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Clinical and Molecular Profile of Dystrophin Gene Deletions in Eastern Morocco

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Abstract

Dystrophinopathies are genetic muscular disorders with recessive inheritance linked to the X chromosome due to mutations in the dystrophin gene, the *DMD* gene located in Xp21. The best-known forms are Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD). Our study aims to determine the molecular profile and genotype/phenotype correlations of dystrophinopathies in the eastern region of Morocco. We report patients

referred for 4 years (2020-2023) to the Medical Genetics Laboratory of the Mohammed VI University Hospital in Oujda for suspected dystrophinopathy and confirmed by a genetic study of the *DMD* gene using the PCR- Multiplex technique. A total of 15 patients were recruited. They had a mean age of 7.3 years at diagnosis, with a mean age of symptom onset of 3.37 years. Consanguinity was estimated at 46.66%, with 13.33% of familial forms. All patients have a DMD phenotype; calf hypertrophy was present in 86.66% of cases, a positive GOWERS sign, and elevated CK levels were present in all patients. Analysis of the *DMD* gene using the PCR-Multiplex technique showed that 80% of deletions are located in the central region of the gene, exon 50 being the most frequently deleted. 80% of patients had a deletion disrupting the reading frame, and the genotype/phenotype correlation in these cases was explained by the Monaco rule. The molecular analysis of the *DMD* gene is essential for accurate diagnosis, appropriate genetic counseling, and improved patient care. The PCR-Multiplex technique remains a good first-line strategy in the public health system, with a good cost/benefit ratio, enabling the detection of large deletions by analyzing the most frequently deleted exons. Dystrophinopathies represent a frequent reason for requesting genetic analysis in our practice. Multiplex PCR being a simple, rapid, non-invasive and cost-effective tool allowed us to provide a molecular description of this pathology in Eastern Morocco.

Keywords
Dystrophinopathies; Duchenne; Becker; molecular analysis; *DMD* gene; deletions

1. Introduction

Dystrophinopathies are neuromuscular disorders of a genetic origin, represented essentially by Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD). These X-linked recessive diseases are caused by mutations in the *DMD* gene, resulting in a deficiency or alteration of a structural protein of the muscle cell cytoskeleton: dystrophin [1]. The *DMD* gene is one of the largest human genes, located in Xp21 and comprising 2.5 million base pairs with 79 exons transcribed into a 14kb mRNA encoding dystrophin [2]. DMD and BMD are differentiated by clinical phenotype and causal mutation. DMD is the most frequent and most severe, affecting 1/3500-5000 live male newborns [3], characterized by a complete absence of dystrophin, patients present muscle weakness with gait disorders at the age of 3 to 5 and loss of gait around the age of 12, the evolution is characterized by the onset of cardio-respiratory failure leading to death before the age of 25 [4]. On the other side, BMD is less frequent, affecting 1/18500 live male newborns [5], with a slower and later progression due to partial dystrophin deficiency. A molecular study is indicated in the presence of a phenotype suggestive of dystrophinopathy and is essential for an accurate diagnosis, appropriate genetic counseling, and improved patient management. The mutations involved in dystrophinopathy can be large rearrangements including exon deletions and duplications found in around 70% of cases, or point mutations constituting around 30% of mutations [6]. Therefore, deletions and duplications analysis constitute the first step in genetic diagnosis, and they are detected using two techniques. The first is Multiplex PCR, a simple tool providing a rapid and effective diagnosis, allowing the co-amplification of the 19 most frequently deleted exons, enabling

98% of deletions to be detected. The second is MLPA (Multiplex ligation-dependent probe amplification), a semi-quantitative molecular technique for detecting deletions and duplications, with the ability to analyze all 79 exons of the *DMD* gene [7]. Point mutations are suspected when no large anomalies have been detected. These mutations are detected either by conventional PCR followed by Sanger sequencing, or by next-generation sequencing (NGS) [4, 6, 7].

The objective of this study is to describe the clinical and molecular profile of dystrophinopathies in the eastern region of Morocco, to highlight the contribution of Multiplex PCR in its diagnosis and to discuss possible genotype-phenotype correlations.

2. Material and Methods

We conducted a retrospective descriptive study over 4 years between 2020 and 2023 on a series of patients referred to the Medical Genetics Laboratory of the Mohammed VI University Hospital of Oujda for suspected dystrophinopathy confirmed by genetic testing.

