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MLPA analysis of an Argentine cohort of patients with dystrophinopathy: Association of intron breakpoints hot spots with STR abundance in *DMD* gene

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ABSTRACT

Dystrophinopathies are X-linked recessive diseases caused by mutations in the *DMD* gene. Our objective was to identify mutations in this gene by Multiplex Ligation Probe Amplification (MLPA), to confirm the clinical diagnosis and determine the carrier status of at-risk relatives. Also, we aimed to characterize the Dystrophinopathies argentine population and the *DMD* gene. We analyzed a cohort of 121 individuals (70 affected boys, 11 symptomatic women, 37 at-risk women and 3 male villus samples). The MLPA technique identified 56 mutations (45 deletions, 9 duplications and 2 point mutations). These results allowed confirming the clinical diagnosis in 63% (51/81) of patients and symptomatic females. We established the carrier status of 54% (20/37) of females at-risk and 3 male villus samples. We could establish an association between the most frequent deletion intron breakpoints and the abundance of dinucleotide microsatellites loci, despite the underlying mutational molecular mechanism remains to be elucidated. The MLPA demonstrate, again, to be the appropriate first mutation screening methodology for molecular diagnosis of Dystrophinopathies. The reported results permitted to characterize the Dystrophinopathies argentine population and lead to better understanding of the genetic and molecular basis of rearrangements in the *DMD* gene, useful information for the gene therapies being developed.

1. Introduction

Dystrophinopathies are X-linked recessive diseases caused by mutations in the *DMD* gene (OMIM ID: 300377). This gene spans 2.4 Mb and has 79 exons [1]. Also, it has seven different promoters that regulate tissue-specific expression of *DMD*, and together with multiple alternative splicing and polyadenylation sites give rise to, at least, 15 isoforms [2].

The dystrophin protein interacts with actin from cytoskeleton by its N-terminal domain and with the Dystrophin-Associated Glycoproteins complex (DAG) by its cysteine rich and C-terminal domains. These interactions establish a link between the internal and external regions of the cell and play a major role in maintaining membrane stability and organization of membrane specializations. In addition, the protein has a central rod domain which, in skeletal muscle, allows dystrophin to participate in the transduction of muscle strength, protecting the fibers from damage induced by muscle contraction [3,4].

According to the expression pattern of the gene can be distinguished 3 different clinical conditions: 1. Duchenne Muscular Dystrophy (DMD) generated by a complete absence of the dystrophin protein; 2. Becker Muscular Dystrophy (BMD), allelic disease of DMD, produced by a decrease in the amount or function of the protein; and, 3. X-linked Dilated Cardiomyopathy (XLDC) caused by a selective loss of dystrophin in the heart, without significantly signs of muscle dystrophy [5,6].

DMD affects 1:3500 born males and produces early muscle degeneration, leading to increase serum levels of creatine kinase and lactate dehydrogenase. Symptoms begin to show at 2–3 years old, patients become wheelchair-bound at approximately 12 years old and, in the second/third decade of life, they die due to heart or respiratory failure. BMD affects 1:18.000 born males and has similar symptomatology than DMD but with a slower progression rate, thus patients may reach an advanced age [7]. *De novo* mutations and germline mosaicism are responsible for 1/3 of DMD/BMD cases, while family history of Dystrophinopathies with several affected males accounts for 2/3 of cases [8–10].

Due to the X-linked inheritance pattern of Dystrophinopathies females are asymptomatic carriers. However, some women can reveal

Abbreviations: BMD, Becker Muscular Dystrophy; CVS, Chorionic Villus Sample; DMD, Duchenne Muscular Dystrophy; MLPA, Multiplex Ligation-dependent Probe Amplification; PCR, Polymerase Chain Reaction; STR, Short Tandem-Repeat; XLDC, X-linked Dilated Cardiomiopathy.

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symptoms that vary from mild muscle weakness to a more severe clinical course, being classified as manifesting or symptomatic carriers [11]. These female dystrophinopathy patients are hypothesized to have a skewed X inactivation pattern, where the X chromosome carrying a normal copy of the *DMD* gene is being preferentially inactivated. This is also supported by the fact that females heterozygous for *DMD* mutations that have most of their normal X chromosomes randomly inactivated manifest mild *DMD* symptoms [12].

Mutations responsible for the DMD/BMD phenotype are gross deletions (1 or more exons) in 65% of cases, gross duplications in 5% and point mutations in the remaining 30% [13,14]. Large deletions are preferentially clustered on two areas: one mayor deletion hotspot in the central part of the gene, around exons 45 and 53, and a minor hotspot in the 5′end (exons 2–20) [15,16]. Recent studies have reported the existence of a duplication hotspot in the 5′end of the *DMD* gene, being exons 6 and 7 the most frequently duplicated [17]. Different molecular mechanism such as homologous recombination, Non-homologous end joining, presence of replication origins, ALU sequences, microsatellites and matrix attachment regions, has been proposed to explain how these rearrangements in the *DMD* gene arise [17,18]. It has been also established a correlation between deletions generated by aberrant homologous recombination and a preferential maternal origin, and a paternal predisposition to generate small mutations due to replication errors [19].

The "reading frame" theory establishes a correlation between phenotype and mutation type, which agrees with the observed phenotype in 92% of cases [20,21]. 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.

XLDC affects males around 10 and 30 years old and has a similar clinical criteria to others conditions of dilated cardiomyopathy, but with an X-link inheritance pattern and increase levels of serum creatine kinase. Cohen et.al. (2003) [22] classify mutations responsible of XLDC into two groups: one includes mutations affecting transcription and splicing preferentially in the heart and the other group includes mutations altering specific domains of the protein, leading to a loss of functionality in the heart but not in the skeletal muscle [22].

