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TESI DI SPECIALIZZAZIONE

Cardiac Involvement and Management of Duchenne Muscular Dystrophy.
The Experience of the Gaslini Institute.

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1 Purpose of the Study

Duchenne muscular dystrophy (DMD) is an X-linked recessive disease due to mutation in the dystrophin gene, resulting in lack or defects in the homonymous protein.

Dystrophin plays a key role in the muscle membrane stability, both in skeletal and cardiac muscle.

Cardiac involvement in DMD is inescapable, presenting as dilated cardiomyopathy, progressing to heart failure in the advanced stages of the disease.

Heart disease is now recognized as the leading cause of death in DMD, thus requiring optimal management.

The introduction of high-sensitivity cardiac troponin I (hs-cTnI) assay has revolutionized troponin testing and scientific literature suggests that hsTnI analysis should be incorporated into the evaluation and management of patients with DMD.

Its utilization could provide new data to aid in clinical decision-making and risk stratification even in asymptomatic.

The primary objective of this study is to provide a comprehensive description of cardiac function in patients with DMD in follow-up at our Institute, by a longitudinal analysis of echocardiographic data collected over the years, determining the average age at which cardiac dysfunction occurs in these patients.

These data have been correlated with clinical variables and ongoing treatment regimens and compared with those present in the literature.

Ultimately, in order to identify potential biomarkers of early cardiac damage, an initial measurement of high-sensitivity cardiac troponin I (hs-cTnI) has been conducted in a subset of patients.

While the initial assessment of hs-cTnI was conducted at time zero, a subsequent evaluation is scheduled at the one-year mark.

The objective of this follow-up hs-cTnI measurement is to further elucidate its clinical significance in the context of DMD.

2 Introduction

Muscular dystrophies constitute a broad and heterogeneous group of genetically determined myopathies characterized by progressive deficits in muscle strength and trophism due to a primary degenerative process of skeletal muscle tissue.

2.1 Duchenne Muscular Dystrophy

2.1.1 Epidemiology and Etiopathogenesis

Duchenne muscular dystrophy (DMD) represents one of the most common and severe form of muscular dystrophy and occurs in 1/3500 male births [1].

DMD is an X-linked inherited myogenic disorder due to mutations in the *dystrophin* gene, encoding dystrophin, a 427 kda protein, composed of 3685 amino acids, located on the inner surface of muscle fiber sarcolemma.

The *DMD* gene, the largest known human gene, containing 79 exons spanning 2.2 Mb, is located on the short arm of the X chromosome (Xp21.2-p21.1) [2].

DMD is caused by mutations in DMD gene that disrupt the reading frame (“out of frame”) or generate a premature stop codon, leading in the absence of a functional dystrophin protein.

Mutations in the same gene but of a different type ("in frame"), leading in a reduced amount or shortened dystrophin protein, cause the milder form of Becker muscular dystrophy (BMD) [3]

The majority of mutations causing DMD are deletions (~68%) or duplications (~11%) of one or more exons, while small mutations are involved in ~20% of patients [4].

The mutation rate is high as a de novo mutation is present in one-third of cases.

Dystrophin is part of a larger complex of sarcolemma proteins and glycoproteins found in all three types of muscle: skeletal, cardiac and smooth (Figure 1).

It serves as a physical link between the intracellular actin cytoskeleton and the extracellular matrix, serving as a structural support for the membrane to ensure its stability and flexibility [5].

It binds through the amino terminal to the F-actin of the cytoskeleton and through the carboxy terminal to β-dystroglycan, a transmembrane component of a

multimolecular complex called the "dystrophin-associated glycoprotein complex," which also includes syntrophins, α-dextroglycan, and α, β, γ, and δ sarcoglycans. This complex protects the muscle cell from contraction-induced damage [6].

Loss or abnormal dystrophin destabilizes the sarcolemma making the muscle fibers susceptible to contraction injury and triggering a cascade of events that ultimately lead to muscle necrosis [7].

In advanced stages of the disease, the repeated episodes of necrosis followed by regeneration lead to replacement of muscle cells by fat and connective tissue.

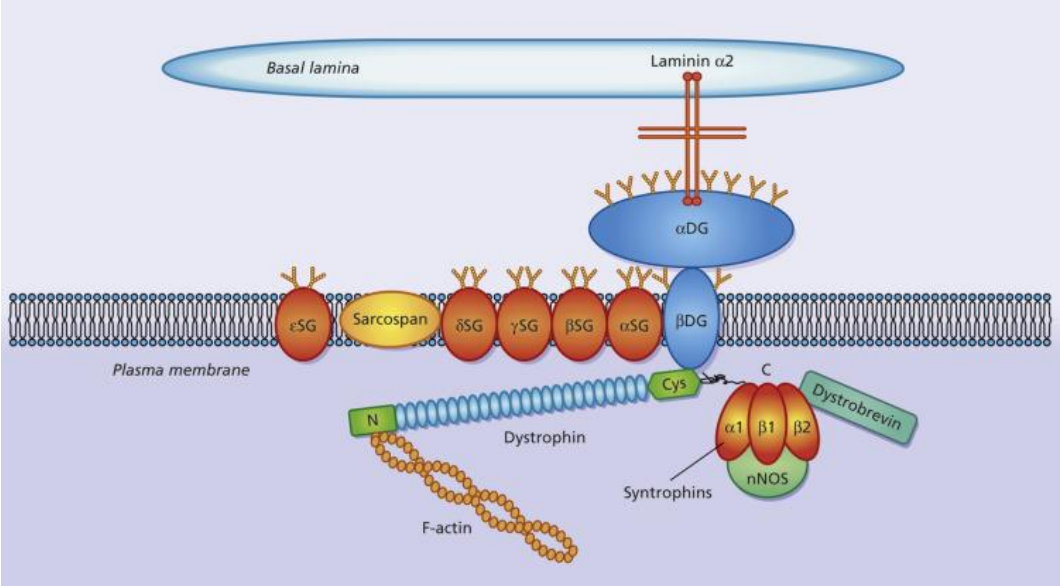


Figure 1. Interaction of the dystrophin-associated protein complex with the actin cytoskeleton (The Dictionary of Cell & Molecular Biology, Fifth Edition, 2013)

2.1.2 Clinic

Children affected by DMD do not exhibit symptoms at birth but progressively lose previously acquired skills.

DMD typically manifests between the ages of 18 and 36 months, with initial signs including motor clumsiness and difficulties such as trouble climbing stairs, running, and jumping. Furthermore, children with DMD often experience frequent falls.

However, before the age of two, it is imperative to recognize certain minor signs, such as delayed walking, speech, or cognitive abilities, which should be viewed as crucial warning signs [8].

As the condition progresses, muscle weakness and exercise intolerance become increasingly prominent.

On physical examination, notable findings include calf pseudohypertrophy, which results from the gradual replacement of muscle tissue with connective and adipose tissue. Additionally, there is evidence of hypotonia, muscle weakness, and atrophy in the pelvic girdle, leading to hyperlordosis in the lower back, and in the scapular girdle, causing winged scapulae.

Walking patterns evolve as the disease advances.

Deambulation becomes broad-based with lateral trunk oscillations, often described as the "waddling gait." This is a consequence of progressive weakness in the pelvic girdle. Patients also exhibit forefoot support during walking (toe-walking) due to the gradual contracture of calf muscles.

By the age of 5, muscle weakness becomes increasingly evident during strength assessments. Transitioning from a supine to an upright position becomes challenging and necessitates compensatory maneuvers (such as Gower's sign or the climbing maneuver).

Osteotendinous reflexes progressively become hypoactive, eventually leading to their complete absence.

Between the ages of 8 and 10, walking may require the use of assistive devices. Joint contractures and limitations in hip flexion, knee extension, elbow extension, and wrist extension are exacerbated by prolonged periods of sitting.

The disease's progression is rapid, with the loss of independent walking typically occurring between the ages of 12 and 14.

As immobility sets in, there is a swift progression of skeletal deformities, notably dorso-lumbar scoliosis. Motor function deterioration is monitored using outcome measures that assess muscle strength at various stages of the disease.

Around the second decade of life, restrictive respiratory insufficiency emerges due to weakness in accessory respiratory muscles, exacerbated by thoracic deformities. Cardiac involvement is a part of the disease's natural history, presenting as dilated cardiomyopathy, often marked by sinus tachycardia, atrial arrhythmias, and

conduction defects. Severe heart failure typically occurs only in advanced stages of the disease.

Approximately 30% of patients also exhibit cognitive deficits, primarily affecting verbal abilities. Attention deficit hyperactivity disorder (ADHD) and obsessive-compulsive disorder are also observed in some cases.

Cardiorespiratory complications, particularly when left untreated, are the primary causes of mortality, which generally occurs between the second and third decades of life.

2.1.3 Diagnosis

Laboratory-wise, serum creatine phosphokinase (CK) values are invariably elevated, 20-100 times the normal range. These values are abnormal at birth, peak at the clinical onset of the disease but tend to decrease to nearly normal levels in the advanced stages due to inactivity and muscle loss [9].

The discovery, often incidental, of CK values exceeding 10,000 IU/L must prompt a request for specific genetic analysis.

In recent years, the use of muscle biopsy has decreased in favor of genetic investigations, although it still remains a frequently employed tool as an outcome measure in certain therapeutic trials.

Muscle biopsy in affected children shows fiber caliber heterogeneity and small groups of necrotic fibers and regenerating fibers, interspersed with hyaline fibers, histological markers of the disease (Figure 2). Connective and adipose tissues replace muscle fibers. Immunohistochemical techniques detect the absence of dystrophin [10,11].

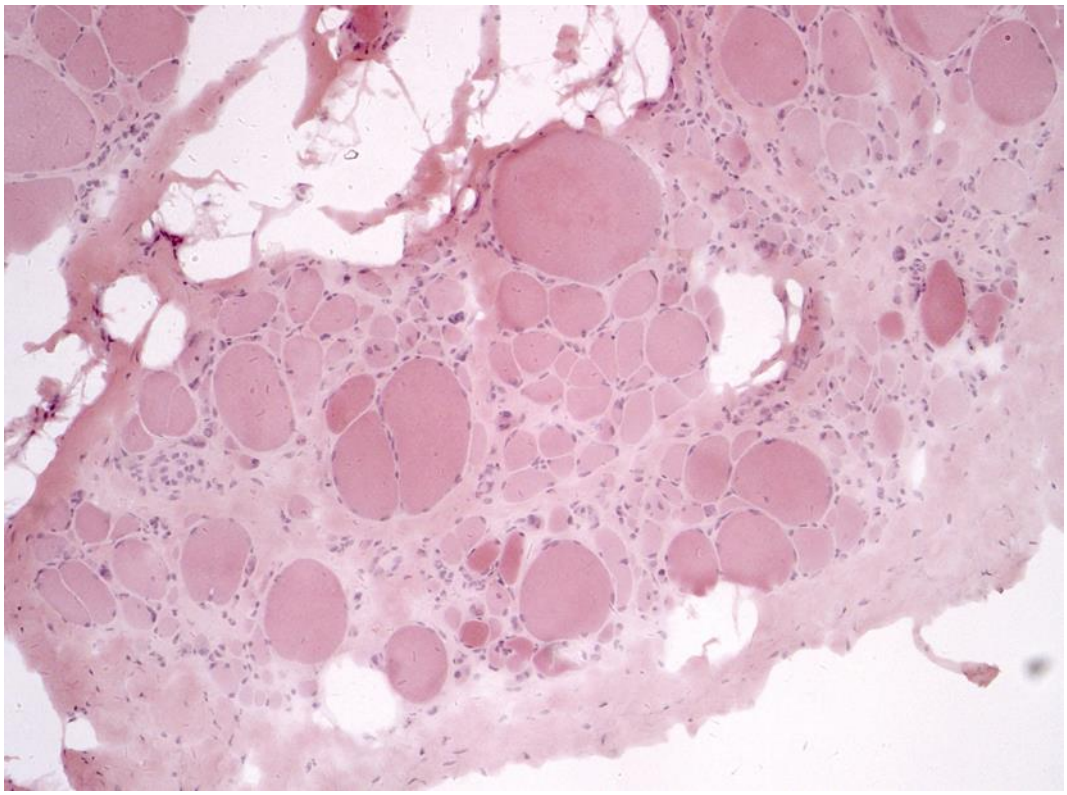


Figure 2. A histological hematoxylin and eosin-stained specimen from a muscle biopsy of a DMD patient

Genetic analysis is crucial for diagnosis and for determining if the patient may be a candidate for mutation-specific therapies [12].

