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ARTICLE

SMN transcript levels in leukocytes of SMA patients determined by absolute real-time PCR

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Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder caused by homozygous mutations of the *SMN1* gene. Three forms of SMA are recognized (type I–III) on the basis of clinical severity. All patients have at least one or more (usually 2–4) copies of a highly homologous gene (*SMN2*), which produces insufficient levels of functional SMN protein, because of alternative splicing of exon 7. Recently, evidence has been provided that *SMN2* expression can be enhanced by pharmacological treatment. However, no reliable biomarkers are available to test the molecular efficacy of the treatments. At present, the only potential biomarker is the dosage of *SMN* products in peripheral blood. However, the demonstration that SMN full-length (*SMN*-fl) transcript levels are reduced in leukocytes of patients compared with controls remains elusive (except for type I). We have developed a novel assay based on absolute real-time PCR, which allows the quantification of *SMN1*-fl/*SMN2*-fl transcripts. For the first time, we have shown that *SMN*-fl levels are reduced in leukocytes of type II–III patients compared with controls. We also found that transcript levels are related to clinical severity as in type III patients *SMN2*-fl levels are significantly higher compared with type II and directly correlated with functional ability in type II patients and with age of onset in type III patients. Moreover, in haploidentical siblings with discordant phenotype, the less severely affected individuals showed significantly higher transcript levels. Our study shows that *SMN2*-fl dosage in leukocytes can be considered a reliable biomarker and can provide the rationale for SMN dosage in clinical trials.
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INTRODUCTION

Proximal spinal muscular atrophies (SMA) are a group of clinically variable motor neuron disorders characterized by the degeneration of the anterior horn cells of the spinal cord. On the basis of age of onset and severity of the clinical course, childhood-onset SMA can be classified into three forms (type I–III). SMA III patients can be further divided into type IIIa and IIIb on the basis of whether the onset is below or over the age of 3 years, respectively.[1] SMAI-III are autosomal recessive, and are caused by loss of function of the survival motor neuron (*SMN1*) gene.[2] *SMN1* and a nearly identical copy, *SMN2*, are located in a duplicated inverted region at 5q13. Both genes encode the SMN protein but, because of alternative splicing, the majority of *SMN2* transcripts lack exon 7 (SMN-delta7), and are unable to produce a sufficient amount of protein to prevent the onset of the disease. The SMN protein is expressed in most tissues and is localized in the cytoplasm and in the nucleus. It has been shown that the level of SMN protein is markedly reduced in SMA patients, both in spinal cord and in cell cultures and inversely correlate with phenotypic severity.[3–5] Patients can carry a variable copy number of the *SMN2* gene, higher copy numbers being generally associated with milder phenotypes.[6–8]

At present, no cure for SMA is available. Recently, evidence has been provided that *SMN2* gene expression can be enhanced by pharmacological treatment *in vivo* and/or *in vitro*, using different compounds.[9–17] The clinical efficacy of some of these compounds has been tested also in clinical trials.[18–21]

The advances in SMA clinical research highlight the need of reliable biomarkers to monitor the efficacy at the molecular level of treatments during trials, the dosage of *SMN* transcripts or protein in peripheral blood samples being the only one potentially available. However, possible variations of *SMN* transcripts/protein levels as evaluated in leukocytes may not reflect the real effect of pharmacological treatment in target tissues, such as spinal cord and, eventually, skeletal muscle. So far, some assays have been developed and validated for *SMN2* transcript[14,22–24] or protein[25–26] quantification. However, to date it has not been shown whether *SMN* full-length (*SMN*-fl) transcript or protein levels in leukocytes differ significantly among controls, carriers, and patients. In particular, a reduction of *SMN*-fl levels has been shown only for type I patients.[14,22] The reported *SMN* mRNA assays are mainly based on relative semiquantitative PCR in which transcript levels are determined by normalizing with respect to housekeeping gene transcript levels, used as endogenous controls.[22–24] However, it has been shown that the expression levels of these genes vary widely in the general population and/or can be putatively affected by pharmacological treatments or metabolic status, thus reducing the

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sensitivity of the earlier published assays.[14] Brichta *et al*[14] have developed a real-time PCR assay based on the measurement of SMN levels relative to the amount of RNA. We have developed an alternative molecular test based on absolute real-time PCR that allows the quantification of the number of *SMN1*-fl and *SMN2*-fl mRNA molecules per nanogram of total RNA (mol/ng) and is suitable for measuring *SMN* transcripts, thus avoiding possible biases because of the variations in endogenous control transcript levels. In our assay, *GAPDH* transcripts quantification has been included as positive control for PCR amplification and to rule out that possible differences between patients and controls could be related to PCR efficiency or RNA quality (see Supplementary information). We have used this novel assay to investigate whether *SMN*-fl transcript levels are reduced in patients compared with controls, and to assess whether transcript levels correlate with phenotypic severity in patients, which are the prerequisites for using SMN dosage as a biomarker for future clinical trials in SMA patients.

