

Small mutation screening in the *DMD* gene by whole exome sequencing of an argentine Duchenne/Becker muscular dystrophies cohort

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Abstract

Dystrophinopathies are neuromuscular X-linked recessive diseases caused by mutations in the *DMD* gene. This study aimed to identify *DMD* gene small mutations by Whole Exome Sequencing (WES), in order to confirm clinical diagnosis, identify candidates for Ataluren treatment and perform carrier status testing. Furthermore, was our goal to characterize the *DMD* sequence variants and identify ancestral haplotypes. We analyzed 40 non-related individuals (38 affected boys with dystrophinopathy presumptive clinical diagnosis and 2 at-risk women) with negative MLPA results. Pathogenic *DMD* variants were found in 32 boys. Surprisingly, in another 4 patients with absence/deficiency of dystrophin in muscle biopsy, pathogenic variants were found in Limb-girdle muscular dystrophy genes. Therefore, the WES detection rate resulted ~94% (36/38). We could identify 15 Ataluren candidates and exclude 2 at-risk women. The characterization of the occurrence and diversity of *DMD* sequence variants from our cohort and from LOVD database, revealed no hotspots but showed exons/introns unlikely to carry small molecular alterations and exons presenting a greater mutagenic abundance than others. Also, we have detected the existence of 2 co-segregating haplotypes blocks. Finally, this work represents the first *DMD* gene small mutations screening applying WES in an argentine cohort, contributes with the characterization of our population and collaborates with the *DMD* small mutation's knowledge.

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1. Introduction

Dystrophinopathies are X linked recessive diseases caused by mutations in the *DMD* gene (OMIM ID: 300377). This gene is one of the largest of the Human genome, spanning approximately 2.4Mb and having 79 exons [1]. It encodes the dystrophin protein, which in the skeletal muscle, plays a major role in maintaining membrane stability, organization of membrane specializations and participates in the transduction

of muscle strength. Therefore, dystrophin protects the fibers from damage induced by muscle contraction [2,3].

Dystrophinopathies consist of a continuous gradient of severity, however two distinctive clinical manifestations can be distinguished. On the one hand, Duchenne muscular dystrophy (DMD) affects 1:3.500–5.000 born males, being the most frequent neuromuscular disease in childhood [4,5]. This pathology is generated by a complete absence of the dystrophin protein which produces early muscle degeneration, leading to increase serum levels of creatine kinase (CK) and lactate dehydrogenase (LDH) [6,7]. On the other hand, Becker muscular dystrophy (BMD) affects 1:18.000 born males and has a similar pattern than DMD but with a slower progression rate as it is caused by a decrease in the amount or function of dystrophin [8].

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The “reading frame” theory establishes a correlation between phenotype and mutation type, which agrees with the observed phenotype in 92% of cases [9,10]. According to this theory, patients carrying a mutation causing a disruption on the translational reading frame (out-of-frame mutation) show a clinical progression to DMD, while patients with a genetic alteration that do not affect the translational reading frame (in-frame mutation) develop a milder phenotype, BMD-like.

Spectrum of *DMD* gene mutations comprises gross deletions (1 or more exons) in ~68% of cases, gross duplications in ~11% and point mutations in the remaining. Small mutations can also be divided into: ~10% nonsense mutations, ~7% insertion/deletion and ~3% splice site mutations [11]. Genetic testing for the *DMD* gene initiates with the screening of large mutations, for what the method of choice is the quantitative technique Multiplex Ligation-dependent Probe Amplification (MLPA). When no deletion/duplication has been identified, the diagnostic algorithm must proceed with the screening of small mutations by sequencing the coding region and the donor/acceptor splice sites [12]. This was generally performed by Polymerase Chain Reaction (PCR) amplification and Sanger sequencing of every exon of the gene. However, nowadays, the decrease in Next Generation Sequencing (NGS) Technology prices has turned it into a rapid, accurate and cost/effective diagnostic alternative.

Recently, two gene therapies for DMD were conditionally approved: The U.S. Food and Drug Administration (FDA) and The European Medicines Agency (EMA) gave their consent to exon skipping of exon 51 (Eteplirsen, Sarepta) and to the premature stop codon read-through (Ataluren, PTC), respectively [13,14]. Moreover, several lines of research on *DMD* gene therapies continue being developed, for example the ones based on Utrophin upregulation and *DMD* gene editing [15,16]. Therefore, accurate detection and characterization of the causing genetic abnormality is essential to allow precise genetic counseling, patient follow-up and to determine the most suitable gene therapy for each individual.

The present study aimed to identify small mutations in the *DMD* gene by Whole Exome Sequencing (WES), in order to confirm the clinical diagnosis in patients, identify candidates for Ataluren treatment and offer genetic assessment to their families. It was also our goal to evaluate the sensitivity and applicability of the WES methodology for the *DMD* small mutation detection. Furthermore, we performed a deep analysis of the diversity of the *DMD* sequence variants identified in our population. Finally, was also our objective to detect the existence of an ancestral haplotype within the DMD/BMD Argentine cohort and small mutation hotspots within the *DMD* gene.

2. Materials and methods

2.1. Patients

A total amount of 168 patients were referred to our laboratory to confirm a clinical diagnosis of Dystrophinopathy. MLPA was performed as the first step of the molecular

algorithm, detecting deletion/duplication in 96 of them. From the remaining 72 patients without causative mutation found, 38 could proceed to the sequencing step which was performed by Whole Exome Sequencing. Must be highlighted that these NGS studies are still very expensive in underdeveloped countries such as Argentina, and this is why not all of the patients could reach the small mutation screening.

Clinical diagnosis of Dystrophinopathy in the affected boys was done according to the following criteria: progressive muscular weakness since childhood; high levels of serum CK; myopathic changes on electromyography; and, in some cases, a muscle biopsy showing absence or decreased dystrophin levels [6,7,17].

On the other hand, 2 at-risk women with a previous negative MLPA, were included in the study to assess their carrier status. Both belong to sporadic cases, the only DMD affected member were their uncles. Not only were the two affected relatives deceased, but also didn't have their causative mutation identified. Serum CK levels of these girls resulted within the reference intervals (#332: 100UI/L and #377: 84UI/L; reference value: 30–145 UI/L).

The protocol was approved by the institutional ethics committee. Informed consent was obtained for all study subjects prior to the molecular studies.

2.2. Samples

Whole blood was drawn by venipuncture with 5% ethylene-diamine tetraacetic acid (EDTA) as anticoagulant for all study subjects. Genomic DNA was isolated using the cetyl-trimethyl-ammonium bromide (CTAB) method [18]. DNA concentration and quality were measured by absorbance at 260nm and by the ratios of A260nm/A280nm and A260nm/A230nm, respectively. All samples were stored at –20 °C.