The primary-level genetic-molecular investigation is conducted using the MLPA method (Multiplex Ligation-Dependent Probe Amplification), which is able of detecting deletions and duplications [13].

In the event of a negative MLPA result, particularly in cases with a strong clinical suspicion of DMD, it is advisable to perform direct gene sequencing for the detection of point mutations and small deletions. A final subset, constituting a minor proportion (~5%), consists of complex rearrangements or intronic alterations, which can be investigated through RNA analysis extracted from muscle tissue utilizing RT-PCR (Figure 3) [14].

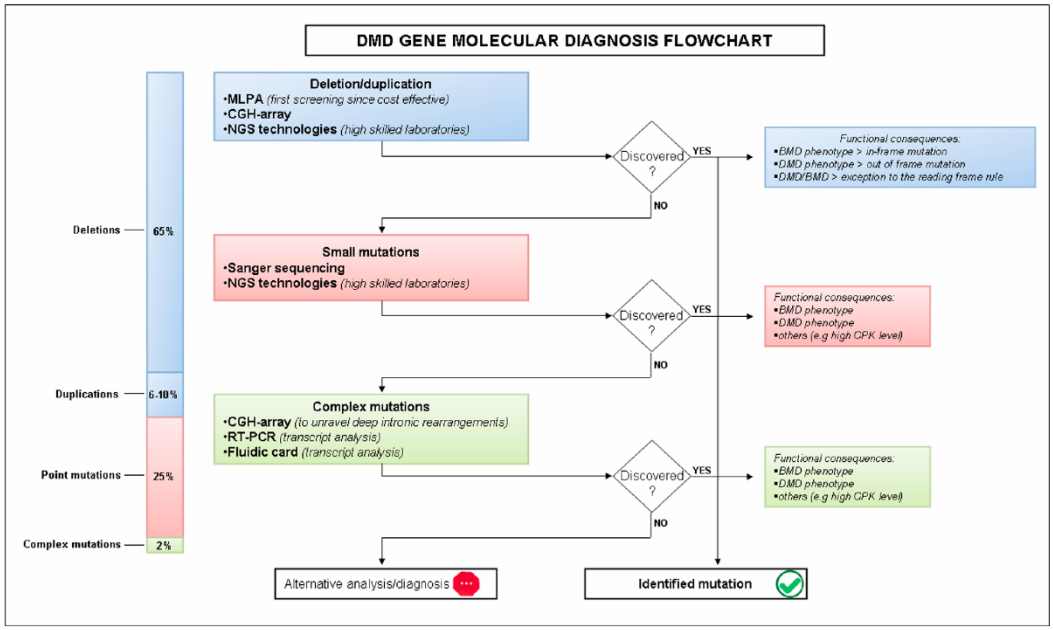


Figure 3. Flowchart of DMD diagnosis from suspicion to its confirmation. Procedures and tools for the identification of the mutation are shown. The phenotype depends on the type of mutation: DMD phenotype when the mutation alters the reading frame and leads to the complete absence of the dystrophin protein; and BMD when the mutation preserves the reading frame and allows translation of a partially functional dystrophin protein. Although, mutations that do not follow the reading frame rule are also identified (Falzarano M, Scotton C, Passarelli C, et al. Duchenne Muscular Dystrophy: From Diagnosis to Therapy. Molecules. 2015)

2.1.4 Clinical and Instrumental Follow-up

The follow-up of DMD patients entails a multidisciplinary approach that involves various healthcare professionals. Their collective expertise is essential for the timely detection of potential complications and the implementation of necessary therapeutic measures. Recent updates and expansions in guidelines encompass the management of cardiac, pulmonary, nutritional, endocrine, rehabilitative, physiatric, and orthopedic aspects, as well as recommendations regarding vaccinations and the utilization of oral glucocorticoids [1, 15, 16].

Over the past decade, numerous functional tests have been developed to monitor the progression of muscle weakness, providing crucial insights into disease advancement and treatment response:

Motor outcome measures

-Timed tests: time taken to ascend and descend 4 steps; time taken to rise from supine position; time taken to cover 10 meters running; 6-Minute Walking Test (6-MWT) [17].

-Tests assessing global motor function: North Star Ambulatory Assessment (NSAA) and Performance of the Upper Limb (PUL) [18,19].

Respiratory function tests

-Forced vital capacity (FVC).

-Forced expiratory volume in the 1st second (FEV1).

-Peak expiratory flow (PEF).

-Cough peak flow (CPF).

2.1.5 Therapies

Available therapies

Corticosteroid Drugs

Corticosteroid drugs have shown benefits in symptom control and currently oral corticosteroid treatment constitute the “gold standard” therapy for DMD. Their primary role appears to be the reduction of inflammation [20].

The standard dosage is 0.75 mg/kg/day for prednisone and 0.9 mg/kg/day for deflazacort. Their administration yields a beneficial impact not solely on motor function, delaying the loss of autonomous ambulation, but also on respiratory and cardiac functions [21, 22].

Randomized comparison studies have demonstrated that deflazacort results in less weight gain compared to prednisone, making it the preferred corticosteroid therapeutic choice [23].

Corticosteroid use is recommended from the age of 3-4 years and should not be discontinued after the loss of ambulation, considering their effectiveness on the cardiorespiratory system.

However, this therapy still induces some adverse effects such as Cushingoid appearance, skin disorders, headache, increased incidence of upper respiratory tract infections, increased blood pressure, risk of gastrointestinal perforation, behavioral and mood changes, growth delay, cataracts and decreased bone density, leading to an increased risk of vertebral and long bone fractures. Additionally, patients receiving immunosuppressive doses of corticosteroids should not be given live or live attenuated vaccines.

More recently, research has focused on new steroids such as vamorolone, which may offer a better alternative by reducing the adverse effects associated with traditional steroids [24].

Ataluren

Ataluren is a medication indicated for a specific and limited group of patients with DMD caused by a nonsense mutation in the dystrophin gene. This type of mutation occurs in only about 10-15% of DMD patients.

This type of mutation, also known as a stop mutation, leads to the production of an early termination signal in dystrophin synthesis. Consequently, the dystrophin protein produced is truncated and nonfunctional. Ataluren is a small molecule that interacts with mRNA and can promote the read-through of the premature stop codon, allowing for the complete and functional synthesis of the dystrophin protein through a process called "read through."

Several randomized clinical trials have demonstrated safety and efficacy characteristics of Ataluren [25].

In 2014, the European Commission granted conditional approval for the marketing of Ataluren for the treatment of DMD in patients with nonsense mutations who are ambulatory and over 5 years of age. In Italy, the medication has been available since 2014 and is funded by the National Health Service for eligible patients.

Ataluren is administered orally in the form of granules and should be taken three times a day at the recommended dose of 40 mg/kg/day. It can be used concomitantly with corticosteroids but requires careful monitoring of blood pressure.

Therapies in clinical trials

While Ataluren has generated significant enthusiasm as the first approved drug for DMD, ongoing research into other potential treatments, as indicated by the investigation of drugs not yet on the market but in advanced stages of development, remains crucial.

In recent years, various molecules have been tested, conventionally categorized based on their therapeutic target into:

-molecules aimed at restoring dystrophin production (exon skipping and gene therapy)

-molecules that target secondary defects resulting from the dystrophin deficiency itself (pro-regenerative drugs, non-steroidal anti-inflammatory drugs, anti-fibrotic drugs).

Exon-skipping

In exon-skipping therapy, antisense oligonucleotides (ASOs) are used.

These are small molecules that function like pre-mRNA and are capable of skipping the copying of the mutated exon. In this way, the correct reading frame of the mRNA is restored, leading to the production of a shortened but functional dystrophin, similar to what is produced in Becker's disease [26].

The ASOs are administered once a week intravenously in patients with out-of-frame deletions, where the removal of an exon adjacent to the deletion is capable of generating an in-frame mutation.

Among these, we particularly mention exon 51, exon 45, and exon 53 skipping.

These molecules have been approved by the FDA and are currently marketed only in the United States, in order of approval: Eteplirsen for exon 51 skipping (applicable to 13% of patients), Golodirsen and Viltolarsen for exon 53 skipping (8% of patients), Casimersen for exon 45 skipping (8% of patients). They are still being administered as part of clinical trials in the rest of the world.

Gene therapy

Over the past three decades, significant efforts have been made to develop gene therapy for Duchenne muscular dystrophy. In concept, replacing the mutated gene with a normal one would cure the disease. However, the development of these therapies has faced significant challenges due to the large size of the gene. Therefore, shorter constructs than the endogenous dystrophin were developed to fit within the capacity of viral vectors, and they are called 'mini' or 'micro' dystrophins [27].

The viral vectors used are adeno-associated viruses (AAVs), specifically serotypes AAV9 and AAVrh74, which have a high affinity for skeletal and cardiac muscles [28].

These viral vectors are stripped of essential replication genes, rendering them incapable of reproducing in the body but capable of transferring the modified genetic material into the cell.

Through engineering techniques, the genetic material does not integrate with the host's DNA, remaining in an 'episomal' form, reducing geno-toxicity but increasing

the risk that the modified genetic material may be lost with subsequent cell replication cycles.

Another challenge for the applicability of any gene therapy is that a significant number of individuals may have already been exposed to natural AAV variants or adenoviruses and may have developed antibodies or other forms of cell-mediated immunity against the viral capsid [27].

Currently, studies are underway to determine the long-term efficacy and safety of gene therapy years after its administration (ClinicalTrial.gov: NCT03362502, NCT03769116, NCT0336874).

Among the molecules that target secondary defects resulting from dystrophin deficiency, there are pro-regenerative drugs like givinostat, non-steroidal anti-inflammatory drugs like vamorolone, and anti-fibrotic drugs like pamvlevlumab. These therapies are in the experimental phase and have the potential to be applicable to all patients with DMD, regardless of the type of mutation.

Givinostat

This drug, due to its evident anti-inflammatory activities, received orphan drug designation from the EMA for rare diseases such as juvenile idiopathic systemic arthritis (January 28, 2010), polycythemia vera (February 3, 2010), and finally, Duchenne muscular dystrophy (July 4, 2012).

The wide range of indications for this drug can be attributed to its mechanism of action. Givinostat is an orally administered small molecule that acts as an inhibitor of histone deacetylase (HDAC). HDAC is an enzyme involved in the activation or deactivation of genes within cells. In the case of DMD, it is presumed that givinostat may work by activating the follistatin gene, which would, in turn, act as an inhibitor of myostatin, increasing muscle mass.

Vamorolone

It is a dissociative steroid anti-inflammatory drug that binds to the same receptors as corticosteroids, but it has a different chemical structure and a distinct mechanism of action. This drug minimizes the side effects associated with chronic steroid therapy while retaining the inhibition of pro-inflammatory pathways involving nuclear factor κB (NF-κB) [29].

Pamvlevlumab

It is a monoclonal antibody targeting connective tissue growth factor (CTGF), a protein that promotes the deposition of fibrotic tissue [30].

