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Ethnicity-related DMD Genotype Landscapes in European and Non-European Countries

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Abstract

Objective

Genetic diagnosis and mutation identification are now compulsory for Duchenne (DMD) and Becker muscular dystrophies (BMD), which are due to dystrophin (*DMD*) gene mutations, either for disease prevention or personalized therapies. To evaluate the ethnic-related genetic assortments of *DMD* mutations, which may impact on DMD genetic diagnosis pipelines, we studied 328 patients with DMD and BMD from non-European countries.

Methods

We performed a full DMD mutation detection in 328 patients from 10 Eastern European countries (Poland, Hungary, Lithuania, Romania, Serbia, Croatia, Bosnia, Bulgaria, Ukraine, and Russia) and 2 non-European countries (Cyprus and Algeria). We used both conventional methods (multiplex ligation-dependent probe amplification [MLPA] followed by gene-specific sequencing) and whole-exome sequencing (WES) as a pivotal study ran in 28 patients where *DMD* mutations were already identified by standard techniques. WES output was also interrogated for *DMD* gene modifiers.

Results

We identified *DMD* gene mutations in 222 male patients. We identified a remarkable allele heterogeneity among different populations with a mutation landscape often country specific. We also showed that WES is effective for picking up all *DMD* deletions and small mutations and

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Glossary

ACMG = American College of Medical Genetics and Genomics; **BMD** = Becker muscular dystrophy; **CK** = creatine kinase; **DMD** = Duchenne muscular dystrophy; **IGV** = Integrative Genomics Viewer; **LGMD** = limb-girdle muscular dystrophy; **NGS** = next-generation sequencing; **OMIM** = Online Mendelian Inheritance in Man; **VUS** = variant of uncertain/unknown significance; **WES** = whole-exome sequencing; **WGS** = whole-genome sequencing.

its adoption could allow a detection rate close to 90% of all occurring mutations. Gene modifiers haplotypes were identified with some ethnic-specific configurations.

Conclusions

Our data provide unreported mutation landscapes in different countries, suggesting that ethnicity may orient genetic diagnosis flowchart, which can be adjusted depending on the mutation type frequency, with impact in drug eligibility.

Dystrophinopathies are a group of X-linked allelic diseases caused by mutations in the dystrophin gene (*DMD* gene, Online Mendelian Inheritance in Man [OMIM] *300377) affecting every 1 in 5,000 male births worldwide. Mutations in the *DMD* gene lead to a spectrum of diseases, from the severe Duchenne (DMD, #310200) and the milder Becker (BMD, #300376) muscle dystrophies to the isolated dilated cardiomyopathy (#302045) and other mild phenotypes or asymptomatic males.[1–3] The frame rule assumes that the functional consequences of *DMD* mutations are related to the maintaining of the open reading frame, allowing a shorter and partially functional dystrophin to be translated. Therefore, mutations that maintain the frame result in the milder BMD phenotype, whereas out-of-frame mutations cause multiple premature stop codons downstream and are associated with the severe DMD phenotype, although some exceptions occur.[4] Genetic testing is required to confirm a dystrophinopathy diagnosis and allows genetic counseling, prevention, and patients eligibility for already-approved drugs or novel personalized therapeutic treatments.[5,6]

We approached 328 patients referred to us by 10 Eastern European countries (Poland, Hungary, Lithuania, Romania, Serbia, Croatia, Bosnia, Bulgaria, Ukraine, and Russia) and 2 non-European countries (Algeria and Cyprus) with the aim of identifying *DMD* mutations. We also validated whole-exome sequencing (WES) as an accurate and highly sensitive method of detecting all *DMD* deletions and small mutations, although not duplications, and also gene modifiers. Our study allowed to identify different mutation landscapes in the studied populations and primed up considerations for personalized treatments and readjusted diagnostic flowcharts based on the ethnicities.

Methods

Patient Enrollment

Patient enrollment was conducted based on the clinical diagnosis with a suspicion of a dystrophinopathy. We enrolled 328 male patients (June 2016 and December 2019) from Algeria (68), Cyprus (2), Bosnia (1), Bulgaria (12), Croatia (5), Hungary (14), Lithuania (6), Poland (66), Romania (62), Russia (1), Serbia (2), and Ukraine (89). A total of 328 affected males and 28 females were tested for *DMD* gene mutations. Among the 328 males, 229 were clinically diagnosed as DMD, 27 as BMD, 33 as dystrophinopathy, and 39 as high CK. The 28 at-risk females were from Bosnia (1), Bulgaria (1), Croatia (3), Hungary (4), Poland (1), Romania (13), Ukraine (1), and Algeria (4). The clinical diagnosis was conducted according to established standard clinical outcome measures and scales for *DMD* mutation.[7]

Ethical Issues

Ethical consent forms for genetic testing were collected at each clinical center, which referred the patients to our institution as part of the routine diagnostic procedures for DMD genetic diagnosis approved by each local ethical committee. Whole-exome sequencing data were analyzed for *DMD* mutations and gene modifiers (for scientific purpose) only, as per the ethical consent forms. Study design and data analyses described in this article were performed based on Ethical Approval N. 55/2016 and N. 66/2020/Oss/AOUFe.

