Title: Longitudinal evaluation of SMN levels as biomarker for Spinal Muscular Atrophy Molecular: results of a phase-IIb double-blind study of salbutamol

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ABSTRACT:

Background: Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder, due to the loss of function of the *SMN1* gene. The first treatment for the condition, recently approved, is based on the reduction of exon 7 skipping in mRNAs produced by a highly homologous gene (*SMN2*). The primary objective of the present study was to evaluate the applicability of *SMN* dosage in blood, as biomarker for SMA, and the safety of oral salbutamol, a beta-2 adrenergic agonist modulating *SMN2* levels.

Methods: We have performed a 1-year multicenter, double-blind, placebo-controlled study with salbutamol in forty-five SMA adult patients. Patients assumed 4mg of salbutamol or placebo/3 times a day. Molecular tests were *SMN2* copy number, SMN transcript and protein levels. We have also explored the clinical effect, by the outcome measures available at the time of study design.

Results: Thirty-six patients completed the study. Salbutamol was safe and well tolerated. We observed a significant and progressive increase in *SMN2* full-length levels in peripheral blood of the salbutamol-treated patients ($p<0.00001$). The exploratory analysis of motor function showed an improvement in most patients.

Conclusions: Our data demonstrate safety and molecular efficacy of salbutamol. We provide the first longitudinal evaluation of SMN levels (both transcripts and protein) in placebo and in response to a compound modulating SMN levels: *SMN* transcript dosage in peripheral blood is reliable and may be used as pharmacodynamic marker in clinical trials with systemic compounds modifying *SMN2* expression.

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INTRODUCTION

Type I-III spinal muscular atrophies (SMA) are autosomal recessive conditions, caused by the loss of function of the survival motor neuron (*SMN1*) gene, and characterized by the degeneration of the lower motor neuron.[1] Irrespective of the phenotypic severity, both *SMN1* copies are absent in about 95% of patients, while subtle pathogenic variants are relatively uncommon (2-3% of cases).[2] In patients, reduced amounts of the SMN protein are produced by *SMN2*, a nearly identical copy gene. *SMN2* is present in variable copy number in patients, generally two-four, being higher *SMN2* copies grossly related to milder phenotypes.[3,4] *SMN2* genes mainly produce mRNAs lacking exon 7 (*SMN*-del7), due to an alternative splicing, that give rise to an unstable protein that is rapidly degraded.[5] Nusinersen (Spinraza®), an antisense oligonucleotide based therapeutic approach with intrathecal administration, has been recently approved by FDA and EMA as the first effective treatment in children with SMA.[6,7] Further, a number of human trials with different therapeutic approaches are in the pipeline. The most are aimed at increasing the production of the SMN protein from *SMN2*, or at providing *SMN1* by gene therapy.[8,9]

In the past, beta-2 adrenoceptor agonists have been evaluated as possible candidates to the treatment of SMA: in two small open pilot trials of SMA type III and/or SMA type II children, albuterol/salbutamol determined a clinical improvement of patients;[10,11] we have shown that the compound increases SMN levels both *in vitro* (in fibroblast cultures of patients[12]) and *in vivo* (in a small open label study[13]).

The widest use of salbutamol has been for over thirty years in the treatment of asthma. Consequently, the pharmacodynamics of the compound has been extensively studied, including genetic factors predisposing to the receptor desensitization. For example, the lack of response to

beta2-adrenoceptor agonists in asthmatic patients has been reported to occur more frequently in the presence of the Gly allele of the p.Arg16Gly variant, a common polymorphism in the adrenoceptor beta 2 gene [*ADRB2*, rs1042713].[14]

In this study, we report on the results of a randomized, double-blind, placebo-controlled trial of salbutamol in a group of SMA type III adult patients. The primary aims were a) to assess safety and tolerability of orally administered high dosage of salbutamol, b) to verify its efficacy in increasing SMN levels in peripheral leukocytes, and c) to confirm reliability and applicability of *SMN2* product dosage in peripheral blood [*SMN2*-full length (*SMN2*-fl), *SMN*-del7, *SMN2*-total transcripts (*SMN2*-tot)] as a response biomarker/pharmacodynamic markers for SMA. Additional objectives were to explore the potential benefit of the drug in improving the motor function of treated patients and to identify possible correlations between the clinical and molecular responses.

