Importance of *SPP1* genotype as a covariate in clinical trials in Duchenne muscular dystrophy

Luca Bello, MD
Luisa Piva, MS
Andrea Barp, MD
Antonella Taglia, MD
Esther Picillo, PhD
Gessica Vasco, MD
Marika Pane, MD
Stefano C. Previtali, MD, PhD
Yvan Torrente, MD
Elisabetta Gazzerro, MD
Maria Chiara Motta, MD
Gaetano S. Grieco, MS
Sara Napolitano, MD
Francesca Magri, MD
Adele D'Amico, MD
Guja Astrea, PhD
Sonia Messina, MD
Maria Sframeli, MD
Gian Luca Vita, MD
Patrizia Boffi, MD
Tiziana Mongini, MD
Alessandra Ferlini, MD
Francesca Gualandi, PhD
Gianni Soraru', MD, PhD
Mario Ermani, MD
Giuseppe Vita, MD
Roberta Battini, MD
Enrico Bertini, MD
Giacomo P. Comi, MD
Angela Berardinelli, MD
Carlo Minetti, MD
Claudio Bruno, MD
Eugenio Mercuri, MD
Luisa Politano, MD
Corrado Angelini, MD
Eric P. Hoffman, PhD
Elena Pegoraro, MD, PhD

Correspondence & reprint requests to Dr. Pegoraro: elena.pegoraro@unipd.it

ABSTRACT

Objective: To test the effect of the single nucleotide polymorphism −66 T>G (rs28357094) in the osteopontin gene (*SPP1*) on functional measures over 12 months in Duchenne muscular dystrophy (DMD).

Methods: This study was conducted on a cohort of ambulatory patients with DMD from a network of Italian neuromuscular centers, evaluated longitudinally with the North Star Ambulatory Assessment (NSAA) and the 6-Minute Walk Test (6MWT) at study entry and after 12 months. Genotype at rs28357094 was determined after completion of the clinical evaluations. Patients were stratified in 2 groups according to a dominant model (TT homozygotes vs TG heterozygotes and GG homozygotes) and clinical data were retrospectively compared between groups.

Results: Eighty patients were selected (age 4.1–19.3 years; mean 8.3 ± 2.7 SD). There were no differences in age or steroid treatment between the 2 subgroups. Paired *t* test showed a significant difference in both NSAA ($p = 0.013$) and 6MWT ($p = 0.03$) between baseline and follow-up after 12 months in patients with DMD carrying the G allele. The difference was not significant in the T subgroup. The analysis of covariance using age and baseline values as covariate and *SPP1* genotype as fixed effect showed that these parameters are significantly correlated with the 12-month values.

Conclusions: These data provide evidence of the role of *SPP1* genotype as a disease modifier in DMD and support its relevance in the selection of homogeneous groups of patients for future clinical trials. ***Neurology*® 2012;79:159–162**

GLOSSARY

6MWT = 6-Minute Walk Test; **ANCOVA** = analysis of covariance; **DMD** = Duchenne muscular dystrophy; **NSAA** = North Star Ambulatory Assessment.

Osteopontin (secreted phosphoprotein, SPP1), a 35–60 kDa secreted glycoprotein, functions as a pleiotropic cytokine in several pathologic and reparative processes.[1] Lately, evidence has emerged that in the *mdx* mouse model SPP1 modulates muscle inflammation and regeneration,[2] and that *SPP1* genetic ablation ("double mutant" mouse) induces a milder phenotype.[3]

We recently found that the polymorphic G genotype at position −66 in the *SPP1* promoter (rs28357094) is associated with earlier loss of ambulation and more rapid weakness progression in Duchenne muscular dystrophy (DMD).[4] This association needs further confirmation and assessment of magnitude and statistical power.[5]

