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RESEARCH ARTICLE

Genetic modifiers of respiratory function in Duchenne muscular dystrophy

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Abstract

Objective: Respiratory insufficiency is a major complication of Duchenne muscular dystrophy (DMD). Its progression shows considerable interindividual variability, which has been less thoroughly characterized and understood than in skeletal muscle. We collected pulmonary function testing (PFT) data from a large retrospective cohort followed at Centers collaborating in the Italian DMD Network. Furthermore, we analyzed PFT associations with different *DMD* mutation types, and with genetic variants in *SPP1*, *LTBP4*, *CD40*, and *ACTN3*, known to modify skeletal muscle weakness in DMD. Genetic association findings were independently validated in the Cooperative International Neuromuscular Research Group Duchenne Natural History Study (CINRG-DNHS). **Methods and Results**: Generalized estimating equation analysis of 1852 PFTs from 327 Italian DMD patients, over an average follow-up time of 4.5 years,

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estimated that forced vital capacity (FVC) declined yearly by −4.2%, forced expiratory volume in 1 sec by −5.0%, and peak expiratory flow (PEF) by −2.9%. Glucocorticoid (GC) treatment was associated with higher values of all PFT measures (approximately + 15% across disease stages). Mutations situated 3' of *DMD* intron 44, thus predicted to alter the expression of short dystrophin isoforms, were associated with lower (approximately −6%) PFT values, a finding independently validated in the CINRG-DNHS. Deletions amenable to skipping of exon 51 and 53 were independently associated with worse PFT outcomes. A meta-analysis of the two cohorts identified detrimental effects of *SPP1* rs28357094 and *CD40* rs1883832 minor alleles on both FVC and PEF. **Interpretation**: These findings support GC efficacy in delaying respiratory insufficiency, and will be useful for the design and interpretation of clinical trials focused on respiratory endpoints in DMD.

Introduction

Duchenne muscular dystrophy (DMD) is caused by *DMD* mutations leading to complete or near-complete dystrophin defects in skeletal muscle,[1] resulting in its fibro-fatty degeneration.[2] Skeletal muscle weakness is the most distinctive clinical feature; however, respiratory muscle weakness impacts life quality and expectancy even more heavily.[3] Inspiratory/expiratory dysfunction appears around the age of 10, and progresses over the years,[4,5] eventually leading to nocturnal ventilatory insufficiency requiring noninvasive ventilatory assistance (NIV), reduced airway clearance, and infections. Eventually, daytime or continuous NIV becomes necessary, and sometimes tracheostomy.[3]

As novel treatments emerge for DMD, a quantitative description of its respiratory "natural history" is paramount for the design and interpretation of trials including respiratory endpoints. Current standards of care recommend pulmonary function tests (PFTs) yearly in ambulatory patients, and twice yearly in nonambulatory, as forced vital capacity (FVC) below 1 L or below 50% of the predicted value by age and height[6] indicates a risk of nocturnal hypoventilation. It has been shown in different DMD cohorts that percent-predicted FVC and peak expiratory flow (PEF) decrease gradually and colinearly, especially during intermediate disease stages (i.e. approximately before 10 or after the 20 years of age; or above 80% and below 30% of predicted FVC values).[4,5,7]

As seen for skeletal muscle, a striking interindividual variability is observed in PFT deterioration.[7] Other than glucocorticoid (GC) treatment and standards of care,[6,8,9] several genetic factors have been called into play as modifiers of DMD severity. These include "cis-acting" modifiers, such as specific *DMD* mutations which may lead to the expression of minimal, but clinically relevant quantities of dystrophin[10–13]; and "trans-acting" modifiers, that is, polymorphisms in genes different from *DMD*, that may influence the pathological consequences of dystrophin deficiency.[14–17] As specific *DMD* mutations are now amenable to targeted molecular treatments,[18] such as "exon skipping" antisense oligonucleotides, or small molecules promoting stop codon readthrough, delineating "natural history" trajectories in corresponding subgroups are useful.

Here we aimed at describing the natural history of, and effect of GC treatment on respiratory insufficiency, in a large retrospective cohort followed by the Italian DMD Network. Furthermore, we analyzed the respiratory effects of several genetic variables, previously associated with differential DMD expressivity. As replication of genetic association findings in independent cohorts is crucial,[19] we validated findings in the Cooperative International Neuromuscular Research Group Duchenne Natural History Study (CINRG-DNHS) cohort.

Methods

Cohorts

Collaborating Italian Centers collected retrospective PFT data from clinical records of patients followed from January 1990 to May 2018 (henceforth: "Italian cohort"), with the aim of describing respiratory natural history and GC effects. These have been described elsewhere for the CINRG-DNHS cohort,[6] which was used here to validate genetic associations.

Ethics statement

All participants or their parents/guardians provided informed consent to study procedures, which were carried out in accordance with the Declaration of Helsinki and approved by Ethics Committees/Institutional Review Boards in the participating Institutions.

Inclusion criteria

We selected Italian cohort patients with frameshifting or nonsense *DMD* mutations; or absent or <3% dystrophin detected by immunohistochemistry (except revertant fibers) or immunoblot; or any *DMD* mutation, plus absent dystrophin as above and/or overt muscle weakness by 5 years, or loss of independent ambulation (LoA) by 13 years without GC treatment, or 16 with GCs. CINRG-DNHS criteria were similar.[20]

PFTs

PFTs were performed according to international guidelines,[21] and available measurements of FVC, FEV1 (forced expiratory volume in 1 sec), and PEF, expressed as % of predicted, were collected retrospectively. Age and GC treatment status at the time of PFTs, and age at commencement of NIV were collected when available. PFTs in the CINRG-DNHS were performed longitudinally as described.[4]

DMD genotype

Information about pathogenetic *DMD* mutations was collected when available from clinical records or genetic reports. We classified deletions based on amenability to molecular treatments, that is, skipping of exons 8, 44, 45, 51, and 53 (henceforth: "skip 8", "skip 44", etc.). Nonsense and splice mutations were also considered as separate groups. Moreover, mutations were subdivided into "proximal", that is, situated 5' of intron 44, and therefore not predicted to alter the expression of short dystrophin isoforms (Dp140, Dp116, and Dp71); and "distal", that is, involving intron 44 and/or regions 3' of it, thus disrupting these isoforms. The same criteria were adopted to classify CINRG-DNHS participants with available *DMD* mutation data, as described.[10]

Modifier genotypes

Patients with available DNA samples were genotyped by TaqMan probes at the main known DMD modifier loci: *SPP1* rs28357094,[14] *LTBP4* rs10880,[15] *CD40* rs1883832,[16] and *ACTN3* rs1815739.[17] For tests of genotype/phenotype association, we used the same inheritance models as in published reports.