We noted for each patient: sex, age at diagnosis, presence or absence of consanguinity, presence or absence of similar cases in the family, age of the first symptom, mode of onset, clinical signs, CK assay, electromyography (EMG), muscle biopsy and results of the genetic testing.

Patient anonymity and confidentiality were respected during data collection. The Biomedical Research Ethics Committee of Oujda (CERBO) approved the ethical protocol for this study.

DNA of the patients was extracted from a venous blood sample collected in an EDTA tube using the QIAamp DNA extraction kit (QIAGEN). DNA quantity and quality were controlled by spectrophotometry.

The primers of the *DMD* gene were designed to target the most frequent deletions (Table 1, Table 2, Table 3). We amplified 19 exons of the *DMD* gene using a Multiplex PCR according to three primer mixes. Mixture I amplified exons 12, 13, 19, 45, 47, 51, and 52. Mixture II covered exons 6, 8, 17, 46, 50 and the promoter, while Mixture III amplified exons 3, 4, 43, 44, 48 and 60 [8].

Table 1 Sequence of oligonucleotide primers for the *DMD* gene. (Set I: *DMD* gene primers of mix I).

Exon	Forward primer (5 → 3)	Reverse primer (5 → 3)
Exon 12	GATAGTGGGCTTTACTTACATCCTTC	GAAAGCACGCAACATAAGATACACCT
Exon 13	AATAGGAGTACCTGAGATGTAGCAGAAAT	CTGACCTTAAGTTGTTCTTCCAAAGCAG
Exon 19	GATGGCAAAAGTGTTGAGAAAAAGTC	TTCTACCACATCCCATTTTCTTCCA
Exon 45	AAACATGGAACATCCTTGTGGGGAC	CATTCCTATTAGATCTGTCGCCCTAC
Exon 47	CGTTGTTGCATTTGTCTGTTTCAGTTAC	GTCTAACCTTTATCCACTGGAGATTTG
Exon 51	GAAATTGGCTCTTTAGCTTGTGTTTC	GGAGAGTAAAGTGATTGGTGGAAAATC
Exon 52	AATGCAGGATTTGGAACAGAGGCGTCC	TTCGATCCGTAATGATTGTTCTAGCCTC

Table 2 Sequence of oligonucleotide primers for the *DMD* gene. (Set II: *DMD* gene primers of mix II).

Exon	Forward primer (5 → 3)	Reverse primer (5 → 3)
DMD Pm	GAAGATCTAGACAGTGGATACATAACAAATGCATG	TTCTCCGAAGGTAATTGCCTCCCAGATCTGAGTCC
Exon 6	CCACATGTAGGTCAAAAATGTAATGAA	GTCTCAGTAATCTTCTTACCTATGACTATGG
Exon 8	GGCCTCATTCTCATGTTCTAATTAG	GTCCCTTACACACTTTACCTGTTGAG
Exon 17	GACTTTCGATGTTGAGATTACTTTCCC	AAGCTTGAGATGCTCTCACCTTTTCC
Exon 46	GCTAGAAGAACAAAAGAATATCTTGTC	CTTGACTTGCTCAAGCTTTTCTTTTAG
Exon 50	CACCAAATGGATTAAGATGTTCATGAAT	TCTCTCTCACCCAGTCATCACTTCATAG

Table 3 Sequence of oligonucleotide primers for the *DMD* gene. (Set III: *DMD* gene primers of mix III).

Exon	Forward primer (5 → 3)	Reverse primer (5 → 3)
Exon 3	TCATCCATCATCTTCGGCAGATTAA	CAGGCGGTAGAGTATGCCAAATGAAAATCA
Exon 4	TTGTCGGTCTCTCTGCTGGTCAGTG	CAAAGCCCTCACTCAAACATGAAGC
Exon 43	GAACATGTCAAAGTCACTGGACTTCATGG	ATATATGTGTTACCTACCCTTGTCGGTCC
Exon 44	CTTGATCCATATGCTTTTACCTGCA	TCCATCACCCTTCAGAACCTGATCT
Exon 48	TTGAATACATTGGTTAAATCCCAACATG	CCTGAATAAAGTCTTCCTTACCACAC
Exon 60	AGGAGAAATTGCGCCTCTGAAAGAGAACG	CTGCAGAAGCTTCCATCTGGTGTTCAGG

In a total volume of 20 µl, the reaction mixture comprised 10 µl of Multiplex master mix, 3 µl of primers, 6 µl of molecular biology grade water and 1 µl of patient DNA (Table 4). The Multiplex PCR reaction was performed in a thermocycler (Biometra TRIO), with a primer melting temperature of 55°C (for set I; II) and 58°C (for set III) for 35 cycles (Table 5). The amplification of the products was controlled by 3% agarose gel electrophoresis, and visualized under UV light. The results obtained are interpreted according to the presence or absence of the bands expected under UV and using a size marker as a reference.