METHODS

Study design and participants

We designed a multi-centric, 12 months, prospective, randomized, placebo-controlled, double blind, phase IIb trial of salbutamol in type III SMA adults. The primary endpoint of the study was to verify safety and tolerability of salbutamol and the molecular efficacy of the compound on *SMN2* gene expression. The exploratory evaluation of the clinical outcome was also performed.

Inclusion criteria were: adult male or female type III SMA out-patients with molecular confirmation of the diagnosis, ability to understand and provide an informed consent, and to

perform the evaluation tests. Exclusion criteria were: presence of respiratory failure, asthma, cardiovascular, hepatic or renal disorders, convulsive disorders, hyperthyroidism, diabetes mellitus; concomitant treatment with drugs known to interfere with salbutamol; use of drugs known to modulate *SMN2* gene expression, within 6 months before starting the study; pregnancy or lactation; subjects who have participated in clinical trials within three months prior to screening.

After screening at the baseline visit (V0), patients started oral treatment with salbutamol or placebo. The scheme of titration was: 4 mg once-a-day for two weeks, 4 mg twice-a-day for the following week, 4 mg three times-a-day for the whole duration of the study.

After V0, patients were evaluated at one (V1), three (V2), six (V3), and twelve months (V4) of full dosage of salbutamol/placebo.

Safety assessment

Safety of salbutamol was evaluated by the following laboratory and cardiologic assessments: blood count, electrolytes, liver and kidney function evaluation, plasma glucose, serum total creatine kinase (CK), and urine analysis (each visit); standard-ECG (each visit), Holter-ECG and blood pressure 24-hour recording (V0, V1, V3, and V4), and echocardiogram (V0, and V4). Adverse events were graded using Common Terminology Criteria for Adverse Events (CTCAE v3.0, www.ctep.cancer.gov).

Randomisation

After the screening visit and the evaluation of inclusion/exclusion criteria of each patient, every Centre required randomization to the Research and Development Office (RDO) of the Institute

"Carlo Besta", the coordinating Centre, by emailing through the electronic CRF. The RDO balanced the randomization, assigned a code to each patient, and requested to the Doppel Industry (Rozzano, Milano, Italy) to send the assigned treatment to the recruiting Centre. Patient randomization (1:1) was performed per blocks of 6 consecutive numbers (each block contained three salbutamol and three placebo treatments). One or more treatment blocks were assigned to each Clinical Centre.

Molecular studies

Molecular studies were performed on peripheral blood (sampled at V0, V2, V3, and V4), and were: *SMN2*-fl, *SMN*-del7, total *SMN2* (*SMN2*-fl plus *SMN*-del7, *SMN2*-tot), *GAPDH* (used as positive control) transcript levels by absolute real time PCR.[15] Molecular responders were defined for increases in *SMN2* transcript levels above the experimental variation. "Early molecular responders" identifies patients who responded at the molecular levels at V2; "late molecular responders" did not respond to the treatment at V2. SMN protein levels were assessed at V0, V2, and V3 by ELISA assay commercially available (Enzo Life Science).[16]

Out of the study protocol, we investigated the pharmacodynamics of salbutamol by evaluating the variation of *SMN2* transcript levels in response to a single dose of 4mg, in two further type III naïve patients. We performed blood samplings at the following time points: 30 min before salbutamol administration (T-1), at the assumption (T0), after 30 min (T1), and one (T2), two (T3), and four hours (T4).

Genomic DNA was collected at V0 to determine *SMN2* copy number, and to assess the presence of the p.Gly287Arg variant of the *SMN2* gene in the whole cohort. These data have already been

reported.[17] In the salbutamol arm, we also performed the genotype of the p.Arg16Gly variant of *ADRB2*, as previously reported.[18]

Clinical and instrumental evaluation

The following outcome measures were evaluated at each visit, except for V1: Forced Vital Capacity (FVC), Medical Research Council (MRC) score, North Star Ambulatory Assessment (NSAA), Six Minutes Walk Test (6MWT).[19-21] The muscle groups we have evaluated were previously described.[14] The last two evaluations were performed in ambulant patients only (11 patients in salbutamol group and 11 in placebo group). We considered "clinical responders" salbutamol treated patients who had an increase in outcome measure scores above the median of placebo in at least two visits. NSAA discrete raw scores were transformed into linear measures, according to the Rasch model.[17] Muscle mass was assessed by Dual X-Ray Absorption (DXA) at V0, and V4.[22]

Statistical analyses

Mean and standard deviation (SD), and median and interquartile range (IQR) were calculated for continuous variables; proportions were used for categorical variables. Correlations of *SMN2*-fl, *SMN*-del7, *SMN2*-tot transcript levels, and SMN protein levels, with clinical characteristics were assessed by linear regression models. A multivariate model was used to evaluate the influence of other covariates. Due to the sample size and to the non-normal distribution of *SMN2* transcript levels the non-parametric Kruskal–Wallis 'ANOVA' by ranks (KW) and Mann–Whitney U-test

(MW) were used.[19] The coefficient of variation (CV) was used to measure the longitudinal variation of *SMN2* transcript levels over time.