Other therapies

It is important to emphasize that medical therapy is always accompanied by appropriate physical therapies, which are usually started at the age of 3-4 years and can extend the ambulatory phase of the disease through the use of suitable orthoses and targeted surgical measures. The systematic use, although not always well-tolerated, of nighttime braces is more effective than passive stretching in preventing Achilles tendon contractures. The application of rigid bracing to the lower limb (knee-foot orthosis) can help reduce falls caused by quadriceps weakness. The goal of surgery is to maintain the lower limb in extension and prevent contractures of the iliotibial band and hip flexors. Useful surgical procedures to delay loss of ambulation include percutaneous tenotomies of the Achilles tendon, knee flexors, and hip flexors, as well as iliotibial band release. Spinal stabilization techniques, leading to improved respiratory function, are indicated to correct scoliosis with curves greater than 35 degrees.

Also fundamental are respiratory physiotherapy, non-invasive ventilation, cardioprotective therapy, osteopenia/osteoporosis treatment, and gastrostomy nutrition according to the timing and modalities suggested by the most recent guidelines [1, 15, 16].

2.1 Cardiac Involvement in Duchenne Muscular Dystrophy

Medical advancements, especially in the fields of non-invasive ventilation and spinal stabilization, have improved survival rates in DMD (Figure 4) [31, 32].

As a result, DMD cardiomyopathy has become more prevalent and is currently acknowledged as the primary cause of mortality in DMD patients. [33, 34].

2.2.1 Epidemiology, Pathophysiology and Clinic

The frequency of cardiomyopathy in DMD rises with advancing age [35].

While approximately 25% of patients exhibit cardiomyopathy at the age of 6, and this percentage increases to ~ 59% by the age of 10, it's important to note that

cardiac involvement becomes nearly universal among older DMD patients. In fact, over 90% of young individuals aged 18 and older show signs of cardiac dysfunction [36].

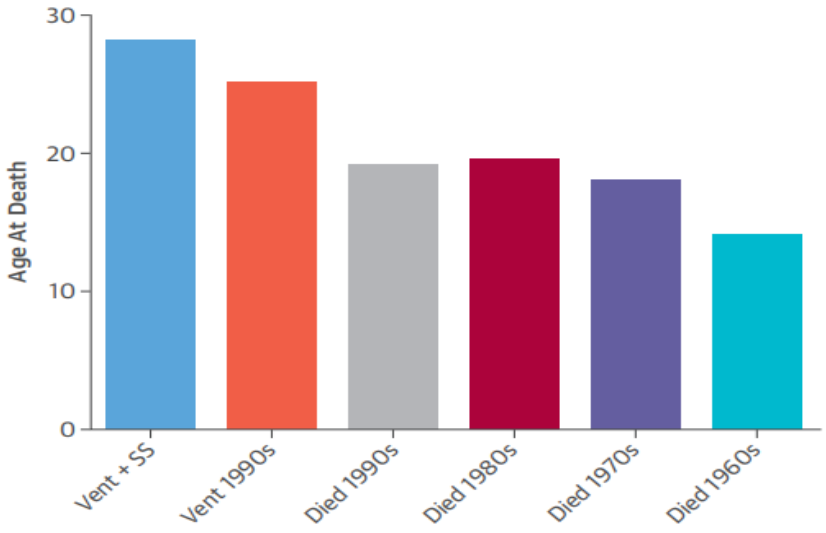


Figure 4 A marked increase in survival of patients with DMD has been achieved with the addition of ventilation strategies (vent) and spinal stabilization surgeries (SS). (Kamdar F, Garry DJ. Dystrophin-Deficient Cardiomyopathy. J Am Coll Cardiol. 2016; Eagle M, Baudouin SV, Chandler C, et al. Survival in Duchenne muscular dystrophy: improvements in life expectancy since 1967 and the impact of home nocturnal ventilation. Neuromuscul Disord 2002)

Myocardial impairment, primarily attributed to the lack of dystrophin, develops, akin to what occurs in skeletal muscle, through the gradual replacement of cardiac cells with fibro-adipose tissue, resulting in the loss of contractile capacity and cardiac geometry.

Myocardial damage initiates in the inferolateral wall and gradually extends to encompass the entire left ventricle (LV) by the conclusion of the second decade [37]: its progression is closely linked to the development of myocardial fibrosis [38, 39]. As this last becomes more pronounced, the LV undergoes progressive dilation, resulting in an elevated cardiac workload and the triggering of both the renin-angiotensin system and the sympathetic nervous system. This sequence of events exacerbates heart failure (Figure 5, Figure 6) [40].

In addition, the increased heart rate (HR) resulting from autonomous system dysfunction [41], combined with the LV dyssynchrony, has the potential to further deteriorate LV function as time progresses [40].

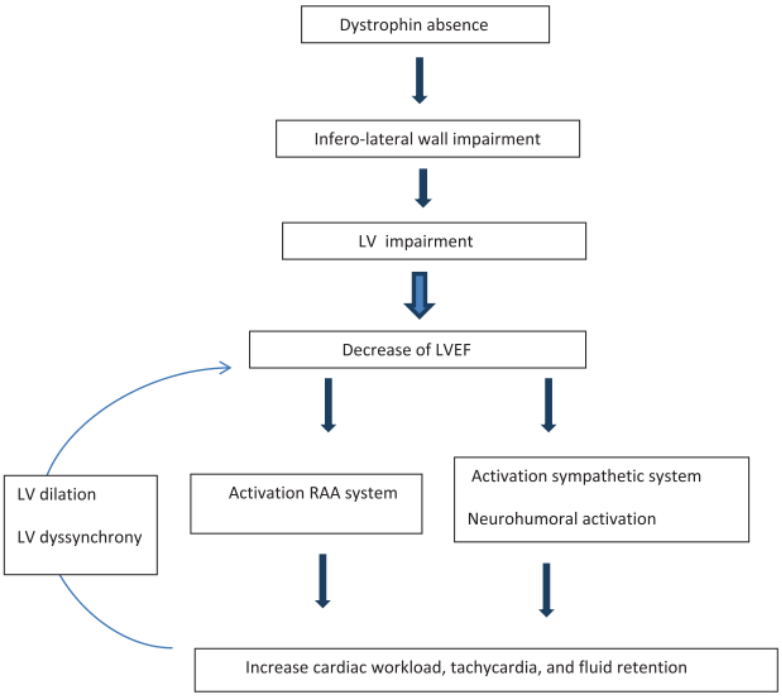


Figure 5. Pathophysiology of heart failure in DMD. LVEF: left ventricular ejection fraction. LV: left ventricle. RAA: renin angiotensin aldosterone (Fayssoil A, Abasse S, Silverston K. Cardiac Involvement Classification and Therapeutic Management in Patients with Duchenne Muscular Dystrophy. J Neuromuscul Dis. 2017)

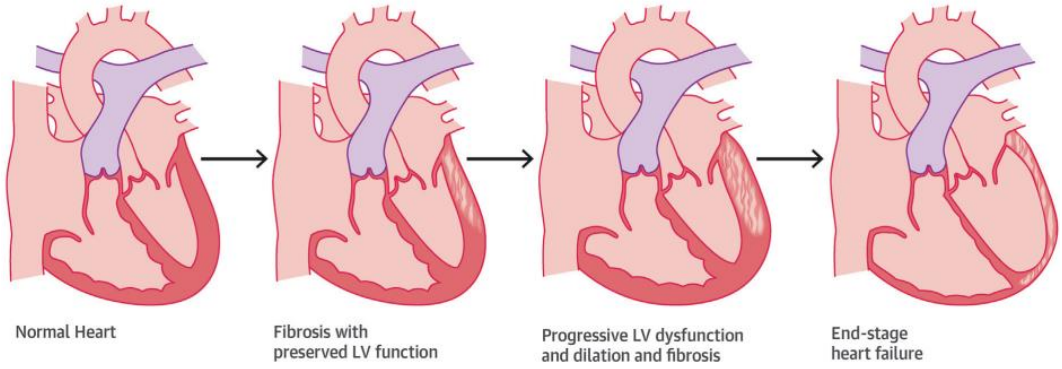


Figure 6. Schematic outlining DMD cardiomyopathy disease progression (Kamdar F, Garry DJ. Dystrophin-Deficient Cardiomyopathy. J Am Coll Cardiol. 2016)

Due to DMD patients' limited mobility, symptoms associated with cardiomyopathy are frequently not easily detectable. Arrhythmia can manifest clinically with palpitations [41]. In older DMD patients receiving mechanical ventilation, typical

indicators of right-sided heart failure include peripheral edema and ascites, along with pleural effusion in the late stages of the disease [42].

2.2.2 Cardiac Diagnostic Methods

Electrocardiogram (ECG) anomalies documented in DMD often exhibit a classical pattern. This pattern typically encompasses sinus tachycardia, shortened PR intervals, tall R waves in the right precordial leads, right axis deviation, complete right bundle branch block, Q waves in inferolateral leads (distinct from those characteristic of myocardial ischemia), as well as flat and inverted T waves [43-46]. Within DMD patients, ECGs can also exhibit a pattern resembling Wolff-Parkinson-White (WPW) syndrome [47].

The observed ECG patterns are consistent with studies that have revealed a tendency for fibrosis in the basal posterior ventricular wall in DMD patients, resulting in a reduced electrical activity (Figure 7) [37].

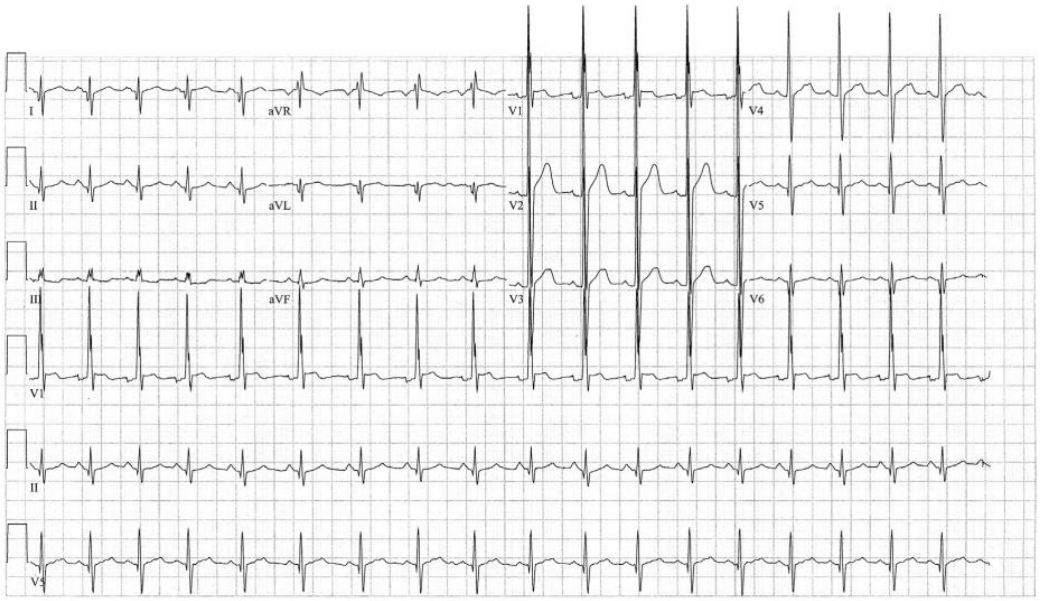


Figure 7. ECG from a patient with DMD. The ECG demonstrates sinus tachycardia, Q waves in leads I, aVL, and V4 to V6, and large R waves in V1 and V2 (Kamdar F, Garry DJ. Dystrophin-Deficient Cardiomyopathy. J Am Coll Cardiol. 2016)

Standard Doppler echocardiography should be conducted following the guidelines provided by the American Society of Echocardiography [48]. M-mode is utilized to analyze parameters including left atrial diameter, septal and posterior wall thickness, and the motion of the LV. It is also used for measuring LV end-systolic and end-

diastolic diameters and for calculating the LV shortening fraction and left ventricular ejection fraction (LVEF). In 2D mode, it’s possible to evaluate cardiac anatomical structures and function, with a particular focus on LV function using a four-chamber apical view [48]. Doppler is employed to evaluate both LV systolic function (through measurement of LV aortic outflow tract systolic velocity) and LV diastolic function (through measurement of trans-mitral flow velocities). Additionally, it is utilized to evaluate pulmonary arterial pressures [49].