SUBJECTS AND METHODS

Subjects

Blood samples were obtained from 51 SMA patients (2 type I, 16 type II, and 33 type III), 23 carriers, and 28 controls. The characteristics of age and sex ratio of the three groups are summarized in Table 1. All patients had homozygous absence of *SMN1* exon 7. Type III SMA patients have been subgrouped according to the classification proposed by Zerres[1] in type IIIa and IIIb. For type II patients between 2.5 and 12 years of age, functional ability was evaluated using the Hammersmith functional motor scale.[27]

Among patients, six sib pairs were analyzed: five pairs were phenotypically discordant (type IIIa sister/II brother; type IIIa/II sisters; asymptomatic/IIIb sisters; type IIIb brother/IIIa sister; oligosymptomatic/type IIIb brothers), and one further pair of sibs who were phenotypically similar (type IIIb brother and sister). Carriers were either parents of patients or were selected among relatives of patients or other relatives who tested positive for carrier status. For patients and carriers, both total RNA and genomic DNA were extracted. Controls were healthy individuals, seen at the Genetics Clinic of the Catholic University Hospital for karyotype analysis. Blood samples from controls were rendered anonymous and used for RNA extraction only. Individuals taking drugs known to modify *SMN* expression were excluded from our cohorts. For repetitive samplings, blood samples were drawn approximately at the same hour during the morning, to rule out possible biases because of circadian variations in *SMN*/*GAPDH* transcript levels or in feeding. Finally, four fibroblast cell cultures were analyzed in this study, one from a control and three from patients (one for each type of SMA).

DNA extraction, SMN2 gene copy number assessment, RNA extraction, and RT-PCR. The DNA, extracted by the standard salting-out procedures, was quantified by absorbance at 260 and 280 nm (GeneQuant Pro, Pharmacia Biotech, Arlington Heights, IL, USA). *SMN2* gene copy number, as well as carrier status, was determined as reported earlier.[12] For RNA extraction from peripheral blood, PAXgene blood RNA tubes (BD Biosciences, San Jose, CA, USA) and kit (Qiagen) were used. In the case of fibroblast cultures, total RNA was extracted by RNeasy mini kit (Qiagen, Duesseldorf, Germany). For all RNA samples, concentration was established by absorbance determination and quality was assessed by agarose gel electrophoresis. A total of 2 μg of total RNA were used for RT-PCR using a High Capacity cDNA Archive Kit (Applied Biosystems, Carlsbad, CA, USA) in a final reaction volume of 25 μl, using random primers for reverse transcription.

External standard constructs design. Three plasmids were constructed for *SMN1*-fl, *SMN2*-fl, and *GAPDH*, respectively, by amplifying a control cDNA. *SMN1* and *SMN2* were amplified by using the primer pair: SMN_exst-F: 5′-GCTTTGGGAAGTATGTTAATTTCA-3′ and SMN_exst-R: 5′-CTATGCCA GCATTTCTCCTTAATT-3′, located in exon 6 and exon 7/8 junction, respectively. For *GAPDH*, primer pair GAPDH_exst-F: 5′-CTCTGCTGATGCCCCC ATGTTCGT-3′ and GAPDH_exst-R: 5′-CAAAGTTGTCATGGATGACCTTGG-3′, located in exon 5 and exon 6, respectively, was used. For *SMN1*/*SMN2* and *GAPDH* genes, PCR products of 129 and 133 bp, respectively, were obtained. Subsequently, PCR products were cloned by using TA cloning kit (Qiagen). Plasmid DNA was extracted by the QIAprep Spin Miniprep Kit (Qiagen) and

Table 1 Characteristics of each group included in this study (number of individuals, age range, sex ratio, and transcript levels)

	Controls	*Carriers*	*SMA patients*			
			Type I	*Type II*	*Type III*	*Total*
n (M/F)	28 (14/14)	23 (9/14)	2 (1/1)	16 (8/8)	33 (16/17)	51 (25/26)
Age range (years)	19–45	18–73	0.5–1.5	3–33	2–68	0.5–68
SMN1-fl						
Mean ± SD	78.27 ± 50.61	72.28 ± 36.72	—	—	—	—
Median	65.13	68.88	—	—	—	—
Min–max	28.25–217.25	18.05–178.00	—	—	—	—
SMN2-fl						
Mean ± SD	41.65 ± 25.62	58.73 ± 41.63	37.13 ± 4.77	52.50 ± 24.78	73.67 ± 29.68	67.33 ± 29.36
Median	37.75	47.50	37.13	47.50	72.50	61.25
Min–max	9.95–100.75	13.08–209.50	33.75–40.50	26.75–102.75	28.50–123.00	26.75–123.00
SMN-fl						
Mean ± SD	119.92 ± 72.98	127.44 ± 67.28	—	—	—	—
Median	107.63	120.88	—	—	—	—
Min–max	39.45–318.00	43.55–323.75	—	—	—	—
GAPDH						
Mean ± SD	5714 ± 2929	5121 ± 1566	5412 ± 760	4879 ± 1820	5392 ± 1469	5227 ± 1552
Median	4725	5100	5412	4500	5600	5225
Min–max	1092–13025	2975–8975	4875–5950	2500–8925	2310–7925	2310–8925