Clinical outcome measures at the different time points were compared by the signed and the signed rank test for paired samples. The variations at the different time points as a group were evaluated by the Mann-Whitney U-Test.

Study approval

The study was approved by the Institutional Review Board of each participating Centre; a written informed consent was obtained from all subjects. The study was done in accordance with the Declaration of Helsinki and the principles of Good Clinical Practice.

RESULTS

Salbutamol is safe and well tolerated

Forty-nine patients were screened and forty-five were enrolled between July 10th 2009 and September 9th 2010 (twenty-nine were males). The overall characteristics of the whole population at baseline have already been reported elsewhere, and will not be further discussed.[17] Twenty-three patients were randomized to the salbutamol and twenty-two to the placebo group (Fig. 1). At V0 the two groups were homogenous for demographic data and clinical assessments (Table 1). Nine patients did not complete the study, five in the salbutamol and four in the placebo arm. One patient of the salbutamol group dropped out before V4, due to a

skin erythema. The other patients withdrew from the study for personal or logistic issues, and not for adverse events.

In general, salbutamol was well tolerated: occasionally, patients reported an increase of tremors, tachycardia, and/or cramps, that were transient and did not require specific treatment, nor interruption or reduction of salbutamol dosage. One patient of the placebo group reported transient tachycardia.

No significant abnormalities in cardiologic assessments or laboratory tests were observed, with the exception of a statistically significant increase in serum CK levels in the salbutamol group (16/19 patients vs. 2/14 in the placebo arm), at V4 vs. V0 (p=0.0002 vs p=0.18, in salbutamol and placebo, respectively; Fig. 2).

Table 1. Comparison of placebo and active compound patients at baseline

	Placebo					Salbutamol					p-value
	N patients	Mean	Median	Range	SD	N patients	Mean	Median	Range	SD	
MALE	11					17					
FEMALE	11					6					
AGE (years)	22	34.71	37	18-55	11.41	23	35.39	34	21-53	11.04	0.83
AMBULANT	11					11					
AGE AT ONSET	22	10.7	10	1-32	10.96	23	14.3	6	2-50	14.96	0.36
DISEASE DURATION	22	27.7	29	2-47	14.92	23	21	21	3-47	10.18	0.08
MCR TOTAL SCORE	22	61.4	58	25-89	17.36	23	59	60	34-82	14.6	0.68
NSAA SCORE	11	21.6	25	7-31	85	11	17.5	18	7-23	5.28	0.19
6MWT (meters)	11	391.6	363	212.5-543	112.44	11	323.1	355	85.9-476.1	126.13	0.19
FVC (%)	22	91.6	92	64-121	20.79	23	81.9	82	43-110	16.28	0.09
LEAN BODY MASS (gr)	10	37336.2	35325.9	25198-52783.2	10275.3	7	33671.3	32900	23751.3-45282.5	6650.89	0.42
***SMN2* COPY NUMBER**	22	3.818	4	3-5	0.583	23	3.565	4	1-4	0.728	0.21

***SMN2*-fl TRANSCRIPTS (mol/ng RNA)**	22	72.17	65.14	44.15-138.92	23.86	23	62.93	64.9	28.54-94.53	16.43	0.314
***SMN-Δ7* TRANSCRIPTS (mol/ng RNA)**	22	233.54	231.22	74.32-534.78	100.69	23	237.03	196.41	90-513.12	120.42	0.92
***SMN*-tot TRANSCRIPTS (mol/ng RNA)**	22	305.71	288.18	131.12 - 628.85	114.19	23	302.44	263.54	149.86-582.41	123.7	0.93
SMN PROTEIN (pg/10^6 cells)	21	211.53	104.5	18.73-755.2	226.35	22	248.65	197.07	13.41-1076.73	252.51	0.62

Salbutamol increases *SMN2* transcript levels

In the placebo group, the longitudinal variation of *SMN2* transcript levels was very low (mean CV±SD: 0.076±0.091, 0.173±0.117 for *SMN2*-fl, and *SMN*-del7, respectively), with no statistically significant differences across the different time points ($p>0.09$; Fig. 3-A, C); *GAPDH* transcript levels were more variable than those of *SMN2* (mean CV±SD: 0.202±0.097, data not shown).

