A manifesting female carrier of Duchenne muscular dystrophy: importance of genetics for the dystrophinopathies

INTRODUCTION

Dystrophinopathies are a group of X-linked neuromuscular disorders arising from mutations in the dystrophin (*DMD*) gene. The *DMD* gene encodes dystrophin protein, which plays an integral role in stabilising the subsarcolemmal membrane. These disorders can be differentiated based on the degree of *DMD* gene expression, which determines disease severity and clinical phenotype. The absence of dystrophin protein leads to a more severe phenotype of Duchenne muscular dystrophy (DMD), while reduced amount or function of dystrophin causes the milder phenotype of Becker muscular dystrophy (BMD). Although these disorders primarily affect males, females can be carriers that manifest symptoms in rare cases.[1] Traditionally, diagnosis of dystrophinopathies relied on clinical features and muscle biopsy. However, there has been a shift towards genetic diagnosis in recent years.[2] With advancements in genetic therapies, genetic testing has become increasingly important for the diagnosis, prognostication and identification of individuals who may benefit from such targeted treatment.[1] We present the case of a manifesting carrier of DMD, and discuss disease pathogenesis, genetic testing and available treatments for dystrophinopathies.

CASE PRESENTATION

A 55-year-old Chinese woman presented at our centre with a fall. She had a background of lower limb weakness since her adolescence, and recently started ambulating with a walking stick. She also had a family history of neuromuscular disease, with a son who was diagnosed with DMD through muscle biopsy. The examination revealed normal cognitive function and proximal lower limb weakness. Gower's sign and bilateral calf muscle pseudohypertrophy were observed [Figure 1]. Serum creatine kinase (CK) level was elevated (610 U/L). Collectively, these findings of proximal limb weakness, elevated serum CK and family history of DMD raised clinical suspicion of dystrophinopathy.

Spirometry was subsequently performed to screen for respiratory muscle involvement. This revealed low forced expiratory volume in 1 second (FEV1) and forced vital capacity (FVC) (62% and 53% predicted, respectively), and a normal FEV1/FVC ratio (0.85) that is suggestive of restrictive lung disease [Figure 2]. Maximal expiratory pressure (MEP) and inspiratory pressure (MIP) were reduced (MEP: 48 cm H_2O; MIP: 40 cm H_2O). Screening electrocardiogram was unremarkable, while transthoracic

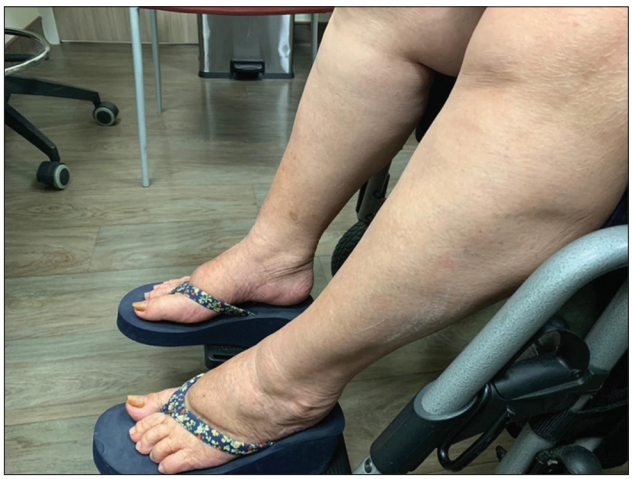

Figure 1: Photograph shows bilateral calf muscle pseudohypertrophy.

echocardiogram demonstrated dilated left atrium and left ventricular ejection fraction of 70%. Cardiac magnetic resonance (CMR) imaging was performed to assess for subclinical cardiomyopathy [Figure 3]. On CMR imaging, asymmetric thickening of inter-ventricular septum was demonstrated in four-chamber view [Figure 3a, white arrow]. Short-axis view at the mid-ventricular level revealed mid-wall and subepicardial late gadolinium enhancement (LGE) in the left ventricular myocardium [Figure 3b, white arrows]. Notably, these mid-wall and subepicardial patterns of LGE characteristically reflect non-ischaemic myocardial fibrosis, typically seen in dystrophinopathy-related cardiomyopathies.[3] She was subsequently diagnosed as a manifesting carrier of DMD, with subclinical respiratory and cardiac involvement.