The strain analysis method has been employed in recent years to evaluate early-stage regional myocardial dysfunction [50].

Strain imaging, in the context of medical imaging and echocardiography, is a technique used to evaluate the deformation or strain of cardiac tissues, typically the myocardium (the muscular walls of the heart). It provides a quantitative assessment of how much the heart muscle contracts and relaxes during each heartbeat, offering insights into myocardial function and identifying abnormalities in cardiac mechanics.

In DMD patients, there is a progressive deterioration in cardiac function with dilation and widespread alterations in segmental contractility, ultimately leading to a confirmed state of dilated hypokinetic cardiomyopathy. Early-stage myocardial abnormalities affecting primarily the inferolateral region have been documented in DMD patients [51]. Akinesia in the LV inferobasal wall [52], along with LV dilation and LV systolic dysfunction, can be observed in older patients.

An important limitation of the echocardiographic technique is the challenge posed by thoracic deformities and the difficulty in obtaining high-quality images in wheelchair-bound patients.

Cardiac magnetic resonance imaging (CMR) is considered the "gold standard" diagnostic method due to its precision, operator-independence, and its ability to overcome the limitations of the "acoustic window." However, in clinical practice, Cardiac MRI is typically employed as the first approach when ultrasound is not conclusive due to inadequate acoustic windows. Its use should be considered in the course of managing cardiac pathologies.

CMR is employed in DMD patients to assess LVEF and identify cardiac remodeling as well as abnormalities in wall motion. It is capable of defining not only functional data but also myocardial tissue characteristics. CMR can potentially identify cardiac abnormalities in early stages, even when Doppler echocardiography shows a normal LVEF [53].

Late gadolinium enhancement (LGE) imaging is utilized in CMR to visualize free wall segments [54] and assess myocardial fibrosis (Figure 8, Figure 9). Myocardial fibrosis is associated with a decline in LVEF [54, 55] together with which it constitutes a prognostic factor [56].

While enabling early detection of cardiovascular involvement and facilitating early cardioprotective treatment [57], its use in the pediatric population may be hindered by the need for sedation, cost considerations, and limited accessibility.

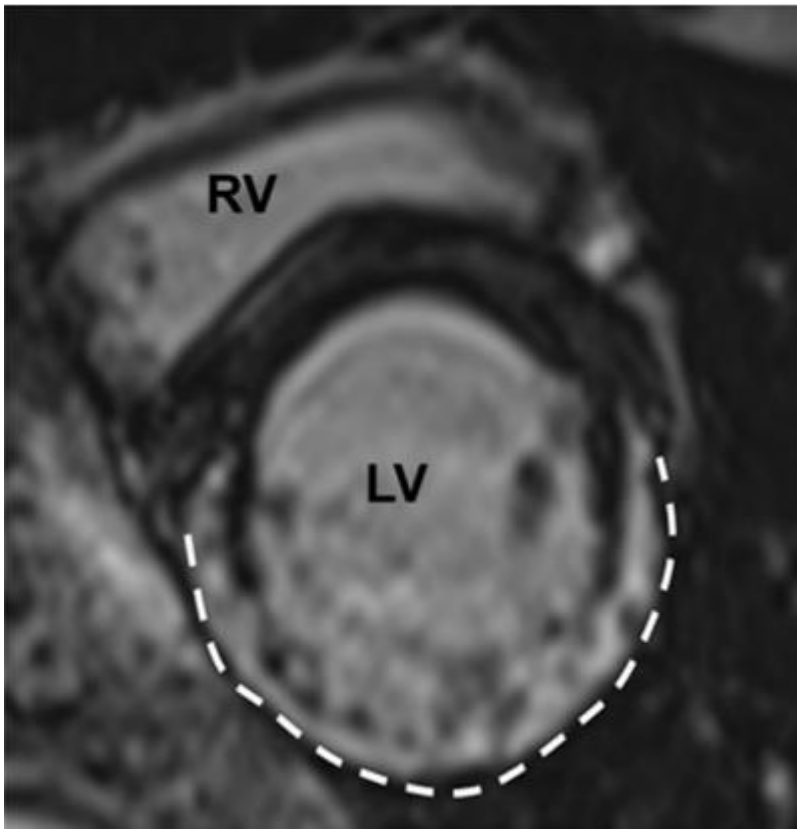


Figure 8 This image demonstrates near transmural enhancement (gray) in the left ventricular (LV) basalmidanterolateral, inferolateral, and lateral wall consistent with myocardial scar or fibrosis (Kamdar F, Garry DJ. Dystrophin-Deficient Cardiomyopathy. J Am Coll Cardiol. 2016)

Stage	Clinic	Echocardiography CMR	Treatment
Stage 1	Asymptomatic	Echo: LVEF>55% Possible 2D strain abnormalities CMR: Myocardium LGE often negative	ACE inhibitors
Stage 2	Tachycardia	Echo: 45%< LVEF <55% 2D strain abnormalities Infero-lateral wall impairment CMR: Myocardium with positive LGE	ACE inhibitors Beta blockers Mechanical ventilation at the end of the second decade
Stage 3	Peripheral edema Sometimes Dyspnea (patients without MV) Tachycardia	Echo 35%<LVEF <45% 2D strain abnormalities CMR: Myocardium with Positive LGE+++	ACE inhibitors Beta blockers Anti-aldosterone Diuretic (congestion) Intermittent mechanical ventilation
Stage 4	Anasarca Peripheral edema Ascites Lipothymia Tachycardia	Echo: LVEF<35% 2D stain abnormalities CRM: Diffuse myocardial LGE+++	ACE inhibitors Beta blockers Anti-aldosterone Diuretic (congestion) +- CRT or CRT-D Permanent mechanical ventilation

LVEF: left ventricular ejection fraction. CRT-D: cardiac resynchronization therapy defibrillator. CRT-P: cardiac resynchronization therapy pacemaker. ACE: angiotensin converting enzyme. LGE: late gadolinium enhancement. CMR: cardiac magnetic resonance.

Figure 9 Proposal for staging of heart involvement in DMD (Fayssoil A, Abasse S, Silverston K. Cardiac Involvement Classification and Therapeutic Management in Patients with Duchenne Muscular Dystrophy. J Neuromuscul Dis. 2017)

2.2.3 Genotype and Cardiomyopathy

Specific dystrophin mutations have been associated with a higher prevalence of cardiomyopathy: in particular, there is a well-documented association between severe cardiomyopathy and deletions involving exons 48–49 in both DMD and BMD patients. It's reported that patients with deletions in exons 2–9 exhibit the earliest onset of cardiomyopathy and that individuals with deletions in exons 45–49 tend to develop cardiomyopathy earlier when compared to those with deletions in exons 50–51. Jefferies et al. also found evidence indicating a decreased likelihood of cardiac involvement in individuals with DMD and BMD who have deletions in exons 51 and 52. Additionally, the earliest onset of cardiomyopathy has been observed in cases involving deletions encompassing exons 12 and 14–17 [58, 59].

2.2.4 Clinical and Instrumental Follow-up

At present, clinical guidelines recommend the first cardiac screening, which includes a cardiological history, physical examination, ECG, and non-invasive imaging, at the time of DMD diagnosis. Up to the age of 10, this cardiac screening, sometimes also including ECG Holter, should be conducted annually [60]. After the age of 10,

asymptomatic individuals should undergo at least annual evaluations, while symptomatic patients should be assessed more frequently [60].

A cardiological evaluation should be conducted prior to major surgical procedures, including scoliosis correction, in patients with DMD [60]. This is crucial because DMD is linked to specific anesthesia-related risks, and it is imperative for the anesthetist to have a thorough understanding of the patient's cardiac history [61].

Female carriers of DMD are advised to undergo a baseline cardiac examination in early adulthood, which should encompass an ECG and non-invasive imaging [60]. This assessment should be repeated at intervals of every 3 to 5 years, aligning with screening recommendations for other genetic cardiomyopathies [62].

2.2.5 Treatment

In 2014, the NHLBI working group recommended the utilization of ACE inhibitors or angiotensin receptor blockers (ARBs) in asymptomatic DMD patients with normal left ventricular systolic function by the age of 10 [63]. However, opinions vary regarding the use of ACE inhibitors in asymptomatic individuals under the age of 10 [60]. Regardless of age, the commencement of pharmacological therapy should occur when heart failure symptoms manifest or when CMR or echocardiogram detect abnormalities, such as a reduced LVEF, anomalous chamber dimensions, or myocardial fibrosis [60].

The mechanism of action of ACE inhibitors involves inhibiting the conversion of angiotensin I to angiotensin II, thus leading to a decrease in arterial vascular resistance resulting in an elevation of stroke volume [40].

ACE inhibitors have been linked to a reduction in morbidity and mortality in individuals experiencing chronic heart failure, among the general population [64].

In individuals with DMD, ACE inhibitors, such as perindopril, have demonstrated the ability to postpone the onset of cardiomyopathy, an effect that might be associated with their anti-fibrotic properties [65, 66].

Beta-blockers work by inhibiting the beta-adrenergic receptors, leading to a decrease in sympathetic activity, heart rate, and both heart contractility and relaxation [40]. A positive outcome of administering beta-blockers in conjunction with ACE inhibitors has been documented in relation to the reduction of cardiac morbidity and mortality in individuals with DMD [67, 68].

Eplerenone, an aldosterone antagonist, has recently demonstrated a favorable impact in DMD when used in conjunction with either an ACE inhibitor or an angiotensin receptor blocker [69].

Steroids can also have a a favorable effect on myocardial function, leading to reduced mortality and a decreased risk of new-onset cardiomyopathy [70].

Idebenone is chemically a derivative of benzoquinone. It is a short-chain drug with analogies to Coenzyme Q10. In DMD the rationale for using the drug lies in reducing oxidative stress, which has been correlated with muscle damage.

The administration of idebenone has been linked to a marginal increase in peak systolic radial strain in the inferolateral wall of the LV in 2D strain echocardiography studies, indicating a potential positive influence on LV function [71]. It's important to note that idebenone is not currently approved for this use.

Instrumental treatment primarily involves cardiac resynchronization therapy (CRT) and the use of non-invasive ventilation (NIV).

CRT can be advantageous for individuals experiencing symptomatic heart failure, even when they are receiving optimal drug therapy, with a QRS duration exceeding 130 milliseconds [72]. Implantable cardioverter defibrillator placement is recommended for adult patients with an ejection fraction below 35% [73], except for individuals with chest wall deformities or sedation risk [60].

DMD patients often experience restrictive respiratory failure, necessitating mechanical ventilation. This mechanical ventilation (MV) may affect heart performance, as positive pressure ventilation reduces the afterload [74]. The ATS consensus has recommended NIV as the preferred therapy in DMD for respiratory failure [68]. In the context of DMD, there is a suggestion of potential positive effects of chronic MV on cardiac performance [1].

2.2.6 Cardiac Troponin Testing in Duchenne Muscular Dystrophy: state of the art

Troponins are proteins present in both skeletal and cardiac muscle. They play a critical role in regulating muscle contraction at the cellular level, primarily by controlling the calcium-mediated interaction between actin and myosin [75].

The troponin complex consists of three subunits: Troponin C (TnC), which binds to calcium ions, Troponin I (TnI), which binds to F-actin, and Troponin T (TnT), which assembles the troponin complex by binding to tropomyosin and promotes muscle contraction (Figure 10).