SMN-fl, *SMN1*-fl, *SMN2*-fl, and *GAPDH* indicate transcript levels, measured as no. of molecules per nanogram of total RNA.

quantified both by absorbance and by agarose gel electrophoresis with scaling serial dilution of lambda DNA. The presence of possible sequence variations in plasmids, randomly introduced by Taq polymerase, was ruled out by sequence analysis of the clones, performed using the ABI-Prism 3130 instrument and BigDye terminator v3.1 Cycle Sequencing kit (Applied Biosystems). On the basis of plasmid length (3980 and 3984 bp for *SMN1*/*SMN2* and *GAPDH*, respectively), the molecular weight and the number of plasmid molecules per nanogram of DNA (around 2.29×10^8/ng for the three plasmids) were determined. Single use serial dilutions of the three external standards, ranging from 10^4 to 10^7 molecules, were aliquoted and kept frozen at -80°C.

Primers and MGB-probes. Primer Express v1.5 software (Applied Biosystems) was used to design optimized minor groove binder (MGB) probes and primers for real-time RT-PCR. *SMN1* and *SMN2*-fl transcripts were amplified by using the same primer pair (SMN_abs-F: 5′-TACATGAGTGGCTATCA TACTGGCTA-3′ and SMN_abs-R: 5′-AATGTGAGCACCTTCCTTCTTTTT-3′, located in exons 6 and 7, respectively), obtaining 72 bp PCR products. Full-length transcripts of the two genes were specifically distinguished by two different Taqman MGB probes, labeled with different fluorochromes, on the basis of the C-T transition located in exon 7 (SMN1_abs: 5′-NED-TATGGGTTT**C**AGACAAA-NFQ-3′ and SMN2_abs: 5′-VIC-ATATGGGTTT **T**AGACAAAA-NFQ-3′). For *GAPDH*, an amplicon of 73 bp was obtained by using the primer pair GAPDH_abs-F: 5′-GGGTGTGAACCATGAGAAGTAT GA-3′ and GAPDH_abs-R: 5′-CTAAGCAGTTGGTGGTGCAGG-3′. MGB probe sequence was: 5′-FAM-CAAGATCATCAGCAATGC-NFQ-3′.

Real-time PCR assay and construction of standard curves. The PCR reactions were performed in a final volume of 20 μl, containing 2× Taqman Universal Mastermix (Applied Biosystems), 40 ng of cDNA (or appropriate dilutions of external standards), appropriate concentrations of *SMN* primers, *SMN1* and *SMN2* probes, or of *GAPDH* primers and probe. Each sample was amplified in quadruplicate and each experiment repeated at least twice. All reactions were performed using 7900HT Fast Real-Time PCR System (Applied Biosystems). Optimal primer and probe concentrations were as follows: SMN_abs_F and SMN_abs_R: 900 nM; GAPDH_abs_F and GAPDH_abs_R: 30 nM; SMN1_abs, SMN2_abs, GAPDH_abs: 200 nM. Serial dilutions of external standards, ranging from 10^4 to 10^7 copies were used to construct the standard curves. We did not use plasmid concentrations lower than 10^4 copies because of the poor stability of such dilutions. The number of *SMN1*-fl, *SMN2*-fl, and *GAPDH* mRNA molecules was extrapolated automatically by the Sequence Detection System v2.2.2 software (Applied Biosystems).

Statistical analysis. Statistical analysis was performed by using Statgraphics-Centurion XV.II (Statpoint Technologies, Warrenton, VA, USA) software. The experimental variability and the reproducibility of the real-time PCR assay were assessed by determining the mean and standard deviation (SD) of coefficient of variation (CV) of repeated experiments. For each sample, the CV was determined as the ratio between the SD and mean transcript levels of repeated amplifications. The distribution of *SMN1*-fl, *SMN2*-fl, total *SMN*-fl (*SMN1*-fl plus *SMN2*-fl), and *GAPDH* transcripts was analyzed by using Kolmogorov–Smirnov, Shapiro–Wilks' W, and Lilliefors tests for normality. Possible alternative distributions of *SMN* transcripts were also evaluated by using goodness-of-fit tests.