From the Neuromuscular Center (L.B., L. Piva, A.B., G.S., M.E., C.A., E. Pegoraro), Department of Neurosciences, University of Padova, Padova; Department of Experimental Medicine, Cardiomyology and Medical Genetics (A.T., E. Picillo, L. Politano), Second University of Napoli, Naples; Department of Paediatric Neurology (G.V., M.P., E.M.), Catholic University, Rome; Department of Neurology and INSPE (S.C.P., S.N.), San Raffaele Scientific Institute, Milan; Dino Ferrari Centre (Y.T., F.M., G.P.C.), Department of Neurological Sciences, University of Milano, I.R.C.C.S. Foundation Cà Granda, Ospedale Maggiore Policlinico, Milan; Neuromuscular Disease Unit (E.G., C.M., C.B.), G. Gaslini Institute, Genova; Child Neurology and Psychiatry Unit (M.C.M., A.B.), IRCCS "C. Mondino" Foundation, University of Pavia, Pavia; Laboratory of Experimental Neurobiology (G.S.G.), IRCCS, National Neurological Institute "C. Mondino," Pavia; Department of Laboratory Medicine (A.D., E.B.), Unit of Molecular Medicine, Bambino Gesù Hospital, Rome; Department of Developmental Neuroscience (G.A., R.B.), Stella Maris Institute, University of Pisa, Pisa; Department of Neurosciences, Psychiatry and Anaesthesiology (S.M., M.S., G.L.V., G.V.), University of Messina, Messina; Neuromuscular Center (P.B., T.M.), S.G. Battista Hospital, University of Torino, Torino; Department of Experimental and Diagnostic Medicine (A.F., F.G.), University of Ferrara, Italy; and Research Center for Genetic Medicine (E.P.H.), Children's National Medical Center, Washington, DC.

Study funding: Supported by grants from NIH 1U54HD053177 (Wellstone Muscular Dystrophy Center), Italian Telethon UILDM grant (GUP07009), the Eurobiobank network (QLRT2001-027769 to C.A.), Telethon Bank (GTF05003), and NHGMBB (Second University of Napoli), member of Eurobiobank and Telethon Network of Genetic Biobanks.

Go to Neurology.org for full disclosures. Disclosures deemed relevant by the authors, if any, are provided at the end of this article.

In the present study, we test the hypothesis of the influence of the G genotype on disease progression on a DMD cohort from 11 Italian centers (the Italian "North Star" Group). This group has described various aspects of DMD functional measures, such as the inter-operator reliability of the North Star Ambulatory Assessment (NSAA),[6] its correlation with the 6-Minute Walk Test (6MWT),[7] and the longitudinal evaluation of these measures over 12 months.[8]

METHODS Inclusion criteria were as previously described[8]: molecularly confirmed DMD diagnosis, ability to walk 75 meters, absence of severe/moderate learning or behavioral problems, and availability of a DNA sample.

Clinical evaluation with NSAA (score 0–34) and 6MWT was performed at baseline (T0) and after 12 months if still ambulatory (T12), as previously described.[6,9]

Genotype at rs28357094 was determined by amplification refractory mutation system,[4] after clinical evaluations were completed (evaluators blind to genotype).

Patients were divided into 2 subgroups (dominant model): wild-type homozygotes vs heterozygote/homozygote carriers of rs28357094 (TT vs TG/GG). A recessive model (TT vs TG vs GG) could not be adopted due to numerosity issues.

Age differences and functional changes over 12 months were compared between subgroups with a *t* test for independent groups. Distribution by steroid treatment was compared with a χ^2 test. Data normality was tested using the Kolmogorov-Smirnov test. Paired *t* test was used to compare functional measures at T0 and T12 within each subgroup. Linear correlations were tested using Pearson *r*. Analysis of covariance (ANCOVA) was used to determine the cumulative effect of T0 functional measures, age (covariates), and genotype (fixed effect) on functional outcome at T12. A parallelism test was used to compare linear regression slopes of functional measure changes relative to age between subgroups. All measures are expressed as mean ± SD.

Standard protocol approvals, registrations, and patient consents. Ethical standards committees on human experimentation granted their approval in each institution, and informed consent for research was obtained from all participating patients or guardians of patients.