The method of choice for screening large mutations is the quantitative technique Multiplex Ligation-dependent Probe Amplification (MLPA). It is a highly sensitive and rapid alternative to multiplex PCR. The main advantages of the MLPA are the possibility to detect dosage increase and decrease of all *DMD* exons and the application for female carrier analysis [23]. This method can be applied on blood and chorionic villi samples, which allow the usage on prenatal tests also [24].

Nowadays, there is not an effective treatment or rehabilitation of progressive muscular dystrophy. However, several lines of research on *DMD* gene therapies are being developed, for example the ones based on exon skipping, premature stop codon read-through and Utrophin upregulation [25–28]. So, accurate detection and characterization of the causing genetic abnormality is essential to predict the clinical course of the disease which, in turn, allows for a precise genetic counseling and patient follow-up, and to determine the most suitable gene therapy for each case. Yet, until now, only genetic counseling and prenatal diagnosis can be offered to affected families, in order to prevent the birth of new affected boys.

This study aimed to identify mutations in the *DMD* gene by MLPA, in order to confirm the clinical diagnosis in patients and to estimate the probability of developing or transmitting the disease in patient's relatives. Furthermore, our objective was to characterize the Dystrophinopathies argentine population and the *DMD* gene so as to get a better understanding of the genetic and molecular basis of mutations.

2. Materials and methods

2.1. Patients

A group of 121 individuals from 103 non-related families were referred to our laboratory to confirm a clinical diagnosis of Dystrophinopathies or to assess carrier status using MLPA analysis. Samples correspond to 70 affected boys, 11 symptomatic women, 37 at-risk women and 3 prenatal diagnoses. Some of the males from our cohort have a previous mutational screening performed by multiplex PCR.

The following criteria was used for clinical diagnosis of muscle dystrophy in boys and symptomatic females: progressive muscular weakness since childhood; high levels of serum creatine kinase; myopathic changes on electromyography; and, in some cases, a muscle biopsy showing absent or decreased dystrophin levels [7,29,30]. Whereas clinical diagnosis of XLDC was done according the following criteria: dilatation of ventricle diameter; decrease in myocardial contractile function; high creatine kinase levels; and X-linked inheritance pattern [22].

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 [31]. For the fetuses, a chorionic villus sample (CVS) was obtained by trained personnel and DNA was isolated by QIAGEN DNeasy Blood and tissue kit [Redwood City, California (www.qiagen.com)]. DNA concentration and quality were measured by absorbance at 260 nm and by the ratio of A260 nm/A280 nm, respectively. All samples were stored at −20 °C.

2.3. Multiplex Ligation-dependent Probe Amplification assay

The commercially available Multiplex Ligation-dependent Probe Amplification kit for the *DMD* gene was used to determine gene deletion/duplication [23,32,33]. The assay conditions and reactions were performed according to the manufacturer's recommendations [MRC Holland, Amsterdam, The Netherlands (www.mlpa.com)]. The reaction products were analyzed using a DNA analyzer [ABI 3730 XL; Applied Biosystems, Foster City, California] and 500Liz as internal size standard. Data analysis was performed using Coffalyser [MRC Holland, Amsterdam, The Netherlands] and GeneMarker [Softgenetics, State College, Pennsylvania] softwares for MLPA. Wildtype, deleted, and duplicated DNA controls were included in all reactions.

When the MLPA result suggested a single-exon deletion, the result was confirmed by an alternative molecular technique [34]. Whereas cases with single-exon duplication were tested by two independent MLPA studies.

2.4. Polimerase Chain Reaction

Cases of single-exon deletion in males were analyzed by Polimerase Chain Reaction (PCR). The method was performed as previously described elsewhere, with minor modifications [35]. 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 1× TBE buffer and dyed with SYBR Safe [Life Technologies]. Gels were photographed and analyzed with specific software. Positive controls (wild-type DNA), deletion controls (DNA carrying a deletion in the analyze exon) and negative controls (no DNA) were included in all reactions.

2.5. Sanger sequencing

Point mutation screening was performed in cases where the MLPA showed single-exon deletion but the PCR amplified correctly. 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].

