

Genetic Modifiers of Ambulation in the Cooperative International Neuromuscular Research Group Duchenne Natural History Study

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Objective: We studied the effects of *LTBP4* and *SPP1* polymorphisms on age at loss of ambulation (LoA) in a multi-ethnic Duchenne muscular dystrophy (DMD) cohort.

Methods: We genotyped *SPP1* rs28357094 and *LTBP4* haplotype in 283 of 340 participants in the Cooperative International Neuromuscular Research Group Duchenne Natural History Study (CINRG-DNHS). Median ages at LoA were compared by Kaplan–Meier analysis and log-rank test. We controlled polymorphism analyses for concurrent effects of glucocorticoid corticosteroid (GC) treatment (time-varying Cox regression) and for population stratification (multi-dimensional scaling of genome-wide markers).

Results: Hispanic and South Asian participants ($n = 18, 41$) lost ambulation 2.7 and 2 years earlier than Caucasian subjects ($p = 0.003, <0.001$). The TG/GG genotype at *SPP1* rs28357094 was associated to 1.2-year-earlier median LoA ($p = 0.048$). This difference was greater (1.9 years, $p = 0.038$) in GC-treated participants, whereas no difference was observed in untreated subjects. Cox regression confirmed a significant effect of *SPP1* genotype in GC-treated participants (hazard ratio = 1.61, $p = 0.016$). *LTBP4* genotype showed a direction of association with age at LoA as previously reported, but it was not statistically significant. After controlling for population stratification, we confirmed a strong effect of *LTBP4* genotype in Caucasians (2.4 years, $p = 0.024$). Median age at LoA with the protective *LTBP4* genotype in this cohort was 15.0 years, 16.0 for those who were treated with GC.

Interpretation: *SPP1* rs28357094 acts as a pharmacodynamic biomarker of GC response, and *LTBP4* haplotype modifies age at LoA in the CINRG-DNHS cohort. Adjustment for GC treatment and population stratification appears crucial in assessing genetic modifiers in DMD.

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Duchenne muscular dystrophy (DMD) is caused by the absence of the protein dystrophin in myofibers, due to truncating dystrophin gene mutations.¹ Despite

this homogeneous molecular defect, variability in phenotype severity is commonly observed, for example, variable age at loss of ambulation (LoA). This is due to

Collaborating authors (Cooperative International Neuromuscular Research Group investigators) are listed in the Appendix on page 695.

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environmental factors, such as implementation of standards of care (glucocorticoid corticosteroid [GC] treatment, physical therapy, management of contractures, fracture prevention),^{2,3} and to the genetic background. Two genetic modifiers of DMD, that is, common polymorphisms that modulate disease severity combined with a pathogenic mutation, have been described: a single nucleotide polymorphism (SNP) in the promoter of the *SPP1* (secreted phosphoprotein 1, or osteopontin) gene, and a coding *LTBP4* (latent transforming growth factor β binding protein 4) haplotype. The association of the *SPP1* rs28357094 rare G allele with earlier LoA, in a dominant inheritance model, was originally reported in 106 Italian DMD patients.⁴ *SPP1* encodes an inflammatory cytokine involved in tissue damage response, and is part of the transforming growth factor β (TGF β) pathway.⁵ The rs28357094 polymorphism alters transcription at baseline⁶ and in response to steroid hormones.⁷

The *LTBP4* locus was identified by genome-wide mapping in a murine model of muscular dystrophy.⁸ Subsequently, a *LTBP4* haplotype was associated with variable LoA in 254 patients with severe dystrophinopathy (United Dystrophinopathy Project).⁹ The haplotype consists of 4 coding SNPs in strong linkage disequilibrium (LD), 1 of which, rs10880, was independently associated with age at LoA. Homozygotes for the minor allele T at rs10880 (T1140M), in LD with the haplotype IAAM, showed later LoA. The proposed mechanism is that the IAAM protein isoform results in a more stable latent TGF β complex, reducing TGF β signaling. In the same paper, the authors found no association of *SPP1* genotype with age at LoA.

Validation of genetic associations in independent cohorts is essential to establish genetic modifiers of Mendelian diseases,¹⁰ but may be exaggerated or obscured by confounding variables, such as ancestry-dependent differences in allele frequency and haplotype configuration, which associate with variations of standards of care and other environmental factors, and lead to population stratification.^{11–13} Disparities in diagnostics,¹⁴ standards of care,¹⁵ and phenotype severity^{16,17} between DMD patients of different ethnic backgrounds have been reported. The Cooperative International Neuromuscular Research Group Duchenne Natural History Study (CINRG-DNHS)¹⁸ comprises participants from 20 centers on 4 continents, constituting an ethnically diverse cohort.

We have expanded analysis of the CINRG-DNHS cohort, from the baseline cross-sectional analysis of grip strength in 156 participants⁴ to a longitudinal study (average follow-up 4 years) of all 340 participants.^{18,19} Here we sought to test the effect of *SPP1* and *LTBP4*

genotypes on LoA in the CINRG-DNHS population, controlling for GC treatment and population stratification. After controlling for these confounding factors, we find an association of both loci with LoA.

Subjects and Methods

The institutional review board or ethics review board at each participating institution approved the study protocol, and consent and assent documents. Informed consent/assent was obtained for each participant prior to conducting study procedures.

Inclusion and Exclusion Criteria

The inclusion and exclusion criteria for the CINRG-DNHS have been previously described.^{18,19} Recruitment was aimed at obtaining a population representing an age span from very young to adult (age 2–28 years at baseline). Conversely, recruitment was not specifically aimed at obtaining subpopulations with homogeneous ancestry for genetic association analysis. For all analyses focused on *SPP1* and *LTBP4* genotypes, we excluded patients with no available genomic DNA for SNP genotyping.

LoA and GC Treatment Definitions

LoA was defined as patient-reported continuous wheelchair use, verified by inability to walk 10m unassisted. GC treatment history was recorded both retrospectively at baseline, and longitudinally during the study, and the population was dichotomized into treated at least 1 year with GCs before LoA, and untreated or treated <1 year before LoA. This included patients who had gone on and off GC treatment 1 or more times, but were cumulatively treated for at least 1 year before LoA. The 1-year treatment threshold was chosen based on the clinical rationale that a long-term effect of GC treatment, such as delaying disease milestones, cannot be reasonably expected from short-term treatment. Data were also reanalyzed with a 6-month treatment threshold, as in Flanigan et al,⁹ to compare *LTBP4* data with the same methodology as the original report of this modifier.