In the salbutamol group we observed a progressive and statistically significant increase in *SMN2*-fl levels only, from V0 through V4 ($p<0.0001$, Fig 3-B; *SMN*-del7, $p=0.38$, Fig. 3-D; *SMN2*-tot, $p=0.10$, data not shown). The ratio fl/del7 significantly increased with the treatment, particularly at V4 vs. V0 ($p=0.02$, data not shown). The number of patients responding to the treatment progressively increased from V0 to V4, achieving 100% at V4 (n=12/21, 13/20, and 15/15 at V2, V3, and V4, respectively; fig 4A). Regarding the mode of action of the compound, we observed unexpected mechanisms in a consistent proportion of samples (Fig. 4B). We observed a reduction of *SMN2*-tot transcript levels with an increase in the fl/del7 ratio in 19/56 (34%)

drawings (V2+V3+V4), and the opposite in 6/56 (10.7%), i.e. the increase in *SMN2*-tot transcript levels and the reduction in the fl/del7 ratio.

At V2, only 50% of patients were early molecular responders. We tested whether this finding could be related to the beta2-adrenoceptor desensitization: the ArgGly or GlyGly genotypes were more common among the early molecular responders, whereas 8/9 late molecular responders were Arg/Arg (OR=16,0; 95%CI=1.5-176.5; p=0.01).

We then assessed *SMN2* transcript level variations during the first hours after salbutamol assumption in two naïve type III SMA patients, who were not participating at the study. The first post-treatment sampling was performed after 30 minutes, when salbutamol raises therapeutic plasmatic concentrations.[23] In these two subjects, the molecular response was very similar to that observed *in vitro* and is summarized in fig. 5.[12] In patient 1 (Fig. 5A) *SMN2*-fl levels increase was about 65% after 30 minutes, *SMN2*-tot levels increased by 40-50% and the fl/del7 ratio remained substantially stable. In patient 2 (Fig. 5B), we observed a faster increase of about 100% between 30 and 120 minutes; after 4 hours, *SMN2*-fl levels returned back to the baseline. Protein samples were not available for all drawings, due to insufficient blood amount or extraction failure. In the placebo group, we tested twenty-two, fourteen, and thirteen samples at V0, V2, and V3, respectively. In the salbutamol arm, we analysed twenty-one, fifteen, and thirteen samples, respectively. The CV ranged from 0.49 to 1.16 in the placebo group, and from 0.66 to 0.82 in the salbutamol arm. The longitudinal variation of SMN protein levels was very wide in both groups, as shown in fig 6. Nonetheless, in the active compound group, we observed a trend towards a progressive increase in SMN levels, although non-significant (sign test, V2 and V3 vs. V0, p>0.149).

Exploratory evaluation of the clinical outcome

The clinical outcome of salbutamol treatment was evaluated for possible correlations with the molecular response. We used a combination of different measures that were appropriate for the different levels of patients' ability. The median variation of the individual clinical outcome measures in the placebo group was very low and not statistically significant over the different time points. In Supplemental Figure 1 (A, D, and G) the variations vs. V0 are represented: MRC (V2-V4 vs. V0, sign test $p \geq 0.92$), NSAA (V2-V4 vs. V0, sign test $p \geq 0.96$); 6MWT (V2-V4 vs. V0, sign test $p \geq 0.1$). Among all items, 6MWT displayed the highest variability.

All salbutamol-treated patients reported a subjective improvement in motor performance, due to a reduction of the perceived fatigability.

While the global variation of the MRC score was not significant (Supplemental Fig. 1C), we identified a group of clinical responders who had a median improvement of 4 points (11/18, Supplemental Fig. 1B). The increase was statistically significant either by tests for paired samples (signed rank test p=0.001, V2-V4 vs V0), or by taking into account the MRC score variation at each time point vs. V0 (p=0.004, 0.03, 0.007 at V2, V3 and V4, respectively).