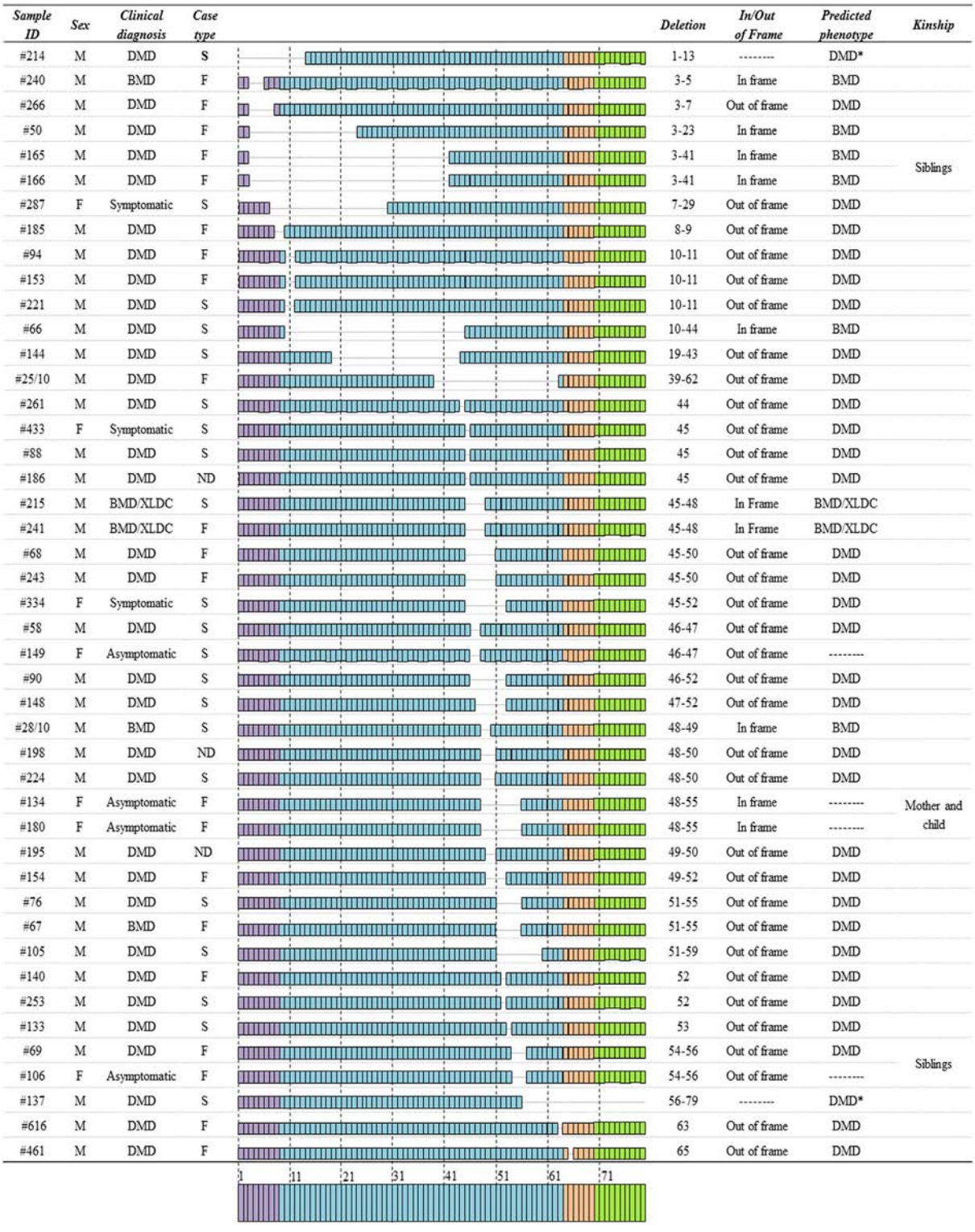

Fig. 1. Subjects with deletion. Patients, symptomatic females and at-risk women with identified deletions are shown. For each individual, the deletion found is represented in an illustration of the Dystrophin cDNA, where each box represents an exon and the color indicates the encoding protein domain (violet: Actin Binding Domain, blue: Rod Domain, orange: Cysteine Rich Domain and green: C-Terminal Domain). The clinical phenotype was stablished according the criteria presented in Materials and methods section (Subjects). The predicted phenotype was determined applying the Reading Frame Rule, except for two cases marked with an asterisk where the phenotype was estimated by the importance of the affected domains. Abbreviations: M, male; F, Female (Sex); F, Familial Case; S, Sporadic Case (Case type) ND, Not determined. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Sequence results were analyzed by comparison with the Genbank RefSeq of *DMD* (NM_004006.1).

2.6. Maternal cell contamination test by Short Tandem Repeats

Fetal DNA obtain from chorionic villus sample were tested for maternal cell contamination due to its presence at a significant level can affect the interpretation of the prenatal study [36]. This was carried out using a segregation analysis of intragenic Short Tandem Repeats (STR), analyzing in parallel the DNA samples from the fetus and his mother. The molecular markers were amplified by fluorescent PCR. Primer sequences were obtained from the Leiden Muscular Dystrophy site [Leiden Muscular Dystrophy webpages (www.dmd.nl)]. The forward primers were label with 6-carboxyfluorescein (6-FAM). The method was performed as previously described by Luce et al. (2014) [37], with minor modifications. All PCR reactions were performed as mentioned above. The reaction products were analyzed using a DNA analyzer as mentioned in Multiplex Ligation-dependent Probe Amplification Assay. Data analysis was performed using Peak Scanner [Applied Biosystems, Foster City, California] software. If the fetus sample did not show extra STR peaks than expected according to the baby sex, the sample was considered to be not contaminated and was used for further prenatal testing.

3. Results

3.1. Molecular diagnosis

Molecular alterations were identified in 56 of the 121 individuals referred. We characterized 45 deletions, 9 duplications and 2 point mutations.

3.1.1. Deletions

Deletions in the *DMD* gene were found in 45 of the studied individuals, corresponding to 42 non-related families. Of them, 38 were affected boys, 3 symptomatic and 4 at-risk women (Fig. 1).

The presence of deletions in the affected children allowed confirmation of the clinical diagnosis of dystrophinopathy. The molecular alterations detected in 28 of them were out-of-frame, which correlates with a *DMD* phenotype according to the reading frame rule. While, 8 boys presented an in-frame deletion, that are associated with BMD. The reading frame rule could not be applied in the remaining 2 cases, due to lack of 5′end of the gene in sample #214 and the absence of 3′ end in sample #137. However, both boys are predicted to have a DMD phenotype because of the absence of important functional domains of the dystrophin protein, actin binding domain (#214) and cysteine rich and C-terminal domain (#137).

The predicted and the observed phenotypes agree in 33 of the patients. In 5 patients the reading frame rule did not coincide with the clinical phenotype. The children #50, #66 and the siblings #165–#166 presented an in-frame deletion affecting exons 3–23, 10–44 and 3–41 respectively but had a DMD phenotype. On the other hand, patient #67 had an out-of-frame deletion of exons 51–55 while having a BMD clinical phenotype.