Test/validation and "meta-analysis"

Forgenotype/phenotype associations, we used both a test/validation approach, that is, same statistical tests in the Italian and CINRG-DNHS cohorts; and a "meta-analysis" approach, that is, same tests in the merged Italian and CINRG datasets (including all individual data points). This is justified by the consideration that there is no existing estimate of mutation/SNP effect sizes on the respiratory phenotype, so that formal power calculations are not feasible, leaving the possibility that the test and/or validation cohorts alone may be undersized to identify relevant effects.

Statistical analysis

Quantitative variables were summarized as mean ± standard deviation and median (range), unless otherwise specified. Intervals of linear decline of PFT measures were defined by piecewise regression, using baseline data and choosing a two-break model for FVC/FEV1, and a one-break model for PEF after visual inspection of the data. Generalized Estimating Equations (GEEs), a regression model adequate for clustered longitudinal data were used to estimate effects of several covariates: age; GC treatment (on vs. off at each PFT); *DMD* mutation (tested separately: each specified mutation group vs. "other" mutations; or "distal" vs. "proximal"); and SNP genotypes (dominant, recessive, or additive as appropriate). GEEs were applied within the "linear" age range defined by piecewise regression, using the same range for test/validation, and recalculating it for meta-analysis. NIV commencement was studied with time-to-event analyses, using age as the time variable. NIV-free participants were censored. Median age at NIV was estimated with the Kaplan–Meier method. Statistical significance was set at $P < 0.01$ (Bonferroni correction for five loci: *DMD*, *SPP1*, *LTBP4*, *CD40*, *ACTN3*). No Bonferroni correction was applied to multiple PFT outcomes as these are strongly intercorrelated and reflect inspiratory/expiratory strength. Statistical analyses were performed using R v.3.5.2.

Results

Demographics

For the Italian cohort, we collected data from 1852 PFT evaluations performed by 327 DMD patients, mean age: 11.7 ± 5.3 years, from 1990 to 2018. On average, participants underwent 5.7 ± 4.5 evaluations (maximum 19), performed every 0.97 ± 0.88 years, with a follow-up time of 4.5 ± 3.9 years (maximum 19.4). At last evaluation, average age was 16.3 ± 6.6 years. Features of the CINRG-DNHS cohort have been described.[4] All patients were male.

GC treatment

During follow-up, 134 patients (41.0%) were continuously on GC treatment, while 116 (35.5%) were

continuously off. Untreated patients were older than those treated (approximately 5-year difference). GC treatment in the CINRG-DNHS cohort has been described (Table 1).[4]

DMD mutations

DMD mutations were defined in 274 (83.8%) patients in the Italian cohort. As expected, single- or multiexon deletions represented the majority of mutations (70.1%), followed by duplications (11.3%) and nonsense mutations (9.5%). The mutations were "distal" (3' of exon 44) in 168/274 participants (61.3%), and "proximal" (5' of intron 44) in 103/274 (37.6%). Three mutations (1.1%) were reported as "nonsense" or "splice site", but no nucleotide position was available, so that "distal" versus "proximal" could not be determined. Distribution by mutations in the CINRG-DNHS was similar, as described previously,[10] with 66.9% "distal" and 33.1% "proximal" mutations. The CINRG-DNHS subpopulation with available mutational and PFT data (n = 175) is also recapitulated in Table 2.

Table 2. Distribution by *DMD* mutation type.

Mutation group		Italian cohort *n* (%)		CINRG-DNHS cohort *n* (%)	
Deletions	Skip 8	4 (1.5%)	192	5 (2.9%)	138
	Skip 44	16 (5.8%)	(70.1%)	16 (9.1%)	(78.9%)
	Skip 45	19 (6.9%)		22 (12.6%)	
	Skip 51	27 (9.9%)		36 (20.6%)	
	Skip 53	25 (9.1%)		12 (6.9%)	
	Other	101 (36.9%)		47 (26.9%)	
Duplications		31 (11.3%)		12 (6.9%)	
Nonsense mutations		26 (9.5%)		16 (9.1%)	
Small FS mutations		13 (4.7%)		9 (5.1%)	
Splice site mutations		10 (3.6%)		0 (0.0%)	
Total (molecularly defined)		274 (100%)		175 (100%)	

CINRG-DNHS, Cooperative International Neuromuscular Research Group Duchenne Natural History Study; "skip 8", deletion amenable to treatment by antisense oligonucleotide promoting the skipping of exon 8; same for other exon numbers; FS, frameshifting.

Modifier genotypes

Genotyped SNPs in the Italian cohort showed expected allele frequencies in populations of European ancestry, and Hardy–Weinberg Equilibrium (HWE) was respected except for a slight violation for*ACTN3*. Genotype distributions in the CINRG-DNHS cohort have been described,[16,17,22] and did not deviate from HWE (Table 3).

Table 1. Distribution by glucocorticoid treatment and demographics of treatment subgroups.

Treatment subgroup	*n*	Mean age in years ± SD Median age in years (min–max)
Continuously off GCs	116 (35.5%)	14.6 ± 5.9 13.7 (3.6–44.5)
Continuously on GCs	134 (41.0%)	9.4 ± 3.5 8.6 (4.2–24.8)
Started GCs during FU	9 (2.8%)	6.3 ± 1.4 6.3 (3.7–8.1)
Stopped GCs during FU	26 (8.0%)	10.5 ± 3.3 9.5 (6.9–21.6)
Multiple switches	3 (0.9%)	12 ± 4.8 10.6 (8–17.3)
Unknown	39 (11.9%)	13.0 ± 5.6 13.2 (5.5–28.0)
Total	327 (100%)	11.7 ± 5.3 10.4 (3.6–44.5)

SD, standard deviation; GCs, glucocorticoids; FU, follow-up.