Race and Ethnicity Definitions

Self-identification of participants into 1 of the following racial categories was recorded: African American, Asian, Caucasian, Mixed, or Other; self-identification into non-Hispanic or Hispanic ethnicity was specified as a separate option, according to official US Census categories (http://www.whitehouse.gov/omb/fedreg_1997standards/). Here, we clustered this information into the following groups: African American, Asian, non-Hispanic Caucasian, Hispanic Caucasian, Hispanic (ie, participants self-identifying their ethnicity as Hispanic, and race as Mixed/Other), and Other (ie, participants self-identifying their ethnicity as non-Hispanic, and race as Mixed or Other); and distinguished as South Asian participants recruited at the Study Center in Chennai, India (for these participants, no DNA samples were available because of local regulations that did not allow the shipment of DNA for the purposes of our study).

Targeted Genotyping

Targeted genotyping was carried out by TaqMan allele discrimination assays. For *SPP1* rs28357094, a dominant model for the minor allele G was adopted.⁴ For *LTBP4* association studies, we focused mainly on the rs10880 SNP (T1140M), which is in strong LD with the IAAM haplotype, and showed an independent association with LoA in the original report, in a recessive model.⁹ Focusing initially on a single significant SNP allowed inclusion of larger numbers of participants in the analyses. To confirm association with the full haplotype in carriers of the rs10880 T allele, we genotyped rs2303729 (V194I), rs1131620 (T787A), and rs1051303 (T820A). *LTBP4* haplotypes were phased with PLINK,²⁰ and median ages at LoA were calculated for all observed haplotype configurations, in all those patients for whom haplotypes could be phased with at least 90% probability.

Multidimensional Scaling Analysis

Genotype data from the Illumina (San Diego, CA) HumanExome Chip was available for 175 participants. These were not selected by ethnicity, nor any phenotype-related data points, but solely on the base of available DNA quantity and quality. Although mainly focusing on coding regions, the chip contains ancestry markers and other common variant markers (~30,000) that ensure sufficient genome-wide coverage for multidimensional scaling (MDS) analysis. Chip design information is publicly available at http://genome.sph.umich.edu/wiki/Exome_Chip_Design#Illumina_Exome_Arrays. Genotypes were called from raw intensity data with Genome Studio software, and data was exported into PLINK format with the dedicated plug-in software by Illumina. PLINK was used for data cleaning and MDS analysis.²⁰ Subjects with >10% missed calls and SNPs with >5% missed calls were removed to ensure data quality.²¹ MDS analysis was based on the calculation of genome-wide identity-by-state pairwise distances, on a set of “pruned” genome-wide markers in no significant LD with each other, using the PLINK whole genome association analysis toolset.²⁰ LD-based pruning parameters were the following: 50-SNP window size, 5-SNP window slide at each step, variance inflation factor threshold = 2. The 2 highest ranking principal components were plotted.

Grouping Criteria for Analyses of Race/Ethnicity Differences in GC Treatment and LoA

Median LoA and GC treatment rates were calculated in participants with different self-identified race/ethnicities in the whole DNHS cohort of 340 patients, grouped as defined above.

Grouping Criteria for Analyses of SNP Effect on LoA

SNP effects on LoA were analyzed in the whole cohort with available genomic DNA for genotyping, and in a subcohort of participants of Caucasian ancestry, identified by MDS analysis. In both cohorts, analyses were carried out in 3 groupings based on GC treatment: all participants regardless of treatment; GC-treated participants (at least 1 year while ambulatory as defined

above); GC-untreated participants (including those treated <1 year while ambulatory, as defined above). Data were also reanalyzed with a 6-month GC treatment threshold, as explained above.

A flow diagram of participant grouping, with corresponding planned analyses, participant numbers, and GC treatment rates, is shown in Figure 1.

Statistical Methods

Rates of GC treatment between self-identified racial and ethnic subgroups were compared by chi-square test. Loss of ambulation was studied in a time to event model with age as the time variable, and LoA as the failure event. Ambulatory participants were censored at the age of last follow-up. Median ages at LoA in race/ethnicity and genotype subgroups were based on the empiric survival curve from a Kaplan–Meier (KM) curve calculation, and compared by log-rank test. For *SPP1* and *LTBP4* genotypes, patients were grouped based on inheritance models specified above. Additionally, concurrent effects of GC treatment and SNP genotype were analyzed in a Cox regression model, with a time-varying GC treatment covariate (on/off treatment defined for all participants at the time of each event). Statistical significance was set at $p < 0.05$. Genomics Suite 6.6 (Partek, St Louis, MO) and Stata v13 (StataCorp, College Station, TX) were used for statistical analyses.

Results

Distribution by Race/Ethnicity and GC Treatment

Self-identified race/ethnicity in the 340 participants enrolled in the CINRG-DNHS was distributed as follows: 225 (66%) Caucasian, 23 (7%) Hispanic-Caucasian, 41 (12%) South Asian, 18 (5%) Hispanic, 14 (4%) Asian, 6 (2%) African American, and 13 (4%) Other. Of note, the South Asian group was the only one entirely referring to a single study center (Chennai, India).

GC treatment was administered for at least 1 year before LoA to 252 of 340 participants (74%). There were differences in the proportion of participants treated for at least 1 year with GCs between self-identified racial–ethnic subgroups. Overall, participants self-identifying as Caucasian (both Hispanic and non-Hispanic ethnicity) were more often treated for at least 1 year than other participants: 191 of 248 (77%) versus 61 of 92 (65%), chi-square $p = 0.045$ (Table 1). This comparison might be biased by different proportions of younger, ambulatory GC-naïve participants, as opposed to nonambulatory participants who did not receive treatment before LoA. When analyzing nonambulatory participants only, we found a trend in the same direction; nonambulatory Caucasians treated at least 1 year while ambulatory were 113 of 162 (70%) versus other ethnicities 39 of 67 (58%, chi-square $p = 0.09$, see Table 1).

