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Salbutamol increases SMN transcript levels in leukocytes of spinal muscular atrophy patients: relevance for clinical trial design

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ABSTRACT

Background: Spinal muscular atrophy (SMA) is a neuromuscular disorder caused by mutations of the *SMN1* gene. Based on severity, three forms of SMA are recognized (type I-III). All patients have usually 2-4 copies of a highly homologous gene (*SMN2*) which produces insufficient levels of functional SMN protein. Recently, we have provided evidence that *SMN2* expression can be enhanced *in vitro* by salbutamol, a beta2-adrenergic agonist. This compound has also been shown to improve motor function of SMA patients in two different pilot trials. **Aim**: In the present study, we have evaluated the *in vivo* molecular efficacy of salbutamol in SMA patients.

1 **Methods**: 12 type II-III patients took the compound orally for six months. *SMN2* full length transcript levels have been determined in peripheral blood leukocytes by absolute real time PCR, at baseline and after 3 and 6 months of treatment. **Results**: A significant and constant increase in *SMN2*-full length transcript levels was detected; the response was directly proportional to *SMN2* gene copy number. **Conclusions**: Our data strongly support salbutamol as candidate for SMA treatment, suggest that *SMN2* copy number may predict the molecular response to treatment and may be a useful randomization parameter in double blind placebo-controlled clinical trial design.

INTRODUCTION

Spinal muscular atrophy (SMA) is one of the most common autosomal recessive neuromuscular disorders and a leading genetic cause of infant mortality. The disease presents with variable phenotype ranging from severe to mild (type I to III). SMA is caused by a deficit of the survival motor neuron (SMN) protein encoded by two closely related genes, *SMN1* and *SMN2*. Patients have no functional *SMN1* genes, although retain at least one copy of the *SMN2* gene [1]. The *SMN2* gene produces insufficient levels of functional protein due to a C-T transition in exon 7, determining

the exclusion of this exon in the majority of mature transcripts. With the aim to increase functional SMN protein levels, several compounds have been investigated for their effect on *SMN* expression *in vitro* and/or *in vivo* [2-6]. We have recently shown that salbutamol (albuterol), a β2-adrenoceptor agonist, increases *SMN2*-full length (*SMN2*-fl) transcripts in SMA fibroblasts, mainly by promoting exon 7 inclusion in *SMN2* transcripts [7]. This finding and the data of two pilot trials [8-9] suggest that salbutamol may be useful for therapeutic intervention. In the present study we have investigated the *in vivo* molecular effect of this compound by determining *SMN2*-fl transcript levels in leukocytes of SMA patients who received salbutamol over a period of 6 months.

METHODS

The present study was approved by the ethical committee of the Catholic University. Patients were included upon signing a written informed consent. Age, sex and type of SMA are indicated in Table 1.

Table1. Characteristics of sex, type of SMA, age, number of *SMN2* genes and transcript levels (no. of molecules/ng of total RNA) of patients included in the present study

Patient no.	Sex	Type of SMA	Age at baseline (yrs.)	No. of *SMN2*	*SMN2*-fl (no. of mol/ng of RNA)		
					T0	T1	T2
1	M	II	8	3	57,50	80,59	144,20
2	M	II	2.8	3	102,94	-	130,85
3	F	II	7.5	3	51,18	68,94	113,73
4	M	II	4	3	76,18	88,77	104,30
5	F	II	2.5	3	100,30	-	136,22
6	M	II	4.5	3	80,27	142,82	152,18
7	M	II	2.5	3	102,05	119,76	197,70
8	F	III	54	4	88,25	214,21	240,87
9	M	III	58	4	80,63	145,57	163,77
10	F	III	38	3	59,42	83,59	161,85
11	M	III	30	4	103,75	-	193,84
12	M	III	3	3	120,30	119,58	151,10
				Mean	85,73	117,79	157,23
				Median	84,44	116,00	150,55
				SD	22,07	45,39	38,13

2 Seven type II and 5 type III patients took oral salbutamol (2 mg three times/day for body weight ≤20 kg, four times/day >20 kg) for six months. All patients had homozygous absence of *SMN1*. *SMN2* copy number was assessed as previously reported [3]. Blood was drawn into PAXgene blood RNA tubes (BD Biosciences). Blood draws were performed at baseline (T0), at 3 months of salbutamol