DMD Deletion and Duplication Identification

Genomic DNA was extracted from EDTA-preserved whole blood using QIAsymphony Instrument and Kits following the manufacturer's instructions. Multiplex ligation-dependent probe amplification (MLPA) assay was performed on all 328 male patients using the P034/P035 DMD Kit (MRC Holland, Amsterdam, the Netherlands) according to the manufacturer's instructions. The reaction products were analyzed using a DNA analyzer (ABI 3130 XL, Applied Biosystems, Foster City, CA, USA), and data analysis was performed using Coffalyser (MRC Holland, Amsterdam, the Netherlands) and GeneMarker (SoftGenetics, State College, PA, USA) software for MLPA. When the MLPA result suggested a single exon deletion or duplication, the result was always confirmed by an alternative molecular technique, which was PCR and sequencing for single-deleted exons (including the 5′ and 3′ adjacent exons) and exon-specific real-time PCR for single exon duplications, according to current DMD diagnosis guidelines.[8,9]

DMD Small Mutation Identification

The DMD gene was sequenced in all patients resulted negative by MLPA testing using next-generation sequencing (NGS) approach. DMD MASTR™ assay (Multiplicom, Niel, Belgium) was used according to the manufacturer's instructions. All 79 exons were sequenced with a minimal coverage per allele of 50X and run on MiSeq (Illumina, San Diego, CA, USA). Data analyses were performed using Sophia Genetics pipeline. All detected variants were validated by Sanger sequencing.

WES Analysis

WES pilot validation study was performed on 28 male patients already tested using MLPA and NGS sequencing and proven positive for the presence of a *DMD* mutation. MGIEasy Exome Capture V4 Probe Set (MGI, Shenzhen, China) was performed to enrich the coding regions, and constructed libraries were then sequenced by using Illumina HiSeq2000 platform with 90-bp paired-end reads. Standard analysis of the raw data was performed as previously described,[10] including alignment, variant (single nucleotide polymorphism [SNP], insertion/deletion polymorphism [InDel], and copy number variation [CNV]) calling, and annotation. Integrative Genomics Viewer (IGV) software[11] was also used to determine the coverage of every exon of the DMD gene and the quality of the reads.

Data Analysis and Bioinformatics Tools

All mutations/variations recorded in this study were annotated according to the standards established by the Human Genome Variation Society (HGVS, hgvs.org) and available databases established by the Leiden Muscular Dystrophy pages (dmd.nl), the Leiden Open Variation Database 3.0 (lovd. nl/3.0/home), and UMD-DMD (umd.be/DMD/).[12] All variations were checked for their presence in databases (LOVD, ClinVar, and gnomAD), and their possible pathogenic meaning was verified using the MutationTaster, PolyPhen, and Human Splicing Finder in silico prediction tools. All known and novel variations (known pathogenic, likely pathogenic, and variant of uncertain/unknown significance [VUS]) identified in this study were deposited in the LOVD database.

Data were analyzed looking for mutation type (deletions, duplications, and small mutations), frequency, and distribution along the *DMD* gene and mutation hotspots. We considered mutations occurring in DMD and BMD as main clinical categories. Patients were categorized based on their country of origin to identify mutation type frequency differences related to ethnicity.

We also categorized mutations amenable for personalized therapies (exon 44, 45, 51, and 53 skipping by antisense oligoribonucleotides and stop codon reversion).[13,14] We categorized previously unreported variants based on the ACMG classification[15] as likely pathogenic and variant of uncertain significance and reported them separately.

Data Availability

The data generated and analyzed in the current study will be available and shared by request to the corresponding author (A.F.) from any qualified investigator. These data include clinical data and raw genome files (NGS, MLPA, WES, and Sanger sequences).

Results

Table 1 shows the result of *DMD* testing in all patients with DMD/BMD. Of the 328 male patients, 222 resulted positive for carrying a hemizygous *DMD* variation (the detection rate was 68%), whereas 106 resulted negative. Among the 222 patients carrying a *DMD* mutation/variation, DMD were 188, and BMD were 24. In 10 patients with DMD (1), BMD (1), high CK (7), and dystrophinopathy (1) phenotypes, we identified VUS. Dystrophinopathy definition was adopted by clinicians when a phenotype was not typical or when the boys' age (younger than 7 years) did not allow unequivocal clinical diagnosis. Among the 106 negative cases, 34 were defined as DMD, 8 as BMD, 39 as high CK, and 25 as dystrophinopathy. Among all identified variants, 26 (all from Eastern European patients) were previously unreported and categorized as likely pathogenic (table e-1, links.lww.com/NXG/A338). Another 9 variations (in 10 patients) were categorized as VUSs (table e-2, links.lww.com/NXG/A339). Novel, likely pathogenic variants were included in the data analysis, whereas VUSs were not included in the statistics.