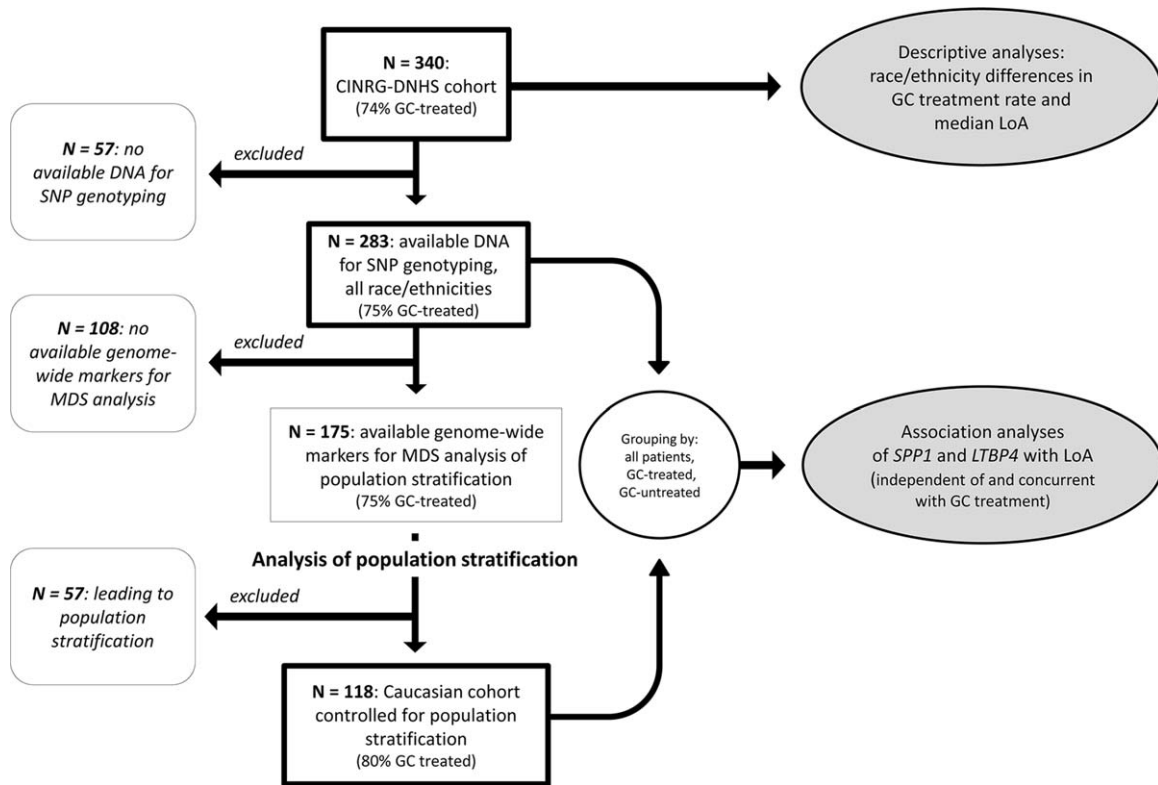


FIGURE 1: Flow diagram of analysis plan and population grouping. Subgroups included in different analyses are shown, starting from the top with the whole Duchenne Natural History Study (DNHS) cohort, and in subsequent steps excluding patients with no available DNA for genotyping; subjects with no available genome-wide markers for multidimensional scaling analysis for population stratification; and subjects leading to population stratification. Thick-border boxes indicate groups selected for specific analyses. CINRG = Cooperative International Neuromuscular Research Group; GC = glucocorticoid corticosteroid; LoA = loss of ambulation; MDS = multidimensional scaling; SNP = single nucleotide polymorphism.

Age at LoA and GC Treatment Show Differences between Self-Reported Ethnic Subgroups

Median age at LoA was 12.4 years in the non-Hispanic Caucasian population (n = 225). Compared to this

numerically predominant group, median age at LoA was significantly earlier in the Hispanic (n = 18, 9.7 years, log-rank $p = 0.003$) and South Asian (n = 41, 10.4 years, $p < 0.001$) subpopulations. Median LoA was earlier in the Asian subpopulation (n = 14, 11.3 years), and later

TABLE 1. Number and Percentage of Participants Treated with Glucocorticoid Corticosteroids at Least 1 Year While Ambulatory, Grouped by Self-Identified Race and Ethnicity

Self-Identified Race and Ethnicity	All Participants		Nonambulatory		Ambulatory	
African American	2/6	(33%)	0/4	(0%)	2/2	(100%)
Asian	9/14	(64%)	8/13	(62%)	1/1	(100%)
Caucasian						
Non-Hispanic	173/225	(77%)	102/146	(70%)	71/79	(90%)
Hispanic	18/23	(78%)	11/16	(69%)	7/7	(100%)
Hispanic	8/18	(44%)	6/16	(37%)	2/2	(100%)
Other	12/13	(92%)	5/6	(83%)	7/7	(100%)
South Asian	30/41	(73%)	20/28	(71%)	10/13	(77%)
Overall	251/340	(74%)	151/229	(66%)	100/111	(90%)

TABLE 2. MAFs for *SPP1* rs28357094 and *LTBP4* rs10880 Compared to 1000G MAFs

Population	<i>SPP1</i> rs28357094		<i>LTBP4</i> rs10880	
	DNHS	1000G	DNHS	1000G
African American, n = 6	0.10	0.04 (AFR)	0.30	0.51 (AFR)
Asian, n = 14	0.08	0.00 (ASN)	0.31	0.29 (ASN)
non-Hispanic Caucasian, n = 225	0.18	0.24 (EUR)	0.36	0.41 (EUR)
Hispanic Caucasian, n = 23	0.11	0.24 (EUR)	0.34	0.41 (EUR)
Hispanic, n = 18	0.18	0.14 (AMR)	0.31	0.27 (AMR)
Other, n = 13	0.13	0.12	0.31	0.38
Overall	0.17	0.12	0.35	0.38

MAFs for 1000G refer to a continental reference population (in parentheses), or to the whole project if not otherwise specified. 1000G = 1000 Genomes project; DNHS = Duchenne Natural History Study; MAF = minor allele frequency.

in Hispanic-Caucasian (n = 23, 13.0 years) and African American subpopulations (n = 6, 14.2), but differences with non-Hispanic Caucasians were not statistically significant.

Genotyping Results

Genomic DNA samples for targeted genotyping were available for 283 of 340 participants. The 57 patients excluded because of unavailability of DNA samples comprised all 41 patients followed at the study center in Chennai, India, as regulatory authorities did not allow participation in this part of the study.

Minor allele frequencies (MAFs) for *SPP1* rs28357094 and *LTBP4* rs10880 in the CINRG-DNHS population, broken down by ethnic subgroups, are shown in Table 2, compared to MAFs in continental reference populations from the 1000 Genomes project (<http://www.1000genomes.org/>). For both SNPs, the MAF in the numerically preponderant Caucasian population was slightly lower than in the 1000 Genomes reference EUR population (0.18 vs 0.24 for *SPP1* rs28357094, and 0.36 vs 0.41 for *LTBP4* rs10880).