2.3. Whole exome sequencing and bioinformatic filtering

WES was carried out by MacroGen Services [Republic of Korea]. Exome libraries were captured by hybridization with the Agilent SureSelect V4 Target Enrichment Kit in 20 samples and Agilent SureSelect V5 Target Enrichment Kit in the other 20 [Agilent Technologies, Santa Clara, United States]. All WES were performed on an Illumina HiSeq 4000 [Illumina, San Diego, United States], according to the manufacturer's recommendations. FASTQ sequencing files were aligned to the Human Reference Genome hg19 from UCSC (original GRCh37 from NCBI, Feb. 2009) applying Burrows-Wheeler Alignment Tool (BWA –0.7.12). Analysis proceeded using Picard (picard-tools-1.130) and Genome Analysis Toolkit (GATK3.v4). Finally, variant annotation was carried out applying SnpEff (SnpEff_v4.1), dbSNP database (version 142), 1000Genomes Phase 3, ClinVar database (version 05/2015) and ESP database (ESP6500SI_V2). On the other hand, the .bam files were also analyzed using the Integrative Genomics Viewer (IGV) software [Broad Institute, University of California, United States] so as to determine the

coverage of every exon of the *DMD* gene and the quality of the reads.

The data obtained by the Agilent SureSelect V4 had a mean depth of target region of 80 and 20-fold coverage in 92% of the targeted regions. Whereas the results of Agilent SureSelect V5 had a mean depth of target region of 118 and 20-fold coverage in more than 95% of the targeted regions.

2.4. Selection of pathogenic candidate variants and in silico analysis of their impact

Candidate pathogenic variants in the *DMD* gene were selected according to the following criteria: (1) Sequence variants were filtered based on population frequency in 1000Genomes and Exome Aggregation Consortium (ExAC), being discarded the ones with a frequency > 1% in any population; (2) Variants predicted to have a functional impact on coding regions (predicted missense, nonsense, consensus donor/acceptor splice site mutations and insertions/deletions); (3) Variants previously reported in the Leiden Open Variant Database (LOVD) as *DMD*/*BMD* causative molecular alteration; and, (4) Variants determined as Damaging or Probably Damaging by at least 4 *in silico* predictive mutation impact software: PolyPhen-2 [<http://genetics.bwh.harvard.edu/pph2/>], SIFT [<http://sift.jcvi.org/>], Mutation Taster [<http://www.mutationtaster.org/>], Mutation Assessor [<http://mutationassessor.org/r3/>], CADD [<http://cadd.gs.washington.edu/>] and UMD Predictor [<http://umd-predictor.eu/analysis.php>].

2.5. Polymerase chain reaction and Sanger sequencing

Every genetic variation identified as damaging or probably damaging were corroborated by PCR and Sanger Sequencing. The method was performed as previously described elsewhere, with minor modifications [19]. Primer sequences were obtained from the Leiden Muscular Dystrophy site [Leiden Muscular Dystrophy webpages (www.dmd.nl)]. All PCR reactions were performed in a thermal cycler [Veriti; Applied Biosystems, Foster City, California]. PCR amplicons were analyzed by 2% agarose [Genbiotech SRL] gel electrophoresis in 1X TBE buffer and dyed with GelGreenTM [Biotium]. Positive controls (wild-type DNA) and negative controls (no DNA) were included in all reactions.

The exons were sequenced using both PCR primers and the reaction products were analyzed using a DNA analyzer [ABI 3730 XL; Applied Biosystems, Foster City, California]. The quality of the obtained sequence was determined using FinchTV software [Geospiza, Seattle, USA] and the results were analyzed by comparison with the GenBank sequence of the *DMD* gene (NM_004006.2).

2.6. Identification of small mutation hotspots in *DMD* gene

The analysis centered on the reported small mutations from the LOVD database [Leiden Muscular Dystrophy webpages (www.dmd.nl)]. We have analyzed the frequency, the type of

mutation (substitutions, deletions, insertions, duplications and indel) and the localization of the molecular alteration (exonic or splice site variants).

2.7. Linkage disequilibrium and haplotype blocks analysis

This study was carried out implementing 45 SNPs identified by the WES methodology in the 40 individuals tested. Were excluded from the analysis the mutations rendered as pathogenic and the singletons (variants observed in a single individual from our cohort). So as to determine the existence of Hardy-Weinberg (HW) equilibrium or Linkage Disequilibrium (LD) between the studied loci and to identify the presence of ancestral co-segregating haplotypes in this population, we have implemented the Haploview 3.2 software [<https://www.broadinstitute.org/haploview>] [20]. The null hypothesis of HW equilibrium was rejected applying a *p*-value < 0,001. Only 8 of the SNPs were found to be in HW equilibrium (rs1800264, rs41303183, rs190527338, rs1800265, rs182502235, rs72468689, rs41303181 and rs1800279). Visualization of the LD relationships was performed on the basis of the D'parameter (Tajima's D normalization), which varies from 0 (absence of LD) to 1 (complete LD or, in other words, absence of recombination between the analyzed loci).

3. Results

3.1. Molecular diagnosis and prognosis

The above mention methodology was able to detect the *DMD* gene pathogenic variant in 32 of the 38 affected boys studied, allowing us to confirm the clinical diagnosis of the patients and provide genetic assessment to their families (Table 1). In addition, we have determined the expected phenotype for the patients carrying nonsense mutations, deletions and duplications applying the “reading frame” theory, however, we did not establish the expected phenotype for those carrying splice sites mutations given the difficulty to predict their effect on the maturation of the messenger RNA (mRNA). The expected and observed phenotype agreed in all cases with the exception of three boys (#70, #182 and #398).

The *DMD*/*BMD* causative mutations could not be found in 6 boys. In order to discard the chance of having filtered the pathogenic sequence variants and also check if all the exons had been correctly captured and sequenced, the .bam file was analyzed using the IGV software. All exons of the *DMD* gene probed to be well captured and no molecular alteration was found in these 6 patients. Therefore, as all of these patients had confirmed diagnosis by biopsy and immunohistochemistry, they might carry a regulatory/promoter mutation or a deep intronic alteration.

On the other hand, the 2 women at-risk of being carriers did not present any pathogenic molecular alteration in the *DMD* gene. Therefore, both could be excluded from being carriers with a ~99% certainty due to the fact that regula-

Table 1
Affected boys results.

