European Journal of Human Genetics (2013) 21, 630–636

www.nature.com/ejhg

ARTICLE

Clinical and molecular cross-sectional study of a cohort of adult type III spinal muscular atrophy patients: clues from a biomarker study

Francesco D Tiziano*[,1], Rosa Lomastro[1], Lorena Di Pietro[1], Maria Barbara Pasanisi[2], Stefania Fiori[1], Carla Angelozzi[1], Emanuela Abiusi[1], Corrado Angelini[3], Gianni Sorarù[3], Alessandra Gaiani[3], Tiziana Mongini[4], Liliana Vercelli[4], Gessica Vasco[5], Giuseppe Vita[6], Gian Luca Vita[6], Sonia Messina[6], Luisa Politano[7], Luigia Passamano[7], Grazia Di Gregorio[7], Cristina Montomoli[8], Chiara Orsi[8], Angela Campanella[2], Renato Mantegazza[2] and Lucia Morandi[2]

Proximal spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder caused by mutations of the *SMN1* gene. Based on severity, three forms of SMA are recognized (types I–III). All patients usually have 2–4 copies of a highly homologous gene (*SMN2*), which produces insufficient levels of functional survival motor neuron (SMN) protein due to the alternative splicing of exon 7. The availability of potential candidates to the treatment of SMA has raised a number of issues, including the availability of biomarkers. This study was aimed at evaluating whether the quantification of SMN2 products in peripheral blood is a suitable biomarker for SMA. Forty-five adult type III patients were evaluated by Manual Muscle Testing, North Star Ambulatory Assessment scale, 6-min walk test, myometry, forced vital capacity, and dual X-ray absorptiometry. Molecular assessments included *SMN2* copy number, levels of full-length *SMN2* (*SMN2*-fl) transcripts and those lacking exon 7 and SMN protein. Clinical outcome measures strongly correlated to each other. Lean body mass correlated inversely with years from diagnosis and with several aspects of motor performance. *SMN2* copy number and SMN protein levels were not associated with motor performance or transcript levels. *SMN2*-fl levels correlated with motor performance in ambulant patients. Our results indicate that *SMN2*-fl levels correlate with motor performance only in patients preserving higher levels of motor function, whereas motor performance was strongly influenced by disease duration and lean body mass. If not taken into account, the confounding effect of disease duration may impair the identification of potential SMA biomarkers.
European Journal of Human Genetics (2013) **21,** 630–636; doi:10.1038/ejhg.2012.233; published online 17 October 2012

Keywords: spinal muscular atrophy; SMN; biomarker; outcome measure; real-time PCR

INTRODUCTION

Proximal spinal muscular atrophies (SMAs) are a group of clinically variable motor neuron disorders characterized by degeneration of spinal cord anterior horn cells. SMAs are generally classified into types I to III according to age at onset and highest motor milestone achieved.[1,2] SMA type III is the most clinically variable form, with symptoms onset before (type IIIa) or after (type IIIb) age 3 years,[3] normal achievement of motor milestones, variable severity of scoliosis, tendon retractions and joint contractures, and eventual loss of walking ability.

Type I–III SMAs are autosomal recessive conditions caused by loss of function of the survival motor neuron (*SMN1*) gene.[4] Irrespective of phenotypic severity, both copies of the *SMN1* gene are absent in about 95% of cases, whereas 2–3% of patients are compound heterozygotes typically with one allele deleted and subtle mutations in the other.[5] Complete loss of the SMN protein is embryonically lethal,[6] but SMA patients obtain reduced amounts of the protein from a nearly identical gene copy, *SMN2*, present (with *SMN1*) in a duplicated and inverted region of 5q13.[4] Because of alternative splicing, most *SMN2* transcripts lack exon 7 (SMN-del7) so that insufficient amounts of functional protein are produced. In fact, SMN protein levels are reduced in spinal cord and cell cultures from SMA patients, and correlate inversely with phenotypic severity.[7–9] *SMN2* copy number can also vary, and patients with high copy number often have a milder phenotype.[10–12]

At present, there is no effective treatment for SMA. Some therapeutic approaches aim to increase the amount of SMN protein produced by *SMN2* through promoter activation, reduction of exon 7 alternative splicing, or both.[13–22] Some of these approaches are being investigated in ongoing or planned clinical trials, and great efforts have been done to identify the most appropriate clinical outcome measures for patients affected from various severities.[23] In this view,

[1]Medical Genetics Institute, Catholic University, Rome, Italy; [2]Muscle Pathology and Neuroimmunology Unit, Foundation Neurological Institute Carlo Besta, Milan, Italy; [3]Department of Neurosciences, University of Padua, Padova, Italy; [4]Neuromuscular Disorders, Molinette Hospital, Turin, Italy; [5]Department of Pediatric Neurology, Catholic University, Rome, Italy; [6]Department of Neurosciences, Psychiatry, Anesthesiology, University of Messina, Messina, Italy; [7]Cardiomyology Genetics Section, Second University of Naples, Naples, Italy; [8]Department of Public Health, Neuroscience, Experimental and Forensic Medicine – Section of Biostatistics and Clinical Epidemiology, University of Pavia, Pavia, Italy

*Correspondence: Dr FD Tiziano, Institute of Medical Genetics, Catholic University, Largo Francesco Vito, 1, 00168 Rome, Italy. Tel: +39 0630154606; Fax: +39 0630157223; E-mail: fdtiziano@rm.unicatt.it

Received 1 May 2012; revised 22 August 2012; accepted 18 September 2012; published online 17 October 2012

it would be very useful to have reliable biomarkers of disease severity and response to treatment.[23]

In the present study, we investigated associations between clinical phenotype and molecular characteristics in adult patients with type III SMA, with the aim of evaluating available molecular biomarkers for possible use as surrogate endpoints in clinical trials on SMA. Clinical phenotype was assessed by tests of muscle strength and function. Molecular evaluation comprised determination of *SMN2* copy number, SMN transcript levels (full length and del7), and SMN protein levels.