Frame Rule

In the 72 patients with DMD and 16 patients with BMD carrying DMD deletions, the frame rule applies to 98.5% of DMDs (being out of frame) and 95% of BMDs (being in frame). For duplications, the frame rule applies to 99% of DMDs (18 of 19 patients) and to the only 1 duplicated BMD we have identified, being in frame.

Deletion and Duplication Hotspots

We observed a total of 88 deletions (regardless of the country of origin), 72 in patients with DMD and 16 in patients with BMD with a very low frequency of deletions in Eastern European countries. Deletion distribution shows the absence of the usual hotspot around exons 2–7, with all exons until exon 37 rarely involved in deletion events. The well-known hotspot with breakpoint in intron 44 is present in 21 patients. Also evident is a recurrent breakpoint in intron 45 (18 patients) and intron 50 (25 patients), with therefore 73% of deletions having 1 breakpoint within introns 44, 45, and 50 (figure e-1a, links.lww.com/NXG/A342). The statistical analysis of duplications is hampered by the very low number of duplicated patients (18 DMDs and only 1 BMD) (figure e-1b, links.lww.com/NXG/A342).

Mutation Frequency and Distribution in Patients With DMD

We considered our patients as belonging to 2 distinct groups: Eastern Europe (Bosnia, Bulgaria, Croatia, Hungary, Serbia, Lithuania, Poland, Romania, Ukraine, and Russia) and Algeria. Cyprus was not included in the statistics (only 2 cases).

The ethnicity of Eastern Europe is quite heterogeneous, being composed by more than 40 different ethnic groups[16]; nevertheless, a few studies demonstrated high genetic background

Table 1 Centers and Genotypic Data of the Patients

	Patients enrolled	Negative for DMD mutations	Positive for DMD mutations	DMD	BMD	VUS [a]	Deletions all	Deletions DMD	Deletions BMD	Duplications all	Duplications DMD	Duplications BMD	Nonsense all	Nonsense DMD	Nonsense BMD	Missense all	Missense DMD	Missense BMD	Frameshifting all	Frameshifting DMD	Frameshifting BMD	Canonical splice sites all	Canonical splice sites DMD	Canonical splice sites BMD	Splicing consensus sites all	Splicing consensus sites DMD	Splicing consensus sites BMD
Algeria	68	16	52	39	9	4	36	30	6	2	2	0	4	2	2	3	1	1	2	2	0	2	2	0	3	0	0
Bosnia	1	0	1	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Bulgaria	12	0	12	11	1	0	1	1	0	1	1	0	6	6	0	0	0	0	0	0	0	4	3	1	0	0	0
Croatia	5	0	5	4	1	0	0	0	0	0	0	0	3	3	0	0	0	0	1	0	1	1	1	0	0	0	0
Hungary	14	5	9	9	0	0	1	1	0	0	0	0	4	4	0	0	0	0	3	3	0	1	1	0	0	0	0
Lithuania	6	2	4	4	0	0	0	0	0	1	1	0	2	2	0	0	0	0	1	1	0	0	0	0	0	0	0
Poland	66	30	36	31	1	4	2	2	0	2	2	0	13	12	1	3	0	0	11	11	0	5	5	0	1	0	0
Romania	62	25	37	29	7	1	23	17	6	3	3	0	4	4	0	0	0	0	4	4	0	2	1	1	1	0	0
Russia	1	0	1	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Serbia	2	0	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	2	0	0	0	0	0	0	0
Ukraine	89	27	62	56	5	1	25	21	4	9	8	1	15	15	0	2	1	0	7	7	0	3	3	0	1	1	0
Cyprus	2	1	1	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	328	106	222	188	24	10	88	72	16	19	18	1	53	50	3	7	2	1	31	30	1	18	16	2	6	1	0

Abbreviations: BMD = Becker muscular dystrophy; DMD = Duchenne muscular dystrophy; VUS = variant of uncertain/unknown significance.
[a] See table e-2 (links.lww.com/NXG/A339).
All the variants identified were submitted to the LOVD database (lovd.nl). The table reports on countries and clinical centers, which have referred the patients to our Unit, and the mutations identified in DMD and BMD phenotypes only. Patients with BMD showing a *DMD* mutation were only 24. Among these, 16 patients resulted as carrying a *DMD* deletion and only 1 patient carried a duplication, 3 BMD carried a nonsense, 1 a missense mutation, 1 a small in frame deletion, and 2 patients with BMD showed splicing mutations. Algerian patients showed 6 deletions (5 in frame and 1 out of frame), Romanian patients showed 6 in-frame deletions and 1 canonical splice site mutation; Ukrainian patients showed 4 in-frame deletions and 1 duplication; Polish patients showed 1 nonsense; patients from Bulgaria and Croatia showed 1 canonical splice site and 1 small in-frame deletion, respectively.

admixture for which we estimated these countries as a single group for our *DMD* mutation statistical analysis. From the Eastern European countries, a total of 258 patients were genotyped, and 169 patients were diagnosed with a *DMD* mutation. Mutations were predominantly nonsense (31%) and deletions (29%), followed by small frameshifting variations (18%), duplications (11%), and canonical site splicing variants (9%). Missense and consensus splicing variations were also identified (1%). Eighty-nine patients did not show a typical *DMD* mutation (detection rate 65%) (figure 1A).