Ranges of linear decline

Using piecewise regression, we estimated in the Italian cohort that expiratory volumes decreased linearly from the age of 8.6 (FVC) or 8.5 (FEV1),to the age of 22.7 years; while PEF decreased beginning with the earliest PFTs, until the age of 27.1 (Fig. 1). In the "meta-analysis" cohort the boundaries of linear decrease were similar: 8.7–22.6 years for FVC, 8.8–19.0 for FEV1, 0–25.4 for PEF.

Effects of age and GCs

In the Italian cohort, the GEE model estimated the rate of yearly decline (± standard error) of FVC as −4.2 ± 0.2% of predicted, while the decline of FEV1 was −5.0 ± 0.3%, and that of PEF was −2.9 ± 0.3%. GC treatment was associated with increased FVC (4.6 ± 2.1%), FEV1 (15.5 ± 2.0%), and PEF (14.2 ± 1.9). As seen in Figure 1, GC treatment was associated with higher PFT measures beginning at a younger age, but with similar decline rates over the years. Decline rates of FVC and FEV1 in the CINRG-DNHS were similar (within 0.6%), while the decline of PEF appeared somewhat faster (−3.8 ± 0.2%) (Table 4).

DMD mutation effects

In the Italian cohort, "distal" mutations downstream of exon 44 were associated with lower FVC (−6.1 ± 2.3%,

Table 3. Allele frequencies at genotyped modifier loci.

		Italian cohort						CINRG-DNHS cohort					
		Genotype						Genotype					
Gene and SNP effect	SNP	AA	AB	BB	NA	MAF	HWE deviation	AA	AB	BB	NA	MAF	HWE deviation
SPP1 promoter	rs28357094 (T/G)	115	62	13	137	0.23	n.s.	186	72	10	9	0.17	n.s.
LTBP4 missense	rs10880 (C/T)	72	85	29	141	0.38	n.s.	106	129	29	13	0.35	n.s.
CD40 5′UTR	rs1883832 (C/T)	106	64	8	149	0.22	n.s.	150	98	19	10	0.25	n.s.
ACTN3 nonsense	rs1815739 (C/T)	39	97	30	161	0.47	$P = 0.03$	74	114	70	19	0.49	n.s.

SNP, single nucleotide polymorphism; CINRG-DNHS, Cooperative International Neuromuscular Research Group Duchenne Natural History Study; AA, major allele homozygote; AB, heterozygote; BB, minor allele homozygote; NA, undetermined genotype (DNA unavailable or insufficient); MAF, minor allele frequency; HWE, Hardy–Weinberg equilibrium; *SPP1*, Secreted PhosphoProtein 1 (osteopontin); *CD40*, Cluster of Differentiation 40 protein, also known as *TNFRSF5* (tumor necrosis factor receptor superfamily member 5); *LTBP4*, Latent Trasforming growth factor beta Binding Protein 4; *ACTN3*, actinin alpha 3.

$P = 0.008$), lower FEV1 ($-6.3 \pm 2.5\%$, $P = 0.011$), and lower PEF ($5.8 \pm 2.3\%$, $P = 0.010$) (Table 4). Validation in the CINRG cohort showed similar estimates with nominal significance for FVC and FEV1. With the meta-analysis approach, the detrimental effect of distal mutations showed *P*-values of 0.001 (FVC), 0.0094 (FEV1), and 0.013 (PEF). FVC was negatively correlated with "skip 51" and "skip 53"mutations, the latter with large effect sizes (approximately −10%) in all cohorts. "Skip 44"mutations showed nominally significant increases of FVC with the meta-analysis approach ($+7.1 \pm 3.3\%$, $P = 0.016$). "Skip 8" mutations were associated with dramatic increases of PEF ($+20.0 \pm 4.5\%$) and nominally significant increases of FVC ($+13.8 \pm 8.3\%$) and FEV1 ($+15.3 \pm 7.9\%$). Interestingly, splice site mutations were associated with higher expiratory volumes in the Italian cohort (nominally significant; no such mutations were reported in the CINRG-DNHS). FVC and PEF trajectories were associated with different *DMD* and SNP genotypes (merged meta-analysis cohort) are shown in Figure 2.

SNP effects

The dominant G genotype at rs28357094 in the *SPP1* promoter, associated with earlier LoA in DMD,[14,22,23] showed a nominally significant negative effect on FVC ($-4.5 \pm 2.5\%$) in the meta-analysis. A significant effect on PEF was observed in the CINRG ($-8.7 \pm 3.1\%$, $P = 0.005$) and meta-analysis ($-6.3 \pm 2.4\%$, $P = 0.0088$) cohorts. The additive T genotype at rs1883832 in the *CD40* 5' untranslated region, also causing earlier LoA,[16] showed a significant negative effect on FVC in the CINRG ($-6.1 \pm 2.2\%$, $P = 0.005$) and meta-analysis ($-4.8 \pm 1.7\%$, $P = 0.006$) cohorts; and nominally significant effects on FEV1 in the CINRG-DNHS, and on PEF in the meta-analysis cohort. *LTBP4* and *ACTN3* SNPs showed no relevant associations with PFTs (Table 4).

NIV

NIV was initiated in 87/318 participants with available data in the Italian cohort, and 31/276 in the CINRG-DNHS, at a median age of 23.2 and 22.2 years respectively (Table 5). The effect of GC treatment in delaying NIV was estimated at around 2 years in the Italian cohort (P = n.s.), but not validated in the CINRG-DNHS. There was no clear effect of the *DMD* mutation type on NIV commencement. A nominally significant effect was observed for *SPP1*rs28357094 with the meta-analysis approach, with a hazard ratio (HR) of 1.75 (detrimental). For *CD40* rs1883832, there were nominally significant effects in the CINRG-DNHS and meta-analysis cohorts (HR 1.71 and 1.50, $P = 0.044$ and 0.0498 respectively). Cumulative incidence plots for suggestive associations are illustrated in Figure 3.