MATERIALS AND METHODS

Patients and clinical evaluation

A total of 45 patients (29 male, 16 female, Table 1), mean age 36.8 years (range 18–56 years) with diagnosis confirmed by molecular analysis were recruited to an ongoing double-blind placebo-controlled multicenter trial to assess the safety of salbutamol (EudraCT No. 2007-001088-32). All patients enrolled in the double-blind trial were included in the present study. At clinical evaluation, 26 were ambulant and 19 were wheelchair bound (mean age at loss of walking, 20 years). Based on age of onset, 15 were type IIIa and 30 were type IIIb. No patients reported onset of symptoms over 18 years of age. Written informed consent was obtained from all patients, and the study was approved by the Ethics Committee of each participating Centre.

Patients were comprehensively evaluated at baseline. Only selected variables are reported here as potential outcome measures. Muscle strength was assessed by manual muscle testing of 18 muscle groups (elbow flexors and extensors, finger flexors and extensors, thigh flexors and extensors, leg flexors and extensors, foot dorsiflexors) and graded from 0 to 5 according to the Medical Research Council (MRC) scale.[24] The force of maximum voluntary isometric contraction (Newtons, N) was assessed in elbow flexor, handgrip, three-point pinch, knee flexor, and knee extensor[25] for 30 of the 45 patients, using a hand-held myometer (CIT Technics, Groningen, The Netherlands).

In ambulant patients, motor function was assessed by the North Star Ambulatory Assessment (NSAA) scale.[26] Ambulant patients also performed the 6-Min walk test (6MWT) recently shown to be reliable for assessing type III SMA patients.[27,28]

Forced vital capacity (FVC, % of predicted) was measured in all patients using a standard spirometer in the sitting position. Lean body mass (grams) was assessed by dual X-ray absorptiometry (DXA)[29,30] and normalized to height (expressed in cm); this evaluation was performed in 20 patients only, in those neuromuscular Centres where the tool was available. Furthermore, DXA was feasible only for patients who did not have severe contractures preventing the access to the examination bed of the instrument.

Table 1 Selected clinical and molecular characteristics in a cohort of type III adult SMA patients

Characteristic	*No. of patients*	*Mean*	*Median*	*Range*	*SD*
Clinical features					
Age (years)	45	36.8	38	18–56	25.8
MRC total score	45	58.6	58	21–89	16.8
MRC upper limb score	45	31	32	18–40	6.4
MRC lower limb score	45	27.6	29	3–18	11.3
Forced vital capacity (% of predicted)	36	86.5	84.5	43–121	18.9
Elbow flexion (Newtons)	30	60.2	51	3–222	53.5
Handgrip (Newtons)	30	54.7	42	4–187	50.0
Three-point pinch (Newton)	30	38.3	30	3–110	30.3
Knee flexion (Newtons)	30	42.3	31	0–127	37.1
Knee extensor (Newtons)	30	19.8	10	1–88	22.2
NSAA score (ambulatory patients)	26	19.8	20	7–31	6.9
6-min walk test (meters)	26	363.5	362.8	85.9–543	116.6
Lean body mass/height (g/cm)	20	210.3	197.7	146.6–305.1	47.1
Molecular findings					
SMN2 copy number	45	3.7	4	1–5	0.7
SMN2-fl transcripts (mol/ng RNA)	45	67.5	64.9	28.5–138.9	20.7
SMN-delta7 transcripts (mol/ng RNA)	45	235.3	222.4	74.3	534.8
SMN-tot transcripts (mol/ng RNA)	45	304.0	282.1	131.1–628.9	117.8
SMN protein (pg/10^6 cells)	43	230.5	155.3	13.4–1076.7	238.0

Molecular assessments

Blood samples were collected into EDTA tubes for DNA extraction, sodium citrate tubes for protein extraction, and PAX blood RNA tubes (BD Biosciences, San Jose, CA, USA) for RNA. The samples were analysed at the Institute of Medical Genetics of Catholic University in Roma.

Genomic DNA was extracted by standard salting out, and conventional RFLP-PCR used to verify SMA diagnosis.[31] For patients testing negative for *SMN1* mutation by RFLP-PCR, *SMN1* copy number was determined (same method as *SMN2* copy number); for patients with a single *SMN1* copy, sequence analysis of exons 1–7 and exon–intron boundaries was performed (sequence of primers and PCR conditions are available on request).

SMN1 and *SMN2* copy number was determined by relative real-time PCR as reported elsewhere.[14] SMN2 copy number was determined in all patients.

The presence of the p.G287R (c.G859C)[32] variant in *SMN2* was determined in all patients by RFLP-PCR. Briefly, 50 ng of genomic DNA were amplified with R111[4] as forward primer and G287R*Dde*R: 5′-ATTTAAGGAA TGTGAGCACCTT**A**-3′ as reverse primer. The latter contains a mismatch (bold) that introduces a restriction site for *Dde*I in the variant allele. Amplification conditions were: 30 cycles of 94 °C for 1 min, 55 °C for 1 min, 72° C for 1 min. The PCR products were digested with 3U of restriction enzyme *Dde*I overnight at 37 °C. Next day, the digestion products were separated by electrophoresis on 4% agarose gels. If the G287R variant was present, two bands (208 and 185 bp) were obtained.