RESULTS Eighty patients were selected. Seventy-seven were previously included in the multicentric natural history study[8] and 3 of these were also included in the original report of *SPP1* as a genetic modifier of DMD.[4] Mean age at T0 was 8.3 ± 2.7 years (range 4.1–19.3).

Seventy-five patients (94%) were treated with steroids; 49 (61%) received a continuous regimen (0.75 mg/kg prednisolone–0.9 mg/kg deflazacort daily or equivalent[8]), and 26 (33%) an alternate regimen (10 days on-off, alternate days-weeks). Five patients (6%) received no steroids. No patients were treated with Losartan.

rs28357094 genotypes. A total of 57 patients were TT homozygotes (71%, T subgroup), 18 TG heterozygotes (23%), 5 GG homozygotes (6%). TG/GG patients were clustered in a subgroup of 23 patients (29%, G subgroup).

In the T subgroup mean age was 8.5 ± 2.9 years (range 4.5–19.3). Fifty-four of 57 patients (95%) received steroid treatment, 36/57 (63%) with a continuous regimen and 18/57 (32%) with an alternate regimen; 3/57 (5%) were not treated. In the G subgroup mean age was 8.0 ± 2.0 years (range 4.5–11.8). Twenty-one of 23 patients (91%) received steroid treatment, 13/23 (56%) with a continuous regimen and 8/23 (35%) with an alternate regimen; 2/23 (9%) were not treated. Age (*t* test $p = 0.48$) or steroid treatment (χ^2 test $p = 0.79$) did not differ significantly between the subgroups. No patients lost ambulation during follow-up, and all were able to complete NSAA and 6MWT at T12.

The Kolmogorov-Smirnov test showed that both NSAA and 6MWT values were normally distributed.

Mean NSAA T0–T12 scores were 23.3 ± 7.2 and 22.2 ± 8.2 (paired *t* test $p = 0.08$) in the T subgroup, and 24.6 ± 6.3 and 21.5 ± 7.6 (paired *t* test $p = 0.013$) in the G subgroup. Mean T0–T12 changes in NSAA score were −1.0 ± 4.3 in the T subgroup and −3.1 ± 5.5 in the G subroup ($p = 0.08$) (table 1; figure).