A *DMD* mutation was identified in 52 of the 68 samples from Algeria (detection rate 76%) (figure 1B). The vast majority of Algerian patients carried deletions (77%), followed by all other mutations in very similar percentages. In a previous publication, a similar mutation landscape was reported.[17] By merging these data, 86% of Algerian patients carry deletions and 14% all others.

Figure 2 shows the overview of the mutation landscape in each Eastern European country and in Cyprus. The number of patients with BMD (24) is too low to allow statistics. Among the 28 at-risk females, 11 were identified as heterozygous carriers.

WES Analysis

Twenty-eight patients with proven *DMD* mutations (12 deletions, 15 small mutations, and 1 duplication) were used to validate WES analysis (table e-3, links.lww.com/NXG/A340).

As expected, all *DMD* small variants previously detected using NGS sequencing were identified by WES analysis. In addition, all MLPA identified deletions were also picked up by WES. Figure 3, A and B shows software visualization of 2 deletions (exon 51 and exons 18–19), where no reads were visible within the deleted interval. In both cases, WES analysis defined the deletion intervals within intron borders, thus excluding intraexonic deletions. The duplication was not identified by WES (data not shown).

DMD Gene Modifier Haplotyping

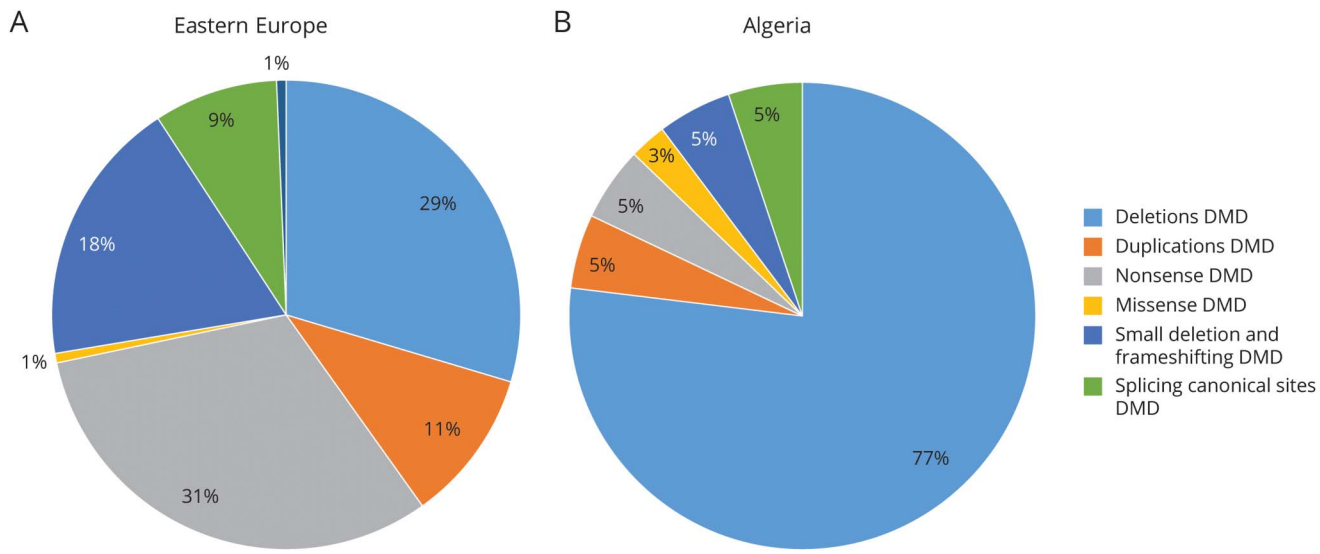
WES analysis performed in the 28 DMDs allowed to call SNPs variations in genes known to be DMD genetic modifiers as SPP1, LTBP4, CD40, *THBS1*, and *ACTN3*.[18] We could not obtain genotypes of *SPP1* and *THBS1* SNP regions (rs28357094 and rs2725797, respectively) because they were not covered by WES enrichment. *LTPB4, CD40*, and *ACTN3* were fully covered, and we profiled the *LFTB4* haplotypes and the *CD40* and *ACTN3* genotypes. Results are shown in table 2.

We also identified in 8 patients other exonic SNPs occurring in 3 gene modifiers, namely 3 SNPs within the *LTPB4* gene, 9 SNPs in *ACTN3*, and 2 SNPs in *THBS1* (table e-4, links.lww.com/NXG/A341). The majority are synonymous (6) and missense changes (7), whereas 1 was a nonsense variant in exon 21 of the *ACTN3* gene.