RNA was extracted by PAX blood RNA extraction kit (Qiagen, Dusseldorf, Germany), according to the manufacturer's instructions. *SMN2* full length (SMN2-fl), lacking exon 7 (SMN-del7) and total (SMN-fl plus SMN-del7, SMN-tot) transcript levels were assessed by absolute real-time PCR (Tiziano *et al*[33] and Angelozzi *et al*, in preparation). In patients with the G287R variant, full-length transcripts were determined by an alternative set of Taqman MGB probe and primers.[33] *GAPDH* transcript levels were determined as quality control for RT-PCR and real-time PCR.

For SMN protein analysis, time between blood collection and preparation of samples ranged from a few hours to 2 days. Samples from 43 patients were analyzable. PBMCs were separated through Lympholyte M medium (Macherey-Nagel, Duren, Germany). The pellet was washed in PBS and frozen in fetal calf serum containing 10% DMSO. After thawing, PBMCs were counted by NucleoCounter (Chemometec, Allerod, Denmark) and resuspended in lysis buffer at 2×10^6 cells/ml (instead of 10^8 cells/ml, as in ELISA protocol, Enzo Life Science, Farmingdale, NY, USA); 2×10^5 cells were loaded onto each ELISA plate. The ELISA kits were kindly provided by the SMA Foundation. SMN protein concentrations were expressed as pg of protein/10^6 cells.

Statistical analysis

Means, medians, and SD for continuous variables and proportions for categorical variables were calculated. Associations of *SMN2*-fl, SMN-del7, SMN-tot transcript levels, and SMN protein levels, with clinical characteristics were assessed by linear regression models. A multivariate model was used to take account of the influence of other covariates. Because of small sample size and non-normal distribution of SMN transcript levels,[33] the non-parametric Kruskal–Wallis 'ANOVA' by ranks (KW) and Mann–Whitney *U*-test (MW)

were used to compare transcript levels between groups (ambulant *vs* non-ambulant; type IIIa *vs* type IIIb). Correlations between clinical characteristics were evaluated by Pearson's *r* (*R*) test. The *t*-test for paired samples was used to compare the performance of groups at different time-points of the 6MWT. Statgraphics (Centurion XV.II, Statpoint Technologies, Warrenton, VA, USA) and SPSS 18.0 (SPSS, Inc., Chicago, IL, USA) were used to carry out the analyses. Differences associated with $P<0.05$ or, after Bonferroni correction for multiple testing, with $P<0.016$ were considered significant.

RESULTS

Genotypic characterization of patients at the SMN locus

In 43 of the 45 patients, *SMN1* exon 7 was absent. The remaining two patients were compound heterozygotes, missing one copy of *SMN1*, and with the W102X mutation[34] in one case, and the S262I mutation[35] in the other (Supplementary Figure 1a).

SMN2 gene copy number was determined in all patients. There were five *SMN2* copies in 2 patients, 4 in 29 patients, three in 13 patients and a single copy in the patient with the S262I mutation. Among type IIIa patients, 7 out of 15 (46.7%) had three *SMN2* copies, the others had 4 genes. Of the 30 type III b subjects, 21 had 4 *SMN2* genes (70%).

The G287R variant (Supplementary Figure 1b) of *SMN2* was found in 4/45 (8.9%) patients, all type IIIb with three *SMN2* copies. One of these patients was homozygous for the G287R variant, being present in both parents.

Correlations between clinical characteristics

Selected baseline clinical features and molecular characteristics of the patients are shown in Table 1. Total MRC score correlated with handgrip ($R=0.78$, $P<0.00001$, $n=30$, data not shown), elbow flexion ($R=0.68$, $P<0.00001$, $n=30$, data not shown), knee extension ($R=0.59$, $P=0.0006$, $n=30$, data not shown), and knee flexion ($R=0.74$, $P<0.00001$, $n=30$, Figure 1a). Total MRC score correlated weakly with predicted forced vital capacity ($R=0.28$, $P=0.06$, $n=45$, Figure 1b). In ambulant patients, total MRC score correlated strongly with NSAA ($R=0.77$, $P<0.00001$, $n=26$, Figure 1c) and 6MWT ($R=0.67$, $P=0.0002$, $n=26$, Figure 1d). Distance covered during the sixth minute (mean 60.37 ± 20.36 m) was significantly less ($P=0.001$, $n=26$) than in the first minute (mean 67.39 ± 18.96 m).

There was no correlation between muscle strength (total MRC scale score) or motor function (NSAA score, ambulant patients only) and patient age (MRC: $R=-0.065$, $P=0.67$; NSAA: $R=-0.26$, $P=0.18$). However, total MRC and NSAA scores (in ambulant patients) did correlate inversely with disease duration (MRC: $R=-0.57$, $P<0.00001$, $n=45$; NSAA: $R=-0.48$, $P=0.01$, $n=26$, Figures 2a and b). Forced vital capacity also correlated inversely with disease duration ($R=-0.31$, $P=0.038$, $n=45$; data not shown).

Correlations between motor performance and lean body mass

In all patients tested, lean body mass correlated with total MRC score ($R=0.66$, $P=0.0015$, $n=20$, Figure 2c). In ambulant patients, correlations of lean body mass with other aspects of motor performance were strong (MRC: $R=0.82$, $P=0.0005$, $n=11$; NSAA: $R=0.71$, $P=0.006$, $n=11$; 6MWT: $R=0.69$; $P=0.009$, $n=11$; Figure 2d and data not shown). Lean body mass also correlated inversely with disease duration ($R=-0.50$, $P=0.025$, $n=20$) and the correlation remained after correcting for height ($R=-0.52$, $P=0.019$, $n=20$, data not shown).