Table 4 Reaction mixture for multiplex PCR.

Reagents	Volume
PCR Master Mix	10 µl
Primer mix	3 µl
Molecular biology grade water	6 µl
DNA	1 µl
Total volume of the reaction	20 µl

Table 5 PCR conditions in the thermocycler.

Step	Temperature	Time	Number of cycles
Initial denaturation	95°C	10 min	1 cycle
Denaturation	95°C	30 sec	
Annealing	55°C or 58°C	45 sec	35 cycles
Extension	72°C	1 min	
Final Extension	72°C	15 min	1 cycle

The DOVE (DMD Open-access Variant Explorer) tool, generated by Leiden University Medical Center (https://www.dmd.nl/), was used to interpret *DMD* gene deletions and predict their effect on the reading frame.

3. Results

A total of 15 patients were recruited in the study. The average age of patients at genetic diagnosis was 7.3 years. 11 of 15 patients (73.33%) were between 6 and 10 at the moment of genetic diagnosis. The onset of the first clinical signs in our series ranged from 1.5 to 6 years, with an average age of 3.37 years. The onset of clinical signs was before 5 years of age for 12 patients (80%), and between 5 and 10 years of age for three patients (20%). Consanguinity was noted for 7 patients (46.66%). Two patients had similar cases in the family (13.33%) and 13 cases were sporadic (86.6%). All patients in our study had normal psychomotor development, with walking difficulties in 7 cases (46.66%). Difficulty running and climbing stairs was the first clinical sign in 5 patients (33.33%). Muscular fatigability was the first symptom for 3 patients (20%). 13 patients in our series (86.66%) had calf hypertrophy. All patients in our series had a positive Gowers sign. None of the patients had cardiac and/or respiratory involvement. 14 patients (93.33%) performed CK testing, and all had elevated levels. The lowest value was 1125 IU/L and the highest was 21550 IU/L. EMG was performed for 4 patients (26.66%), resulting in a myogenic tracing. 2 patients (13.33%) had a muscle biopsy showing a dystrophic appearance with regeneration, fibrosis and adiposis of muscle fibers (Table 6).

Table 6 Clinical and molecular features of the patients.

	N1	N2	N3	N4	N5	N6	N7	N8	N9	N10	N11	N12	N13	N14	N15
Age at the time of the study (years)	2	5	8,5	5,2	11	8,6	7	8,4	10	5,5	8	7,7	10	6	8
Consanguinity	Yes	Yes	No	No	No	Yes	Yes	Yes	No	No	Yes	No	Yes	No	No
Similar cases in family	Yes	No	No	No	No	No	No	No	No	No	No	No	Yes	No	No
Age of onset of symptoms (years)	1,8	1,5	4	3	-	3	4	6	4	5	4	2	3	2	4
Symptoms at the time of study	Walking difficulty	Muscular fatigability	Muscular fatigability	Muscular fatigability	Walking difficulty	Walking difficulty	Walking difficulty	Difficulty in running and climbing stairs	Difficulty in running and climbing stairs	Muscular fatigability	Walking difficulty	Muscular fatigability	Muscular fatigability	Muscular fatigability	Difficulty in running and climbing stairs
Calf hypertrophy	Yes	-	Yes	Yes	Yes	-	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
GOWERS sign	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CK IU/L (30-200 IU/L)	16880	1125	18427	9985	15030	10650	9423	9148	9658	6000	9028	8300	-	21550	2863
EMG	-	-	Myogenic damage	-	Myogenic damage	Myogenic damage	-	-	-	-	-	-	-	-	Myogenic damage

Muscle biopsy	-	-	Dystrophic appearance with regeneration, fibrosis and adiposis of muscle fibers	-	Dystrophic appearance with regeneration, fibrosis and adiposis of muscle fibers	-	-	-	-	-	-	-	-	-	-
Deleted exons	48-52	50	45-52	19-43	8-19	46-51	46-47	45	46-50	45-51	52	45	48-52	12-13	52

Abbreviations: N: Patient Number, CK: Creatine kinase, EMG: Electromyography.