Mutations Amenable of New Therapeutic Approaches

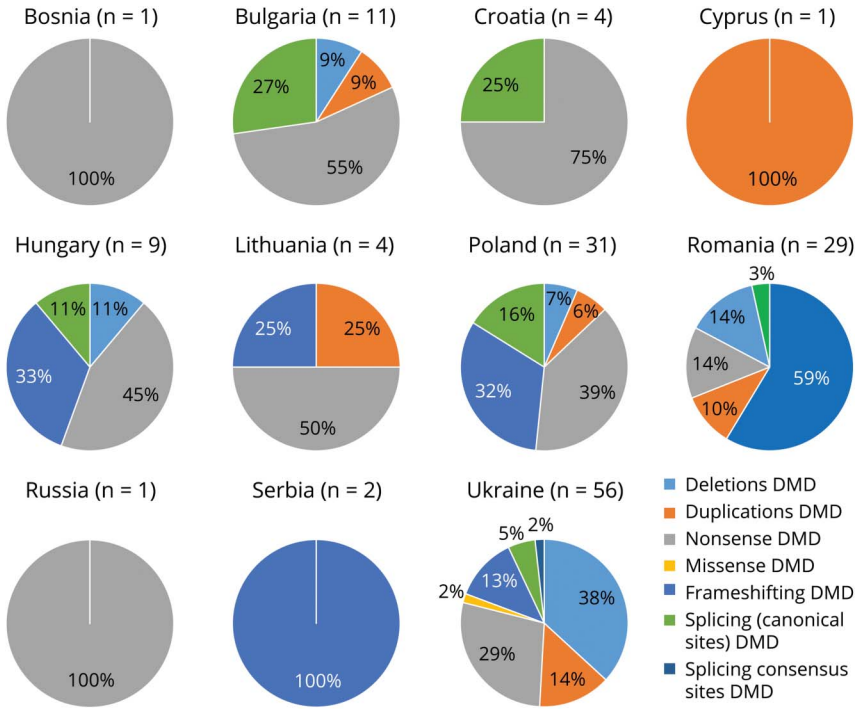
Patients carrying deletions amenable of exon-skipping therapeutic approaches were evaluated. In total, 41% of patients are

Figure 1 Distribution of *DMD* Mutations in Patients With DMD in Countries



Overview of mutation distribution in patients with DMD from Eastern Europe (A) and Algeria (B). Nonsense mutations were the most frequently occurring mutations in Eastern European patients, accounting for 31% of mutation types in the patients with DMD, followed by deletions (29%), frameshifting (18%), duplications (11%), and splicing canonical sites (9%); missense and consensus splicing are the least frequent (1% each). Deletions were the most frequent mutations in Algerian patients (77%), whereas nonsense (5%), frameshifting (5%), splicing canonical sites (5%), duplication (5%), and missense (3%) variations were the least frequent. No splicing consensus sequence mutations were identified in patients from Algeria. The reported numbers include known and novel, likely pathogenic, mutations/variations, but not VUS. Among the 28 females tested for carrier detection, we found 11 carriers, all heterozygous for large deletions (6) and small mutations (5). The remaining females were not carriers. The 2 patients from Cyprus were not included in the statistical analysis as 1 patient showed an exon 2 duplication, whereas the other resulted negative. DMD = Duchenne muscular dystrophy; VUS = variant of uncertain/unknown significance.

Figure 2 Country Distribution of *DMD* Mutations in Patients With DMD in Eastern European Countries



Geographical distribution of *DMD* mutations in Eastern European countries and relative number of patients (in brackets). The percentages for each type of DMD mutation are shown for each country. In countries with more than 30 patients with DMD carrying a DMD mutation, differences can be noted because Polish and Ukrainian patients have a similar mutation landscape with a very high frequency of small mutations (average of 55%), whereas in Romania, 31% of mutation types are small mutations with deletions, and 69% account for duplications. DMD = Duchenne muscular dystrophy.

eligible for exon 51 skipping (orphan drug being eteplirsen), 30% might be treated by exon 45 skipping, 18% by exon 53 skipping, and 11% by exon 44 skipping. Considering nonsense mutation correction by read-through mechanisms for stop codon reversion, 53 patients overall (50 DMDs or 27%) were found to be carrying a nonsense mutation for which ataluren is an approved orphan drug (figure 1).