Associations between clinical and molecular data

By linear regression modeling, *SMN2* copy number was unrelated to any clinical variable (in all cases $P\geq0.27$, data not shown), or to *SMN2* transcript or SMN protein levels (in all cases $P\geq0.08$, data not shown). Similarly, neither *SMN2* transcript nor protein levels were influenced by age, years from symptoms onset, or lean body mass (in all cases $P\geq0.12$, data not shown). Transcript and protein levels did not differ between SMA types IIIa and IIIb (MW and KW, $P>0.20$, data not shown).

In the entire group, neither total nor delta7 SMN2 transcript levels correlated significantly with any clinical characteristic, although

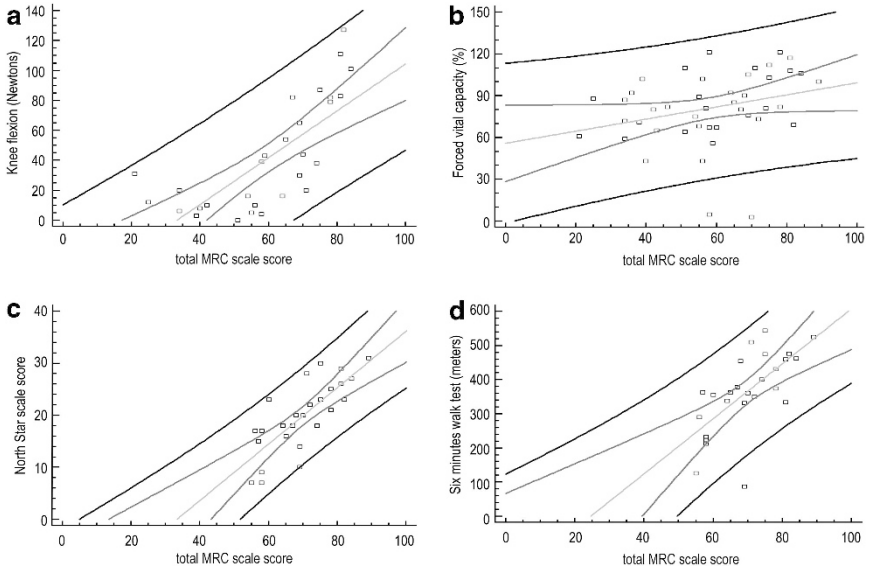

Figure 1 Scatter plots showing associations between total MRC score and (**a**) force of knee flexions in Newtons ($n=30$, $R=0.74$, $P<0.00001$); (**b**) Forced vital capacity (% of predicted) ($n=45$, $R=0.28$, $P=0.06$); (**c**) NSAA score ($n=26$, $R=0.77$, $P<0.00001$); and (**d**) 6-min walk test (meters, $n=26$, $R=0.67$, $P=0.0002$). Straight line: expected distribution; flanking lines, 95% confidence limits; black lines: limits of distribution. (**a**) and (**b**) refers to the whole cohort, whereas (**c**) and (**d**) to ambulant patients only.

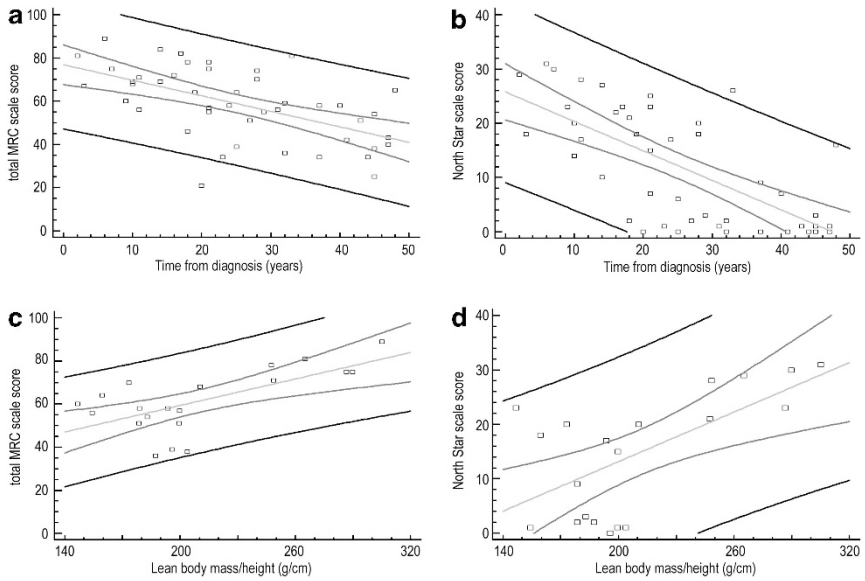

Figure 2 Scatter plots showing associations between time from diagnosis and (**a**) total MRC score ($n=45$, $R=-0.57$, $P<0.00001$) and (**b**) NSAA score ($n=26$, $R=-0.48$, $P=0.01$); associations between lean body mass/height and (**c**) total MRC ($n=20$, $R=0.66$, $P=0.0015$), and (**d**) NSAA ($n=11$, $R=0.71$, $P=0.006$). Straight line, expected distribution; flanking lines, 95% confidence limits; black lines: limits of distribution. (**a**) and (**c**) refers to the whole cohort, whereas (**b**) and (**d**) to ambulant patients only.