Among the 15 cases in our study, 12 patients (80%) carry deletions in the central region of the *DMD* gene from exon 43 to exon 52, two patients (13.33%) present a deletion in the proximal region from exon 8 to exon 19, and one patient (6.6%) has a deletion affecting both regions (Figure 1). Exon 50 was the most frequently deleted, notably for 7 patients (46.66%), followed by exon 48 in 6 patients (40%). Using the DOVE tool on 15 DMD cases, we retained 12 patients (80%) with a frame-disrupting deletion, 2 patients with an in-frame deletion and one patient with a nonsense deletion (Table 7).

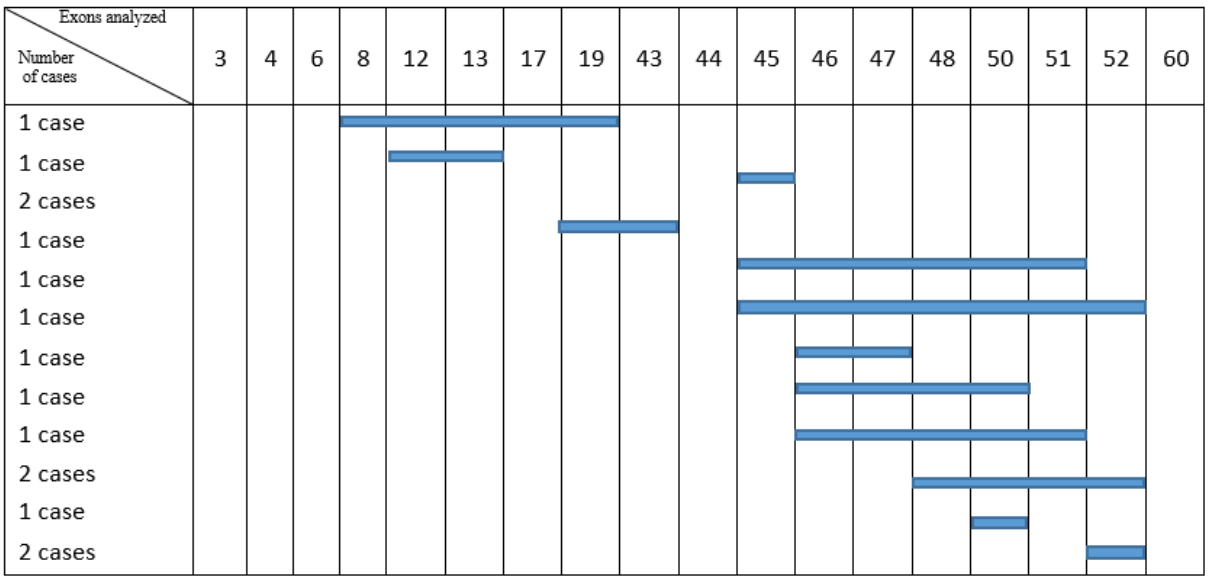

Figure 1 Combinations of *DMD* gene deletions in our patients.

Table 7 Consequence of deletions in patients according to the DOVE tool.

Patients	Deletion	Length of mutated sequence	consequence
N1, N13	48-52	748 Nucleotides	Frameshift
N2	50	109 Nucleotides	Frame shift
N3	45-52	1222 Nucleotides	Frame shift
N4	19-43	3998 Nucleotides	Non-sense
N5	8-19	1731 Nucleotides	In frame
N6	46-51	928 Nucleotides	Frame shift
N7	46-47	298 Nucleotides	Frame shift
N8, N12	45	176 Nucleotides	Frame shift
N9	46-50	695 Nucleotides	Frame shift
N10	45-51	1104 Nucleotides	In frame
N11, N15	52	118 Nucleotides	Frame shift
N14	12-13	271 Nucleotides	Frame shift

4. Discussion

The DMD and DMB are the most frequent genetic myopathy in children. The mean age of onset of symptoms and the mean age of genetic diagnosis in our patients are similar to the data collected in developing countries, 3.37 and 7.30 years respectively, but higher than those collected in

developed countries (Table 8). This difference in results is probably due to the fact that in developed countries, transaminases and CK are frequently measured in children. Once an elevated CK level is detected, boys are often referred directly to a specialized center, probably explaining the shorter time to diagnosis in these cases [9].

Table 8 Mean age of appearance symptoms, diagnosis and delay of diagnosis for DMD in our series compared to the literature.