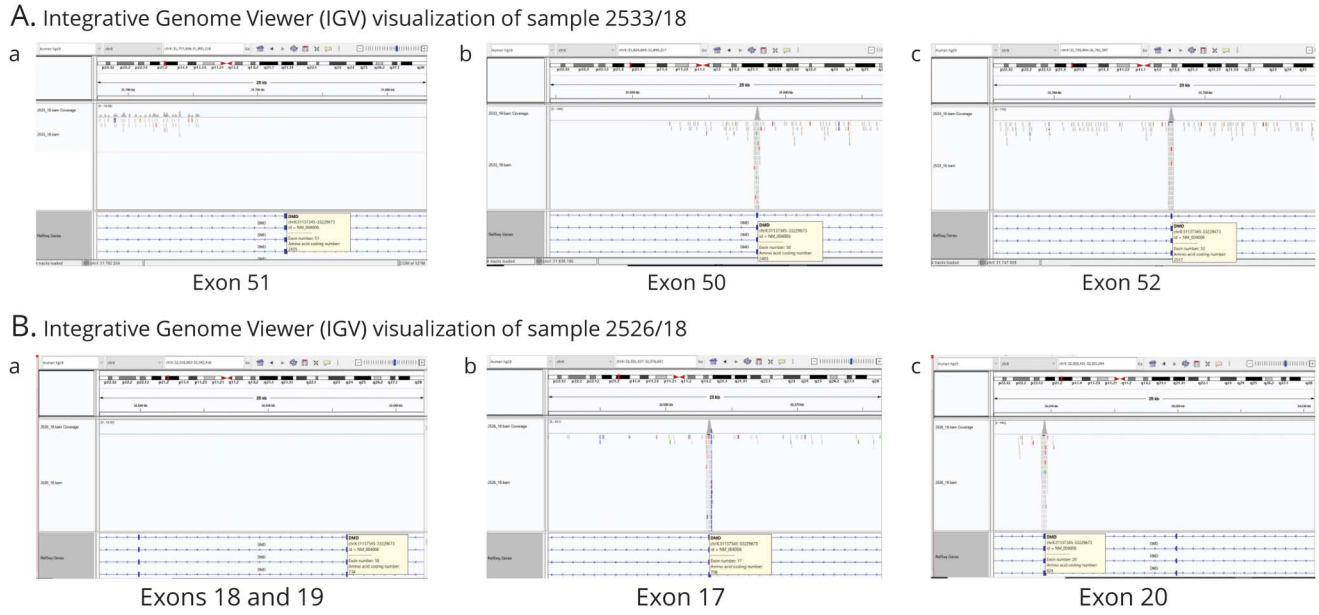
Discussion

DMD mutation identification is an integral part of the diagnostic flowchart for patients and their family, allowing genetic diagnosis, family planning, prenatal testing, and eligibility for personalized treatments.[19] Because of the enormous size of the dystrophin gene, the mutational spectrum is tremendously heterogeneous, and the genetic diagnostic approach must be both accurate and sensitive.

Here, we provide *DMD* gene genetic characterization in a large group of 328 patients with a clinical diagnosis of DMD/BMD, from Eastern European and non-European patients diagnosed between June 2016 and December 2019. Standard diagnostic approach based on best practice guidelines identified mutations in 222 patients.

Our analysis highlighted a relevant diversity in the mutation type across countries. Indeed, the frequency of deletions in Algeria (77%) vs Eastern Europe (29%) greatly varies. Conversely, nonsense mutations (31%) and small mutations (29%) represent the most frequent mutation type in these last countries, while rarely occurring in Algeria (18%). Ethnic origin and genotype assortments may play a role in this evident variable mutation distribution. The high percentage of still undiagnosed negative cases (34% Eastern Europe and 27.4% Algeria) is remarkable. We may firstly consider that some *DMD* mutations may have escaped MLPA and sequencing testing, as for atypical mutations occurring in regulatory regions or due to deep intronic variants/rearrangements.[20] Because these are estimated to be less than 1% in various patient series,[21,22,23,24] their impact on the detection rate should be low, and only a few cases might have been missed. Ethnicity may also play a role in mutation type frequency, as already described[23]; only the adoption of a single and fully accurate method able to detect all mutation types, such as whole-genome sequencing (WGS), will allow us to unravel these events and their frequency in the various populations.[25] Second, a few patients referred as DMD/BMD or generally as possible dystrophinopathies may represent phenocopies of other clinical entities like limb-girdle muscular dystrophies (LGMDs) or other rare hereditary myopathies.[26,27] Indeed, many patients with high CK-related phenotypes and resulted as not carrying a *DMD* mutation showed *DMD* VUSs which might be difficult to interpret (see below). LGMDs may indeed deserve a more intensive investigation, but this requires a multigene approach, for example, gene panel testing, WES, or, in the near future, WGS.[28] Finally, in countries with high consanguinity like Algeria,[29] a lower detection rate due to the occurrence of many recessive

Figure 3 Integrative Genome Viewer (IGV) Visualization



(A) IGV visualization of sample 2533/18. Visualization of sample 2533/18 with deletion of exon 51: (a) there is no coverage or readings of exon 51. (b and c) Visualization of coverage and reads of exons 50 and 52, which precede and follow the deletion of exon 51, respectively. (B) IGV visualization of sample 2526/18. Visualization of sample 2526/18 with deletion of exons 18–19. (a) This figure shows that there is no coverage or readings corresponding to exons 18 and 19. (b and c) Visualization of coverage and reads of the flanking exon 17 preceding the deletion and of exon 20 following the deletion.