For *SPP1* rs28357094, MAFs in Asian and Hispanic populations were higher than reference. These findings might be suggestive of population admixture. Both SNPs were in Hardy-Weinberg equilibrium (HWE).

LTBP4 haplotypes could be phased with 90% probability in 242 participants, including 28 of 32 TT homozygotes for *LTBP4* rs10880. Of these, 24 of 28 were homozygotes for the full IAAM haplotype based on rs2303729, rs1131620, and 1051303 genotypes, whereas 4 of 28 participants were heterozygotes for other rare haplotypes (VAAM or VTTM). Haplotype distributions

in different ethnic subgroups, along with median age at LoA calculated for all observed haplotype configurations (with standard errors [SEs] and 95% confidence intervals), are shown in detail in Supplementary Table 1. All SNPs in the *LTBP4* haplotype were in HWE.

Association Analyses in the Whole Genotyped Cohort: Effect of *SPP1* on LoA Is GC Treatment-Dependent in the DNHS Population

Analyses relative to *SPP1* and *LTBP4* genotypes were limited to 283 patients with available genomic DNA samples (see Fig 1). Of these, 279 (because of limited availability of genomic DNA for a few participants) were successfully genotyped for *SPP1* rs28357094. Median ages at LoA for genotype groups and results of log-rank and Cox regression analyses are summarized in Table 3. Median ages at LoA were 11.8 years in 84 participants carrying the minor allele (TG/GG), and 13.0 years in 195 participants carrying the TT genotype (log-rank $p = 0.048$, Fig 2A). This closely reproduces the methodology of the previously reported association of rs28357094 genotype with LoA in 106 Italian patients,⁴ representing an independent validation of association with this phenotype. In the Cox regression model with GC treatment as time-varying covariate, the hazard ratio (HR) \pm SE for TG/GG genotype was 1.22 ± 0.20 ($p =$ nonsignificant [n.s.]). The HR for GC treatment was 0.41 ± 0.07 ($p < 0.001$).

In 274 participants genotyped for *LTBP4* rs10880, median ages at LoA were 12.0 years in 242 participants with the CC/CT genotype, and 13.9 years in 32 homozygotes for the minor allele T (log-rank $p = 0.20$, Fig 3A). In the Cox regression model with GC treatment as time-varying covariate, HR for the TT genotype was

TABLE 3. KM and Cox Regression Models for LoA: Median Age at LoA, Log-Rank p-Values, Cox Hazard Ratios, and p-Values by SPP1-LTBP4 Genotypes and GC Treatment

Cohort	All Participants				GC Treated				GC Untreated				
	No.	Median Age at LoA, yr	p, KM Log-Rank	HR (95% CI), Cox p	No.	Median Age at LoA, yr	p, KM Log-Rank	HR (95% CI), Cox p	No.	Median Age at LoA, yr	p, KM Log-Rank	HR (95% CI), Cox p	
Whole genotyped DNHS cohort, n = 283 ^a													
<i>SPP1</i> rs28357094			0.048 ^b	1.22 (0.89–1.68), 0.22			0.032 ^b	1.61 (1.09–2.37), 0.016 ^b					0.6
TT	195	13.0			150	13.9				45	10.0		
TG/GG	84	11.8			63	12.0				21	10.0		
<i>LTBP4</i> rs10880			0.20	0.78 (0.49–1.24), 0.29			0.27	0.74 (0.44–1.26), 0.27					—
CC/CT	242	12.0			178	13.3				64	10		
TT	32	13.9			27	13.9				5	9.1		
Caucasian cohort, controlled for population stratification by MDS, n = 118 ^a													
<i>SPP1</i> rs28357094			0.047 ^b	1.54 (0.93–2.54), 0.09			0.07	1.85 (1.01–3.38), 0.047 ^b					0.7
TT	81	13.9			67	13.9				14	10.0		
TG/GG	35	12.0			26	12.0				9	9.0		
<i>LTBP4</i> rs10880			0.024 ^b	0.49 (0.23–1.07), 0.07			0.046 ^b	0.47 (0.20–1.09), 0.08					—
CC/CT	103	12.6			80	13.8				23	10		
TT	12	15.0			11	16.0				1	N/A		

^aTotal number may not correspond exactly to genotype group number because of a few ungenotyped patients (limited DNA availability).

^bStatistically significant effect of genotype on LoA.

CI = confidence interval; DNHS = Duchenne Natural History Study; GC = glucocorticoid corticosteroid; GC treated = GC treatment at least 1 year while patient was ambulatory; GC untreated = no or <1 year of GC treatment while patient was ambulatory; HR = hazard ratio for genotype in Cox regression model with GC treatment as a time-varying covariate; KM = Kaplan–Meier survival analysis with log-rank comparison of median age at LoA; LoA = loss of ambulation; MDS = multidimensional scaling; N/A = not available.