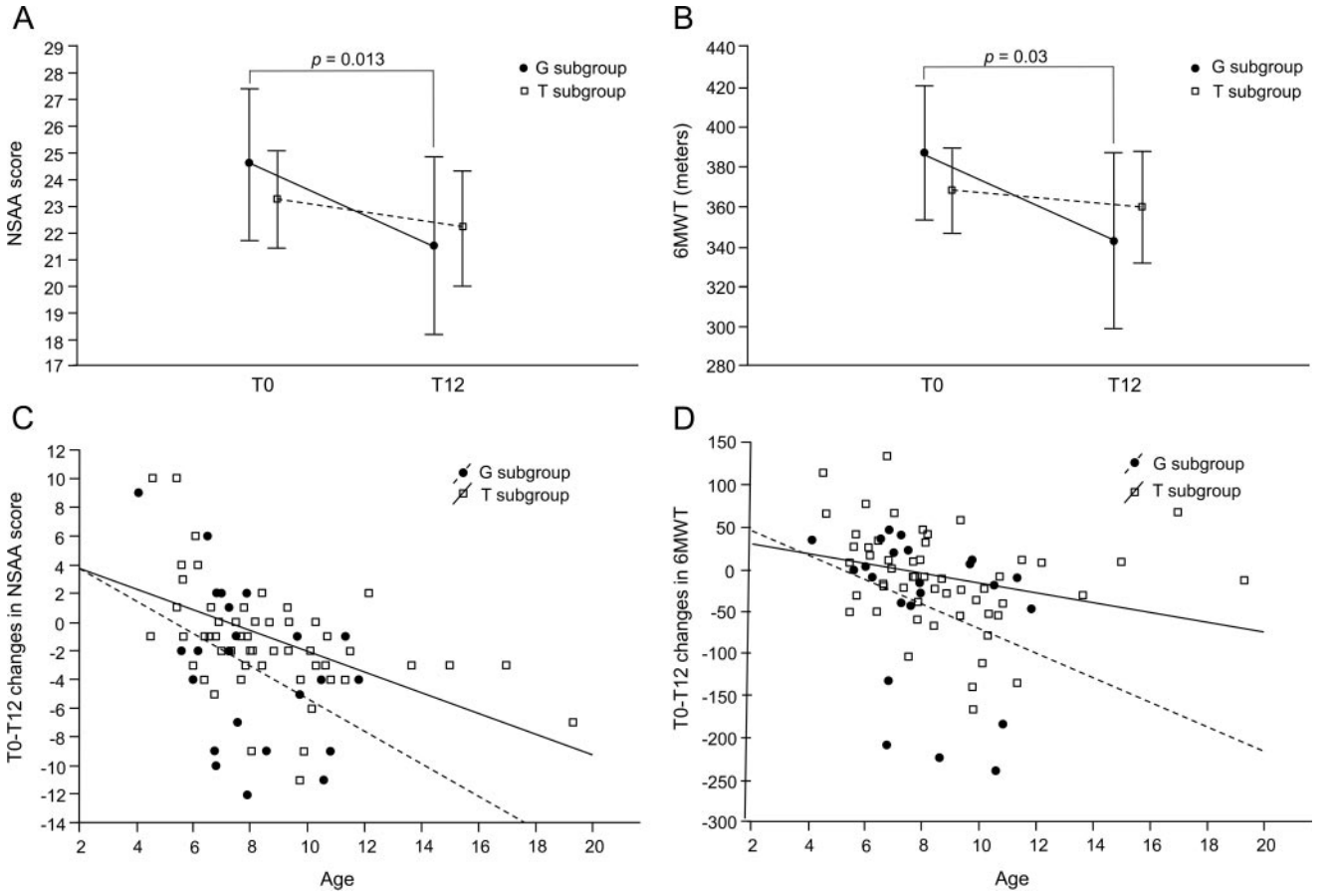
Mean 6MWT T0–T12 distances were 368 ± 86 and 360 ± 98 meters (paired *t* test $p = 0.31$) in the T subgroup, and 387 ± 67 and 343 ± 124 meters (paired *t* test $p = 0.03$) in the G subgroup. Mean 6MWT T0–T12 changes were −8 ± 61 meters in the T subgroup and −44 ± 89 meters in the G subroup ($p = 0.04$) (table 1; figure).

Table 1 NSAA and 6MWT at T0 and T12, mean ± SD

	NSAA score			6MWT, m		
	Baseline	12 mo	Difference	Baseline	12 mo	Difference
TT	23.3 ± 7.2	22.2 ± 8.2	−1.0 ± 4.3	368 ± 86	360 ± 98	−8 ± 61
TG/GG	24.6 ± 6.3	21.5 ± 7.6	−3.1 ± 5.5	387 ± 67	343 ± 124	−44 ± 89

Abbreviations: 6MWT = 6-Minute Walk Test; NSAA = North Star Ambulatory Assessment.

Figure Patients with Duchenne muscular dystrophy carrying the G allele at rs28357094 deteriorated faster than patients carrying the T allele



The mean values for North Star Ambulatory Assessment (NSAA) (A) and 6-Minute Walk Test (6MWT) (B) at T0 and T12 in the T and G subgroups are shown. T0–T12 mean differences in NSAA and 6MWT are significant only in the G subgroup of patients. Vertical bars represent 95% confidence intervals. A scatterplot of T0–T12 changes in NSAA (C) and 6MWT (D) in the T and G subgroups are shown. The superimposed linear regression shows a steeper decline in the G subgroup (NSAA slope estimates: T −0.17, G −1.12, SE: 0.13, $p < 0.001$; 6MWT slope estimates: T −5.76, G −14.44, SE 2.02, $p < 0.001$).

