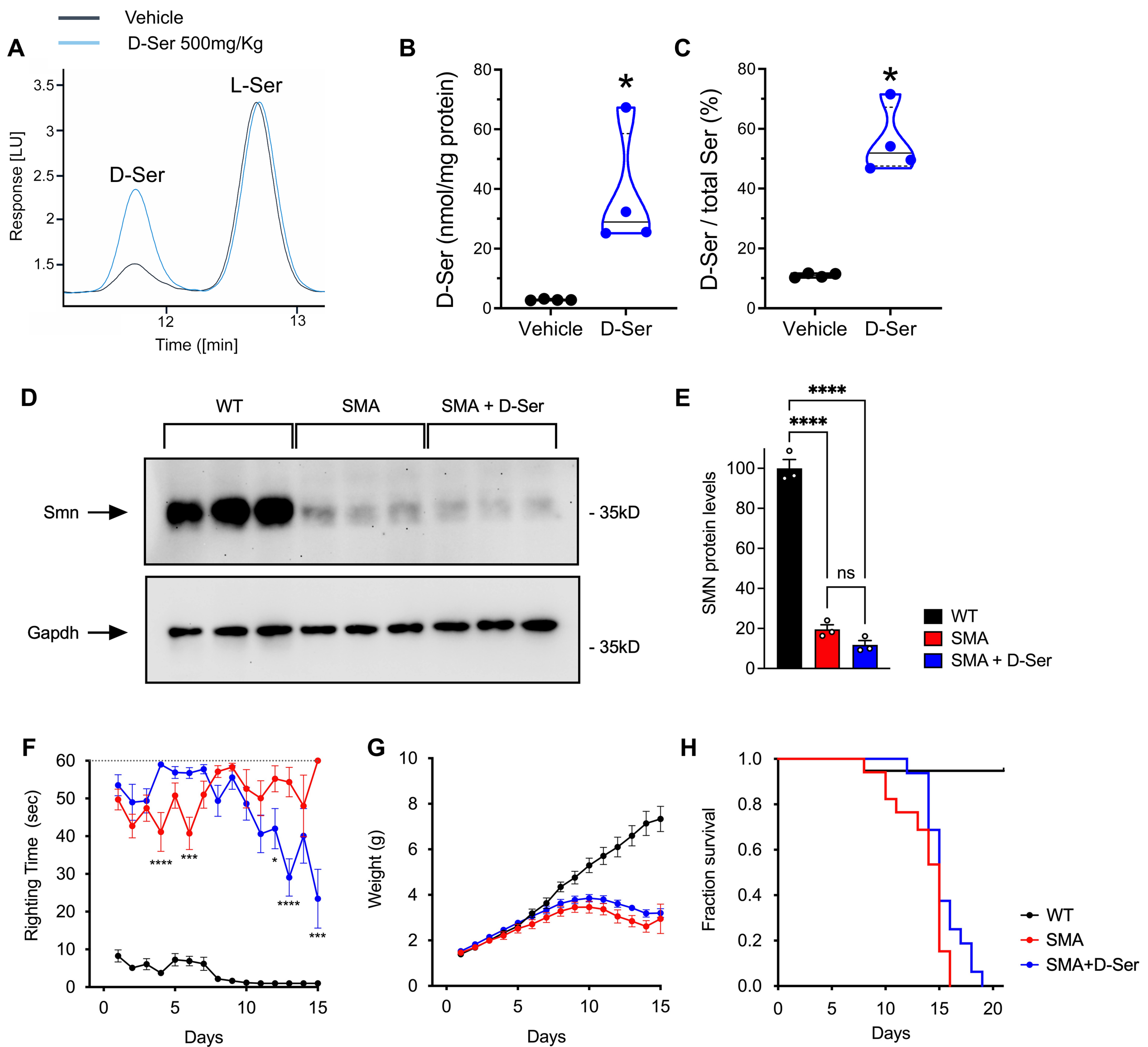

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808 **Figure 4. SMN-independent amelioration of motor function by D-serine supplementation in**
809 **SMA mice.** (**A**) Representative chromatogram showing the peaks of D-serine (D-Ser) and L-serine (L-
810 Ser) in the spinal cord homogenate of SMNΔ7 mice after a single injection of vehicle (black line) or
811 500 mg/kg D-Ser treatment (blu line) at P3. (**B-C**) D-Ser levels and D-Ser/toral Ser ratio in the spinal
812 cord of WT mice treated with a single injection of 500 mg/kg D-serine compared to vehicle-treated
813 mice at P3. *P < 0.05, compared to vehicle (Mann-Whitney test) (**D**) Western blot analysis of SMN
814 protein levels in spinal cords from WT and either untreated or D-Ser-treated SMNΔ7 mice at
815 P11. GAPDH was probed as a loading control. Three independent mice per group were analyzed.
816 (**E**) Quantification of SMN levels normalized to GAPDH from the data in (D). Ordinary one-way
817 ANOVA with Šídák's multiple comparisons test. ****P < 0.0001, ns: not significant. Values are means
818 and SEM. (**F-H**) Analysis of righting time (F), body weight (G), and survival (H) in WT mice (n=19)
819 and either untreated (n=17) or D-Ser-treated (n=16) SMNΔ7 mice. Two-way ANOVA with
820 Tukey's multiple comparisons test. ****P < 0.0001, ***P < 0.001, *P < 0.05. Values are means and
821 SEM.