All the deletions found in the symptomatic females were out of frame, so they correlate with a severe progression of the disease. The presence of deletion in these 3 women had been deduced by hemizygote patterns detected by segregation analysis years before (#287 deletion of STR7A, #334 deletion of STR 45 and STR 50 and #433 deletion of STR45) (data not shown). Then, the MLPA allowed corroborating the previous results and to limit the deletions borders. On the other hand, all women had a skewed X chromosome inactivation pattern as we previously reported in Giliberto et al. [38]. The mutations found and the inactivation pattern verified the clinical diagnosis of Dystrophinopathies and gave an explanation of the symptomatology in these females.

The at-risk women were members of families with at least one affected child carrying an already identified deletion. Due to the fact that these girls presented the familial deletion in the MLPA analysis, it could be determined their carrier status with a 100% certainty.

3.1.2. Duplications

Gross duplications were detected in 9 of the analyzed individuals, corresponding to 8 non-related families. They were 7 affected boys, 1 symptomatic and 1 at-risk woman (Fig. 2).

The duplication detection in the *DMD* gene made possible to confirm the clinical diagnosis in the affected individuals. Even though the rearrangements were not characterized at nucleotide level, we applied the reading frame rule assuming that the duplication had occurred in *tandem* [39]. According to this criterion, all the affected boys are expected to have a *DMD* progression. The clinical phenotype was the same that the one predicted in all 7 cases.

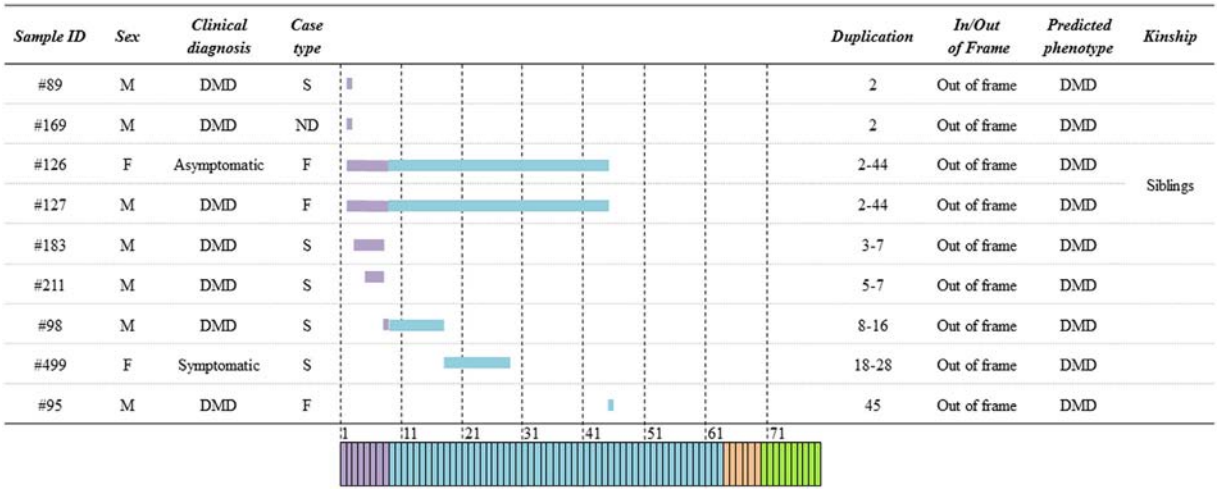

Fig. 2. Individuals with duplication. Patients, symptomatic females and at-risk women carrying a duplication in the *DMD* gene are listed. An illustration of the *DMD cDNA* is shown, where each box represents an exon and the color indicates the encoding protein domain (violet: Actin Binding Domain, blue: Rod Domain, orange: Cysteine Rich Domain and green: C-Terminal Domain). The exons duplicated in each case are represented by a line, colored according to the implicated domains. The clinical phenotype was stablished according the criteria presented in Materials and methods section (Subjects), whereas the predicted phenotype was determined applying the Reading Frame Rule considering an in *Tandem* duplication. Abbreviations: M, male; F, Female (Sex); F, Familial Case; S, Sporadic Case (Case type) ND, Not determined. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