Discussion

Estimating yearly rates of decline of PFTs is relevant for clinical management, as it allows to gauge the relative severity of individual decline trajectories, and for the design and interpretation of clinical trials. The FVC decline slope identified in the Italian cohort (−4.2%/year) is in line with previous reports[4,24,25] which estimated values of decline around 5%/year for both FVC and PEF. The decline of PEF, on the other hand, appeared somewhat slower in the Italian cohort (−2.9%/year). This may be partly due to methodological aspects of our study, in which we applied linear models only within the linear age range of decline estimated by piecewise regression. PEF appeared to decline linearly over a considerably longer time, hence the smaller yearly decline. We suggest that PEF may be a more sensitive endpoint in studies involving younger boys or older adults, while FVC remains the most sensitive outcome in the approximately 10- to 20-year range.

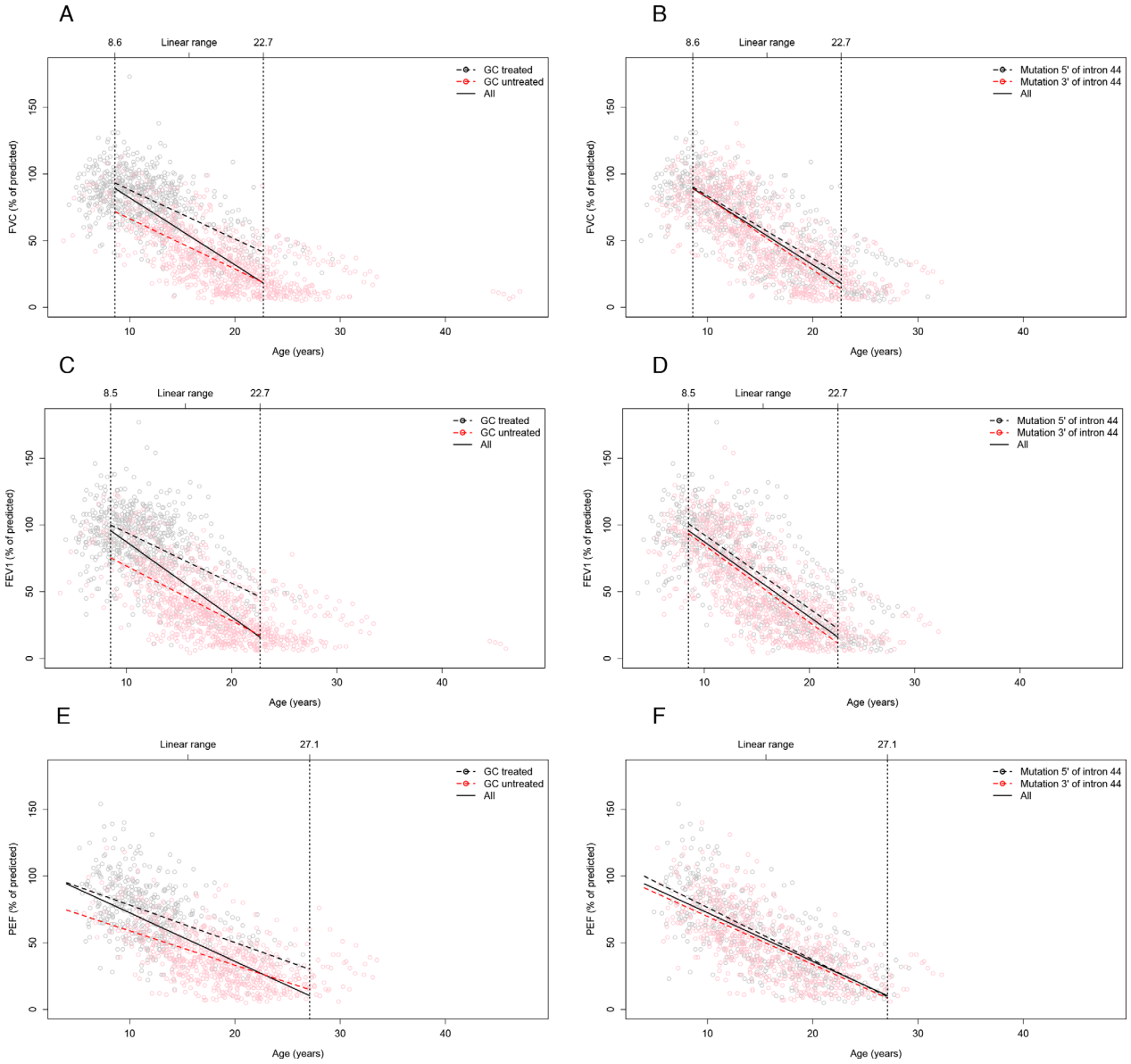

Figure 1. Scatter plots of PFT measures (FVC, panels A and B; FEV1, panels C and D; PEF, panels E and F) by age, in the Italian cohort grouped by GC treatment at the time of spirometry (panels A, C, and E) and by *DMD* mutation type ("proximal" or "distal", panels B, D, and F). Vertical lines indicate the limits of age ranges of linear decrease of corresponding measures, as identified by piecewise regression. Within these boundaries, regression lines represent the slope of decrease in the linear model (derived by regressing measure on age in the overall population and defined subgroups). PFT, pulmonary function testing; FVC, forced vital capacity.

We confirm a large effect of GCs in delaying respiratory decline in DMD. We recognize potential sources of bias which might lead to a partial overestimation of the GC effect: first, the treated population was younger, and might have benefited from an improvement of overall standards of care. Second, percent-predicted PFT values are influenced by height (denominator in the formula), and patients treated chronically with GC display stunted growth.[26] However, the beneficial effect of GCs appears uncontroversial. Interestingly, treated and untreated participants show similar decline slopes (Fig. 1), but starting from a higher plateau in treated participants. This advantage is maintained over time, delaying the age at which functional thresholds related to nocturnal and diurnal respiratory insufficiency are reached. Overall, our findings support of the efficacy of GCs in preserving respiratory function in a teenage to adult age range, at doses tailored to individual tolerability.[9,27]

Table 4. Coefficients of GEE analyses.