822

Table 1. Demographic and clinical characteristics of naïve SMA subjects and controls enrolled in the study.

Demographic and clinical information	Controls (n=7)	SMA1 (n=34)	SMA2 (n=22)	SMA3 (n=17)
Sex (M:F, %)	43 : 57	40 : 60	41: 59	37 : 63
Age, years	7.0 (4.0-12.0)	3.0 (0.7-4.9)	5.5 (2.0-11.8)	13.7 (7.6-17.2)
SMN copy number (2:3:4, %)		91:9:0	10:90:0	21.4: 64.3 14.3
BMI		13.4 (12.4-15.3)	17.9 (15.9-19.8)	19.4 (16.2-22.1)
CHOP-INTEND		14 (3-21)	-	-
HFMSE		-	9.5 (6.5-15)	52 (35-57)
Gastrostomy (Yes:No, %)		51 : 49	0 :100	0 :100
NIV (Yes:No, %)		41 : 59	33 : 67	8 : 92
Tracheostomy (Yes:No, %)		34 :66	0 : 100	0 :100

Data are expressed as percentage frequencies or median (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.

Table 2. Correlation between amino acids and clinical or demographic variables.

		CHOP-INTEND/ HFMSE	L-Gln	L-Glu	L-Gln/ L-Glu	L-Asp	L-Ser	D-Ser	D-Ser/ total Ser
SMA1	Age	ρ=-0.540 ***P*=0.001** n=32	ρ=-0.026 *P*=0.888 n=32	ρ=-0.086 *P*=0.641 n=32	ρ=0.171 *P*=0.349 n=32	ρ=0.067 *P*=0.714 n=32	ρ=-0.044 *P*=0.811 n=32	ρ=-0.428 ***P*=0.015** n=32	ρ=-0.720 ***P*<0.001** n=32
	CHOP-INTEND	-	ρ=0.07 *P*=0.971 n=32	ρ=0.196 *P*=0.281 n=32	ρ=-0.243 *P*=0.180 n=32	ρ=0.233 *P*=0.199 n=32	ρ=0.116 *P*=0.528 n=32	ρ=0.377 ***P*=0.034** n=32	ρ=0.452 ***P*=0.009** n=32
SMA2	Age	ρ=-0.638 ***P*=0.002** n=20	ρ=0.131 *P*=0.571 n=21	ρ=-0.034 *P*=0.882 n=21	ρ=0.076 *P*=0.743 n=21	ρ=0.073 *P*=0.752 n=21	ρ=-0.199 *P*=0.388 n=21	ρ=-0.470 ***P*=0.032** n=21	ρ=-0.577 ***P*=0.006** n=21
	HFMSE	-	ρ=0.049 *P*=0.837 n=20	ρ=-0.032 *P*=0.892 n=20	ρ=0.077 *P*=0.747 n=20	ρ=-0.328 *P*=0.158 n=20	ρ=0.165 *P*=0.488 n=20	ρ=0.378 *P*=0.100 n=20	ρ=0.381 *P*=0.097 n=20
SMA3	Age	ρ=0.338 ***P***=0.238 n=14	ρ=0.178 *P*=0.543 n=14	ρ=-0.231 *P*=0.427 n=14	ρ=0.270 *P*=0.350 n=14	ρ=-0.292 *P*=0.311 n=14	ρ=-0.257 *P*=0.375 n=14	ρ=-0.464 *P*=0.095 n=14	ρ=-0.692 ***P*=0.006** n=14
	HFMSE	-	ρ=0.201 *P*=0.491 n=14	ρ=0.020 *P*=0.946 n=14	ρ=0.029 *P*=0.922 n=14	ρ=0.174 *P*=0.551 n=14	ρ=0.024 *P*=0.934 n=14	ρ=0.082 *P*=0.781 n=14	ρ=0.117 *P*=0.690 n=14
Controls	Age	-	ρ=0.286 *P*=0.535 n=7	ρ=-0.321 *P*=0.482 n=7	ρ=0.429 *P*=0.337 n=7	ρ=0.071 *P*=0.879 n=7	ρ=-0.750 *P*=0.052 n=7	ρ=-0.714 *P*=0.071 n=7	ρ=-0.484 *P*=0.294 n=7

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.

Table 3. Clinical and demographic characteristics of SMA patients treated with Nusinersen.