Country	Year of Study	Mean age of onset symptoms	The mean age of diagnosis	Diagnosis timeframe	Reference
India	2015	3.93	7.74	3.81	Dey et al., [9]
France	2013	2.8	4.8	2	Humbertclaude et al., [10]
Italy	2016	2.6	3.4	0.8	D'amico et al., [11]
USA	2019	2.89	4.43	1.54	Counterman et al., [12]
Morocco (Oujda)	2023	3.44	7.20	3.76	Our series

In our study, 13.33% of DMD patients (2/15) had a family history of Duchenne muscular dystrophy. The study by Gowda VL et al., which collected data from the UK and Ireland, reported 21% of familial cases [13]. However, the study by Tong et al. in China reported a DMD family history in 8.5% of cases [14].

In our study, the diagnosis of Duchenne muscular dystrophy was revealed by motor signs in all patients, dominated by difficulty walking in 46.66% of patients, followed by difficulties in running and climbing stairs in 33.33% of patients and muscle weakness in 20% of patients. In the study by Humbertclaude et al. [10], the first clinical sign is most often an abnormality in motor function (69% of cases), with difficulty walking, climbing stairs, running or overall muscle weakness. However, a delay in psychomotor acquisition is the revealing sign in 28% of patients, with the occasional purely cognitive delay (3.5% of cases). According to D'Amico et al. [11], the revealing mode of the disease was dominated by elevated CK levels (44.3% of patients), motor deficit (15.9% of cases) and muscle weakness in 14% of cases. Our results are in line with those reported in the literature, with the exception of CK levels, which can be a revealing sign enabling early diagnosis.

86.66% of patients in our series presented with calf hypertrophy, similar to the literature. Dey et al. reported 93.8% of patients with calf hypertrophy [9].

All our patients have a positive GOWERS sign. Dey et al. found a positive GOWERS sign in 70.37% of patients [9] and Mekaoui et al. in 67% of patients [15].

All patients with CK testing in our study had elevated levels, in accordance with the literature and the results of other studies. CK levels varied between patients in our study from 1125 IU/L to 21550 IU/L, depending on the degree of muscle destruction. Since the advent of molecular genetics, recourse to other complementary examinations, notably EMG and muscle biopsy, has been considerably reduced. However, immunohistochemistry remains essential especially in cases where the molecular results are negative. In our study, only 26.66% of patients had EMG showing myogenic involvement. Similarly, only 13.33% of patients had a muscle biopsy revealing muscular dystrophy (Table 6).

Multiplex PCR analysis of the *DMD* gene was performed on all patients in our study, revealing deletions of one or more exons. Multiplex PCR is a molecular biology method with primordial interest in determining deletions of one or more exons in large genes such as the *DMD* gene. This technique is based on the amplification of specific intragenic regions known to be the most frequently incriminated in the molecular mechanism of the disease.

According to previous studies (Table 9), we noted a non-random distribution of large deletions. Indeed, 80% of deletions are located in the central region (distal hotspot) of the gene. In comparison, 13.33% of deletions are located in the proximal region and 6.6% of cases have a large deletion affecting both hotspots. In our cohort, deletion of exon 50 was the most frequent, followed by exon 48. This is almost in line with some of the results reported in the study by El Kadiri et al. in Morocco (exon 50 then exon 48) [16], the study by Zhao et al. in China (exon 47 and exon 50) [17] and the study by Barzegar et al. in Iran (exon 50 and exon 49) [18]. In our samples, single-exon deletions represented 33.33% of cases, and deletions of two or more exons occurred in 66.66% of patients. El Kadiri et al. reported the same results, with 29.81% of cases having a single exon deletion [16], and Barzegar et al. reported 33.3% of cases with a single exon deletion [18]. Deletion of exons 48 to 52 and deletion of exon 45 are the most frequently found in patients in our series.

Table 9 Distribution of deletions in Hotspots compared to other studies.

Study	Distribution of deletions	
	Central Region	Proximal Region
Dey et al., 2015 [9]	72.6%	10.9%
El Kadiri et al., 2020 [16]	74.5%	18%
Our study	80%	13.33%

Multiplex PCR is a fast and efficient method that detects 90 to 98% of deletions in the *DMD* gene responsible for Duchenne and Becker muscular dystrophies. However, it is limited in detecting duplications, which account for approximately 5 to 7% of the remaining mutations. The MLPA technique, on the other hand, allows screening of all exons in the *DMD* gene and can detect both deletions and duplications. However, like multiplex PCR, MLPA has limitations in detecting small point mutations, such as base substitutions or small insertions/deletions, which are not always identifiable with these techniques [19].