Sample	Exon / Intron	Mutation	Protein change	Reported	Phenotype		Case	Age	CK	Biopsy	AO
				in LOVD	Pred.	Obs.	Type			Determination	
#392	6	c.433C>T	p.Arg145*	Yes	DMD	DMD	Sporadic	12 y	---	DMD	5 y
#461	15	c.1777C>T	p.Gln593*	No	DMD	DMD	Sporadic	6 y	---	Not performed	4 y
#362	15	c.1793C>G	p.Arg3190*	Yes	DMD	DMD	Familial	5 y	38.525 UI/L	DMD	2 y
#307	16	c.1928G>A	p.Trp643*	Yes	DMD	DMD	Sporadic	6 y	35.000 UI/L	Not performed	4 y
#372	16	c.1978_1979delAA	p.(Lys660Glnfs*59)	Yes	DMD	DMD	Sporadic	4 y	17.900 UI/L	Not performed	3 y
#288	18	c.2270C>G	p.Ser757*	Yes	DMD	DMD	Sporadic	10 y	24.992 UI/L	DMD	4 y
#435	18	c.2293-2A>G	---	No	---	DMD	Familial	18 y	---	Not performed	2 y
#110	20	c.2407C>T	p.Gln803*	Yes	DMD	DMD	Familial	---	---	DMD	---
#326	23	c.3151C>T	p.Arg1051*	Yes	DMD	DMD	Sporadic	10 y	36.567 UI/L	DMD/BMD	4 y
#394	23	c.3151C>T	p.Arg1051*	Yes	DMD	DMD	Sporadic	9 y	---	DMD	5 y
#460	23	c.3151C>T	p.Arg1051*	Yes	DMD	DMD	Sporadic	25 y	---	DMD	---
#39	25	c.3427dupC	p.(Gln1143Profs*34)	No	DMD	DMD	Sporadic	15 y	9.008 UI/L	DMD	5 y
#419	26	c.3603+1G>T	---	Yes	---	DMD	Sporadic	9 y	10.424 UI/L	Not performed	2 y
#258	32	c.4375C>T	p.Arg1459*	Yes	DMD	DMD	Sporadic	11 y	10.676 UI/L	---	8 y
#291	32	c.4518+1G>T	---	Yes	---	DMD	Sporadic	15 y	10.392 UI/L	DMD	7 y
#396	42	c.6118-2A>T	---	No	---	DMD	---	7 y	---	DMD/BMD	4 y
#428	44	c.6391_6392delCA	p.(Gln2131Asnfs*3)	Yes	DMD	DMD	Sporadic	10 y	8.197 UI/L	Not performed	2 y
#338	48	c.6973C>T	p.Gln2325*	No	DMD	DMD	Familial	15 y	2.662 UI/L	DMD	9 m
#420	54	c.8027+2T>C	---	Yes	---	DMD	Sporadic	15 y	12.330 UI/L	DMD	6 y
#436	55	c.8138delA	p.(Asn2713Metfs*13)	No	DMD	DMD	---	12 y	7.599 UI/L	DMD	5 y
#368	55	c.8217+2T>G	---	No	---	BMD	Sporadic	11 y	---	BMD	4 y
#459	62	c.9225-1G>A	---	Yes	---	---	Sporadic	7 y	---	Not performed	3 y
#227	66	c.9568C>T	p.Arg3190*	Yes	DMD	DMD	Familial	8 y	6.680 UI/L	---	---
#196	68	c.9928C>T	p.Gln3310*	Yes	DMD	DMD	Sporadic	7 y	20.780 UI/L	---	3 y
#292	68	c.9890_9894dupTCCTG	p.His3299Serfs*33	No	DMD	DMD	Sporadic	11 y	---	Not performed	9 m
#182	70	c.10101_10103delAGA	p.Glu3367del	Yes	<i>BMD</i>	<i>DMD</i>	Familial	10 y	4.260 UI/L	BMD	3 y
#437	70	c.10108C>T	p.Arg3370*	Yes	DMD	DMD	Familial	6 y	---	Not performed	11 m
#250	70	c.10141C>T	p.Arg3381*	Yes	DMD	DMD	Sporadic	8 y	23.062 UI/L	Not performed	5 y
#393	70	c.10223+1G>A	---	Yes	---	DMD	Familial	9 y	20.003 UI/L	Not performed	2,5 y
#70	71	c.10227delC	p.(Val3410Leufs*3)	No	<i>DMD</i>	<i>BMD</i>	Familial	11 y	8376 UI/L	BMD	2 y
#398	74	c.10453dupC	p.(Leu3485Profs*6)	Yes	<i>DMD</i>	<i>BMD</i>	Sporadic	18 y	26.000 UI/L	BMD	14 y
#438	75	c.10785delG	p.(Gln3595Hisfs*12)	No	DMD	DMD	Familial	8 y	---	Not performed	2 y
#323	---	Not found	---	---	---	BMD	Sporadic	23 y	15.000 UI/L	BMD	3 y
#369	---	Not found	---	---	---	DMD	Sporadic	10 y	1.371 UI/L	DMD	2,5 y
#374	---	Not found	---	---	---	DMD	Sporadic	11 y	---	DMD	6 y
#395	---	Not found	---	---	---	DMD	---	---	---	DMD/BMD	---
#397	---	Not found	---	---	---	DMD	Familial	5 y	23.000 UI/L	DMD	3 y
#501	---	Not found	---	---	---	DMD	Sporadic	31 y	669 UI/L	DMD/BMD	5 y

Mutations and Protein Changes that apply for Ataluren are marked in bold. Differences between observed (Obs.) and predicted (Pred.) phenotype are marked in italics. y: years; m: months.

tory and deep intronic sequences are not analyzed by this methodology.

We have identified 15 nonsense mutations (47%, 15/32), 8 consensus donor/acceptor splice site mutations (25%, 8/32), 6 deletions (19%, 6/32) and 3 duplications (9%, 3/32) (Table 1). None of them were found in 1000Genomes nor

ExAC consortiums, however, 21 of them were previously reported in the LOVD database as pathogenic. Noteworthy, for 1 of these variants, changes were reported in the same position but with a different nucleotide change. We have submitted all the mutations identified in the LOVD database, including 11 not previously reported. Furthermore, *in silico* analysis using

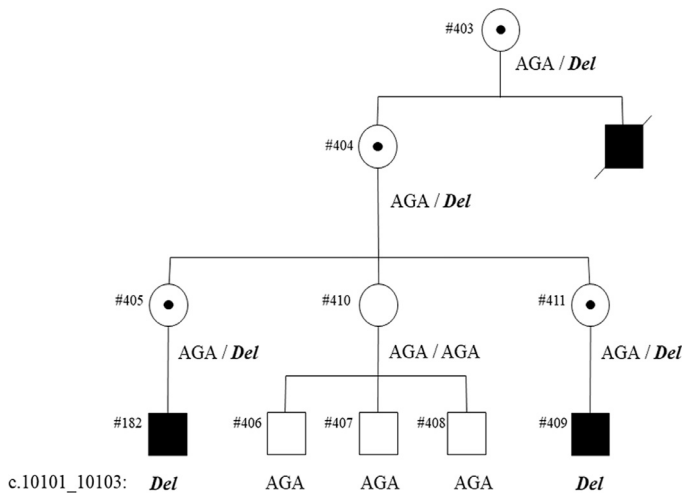


Fig. 1. Segregation analysis of the c.10101_10103delAGA variant in patient's #182 family.