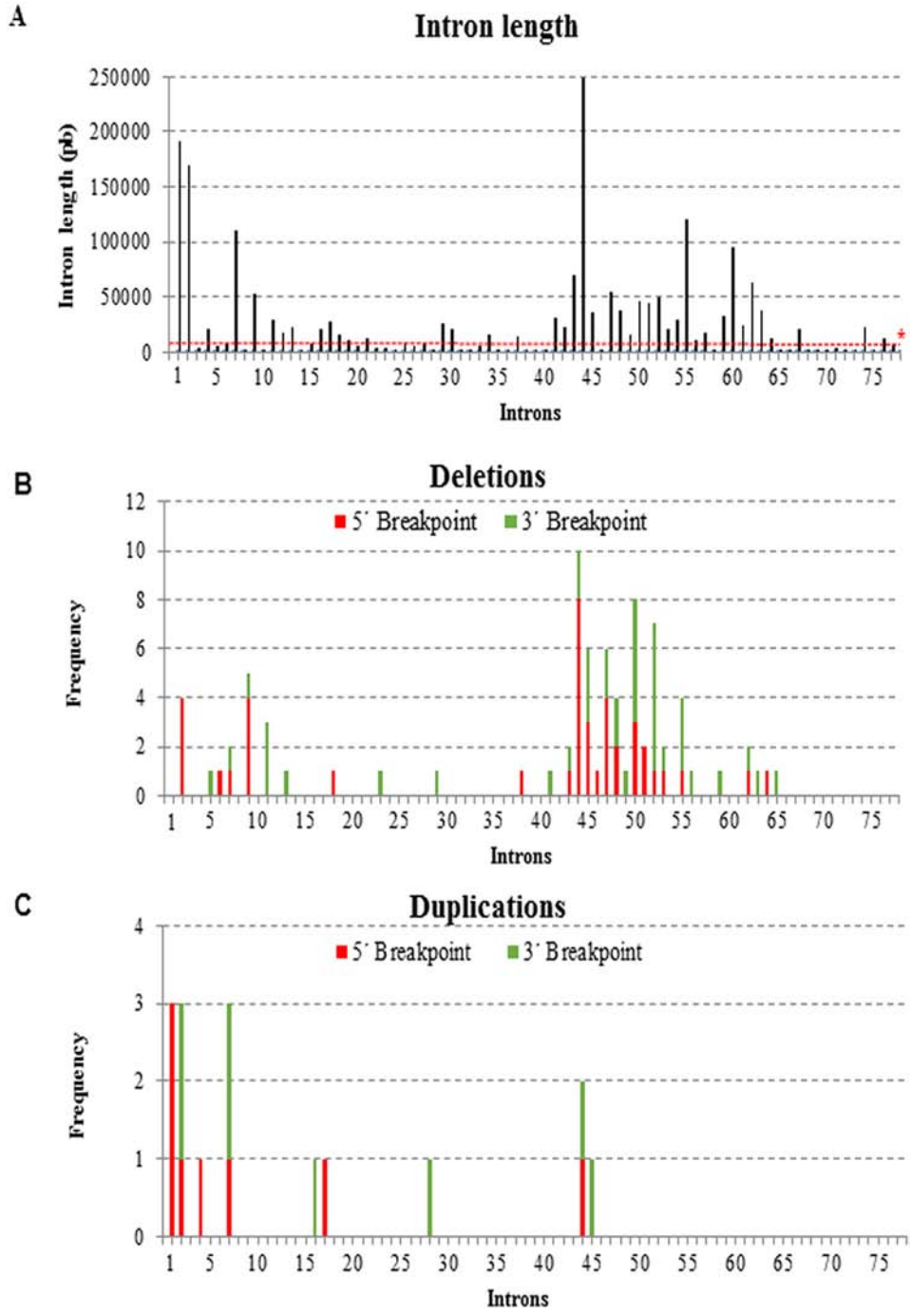

Fig. 3. Breakpoint analysis and correlation with intron length. A. Length of the 78 introns in the *DMD* gene. The red dotted line marked with an asterisk indicates the introns with a length > 10 Kb. Distribution of intronic breakpoints of large deletions (B) and large duplications (C) within the *DMD* gene is shown. The frequency of deletions and duplications 5′ breakpoints (red bars) and 3′breakpoints (green bars) in each intron in indicated. Deletions breakpoints shows a mayor cluster spanning introns 43–55, being intron 44 the most frequently involved in deletions. Even though the amount of duplication intron breakpoints is low, can be noticed a cluster spanning introns 1–7. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The symptomatic female showed an out of frame duplication correlated with a severe disease progression, using the same criteria for the application of the reading frame rule than with boys. Furthermore, just as the symptomatic females with deletion, this patient presented a skewed X chromosome inactivation pattern as previously reported [38]. Therefore, the duplication detected together with the inactivation pattern, verified the clinical diagnosis and explained her symptomatology.

The at-risk woman (#126) analyzed had two affected relatives, her brother (#127) and her uncle. The disease causing mutation was previously identified in her brother, so the finding of this duplication allowed determining her carrier status with a 100% certainty.

3.1.3. Point mutations

The MLPA analysis allowed detecting single exon deletions in two *DMD* affected boys, exon 23 in #49 and exon 58 in #97. In order to verify these results, the implicated exons where amplified by PCR. In both cases, the supposed deleted exons amplified properly whereas the deletions controls did not, so the reaction products were sequenced.

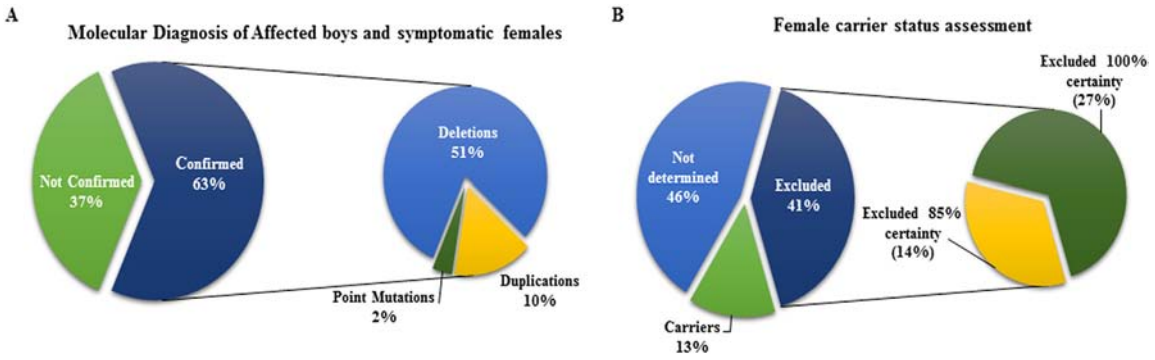

Fig. 4. Genetic assessment analysis. A. Molecular diagnosis of Dystrophinopathy patients and symptomatic females. Also, a distribution of the rearrangements identified in the ones with confirmation of the clinical diagnosis is shown. B. Carrier status assessment of at-risk female relatives of affected boys. In addition, the percentage of certainty with which the women were discarded of being carriers is indicated.

In patient #97 a nonsense mutation was detected in exon 58: c.8608C > T, p.(Arg2870*). In patient #49 a nonsense mutation was detected in exon 23: c.2991C > G, p.(Tyr997*).

These point mutations mapped in the hybridization zone of the MLPA probes, so it was these mismatches that prevented the amplification of the exons by the MLPA technique.

The mutations found confirmed the clinical diagnosis of dystrophinopathy. The appearance of a premature stop codon correlates with a *DMD* phenotype and both mutations were previously described in *DMD* patients by Todorova et al. (2006) and Taylor et al. (2007) [40, 41].