	Italian cohort		CINRG-DNHS cohort		Meta-analysis	
Coefficient	Estimate ± SE	*P*-value	Estimate ± SE	*P*-value	Estimate ± SE	*P*-value
FVC (% predicted)						
Intercept	111.8 ± 3.9	**<0.0001**	125.0 ± 4.7	**<0.0001**	119.4 ± 3.0	**<0.0001**
Age (per-year decrease)	−4.2 ± 0.2↓	**<0.0001**	−4.8 ± 0.3↓	**<0.0001**	−4.6 ± 0.2↓	**<0.0001**
GC treatment	14.5 ± 2.1↑↑	**<0.0001**	9.4 ± 2.3↑	**<0.0001**	11.7 ± 1.6↑↑	**<0.0001**
Mutation 3′ of exon 44	−6.1 ± 2.3↓	**0.008**	−5.9 ± 2.7↓	0.029	−5.8 ± 1.8↓	**0.001**
Mutation type						
Skip 8	12.5 ± 8.9	n.s.	13.2 ± 12.0	n.s.	13.8 ± 8.3↑↑	0.049
Skip 44	6.5 ± 5.0	n.s.	7.8 ± 4.5	0.04	7.1 ± 3.3↑	0.016
Skip 45	−3.4 ± 5.2	n.s.	0.1 ± 3.3	n.s.	−0.8 ± 2.8	n.s.
Skip 51	−6.9 ± 3.8↓	0.035	−5.7 ± 3.1↓	0.031	−5.7 ± 2.3↓	**0.007**
Skip 53	−9.2 ± 3.3↓	**0.003**	−11.6 ± 4.7↓↓	**0.007**	−10.3 ± 2.7↓↓	**<0.0001**
Nonsense	2.3 ± 3.2	n.s.	−6.3 ± 4.0	0.059	−1.1 ± 2.6	n.s.
Splice site	13.9 ± 7.0↑	0.023	NA	NA	13.6 ± 7.0↑	0.069
SNP modifiers						
rs28357094 dom	−5.3 ± 3.8	n.s.	−4.4 ± 3.2	n.s.	−4.5 ± 2.5↓	0.020
rs10880 rec	−6.8 ± 4.2	n.s.	3.4 ± 3.6	n.s.	−1.4 ± 2.8	n.s.
rs1883832 add	−0.1 ± 2.9	n.s.	−6.1 ± 2.2↓	**0.005**	−4.8 ± 1.7↓	**0.006**
rs1815739 add	4.2 ± 2.4	n.s.	0.3 ± 1.7	n.s.	0.8 ± 145	n.s.
FEV1 (% predicted)						
Intercept	125.8 ± 4.3	**<0.0001**	122.6 ± 4.6	**<0.0001**	127.0 ± 3.9	**<0.0001**
Age (per-year decrease)	−5.0 ± 0.3↓	**<0.0001**	−4.6 ± 0.3↓	**<0.0001**	−5.1 ± 0.2↓	**<0.0001**
GC treatment	15.1 ± 2.0↑↑	**<0.0001**	8.5 ± 2.4↑	**0.0003**	13.4 ± 1.7↑↑	**<0.0001**
Mutation 3′ of exon 44	−6.3 ± 2.5↓	0.011	−6.6 ± 2.9↓	0.025	−5.3 ± 2.1↓	**0.0094**
Mutation type						
Skip 8	9.7 ± 7.5	n.s.	17.9 ± 11.4	0.058	15.3 ± 7.9↑↑	0.027
Skip 44	0.9 ± 6.0	n.s.	7.0 ± 4.5	0.059	3.2 ± 4.0	n.s.
Skip 45	−0.7 ± 5	n.s.	−2.7 ± 4.4	n.s.	−1.9 ± 3.3	n.s.
Skip 51	−7.5 ± 4.3↓	0.042	−4.8 ± 3.4	n.s.	−5.3 ± 2.9↓	0.032
Skip 53	−6.1 ± 3.8	0.053	−10.9 ± 4.7↓↓	0.010	−6.6 ± 3.2↓	0.021
Nonsense	2.7 ± 4.5	n.s.	−7.1 ± 4.3↓	0.048	−1.5 ± 3.6	n.s.
Splice site	5.8 ± 4.3	n.s.	NA	NA	11.6 ± 5.1↑↑	0.011
SNP modifiers						
rs28357094 dom	−3.3 ± 4.2	n.s.	−5.8 ± 3.6	0.055	−2.9 ± 2.9	n.s.
rs10880 rec	−4.3 ± 5.2	n.s.	5.4 ± 3.8	n.s.	−2.6 ± 1.9	n.s.
rs1883832 add	2.0 ± 3.1	n.s.	−4.8 ± 2.2↓	0.030	−2.1 ± 3.2	n.s.
rs1815739 add	1.3 ± 3.2	n.s.	1.2 ± 1.8	n.s.	0.6 ± 1.7	n.s.
PEF (% predicted)						
Intercept	89.3 ± 3.6	**<0.0001**	109.4 ± 4.6	**<0.0001**	90.7 ± 3.7	**<0.0001**
Age (per-year decrease)	−2.9 ± 0.2↓	**<0.0001**	−3.8 ± 0.2↓	**<0.0001**	−3.0 ± 0.2↓	**<0.0001**
GC treatment	14.2 ± 1.9↑↑	**<0.0001**	8.0 ± 1.9↑	**<0.0001**	14.1 ± 1.9↑↑	**<0.0001**
Mutation 3′ of exon 44	−5.8 ± 2.3↓	0.010	−4.7 ± 2.8	n.s.	−5.6 ± 2.3↓	0.013
Mutation type						
Skip 8	23.0 ± 4.2↑↑	**<0.0001**	16.1 ± 6.4↑↑	**0.006**	20.0 ± 4.5↑↑	**<0.0001**
Skip 44	−0.6 ± 5.4	n.s.	5.9 ± 4.2	n.s.	3.9 ± 3.5	n.s.
Skip 45	−7.8 ± 5.2	n.s.	−0.7 ± 4.4	n.s.	−2.9 ± 3.4	n.s.
Skip 51	−5.7 ± 3.4↓	0.048	−2.2 ± 3.8	n.s.	−2.9 ± 2.6	n.s.
Skip 53	−5.5 ± 2.9↓	0.027	−5.3 ± 5.2	n.s.	−5.9 ± 2.7↓	0.014
Nonsense	0.5 ± 2.8	n.s.	−6.0 ± 5.1	n.s.	−2.3 ± 3.0	n.s.
Splice site	−0.9 ± 5.1	n.s.	NA	NA	−3.0 ± 5.1	n.s.
SNP modifiers						
rs28357094 dom	−2.3 ± 3.5	n.s.	−8.7 ± 3.1↓	**0.005**	−6.3 ± 2.4↓	**0.0088**
rs10880 rec	−6.2 ± 4.0	n.s.	4.9 ± 4.1	n.s.	1.3 ± 3.1	n.s.
rs1883832 add	−3.7 ± 3.0	n.s.	−3.0 ± 2.2	n.s.	−4.1 ± 1.8↓	0.024
rs1815739 add	4.0 ± 2.5	n.s.	0.1 ± 1.8	n.s.	1.2 ± 1.5	n.s.