Demographic and clinical information	SMA1 (n=18)	SMA2 (n=17)	SMA3 (n=14)
Sex (M:F, %)	39 : 61	30 : 70	30 : 70
Age	2.7 (0.7-4.9)	5.5 (3.1-11.8)	13 (9-16)
SMN copy number (2:3:4, %)	94:6:0	94:6:0	23:63:14
BMI	13.2 (11.7-14.6)	18.2 (16.3-19.8)	19.4 (16.2-22.1)
CHOP-INTEND	6.5 (3-17)		
HFMSE		9 (8-13)	55 (36-56)
Gastrostomy (Yes:No, %)	66:34	0:100	0/100
NIV (Yes:No, %)	39: 61	35:65	8:92
Tracheostomy (Yes:No, %)	33: 67	0:100	0:100

Data are expressed as percentage frequencies or median (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.

Table 4. Correlation between amino acids and clinical or demographic variables in SMA1, SMA2 and SMA3 patients before and after treatment with Nusinersen.

Patients	Therapy phase	Parameter	CHOP-INTEND / HFMSE	L-Gln	L-Glu	Gln/Glu	L-Asp	L-Ser	D-Ser	D-Ser/tot Ser
SMA1 (N=18)	T0	Age	ρ=-0.636 ***P*=0.005**	ρ=0.084 *P*=0.741	ρ=0.071 *P*=0.779	ρ=0.018 *P*=0.945	ρ=0.237 *P*=0.343	ρ=-0.005 *P*=0.984	ρ=-0.344 *P*=0.162	ρ=-0.649 ***P*=0.004**
		CHOP-INTEND	-	ρ=0.113 *P*=0.656	ρ=0.292 *P*=0.240	ρ=-0.326 *P*=0.187	ρ=0.325 *P*=0.188	ρ=0.257 *P*=0.304	ρ=0.408 *P*=0.093	ρ=0.389 *P*=0.110
	T302	Age	ρ=-0.735 ***P*=0.001**	ρ=0.141 *P*=0.576	ρ=0.189 *P*=0.453	ρ=0.001 *P*=0.997	ρ=0.352 *P*=0.152	ρ=-0.005 *P*=0.984	ρ=-0.257 *P*=0.303	ρ=-0.364 *P*=0.137
		CHOP-INTEND	-	ρ=-0.303 *P*=0.237	ρ=-0.225 *P*=0.386	ρ=-0.039 *P*=0.881	ρ=-0.184 *P*=0.479	ρ=-0.139 *P*=0.596	ρ=0.264 *P*=0.306	ρ=0.528 ***P*=0.030**
SMA2 (N=17)	T0	Age	ρ=-0.511 ***P*=0.036**	ρ=0.020 *P*=0.940	ρ=0.025 *P*=0.926	ρ=-0.065 *P*=0.804	ρ=0.040 *P*=0.877	ρ=-0.207 *P*=0.425	ρ=-0.415 *P*=0.098	ρ=-0.471 *P*=0.056
		HFMSE	-	ρ=0.102 *P*=0.696	ρ=-0.119 *P*=0.648	ρ=0.217 *P*=0.404	ρ=-0.437 *P*=0.079	ρ=0.053 *P*=0.840	ρ=0.233 *P*=0.369	ρ=0.177 *P*=0.496
	T302	Age	ρ=-0.845 ***P*<0.001**	ρ=-0.422 *P*=0.091	ρ=-0.329 *P*=0.197	ρ=-0.145 *P*=0.579	ρ=-0.188 *P*=0.471	ρ=-0.677 ***P*=0.003**	ρ=-0.729 ***P*=0.001**	ρ=-0.218 *P*=0.400
		HFMSE	-	ρ=0.402 *P*=0.109	ρ=0.330 *P*=0.196	ρ=0.028 *P*=0.914	ρ=0.098 *P*=0.707	ρ=0.522 ***P*=0.032**	ρ=0.568 ***P*=0.017**	ρ=0.038 *P*=0.884
SMA3 (N=13)	T0	Age	ρ=0.337 *P*=0.259	ρ=0.206 *P*=0.499	ρ=-0.124 *P*=0.687	ρ=0.157 *P*=0.609	ρ=-0.217 *P*=0.476	ρ=-0.248 *P*=0.415	ρ=-0.437 *P*=0.135	ρ=-0.710 ***P*=0.007**
		HFMSE	-	ρ=0.210 *P*=0.491	ρ=0.017 *P*=0.957	ρ=0.039 *P*=0.900	ρ=0.210 *P*=0.491	ρ=0.033 *P*=0.914	ρ=0.110 *P*=0.719	ρ=0.110 *P*=0.719
	T302	Age	ρ=0.262 *P*=0.388	ρ=0.393 *P*=0.184	ρ=0.025 *P*=0.936	ρ=0.165 *P*=0.590	ρ=0.201 *P*=0.511	ρ=0.044 *P*=0.886	ρ=-0.105 *P*=0.734	ρ=-0.746 ***P*=0.003**
		HFMSE	-	ρ=0.536 *P*=0.059	ρ=0.377 *P*=0.204	ρ=-0.094 *P*=0.761	ρ=0.198 *P*=0.517	ρ=0.272 *P*=0.368	ρ=0.358 *P*=0.230	ρ=0.039 *P* =0.901

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.