The Monaco rule described in 1988 showed that the phenotype of dystrophinopathies was not correlated with the size of the deletion but with the consequence of the deletion on the reading frame [20]. Mutations that maintain an open reading frame (in-frame mutation) in the mutated transcript allow the synthesis of normal or reduced quantities of partially functional truncated dystrophin, leading to a Becker-type phenotype. However, in the DMD phenotype, frameshift mutations resulted in unstable RNA and the production of an almost undetectable level of truncated dystrophin [21, 22]. In our cohort, the Monaco rule applied to 80% of *DMD* mutations. These results agree with previous studies where exceptions to the reading frame rule have been reported. The study by Mohammed et al. in Kuwait reported that 75.6% with a DMD phenotype had a reading frame-disrupting deletion, whereas 24.4% of DMD patients had a reading frame-preserving deletion [23]. Similarly, in the study by Guo et al. in China, the Monaco rule can explain 88.6% of *DMD* gene mutations [24]. Exceptions to the reading frame rule have been reported and explained by certain

deletions that do not alter the reading frame but affect functional domains of dystrophin such as the N-terminal actin-binding domains or the Cys-rich domain affecting dystrophin binding to F-actin or ß-dystroglycan, respectively, which would severely impair the functionality of this protein [25].

Deletions of one or more exons that alter the reading frame generally correlate with the DMD phenotype as reported in our patients. Among patients with DMD, the incidence of dilated cardiomyopathy (DCM) increases with disease progression. Approximately one-third of individuals are affected by the age of 14 years, half by the age of 18 years, and all individuals after 18 years [26]. Like DMD, individuals with BMD are at risk for developing DCM [27]. DCM may be associated with mutations in genes encoding structural proteins of the heart muscle, including dystrophin in some cases. *DMD* gene is a relatively rare cause of DCM. In a cohort of 99 Japanese individuals with DCM, pathogenic variants in the *DMD* gene were identified in only three patients [28].

Dystrophinopathies are inherited in an X-linked recessive pattern. Female carriers have a 1-in-4 risk of having an affected boy and a 1-in-4 risk of having a carrier girl in each pregnancy. The daughters of a man with dystrophinopathy will all be carriers, while none of his sons will be affected. Sporadic cases are reported in a third of cases. All families of affected patients in our series benefited from genetic counseling, during which the risk of recurrence was explained, and the value of screening, follow-up, and monitoring of female carriers was recommended [29].

5. Conclusion

The molecular analysis of the *DMD* gene is an important step toward accurate diagnosis and better patient care. The Multiplex PCR technique remains a good first-line strategy in the public health system, with a good cost/benefit ratio, enabling the detection of large deletions by analyzing 19 of the most frequently deleted exons. Molecular analysis in our study revealed that deletions had a non-random distribution and were concentrated mainly at the distal region (exon 43-52). The phenotype/genotype correlation depends on the reading frame rule described in Monaco in 1988. Genetic counseling is an important element in the care of the disease in order to diagnose female carriers, assess the risks for the descendants, and discuss the different possibilities for having an unaffected child, including prenatal and preimplantation genetic diagnosis.

5.1 Study Limitations

Deletions in the *DMD* gene represent the majority of mutations identified in Becker and Duchenne muscular dystrophies. Multiplex PCR can detect 98% of the principal frequent deletions by studying 19 specific exons of the *DMD* gene. Complementary techniques such as MLPA and NGS are needed to identify deletions of other rarely deleted exons and other types of mutations. The genotype-phenotype correlation in our study is limited by the small size of our series and by the particularities of certain primers (intra-exonic primers and overlap with common SNPs).

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Author Contributions

Smaili Fatimazahra: conducted data collection, performed the experiments and wrote the manuscript with input from all authors. Zerrouki Khawla: conducted data collection, contributed to the interpretation and the analysis of the results and to the writing of the manuscript. Aouni Fatima Ezzahra: aided in interpreting the results and contributed to the final manuscript. Ghanam Ayad: provided the study materials, aided in the analysis, discussed the results and contributed to the final manuscript. Rkain Maria: aided in the analysis, discussed the results and contributed to the final manuscript. Babakhouya Abddeladim: provided the study materials, aided in the analysis, discussed the results and contributed to the final manuscript. Tajir Mariam: designed and supervised the project, coordinated the execution of research activities, contributed to writing the manuscript.

Competing Interests

The authors have declared that no competing interests exist.

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