To compare transcript levels in the three populations, both parametric (*t*-test for independent variables and one-way ANOVA) and non-parametric tests (Kruskal–Wallis ANOVA by ranks (KW) and Mann–Whitney *U*-test (MW)) were used. Possible correlations between *SMN1*-fl and *SMN2*-fl transcript levels, between *SMN2* gene copy number and *SMN2*-fl transcript levels, as well as between Hammersmith's functional motor scale score and *SMN2*-fl transcript levels were analyzed by a linear regression model. Contingency tables and two-tailed Fisher's F-test were used to calculate the relative risk (RR) and 95% confidence interval (CI) for correlations of age of onset and *SMN2*-fl transcript levels. To evaluate possible differences in *SMN2*-fl levels in siblings, the hypothesis test was used: pairs were divided on the basis of phenotype and the relatively less severe sibs were compared with more severe ones. For all tests, significance cutoff was fixed at *P*-values ≤ 0.05.

RESULTS

Validation, specificity, and reproducibility of the assay are described in Supplementary information. The exact number of *SMN2* genes, *SMN*-fl, *SMN1*-fl, *SMN2*-fl, and *GAPDH* transcript levels of single individuals are indicated in Supplementary Table 1.

SMN-fl transcripts do not show a normal distribution

The normality tests reported in the Subjects and methods section indicated that in carriers and controls, *SMN1*-fl, *SMN2*-fl, and *SMN*-fl levels do not show a normal distribution ($P < 0.05$, Supplementary Figure 1a-b and data not shown). Similar results were obtained for *SMN2*-fl levels in patients ($P < 0.04$, Supplementary Figure 1c and Table 1). Therefore, we considered median, quartiles, minimum and maximum as more appropriate to describe *SMN* levels than mean ± SD.

SMN2-fl levels are more stable over time than *SMN1*-fl

To evaluate physiological fluctuations of *SMN1*-fl, *SMN2*-fl, and *GAPDH* transcripts, we performed 2–4 blood samplings in seven controls during a period of 1 month (at days 0, 1, 14, and 30) and two blood draws in six patients (at day 0 and 30). The results are summarized in Figure 1. Although a certain degree of fluctuation was observed, *SMN2*-fl and *GAPDH* transcript levels seemed to be more stable over time than *SMN1*-fl. *SMN2*-fl levels seemed to be less variable in SMA patients than in controls. To confirm this observation, we have evaluated the mean CV (0.19 ± 0.11 and 0.14 ± 0.06 for *SMN2*-fl and *GAPDH*, respectively), which was more similar to that expected for experimental variation of the assay (see Assay development and validation section in online Supplementary information). In contrast, *SMN1*-fl transcripts showed wide day-to-day variations (mean CV: 0.35 ± 0.17). We also evaluated the total *SMN*-fl level variations and observed that transcript level fluctuations reflect that observed for *SMN1*-fl levels (data not shown). As *SMN1*-fl and *SMN2*-fl transcripts are amplified by the same primer pair, the different mean CV of the two amplicons cannot be ascribed to the PCR artifact.

Patients have lower *SMN* levels compared with controls and carriers

To assess whether *SMN*-fl transcripts are reduced in SMA subjects, we compared *SMN2*-fl levels in patients (n=51) with *SMN*-fl in controls (n=28, Table 1 and Figure 2). The difference between the two groups was statistically significant (MW: $P=4.3\times10^{-5}$, KW: $P=4.2\times10^{-5}$); also, when excluding the two type I patients, the *P*-values remained highly significant (MW=KW, $P=8\times10^{-5}$). We subsequently subdivided the patients according to their SMA type. Although the number of samples from type I patients (n=2) was insufficient for statistical analysis, the difference in transcript levels between patients and controls was statistically significant both for SMA type II (n=16, MW: 2.4×10^{-5}, KW: $P=2.22\times10^{-5}$) and type III (n=33, MW and KW: P=0.0042). No significant differences in *SMN2*-fl levels were observed when dividing patients by sex (MW and KW: P=0.16) or age ($<$ or $\geq$14 years, MW: P=0.07, KW: P=0.06).

SMN2-fl levels are not related to *SMN2* gene copy number

To evaluate whether *SMN2*-fl levels correlate with *SMN2* gene copy number, we have determined the gene copies in 35 of 51 patients. The two SMA type I patients had two copies, 25 SMA type II and III patients had three copies, and eight type III patients had four copies. By using a linear correlation model, indicating *SMN2* copy number as an independent variable, no evidence for a correlation between