Nominally significant effects have been marked with arrows, upward for positive effects and downward for negative. Double arrows indicate "strong" effects (arbitrarily: above 10% of the corresponding measure). $P < 0.06$ (nominally significant or close) are indicated in numbers; $P < 0.01$ are highlighted in bold. GEE, Generalized Estimating Equation; FVC, forced vital capacity; FEV1, forced expiratory volume in 1 sec; PEF, peak expiratory flow; CINRG-DNHS, Cooperative International Neuromuscular Research Group Duchenne Natural History Study; SE, standard error; GC, glucocorticoid corticosteroids; Skip 8, mutations amenable to treatment with skipping of exon 8 (same for other exon numbers); SNPs, single nucleotide polymorphisms; NA, not available (no participants included in the corresponding category); n.s., not significant.

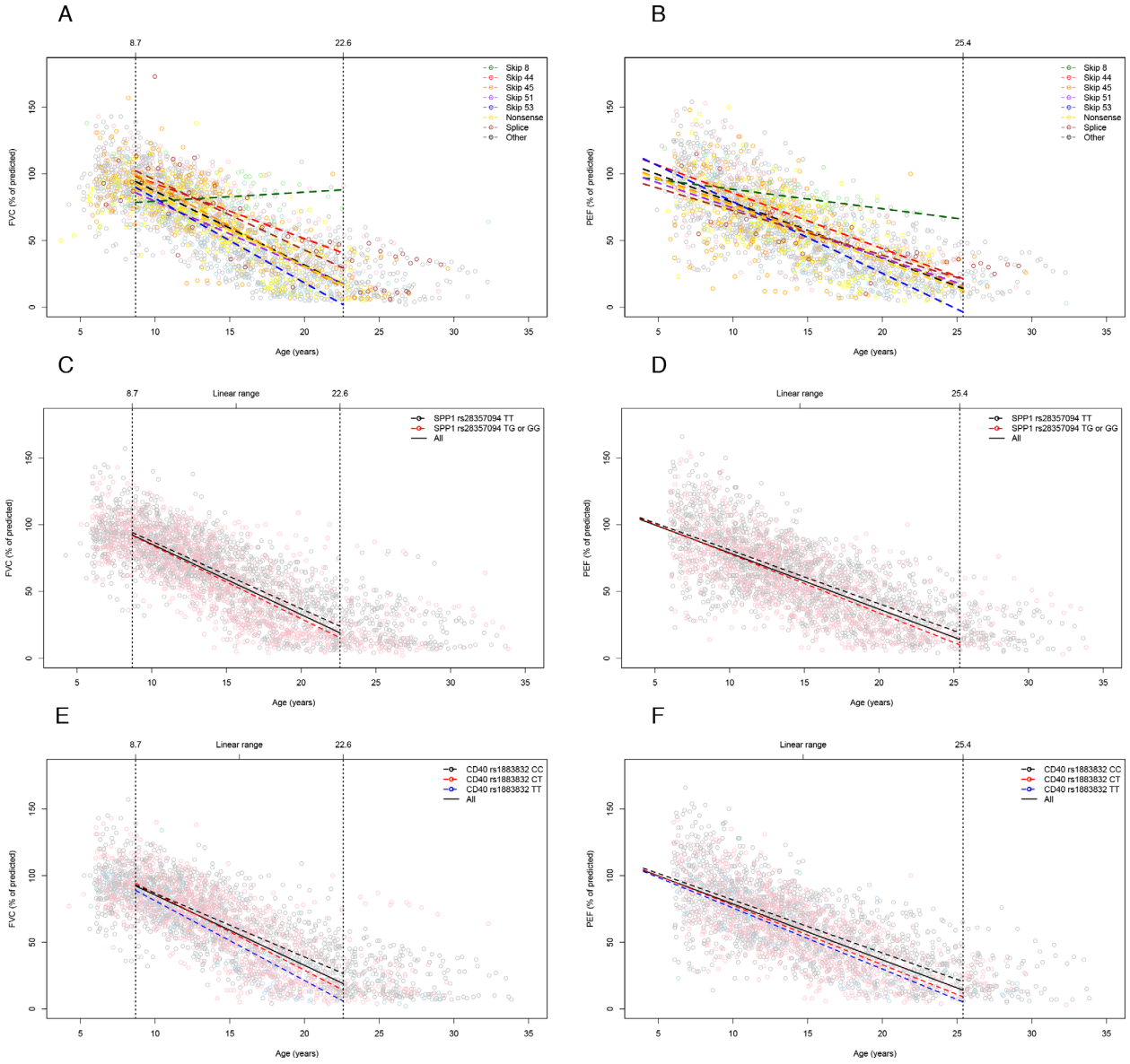

Figure 2. Scatter plots of PFT measures (FVC, panels A, C, and E); PEF, panels (B, D, and F) by age, in the merged Italian and CINRG-DNHS cohorts grouped by *DMD* mutation type (groups defined in Methods, panels A and B) and by modifier SNP genotypes (*SPP1* rs28357094: panels C and D; *CD40* rs1883832: panels E and F). Vertical lines indicate the limits of age ranges of linear decrease of corresponding measures, as identified by piecewise regression. Within these boundaries, regression lines represent the slope of decrease in the linear model (derived by regressing measure on age in the overall population and defined subgroups). PFT, pulmonary function testing; FVC, forced vital capacity; PEF, peak expiratory flow; CINRG-DNHS, Cooperative International Neuromuscular Research Group Duchenne Natural History Study.