The figure shows the pedigree of patient's #182 family. References: c.10101_10103, bases implicated in the deletion nomenclated according to the cDNA sequence; *Del*, c.10101_10103delAGA; AGA, reference or wild type sequence.

predictive mutation impact software were performed for the mutations detected, resulting all of them classified as disease causing.

We have found the variant c.10101_10103delAGA in patient #182, which had been reported in 5 affected boys in the LOVD database with unknown concluded pathogenicity. Aiming to validate its damaging role, we have performed a segregation analysis in the patient's family (Fig. 1). As expected, the molecular alteration was detected by Sanger sequencing in the 3 obligate carriers (#403, #404 and #405), but was absent in the healthy boys (#406, #407 and #408) and their mother (#410). Furthermore, patient #409, who only presented frequent falling downs, could be early diagnosed with dystrophinopathy and his mother (#411) could be established as an obligate carrier.

3.2. DMD variants, gene characterization

3.2.1. Analysis of missense and synonymous variants: VUS or not to VUS?

We have detected 16 variants located within exons of the *DMD* gene, apart from the ones rendered as pathogenic (Table 2). The majority were missense mutations (11/16), while the remaining 5 were synonymous variants.

In order to predict their potential effect, we have performed an exhaustive analysis of their allele frequency on 1000Genomes and ExAC consortiums, their pathogenic implication according to ClinVar and the LOVD database and, finally, the results of 6 different predictive software. The above mention study allowed us to catalogue 12 of these variants as benign, whereas the remaining 4 were classified as Variants of Uncertain Significance (VUS).

Even though, the 12 polymorphisms had an allele frequency >1% and were reported in ClinVar and LOVD as

benign/Not affects function, not all of the predictive software agreed with their non-deleterious effect. Furthermore, a similar disagreement was seen for the mutations rendered as VUS, mutations with an allele frequency <1%. The variants c.2367A > G, c.3936G > C and c.7244G > A were determined as benign by most of the software implemented (3/4; 3/5/6 and 5/6 respectively), while variant c.821A > G was classified as pathogenic by 5/6 of the software.

3.2.2. Identification of small mutation hotspots in *DMD* gene

When analyzing the distribution within the *DMD* gene of the pathogenic small mutations identified in our cohort, caught our attention that even though there were a little number of sequence variants, several of them coincide in the exon/intron in which they have occurred. As can be seen in Table 1, 4 mutations took place in exon/intron 70, 3 mapped in exon/intron 23 and 2 variants were found in exons/introns 15, 16, 18, 32, 55 and 68. This fact made us wonder if there are hotspots of small mutations in the *DMD* gene or if, at least, there are some exons/introns more frequently affected by point mutations. Thus, so as to answer these queries, we have performed a characterization of the small mutations occurrence in the *DMD* gene implementing the variants reported in the LOVD database, as we only counted with a limited amount of mutations.

The LOVD database has a total amount of 3.060 exonic small mutations, among them can be found some with confirmed pathogenic effect, some with probable deleterious effect and others without certain pathogenicity. The most frequent point mutations type were the substitutions (70.6%; 2.159/3.060), followed by small deletions (20.2%; 618/3.060) and small duplications (6.6%; 203/3.060) (Supplementary Fig. 1A). Insertions and deldup were the less frequent, both being responsible for the remaining 2.6% of small mutations. This relation found for the frequency of the different exonic mutation types coincide with the results from our cohort, as we have detected 62.5% (15/24) exonic substitutions, 25% (6/24) deletions and 12.5% (3/24) duplications.

On the other hand, albeit not being evidenced small mutation hotspots in the *DMD* gene, some exons, such as exon 50, 72, 73, 77, 78 and 79, can be highlighted because of showing complete absence or small number of mutagenic events (Supplementary Fig. 1A). Oppositely, exons 6, 20, 21, 23, 37, 48, 59 and 70 can be distinguished for carrying a large amount of sequence variants. Particularly, exons 59 and 70 presented the highest quantity of mutations (124/3.060 each).

As for mutations affecting the consensus splice sites, the database counts with 374 substitution reports. Given the fact that the number of submissions of other types of molecular alterations disturbing the splice sites were negligible, these reports were excluded from the analysis.

The wide amount of substitutions (65.5%) mapped on the donor consensus splice site, while the remaining 34.5% affected the acceptor splice site (Supplementary Fig. 1B). Despite the fact that in our cohort we have identified only 8 sequence variants affecting the splicing process, they seem to mimic this pattern. We have observed a 62.5% (5/8) of muta-

Table 2
Analysis of missense and synonymous variants.