Both the patient and her son were recommended for genetic testing for definitive diagnosis of DMD, but they declined. As genetic testing was not performed, we acknowledge the potential of missing other neuromuscular disorders that mimic DMD. However, the patient was presumed to be a manifesting carrier of DMD in view of her positive family history (affected son with biopsy-proven DMD) and clinical course and manifestations consistent with DMD (e.g. calf pseudohypertrophy, proximal myopathy, Gower's sign and elevated CK levels). Moreover, approximately two-thirds of genetic mutations in DMD are inherited through carrier mothers, while one-third are *de novo* mutations. Hence, every mother of a child with DMD, as in this case with our patient, has a substantial chance (approximately two-thirds) of being a DMD carrier.[1] Close monitoring and counselling regarding

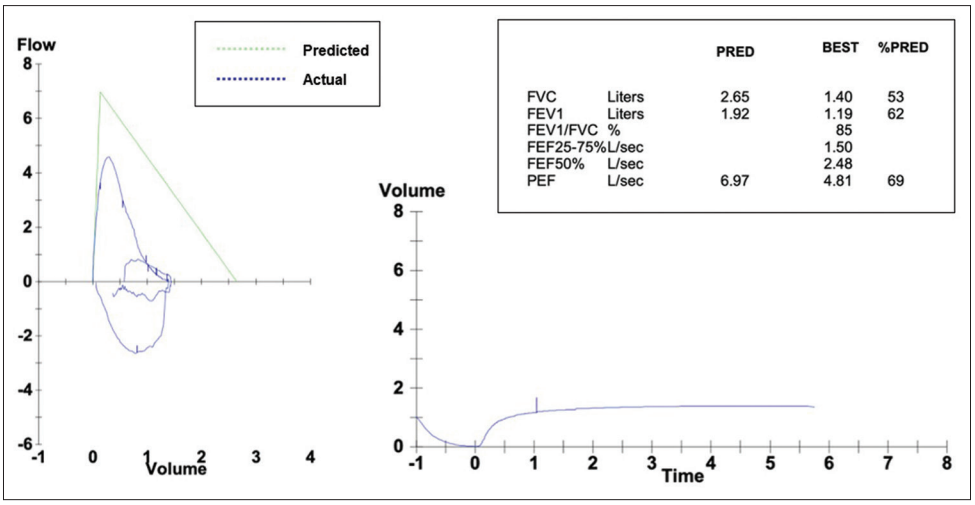

Figure 2: Spirometry graphs of the patient show the flow-volume and volume-time curves.

genetic testing and associated advances will continue to be offered to both the patients and their family.

The patient received care from a multidisciplinary team, including neuromuscular, cardiac and respiratory specialists and physiotherapists. As subclinical cardiomyopathy was demonstrated on CMR imaging, valsartan and bisoprolol were started in view of the potential benefits for delaying the progression of myocardial fibrosis and decline of left ventricular ejection fraction in dystrophinopathies.[4] Although there were no symptoms of nocturnal hypoventilation, pulmonary function testing (PFT) demonstrated FVC approaching <50% predicted and inspiratory muscle weakness (MIP <60 cm H_2O). Hence, she was started on nocturnal BiPAP. Three years later, she became wheelchair-bound and developed proximal upper limb weakness. She continued to not have overt respiratory or cardiac symptoms, with clinical recognition potentially masked by the loss of ambulation from neuromuscular weakness. She continued to receive serial monitoring of disease progression with PFT once every four months, Holter monitoring once a year and CMR imaging once every two years.

DISCUSSION

Dystrophinopathies are a group of X-linked neuromuscular disorders arising from dysfunction of the dystrophin protein. They include DMD, a progressive and fatal muscular dystrophy characterised by the lack of functional dystrophin, and the milder form of BMD, where the partial function of dystrophin is preserved. The prevalence of DMD and BMD is 2–12 and 0.2–9 per 100,000 males, respectively.[5]

Males with DMD usually present after the first year of life with delayed acquisition of gross motor milestones. Early motor manifestations include frequent falls, inability to run, difficulty with stairs and Gower's sign (using arms to climb up the thighs) when getting up from the floor.[1] They may have calf pseudohypertrophy and excessive lumbar lordosis. CK is

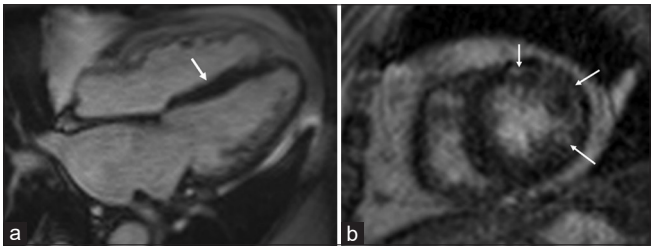

Figure 3: Cardiovascular MR imaging with late gadolinium enhancement (LGE). (a) Four-chamber view shows asymmetric thickening of inter-ventricular septum (white arrow). (b) Short-axis view at the mid-ventricular level shows mid-wall and subepicardial LGE in the left ventricular myocardium (white arrows).