An unexpected finding was the lower PFT performance associated with "distal" DMD mutations, potentially affecting the expression of short dystrophin isoforms. The association in the Italian cohort was consistently validated in the CINRG-DNHS, with an effect size of around 6% for FVC and PEF. The restrictive ventilatory defect in DMD is caused by the absence of full-length dystrophin in the diaphragm and other respiratory muscles. While defects of short dystrophin isoforms have been associated with increased risk of intellectual disability and other central nervous system (CNS) manifestations of DMD,[28–30] they have not been clearly linked to more severe muscle weakness. A possible explanation is that while patients with very severe cognitive deficiencies are excluded from PFTs, because of insufficient cooperation, patients with subtler CNS issues due to Dp140/Dp71 defects still

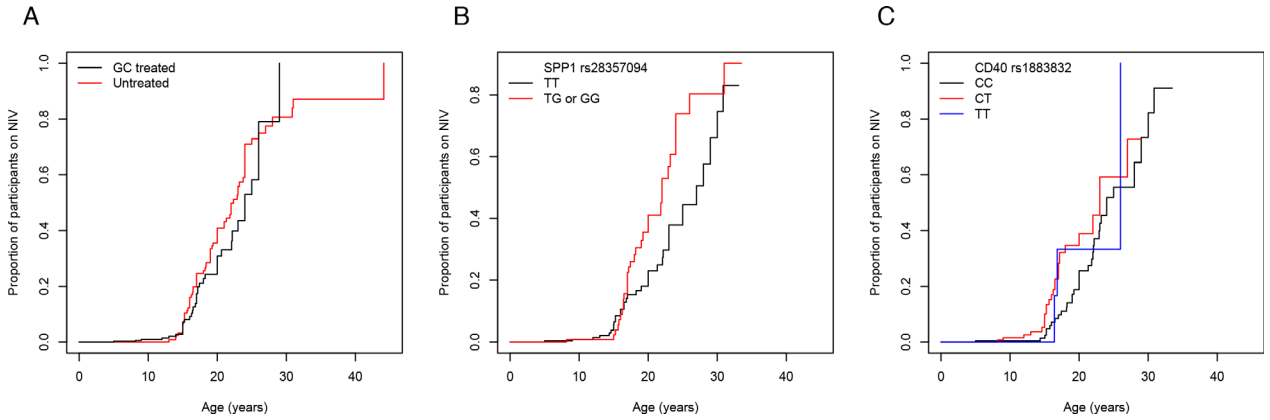

Figure 3. Cumulative incidence plots of NIV use by age in the merged Italian and CINRG-DNHS cohorts, grouped by (A) GC treatment (on vs. off during follow-up), (B) *SPP1* rs28357094 genotype, and (C) *CD40* rs1883832 genotype. NIV, noninvasive ventilatory assistance; CINRG-DNHS, Cooperative International Neuromuscular Research Group Duchenne Natural History Study; GC, glucocorticoid.

perform worse in PFTs, which are largely influenced by volition and effort. However, an influence of short dystrophin isoforms on other physiological variables, such as chest wall compliance or skeletal deformities, cannot be ruled out entirely, and warrants further studies.

When looking into mutations amenable to different molecular treatments,[18] the main anticipated finding of a milder phenotype in participants with "skip 44" deletions[10–13] appeared only marginal, and only nominally significant for FVC in the meta-analysis, possibly because of the relatively small size of these subgroups (n = 16 in the Italian and 16 in the CINRG cohort). Even with regards to ambulatory function, it should be noted, the modifier effect of "skip 44" mutations was in fact variable.[10–13] Proposed underlying mechanisms involve alternative splicing of exon 44 by activation of cryptic splice sites at the deletion breakpoint,[31] which may vary between individuals.

Conversely, the "skip 51" and especially "skip 53" subgroups surprisingly showed worse PFT outcomes (especially PEF) across the Italian and CINRG cohorts. This finding is relevant to ongoing clinical trials of exon skipping drugs such as eteplirsen, which was recently approved in the USA,[32] and shown to modify slopes of FVC change compared to the genotyped CINRG cohort.[33] "Skip 51" and "skip 53" mutations are completely included in the "distal" group, and are predicted to disrupt Dp140 expression. Therefore, these participants may perform worse in PFTs because of the reasons explained above.

The four modifier SNPs tested here have been described because of their effect on muscle strength, mainly ambulatory function.[14–17,22,23,34] Respiratory involvement is more severe in DMD patients with more severe skeletal muscle impairment[7]; thus, modifiers of skeletal muscle strength may be considered candidate modifiers of respiratory insufficiency. In fact, inflammatory, regenerative, and fibrotic events occur in the diaphragm as in skeletal muscle, although with potential differences,[35] and may be modulated by modifier genes. In this study, no loci were validated independently across the two cohorts with Bonferroni-corrected significance. Meta-analysis identified suggestive effects of *SPP1* and *CD40* on FVC and PEF, in the expected direction (minor alleles being detrimental). As previous studies on ambulatory function[22] and in vitro expression of osteopontin[36] by myogenic cells have suggested and interaction between GCs and *SPP1* via a GC receptor-mediated mechanism, a GEE model with an interaction term between rs28357094 genotype and GC treatment was also tested, but yielded no significant results (data not shown).

Both the *SPP1*-encoded cytokineosteopontin[37] and T-cell activation mediated by CD40[38] preferentially influence the earlier inflammatory phase of the dystrophic process, rather than the late, end-stage fibrotic phase.[39] The latter is more affected by latent transforming growth factor β-binding protein LTBP4,[40] whose disease-modifying polymorphisms did not show significant effects on PFTs. For *LTBP4*, this result did not change when taking into consideration the full VTTT/IAAM haplotype.[15] These findings suggest that active dystrophic pathology, that is, necrosis and inflammation within respiratory muscles, may be relevant to PFT measures, at least in the linear decline phase. The clinical meaningfulness of *SPP1* and *CD40* effects was strengthened by findings of earlier NIV associated with risk variants, although only nominally significant. These findings may in part be a consequence of prolonged ambulation, which may improve thoracic dynamics compared to wheelchair-bound patients.