In ambulant patients, the global variation of the NSAA score was not significant vs. V0 (median= 2 points; interquartile range 4.0 and 6.0, at V3 and V4, respectively; Supplemental Fig. 1F). Eight/eleven were clinical responders and had a significant increase at V3 and V4 vs. V0 (median $\geq$ 3 points; signed rank test, $p \leq 0.042$, Supplemental Fig. 1E); this improvement was also confirmed when linearizing the data, according to the Rasch analysis ($p \leq 0.035$). [24]

The 6MWT was performed by 10/11 ambulant patients: the missing patient did not agree to perform this assessment in other visits than V0. The median increase was of 27.85 and 30.41 meters at V3 and V4, respectively (Mann-Withney U-test, $p \geq 0.12$; Supplemental Fig. 1I); in the eight clinical responders, the median increase was $\geq$ 20.5 m (Supplemental Fig. 1H), and was statistically significant at V3 and V4 vs. V0 (signed rank test, $p \leq 0.037$; data not shown).

DXA was performed in 17/36 patients (seven of salbutamol group, ten of placebo); no differences were detected between V0 and V4 in both groups. We did not observe any difference in the respiratory function, as measured by spirometry: FVC value did not vary in the two groups after treatment (data not shown).

We did not observe any correlation between increase in *SMN2*-fl transcript levels in salbutamol patients and changes in clinical outcome measures.

DISCUSSION

This is the first randomised placebo-controlled trial performed in adult type III SMA patients, designed to verify safety and tolerability of high dosages of salbutamol and to confirm the efficacy of the drug in increasing *SMN2*-fl transcripts. Our data show that salbutamol is well tolerated and safe, with no major side effects. The most severe adverse event was skin erythema in a patient who was not assuming other concomitant drugs. The undesired effect was probably related to salbutamol, even though it had occurred after over six months of therapy. The treatment was suspended and the skin erythema promptly remitted with steroids.

The increase in serum CK levels, observed in treated patients, was not unexpected since it is not specific of SMA. HyperCKemia has been reported in asthmatic patients treated with salbutamol or other beta2-agonists.[25,26] A similar finding has been reported also in Kennedy's disease

patients, treated with clenbuterol.[27] Further possible causes of hyper-CKemia in beta2-agonist treatment, include muscle injury, reduction in contraction efficiency due to lower calcium release, or cardiotoxic effect of these drugs.[26] All these mechanisms appear unlikely in our patients, since motor performance or cardiac function were not impaired.

SMN2 transcript levels remained highly stable in blood over one year in the placebo group, indicating that spontaneous *SMN2* mRNA level fluctuations are very tiny; accordingly, most treated patients showed a progressive and significant increase of the -fl isoform.

The lack of response in half patients after three months of treatment was quite unexpected. Based on our *in vitro* data [12] and, more in general, on the receptor mediated mode of action of salbutamol, we expected to observe an early increase in *SMN2* transcript levels in most or all patients. On the other hand, in the two naïve subjects we found a rapid increase in *SMN2*–fl levels about 30-60 minutes after the assumption of the compound, as expected on the basis of the pharmacokinetics of salbutamol. The lack of response at V2 in the late molecular responders could not be explained by the desensitization of the beta2-adrenoceptor, since 90% of these subjects had the Arg/Arg genotype. We hypothesize here that the regulation of *SMN2* transcripts occurs according to a biphasic trend: a short-term transient response followed by a stable progressive long-term increase. Some open questions remain, such as whether this mode of action is specific of salbutamol or it is shared with other *SMN2*-active compounds, how does the long-term regulation occur, or whether this mode of response may impact the clinical outcome.

In our hands, SMN protein dosage in leukocytes is less reproducible than the mRNA quantification. The few similar studies available in the literature had a cross-sectional design and did not provide longitudinal data on SMN protein variations.[16] The main issue in SMN protein

quantification might be related to the insoluble nature of the protein, that hampers the complete extraction from biological specimens.

Regarding the exploratory analysis of the clinical outcome, the muscle performance remained stable in the placebo group, in accordance with other prospective longitudinal studies on SMA type II and III patients, showing a substantial stability of the disease over 1 year.[28-30] Even though the clinical effect of salbutamol was not striking, the significant improvement observed in the clinical responders may be ascribed to the effect of the compound. The clinical efficacy could not be a primary endpoint of our study: a longer duration would have been required, due to the slow progression of the disease in type II-III adult patients. Moreover, we should have designed a protocol including outcome measures more appropriate for adult SMA, such as the Expanded Hammersmith Functional Motor Scale, that are more sensitive to assess muscle strength and functional activity changes both in ambulant and non-ambulant patients. It is now available a map of measures more appropriate for SMA, as a result of an international consensus, that was not stated at the time our study was conceived.[31]

Finally, and more importantly, very few salbutamol crosses the blood brain barrier (around 2% of the plasma concentration): the most part of the effect observed in the clinical responders is likely related to the enhancement of *SMN2* expression in skeletal muscle, rather than in motor neurons. Indeed, beta-2 agonists have been shown to induce skeletal muscle hypertrophy and an increase in the stability of the muscle endplates;[32,33] it is unknown whether these effects could be mediated by the SMN pathway.