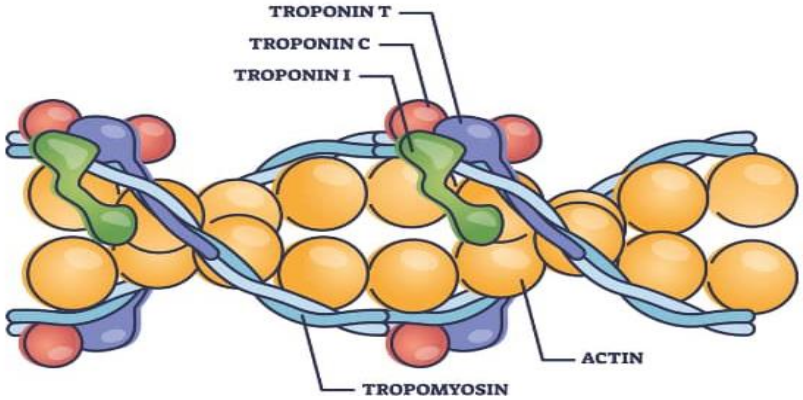


Figure 10 Troponins are classified into three molecular subunits: TnC (Troponin C), TnT (Troponin T), and TnI (Troponin I)

Cardiac troponin I (cTnI) and T (cTnT) are specific to the heart and are normally found in minuscule amounts in the bloodstream. When heart muscle cells sustain damage, these proteins are released into the bloodstream. Because of their heart-specificity and sensitivity, they have become the standard biomarkers for diagnosing and evaluating myocardial injury [76]. Cardiac troponin release can be triggered by various factors, including myocardial ischemia, often associated with atherosclerotic coronary blockages. Additional non-ischemic cardiac factors encompass heart failure, myocarditis, and cardiomyopathies. Furthermore, certain systemic conditions, such as pulmonary embolism, chronic kidney disease, sepsis, and cardiotoxic agents, are also linked to troponin release. Elevated troponin levels have been detected in asymptomatic individuals with DMD well in advance of the onset of the characteristic and clearly defined cardiac complications commonly associated with the condition [77]. Nevertheless, cTnT is regarded as less specific for myocardial injury since its elevation can be observed in various neuromuscular disease conditions, likely stemming from skeletal muscle origins [78, 79].

The more specific cTnI is a fundamental component of the troponin protein complex, with a crucial role in diastolic relaxation through the inhibition of actin-myosin binding interaction. Damage to cardiac myocytes results in the release of cTnI into the bloodstream, allowing for the measurement of serum cTnI concentrations as an indicator of cardiac myocyte damage. Notably, cTnI is not expressed in skeletal muscle, eliminating the confounding factor of progressive skeletal muscle myositis that has impacted the utility of other biomarkers [80]. Troponin serum levels were examined in the dystrophin-deficient mdx mouse. It was established that cTnI is specific to the heart and is not found in regenerating skeletal muscle of *mdx* mice [81]. Elevated cTnI levels were observed in mdx mice, and these levels decreased after idebenone therapy [82]. The dystrophin-deficient golden retriever muscular dystrophy (GRMD) large animal model better replicates certain aspects of the human cardiomyopathy progression.

In a study conducted by Townsend et al., a chronic infusion of a membrane-sealing agent was administered to severely affected GRMD dogs, resulting in decreased troponin I levels and reduced myocardial fibrosis [83].

In ongoing experiments, monthly assessments of troponin I levels in dystrophin-deficient dogs revealed periodic spikes in troponin levels. These spikes increased in frequency as the dogs aged, indicating a correlation with cardiac involvement. Additionally, older dogs with reduced cardiac mass exhibited lower troponin levels [84].

A recent study examining the distribution of TnI in patients with DMD and BMD has revealed significant findings [85]. Among DMD patients, both cTnI levels and the percentage of patients with abnormal cTnI levels were notably higher, especially during the second decade of life. In DMD, the peak cTnI level was observed at the age of 13, and an LVEF was recorded approximately one year later. For BMD patients, the cTnI level reached its peak at the age of 14, and an abnormal LVEF was detected three years later.

Decreased LVEF was consistently observed after the elevation of cTnI levels in both populations. Therefore, the increase in cTnI levels occurring before the onset of cardiac dysfunction could indicate an early stage in the development of cardiomyopathy [85]. This suggests that elevated cTnI may serve as a biomarker for the early detection of cardiomyopathy in individuals with DMD [85].

Evidence has shown that troponin I levels exhibited a significant increase in DMD subjects with mild LGE at cardiac MRI compared to those with no LGE [77]. Troponin I levels have the potential to serve as a valuable minimally invasive marker for monitoring the early stages of myocardial disease progression in DMD [77].

Currently, there are no guidelines for the routine monitoring of cTnI levels in DMD patients.

The Atherosclerosis Risk In Communities Study determined that elevated high-sensitivity cTnI (hs-cTnI) is significantly linked to an increased incidence of cardiovascular diseases in the general population, irrespective of traditional risk factors [86]. As a considerable number of DMD patients will seek care at adult and community hospitals, hs-cTnI levels have supplanted traditional troponin assays and are recommended in the Fourth Universal Definition of Myocardial Infarction [76]. Although the exact implications of the enhanced analytical sensitivity on clinical care in DMD remain uncertain, it will be crucial to incorporate hs-cTnI analyses into future assessments (Figure 11) [87]. This will supply physicians with fresh data to aid in clinical decision-making and potential risk assessment, even in asymptomatic individuals with DMD [84].

A recent study has highlighted the critical diagnostic and prognostic value of hsTnI as part of a comprehensive cardiac evaluation, enhancing the management and treatment of cardiomyopathy in individuals with muscular dystrophy (MD) [88].

Consequently, the study concludes that the analysis of hsTnI should become part of the assessment and care of patients with MD [88].

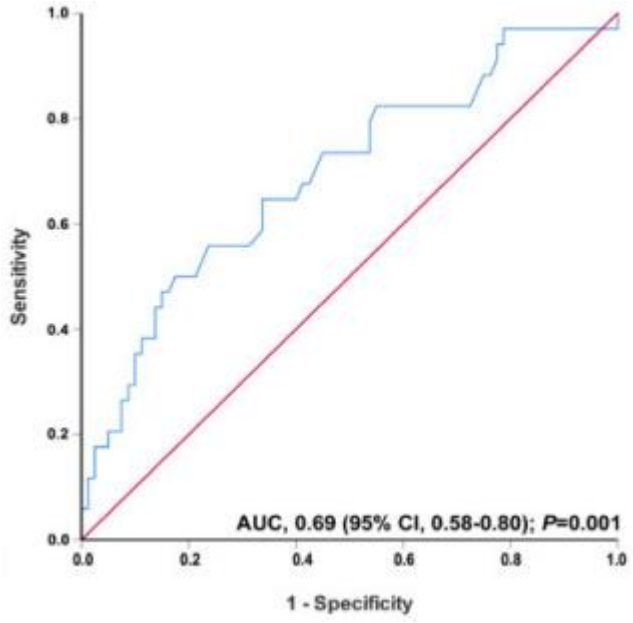


Figure 11 Receiver operating characteristic (ROC) curve analysis of high-sensitive troponin I to assess diagnostic ability of cardiomyopathy. AUC, area under the curve; CI, confidence interval. (Nikhanj A, Miskew Nichols B, Wang K, et al. Evaluating the diagnostic and prognostic value of biomarkers for heart disease and major adverse cardiac events in patients with muscular dystrophy. Eur Heart J Qual Care Clin Outcomes. 2021)

In a separate study, high hs-cTnI levels were observed in ambulatory subjects with myotonic dystrophy type I, and these levels were shown to be predictive of heart dysfunction [89].

Recently, an expert panel comprising cardiologists, scientists, regulatory experts, and industry specialists strongly recommended retrospective analysis of troponin data, prospective long-term troponin monitoring using high-sensitivity cTnI assays, integration of troponin measurements into future clinical trial endpoints, and the development of clinical guidelines for monitoring and managing troponin elevations in DMD patients (Figure 12) [84]. This approach will provide clinicians with enhanced insights into the progression of myocardial disease in individuals with DMD [84].

Elevated troponin levels may indicate a need for adjustments in therapeutic strategies. While the current standard of care involves considering ACE inhibitors at around age 10, the commencement of this medication could be initiated earlier if elevated troponin levels are detected [63, 84].

I. Retrospective data collection

- Integrating and harmonizing *existing* data sets should be an immediate goal
  - Create DMD troponin steering committee to determine priorities and processes for reviewing retrospective data.
  - Use PPMD developed Duchenne Outcomes Research Interchange (DORI), a patient-data warehouse with 10 years of data
  - Leverage existing infrastructure (ACTION; Pediatric Health Information System (PHIS); institutional electronic medical records) to expand data acquisition

II. Prospective data collection

- Develop collaborative multi-center prospective research protocol to collect longitudinal troponin and other potential serum biomarkers
  - Incorporate initial clinician survey through DMD clinical care networks regarding screening practices including troponin assays used and therapeutic responses
  - Obtaining baseline and semi-annual/ annual troponin values in all DMD patients
    - Samples must be unbiased and not obtained only in response to symptoms
    - Recommend use of hs-cTnI assays
  - Consider simultaneously obtaining other meaningful and exploratory serum biomarkers to contextualize troponin values
  - Combining data across systems will require the ability to convert/harmonize assays
    - An immediate goal should be to seek agreement among children's hospitals regarding use of hs-cTnI assay or agreement on a process for standardizing/comparing assays
    - The ACTION network can lead the initial process of determining which assays are currently used by different centers

III. Clinical trials

- Incorporate troponin as an exploratory biomarker, rather than a safety biomarker, in clinical trials
  - Serum samples obtained in clinical trials must be stored appropriately and made available for future analyses
  - A process for sharing data from clinical trials should be established
    - Critical Path Institute can establish a data contribution agreement
  - Longitudinal patient follow-up after clinical trials with appropriate permissions must be established

IV. Development of clinical guideline

- Develop expert consensus clinical guidelines for monitoring and treatment of troponin levels in DMD
  - Determine the lower limit of a concerning elevation in troponin
    - Determine whether absolute values or change(s) in values are more important
  - Consensus regarding treatment recommendations for abnormally elevated troponin values in asymptomatic patients with normal diagnostic testing and symptomatic patients with abnormal diagnostic testing

Figure 12. Recommendations from the expert panel for the further study of the natural history of cardiac troponin I testing in DMD. ACTION, Advanced Cardiac Therapies Improving Outcomes Network; DMD, Duchenne muscular dystrophy; hs-CTnI, high-sensitivity cardiac troponin I; PPMD, Parent Project Muscular Dystrophy (Current state of cardiac troponin testing in Duchenne muscular dystrophy cardiomyopathy: review and recommendations from the Parent Project Muscular Dystrophy expert panel. Open Heart 2021)

3 Methods

This monocentric cross-sectional study was performed on DMD patients followed at IRCCS Gaslini Institute, Genoa, Italy. All patients received a genetically confirmed diagnosis of DMD.

Echocardiographic data collected from the last cardiac evaluation were reviewed. In details, LVEF, fractional shortening (FS), end-diastolic diameter (EDD) and end-systolic diameter (ESD), were evaluated. LVEF values below 55% were considered pathological [40, 59, 93]. Mild systolic dysfunction was defined for $45\% \leq LVEF < 55\%$, moderate systolic dysfunction for $35\% \leq LVEF < 45\%$, while severe dysfunction was defined as $LVEF < 35\%$ [40].

Clinical data, including age, cardiac and non-cardiac therapies, respiratory function, laboratory CK values, and anthropometric data (weight, BMI), were also collected.

For patients who presented a pathological LVEF at last examination, retrospective clinical charts were reviewed to pinpoint the earliest visit with a compromised cardiac function.

At last cardiac evaluation (on the day of visit or within a six-month window), thirty patients underwent plasma hs-cTnI measurements, performed at the Laboratory Medicine Unit, IRCCS Ospedale Policlinico San Martino, Genoa, Italy, directed by Professor Nanni, using a chemiluminescence technique.

All candidate patients and their parents provided their assent and consent, respectively, to participate in the study before enrollment, and the study was conducted in accordance with the 1964 Declaration of Helsinki and its subsequent amendments. Cardiological evaluations and blood sample collections were conducted as part of the routine longitudinal follow-up visits following disease-specific guidelines [15, 60].