3 administration (T1) for 9/12 patients and at 6 months (T2) for all subjects. Only for patient no. 8 *SMN2*-fl baseline levels were assessed twice and were shown to be stable after one month (described in Tiziano et al., 2010). For two patients, blood drawing was performed also 1 year after treatment. *SMN2*-fl levels were evaluated by absolute real time PCR [10]. Similarly, *GAPDH* transcript levels were determined as positive

4 controls both for RT-PCR and real time PCR. Samples from different time points were not analyzed simultaneously but upon arrival to the Institute of Medical Genetics at Catholic University.

Statistical analysis was performed using Statgraphics-CenturionXV.II (Statpoint Technologies) software. Data were analysed by non-parametric tests, both due to small number of patients included and to our previous finding that *SMN*-fl transcript levels do not show a normal distribution in the population [9]. To compare overall variations of transcript levels compared to baseline we used Kruskal-Wallis (KW) and Moods median test (MM). *SMN2*-fl levels at T1 and T2 were compared to baseline by means of sign test (ST) and signed rank test (SR). Transcript level variations were related to SMN2 gene copy number by Mann-Withney W test (MW). Possible correlations between *SMN2*-fl transcript levels and *SMN2* copy number were analyzed by linear correlation model. For all tests, significance cutoff was fixed at P-values≤0.05.

RESULTS

Oral administration of salbutamol induced a significant and persistent increase in *SMN2*-fl levels in the 12 type II/III patients included in the study (Fig. 1A and Table 1, KW p=0.0002; MM p=0.001). At T1, the mean increase was 48.9% (median 40.2%) compared to baseline; all patients except three (no. 4, 7 and 12) showed an increase in *SMN2*-fl transcript levels above experimental variability which was previously shown to be about 13% [10]; the difference between T0 and T1 was significant (ST and SRT $p \leq 0.04$). At T2, all patients showed a marked increase in *SMN2*-fl transcript levels compared to baseline, with a mean of 91.8%, and a median of 86.8% (ST and SRT $p \leq 0.002$).

To assess whether the molecular response to treatment was related to phenotypic severity, data were analyzed in type II and type III patients separately (fig. 1B). Although the increase in transcript levels was higher in type III patients, the difference was not significant both at T1 and T2 (MW p=0.39, fig. 1B). We also evaluated whether the molecular response to salbutamol could be related to the number of *SMN2* (fig. 1C). Nine patients had 3 copies and 3 had 4 copies. While there was no difference in the two groups at baseline levels (3 *SMN2*: mean 84.01$\pm$24.94 mol/ng, median 80.27 mol/ng; 4 *SMN2*: 90.88$\pm$11.78 mol/ng, median 88.25 mol/ng; MW p=0.58), patients with 4 *SMN2* copies showed a better response to salbutamol treatment, compared to patients with 3 genes both at T1 and T2 (T1: MW p=0.05; T2: MW p=0.040; fig. 1C). Moreover, a linear correlation was found between transcript levels and *SMN2* copy number both at T1 and T2 but not at baseline (p=0.66, p=0.015 and p=0.017, at baseline, T1 and T2 , respectively; supplemental data and fig. 1A-C). Also, transcript variations compared to baseline were directly related to *SMN2* copy number at T1 and T2 (MM p=0.04 at T1 and T2; supplemental data and fig. 2A-B).

Finally, salbutamol treatment did not affect *GAPDH* transcript levels (supplemental data and fig. 3).

DISCUSSION

Here, we demonstrate for the first time that salbutamol is effective in increasing *SMN2*-fl transcript levels *in vivo*. The increase cannot be ascribed to fluctuations of transcript levels, since we and others have previously shown that *SMN2-fl* levels in SMA patients are stable over time [10-11]. Except for three patients who showed an increase only at T2, the levels of transcripts increased gradually and were highest after 6 months, indicating that the increase in transcript levels induced by a pharmacological treatment can be maintained for relatively long periods. Moreover, at T2 all patients included in the present study reached *SMN2*-fl transcript levels significantly above median values of controls (~108 mol/ng of total RNA) [10]. For two patients, blood samples were available also after one year of treatment: in one of them (patient 4) *SMN2*-fl transcript levels increased further, in the other (patient 1) the levels remained stable (data not shown).