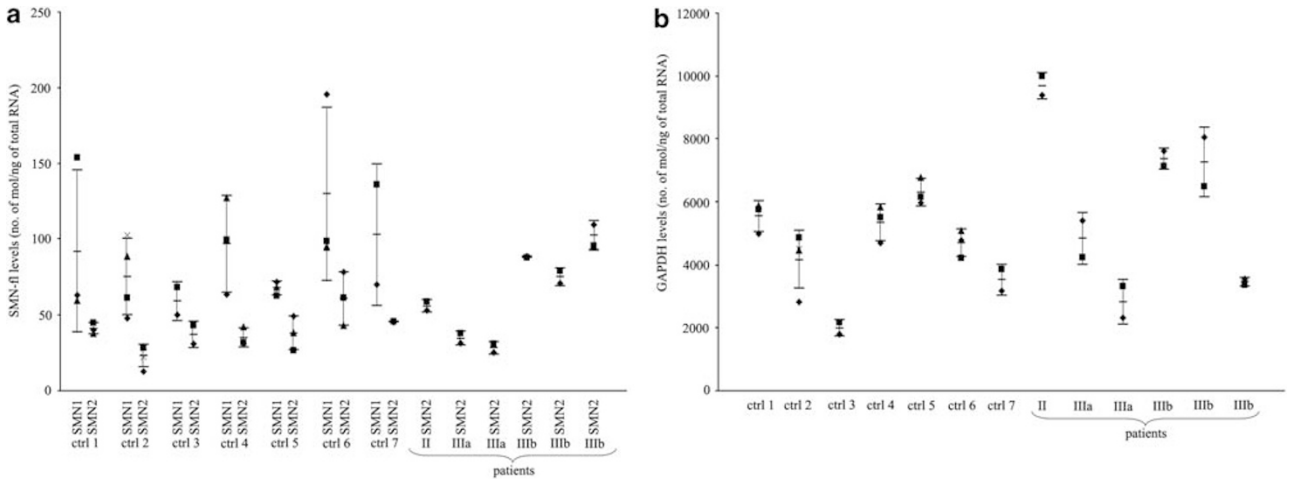

Figure 1 (**a**) Day-to-day variations of *SMN*-fl, *SMN1*-fl, and *SMN2*-fl transcript levels in seven controls (ctrl 1–7) and of *SMN2*-fl 2 type III patients (pt 1–6). *SMN2*-fl level fluctuations were similar to that expected for experimental variability, whereas in controls *SMN1*-fl levels and, consequently, *SMN*-fl levels varied up to threefold. (**b**) *GAPDH* transcript level fluctuations were in the range of experimental variability.

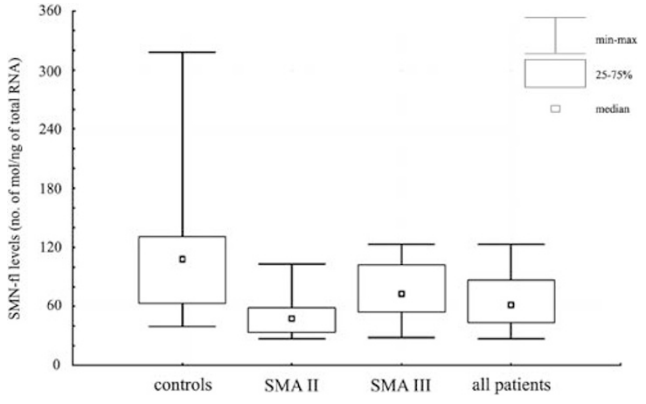

Figure 2 *SMN2*-fl levels in patients *vs* *SMN*-fl in controls. *SMN2*-fl transcript levels in patients are significantly reduced compared with *SMN*-fl levels in controls. Also median *SMN2*-fl levels in patients are significantly lower compared with controls when considering type II and III patients separately. Type II patients showed significantly lower *SMN2*-fl transcript levels compared with type III subjects.

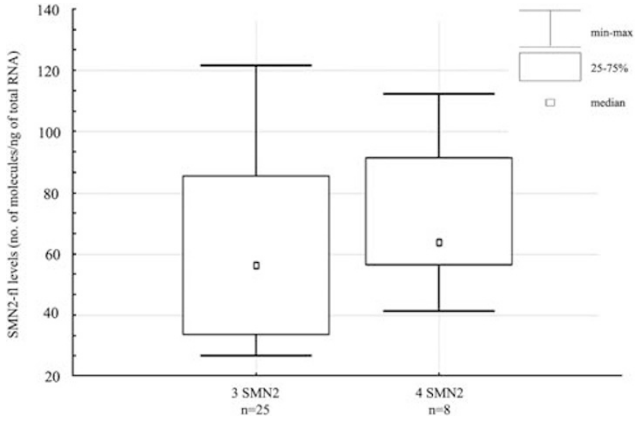

Figure 3 *SMN2*-fl transcript levels are not related to *SMN2* gene copy number. Although patients with four *SMN2* copies showed higher median *SMN2*-fl levels compared with individuals with three *SMN2*, this difference was not statistically significant.

SMN2-fl levels and gene copy number was found (one-way ANOVA $P=0.52$). Moreover, when comparing patients with three and four *SMN2* genes (Figure 3), no statistical difference was found (MW=KW: $P=0.35$), although individuals with four copies had slightly higher median *SMN2*-fl transcript levels (3 *SMN2*: 56.25 mol/ng, range: 26.75–121.5; 4 *SMN2*: 63.75 mol/ng, range: 41.25–112.25).