SMN2-fl levels correlated weakly with total MRC score ($R=0.29$; $P=0.052$, $n=45$, data not shown), as well as with lower limb MRC score ($R=0.29$; $P=0.049$, $n=45$, data not shown). In ambulant patients only, *SMN2*-fl levels correlated with total MRC score ($R=0.46$, $P=0.02$, $n=26$, Figure 3a), and with lower limb MRC score ($R=0.49$, $P=0.01$, $n=26$, Figure 3b), and weakly with 6MWT ($R=0.37$), although this correlation was not significant ($P=0.07$, $n=26$, Figure 3c). SMN protein levels did not correlate with motor performance ($P\geq0.31$, data not shown) or with *SMN2*-fl levels ($R=0.23$, $P=0.18$, $n=43$, Figure 4a); however protein levels did correlate with the SMN2-fl/SMN2-delta7 ratio ($R=0.40$, $P=0.016$, $n=43$, Figure 4b).

DISCUSSION

Several potential therapeutic approaches to SMA are undergoing development or have been tested in recent years.[36] Reliable clinical outcome measures and biomarkers are essential to effectively evaluate these approaches. Different motor function measures have been used and validated in SMA patients,[23,26] but some of them are too long to administer, include tasks unbearable for adult patients, or may be used only for patients with moderate phenotypes due to floor or ceiling effects. Moreover, some complications related to the disease, such as scoliosis, retractions, and weight gain, can further impair motor function. Thus, the identification of reliable biomarkers as surrogate endpoints of disease progression and response to treatment has become a matter of urgency.[23] The aim of our study was to evaluate the applicability of SMN transcript and protein levels, as surrogate outcome measures in adult type III SMA patients, by relating clinical and molecular data. The clinical variables chosen have been previously validated or used in other SMA studies.[23] The molecular techniques we used (absolute real time for transcript analysis, ELISA for protein quantification) are currently considered the most suitable tools for SMN quantification, as they do not make use of normalization against endogenous controls and are therefore unaffected by possible variations in the expression levels of housekeeping genes.

We found that clinical measures correlated strongly with each other, as expected. Similarly, Glanzman *et al*[37] recently found that modified Hammersmith scale score correlated strongly with myometry-measured muscle strength. The motor performance was significantly affected by disease duration but not by age at evaluation, suggesting that in the design of clinical trials this variable could be useful to enroll more homogenous cohorts of patients, rather than age.

Montes *et al*[28] recently evaluated the 6MWT in type III SMA patients spanning a wide age range and found that they showed progressive motor fatigue. We observed similar fatigability in the present series, so it may be also worth investigating whether increased resistance to motor fatigue can be used as a marker of treatment efficacy.

In the present study, we found no correlation between motor performance and *SMN2* copy number. However, similar to our own previous data[11] and those of others,[10,12,37] a higher number of *SMN2* genes was found in type IIIb patients, but was not predictive of the clinical phenotype in individual subjects. In the patient bearing the S262I mutation in exon 6, we found a single *SMN2* copy, suggesting that this point mutation determines only a mild reduction in SMN protein function. This hypothesis is supported by the previous report of the same mutation in another patient,[35] affected by SMA type III as well; however, in that patient, *SMN2* gene copy number was not assessed. Also, the frequency of the G287R variant in our cohort was much higher than previously reported (about 9% *vs* 1%[38]). This variant has a positive effect in the inclusion of exon 7 in *SMN2* transcripts.[32,38] All our patients bearing the G287R variant were type IIIb subjects, thus raising the prevalence of the mutation in this group of patients up to 16% (4/25 individuals). Because of the positive effect of this variant in exon 7 inclusion in *SMN2* transcripts, this finding is not unexpected. It is noteworthy that these four subjects had three