The limit of detection (LoD) for this assay was determined to be 3 ng/L, and the upper reference limit or 99th percentile was established following the latest guidelines: 6 ng/L for subjects up to 18 years [90, 91] and 34 ng/L for subjects over 18 years [92].

Echocardiography

Echocardiographic studies were performed in the Cardiology Unit, IRCCS Gaslini Institute, with last generation machines from Philips IE33 and Philips Epiq. All the cardiac examinations were performed by the same cardiologist. Two-dimensional images and M-mode echocardiograms of atrial and ventricular cavities were obtained in multiple cross-sectional planes, according to the recommendations of the

European Association of Cardiovascular Imaging. LVEF was calculated with Simpson's method.

Statistical Analysis

Descriptive statistics were generated for the whole cohort and data were expressed as mean and standard deviation (SD), median and range for continuous variables and as absolute or relative frequencies for categorical variables. Non parametric analysis (Mann-Whitney U-test or Wilcoxon test) for continuous variables and $\chi 2$ or Fisher's exact test for categorical variables were used to measure differences between groups.

Correlation analysis using Spearman's coefficient rank correlation was used.

A p value ≤ 0.05 was considered statistically significant, and all P values were based upon two tailed tests. Statistical analysis was performed using SPSS for Windows (IBM SPSS Inc., New York, NY, USA).

4 Results

Demographics

This study included 81 DMD patients aged between 2 and 34 years (average age of 16,3 years). At last visit, 41 patients (51%) were non ambulant, and the average age of ambulatory loss was 12 years. Twenty-three patients (28%) required non-invasive ventilatory support (NIV), 4 patients (4%) had percutaneous endoscopic gastrostomy (PEG) tubes, and 5 patients (6%) had undergone corrective surgery for scoliosis (Table 1).

Medication and Therapy Profiles

Sixty-six subjects (81%) were on corticosteroid therapy, 7 subjects (9%) had discontinued steroid treatment prior to the last visit, and 8 subjects (10%) were steroid-naïve. The average age at the initiation of steroid therapy was 6 years. Two patients (2%) were receiving testosterone therapy for delayed puberty (testicular volume < 4 ml at 14 years of age).

Sixty-five patients (80%) were on cardiac therapy, primarily consisting of angiotensin-converting enzyme inhibitors (ACEi) (75%) and beta-blockers (48%). Five patients (6%) were taking diuretics, 3 (4%) were on ivabradine, 2 (2.5%) were prescribed angiotensin receptor blockers (ARBs), and 2 (2.5%) were receiving antiarrhythmic medications. Thirty-eight patients (47%) were on polytherapy for

cardiac conditions. The average age at the initiation of cardiac therapy was 11.5 years; 88% started cardiac therapy for prophylaxis, while 12% initiated it for pathological LVEF. Two patients (2%) had cardioverter-defibrillators implanted.

Cardiac Assessment

The average LVEF at last visit was 50%. Fifty-four patients (67%) had a pathological LVEF. The average age of these patients was 16 years. Among these, 98% of patients were on cardiac therapy, 85% were on steroid therapy, 30% were ambulatory, and 37% required non-invasive respiratory support.

The average age of the onset of LVEF decline was 14 years.

Table 1. Characteristics of patients at last visit

	ALL (N=81)	**Group 1 (<10yrs) *N=16***	**Group 2 (10-18yrs) *N=37***	**Group 3 (>18yrs) *N=28***
Current age, *yrs, median (min, max)*	15.7 (1.9 - 34.3)			
Age at last cardiological examination, *yrs, mean ±SD*		5.7±2.1	13.8±2.2	23.2±3.7
Ambulatory patients, *n(%)*	40 (49.4%)	16 (100%)	22 (59.5%)	3 (10.7%)
Noninvasive ventilation, *n(%)*	23 (28.4%)	0 (0%)	4 (10.8%)	19 (67.9%)
PEG, *n(%)*	4 (4.9%)	0 (0%)	1 (2.7%)	3 (10.7%)
Scoliosis surgery, *n(%)*	5 (6.2%)	0 (0%)	1 (2.7%)	4 (14.3%)
Medication, *n(%)*				
Corticosteroids	66 (81.5%)	13 (81.3%)	37 (100%)	16 (57.1%)
Cardiac medication	65 (80.2%)	1 (6.3%)	36 (97.3%)	28 (100%)
ACEIs	61 (75.3%)		36	25
Beta-blockers	39 (48.1%)	1	12	26
Diuretics	5 (6.2%)		1	4
Ivabradine	3 (3.7%)			3
Antiarrhythmic	2 (2.5%)			2
ARBs	2 (2.5%)			2
Polytherapy	38 (46.9%)		12 (32.4%)	26 (92.9%)
Testosterone	2 (2.5%)		2	
CK, *U/L, median (min, max)*	4702 (180-34549)	14856 (8389-34549)	5518 (521-21412)	964 (180-4060)
Age of ambulation loss, *yrs, mean ±SD*	12.4 ±2.5		12.0±2.5	12.7±2.6
Age at initiation of NIV, *yrs, mean ±SD*	19.3±4.1		16.8±2.2	20.0±4.2
Age at onset of PEG, *yrs, mean ±SD*	30±17.6		17.0	24.3±6.1
Age at scoliosis surgery, *yrs, mean ±SD*	16.3±3.3		18.0	17.3±2.5
Age at initiation of steroids, *yrs, mean ±SD*	6.4±2.1	4.9±0.7	6.7±2.4	6.6±1.8
Age at initiation of cardiac treatment, *yrs, mean ±SD*	11.6±3.1	7.7	10.6±1.7	13.3±3.9
LVEF, *%, mean ±SD*	49.8% ±10.1	60.3% ±2.9	51.7% ±4.7	42.3% ±8.5
Patients with pathological LVEF, *n(%)*	54 (67%)	0 (0%)	27 (73.0%)	27 (96.4%)
Patient LVEF < 55%, *n(%)*	37 (45.7%)		26 (96.3%)	11 (40.7%)

Patient LVEF < 45%, *n(%)*	10 (12.3%)	1 (3.7%)	9 (33.3%)
Patient LVEF < 35%, *n(%)*	7 (8.6%)		7 (25.9%)
Age at onset of pathological LVEF, *yrs, mean ±SD*	14.3±4.2	12.2±2.1	16.9±3.6

Cardiac Function and Other Clinical Features According to Age

We divided the cohort into three subgroups according to age (Table 1).

Group 1 included 16 patients (20%) younger than 10 years (age range: 2-9, mean: 5.7). The group had a mean LVEF of 60.3%, with no patients showing a pathological LVEF. All of these patients were ambulating independently, and none of them required ventilatory support. Only one patient was on cardiac therapy (beta-blocker). Thirteen patients (81%) in this group were on steroid therapy, with an average age of initiation at 4.9 years. The median plasma CK values for this group were 14856 U/L.

Group 2 included 37 patients (46%) between 10 and 18 years (mean: 13.8). In this group, the mean LVEF was 51.7% with 27 patients (73%) having a pathological LVEF: of these, 26/27 had a mild compromise of LVEF, 1/27 (4%) had a moderate compromise. No patient in this group had severe systolic dysfunction. The mean age of onset of LVEF decline for this group of patients was 12.2 years. Forty percent of the patients had lost the ability for independent ambulation at an average age of 12.0 years. Four patients required NIV, which was initiated at an average age of 16.8 years, and one patient received nutritional support through a PEG from the age of 17 years. Thirty-six patients (97%) were on cardiac therapy (ACE inhibitors, beta-blockers, or diuretics), with 12 of them on a combination of these medications. The average age of initiation of cardiac therapy was 10.6 years. All patients in this group were on steroid therapy, which was initiated at an average age of 6.7 years. Two patients were on testosterone replacement therapy for delayed puberty. The median CK level in this group was 5518 U/L.

Group 3 included 28 patients (34%) older than 18 years (age range: 19-34 years, mean: 23.2). The average LVEF for this group was 42.3%, with 96% of patients exhibiting compromised cardiac function: among these, 11 patients (41%) had slightly compromised LVEF, 9 patients (33%) had moderately compromised LVEF, and 7 patients (26%) had severely reduced LVEF (Figure 12, Figure 13). The mean age of the patients with severely reduced LVEF was 24.3 years. For this group of patients, the mean age of onset of LVEF decline was 16.9 years. In this group, only three patients were able to ambulate independently, while the rest had lost this ability at an average age of 12.7 years. Sixty-eight percent of the patients were on non-invasive ventilatory support, which was initiated at an average age of 20.0 years, and none of the patients required mechanical ventilation. Eleven percent of the patients had PEG tubes placed at an average age of 24.3 years. Four patients had undergone surgical scoliosis correction procedures at an average age of 17.3 years. All patients in this group were on cardiac therapy, with 93% of them on multiple cardiac

medications, including ACE inhibitors, beta-blockers, diuretics, ivabradine, antiarrhythmics, and ARBs. Additionally, two patients had cardioverter-defibrillators implanted. The average age of initiation of cardiac therapy was 13.3 years. Fifty-seven percent of the patients were on steroid therapy, and among the remaining 43%, 67% had discontinued it over time. The average age of initiation of steroid therapy was 6.6 years. The median CK level in this group was 964 U/L).

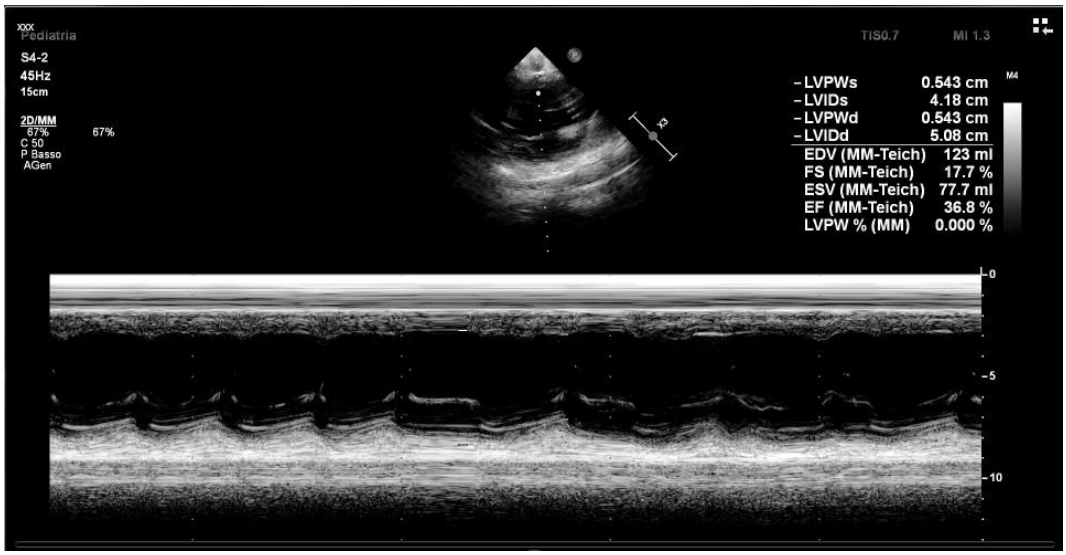


Figure 12. Echocardiographic image of a 25-year-old patient in our cohort, conducted at our Institute, that shows left ventricle dilated, globular, thinning walls, global hypokinesia, resulting in a severe reduction of global systolic function.