Sequence variant	Protein variant	RS	E	P	Patient ID (DMD Pathogenic Mutation)	AF-1000 Genomes ALL	AF-ExAC	ClinVar	LOVD Database		Informatic predictors					
									Reported (quantity)	Effect	Polyphen	SIFT	Mut. Taster	CADD (PHRED)	UMD Predictor	Mut. Assessor
Variants rendered as benign																
c.802T>C	p.Leu268Leu	1800264	8	4	#110 (c.2407C>T) #332 (Not found) #338 (c.6973C>T) #250 (c.10141C>T)	1%	0.7%	B/LB	Yes (4)	NAF	---	T	Pol	B	Pol
c.837G>A	p.Thr279Thr	1800265	9	4	#110 (c.2407C>T) #332 (Not found) #338 (c.6973C>T) #250 (c.10141C>T)	15%	8.6%	B/LB	Yes (9)	NAF	---	T	Pol	Del (16)	Pol	---
c.1635A>G	p.Arg545Arg	5927083	14	6	#307 (c.1928G>A) #70 (c.10227del) #392 (c.433C>T) #394 (c.3151C>T) #372 (c.1978_1979del) #437(c.10108C>T)	11%	11.7%	B/LB	Yes (16)	NAF	---	T	Pol	B	Pol	---
c.2645A>G	p.Asp882Gly	228406	21	36	-	25%	28%	B	Yes** (28)	NAF	B	T	Pol	B	Pol	Neutral
c.3734C>T	p.Thr1245Ile	1800269	27	1	#196 (c.9928C>T)	1%	1,50%	B/LB	Yes (8)	NAF	ProbDam/ PosDam*	T	Pol	PosDel (14)	Path	Low
c.5234G>A	p.Arg1745His	1801187	37	28	-	47%	51%	B/LB	Yes (45)	NAF	ProbDam	T	Pol	Del (32)	ProbPath	Medium
c.7096C>A	p.Gln2366Lys	1800275	48	18	-	82%	72%	B	Yes (55)	NAF	ProbDam**	T	Pol**	Del (16)**	Path**	Neutral
c.7728T>C	p.Asn2576Asn	1801188	53	6	#396 (c.6118-2A>T) #394 (c.3151C>T) #372 (c.1978_1979del) #437 (c.10108C>T) #420 (c.8027+2T>C) #398(c.10453dupC)	18%	17%	B/LB	Yes (30)	NAF	---	T	Pol	B	Pol	---
c.8729A>T	p.Glu2910Val	41305353	59	1	#110 (c.2407C>T)	4%	2,10%	B(6); LB(1); Pat(1)	Yes (13)	NAF	PosDam	Dam	Pol	Del (27)	ProbPol	Low
c.8734A>G	p.Asn2912Asp	1800278	59	1	#110 (c.2407C>T)	4%	2,20%	B(6); LB(1); Pat(1)	Yes (15)	NAF	B	T	Pol	Del (10)	ProbPol	Neutral
c.8762A>G	p.His2921Arg	1800279	59	3	#292 (c.9890_9894dup) #332 (Not found) #437 (c.10108C>T)	1%	2,60%	B(5); LB(2); Pat(1)	Yes (11)	NAF	B	T	Pol	B	ProbPath	Neutral
c.8810G>A	p.Arg2937Gln	1800280	59	36	-	88%	91%	B	Yes** (21)	NAF	B**	T	Pol	Del (17)	Pol	Neutral**
VUS or not to VUS?																
c.821A>G	p.Tyr274Cys	745868830	8	1	#39 (c.3427dup)	0.01%	-	-	Yes (1)	NC	PosDam	Dam	DC	Del (23)	Path	Low
c.2367A>G	p.Glu789Glu	376648723	19	1	#377 (Not found)	0,0006%	-	-	No	-	---	T	DC	B	ProbPol	---
c.3936G>C	p.Leu1312Phe	377156960	29	1	#70 (c.10227del)	0.01%	0.01%	VUS	No	-	PosDam/B*	T	DC	Del (26)	ProbPol	Low
c.7244G>A	p.Arg2415His	373749120	50	1	#362 (c.1793C>G)	0.01%	-	B/LB	No	-	B	T	Pol	PosDel (11)	Pol	Neutral

Abbreviations: AF: Allele Frequency; B: Benign; Dam: Damaging; DC: Disease Causing; Del: Deleterious; LB: Likely Benign; Mut. Assessor: Mutation Assessor; Mut. Taster: Mutation Taster; NAF: Not Affects Function; NC: Not Classified; Pat*: Number of patients carrying the same variant; Patient ID (DMD Pathogenic Mutation): Patients carrying the sequence variant were listed when the total amount was up to 10; Path: Pathogenic; Pol: Polymorphism; PosDam: Possibly Damaging; PosDel: Possibly Deleterious; ProbDam: Probably Damaging; ProbPat: Probably Pathogenic; ProbPol: Probable Polymorphism; T: Tolerated; VUS: Variant of unknown significance. —: The software could not evaluate the impact of the variant; *: Results from Polyphen HumDiv/HumVar; **: The predictive software/database considers the changing nucleotide/amino acid as the reference one.

tions altering the donor splice sites whereas a 37.5% mapped in the acceptors sites.

Just as it was seen for the exonic mutations, introns 23, 31, 35, 37, 39, 53, 72, 73, 74, 76, 77 and 78 can be highlighted because of the absence of molecular alterations disturbing the splicing mechanism (Supplementary Fig. 1B). On the other hand, introns 1 and 70 showed the larger quantity of mutagenic events. Finally, can also be distinguished introns which

carried mutations affecting only the donor sites (introns: 14, 15, 16, 27, 29, 30, 34, 36, 41, 48, 51, 54, 59, 70 and 71) or just the acceptor sites (introns: 4, 9, 28, 38, 40, 42, 49, 57, 58, 59, 74 and 76) (Supplementary Fig. 1B).

3.3. Linkage disequilibrium and haplotype blocks analysis

On the basis of 45 SNPs detected in our patients by the WES technique, was our goal to determine the existence of

Table 3
MAF comparison of the loci implicated in the haplotypes blocks.

Sequence Variant	c.1483–123G > T rs808542	c.1483–110G > A rs808543	c.1483–72T > C rs17309542	c.1635A > G rs5927083	c.1704 + 51T > C rs5927082
MAF CLM	52%	52%	3%	8%	7%
MAF TSI	41%	41%	14%	15%	14%
MAF IBS	41%	41%	12%	14%	14%
MAF argentine cohort	30%	32%	14%	16%	16%

co-segregating haplotypes within the DMD/BMD argentine cohort. Using the Haploview Software, we were able to identify 2 haplotype blocks formed by 3 and 2 sequence variants respectively (Fig. 2). The frequency of the haplotypes generated by the 2 blocks are shown in the bottom right corner of Fig. 2.

Due to the argentine Latin origin and the different migratory events that have taken place during our history, our population consist on a preponderant mixture of 3 evolutionary origins: Amerindian, Italian and Spanish. So, in order to carry out a first approach to the evolutionary origin of the haplotype blocks found, we have compared the calculated Minor Allele Frequency (MAF) of this 5 loci of our cohort with the reported MAFs in 3 populations from the 1000Genomes project: CML (Colombians from Medellin), IBS (Iberian population in Spain) and TSI (Tuscans from Italy). The MAFs from our population resulted consistently more similar to the IBS and TSI values, rather than the frequencies reported for the CML cohort (Table 3). Particularly, for the SNPs c.1483–72T > C (rs17309542) and c.1635A > G (rs5927083), which presented different MAFs in the IBS and TSI populations, the argentinian frequencies resulted more similar to the Italian ones.

On the other hand, a third co-segregating block can be distinguished in Fig. 2, it is formed by 3 SNPs: c.832–53C > T, c.837G > A and c.960 + 166T > C. Apparently, this block would not be segregating with the 2 blocks previously mentioned. Finally, the Haploview LD analysis suggested the presence of a fourth block in the 3' end of the *DMD* gene, which would be encompassing the following SNPs c.8810A > G, c.9361 + 138T > C, c.9564–97C > T, c.9649 + 15T > C, c.9975–79G > A, c.10797 + 82G > A, c.10797 + 135A > G and c.10798–100G > C. Despite presenting *D'* values of 1, this block did not reach the statistical significance ($LOD < 2$) required for being accepted. Probably this haplotype block could acquire the needed statistical power if a larger number of individuals were incorporated to the study.

4. Discussion

The screening of small mutations in the *DMD* gene consists in the second key point in the molecular diagnostic algorithm of DMD/BMD. The improvements in the field of Next Generation Sequencing allowed lowering costs and, therefore, turned it into an accessible, reliable and extremely informative tool in the medical territory. In the present work, we

have introduced the implementation of WES as an alternative methodology to the Sanger sequencing of all *DMD* exons for the detection of point molecular alterations in this gene.