invariably elevated, frequently in the ranges of >6,000 U/L.[6] Without treatment, ambulation is lost around 12–14 years of age.[1,6] They develop orthopaedic issues, such as spinal scoliosis and Achilles tendon contractures because of skeletal muscular weakness. Respiratory insufficiency ensues and is progressive, usually occurring after the loss of ambulation around the age of 15.[1,6] Patients surviving into adulthood develop dilated cardiomyopathy as the dystrophinopathy manifests within cardiac muscles, resulting in cardiac failure and arrhythmias from myocardial fibrosis.[1,6,7]

Historically, the diagnosis of muscular dystrophy was made based on clinical signs and symptoms, and with muscle biopsy. Pathological features of dystrophinopathies include the presence of necrotic and regenerating fibres, myopathic grouping, increased endomysial fibrosis and reduced dystrophin staining on immunohistochemistry [Figure 4].[2]

DMD is the first muscular dystrophy in which the disease gene has been identified.[8] Determining the genetic basis of dystrophinopathies has deepened our understanding of the disease pathogenesis and impacted genetic counselling. It has highlighted nuances in genotype–phenotype correlation, the importance of screening asymptomatic mothers and impacted the management of patients.

Understanding disease pathogenesis and impact on genetic counselling

The gene coding for dystrophin is the largest known gene in humans, with a molecular weight of 2.3 Mb. It is situated on the X chromosome and consists of 79 exons with 11 Kb of coding sequences.[9] Transmission, therefore, occurs from carrier mothers to their sons. However, the rate of *de novo* mutations in the dystrophin gene is high (up to 1 in 3),[9] in part owing to its large molecular size. For non-carrier females with affected first sons, there is an even greater risk of subsequent affected sons of around 4%–12% due to germline mosaicism (a percentage of the oocytes carrying the mutation).[10]

The dystrophin gene encodes for a 427 kD sarcolemmal protein, dystrophin. Dystrophin anchors the cytoskeletal F-actin in myocytes to the extracellular matrix. It consists of an N-terminal domain, a rod domain, a cystine-rich (CR) domain and a C-terminal domain. The N-terminal domain and a second actin binding domain within the rod domain bind to actin, while the CR domain and the C-terminal domain bind to transmembrane proteins and the extracellular matrix. The rod domain consists of 70% of the molecular weight of dystrophin, which is made up of 24 spectrin-like repeats and four intervening hinges and contains a nitric oxide synthase (nNOS) binding domain.[1]

Several mechanisms explain how dystrophin deficiency results in muscle damage. Firstly, the absence of dystrophin, which provides structural integrity by linking the cytoskeleton to the extracellular matrix, exposes the sarcolemma to the risk of damage from the stress of repeated contractions.[1] Secondly, the nNOS binding domain is essential in generating vasodilatory nitric oxide in response to exercise, enhancing perfusion to exercising muscles, the absence of which results in functional ischaemia in myocytes.[11] Thirdly, reactive oxygen species generation is upregulated in DMD due to microtubule disorganisation.[12] Infiltrating inflammatory cells and dysfunctional mitochondria further contribute to free radical generation, causing damage to already vulnerable muscle. Further purported mechanisms include cytosolic calcium overloading triggering degenerative pathways, failure of muscle regeneration due to dysfunctional epigenetic regulation of muscle progenitor cells[13] and repression of autophagy leading to accumulation of uncleared cellular debris.[14]

Understanding genotype–phenotype correlations

The spectrum of clinical phenotypes present in dystrophinopathies is closely correlated with the degree of dystrophin deficiency. Generally, variants that disrupt the reading frame lead to prematurely truncated dystrophin products that are non-functional, giving rise to the severe phenotype of DMD. However, variants that preserve the reading frame lead to altered dystrophin protein that still has its actin and transmembrane binding sites intact, allowing it to remain partially functional. This gives rise to the milder phenotype of BMD.