SMN2-fl transcript levels are related to phenotypic severity

To assess whether *SMN2*-fl levels are related to clinical severity, different parameters were evaluated: type of SMA, age at onset, and the Hammersmith motor scale score (in type II patients with age range 2.5–12 years). Type II patients showed median *SMN2*-fl transcript levels lower than type III, and the difference between the two groups was highly significant (MW=KW: $P=0.0034$, Table 1 and Figure 2). Subsequently, *SMN2*-fl transcript levels were related to the age of onset of type II and III patients. Although in type II patients no correlation was found between the two variables (data not shown), in the case of SMA type III ($n=23$, 1.5–19 years) age of onset was inversely related to *SMN2*-fl levels. Although it was not possible to identify a linear regression model between the two variables, we found that *SMN2*-fl levels ≥ 58 mol/ng are related to a threefold lower risk of disease onset before the age of 3 (RR: 0.31, CI: 0.12–0.76, Fisher's exact test $P=0.02$, Figure 4a). A total of 6 out of 12 type IIIb and 2 out of 10 type IIIa patients had four *SMN2* copies. The other patients had three *SMN2* genes.

The motor ability of type II patients was evaluated by the Hammersmith scale score.[27] The cutoff of 12 years was chosen to avoid bias because of the presence of complications, such as severe scoliosis and contractures, which are more frequent after this age. Only 10 out of 16 type II patients were below the age of 12 years. We compared *SMN2*-fl levels and motor function by using a linear regression model, indicating transcript levels as an independent variable, and found a significant correlation between the two variables ($\beta=0.64$, $P=0.04$, Figure 4b), indicating a moderately strong association between the Hammersmith score and transcript levels. The