Due to the limited permeability of the blood brain barrier, salbutamol is not suitable for the treatment of SMA today, but was the most promising at the time of study design. Actually, the

best treatment options for adult patients are still debated in the scientific community: the use of beta-2 agonists may help to preserve longer the motor function of adult chronic subjects up to the availability of more appropriate guidelines. Beta-2 agonists might be interesting candidates for the treatment of SMA if modified to cross the blood brain barrier.

In conclusion, our data provide evidence that the dosage of *SMN2* transcripts in peripheral blood can be usefully adopted as a pharmacodynamic marker for systemic therapies, aimed at increasing SMN expression. However, our study does not allow to establish whether *SMN2* transcript levels are a response-biomarker for SMA. It is also possible that the restoration of SMN levels to normal range in blood does not necessarily correlate with the functional recovery, but might predict only the long-term effect of a given treatment. Studies on more clinically effective compounds, such as Risdiplam, could clarify whether the molecular response may predict the clinical outcome.[34] Finally, it is still unproven the utility of SMN-independent biomarkers, such as the SMA-MAP, a panel of plasma protein related with the clinical function of patients, or some miRNAs identified by others and ourselves.[35, 36 and unpublished data] These analytes might theoretically correlate better with patients' outcome but have not yet been validated in interventional trials.

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Author contributions: FDT and LM conceived the study, performed statistical analysis, wrote down the manuscript; RL, LDP, EA, SF performed molecular analyses; MBP performed clinical evaluation of patients and contributed to write down the manuscript; GB, CA, GS, AG, TG, LV, EM, GeVa, GiVi, GiaVi, SM, LP, GDG, AC, RM performed enrollment and clinical evaluation of patients. All Authors revised the manuscript before submission.

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FIGURE LEGENDS

Fig. 1. **Scheme of study design.** Patients were randomized 1:1 to salbutamol or placebo.

Fig. 2. Salbutamol induces an increase in CPK levels. In the salbutamol group (n=19), we observed a statistically significant increase in serum CPK levels at V4 vs. V0 (Mood's median test, p=0.0002). In placebo (n=14) the difference was not significant (p=0.18). The percent increase observed in salbutamol was statistically significant when compared with placebo (p=0.00008).

Fig. 3. Salbutamol induces an increase in SMN transcript levels. In the placebo group (A, C) the different *SMN2* isoforms remained stable (mean CV±SD=0.076±0.091, and 0.173±0.117, for *SMN2*-fl and –del7, respectively). In the salbutamol group *SMN2*-fl levels progressively increased (B, Kruskal–Wallis 'ANOVA' by ranks and Mann–Whitney U-test, V4 vs. V0, $p \leq 0.0001$), differently from the -del7 isoform and SMN-tot (p=0.38 and p=0.10, respectively; D and data not shown).

Fig. 4. The molecular response to salbutamol was different based at different time points. (A) After 3 months of treatment, slightly more than 50% of patients showed an increase in *SMN2*-fl levels, beyond the experimental variability (early molecular responders). The number of molecular responders increased over time, up to 100% at 1 year. (B) Also the mode of response was variable. The different mechanisms are indicated.

Fig. 5. Time-response curve to salbutamol in 2 different naïve patients. Two type III patients, who were not included in the study, were treated with salbutamol, up to 4 hrs. We observed a rapid increase in *SMN2*-fl levels, after 30 minutes from drug assumption, when the compound raises therapeutic concentrations in blood.

Fig. 6. Longitudinal variation of SMN protein levels in placebo (A) and salbutamol (B) patients. SMN protein displayed a much wider variability compared to SMN transcript

levels. The levels were not significantly different at the different time points (signed-test V2 and V3 vs V0, $p \geq 0.149$).

Supplemental Fig. 1. Effect of salbutamol treatment on clinical performance of patients. MRC scale score in placebo (A) salbutamol whole group treated (C) patients, (B) clinical responder subgroup. NSAA score in placebo (D) and whole group (F) treated patients, (E) clinical responder subgroup. 6-MWT in placebo (G) and salbutamol treated patients (I), (H) clinical responder subgroup.

*statistically significant value ($p<0.05$).