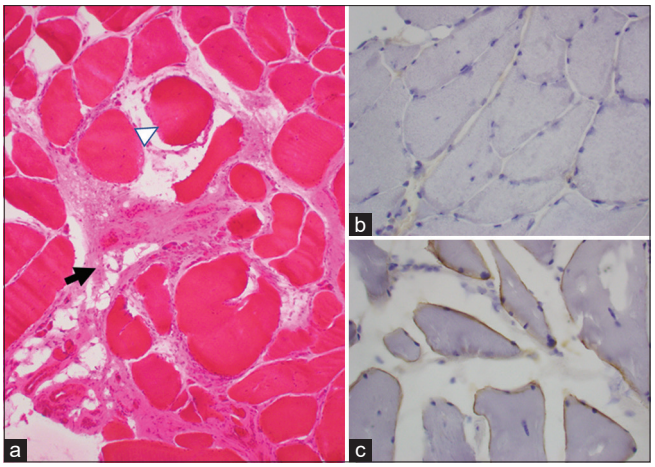

Figure 4: Photomicrographs show examples of muscle biopsy from a different patient with Becker's muscular dystrophy. (a) Muscle fibres with marked variability in fibre sizes with rounded fibres (white arrowhead) and focal interposition of fibroadipose tissue (black arrow) (H&E stain, x200). (b) Negative sarcolemmal immunoexpression for dystrophin-N (immunoperoxidase, x400). (c) Control muscle tissue with positive dystrophin-N immunoexpression (immunoperoxidase, x400).

A majority of the disease-causing variants that give rise to dystrophinopathies are clustered into hot spots along the dystrophin gene, located at exons 45–55[15] and exons 3–9.[16] Variants at these locations comprise 47% and 7% of dystrophin gene mutations, respectively. Many mutation types have been described, with exon deletions (65.8%), duplications (13.6%) and nonsense mutations (9.2%) being the most common. Rarer mutations include microdeletions and insertions (4.9%) and splice site mutations (3.8%).[6]

Mutation type and location affect the severity of BMD to a greater extent than DMD.[6] Depending on the exons involved, the BMD phenotype can range from mild (deletions between exon 10 and 40) to severe (deletions affection first 10 exons; hence, affecting the actin binding sites).[17] Specific variants, for example deletions spanning exons 45–53, can predispose to the development of cardiomyopathy,[18] with proximal deletions presenting with earlier cardiac and muscular involvement and distal mutations correlating with lower IQ scores.[6]

There are also certain exceptions to the reading frame rule in about 10% of cases.[17] In-frame mutations can present as the DMD phenotype when they are located in certain key areas, either abolishing the actin or extracellular binding domains, or being simply too large to form a functional dystrophin molecule.[17] Conversely, out-of-frame mutations presenting as the BMD phenotype can happen when partial dystrophin function is preserved, explained by the following mechanisms. Alternative translation initiation sites can be activated after the out-of-frame mutation (e.g. for variants before exon 8, there is an alternative translation site at exon 8), or when exon skipping occurs (e.g. when the variant disrupts exon recognition sequences resulting in skipping or when there is spontaneous skipping of exon 44).[17]

Carrier females can also experience a range of symptoms of dystrophinopathy, varying from mild weakness and raised CK to more severe manifestations. This variability can be explained by random X-chromosome inactivation or unfavourable lyonisation. Individuals with a greater burden of cells expressing the abnormal dystrophin gene would more likely manifest with clinical symptoms.[19] The incidence of cardiomyopathy in manifesting carriers (up to 40% in DMD mutation carriers) increases with age, even in the absence of muscular symptoms.[20]

In rare cases, carrier females may present with the full phenotype of DMD. This can occur with autosomal translocations involving the *DMD* gene. Cells that inactivated the translocated gene (that still possess an intact *DMD* gene on the active X chromosome) would be non-viable due to the inactivation of the translocated autosome.[1] Hence, all remaining viable cells will possess the translocated autosome with a non-functional *DMD* gene, leading to the full manifestation of DMD. There are also case reports of patients with Turner's syndrome (possessing only a single X chromosome) and patients with bi-allelic mutations that fully manifested the DMD phenotype.[1]

Carrier females manifesting varying degrees of disease are also seen in numerous other X-linked conditions. Examples of X-linked conditions with clinically significant manifestations in heterozygote females include Fabry's disease, Kennedy's disease, fragile X syndrome and adrenoleukodystrophy.[21] Female carrieres of these diseases frequently present with a milder phenotype or later in life as compared to affected males. Furthermore, there can be marked heterogenity in penetrance and severity of the clinical phenotype. Hence, it is essential to keep in mind the possibility of a manifesting female carrier of X-linked diseases when assessing female patients.