A total of 168 clinically Dystrophinopathy patients were referred to our laboratory for molecular confirmation of the presumptive diagnosis. MLPA assay allowed identification of the causative mutation in 96 of these patients. Even though approximately 57.1% (96/168) of the children could be diagnosed by MLPA, its detection rate resulted much lower than the expected by literature [11]. This could be due to the chance of mistaken clinical diagnosis of dystrophinopathy in these boys, mainly because the biopsy procedure was left as the last resource in the international best guidance for DMD/BMD diagnosis recommendations, given its invasiveness [12].

From the remaining 72 with negative MLPA result (no deletions nor duplications), 38 could be tested for small mutations by WES. The combination of the WES technique with the algorithm for the selection of pathogenic candidate variants implemented, proved to be efficient for the identification of small mutations in the *DMD* gene as we have obtained a detection rate of approximately 84% (32/38). The remaining 6 affected children are thought to have a regulatory/promoter or deep intronic alteration, as all of them had muscle biopsy compatible with dystrophinopathy.

Even though, absent or abnormal dystrophin immunohistochemistry detection is frequently used for confirming DMD/BMD diagnosis, must be taken into account that this could also be consequence of alterations in dystrophin related proteins [21,22]. This observation can be demonstrated by the fact that 4 patients with muscle biopsy compatible with dystrophinopathy and without *DMD* molecular alterations found, carried pathogenic variants in other muscular dystrophies genes (#323 / #369: FKRP, #501: SGCG and #395: SGCA). Therefore, these results suggest that 4/6 patients had a misdiagnosis of DMD/BMD, whereas the remaining 2 should have their mRNA analyzed in order to detect the dystrophinopathy causing mutation.

One important conclusion from our work is that dystrophin biopsy alterations cannot be taken as an unequivocal diagnosis, as we have shown that patients with biopsy compatible with dystrophinopathy can have mutations in muscular dystrophy genes other than *DMD*. This observation has important implications in patient management since the standard-of-care and the genetic counseling for each muscular dystrophy is different. Moreover, our work underscores the cost-effective advantage of whole exome sequencing, over a single gene

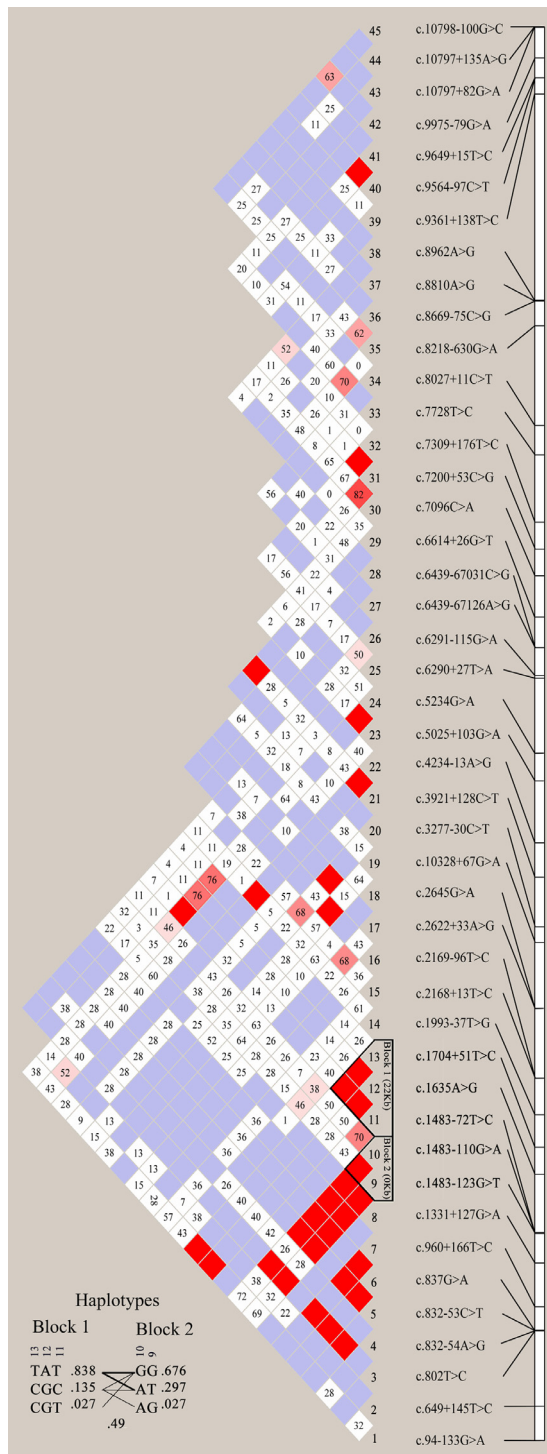


Fig. 2. Linkage Disequilibrium analysis by the Haploview software of the SNPs identified by WES.

The LD diagram was constructed on the basis of the D' statistic. The color of the rhombus depends on the D' and the LOD score values: intense red ($D' = 1$, $\text{LOD} \geq 2$), pink/light red ($D' < 1$, $\text{LOD} \geq 2$), white ($D' < 1$, $\text{LOD} < 2$) and blue ($D' = 1$, $\text{LOD} < 2$). The numbers inside the white and pink rhombus correspond to the D' value divided by 100. The black triangles show the identified haplotype blocks. Finally, in the bottom right of the diagram are depicted the different haplotypes with their corresponding frequency.

analysis or a targeted panel, in analyzing any other genes that had not been considered as first candidates in the diagnostic algorithm.

Considering the aforementioned, the WES detection rate resulted 94.4% (36/38). On the other hand, could also be estimated a frequency of 1.2% (2/164) of regulatory and deep intronic alterations for the *DMD* gene, which coincides with the value reported by Aartsma-Rus et al. [11].

Regarding the evaluation of the sensitivity and the reading depth of the gene, we have performed an analysis of the minimum and maximum number of reads located within the exons and consensus splice sites. The IGV analysis of the .bam files allowed us to determine that all exons of the *DMD* gene had been correctly captured in all of the patients (Supplementary Fig. 2). Furthermore, we could establish that some exons always present a lower read depth than others, showing a maximum average of 10 reads, which may be due to the specific characteristic of their sequence (a high G/C percentage for example). This analysis resulted of utmost importance in the cases where no pathogenic mutations were found, as it has allowed us to discard the possibility of having a sequence variant that had been eliminated during the filtering and annotation process.

Also, WES proved to be a useful technique to perform carrier status testing, especially in cases with deceased affected child and unknown pathogenic alteration. In addition, having found the *DMD/BMD* causative mutation in the affected boys, have enabled us to perform carrier status detection studies by the rapid and reliable Sanger sequencing methodology and, therefore, to assess an even larger amount of individuals with a 100% certainty.