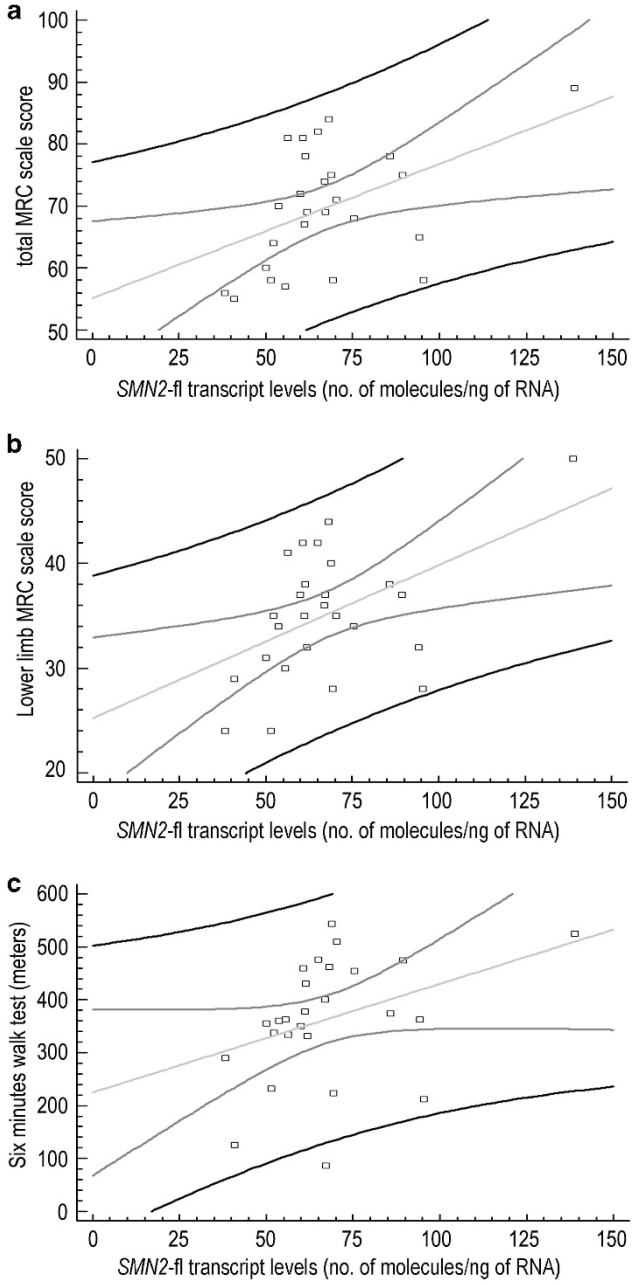

Figure 3 Scatter plots showing associations of *SMN2*-fl transcript levels in ambulant patients with (**a**) total MRC score ($n=26$, $R=0.46$, $P=0.02$), (**b**) lower limb MRC score ($n=26$, $R=0.49$, $P=0.01$), and (**c**) 6-min walk test ($n=26$, $R=0.37$, $P=0.07$). Straight line: expected distribution; flanking lines: 95% confidence limits; black lines: limits of distribution.

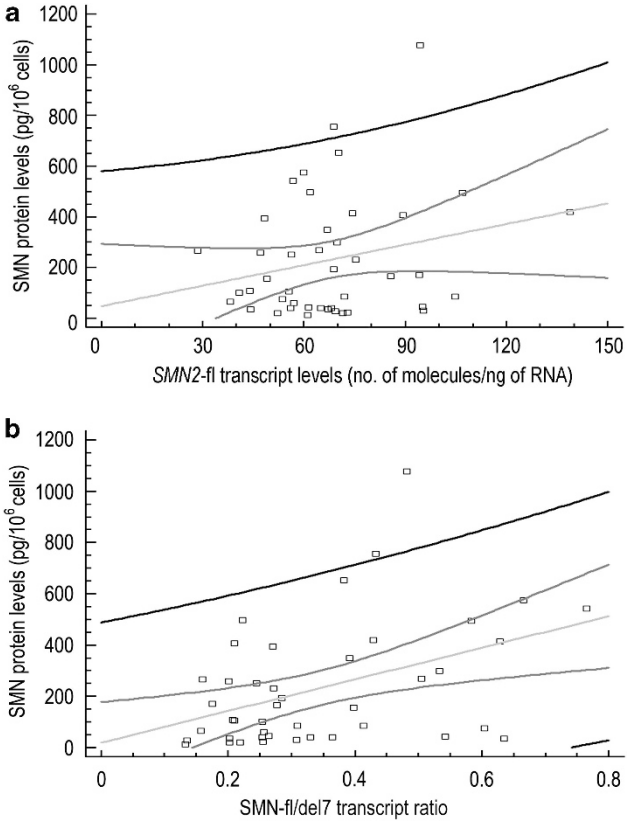

Figure 4 Scatter plots showing associations of levels of SMN protein in peripheral blood with (**a**) *SMN2*-fl transcript levels ($n=43$, $R=0.23$, $P=0.18$), and (**b**) the SMN2-fl/SMN2-delta7 ratio ($n=43$, $R=0.40$, $P=0.016$). Straight line, expected distribution; flanking lines, 95% confidence limits; black lines: limits of distribution.

SMN2 copies, supporting the positive modulating effect of the G287R variant on SMA severity.

We also found a weak correlation between motor performance and *SMN2*-fl transcript levels, when considering all patients, which was much stronger in ambulant patients. It is likely that in non-ambulant patients, the presence of long-term complications of the condition further worsened motor performance. An alternative hypothesis is that *SMN2*-fl levels in blood do not reflect those found in target tissues of the disease, such as spinal cord and/or skeletal muscle. However, in our opinion, this hypothesis is less likely, as the correlation of transcript levels with motor function is stronger in the less severely affected patients. As no other transcript assessed (SMN2-del7, total SMN2 transcripts, or SMN2-fl/ del7 ratio) correlated with any baseline clinical characteristic, even in ambulant patients, *SMN2*-fl appears to have the strongest relation to phenotype. Very recently, some of us (FDT and LR) have collaborated to the BforSMA study.[39,40] Also in that large cohort of young patients spanning the whole phenotypic spectrum of the disease, *SMN2*-fl levels were significantly higher in the less severely affected subjects, although they were not predictive of the motor performance of single individuals. We found similar results also in our previous study.[33] The main difference with the cohort included in the present study is related to the long duration of the disease of our patients and to the associated complications, which may impair the clinical evaluation. Moreover, to our knowledge, longitudinal data regarding SMN level variations with age are not available.

We also found that SMN protein levels were unrelated to baseline clinical characteristics and SMN mRNA levels, except for a weak correlation with the *SMN2*-fl/*SMN*-del7 ratio, whose biological significance remains unclear. Lack of correlation between SMN protein levels and motor performance was also found in the study of Crawford *et al*[39] and remains unexplained. It is possible that SMN protein levels do not reflect those found in target tissues of the disease, or that the ELISA method we used requires optimization. It is

noteworthy that stabilization buffers are not available for protein samples. On the other hand, for RNA extraction, these buffers allow to preserve samples for relatively long time and provide a 'snapshot' of gene expression at the time of sampling. As time between blood sampling and protein extraction (in the context of a multicenter clinical trial) varied considerably, levels of SMN protein could be affected by different variables, such as cell death, sample preservation, higher extractability of SMN protein over time, or post-translational modifications. Indeed, putative loss or increase in SMN protein levels hypothetically related to the factors above cannot be ruled out.