Using genetic testing for diagnosis

Comprehensive genetic testing for dystrophinopathies traditionally requires either multiplex ligation-dependent probe amplification (MPLA) or array comparative genome hybridisation (CGH) to identify large deletions or duplications, and Sanger sequencing to detect single nucleotide variations.

MPLA uses sets of oligonucleotide probes that can hybridise to each of the 79 exons. Each set consists of 2 probes that can hybridise to adjacent segments of each exon and is attached to a tail of a unique length, consisting of 'stuffer' sequences. Probes that could hybridise are subsequently ligated to generate fragments of lengths unique to each exon, which are then amplified by polymerase chain reaction (PCR). The amplified fragments are then analysed for their relative copy number, allowing identification of exon deletions or duplications.[17]

Array CGH uses probes for dystrophin exons and introns conjugated to a glass slide, which are then hybridised to fluorescent-labelled patient and control DNA. This allows for relative quantification of each axon, and further allows for determination of intronic break points.[17]

Should MPLA or CGH not be diagnostic, utilisation of Sanger sequencing for each of the 79 exons and their flanking introns should be performed. It is a painstaking and time-consuming process but allows for the sequencing and identification of point mutations and deletions.[17]

An alternative to MPLA or CGH would be multiplex PCR. It is a cheaper method and may be the only method available in resource-poor settings. It uses primer sets to pick up specific exon deletions, providing detection coverage for the more commonly deleted exon clustering in the two hotspots. It does not cover all 79 exons and is unable to determine the exact exon involved in the deletion, or determine whether the deletion is due to an in-frame or out-of-frame mutation. Hence, MPLA or CGH is preferred over multiplex PCR when available.[17]

Next-generation sequencing (NGS) technologies are increasingly considered more efficient and cost-effective compared to the traditional approach of MPLA/CGH and Sanger sequencing.[22] NGS allows for multiple genes to be sequenced and analysed in parallel, providing high throughput and ability to detect both large deletions/duplications and small point mutations in coding and non-coding regions, leading to the potential of improved molecular diagnosis of DMD.[23] Targeted NGS for dystrophinopathies is available as panels of neuromuscular genes, providing additional coverage for the diagnosis of related neuromuscular conditions or mimics. As different clinical laboratories offering neuromuscular gene panels have variations in the genes covered in their panels, the ordering physician should ensure that the genes of interest are adequately represented.

With the advent of genetic testing, muscle biopsy is now not routinely indicated for diagnosis unless genetic testing fails to detect a relevant mutation. Muscle biopsy with immunohistochemical stating for dystrophin would then be instrumental in determining whether dystrophin is present, reduced or absent in the sampled muscle. Western blotting analysis determines aberrant sizes of dystrophin, which would be diagnostic of BMD. In such cases, the mutation is likely to be deep within the introns, and further analysis of mRNA from the biopsy sample can be considered.[1,17]

Screening asymptomatic mothers

Modalities of cardiac imaging for patients with dystrophinopathy include echocardiography and CMR imaging, where early CMR imaging improves the sensitivity of cardiomyopathy detection.[3]

Patients are recommended to undergo baseline and annual cardiac assessment before the onset of cardiac symptoms. The recommendation for cardiac assessment extends to asymptomatic female carriers, as they are at significant risk of cardiomyopathy.[4,20] Pharmacological treatment includes the use of angiotensin-converting enzyme (ACE) inhibitors and angiotensin II receptor blockers (ARBs), with accepted standard treatment for heart failure and arrhythmias.[4] Genetic testing for asymptomatic mothers is therefore important to

identify those who would benefit from cardiac screening and pharmacological treatment.

Management of dystrophinopathies and gene therapies

With appropriate intervention, the natural history of DMD has been altered with patients frequently surviving past the 4th decade.[1]

As respiratory complications are a major cause of mortality and morbidity in patients with DMD, optimisation of respiratory care over the last decade has improved overall survival. Respiratory management includes proactively monitoring of patient's respiratory function, such that interventions like breath stacking, cough assist devices and non-invasive ventilation can be instituted early.[4]

Long-term corticosteroid treatment can produce sustained clinical improvement with prolongation of the ambulant period, reduced need for spinal surgery, improved cardiac and respiratory function and prolonged survival.[24,25] Steroids are usually initiated around the onset of motor decline, generally at the age of 4–5 years.[1,25]

Currently, available gene therapies for DMD include stop codon read-through therapy and exon skipping therapy. These approaches are mutation-specific, and hence would depend on genetic testing to determine the type and location of the patient's mutation.