Some other compounds have been reported to increase *SMN2*-fl transcripts *in vivo*. In our previous study on the effect of phenylbutyrate, we found considerable variations both among different subjects and among different blood samples from the same subject [4]. In another open-label trial with valproic acid, *SMN2* mRNA levels were found elevated in 7 patients and unchanged or decreased in 13 patients [5]. Similarly, in the most recent open label study of the effect of valproic acid, fluctuation of SMN mRNA levels throughout drug treatment has been reported in patients showing increased, decreased or unaltered levels [6]. The variability observed in the molecular

response to treatment may be related to the different assays used for transcript analysis or to the different molecular efficacy of the compounds. In the present study we applied a novel absolute real time PCR assay for *SMN*-fl transcript quantification [10]. This method allowed an accurate measurement of the molecular efficacy of salbutamol even in those patients whose *GAPDH* transcript levels varied markedly at different time points, supporting its use for *SMN2* transcript quantification in SMA clinical trials.

To evaluate whether the molecular response to salbutamol may depend on phenotypic differences, we subgrouped patients on the basis of the clinical severity. We found that the increase in *SMN2*-fl transcript levels was higher in type III patients compared to type II. This finding is likely related to differences in *SMN2* gene copy number between the two groups (table 1): the analysis of a larger number of patients with the same copy gene number may help to elucidate whether the molecular response to pharmacological treatment is related to the severity of clinical manifestations. Interestingly, we found that patients with 4 *SMN2* genes showed a better response to pharmacological treatment with salbutamol compared to subjects with 3 copies and that *SMN2*-fl transcript levels at T1 and T2 were linearly related to *SMN2* copy number (Supplemental data and suppl. Fig. 1-2). Our data indicate for the first time that at the molecular level patients with more *SMN2* copies respond better to pharmacological treatment and that gene copy number may be evaluated as a randomization parameter in the design of double blind clinical trials for SMA patients, rather than considered an inclusion/exclusion criterion.

5 Previous, uncontrolled open-label studies had suggested a potential benefit of salbutamol [8-9]. The results of this study support that salbutamol has a biological

effect that is consistent with its mechanism of action. However, in the absence of clinical-molecular correlations, it cannot be established whether the molecular efficacy of a given compound can be considered clinically significant and whether *SMN2*-fl quantification is suitable as biomarker or surrogate outcome measure for SMA. In the present study clinical-molecular correlations were not assessable, due to the small size of the color, to the heterogeneity of patients included, both for age and clinical characteristics, and to the absence of a placebo control group. However, all patients chose to continue study drug on their own will. Double blind placebo-controlled trials are necessary to demonstrate possible correlations between clinical and molecular response and to provide the proof of concept of the utility of *SMN2*-fl transcript quantification in peripheral blood leukocytes in the clinical practice of SMA patients.

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Figure legend:

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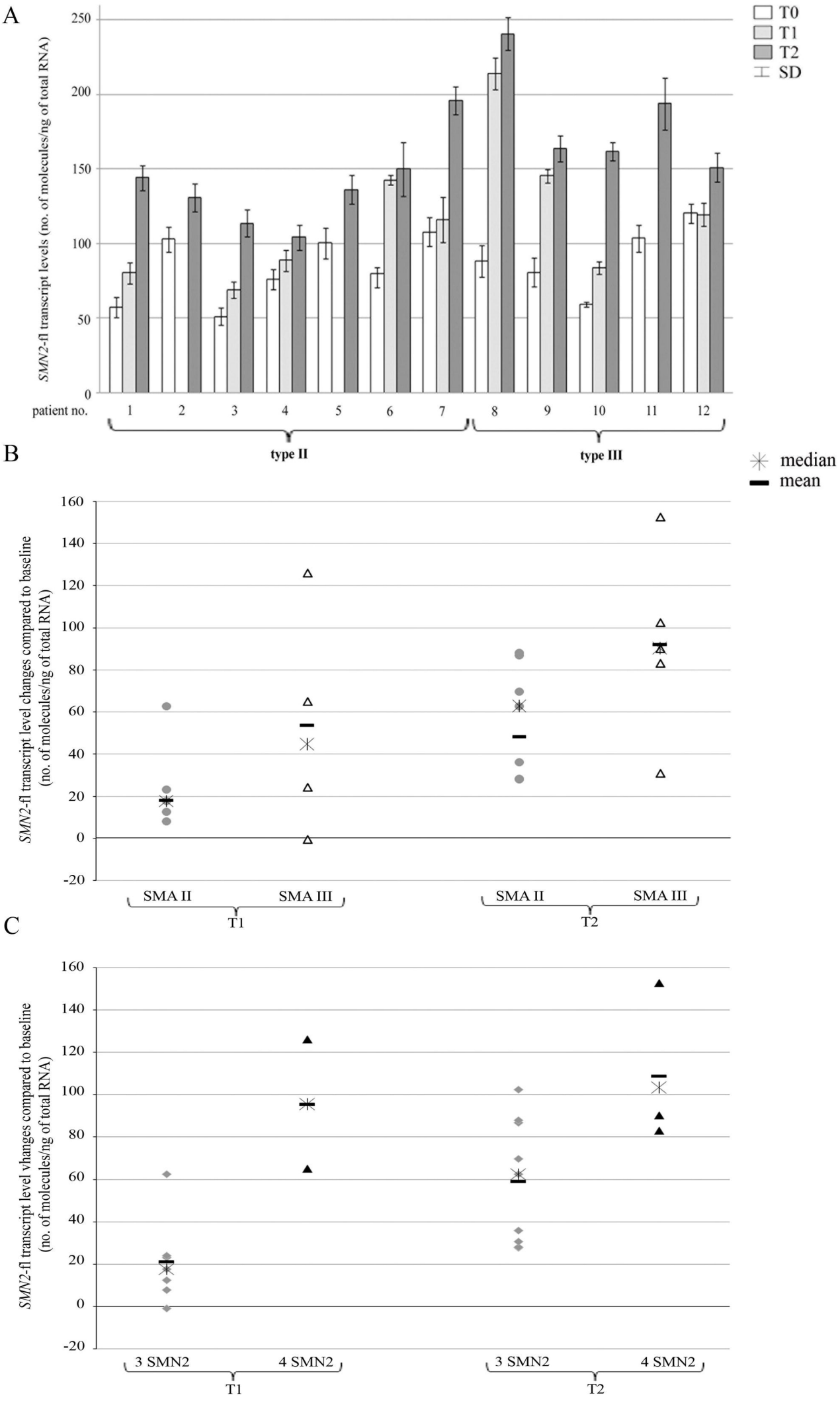
Figure 1. (A) Salbutamol induces a significant increase in *SMN2*-fl transcript levels in peripheral leukocytes. Baseline *SMN2*-fl levels (white column) were compared with those at 3 (T1, light grey columns) and 6 months (T2, dark grey columns) of salbutamol treatment, respectively. (B) Molecular response to salbutamol was evaluated in SMA type II (circles) and III (triangles) patients, at T1 and T2. Although *SMN2*-fl transcript level variations was higher in type III patients, the difference was not significant. (C) Molecular response to pharmacological treatment is related to *SMN2* gene copy number. Individuals with 4 *SMN2* genes had a significantly higher increase in *SMN2*-fl levels during salbutamol treatment compared to patients with 3 *SMN2* copies.


A
T0
T1
T2
SD
SMN2-fl transcript levels (no. of molecules/ng of total RNA)
250
200
150
100
50
0
patient no.
1
2
3
4
5
6
7
8
9
10
11
12
type II
type III
B
median
mean
SMN2-fl transcript level changes compared to baseline (no. of molecules/ng of total RNA)
160
140
120
100
80
60
40
20
0
-20
SMA II
SMA III
SMA II
SMA III
T1
T2
C
SMN2-fl transcript level vhanges compared to baseline (no. of molecules/ng of total RNA)
160
140
120
100
80
60
40
20
0
-20
3 SMN2
4 SMN2
3 SMN2
4 SMN2
T1
T2