Both NSAA and 6MWT values at T12 were linearly correlated with T0 values ($r = 0.81$ for NSAA and $r = 0.73$ for 6MWT, $p < 0.0001$). Moreover, in the T subgroup both NSAA and 6MWT changes over 12 months were inversely correlated with age ($r = -0.48$, $p < 0.0001$; $r = -0.27$, $p = 0.04$), while in the G subgroup the correlation was only valid for NSAA ($r = -0.42$, $p = 0.048$), and not for 6MWT. Parallelism tests of linear regressions between functional changes and age showed a significantly steeper decrease in the G subgroup for both NSAA ($p = 0.001$) and 6MWT ($p = 0.001$) (figure).

ANCOVA analysis showed that age and T0 functional measures strongly determined T12 functional outcome, which was also significantly and independently influenced by genotype (NSAA $p = 0.030$, 6MWT $p = 0.029$, table 2).

DISCUSSION The discovery of *SPP1* as a modifier gene in DMD[4] has relevant clinical implications, but as noted,[5] needs to be confirmed in larger populations. The natural history data collected by the Italian "North Star" network[8] offered an opportunity to test rs28357094 on an independent cohort (except an overlap of 3 patients with our previous report[4]) with validated, clinically meaningful functional measures, homogenous treatment standards, and longitudinal data collection.

As hypothesized based on previous findings,[4] patients in the G subgroup showed a significant decline of NSAA and 6MWT at T12 (despite a slightly, non-significantly better performance at T0), while the T subgroup did not (figure).

We argue that the slight advantage of G patients at baseline may be due to their greater probability of losing ambulation earlier, thus being excluded from the study. As a consequence, relatively older patients with poor baseline performance, but retaining independent ambulation, are included in the T subgroup. This is supported by the narrower age range in the G subgroup: all patients over 12 years carried the TT genotype (figure).

Table 2 Correlation coefficients and analysis of covariance for factors influencing functional outcome

	NSAA at T12			6MWT at T12		
	Correlation coefficients			Correlation coefficients		
	r	*b* (SE)	ANCOVA	*r*	*b* (SE)	ANCOVA
Age	−0.49	−1.44 (0.29)	$p < 10^{-5}$	−0.38	−14.86 (4.12)	$p = 0.013$
Corresponding baseline (T0) value	0.81	0.93 (0.08)	$p < 10^{-6}$	0.73	0.97 (0.10)	$p < 10^{-6}$
rs28357094 genotype (TT vs TG/GG)	NA	NA	$p = 0.030$	NA	NA	$p = 0.029$

Abbreviations: 6MWT = 6-Minute Walk Test; ANCOVA = analysis of covariance; NA = not applicable (in dichotomous variables); NSAA = North Star Ambulatory Assessment.

The need to consider the influence of age and functional performance at baseline together with *SPP1* genotype prompted us to develop a model which could test the effect of different simultaneously acting factors in predicting functional outcome after 12 months. ANCOVA analysis showed that baseline measures and age are, as expected, highly predictive of functional measures at T12, but *SPP1* genotype retains an independent role, significantly influencing the outcome. We could not implement steroid treatment as a covariate in this model because of the very few nontreated patients, who were however equally distributed between the subgroups.

It is well-documented that a high SD is intrinsic to several DMD functional measures,[7,9] and the possible drawbacks of such variability on assessing new treatments are emerging, hence the need for criteria for selecting homogeneous patient populations with a predictable clinical course.

This study adds to the evidence that rs28357094 genotype influences functional outcome in DMD. This may be relevant for the stratification of functionally homogeneous patients when planning clinical trials. Deeper insights into the underlying molecular mechanisms will help our understanding of the biological role of SPP1 in DMD.

AUTHOR CONTRIBUTIONS

Writing team: L.B., E.P.H., E.P. All the others corrected and approved the manuscript. Patient evaluation: L.B., A.B., A.T., G.V., M.P., S.C.P., Y.T., E.G., M.C.M., S.N., F.M., A.D., G.A., S.M., M.S., G.L.V., P.B., T.M., G.S., G.V., R.B., E.B., G.P.C., A.B., C.M., C.B., E.M., L.P., C.A. Genetic screening: L.B., L.P., A.F., F.G. Statistical analysis: M.E. Data analysis: L.B., E.B., E.M., E.P.H., E.P.