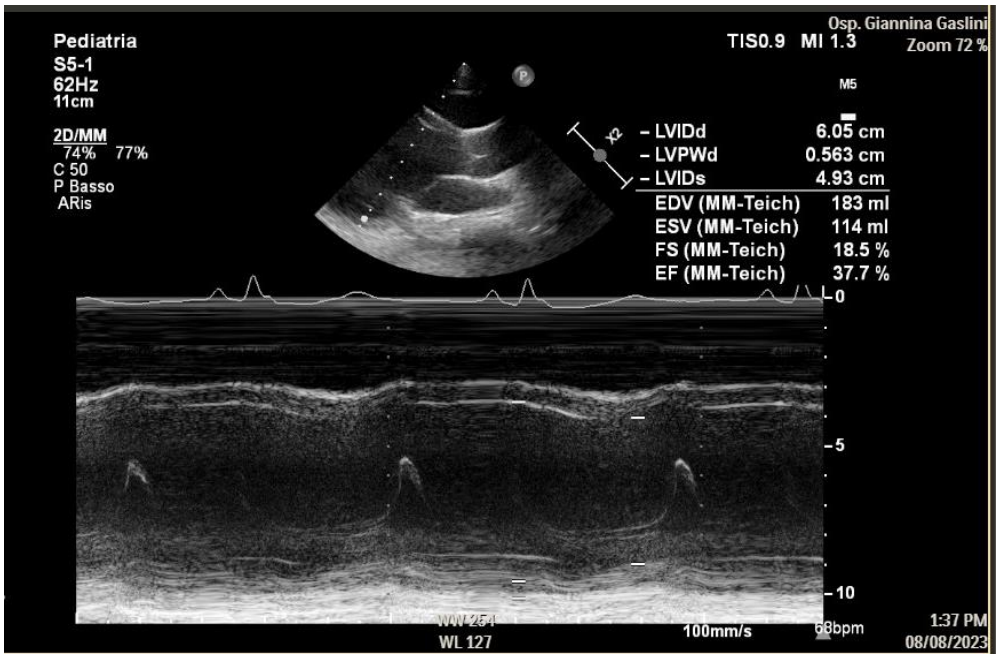


Figure 13. Echocardiographic image of a 23-year-old patient in our cohort, conducted at our Institute, that shows left ventricle dilated, globular, thinning walls, global hypokinesia, resulting in a severe reduction of global systolic function.

Group 2 was further divided into 3 age groups (Table 2): 11 patients (30%) were aged between 10 and 12 years (mean 11.2), with a mean LVEF at last visit of 54.0%. Fifty-five percent had slightly compromised LVEF.

Eighteen patients (49%) were aged between 13 and 15 years (mean 14.0), with a mean LVEF at last visit of 52.0%. 78% had slightly compromised LVEF.

Eight patients (21%) were aged between 16 and 18 years (mean 17.0), with a mean LVEF at last visit of 50.0%. 75% had slightly compromised LVEF, and 1 patient had moderately compromised LVEF.

There was a statistically significant reduction in LVEF values when comparing the first and third groups (p value= 0.02).

Table 2. Subdivision of group 2 into 3 subgroups according to age: LVEF characteristics

	10-12 yrs ***N=11***	**13-15 yrs** ***N=18***	**16-18 yrs** ***N=8***
LVEF, *%, mean ±SD*	54.0% ±3.8	52.0% ±4.5	50.0% ±3.9
Patients with pathological LVEF, *n(%)*	6 (54.5%)	14 (77.8%)	7 (87.5%)
Patient LVEF < 55%, *n(%)*	6 (100%)	14 (100%)	6 (85.7%)
Patient LVEF < 45%, *n(%)*			1 (14.3%)
Patient LVEF < 35%, *n(%)*			

Age at onset of pathological LVEF, *yrs, mean ±SD*	10.8±0.87	11.7±1.6	14.6±1.8

Comparison of Echocardiographic Data Across 3 Age Groups

Echocardiographic data obtained at the last cardiac assessment for all patients were compared across the three age groups in a cross-sectional study (Table 3).

LVEF values showed a significant reduction with increasing age: there was a statistically significant difference (p value ≤0.0001) in LVEF values in Group 1 compared to Group 2 and Group 3, and in Group 2 compared to Group 3.

In addition, other echocardiographic parameters were analyzed for each group: the mean FS for each group was 34.2%, 30.3%, and 27.5%, with a significant reduction as age increased. Statistically significant differences were found between Group 1 and Group 2 (p value = 0.01), Group 1 and Group 3 (p value ≤0.0001), and Group 2 and Group 3 (p value = 0.05).

The mean EDD for each group was 36.8 mm, 46.2 mm, and 51.9 mm, with a significant increase as age increased. Statistically significant differences were observed between EDD values in Group 1 compared to Group 2 and Group 3, and between EDD values in Group 2 compared to Group 3 (p value ≤0.0001).

Similar statistical results were obtained when analyzing ESD values, with mean values for each group being 24.1 mm, 32.0 mm, and 37.5 mm, showing statistically significant differences (p value ≤0.0001) between the values in each group.

Table 3. Comparison of echocardiographic parameters in the 3 groups

	Group 1 (<10yrs) *N=16*	**Group 2 (10-18yrs) *N=37***	**Group 3 (>18yrs) *N=28***	**P_value**		
				Group 1 vs Group 2	Group 1 vs Group 3	Group 2 vs Group 3
LVEF, *%, mean ±SD*	60.3% ±2.9	51.8% ±4.7	42.3% ±8.5	≤0.0001	≤0.0001	≤0.0001
FS, *%, mean ±SD*	34.2% ±3.7	30.3% ±2.6	27.5% ±2.3	0.001	≤0.0001	0.05
EDD, *mm, mean ±SD*	36.8 ±2.3	46.2 ±4.8	51.9 ±5.5	≤0.0001	≤0.0001	≤0.0001
ESD, *mm, mean ±SD*	24.1 ±3.3	32.0 ±5.1	37.5 ±4.8	≤0.0001	≤0.0001	≤0.0001

Cardioprotective Therapy Effect

In group 3 (age > 18 years), we observed three patterns of cardiac dysfunction: mildly, moderately, and severely reduced LVEF (Table 4).

Furthermore, we categorized patients into two subgroups: the first subgroup consisted of 23 patients (82%, mean age: 22.8) who had initiated prophylactic cardiac therapy (mean age at the onset of cardioprotective therapy: 13.0 years).

The second subgroup consisted of 5 patients (18%, mean age: 25.0) who had initiated cardiac therapy with already compromised LVEF (mean age at the onset of cardiac therapy: 14.1).

We observed that among patients with mildly reduced LVEF, 100% had initiated cardioprotective therapy. Among patients with moderately reduced LVEF, 22% had initiated cardiac therapy with LVEF already compromised. Among patients with severely reduced LVEF, 43% had initiated cardiac therapy with LVEF already compromised.

The mean LVEF for the group of patients who had initiated cardioprotective therapy was 44.1%, and for the group that had initiated cardiac therapy with LVEF already compromised at an older age, it was 34.4%, with a statistically significant difference between the two groups (p value 0.01).

As for the other echocardiographic parameters, no statistically significant difference was observed between the two groups.

Finally, the mean age at onset of pathological LVEF for the first group was 17.6 years and 14.0 for the second group and we observed a statistically significant difference between these data (p value 0.04).

Table 4. The role of cardioprotective therapy in the progression of LVEF in group 3

	Patients who initiated cardiological prophylactic therapy N=23	**Patients who initiated cardiological therapy with impaired LVEF N= 5**	**P_value**
LVEF, *%, mean ±SD*	44.1%±7.7	34.4%±7.9	0.01
FS, *%, mean ±SD*	27.9% ±2.2	25.0%	0.50
EDD, *mm, mean ±SD*	51.4±5.7	54.0±4.7	0.30
ESD, *mm, mean ±SD*	36.3±4.3	41.7±4.7	0.11
Age at initiation of cardiac treatment, *yrs, mean ±SD*	13.0±4.0	14.1±3.2	
Age at onset of pathological LVEF, *yrs, mean ±SD*	17.6±3.4	14.0±3.0	0.04

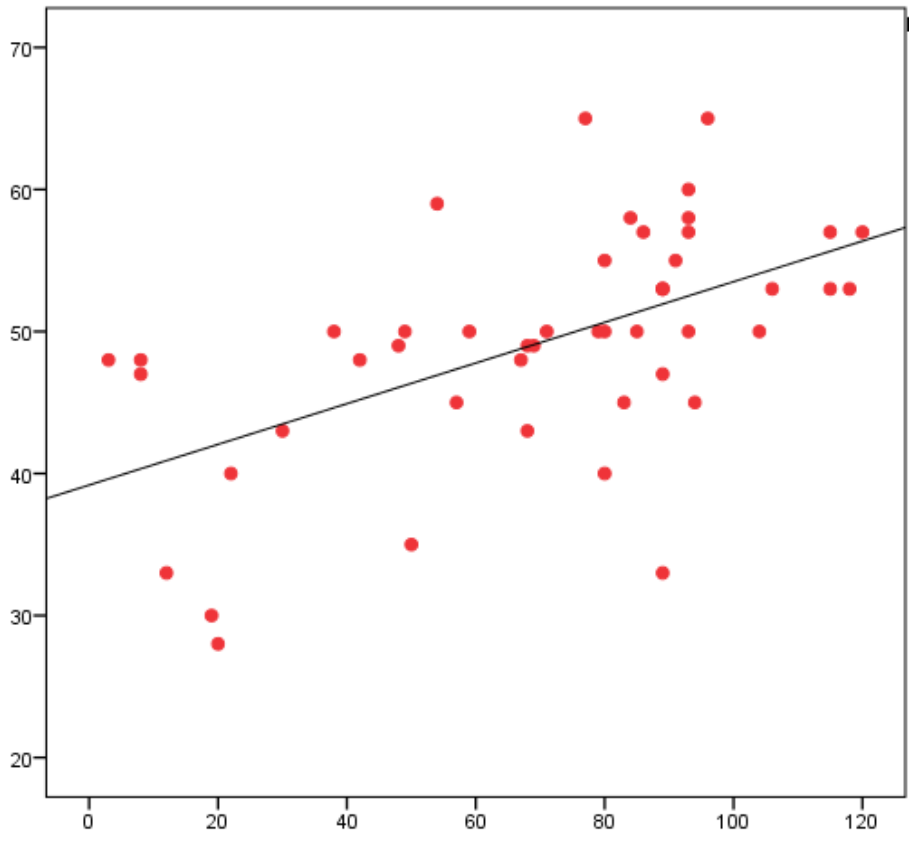
Correlation Between Spirometry and LVEF

The mean values of FEV1% in our patient cohort were 70.3%. The mean values of FVC% in our patient cohort were 66.6%. There was a statistically significant correlation between FEV1% and LVEF values (p-value ≤ 0.0001) and between FVC% and LVEF values (p-value ≤ 0.0001).