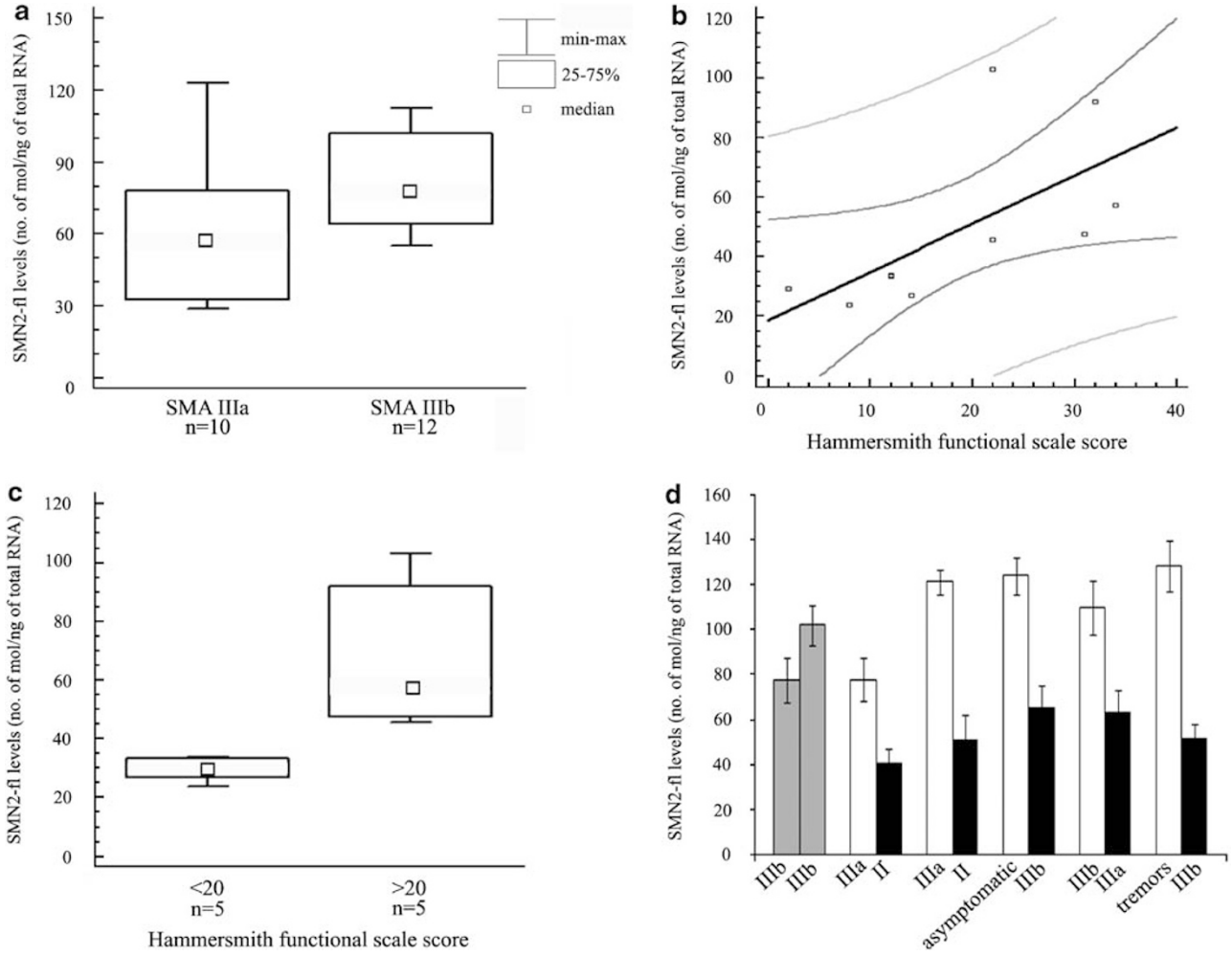

Figure 4 Correlation between *SMN2*-fl levels and clinical severity (**a**) Transcript levels are related to the age of onset of type III patients: patients with *SMN2*-fl levels <58 mol/ng of total RNA showed a threefold higher risk of disease onset below the age of 3 years (type IIIa). (**b**) In type II patients (ranging: 2.5–12 years), a linear correlation was found between *SMN2*-fl levels and Hammersmith functional scale scores. Dots indicate *SMN2*-fl levels and the corresponding functional score for individual patients; the black line is the graphic representation of the equation describing the linear regression model; the dark gray lines indicate 95% confidence interval and the outer light gray lines are 95% prediction limits for new observations. (**c**) Patients with a functional score ≤ 20 showed significantly lower *SMN2*-fl levels. (**d**) In haploidentical sib pairs with discordant SMA phenotype, the less severely affected sib (white columns) showed significantly higher transcript levels compared with the more severely affected one (black columns), whereas phenotypically concordant sibs (gray columns) had similar *SMN2*-fl levels. However, *SMN2nn*-fl transcript quantification is not predictive of phenotypic severity in individual cases, as less severely affected patients may have higher transcript levels. Error bars indicate SD of repeated experiments.

relationship between the two variables can be described by using the following expression:

$$\text{SMN2} - \text{fl} = 18.6455 + 1.61056 \times \text{Hammersmith score}$$

Subsequently, we subgrouped patients on the basis of functional scores ≤ 20 (n=5) or >20 (n=5), and observed that the more severely affected patients had median lower *SMN2*-fl levels (29.25 mol/ng, range: 23.60–33.50), compared with the other group (median: 57.00 mol/ng, range: 45.5–102.75); the difference was statistically significant (MW: P=0.01, KW: P=0.009, Figure 4c).

Finally, to assess whether differences in *SMN2*-fl levels exist between haploidentical SMA siblings, we evaluated transcript levels in six sib pairs, five of which showed marked phenotypic differences, whereas one further sib pair was similar in disease severity. We found that in discordant couples, the less severely affected sib showed significantly higher *SMN2*-fl levels compared with the more severely affected one (P=0.002). The phenotypically similar sib pair showed only slightly different *SMN2*-fl levels (Figure 4d).

DISCUSSION

The recent move in SMA research from basic to clinical has raised the necessity to develop reliable and reproducible clinical tools, and to identify biomarkers useful for monitoring the response of SMA patients to pharmacological treatments. Although validated clinical tools have been developed, at present the quantification of *SMN2* gene products in blood leukocytes, either at protein or transcript levels, is the only potential biomarker available. However, except for type I SMA,[14,22] no clear differences in SMN levels between patients and controls have been shown, thus questioning the reliability of transcript analysis as a biomarker for SMA and its usefulness in monitoring the molecular effects of pharmacological treatment. We have developed and validated a new real-time PCR assay, based on the use of absolute standard curves, which allows the quantification of *SMN*-fl transcripts

as the number of mRNA molecules per nanogram of total RNA, independently from the use of endogenous controls. We have exploited techniques that are widely used to determine the plasmatic load of some RNA viruses, such as HIV[28] or HCV,[29] or of prion protein.[30]

One interesting finding of our study is the difference in day-to-day variability of *SMN1*-fl and *SMN2*-fl levels. Although we have observed moderate variations in *SMN2*-fl levels, which were similar to that expected for experimental variability and comparable with that of *GAPDH* transcripts, *SMN1*-fl levels varied up to threefold from one day to the other. Owing to *SMN1*-fl variability, the total amount of *SMN*-fl transcripts also varied markedly (Figure 1a and b and data not shown; see also Supplementary information). The relevance of this observation is related to the strategy to test the *in vivo* effect of different compounds on SMN expression by administering a given drug to parents of SMA patients, who often offer spontaneously their own collaboration.[13,14] In a study on the molecular effect of valproic acid, 7 of 10 carriers had increased levels of *SMN*-fl transcripts after treatment, whereas only in one-third of the patients a molecular effect was shown.[14] The discrepancy between these observations may be explained by spontaneous fluctuations in *SMN1*-fl transcript levels, as the assay used by these authors does not allow the discrimination of *SMN1* from *SMN2* transcripts. Thus, the results of molecular studies of the *in vivo* efficacy of a given compound on SMN expression in carriers or controls should be interpreted cautiously, and only data relative to *SMN2* transcripts should be taken into account.