3.1.4. MLPAs without genetic alteration

Absence of large mutations was found in 23 boys and 7 symptomatic females. In these cases, the MLPA analysis was not able to confirm the clinical diagnosis of dystrophinopathy, so whole gene sequencing should be done in pursuit of the disease causing mutation.

On the other hand, absence of molecular alteration in the MLPA allowed 15 female relatives of affected boys, with previously identified mutation, to be excluded from being carriers. The certainty of these results was approximately 85% in 5 cases due to the risk of germline mosaicism, and 100% in the remaining 10 women. Also, 3 male fetuses were excluded of being affected, CVS maternal cell contamination could be discarded in all samples by haplotype analysis.

No deletion or duplication were found in 17 at-risk women of being carriers, they belong to families with DMD sporadic cases without alive patient neither identified the causative mutation.

3.2. Characterization of the DMD gene

In order to characterize the occurrence of mutations and their breakpoints in the *DMD* gene, we have analyzed the deletions and duplications found in our cohort considering for the calculations only mutations from unrelated individuals due to it represent independent mutational events.

3.2.1. Deletions

We have detected 42 deletions, comprising 32 different rearrangements in the *DMD* gene. The exon most frequently involved in a deletion was exon 49 (28.6%, 12/42), which is included in the mayor deletion hotspot.

The length of deletions spanned between 1 and 10 exons in the 80.9% (34/42), 11–20 exons in the 2.4% (1/42), 21–30 exons in the 11.9% (5/42) and 31–40 exons in the 4.8% (2/42). Single and two exon deletions were the most frequently found, 21.4% (9/42) and 19% (8/42) respectively. The median deletion length was 4 exons and the longest one cover 39 exons.

The majority of deletions (61.9%, 26/42) mapped, at least in part, in the central deletion hotspot, considered to comprise exons 45–53. Whereas 12 deletions (28.6%) were detected in the 5′ deletion hotspot of the gene, which spans exons 2–20.

All single exon deletions occurred at the central and 3′end of the gene. The nearest to the 5′ end of the gene was exon 44, while the most distal deletion located at exon 65. Exon 45 was the most frequent single exon deletion found (3/9), followed by exon 52 (2/9).

On the other hand, taking into account the domains of the dystrophin protein, the 78.6% (33/42) of the deletions comprised solely the rod domain (Fig. 1). Deletions spanning rod/actin binding domains and rod/cysteine rich/C-terminal domains accounted for 11.9% (5/42) and 2.4% (1/42) respectively. While deletions affecting exclusively the actin binding were 4.8% (2/42) and the cysteine rich domains 2.4% (1/42). No deletions altering only the C-terminal domain were identified.

The most common 5′breakpoint was intron 44 (19.5%, 8/41), being followed by introns 2, 9 and 47 with 9.8% each (4/41), and introns 45 and 50 both with 7.3% (3/41) (Fig. 3). On the other hand, intron 52 was the most common 3′ breakpoint (14.6%, 6/41), followed by intron 50 with 12.2% (5/41) and, introns 11, 45 and 55 with 7.3% each (3/41). In the particular case of 2 deletions (#214 and #137) the 5′ breakpoint and the 3′breakpoint respectively could not be determined.

3.2.2. Duplications

We have identified 8 duplications, comprising 7 different rearrangements in the *DMD* gene, having been found twice duplication of exon 2.

The number of exons involved in the observed duplications ranged from 1 to 10 exons in the 75% (6/8), 11–20 and 41–50 exons in the 12.5% each (1/8). The longest rearrangement spanned 43 exons. Of all, single exon duplications were the most frequent mutation type found (37.5%, 3/8).

Almost all duplications found located in the 5′ end of the *DMD* gene, except for a single exon duplication of exon 45.

The most common 5′ breakpoint was intron 1 (37.5%, 3/8). While, introns 2 and 7 were the most common 3′ breakpoint accounting for 25% each (2/8).

4. Discussion

The MLPA technique development has improved the detection rate of large mutations in the *DMD* gene, allowing to identifying deletions outside the hot spots and duplications rapidly and easily. Thanks to these advantages, it is widely selected as the starting methodology in a *DMD* mutation screening. Also, as it is a quantitative technique, it has become a method of choice in order to detect female carriers and carry out prenatal tests.

In the present work, the MLPA allowed detection of large rearrangements in 47 affected boys and 4 symptomatic females (51/81 63%) (Fig. 4A). In the remaining 30 cases the causing mutations could have been a small mutation or a pure intronic/regulatory mutation [42], being whole gene/whole exome sequence analysis the next screening methodology

Table 1
Intronic Short Tandem Repeats of the dystrophin gene.