Our finding of strong correlations between several aspects of motor performance and lean body mass is potentially important and suggests that lean body mass, as measured by DXA, might be worth further investigation as an outcome measure in clinical trials on potential therapeutic agents in SMA. On the other side, DXA is not easily feasible in patients with severe contractures, the longitudinal variation of lean body mass in relation to age and disease course is at present unavailable, and the time required to observe a lean body mass increase in response to a potentially effective intervention is also unknown.

To conclude, the results of our study suggest that, in adults with type III SMA, *SMN2* copy number, SMN2-del7 transcripts, and SMN protein levels in blood cells are not suitable as markers of phenotypic severity and hence as indicators of response to treatment. *SMN2*-fl transcript levels appear potentially more useful, as they correlate satisfactorily with motor performance in ambulant patients. Importantly we found that lean body mass shows promise as marker of disease severity and possibly also response to treatment. These findings require verification in larger series patients, of wider range of disease severity and age range (including children). Finally, our data suggest that if not taken into account, the confounding effect of disease duration may impair the identification of potential SMA biomarkers.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

The present study is dedicated to the memory of Christina Brahe. We are grateful to the SMA Foundation for kindly providing SMN ELISA kits, and to Dr Dione Kobayashi, Professor Eugenio Mercuri, and Dr Enrico Bertini for critical comments on the manuscript. We are extremely grateful to the patients and their families. The study was funded by the Agenzia Italiana del Farmaco (AIFA). Carla Angelozzi has been granted by ASAMSI.

DISCLAIMER

FDT and LM had full access to all study data and take responsibility for the integrity of the data and the accuracy of the analyses.