The most relevant finding of our study is that *SMN2*-fl transcripts are significantly reduced in type II and III patients. As indicated in Figure 2 and the Table 1, both considering all patients as a group or divided on the basis of the type of SMA, *SMN2*-fl transcripts are significantly reduced in the majority of SMA patients, compared with *SMN*-fl in controls. Moreover, type III patients have significantly higher *SMN2*-fl transcript levels than type II. To our knowledge, this is the first demonstration of a statistically significant reduction of SMN levels in blood leukocytes of type II–III patients. The partial overlap of *SMN*-fl levels between patients and controls is not unexpected: blood leukocytes are not target cells in SMA, and it is conceivable that in target tissues, the cutoff between patients and controls could be sharper. It would be important to study the key cell types involved in SMA pathophysiology, such as motor neurons and/or muscle cells and to relate *SMN2*-fl levels in these cells with those found in blood.

To gain further insights into a possible correlation between transcript levels and clinical severity, we investigated whether *SMN2*-fl levels are related to the age of onset of type II and III patients, and to the Hammersmith motor scale score (for type II patients below the age of 12 years). We did not observe any correlation between age of onset of type II patients and *SMN2*-fl transcript levels; however, in these patients, the first symptoms of the disease are often misrecognized by parents and may be interpreted as a slight delay in ambulation achievement. In the case of type III patients, we observed that higher transcript levels are related to more advanced age at onset (Figure 4a). In particular, we found that patients with *SMN2*-fl levels ≥ 58 mol/ng have a threefold lower risk of disease onset below the age of 3 years. The onset of the disease below or over this age (type IIIa and b) was earlier indicated as an important prognostic factor for walking ability maintenance:[1] in an earlier study, Wirth *et al*[8] reported that the 60% of type IIIb and 35% of type IIIa patients have four *SMN2* copies (50 and 20%, respectively, in our cohort), confirming the correlation between phenotypic severity and *SMN2* gene number. A correlation between clinical severity and *SMN2*-fl transcript levels is further supported by the finding that type II children with higher scores of the Hammersmith functional motor scale show significantly higher *SMN2*-fl transcript levels compared with those with lower scores (Figure 4b-c). In addition, in haploidentical SMA siblings with discordant phenotype, the less severely affected sib showed significantly higher *SMN2*-fl levels compared with the more severely affected one, whereas in a phenotypically similar pair, both sibs had similar *SMN2*-fl levels (Figure 4d).

We and others have earlier found a correlation between *SMN2* copy number and phenotypic severity;[6,7,8] however, *SMN2* gene number alone is not sufficient to explain the phenotypic variability of SMA, as patients with the same gene number have different phenotypes, and haploidentical sibs can be markedly discordant for disease severity. To our knowledge, this is the first *in vivo* molecular study on SMA discordant siblings; only one *in vitro* study has been published earlier,[31] showing that the more severely affected sibs had lower *SMN* levels in lymphoblastoid cell lines but not in fibroblasts. We compared the *SMN2*-fl transcript levels and *SMN2* gene number and did not find a significant correlation (Figure 3). Similar results were also found in the study by Simard *et al*,[23] whereas Sumner *et al*[22] and Vezain *et al*[24] found that *SMN2* transcript levels are related to the number of *SMN2* genes. The discrepancy among different studies can be at least partially accounted for by the use of relative quantification and of different endogenous internal standards that vary widely among different individuals. Our data suggest that the regulation of *SMN2*-fl transcript production (at transcriptional or splicing levels) has greater influence on phenotypic modulation than *SMN2* copy number *per se*, which is further supported by the finding that in discordant sib pairs with identical gene copy number, the less severely affected sib higher transcript levels. However, although *SMN2*-fl transcript quantification appears to be more tightly related to phenotypic variability than *SMN2* copy number assessment, it should not be used as a prognostic marker for individual patients, because of the overlapping between the different phenotypic groups. It would be of interest to have further data on a possible correlation between SMN transcript and protein levels in leukocytes by means of an ELISA assay, which is currently available for cell cultures only.[26]

In conclusion, our data indicate that *SMN2*-fl quantification in blood leukocytes by absolute real-time PCR can be considered suitable as a biomarker in SMA clinical trials as *SMN2*-fl transcripts are both reduced in patients compared with *SMN*-fl in controls and their level may at least partially reflect *SMN* levels in target tissues, being related to disease severity. The present assay, compared with that published earlier, offers the opportunity to standardize and optimize *SMN2*-fl transcript quantification in different laboratories, in the view of upcoming international multicentric clinical trials. Further studies are still necessary to assess whether possible *SMN2*-fl transcript increments may correlate with clinical improvements of SMA patients included in clinical trials, and/or if *SMN2*-fl dosage can predict clinical response to pharmacological treatment.

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