LGMDs might be expected. In Algeria, however, the DMD detection rate was higher than in the Eastern European countries. Although the lack of muscle biopsy availability in negative patients did not allow an RNA study to look for *DMD* mutations, in some negative cases, the new tool of urinary stem cells to profiling the *DMD* transcript might be considered.[30] Because the DNA testing remains the gold standard to achieve a genetic diagnosis, only a genome-based diagnostic approach will allow us to define the full *DMD* mutation scenario.

We identified 9 previously unreported VUSs (1 shared by 2 brothers) in the *DMD* gene. The pathogenic effect of these VUSs is often based on an in silico prediction model (table e-2, links.lww.com/NXG/A339), although VUS requires functional validation, possibly by RNA profiling or segregation studies in larger families to assess their pathogenic meaning. Therefore, their meaning remains hypothetical.

We fully validated WES analysis in our pilot study. All small mutations and large deletions were correctly identified. Therefore, WES may cover more than 90% of all *DMD* gene mutations for certain populations, and MLPA might only be performed in negative cases to identify duplications.

The adoption of this approach, especially if running many patients in parallel, could reduce costs and time to diagnosis, bringing to light more than 90% of the causative mutations of dystrophinopathies. Furthermore, the use of WES as a first approach would not only allow the study of the variations of the *DMD* gene but also all other genes of clinical interest.

WES provides the great advantage to possibly interrogate the data for SNPs in gene modifiers. Indeed, we were able to profile the genotypes of *LTBP4, CD40,* and *ACTN3*. Although the small numbers (28 patients) do not allow a genotype-phenotype correlation, some differences in genotype assortment are visible if comparing Algerian and Eastern Europe (Ukraine) patients. Looking at the allele frequency of *LTBP4* SNPs (which form a 4-SNP haplotype), Ukrainian patients have higher frequency of the A (19/28), G (19/28), G (19/28), and T (18/28) alleles compared with Algerian patients. This implies that the *VAAM* (AGGT) protective haplotype frequency in patients may vary, therefore having a less powerful value for predicting milder DMD phenotype (in terms of loss of ambulation). Indeed, ethnic differences in SNPs allele frequency may hamper a meaningful statistical association in population studies. WES also identified previously unreported SNPs in *LTBP4, ACTN3*, and *THBS1*. The heterozygous *ACTN3* exon 21 nonsense variation is unreported and may have a deleterious effect with a possible role in modulating the DMD phenotype.

The mutation landscape also allows for the evaluation of personalized treatment eligibility.[31] Eteplirsen (Exondys 51) which induces *DMD* exon 51 skipping in amenable patients,[32] is an approved orphan drug for DMD in the USA. Other molecules that induce exon 45 and 53 skipping are also currently in clinical trials.

Table 2 Known Gene Modifiers: Genotyping of Patients Studied by WES

	Country	ID number	DMD mutation	LTBP4 (chr.19) rs2303729 G/A, MAF = 0.47564 (G)	LTBP4 (chr.19) rs1131620 A/G, MAF = 0.49521 (G)	LTBP4 (chr.19) rs1051303 A/G, MAF = 0.49501 (G)	LTBP4 (chr.19) rs10880 C/T, MAF = 0.41713 (T)	CD40 (chr.20) rs1883832 C/T, MAF = 0.22883 (T)	ACTN3 (chr.11) rs1815739 C/T, MAF = 0.40076 (T)
1	Algeria	GM 2173/18	Exon 10: c.1012G>T (p.Glu338*)	GA	AG	AG	CC	CT	CT
2	Algeria	GM 2177/18	Exon 18: c.2253delG (p.Lys752Argfs*8)	GG	AA	AA	CC	CC	CC
3	Algeria	GM 2510/18	Gene deletion exons 45-47	AA	GG	GG	TT	TT	CC
4	Algeria	GM 2511/18	Gene deletion exons 48-50	GG	AA	AA	CC	CC	CC
5	Algeria	GM 2515/18	Gene deletion exons 2-26	AA	GG	GG	TT	CT	CT
6	Algeria	GM 2516/18	Gene duplication exons 52-62	GA	AG	AG	CC	CC	TT
7	Algeria	GM 2517/18	Gene deletion exons 7-12	GA	AA	AA	CC	CT	CT
8	Algeria	GM 2524/18	Gene deletion exons 45-50	GA	AG	AG	TT	CC	CC
9	Algeria	GM 2525/18	Gene deletion exons 18-19	GG	AA	AA	CC	CT	CC
10	Algeria	GM 2526/18	Gene deletion exons 18-19	GG	AA	AA	CC	CC	CT
11	Algeria	GM 2528/18	Gene deletion exons 35-45	GA	AG	AG	CT	CC	CT
12	Algeria	GM 2529/18	Gene deletion exons 52-54	GG	AA	AA	CT	CT	CT
13	Algeria	GM 2530/18	Gene deletion exons 4-7	GA	AG	AG	CT	CC	CC
14	Ukraine	GM 2573/18	Intron 54: c.8027+1G>T	GG	AA	AA	CC	CC	CT
15	Ukraine	GM 2010/18	Exon 38: c.5444A>G (p.Asp1815Glufs*2)	GG	AG	AG	CC	CC	CT
16	Ukraine	GM 2043/18	Exon 4: c.206dupC (p.Arg70Lysfs*19)	AA	GG	GG	TT	CC	CT
17	Ukraine	GM 2046/18	Intron 68: c.9975-2A>T	GG	AA	AA	CC	CC	CT