Intron	Short Tandem Repeats
1	$[CA_{17}]$-$[CA_{12}]$-$[(TG)_6(TA)_7]$-$[TG_{12}]$-$[TG_{14}]$-$[TG_{17}]$-$[(TA)_{18}(GA)_{17}]$-$[TA_{10}]$-$[GA_{11}]$
2	$[AC_{16}]$-$[GT_{10}]$-$[GT_{23}]$-$[GT_{20}]$-$[GT_{18}]$-$[GT_{19}]$-$[GT_{18}]$-$[GT_{24}]$-$[GA_{10}]$-$[CT_{13}]$
4	$[CA_{24}]$-$[GT_{11}]$
6	$[(TA)_3TG(TA)_4T(GT)_8(AT)_{13}]$
7	$[(CT)_5TT(CT)_{12}(CA)_{17}]$-$[AC_{18}]$-$[GT_{11}]$-$[(TG)_5(TA)_9]$
9	$[AC_{11}]$-$[CA_{14}]$-$[(AC)_3(TG)_{18}]$
11	$[TA_{14}]$
12	$[(GT)_5(AT)_{10}]$-$[GT_{13}]$
16	$[CT_{19}]$
21	$[CA_{13}]$- $GT_{14}]$
23	$[(TA)_3(TG)_3(TA)_9]$
25	$[(CA)_8TA(CA)_{19}]$
29	$[GT_{11}]$-$[TA_{10}]$
37	$[GT_{19}]$
41	$[CA_{15}]$
43	$[TA_{22}]$-$[TA_{23}]$-$[(TG)_2(TA)_{11}CTG(TA)_9]$
44	$[CA_{23}]$-$[AC_{17}]$-$[(CA)_3TA(CA)_{19}]$-$[(GT)_4ATA(CA)_9]$-$[CA_{17}]$-$[CA_{16}]$-$[(CT)_{23}(CA)_9]$-$[(TG)_6G(GT)_{16}AT(GT)_2(AG)_{14}]$-$[GT_{18}]$-$[GT_{22}]$-$[GT_{23}]$-$[GT_{13}]$-$[(CT)_{11}(GT)_9]$-$[GA_{10}]$-$[GA_{21}]$-$[(GA)_9TT(GA)_7]$-$[TA_{17}]$-$[(TA)_8TT(TA)_8]$-$[TA_{20}]$-$[AT_{22}]$-$[CT_{10}]$-$[(TG)_5(TA)_3(TG)_2TACA(TA)_{19}]$-$[(CT)_{13}CCCTCC(CT)_8]$
45	$[CA_{28}]$-$[CA_{15}]$-$[TC_{25}]$-$[(TA)_7CA(TA)_7(CA)_2(TA)_9TG(TA)_5]$
46	$[(TA)_3(TG)_6TATT(TA)_4(CA)_5]$
47	$[CA_{14}]$-$[(CG)_4(CA)_{15}]$-$[(GT)_{17}(GA)_{14}CA(GA)_5]$
48	$[GT_{18}]$-$[GT_{15}]$-$[GT_{17}]$-$[AG_{25}]$-$[(GT)_7(AT)_4(AC)_{14}]$
49	$[CA_{23}]$-$[CT_{10}]$
50	$[CA_{16}]$-$[GT_{13}]$-$[GA_{10}]$
51	$[CA_{14}]$-$[GT_{13}]$
52	$[GT_{23}]$-$[AT_{16}]$-$[(CA)_{16}GACA(GA)_{12}]$-$[(TA)_8(TG)_{15}]$-$[(TA)_{13}(GA)_{16}GT(GA)_8]$-$[(TA)_5(CA)_3TACA(GA)_{14}]$
53	$[(CA)_5TA(CA)_7TA(CA)_6(TA)_2]$
54	$[CA_{14}]$-$[TA_{13}]$
55	$[AC_{20}]$-$[GT_{23}]$-$[TA_{11}]$-$[(TG)_{14}(TA)_{16}]$ - $[(GT)_6ATTT(GT)_{10}]$ - $[(TA)_{12}(GA)_{19}]$
57	$[CA_{21}]$
59	$[GT_{17}]$-$[GA_{10}]$- $[TA_{17}]$-$[(GT)_8(AT)_{11}(AC)_6]$-$[(TA)_7(CA)_{20}]$
60	$[TG_{10}]$-$[CT_{11}]$-$[(CT)_{13}(CA)_{20}]$-$[(GT)_{27}(GC)_5]$-$[(AT)_8(GT)_5T(TA)_7]$-$[(TA)_8(TG)_5(TA)_9(TG)_7(TA)_7GAAA(GA)_{12}]$
61	$[GT_{10}]$-$[(CT)_{10}(CA)_{18}]$-$[(GT)_{14}(GC)_5]$
62	$[AC_{21}]$-$[AC_{15}]$-$[AC_{21}]$-$[GT_{19}]$-$[CT_{19}]$-$[(CA)_5(TA)_9TG(GA)_{14}]$-$[(GT)_6AT(GT)_{10}AT(GT)_9]$
63	$[CA_{15}]$-$[CA_{17}]$
64	$[GT_{12}]$-$[(CA)_{11}TA(CA)_5(TA)_{10}]$
67	$[CA_{11}]$-$[CT_{35}]$-$[(GT)_6GC(GT)_{20}]$

to be performed. However, another reason could be a mistaken clinical diagnosis which is probable in patients without muscle biopsy and immunohistochemistry tests performed, where another gene could be involved in the disease. This is due to most clinicians and neurologists have changed their diagnostic scheme, starting with molecular studies because of their availability and the invasiveness of the biopsy procedure.

The MLPA permitted to diagnose as carriers 5 from the 37 at-risk women (4 deletions and 1 duplication) (Fig. 4B). Another 15 were excluded of being carriers, 10 with a 100% certainty and 5 with 85% certainty due to the possibility of germline mosaicism. The remaining 17 at-risk women, with normal MLPA results, could not be excluded of being carriers, so whole gene sequence analysis is recommended to rule out possible inherited small mutations. Also, in these inconclusive cases, haplotyping is an alternative method to solve them when key samples are available. In addition, 3 MLPA prenatal diagnoses allowed excluding 3 male fetuses from being affected.

Taking into the account only affected boys, a 67% detection rate of the MLPA was obtained, which coincide with the reported in literature for this technique (approximately 70%) [14,43]. Whereas, the proportion of large deletions and duplications found was 54.3% (38/70) and 10% (7/70) respectively. Comparing these percentages with the reported ones, we can see a slightly increase in the duplication frequency and a decrease in the deletion value [13,14,43]. We consider that the reason is that many males from our cohort have a previous mutational screening performed by multiplex PCR, so only the one with a negative result were reanalyzed by MLPA. This could be the reason why the deletion detection rate is lower than the expected.