DISCLOSURE

L. Bello, L. Piva, A. Barp, A. Taglia, E. Picillo, G. Vasco, M. Pane, S. Previtali, Y. Torrente, E. Gazzero, M.C. Motta, G. Grieco, S. Napolitano, F. Magri, A. D'Amico, G. Astrea, S. Messina, M. Sframeli, G.L. Vita, and P. Boffi report no disclosures. T. Mongini has served on a scientific advisory board for Telethon Italy; has received funding for travel from Genzyme Corporation; and has received research support from AIFA (Italian Government Drug Agency) and Telethon Italy. A. Ferlini, F. Gualandi, G. Soraru', and M. Ermani report no disclosures. G. Vita serves as an Associate Editor for *Neurological Sciences*. R. Battini reports no disclosures. E. Bertini receives research support from Telethon Italy and SMA Europe. G. Comi receives research support from Telethon Italy and SMA Europe. A. Berardinelli, C. Minetti, and C. Bruno report no disclosures. E. Mercuri serves on scientific advisory boards for Aceleron Pharma, PTC Therapeutics, Inc./Genzyme Corporations; serves on the editorial boards of *Neuromuscular Disorders*, *Annals of Neurology*, *Developmental Medicine & Child Neurology*, and Neuropediatrics; and receives research support from Telethon Italy and SMA Europe. L. Politano reports no disclosures. C. Angelini serves on the editorial board of *Neurology®*, *Neuromuscular Disorders*, *Journal of Neurological Sciences*, *Current Opinion of Neurology*, and *Therapeutic Advances in Neurological Disorders*. E. Hoffman reports no disclosures. E. Pegoraro serves on a scientific advisory board for BioMarin Pharmaceutical Inc.; has received funding from MEDA Pharmaceuticals Inc.; and receives research support from Wellstone Grant 8568-01-01 and Italian Telethon. **Go to Neurology.org for full disclosures.**

Received October 14, 2011. Accepted in final form February 29, 2012.

REFERENCES

1. Scatena M, Liaw L, Giachelli CM. Osteopontin: a multifunctional molecule regulating chronic inflammation and vascular disease. Arterioscl Thromb Vasc Biol 2007;27:2302–2309.
2. Uaesoontrachoon K, Yoo HJ, Tudor EM, Pike RN, Mackie EJ, Pagel CN. Osteopontin and skeletal muscle myoblasts: association with muscle regeneration and regulation of myoblast function in vitro. Int J Biochem Cell Biol 2008;40:2303–2314.
3. Vetrone SA, Montecino-Rodriguez E, Kudryashova E, et al. Osteopontin promotes fibrosis in dystrophic mouse muscle by modulating immune cell subsets and intramuscular TGF-beta. J Clin Invest 2009;119:1583–1594.
4. Pegoraro E, Hoffman EP, Piva L, et al. SPP1 genotype is a determinant of disease severity in Duchenne muscular dystrophy. Neurology 2011;76:219–226.
5. Nelson SF, Griggs RC. Predicting the severity of Duchenne muscular dystrophy: implications for treatment. Neurology 2011;76:208–209.
6. Mazzone ES, Messina S, Vasco G, et al. Reliability of the North Star Ambulatory Assessment in a multicentric setting. Neuromuscul Disord 2009;19:458–461.
7. Mazzone E, Martinelli D, Berardinelli A, et al. North Star Ambulatory Assessment, 6-minute walk test and timed items in ambulant boys with Duchenne muscular dystrophy. Neuromuscul Disord 2010;20:712–716.
8. Mazzone E, Vasco G, Sormani MP, et al. Functional changes in Duchenne muscular dystrophy: a 12-month longitudinal cohort study. Neurology 2011;77:250–256.
9. McDonald CM, Henricson EK, Han JJ, et al. The 6-minute walk test as a new outcome measure in Duchenne muscular dystrophy. Muscle Nerve 2010;41:500–510.