Continued

Table 2 Known Gene Modifiers: Genotyping of Patients Studied by WES *(continued)*

	Country	ID number	DMD mutation	LTBP4 (chr.19) rs2303729 G/A, MAF = 0.47564 (G)	LTBP4 (chr.19) rs1131620 A/G, MAF = 0.49521 (G)	LTBP4 (chr.19) rs1051303 A/G, MAF = 0.49501 (G)	LTBP4 (chr.19) rs10880 C/T, MAF = 0.41713 (T)	CD40 (chr.20) rs1883832 C/T, MAF = 0.22883 (T)	ACTN3 (chr.11) rs1815739 C/T, MAF = 0.40076 (T)
18	Ukraine	GM 2050/18	Intron 26: c.3603+1G>T	AA	GG	GG	CT	CC	CC
19	Ukraine	GM 2097/18	Exon 20: c.2512C>T (p.Gln838*)	GA	AG	AG	CT	CC	CT
20	Ukraine	GM 2131/18	Exon 44: c.6292C>T (p.Arg2098*)	AA	GG	GG	TT	CT	CT
21	Ukraine	GM 2132/18	Exon 38: c.5341A>T (p.Lys1781*)	AA	AG	AG	CT	CC	CT
22	Ukraine	GM 2533/18	Gene deletion exon 51	GA	AG	AG	TT	CC	CC
23	Ukraine	GM 2534/18	Exon 20: c.2521C>T (p.Gln841*)	AA	GG	GG	TT	CT	CC
24	Ukraine	GM 2536/18	Exon 16: c.1961T>G (p.Leu654*)	GA	AG	AG	CT	CC	CC
25	Ukraine	GM 2538/18	Exon 8: c.794delAinsCT (p.His265Profs*22)	AA	GG	GG	TT	CT	TT
26	Ukraine	GM 2539/18	Intron 5: c.358-1G>T	GA	AG	AG	CT	CT	CT
27	Ukraine	GM 2540/18	Gene deletion exons 48-50	GA	AG	AG	CT	CC	CC
28	Ukraine	GM 2541/18	Exon 55: c.8034_8037delTGAG (p.Glu2681Leufs*44)	AA	GG	GG	TT	CT	CT

Abbreviations: DMD = Duchenne muscular dystrophy; SNP = single nucleotide polymorphism; WES = whole-exome sequencing.
The modifier SNPs of genes LTBP4, CD40, and ACTN3, already known to be associated with the DMD phenotypic variability, are reported. Four missense SNPs (rs2303729, rs1131620, rs1051303, and rs10880) along the coding sequence of LTBP4, SNP rs1883832 in the 5′-UTR of CD40, and SNP rs1815739 of ACTN3 (R577X) were studied, and the genotypes are reported. For each sample, ID number, *DMD* mutation, and SNPs genotypes are reported. SNP rs28357094 of the SPP1 gene and SNPs rs2725797 and rs2624259 of the THBS1 gene could not be studied due to their location in the promoter (5 bases upstream of the transcription start site) and in a telomeric region situated about 750 kb flanking a strong enhancer site, respectively.

Indeed, 51 is the most skippable exon (orphan drug eteplirsen), 30% might be treated by exon 45 skipping, 18% by exon 53 skipping, and 11% by exon 44 skipping. Considering nonsense mutation correction by read-through mechanisms for stop codon reversion, 53 patients overall (23.9%) were found to be carrying a nonsense mutation for which ataluren is an approved orphan drug. There were, however, remarkable differences when considering country distribution, with Eastern European countries showing 31% of patients treatable using a nonsense mutation correction approach and Algeria just 5%.[33]

Genetic characterization in different and large patient cohorts prompts many reflections on DMD/BMD prevalence, mutation type, gene modifiers allele frequency, and personalized therapy, as we have explored in Eastern Europe and in Algeria. We suggest that diagnostic flowcharts should be adjusted depending on ethnic characteristics. In some populations where small mutations are more frequent than deletions and/or duplications, like in Eastern European countries, WES might be indeed the preferred first-step diagnostic tool. These studies will have an impact on diagnostic approaches pipelines with repercussions on care and design of new therapies.

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Appendix *(continued)*

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Appendix *(continued)*

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Continued

Appendix *(continued)*

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Ethnicity-related DMD Genotype Landscapes in European and Non-European Countries

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