As the mutation dependent gene therapy of premature stop codon read-through is already in use in Argentina, it is compulsory to identify the *DMD/BMD* causative mutation in all patients in order to discriminate those that are eligible for it. Here, we have determined that 15 of the analyzed children are candidates for the premature stop codon read-through (Ataluren, PTC) treatment, as they carry a nonsense mutation in the *DMD* gene. However, the ones who do not qualify for Ataluren, can still apply for a mutation independent treatment, such as Utrophin upregulation, which is currently on phase 2 clinical trial [23].

As regards prognostics, we have analyzed patients carrying deletions, duplications or nonsense mutations and we have found that the “reading frame” theory was able to explain the observed phenotype in ~88% (21/24) of the cases (Table 1). This proportion is similar to the reported effectiveness rate of this theory [9,10]. Only in 3 cases the clinical and expected phenotype did not agree. Patients #70 and #398 presented frameshift deletions, which are expected to produce a clinical progression to *DMD*, but showed a milder symptomatology (*BMD*) (Table 1). These discrepancies could be explained by the occurrence of natural exon skipping of the exons carrying the small mutations, as if exon 71 and 74 are skipped the translational reading frame is maintained. Yet, these hypotheses should be tested by the analysis of the mRNA.

In the particular case of #182 the expected phenotype was BMD, as the patient carries 1-codon deletion (c.10101_10103delAGA), however the child showed a severe clinical course of the disease. Even though dystrophin was preserved in the muscle biopsy, the patient became wheelchair bound at the age of 10 years. Aiming to validate the pathogenicity of the in-frame deletion, we have discarded the existence of small mutations in other genes associated with the development of muscular dystrophies [24]. Furthermore, we have confirmed that the variant cosegregates with the affected children and obligate carriers. This small deletion affects the Cystein rich domain, especially the dystroglycan binding site, and the C-terminal domain. All these results suggest that this specific aminoacid must have an impact on the wild-type protein function.

The proportion of nonsense mutations identified in our cohort was ~47% (15/32), coinciding to the frequency reported in literature [11]. However, the percentage of deletions/duplications (~28%, 9/32) was comparable to the proportion of consensus splice site mutations (25%, 8/32), which disagrees with the reported values (35% and 15%, respectively) [11]. This could be mainly caused by the small size of our cohort or could be a characteristic proper of our population, so further screening and analysis of small mutations in the *DMD* gene of argentinian patients are needed in order to distinguish between these 2 possibilities.

Despite our small cohort, surprised us that 3 non-related patients presented the same substitution in exon 23 (Table 1). Moreover, according to the LOVD database this mutation is one of the most frequent variants that take place in this exon (Fig. 3). As WES is still an expensive technique for underdeveloped countries and the MLPA has the quality of detecting small mutations located within the hybridization zone of the probes, it would be an outstanding improvement in the MLPA technique to modify the probes in order to target frequent small mutations. This development would allow not only to increase the detection rate of the disease causing mutation by the MLPA but also to reach molecular diagnosis of dystrophinopathy in a wider amount of patients, especially in countries with economic difficulties.

Regarding the analysis of missense and synonymous variants, can be highlighted the fact that all of the variants rendered as benign are reported in the LOVD database of Dystrophinopathy patients (Table 2). Furthermore, 3 of the variants found, were reported as pathogenic and benign in ClinVar. Yet, given their elevated allele frequency in non-affected people, all of them showing a MAF \geq 1%, there is no doubt that they are polymorphisms so they should be reclassified by a careful curating process. In addition, the analysis of these exonic variants served as an example of the need to implement several predictive software, as different results can be obtained owing to their particular analysis algorithm.

As for distribution of small mutations in the *DMD* gene, the analysis of the LOVD database did not suggest the existence of hotspots, it showed not only that some exons/introns are unlikely expected to carry small molecular alterations but also that some exons present a greater abundance of mutations

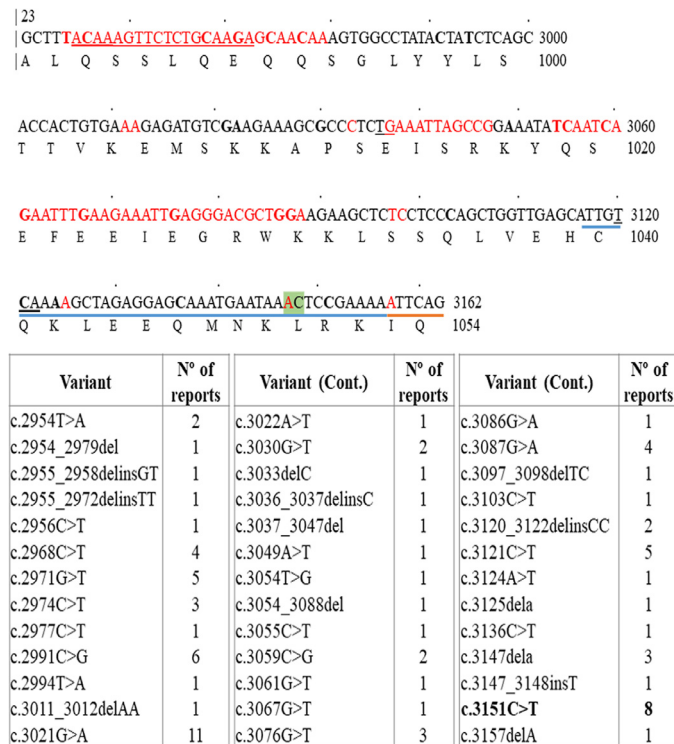


Fig. 3. Distribution and frequency of exon 23 LOVD mutations and the localization of the MLPA probe.

The mutations submitted in the LOVD database are marked in the reference sequence of the exon 23. The different types of molecular alterations are shown as: substitutions (bold), deletions (letters in red), deletions/insertion (delins, underlined) and insertions (green highlighted). When more than a mutation occurred in the same position, both marks were implemented. The blue line depicts the hybridization zone of the 5' hemiprobe of the MLPA while the orange line corresponds to the 3' hemiprobe. Furthermore, variants nomenclature and their frequency are presented in the form of table on the right side of the figure.

than others (Supplementary Fig. 1). Particularly, exon 70 was probed to be an important mutation target, which coincides with the results from our cohort. Lastly, this analysis allowed us to detect the predominance of substitutions at exonic and consensus splice site level, and also the prevalence of mutations at the donor splice sites over the molecular alterations affecting the acceptor splice sites.