Ataluren, a small molecule that promotes stop codon read-through that partially restores dystrophin function, failed to meet its primary end point of an improvement in the 6-minute walk test (6MWD) between patients given Ataluren versus placebo. However, it did show an improvement for a prespecified subgroup of patients with baseline 6MWD of between 300 m and 400 m and an overall improvement in various timed function tests.[26] Ataluren is approved and available in Europe but has not been approved by the US Food and Drug administration.

A number of exon skipping therapies are available, namely Eteplirsen[27] (exon 51), Golodirsen[28] and Viltolarsen[29] (exon 53). They are engineered antisense oligonucleotides designed to bind to a target exon, preventing its inclusion in the mRNA transcript. This allows a mutation on the affected exon that disrupts the reading frame to be bypassed, thereby restoring the reading frame and resulting in the production of a partially functional dystrophin molecule. They are delivered systemically and require frequent repeated dosing. Patients treated with exon skipping therapies have increased dystrophin production and showed a slower rate of decline in ambulation, and respiratory and cardiac functions.[27–29]

Other approaches to restoring dystrophin production are being studied, including stem cell transplantation, gene therapy utilising an adeno-associated viral vector (delivering a micro-dystrophin gene into host cells) or the CRISPR-CAS9 system ('molecular scissors' inducing targeted double stranded DNA breaks potentially correcting errors via repair or deleting exons). These approaches have not yet entered routine clinical use.

In summary, dystrophinopathies cause a wide spectrum of manifestations in affected individuals, from males with DMD and BMD to manifesting female carriers. It is a multisystemic disease, which necessitates multidisciplinary specialised care. With available and upcoming genetic therapies, many of which are exon specific, understanding of its genetic basis is increasingly instrumental. Therefore, genetic testing has become the cornerstone in the diagnosis and management of dystrophinopathies.

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Conflicts of interest

There are no conflicts of interest.

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SMC CATEGORY 3B CME PROGRAMME

Online quiz: https://www.sma.org.sg/cme-programme

Deadline for submission: 6 pm, 28 February 2023

Question	True	False
1. Regarding genotype–phenotype correlation of dystrophinopathies:		
(a) Dystrophinopathies are X-linked genetic disorders arising from reduced or absent expression of dystrophin protein.		
(b) Mutations that disrupt the reading frame of dystrophin lead to more severe clinical phenotypes.		
(c) Partial expression of dystrophin protein leads to Duchenne muscular dystrophy (DMD).		
(d) The presence of some functional dystrophin protein leads to increased muscle membrane damage and more severe disease phenotype.		
2. Regarding the diagnosis of dystrophinopathies:		
(a) Muscle biopsy is routinely required for the diagnosis of dystrophinopathies.		
(b) Deletion and duplication analysis can detect most pathogenic variants of the *DMD* gene.		
(c) Multiplex ligation-dependent probe amplification is widely used as an initial diagnostic test, as it can detect exonal deletions and duplications.		
(d) Sanger sequencing enables detection of point mutations and deletions.		
3. Regarding female carriers of dystrophinopathies:		
(a) Female carriers are often asymptomatic but are at risk of cardiomyopathy.		
(b) All daughters of affected male patients are carriers, while sons are not affected.		
(c) Skewed X-chromosome inactivation explains why female carriers may not express the disease.		
(d) Female carriers may have myalgia and high creatine kinase levels.		
4. Regarding potential multi-organ manifestations:		
(a) Cardiac magnetic resonance (CMR) imaging is a sensitive method for recognising early cardiac involvement in dystrophinopathies.		
(b) Subendocardial and transmural patterns of late gadolinium enhancement on CMR are characteristic features of dystrophinopathies.		
(c) Only a minority of patients with DMD will develop cardiomyopathy.		
(d) Respiratory and cardiac involvements are major causes of mortality in dystrophinopathies.		
5. Regarding the management of dystrophinopathies:		
(a) Stop codon read-through therapy can be used in all genetically confirmed cases of DMD.		
(b) Exon skipping therapies can convert out-of-frame mutations into in-frame mutations, leading to milder clinical phenotypes.		
(c) Eligibility for genetic therapy depends on the type and location of the mutation.		
(d) Angiotensin-converting-enzyme inhibitor, angiotensin-receptor blocker or beta-blocker therapy has been shown to delay progression of myocardial fibrosis.		