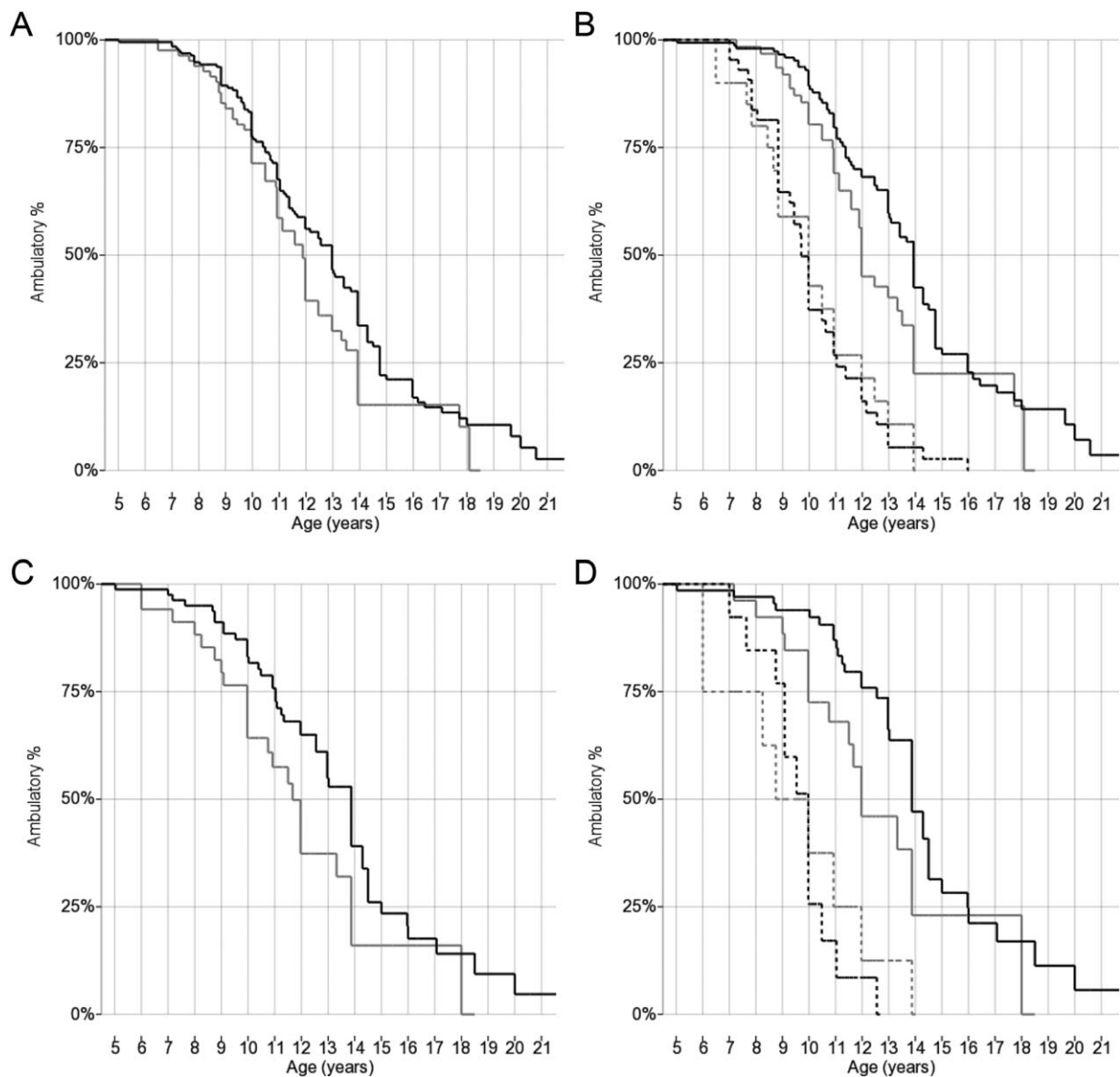


FIGURE 2: Kaplan–Meier plots of age at loss of ambulation grouped by *SPP1* rs28357094 genotype. (A) All patients genotyped for *SPP1* rs28357094, including all races and ethnicities ($n = 279$), grouped 2 ways by rs28357094 genotype (black line = TT; gray line = TG/GG). (B) All patients genotyped for *SPP1* rs28357094, including all races and ethnicities ($n = 279$), grouped 4 ways by rs28357094 genotype (black lines = TT; gray lines = TG/GG) and GC treatment (continuous lines = at least 1 year while ambulatory; dashed lines = <1 year or untreated). (C) Caucasian cohort controlled for population stratification and genotyped for *SPP1* rs28357094 ($n = 116$), grouped 2 ways by rs28357094 genotype (black line = TT; gray line = TG/GG). (D) Caucasian cohort controlled for population stratification and genotyped for *SPP1* rs28357094 ($n = 116$), grouped 4 ways by rs28357094 genotype (black lines = TT; gray lines = TG/GG) and GC treatment (continuous lines = at least 1 year while ambulatory; dashed lines = <1 year or untreated).

0.78 ± 0.18 ($p = \text{n.s.}$). The HR for GC treatment was 0.39 ± 0.06 ($p < 0.001$). In this and the following analyses, participant numbers for the 2 genotyped SNPs differ slightly, because of limited availability of genomic DNA for a few participants.

These data show directions of association as previously reported (*SPP1* TG/GG genotype: earlier age at LoA; *LTBP4* TT genotype: later age at LoA). *SPP1* was statistically significant in the log-rank comparison of

median LoA, but not in the GC treatment–adjusted Cox model, whereas *LTBP4* did not reach statistical significance. Findings for *LTBP4* were similar for 24 of 32 rs10880 TT homozygotes carrying the whole IAAM/IAAM haplotype (data not shown).

When grouping only GC-treated participants (at least 1 year of treatment while ambulatory), we observed a 1.9-year difference in median LoA between *SPP1* rs28357094 genotypes; median ages at LoA were 12.0 and 13.9 years for