Concerning linkage disequilibrium results, the Haploview software identified 2 co-segregating blocks formed by 3 and 2 SNPs respectively (Fig. 2). The similarity of the MAF frequencies from our cohort with the IBS and TSI populations correlates with the migratory history of Argentina, mainly Spanish and Italian immigration. However, as the majority of the patients came from Buenos Aires Province and its surroundings, these observations could not represent the rest of the country, as some areas could have more autochthonous genomic features. Furthermore, the MAF comparison could be uncertain, firstly because we do not count with knowledge about the genomic architecture of the indigenous tribes of Argentina. Secondly, although the CML population from 1000Genomes resulted the closest geographically, this does not mean that should be genetically similar to ours. Lastly,

this highlights the need and importance of counting with national databases and, therefore, creating sequencing consortiums for the characterization of every country's genome.

Finally, the present work consists in the first *DMD* gene small mutations screening performed by WES in an Argentine cohort. This methodology allowed us to confirm the clinical diagnosis of patients and identify candidates for the premature stop codon read-through therapy. Also, we were able to establish the carrier status of females at-risk. Furthermore, we have accomplished a characterization of the occurrence and diversity of *DMD* sequence variants in our Argentinian cohort and in the dystrophinopathy patients reported in the LOVD database. Moreover, we were capable of identifying linkage disequilibrium between 5 loci, which haplotypes could have a European origin. In conclusion, the reported results contributed to the characterization of the Dystrophinopathies Argentine population and lead to a better understanding of the small molecular alterations that take place in the *DMD* gene.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.nmd.2018.08.012.

References

- [1] Kunkel LM, Beggs AH, Hoffman EP. Molecular genetics of Duchenne and Becker muscular dystrophy: emphasis on improved diagnosis. *Clin Chem* 1989;35(7 suppl):B21–4.
- [2] Haenggi T, Fritschy JM. Role of dystrophin and utrophin for assembly and function of the dystrophin glycoprotein complex in non-muscle tissue. *Cell Mol Life Sci* 2006;63:1614–31. doi:10.1007/s00018-005-5461-0.
- [3] Gumerson JD, Michele DE. The dystrophin-glycoprotein complex in the prevention of muscle damage. *J Biomed Biotechnol* 2011;2011:210797. doi:10.1155/2011/210797.
- [4] Emery AEH. Population frequencies of inherited neuromuscular diseases- a world survey. *Neuromuscul Disord* 1991;1(1):19–29.
- [5] Guiraud S, Aartsma-Rus A, Vieira NM, et al. The pathogenesis and therapy of muscular dystrophies. *Annu Rev Genomics Hum Genet* 2015;16:281–308. doi:10.1146/annurev-genom-090314-025003.
- [6] Emery AEH. Muscle histology and creatine kinase levels in the foetus in Duchenne muscular dystrophy. *Nature* 1977;266(5601):472–3.
- [7] Darras BT, Miller DT, Urion DK. Dystrophinopathies. GeneReviews [Internet]. Pagon RA, Bird TD, Dolan CR, editors, Seattle, WA: University of Washington; 2000. Available from: <http://www.ncbi.nlm.nih.gov/books/NBK11119/>.
- [8] Emery AEH, Rimoin DL. Duchenne and other X-linked muscular dystrophies. In: Rimoin DL, Connor JM, Pyeritz RE, Korf BR, editors. *Emery and rimoin's principles and practice of medical genetics*. London: Churchill Livingstone; 1996. p. 2337–54.
- [9] Monaco AP, Bertelson CJ, Liechti-Gallati S, Moser H, Kunkel LM. An explanation for the phenotypic differences between patients bearing partial deletions of the *DMD* locus. *Genomics* 1988;2(1):90–5.
- [10] Koenig M, Beggs AH, Moyer M, et al. The molecular basis for Duchenne versus Becker muscular dystrophy: correlation of severity with type of deletion. *Am J Hum Genet* 1989;45:498–506.
- [11] Aartsma-Rus A, Ginjaar IB, Bushby K. The importance of genetic diagnosis for Duchenne muscular dystrophy. *J Med Genet* 2016;53(3):145–51. doi:10.1136/jmedgenet-2015-103387.
- [12] Abbs S, Tuffery-Giraud S, Bakker E, et al. Best practice guidelines on molecular diagnostics in Duchenne/Becker muscular dystrophies. *Neuromuscul Disord* 2010;20:422–7. doi:10.1016/j.nmd.2010.04.005.
- [13] Haas M, Vlcek V, Balabanov P, et al. European Medicines Agency review of ataluren for the treatment of ambulant patients aged 5 years and older with Duchenne muscular dystrophy resulting from a nonsense mutation in the dystrophin gene. *Neuromuscul Disord* 2015;25(1):5–13. doi:10.1016/j.nmd.2014.11.011.
- [14] Aartsma-Rus A, Krieg AM. FDA approves Eteplirsen for Duchenne muscular dystrophy: the next chapter in the Eteplirsen Saga. *Nucleic Acid Ther* 2017;27(1):1–3. doi:10.1089/nat.2016.0657.
- [15] Guiraud S, Squire SE, Edwards B, et al. Second-generation compound for the modulation of utrophin in the therapy of DMD. *Hum Mol Genet* 2015;24(15):4212–24. doi:10.1093/hmg/ddv154.
- [16] Iyombe-Engembe JP, Ouellet DL, Barbeau X, et al. Efficient restoration of the dystrophin gene reading frame and protein structure in DMD myoblasts using the CinDel method. *Mol Ther Nucleic Acids* 2016;5:e283. doi:10.1038/mtna.2015.58.
- [17] Bonilla E, Samitt CE, Miranda AF, et al. Duchenne muscular dystrophy: deficiency of dystrophin at the muscle cell surface. *Cell* 1988;54(4):447–52.
- [18] Murray MG, Thompson WF. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res* 1980;8:4321–4325.
- [19] Prior TW, Wenger GD, Papp AC, et al. Rapid DNA haplotyping using a multiplex heteroduplex approach: application to Duchenne muscular dystrophy carrier testing. *Hum Mutat* 1995;5(3):263–8. doi:10.1002/humu.1380050312.
- [20] Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005;21(2):263–5. doi:10.1093/bioinformatics/bth457.
- [21] Yamamoto LU, Velloso FJ, Lima BL, et al. Muscle protein alterations in LGMD2I patients with different mutations in the Fukutin-related protein gene. *J Histochem Cytochem* 2008;56(11):995–1001. doi:10.1369/jhc.2008.951772.
- [22] Barresi Rita. From proteins to genes: immunoanalysis in the diagnosis of muscular dystrophies. *Skelet Muscle* 2011;1(1):24. doi:10.1186/2044-5040-1-24.
- [23] Guiraud S, Davies KE. Pharmacological advances for treatment in Duchenne muscular dystrophy. *Curr Opin Pharmacol* 2017;34:36–48. doi:10.1016/j.coph.2017.04.002.
- [24] Bonne G, Rivier F, Hamroun D. The 2018 version of the gene table of monogenic neuromuscular disorders (nuclear genome). *Neuromuscul Disord* 2017;27(12):1152–83. doi:10.1016/j.nmd.2017.10.005.