As mutation dependent gene therapies are being introduced in Argentina, like exon 51 skipping and premature stop codon read-through, the identification of the disease causing mutation is indispensable in order to determine the most suitable therapy for each patient. The MLPA analysis of our patients allowed us to determined that 9 boys apply for these therapies, 7 can be included in the exon 51 skipping therapy and 2 in the premature stop codon read-through treatment. The exon 51 skipping patients' account for 10% (7/70) of our cohort, which correlates with the 13% previously reported [44].

As regards prognostic diagnosis and considering patients with deletions and duplications, we have found that in the 89% of cases the reading frame rule was able to explain the observed phenotype. Only in 5 cases the clinical and expected phenotype did not coincide. Patient #50 and the siblings #165-#166 presented in-frame deletions spanning the actin binding and the rod domains, previously described in *DMD* affected children [45–47]. According to Aartsma-Rus et al. (2006) [48], the *DMD* phenotype could be due to the loss of the actin binding sites of the dystrophin protein, 3 allocated at the acting binding domain and 1 at the rod domain, and thus the impairment of the protein to anchor to the cytoskeleton. Patient #66 carrying an in-frame deletion of exons 10–44, had a DMD phenotype. This mutation was submitted in the Leiden Duchenne Muscular Dystrophy Mutation Database link with both, *DMD* and BMD phenotypes, so complementary studies are necessaries to clarify these discrepancies. The remaining patient #67 showed an out of frame deletion of exons 51–55 but a BMD clinical phenotype, this disagreement could be explained by the occurrence of natural exon skipping restoring the translational reading frame [49]. However, this hypothesis could not be tested due to unavailability of muscle biopsy.

Noteworthy, the non-related #215 and #241 patients presented the same deletion spanning exons 45–48 that correlates with BMD according to the reading frame rule but, also, has been associated with XLDC [22]. Both individuals showed mild skeletal muscle involvement but a significant cardiomyopathy and high levels of creatine kinase, being #215 in the heart transplant waiting list. In addition, #241 had a 4-year-old deceased sister with cardiomyopathy and walking difficulties. Both patients are good examples of the continuous phenotype range between BMD and XLDC, and that the boundary between these 2 clinical conditions is not clear.

The 4 symptomatic females with identified mutation by MLPA (3 deletions and 1 duplication) showed a skewed X chromosome inactivation pattern [38]. This finding suggests a preferential inactivation of the X chromosome carrying the wild type *DMD* gene, leading to expression of the mutated one. It has been reported that the X-chromosome inactivated is selected because of having a deleterious mutation, so in these cases if the active chromosome carries a *DMD* mutation, the other one could have an even more severe alteration, among others things [50,51]. This molecular mechanism explains the occurrence of female with severe clinical symptoms of an X-linked recessive disease as Dystrophinopathies.

Even though the MLPA technique was developed to detect exon deletions and duplications, it was capable to identify 2 point mutations. Both mutations mapped in the hybridization zone of the MLPA probes, so a single mismatch was enough to decrease amplification being evidenced as a single exon deletion. These findings support the need to corroborate single exon deletion by an alternative methodology. On the other hand, although we know that single exon duplications should also be validated by high density CGH, this is not an affordable methodology in our country, so we inform these duplications only if 2 independent MLPA test arise the same result and suggest that should be corroborated by an alternative technique.

Deletion of exon 45 and exons 10–11 were the most frequently found in the analyzed cohort, both identified in 3 unrelated patients. These deletions map in the deletion hot spots of the gene, mayor and minor hot spots respectively. Single exon deletion of exon 45 was reported by Aartsma-Rus et al. (2006) [48], as the most frequently submitted in the Leiden Duchenne Muscular Dystrophy Mutation Database. Intron breakpoints of these 2 most frequent deletions (introns: 9, 11, 44 and 45) share molecular characteristics (length > 10 Kb and presence of microsatellites) that make them more susceptible to suffer rearrangements.

On the other hand, duplication of exon 2 was the most frequently detected in our unrelated patients, which was also previously described as the most common duplication in the Leiden database [48]. Almost all of the duplications found mapped in the 5′ end of the gene, which agree with the duplication hot spot detected with the advent of quantitative techniques [17].

We have observed that some introns have a mayor susceptibility to suffer breakpoints leading to deletions (Fig. 3). It is also known that microsatellites stimulate recombination events and are involved in genic regulation [52,53]. In order to analyze if there is a correlation between the abundance of STRs and these breakpoints, we performed an *in silico* analysis of the sequence of all the *DMD* introns in order to identify simple and complex dinucleotide microsatellites. We have identified dinucleotides STRs in 37 out of the 78 introns (47.4%), some microsatellites were already described in the Leiden Muscular Dystrophy site [Leiden Muscular Dystrophy webpages (www.dmd.nl)] (Table 1). We have estimated a total of 82 (5′and 3′) breakpoints from the analyzed non-related deletion cohort. Dinucleotide STRs has been found in 92.7% (76/82) of the deletion breakpoints. These findings support the role of STRs in the occurrence of deletion events, while further investigation would be required in order to elucidate the underlying molecular mechanism.

Finally, once more, the MLPA analysis demonstrates to be the appropriate first mutation screening methodology for the molecular diagnosis of Dystrophinopathies. It allowed us to confirm the clinical diagnosis of patients and symptomatic females and determine the candidates for the available gene therapies. Also, we were able to establish the carrier status of at-risk women and to perform prenatal diagnosis. Furthermore, the characterization of the identified mutations in the argentine population make possible to establish a link between the most frequent intron breakpoints and the distribution of dinucleotide STRs. In conclusion, our results permitted to characterize the Dystrophinopathies argentine population and lead to a better understanding of the genetic and molecular basis of rearrangements.

Conflict of interest

The authors declare no conflict of interest.

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