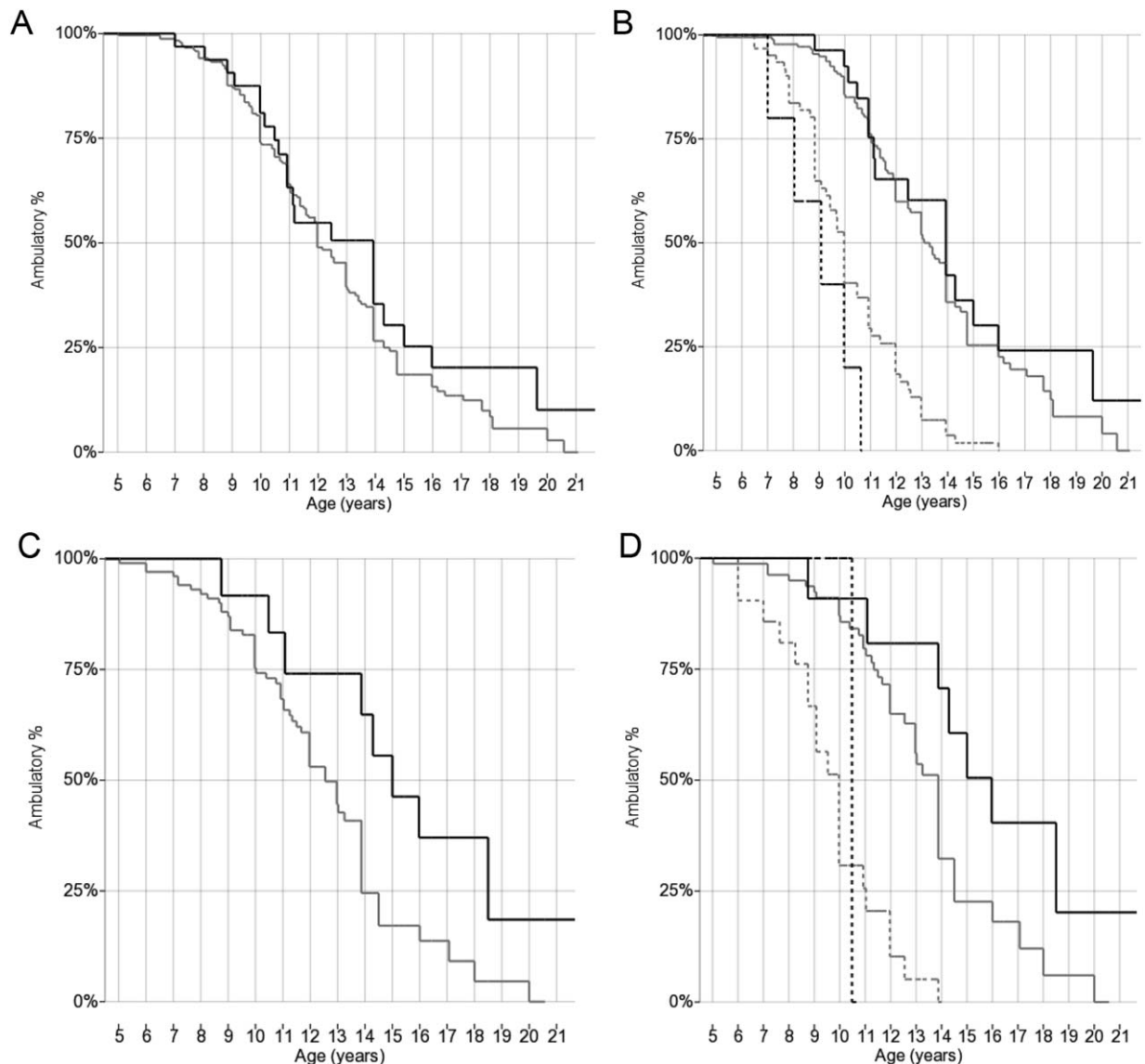


FIGURE 3: Kaplan–Meier plots of age at loss of ambulation grouped by *LTBP4* rs10880 genotype. (A) All patients genotyped for *LTBP4* rs10880, including all races and ethnicities ($n = 274$), grouped 2 ways by rs10880 genotype (black line = TT; gray line = CC/CT). (B) All patients genotyped for *LTBP4* rs10880, including all races and ethnicities ($n = 274$), grouped 4 ways by rs10880 genotype (black line = TT; gray line = CC/CT) and GC treatment (continuous lines = at least 1 year while ambulatory; dashed lines = <1 year or untreated). (C) Caucasian cohort controlled for population stratification and genotyped for *LTBP4* rs10880 ($n = 115$), grouped 2 ways by rs10880 genotype (black line = TT; gray line = CC/CT). (D) Caucasian cohort controlled for population stratification and genotyped for *LTBP4* rs10880 ($n = 115$), grouped 4 ways by rs10880 genotype (black line = TT; gray line = CC/CT) and GC treatment (continuous lines = at least 1 year while ambulatory; dashed lines = <1 year or untreated).

$n = 63$ GG/GT and $n = 150$ TT, respectively (log-rank $p = 0.032$, see Fig 2B). In the Cox regression model with GC treatment as time-varying covariate, the HR for TG/GG genotype was 1.61 ± 0.32 ($p = 0.016$). The HR for GC treatment was 1.30 ± 0.49 ($p = \text{n.s.}$). Median ages at LoA were identical (10.0 years) for untreated participants with different *SPP1* genotypes ($n = 21$ and 45 , respectively, see Fig 2B). This suggests that the *SPP1* locus may be a pharmacodynamic marker for GC response, rather than directly modifying DMD severity.

As for the *LTBP4* rs10880 genotype, median ages at LoA in GC-treated participants were 13.3 and 13.9 years for the CC/CT and TT genotype, $n = 178$ and 27 , respectively (log-rank $p = \text{n.s.}$, see Fig 3B). In the Cox regression model with GC treatment as time-varying covariate, the HR for the TT genotype was 0.74 ± 0.20 ($p = \text{n.s.}$). The HR for GC treatment was 1.08 ± 0.40 ($p = \text{n.s.}$). The number of untreated participants with the rare recessive genotype was too small for a meaningful comparison ($n = 64$ and 5 , see Fig 3B).

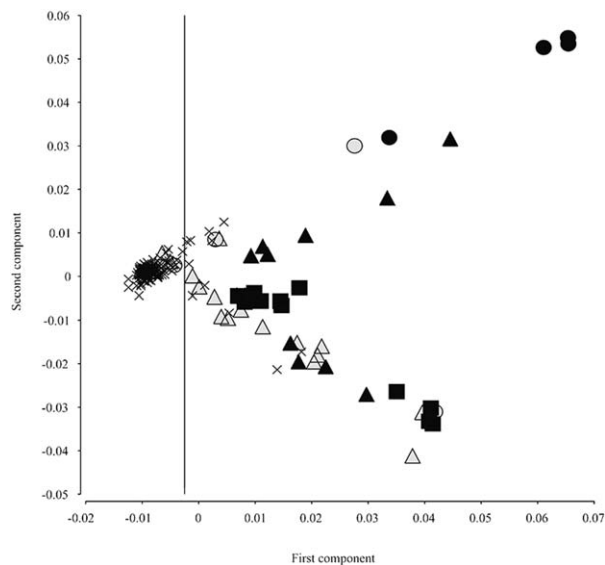


FIGURE 4: Cartesian plot of multidimensional scaling analysis of genome-wide marker population stratification. Values of the 2 highest ranking components are shown (1st on the x-axis and 2nd on the y-axis). Shape and color of the markers indicate self-identified ethnicity. Participants self-identifying as non-Hispanic Caucasian, indicated by x-shaped markers, form a cluster with low values of the first component (< -0.0025 , vertical cutoff line). Forty-five participants with other self-identified races and ethnicities are mostly positioned right of the cutoff line: African American (filled circles), Asian (filled squares), Hispanic Caucasian (empty triangles), Hispanic (filled triangles), and Other (empty circles). Twelve participants self-identifying as non-Hispanic Caucasians appear as outliers, whereas 3 participants self-identifying as Hispanic Caucasian or Other cluster together with non-Hispanic Caucasians and are included in subsequent analyses.

MDS Analysis Shows Admixture and Population Stratification

MDS analysis was based on the calculation of identity-by-state pairwise distances, performed on 175 participants with available genome-wide markers. Compared to 108 patients excluded from this analysis because of unavailability of genome-wide markers (DNA quantity and quality not sufficient for SNP chip analysis), there were no significant differences in GC treatment rate (75.0% vs 76.5%) or median age at LoA (13.0 vs 12.0 years, log-rank $p = 0.12$, Supplementary Fig).