We acknowledge several limitations to this study: the retrospective nature of Italian cohort data, compensated

Table 5. Coefficients of time-to-event analyses of age at commencement of NIV.

	Italian cohort				CINRG-DNHS cohort				Meta-analysis			
	n (events)	Median age at NIV (95% CI)	HR (95% CI)	*P*-value	*n* (events)	Median age at NIV (95% CI)	HR (95% CI)	*P*-value	*n* (events)	Median age at NIV (95% CI)	HR (95% CI)	*P*-value
All participants	318 (87)	23.2 (22.0–24.0)	NA	NA	276 (31)	22.2 (21.8–NA)	NA	NA	594 (118)	23.0 (22.0–24.0)	NA	NA
GC treatment												
Yes	166 (15)	24.0 (23.0–NA)	0.67 (0.37–1.18)	n.s.	211 (23)	22.2 (22.1–NA)	0.91 (0.41–2.06)	n.s.	377 (38)	24.0 (22.2–NA)	0.79 (0.53–1.18)	n.s.
No	113 (62)	22.0 (20.0–24.0)			65 (8)	22.0 (21.8–NA)			178 (70)	22.4 (21.0–24.0)		
DMD mutation												
Prox.	102 (24)	23.0 (21.0–NA)	0.98 (0.59–1.60)	n.s.	58 (3)	NA (17.2–NA)	0.76 (0.21–2.71)	n.s.	160 (27)	23.2 (21.0–NA)	0.92 (0.59–1.46)	n.s.
Dist.	168 (47)	22.9 (20.0–24.0)			117 (13)	21.8 (20.0–NA)			285 (60)	22.9 (20.0–24.0)		
SPP1 rs28357094 (dominant)												
TT	110 (20)	28.0 (25.0–NA)	1.72 (0.91–3.26)	0.094	185 (15)	25.0 (22.2–NA)	1.71 (0.80–3.66)	n.s.	295 (35)	27.0 (25.0–NA)	1.75 (1.08–2.84)	0.023
TG/GG	72 (19)	22.9 (22.0–NA)			82 (13)	20.0 (17.4–NA)			154 (32)	22.0 (20.0–24.0)		
LTBP4 rs10880 (recessive)												
CC/CT	149 (31)	24.0 (23.0–30.0)	1.27 (0.58–2.79)	n.s.	234 (29)	22.2 (21.8–NA)	0.35 (0.05–2.59)	n.s.	383 (60)	24.0 (22.2–28.0)	0.91 (0.45–1.85)	n.s.
TT	29 (8)	23.0 19.2–NA)			29 (1)	NA (NA–NA)			58 (9)	30.9 (23.0–NA)		
CD40 rs1883832 (additive)												
CC	99 (23)	24.0 (22.0–NA)	0.97 (0.47–1.99)	n.s.	150 (11)	25.1 (22.0–NA)	1.71 (1.01–2.88)	0.044	249 (34)	24.0 (22.9–NA)	1.50 (1.00–2.26)	0.0498
CT	63 (10)	27.0 (22.0–NA)			98 (16)	20.0 (17.0–NA)			161 (26)	23.0 (20.0–NA)		
TT	8 (0)	NA (NA–NA)			18 (3)	21.4 (16.4–NA)			26 (3)	26.0 (16.8–NA)		
ACTN3 rs1815739 (additive)												
CC	37 (7)	29.0 (19.0–NA)	1.10 (0.58–2.10)	n.s.	74 (5)	NA (20.0–NA)	0.26 (0.69–1.92)	n.s.	111 (12)	29.0 (20–NA)	1.18 (0.79–1.78)	n.s.
CT	92 (12)	27.0 (23.2–NA)			114 (15)	22.2 (21.8–NA)			206 (27)	27.0 (23–NA)		
TT	29 (6)	22.9 (20.0–NA)			69 (7)	25.0 (22.1–NA)			98 (13)	22.9 (20.0–NA)		

Age is indicated in years. HRs above 1 indicate detrimental effects (i.e., earlier NIV), while HRs below 1 correspond to later NIV. The direction of HRs is calculated for GC treated versus untreated; "distal" versus "proximal" *DMD* mutation; and minor alleles for modifier SNPs (with the indicated inheritance models). CINRG-DNHS, Cooperative International Neuromuscular Research Group Duchenne Natural History Study; *n* (events), number of participants included in the analysis and (in brackets) number of observed events, that is, commencement of NIV; NIV, noninvasive ventilation; HR; hazard ratio; CI, confidence interval.

for by the longitudinal CINRG-DNHS[4]; the fact that variability in the Italian data may be affected by changes in practice during the long time frame of data collection; the unavailability of maximal inspiratory and expiratory pressure (MIP and MEP) data, which we plan to collect in future studies; the concern that missing NIV data adds uncertainty to corresponding conclusions; and finally, lack of genotyping, because of limited DNA availability, at a *THBS1* long-range genomic regulator locus recently added to the list of DMD modifiers.[41]

In conclusion, our findings define linear PFT changes in a large Italian DMD cohort; strengthen the indication of GC treatment for teenagers and adults living with DMD; and identify "distal" *DMD* mutations, and probably *SPP1* and *CD40* variants, as risk factors for worse PFT outcomes. These findings will ultimately be relevant for clinical management and trial design in DMD.

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Conflict of Interest

The research findings described in this article do not have direct relevance to any commercial product.

Author Contribution

LB drafted the manuscript. LB and HGD performed statistical analyses. LB, AV, CB, EPH, CMM, and EP designed the study. GDA, MV, AF, SV, BM, DS, AB, SG, FM,GPC, MP, PT, VL, FT, ADA, EB, GA, LP, RM, GB, EA, EDM, FR, VAS, SP, SM, GLV, AB, TM, AP, MP, EM contributed to data collection and analysis, and revised the manuscript for intellectual content. All authors approved the final version of the manuscript. Cooperative International Neuromuscular Research Group (CINRG) Investigators are listed in a supplementary file.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