1 Pearn J: Classification of spinal muscular atrophies. *Lancet* 1980; **1**: 919–922.
2 Munsat TL, Davies KE: International SMA consortium meeting (26–28 June 1992, Bonn, Germany). *Neuromuscul Disord* 1992; **2**: 423–428.
3 Zerres K, Rudnik-Schöneborn S, Forrest E *et al*: A collaborative study on the natural history of childhood and juvenile onset proximal spinal muscular atrophy (type II and III SMA): 569 patients. *J Neurol Sci* 1997; **146**: 67–72.
4 Lefebvre S, Bürglen L, Reboullet S *et al*: Identification and characterization of a spinal muscular atrophy-determining gene. *Cell* 1995; **80**: 155–165.
5 Wirth B: An update of the mutation spectrum of the survival motor neuron gene (SMN1) in autosomal recessive spinal muscular atrophy (SMA). *Hum Mut* 2000; **15**: 228–237.
6 Frugier T, Tiziano FD, Cifuentes-Diaz C *et al*: Nuclear targeting defect of SMN lacking the C-terminus in a mouse model of spinal muscular atrophy. *Hum Mol Genet* 2000; **9**: 849–858.
7 Lefebvre S, Burlet P, Liu Q *et al*: Correlation between severity and SMN protein level in spinal muscular atrophy. *Nat Genet* 1997; **16**: 265–269.
8 Coovert DD, Le TT, McAndrew PE *et al*: The survival motor neuron protein in spinal muscular atrophy. *Hum Mol Genet* 1997; **6**: 1205–1214.
9 Patrizi AL, Tiziano F, Zappata S *et al*: SMN protein analysis in fibroblast, amniocyte, and CVS cultures from spinal muscular atrophy patients and its relevance for diagnosis. *Eur J Hum Genet* 1999; **7**: 301–309.
10 Feldkötter M, Schwarzer V, Wirth R *et al*: Quantitative analysis of SMN1 and SMN2 based on real-time LightCycler PCR: fast and highly reliable carrier testing and prediction of severity of spinal muscular atrophy. *Am J Hum Genet* 2002; **70**: 358–368.
11 Tiziano FD, Bertini E, Messina S *et al*: The Hammersmith functional score correlates with the SMN2 copy number: a multicentric study. *Neuromuscul Disord* 2007; **17**: 400–403.
12 Wirth B, Brichta L, Schrank B *et al*: Mildly affected patients with spinal muscular atrophy are partially protected by an increased SMN2 copy number. *Hum Genet* 2006; **119**: 422–428.
13 Brichta L, Hofmann Y, Hahnen E *et al*: Valproic acid increases the SMN2 protein level: a well-known drug as a potential therapy for spinal muscular atrophy. *Hum Mol Genet* 2003; **12**: 2481–2489.
14 Andreassi C, Angelozzi C, Tiziano FD *et al*: Phenylbutyrate increases SMN expression *in vitro*: relevance for treatment of spinal muscular atrophy. *Eur J Hum Genet* 2004; **12**: 59–65.
15 Brahe C, Vitali T, Tiziano FD *et al*: Phenylbutyrate increases SMN gene expression in spinal muscular atrophy patients. *Eur J Hum Genet* 2005; **13**: 256–259.
16 Jarecki J, Chen X, Bernardino A *et al*: Diverse small-molecule modulators of SMN expression found by high-throughput compound screening: early leads towards a therapeutic for spinal muscular atrophy. *Hum Mol Genet* 2005; **14**: 2003–2018.
17 Angelozzi C, Borgo F, Tiziano FD *et al*: Salbutamol increases SMN mRNA and protein levels in spinal muscular atrophy cells. *J Med Genet* 2008; **45**: 29–31.
18 Pane M, Staccioli S, Messina S *et al*: Daily salbutamol in young patients with SMA type II. *Neuromuscul Disord* 2008; **18**: 536–540.
19 Mercuri E, Bertini E, Messina S *et al*: Randomized, double-blind, placebo-controlled trial of phenylbutyrate in spinal muscular atrophy. *Neurology* 2007; **68**: 51–55.
20 Tiziano FD, Lomastro R, Pinto AM *et al*: Salbutamol increases SMN transcript levels in leukocytes of spinal muscular atrophy patients: relevance for clinical trial design. *J Med Genet* 2010; **47**: 856–858.
21 Hua Y, Sahashi K, Hung G *et al*: Antisense correction of SMN2 splicing in the CNS rescues necrosis in a type III SMA mouse model. *Genes Dev* 2010; **24**: 1634–1644.
22 Singh NN, Shishimorova M, Cao LC *et al*: A short antisense oligonucleotide masking a unique intronic motif prevents skipping of a critical exon in spinal muscular atrophy. *RNA Biol* 2009; **6**: 341–350.
23 Kaufmann P, Muntoni F: Issues in SMA clinical trial design. The International Coordinating Committee (ICC) for SMA Subcommittee on SMA Clinical Trial Design. *Neuromuscul Disord* 2007; **17**: 499–505.
24 Florence JM, Pandya S, King WM *et al*: Intrarater reliability of manual muscle test (Medical Research Council scale) grades in Duchenne's muscular dystrophy. *Phys Ther* 1992; **72**: 115–122.
25 Merlini L, Mazzone ES, Solari A *et al*: Reliability of hand-held dynamometry in spinal muscular atrophy. *Muscle Nerve* 2002; **26**: 64–70.
26 Mercuri E, Mayhew A, Muntoni F *et al*: Towards harmonisation of outcome measures for DMD and SMA within TREAT-NMD; Report of three expert workshops: TREAT-NMD/ENMC workshop on outcome measures, 12th–13th May 2007, Naarden, The Netherlands; TREAT-NMD workshop on outcome measures in experimental trials for DMD, 30th June–1st July 2007, Naarden, The Netherlands; Conjoint Institute of Myology TREAT-NMD Meeting on physical activity monitoring in neuromuscular disorders, 11th July 2007, Paris, France. *Neuromuscul Disord* 2008; **18**: 894–903.
27 Takeuchi Y, Katsuno M, Banno H *et al*: Walking capacity evaluated by the 6-minute walk test in spinal and bulbar muscular atrophy. *Muscle Nerve* 2008; **38**: 964–971.
28 Montes J, McDermott MP, Martens WB *et al*: Muscle Study Group and the Pediatric Neuromuscular Clinical Research Network. Six-Minute Walk Test demonstrates motor fatigue in spinal muscular atrophy. *Neurology* 2010; **74**: 833–838.
29 Khatri IA, Chaudhry US, Seikaly MG *et al*: Low bone mineral density in spinal muscular atrophy. *J Clin Neuromuscul Dis* 2008; **10**: 11–17.
30 Kinali M, Banks LM, Mercuri E *et al*: Bone mineral density in a paediatric spinal muscular atrophy population. *Neuropediatrics* 2004; **35**: 325–328.
31 van der Steege G, Grootscholten PM, van der Vlies P *et al*: PCR-based DNA test to confirm clinical diagnosis of autosomal recessive spinal muscular atrophy. *Lancet* 1995; **345**: 985–986.
32 Prior TW, Krainer AR, Hua Y *et al*: A positive modifier of spinal muscular atrophy in the SMN2 gene. *Am J Hum Genet* 2009; **85**: 408–413.
33 Tiziano FD, Pinto AM, Fiori S *et al*: SMN transcript levels in leukocytes of SMA patients determined by absolute real time PCR. *Eur J Hum Genet* 2010; **18**: 52–58.
34 Sossi V, Giuli A, Vitali T *et al*: Premature termination mutations in exon 3 of the SMN1 gene are associated with exon skipping and a relatively mild SMA phenotype. *Eur J Hum Genet* 2001; **9**: 113–120.
35 Hahnen E, Schonling J, Raschke H *et al*: Missense mutations in exon 6 of the survival motor neuron gene in patients with spinal muscular atrophy. *Hum Mol Genet* 1997; **6**: 821–825.
36 Sproule DM, Kaufmann P: Therapeutic developments in spinal muscular atrophy *Ther Adv Neurol Disord* 2010; **3**: 173–185.

37 Glanzman AM, O'Hagen JM, McDermott MP *et al*: Pediatric Neuromuscular Clinical Research Network for Spinal Muscular Atrophy (PNCR); Muscle Study Group (MSG). Validation of the Expanded Hammersmith Functional Motor Scale in Spinal Muscular Atrophy Type II and III. *J Child Neurol* 2011; **26**: 1499–1507.
38 Vezain M, Saugier-Veber P, Goina E *et al*: A rare SMN2 variant in a previously unrecognized composite splicing regulatory element induces exon 7 inclusion and reduces the clinical severity of spinal muscular atrophy. *Hum Mutat* 2010; **31**: E1110–E1125.
39 Finkel RS, Crawford TO, Swoboda KJ *et al*: Pilot Study of Biomarkers for Spinal Muscular Atrophy Trial Group. Candidate proteins, metabolites and transcripts in the Biomarkers for Spinal Muscular Atrophy (BforSMA) clinical study. *PLoS One* 2012; **7**: e35462.
40 Crawford TO, Paushkin SV, Kobayashi DT *et al*: Pilot Study of Biomarkers for Spinal Muscular Atrophy Trial Group. Evaluation of SMN protein, transcript, and copy number in the biomarkers for spinal muscular atrophy (BforSMA) clinical study. *PLoS One* 2012; **7**: e33572.

Supplementary Information accompanies the paper on European Journal of Human Genetics website (http://www.nature.com/ejhg)