MDS analysis identified a first principal component with lower values for participants of European ancestry. This component is plotted on the x-axis in Figure 4. The y-axis represents the second principal component. On the left side of the plot (low values of first principal component), 118 participants self-identifying mostly as non-Hispanic Caucasian ($n = 115$), and rarely as Hispanic-Caucasians ($n = 2$) or Other ($n = 1$), are clustered closely together, indicating a subcohort of relatively homogeneous European ancestry. Of the remaining 57 patients

with higher values of the first component, 12 self-identified as non-Hispanic Caucasian, appearing as “outliers” on the right side of the plot, and indicating admixture and population stratification within self-identified Caucasian participants.

Association Analyses in the Caucasian Cohort Controlled for Population Stratification Lead to Validation of Both *SPP1* and *LTBP4* Association with Age of LoA

As MDS analysis showed population stratification within self-identified racial-ethnic groups, to adjust for population-related confounding factors in genetic association, we restricted subsequent analyses to 118 patients showing no evident population stratification (see Fig 1). Of these, 116 were successfully genotyped for *SPP1* rs28357094. Median ages at LoA were 12.0 and 13.9 years, $n = 35$ and 81 for TG/GG and TT, respectively (log rank $p = 0.047$, see Fig 2C). In the Cox regression model with GC treatment as time-varying covariate, the HR for TG/GG genotype was 1.54 ± 0.17 ($p = 0.09$), and the HR for GC treatment was 0.26 ± 0.07 ($p < 0.001$).

Of the participants described above, 93 were treated with GC for at least 1 year while ambulatory. For these patients, KM median ages at LoA were 12.0 and 13.9 years for TG/GG and TT genotypes, $n = 26$ and 67 (log-rank $p = 0.07$, see Fig 2D). When applying the Cox regression model with GC treatment as time-varying covariate, HR for TG/GG genotype was 1.85 ± 0.57 ($p = 0.047$). The HR for GC treatment was 0.72 ± 0.39 ($p = \text{n.s.}$). In 23 GC-untreated patients, KM median ages at LoA were 9.0 and 10.0 years for TG/GG and TT ($n = 9$ and 14, respectively, $p = \text{n.s.}$, see Fig 2D).

Of 118 participants in the Caucasian cohort controlled for population stratification, 115 were genotyped for *LTBP4* rs10880. KM curves plotted for this group showed delayed median LoA (15.0 years) in 12 participants carrying the TT genotype, in contrast to 103 carrying the CC/CT genotype (12.6 years, log-rank $p = 0.024$, see Fig 3C). Of these 12 participants, 9 were homozygotes for the full IAAM haplotype, whereas 3 were heterozygotes for IAAM and other rare *LTBP4* haplotypes (VAAM and VTTM). In the Cox regression model with GC treatment as time-varying covariate, HR for TT genotype was 0.49 ± 0.19 ($p = 0.07$). The HR for GC treatment was 0.26 ± 0.07 ($p < 0.001$).

Of the participants described in the previous paragraph, 91 were GC treated for at least 1 year before LoA. Within this group, KM median age at LoA was 16.0 years for TT genotype and 13.8 for CC/CT, $n = 11$ and 80 (log-rank p -value = 0.046, see Fig 3D). The Cox

regression model with GC treatment as time-varying covariate showed an HR for TT genotype of 0.47 ± 0.20 ($p = 0.08$). The HR for GC treatment was 0.75 ± 0.40 ($p = \text{n.s.}$). The presence of just 1 GC-untreated participant with rs10880 TT genotype precludes statistical analysis of GC-untreated participants for *LTBP4* in this subgroup.

Taken together, these findings support the protective effect of the rs10880 TT genotype described by Flanagan et al.⁹ This was confirmed by reanalyzing CINRG-DNHS data with the 6-month GC treatment threshold used in the original report. All KM and Cox regression parameters for this reanalysis are shown in Supplementary Table 2.

Discussion

We aimed to study the effect of 2 genetic modifiers on DMD phenotype, using age at LoA as a disease severity indicator, in the CINRG-DNHS cohort. This cohort includes 340 participants followed longitudinally for an average of 4 years, and the study design and baseline data have been recently reported.^{18,19} We grouped participants by genotype at 2 loci associated with age at LoA in DMD (*SPP1* rs28357094⁴ and *LTBP4* haplotype⁹). We had previously reported a cross-sectional analysis of grip strength as a function of *SPP1* genotype in a subset of this cohort ($n = 156$), not stratified for ethnicity. Here, we report time to event analyses for age at LoA in the complete CINRG-DNHS cohort (except 57 participants with unavailable DNA samples) for both the *SPP1* and *LTBP4* loci, controlling for population stratification and GC treatment as possible confounders.

It is well established that different ethnic groups show different MAFs for any specific genetic polymorphism, as well as different LD between genetic markers and functional variants. This can lead to hidden population stratification even within self-identified racial/ethnic groups, and thus to false-positive or false-negative findings in genetic association. The CINRG-DNHS cohort recruited participants from 20 clinical centers in 4 different continents, and is ethnically heterogeneous, although with a majority of Caucasian participants. Both previous reports^{14,15,17} and observed tendencies of the phenotype to differ between ethnic groups in our data further stress the importance of accounting for population stratification issues. Conversely, because of inherent study design characteristics of the CINRG-DNHS, which did not purposely recruit representative racial/ethnic subgroups, conclusive statements cannot be made about racial/ethnic disparities in DMD, based on our data.

The second potential confounding factor, GC treatment, is probably the single environmental factor most

heavily affecting age at LoA in DMD.^{22–25} We accounted for it by implementation in time to event models, as a grouping criterion for KM analyses and a time-varying covariate for Cox regression. Because of CINRG-DNHS population characteristics, that is, a numerical predominance of GC-treated participants, our findings can be generalized more confidently to GC-treated DMD populations. Nevertheless, this is more relevant for patient care and clinical trials, as GC treatment is considered a standard of care,² and often an inclusion criterion in clinical trials of new treatments.

We first studied SNP effects in the entire cohort with available DNA samples, without grouping for GC treatment or ethnicity. The *SPP1* rs28357094 G allele, in a dominant model, was associated with 1.2-year earlier median LoA. This reproduces the methodology of the original report⁴ and represents an independent validation of association with LoA. The recessive *LTBP4* rs10880 T allele, in close LD with the IAAM haplotype, showed a direction of association as previously reported,⁹ but not a statistically significant difference.

We then grouped participants genotyped for *SPP1* and *LTBP4* by GC treatment. In our baseline analysis of 156 DNHS participants, the association of *SPP1* genotype with grip strength showed the largest effect in GC-treated participants⁴; and an 80-patient Italian cohort, in which the association was established with longitudinal changes of ambulation-related functional measures,²⁶ was almost entirely GC treated. In line with these previous findings, which suggest a stronger modifier role of *SPP1* in GC-treated patients, an effect of *SPP1* genotype on LoA in the CINRG-DNHS cohort was observed in the GC-treated subgroup (see Fig 2B). This finding supports a role of *SPP1* rs28357094 as a modulator of GC response in DMD, rather than of disease progression itself. This is in concordance with several preclinical studies of *SPP1* promoter function; the minor G allele at rs28357094 decreases transcriptional activity of the gene at baseline,⁶ but shows a 3-fold increase in gene expression in response to steroids, whereas the common allele leaves expression unchanged by steroids.⁷ Consistent with a steroid-induced alteration of *SPP1* expression, differences in *SPP1* mRNA levels between genotypes were not found in vivo in DMD diagnostic muscle biopsies obtained prior to GC treatment.²⁷ Evidence of a sexually dimorphic effect of rs28357094 genotype on muscle size and remodeling in Caucasian women,²⁸ and of an increased transcriptional response of the *SPP1* promoter to estrogen stimuli,⁷ are consistent with a pharmacodynamic role of this genetic biomarker. GCs are well-known transcriptional regulators of inflammation-related genes,²⁹ both directly, through positive or negative GC-

responsive elements (GREs), and indirectly, through suppression of other transcription factors (eg, NF- κ B); and these mechanisms are relevant to GC efficacy and possibly side effects in DMD.^{30,31} The *SPP1* promoter is predicted to contain both GREs and NF- κ B-responsive sites, and further studies are needed to dissect these mechanisms both in vitro and in vivo. To our knowledge, this is the first demonstration of a pharmacodynamic biomarker for response to GCs, and this may be relevant to other conditions where GCs are standard of care. Our data, combined with the recent publication of Barfield et al,⁷ suggest that the 20 to 30% of DMD patients with the rare *SPP1* allele are poor responders to GCs, possibly with an altered balance between beneficial and side effects. Future studies are needed to confirm this association, before routine genotyping of *SPP1* is considered as part of standard of care in DMD.

Flanigan et al⁹ described a larger effect of the protective *LTBP4* variant in GC-treated patients than in untreated patients. In the CINRG-DNHS cohort, when stratifying by GC treatment and *LTBP4* genotype, we did not observe differences in genotype effect between the treated and untreated populations. Reanalyzing data with the same GC treatment threshold (at least 6 months before LoA) as in the original report did not modify these findings (see Supplementary Table 2).

To adjust for potential population stratification bias, we performed MDS analysis on a subgroup of 175 participants. This showed that the correspondence between self-identified ethnicity and unbiased grouping determined by genotype identity was partial, with several outlier self-identifying Caucasian participants (admixture). Subsequently, we selected MDS rather than self-identification as a method to adjust for population stratification. A limitation of this study is the unavailability of genome-wide markers in the whole cohort, which would have allowed the selection of a larger homogeneous subcohort. However, the reason for exclusion of 108 patients was technical (DNA sample quality and quantity) and not linked to any clinically relevant variables. Furthermore, we verified that excluded patients did not significantly differ in terms of GC treatment and age at LoA (see Supplementary Fig). Thus, we expect the cohort analyzed by MDS to be representative of the whole CINRG-DNHS.

In the smaller but more homogeneous MDS-selected Caucasian subcohort, *LTBP4* rs10880 was confirmed as a strong modifier of ambulatory function in DMD, with a median age at LoA in carriers of the protective genotype of 15.0 years overall, and 16.0 years in those with GC treatment (see Fig 3C–D), which positions >50% of these patients within the “intermediate

dystrophinopathy” clinical spectrum. The stronger association of rs10880 in Caucasians did not seem to be in direct relation with a stronger LD with the full IAAM haplotype (see Supplementary Table 1), although numbers are too small to reach a conclusion (ie, very few patients with rare haplotypes). It could also be hypothesized that in Caucasians the T allele for rs10880 might be in stronger LD with another unrecognized, functional variant, than in patients with different ancestries. Conversely, Flanigan et al did not find additional nonsynonymous coding variation by resequencing 40 chromosomes and querying the 1000 Genomes database, and conducted in vitro experiments showing an effect of the coding haplotype on TGF β signaling, in conditions of equal *LTBP4* protein expression.⁹ As for *SPP1* rs28357094, the effect on age at LoA in the Caucasian subcohort appears smaller in magnitude than that of the *LTBP4* haplotype. This is similar to what was described in the single center cohort from Padua,⁴ which was recruited from a homogeneous Caucasian population (great majority from northeastern Italy) with a predominance of GC-treated participants.

Recently, another genetic modifier study in DMD was published by a collaborative European group,³² further confirming the effect of the *LTBP4* IAAM haplotype in delaying LoA. Conversely, the *SPP1* association was not replicated in 336 patients, of whom 102 had been treated with GCs for at least 1 year while ambulatory. If *SPP1* is a modifier of GC response, as our association data and in vitro findings suggest, this low GC treatment rate might have limited the power of this part of the study.

Lastly, although the genetic modifiers described here seem to explain some of the variance of the LoA phenotype in DMD, both from our data and from case reports of outlier DMD phenotypes³³ it appears that several other, yet uncharacterized genetic factors must be at play.

In conclusion, our findings show that *SPP1* rs28357094 acts as a modifier of the long-term effect of GC treatment in the CINRG-DNHS. Furthermore, we confirm that *LTBP4* rs10880 modifies age at LoA in DMD. Our data also stress the importance of adjusting for GC treatment and population substructure in genetic association studies in DMD. These findings are relevant for future analyses of observational and interventional studies involving international, multicentric, ethnically diverse cohorts.

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Authorship

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Potential Conflicts of Interest

EKH: grant, Parent Project Muscular Dystrophy US; past member, CINRG Executive Committee; consultancy, PTC Therapeutics, Genzyme. E.P.: speaking fee, Genzyme; scientific advisory board, Biomarin. C.M.M.: grant, Parent Project Muscular Dystrophy US.

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