Graph 1. Correlation between FEV1% (x-axis) and LVEF (y-axis)

Spearman's rho 0.59

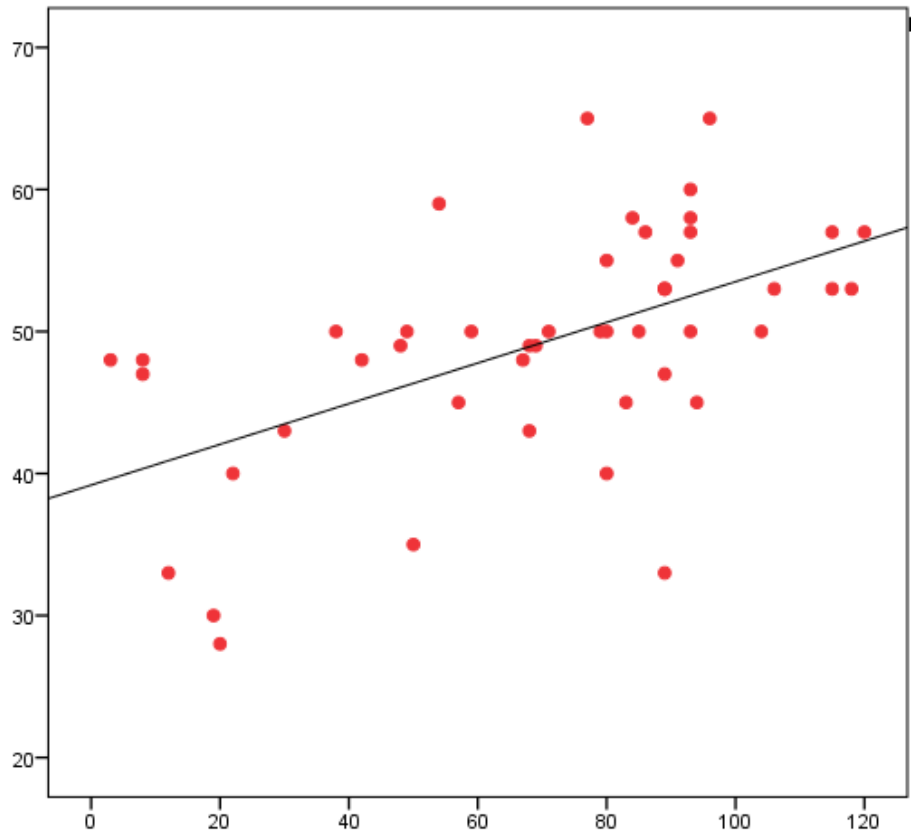
P value ≤0.0001



Graph 2. Correlation between FVC% (x-axis) and LVEF (y-axis)

Spearman's rho 0.59

P value ≤0.0001



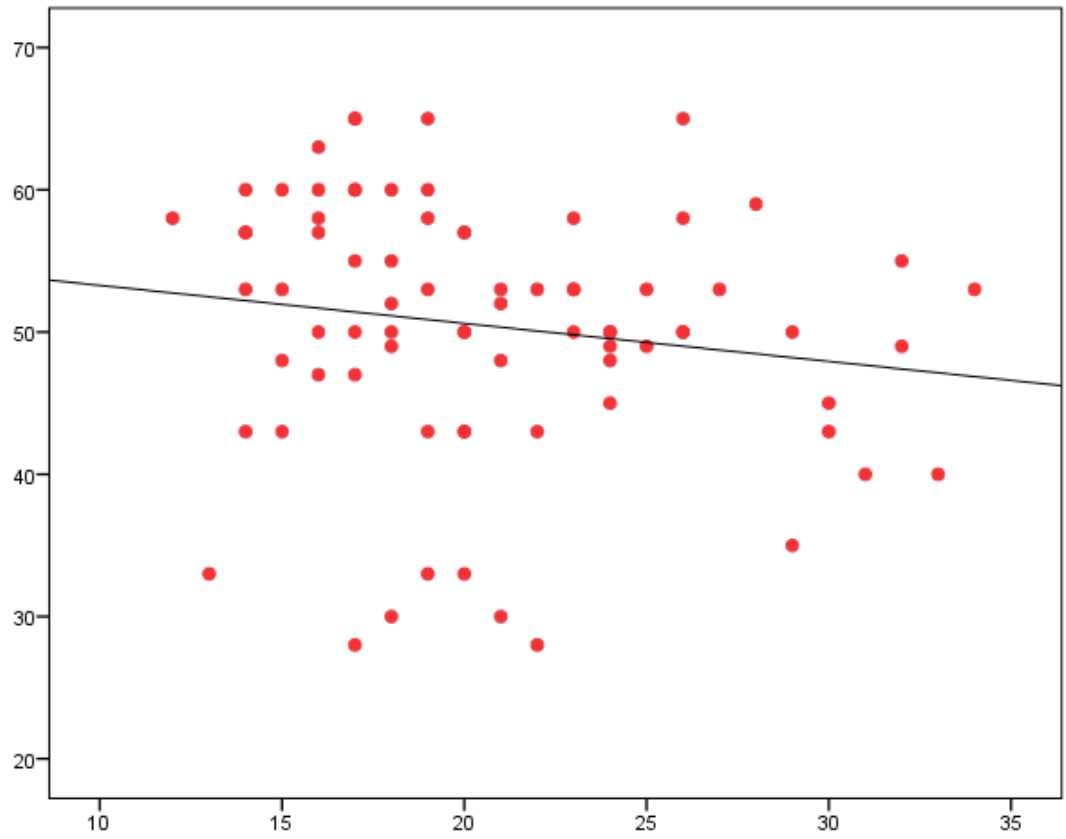
Correlation Between BMI and LVEF

There was a statistically significant inverse correlation between BMI values (mean 20.8 kg/m^2) and LVEF values (P value = 0.04).

Graph 3. Correlation between BMI (x-axis) and LVEF (y-axis)

Spearman's rho -0.23

P value=0.04



Longitudinal Cardiac Assessments

For patients with pathological LVEF at their last visit (n=54), we retrospectively reviewed clinical charts and found that the average age of onset of cardiac dysfunction, as detected by echocardiography, was 14 years. Furthermore, in this retrospective study, we assessed an intermediate time point between the initial visit where pathological LVEF was observed and the latest assessment (Table 5).

The average follow-up period was 4.5 years, and the annual LVEF variation was -1.1%.

The LVEF variation per year for patients who had initiated cardioprotective therapy was -1.09%, and for those who had not initiated cardioprotective therapy, it was -1.24% (non-significant p-value).

The LVEF variation per year for patients on steroid therapy was -1.11%, and for those not on steroid therapy, it was -1.12% (non-significant p-value).

There was a statistically significant reduction in LVEF at all three time points (mean values of 51.0%, 47.1%, and 44.0%, respectively) with a p-value of ≤ 0.0001 in both the comparison between the first and second time points, and between the second and third time points.

There was a statistically significant increase in EDD at all three time points (mean values of 46.7 mm, 50.4 mm, and 50.9 mm, respectively), with a p-value of ≤0.0001 in the comparison between the first and second time points and a p-value of 0.09 in the comparison between the second and third time points.

The mean FS was 29.3%, 28.1%, and 28.4%, respectively, with a statistically significant difference (p-value 0.009) between the first and second time points.

Table 5. Cardiac function progression through echocardiographic parameter evaluation at 3 time points

	T0	**T1**	**T2**	**P_value**		
				T0 vs T1	T0 vs T2	T1 vs T2
LVEF, *%, mean ±SD*	51.0±2.3	47.1±5.1	44.0±7.4	≤0.0001	≤0.0001	≤0.0001
FS, *%, mean ±SD*	29.3±2.6	28.1±3.2	28.4±2.5	0.009	0.37	0.59
EDD, *mm, mean ±SD*	46.7±4.9	50.4±5.5	50.9±5.8	≤0.0001	≤0.0001	0.09

DMD mutations

The distribution of mutations observed in our cohort closely resemble those documented in the literature [4].

Table 6 offers an overview of the distribution of mutations in our cohort. There was no statistically significant correlation between genotype and cardiologic phenotype.

Table 6. Genotypic characteristics of the population

Mutation	**Number of patients (%)**
Deletions	55 (70%)
Duplications	6 (7%)
Point mutations	19 (23%)
Other *	1 (1%)

*: rearrangements investigated through RNA analysis extracted from muscle

High-sensitivity Cardiac Troponin I Results

Thirty patients, divided into three age subgroups, underwent plasma hs-cTnI measurements within 6 months from the last cardiac evaluation (Table 7, Table 8). The limit of detection (LoD) for this assay was determined to be 3 ng/L, and the

upper reference limit coincided with the 99th percentile for age and gender, as illustrated in the methods.

Seven patients (23%) were younger than 10 years (age range: 5-9, mean: 7.0). All of them had preserved cardiac function at the time of sampling (mean LVEF: 58.0%). No patient in this group was on cardiological therapy. Among this group, hs-cTnI was pathological in 2 patients (29%) and detectable in 4 (57%). Three patients had hs-cTnI values below the limit of detection. The mean hs-cTnI for this group was 7.9 ng/L.

Fifteen patients (50%) were between 10 and 18 years (mean: 13.0). The mean LVEF was 51.3%, and 3 patients had preserved LVEF. 12 out of 15 (80%) patients were on cardiological therapy. In this group, hs-cTnI was pathological in 8 patients (53%), one of whom had preserved LVEF, and detectable in 11 (73%). Four patients had hs-cTnI values below the limit of detection. The mean hs-cTnI for this group was 42.9 ng/L.

Eight patients (27%) were older than 18 years (age range: 19-29, mean: 21.0). The mean LVEF was 48.3%. Only one patient had preserved LVEF. All patients in this group were on cardiological therapy. In this group, hs-cTnI was pathological in 4 patients (50%), and detectable in all patients. The mean hs-cTnI for this group was 39.1 ng/L.

Among patients with normal LVEF at the time of sampling, hs-cTnI was pathological in 3 (27%) and detectable in 6 (54.5%).

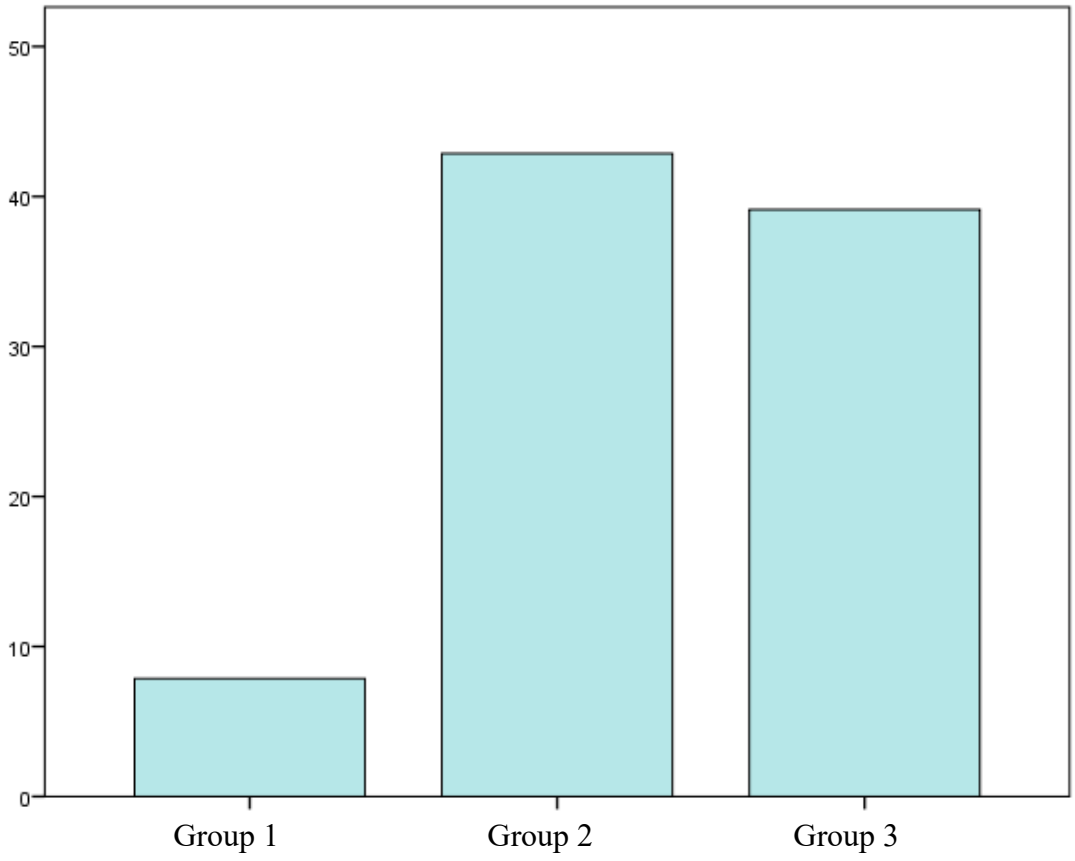
Table 7. High sensitivity cardiac troponin I (hs-cTnI) assay results in the 3 age-stratified patient groups [N=30]

	Group 1 (<10yrs) *N=7*	Group 2 (10-18yrs) *N=15*	Group 3 (>18yrs) *N=8*
Pathological hs-cTnI, *n(%)*	2 (28.6%)	8 (53.3%)	4 (50.0%)
Quantifiable hs-cTnI, *n(%)*	4 (57.1%)	11 (73.3%)	8 (100%)

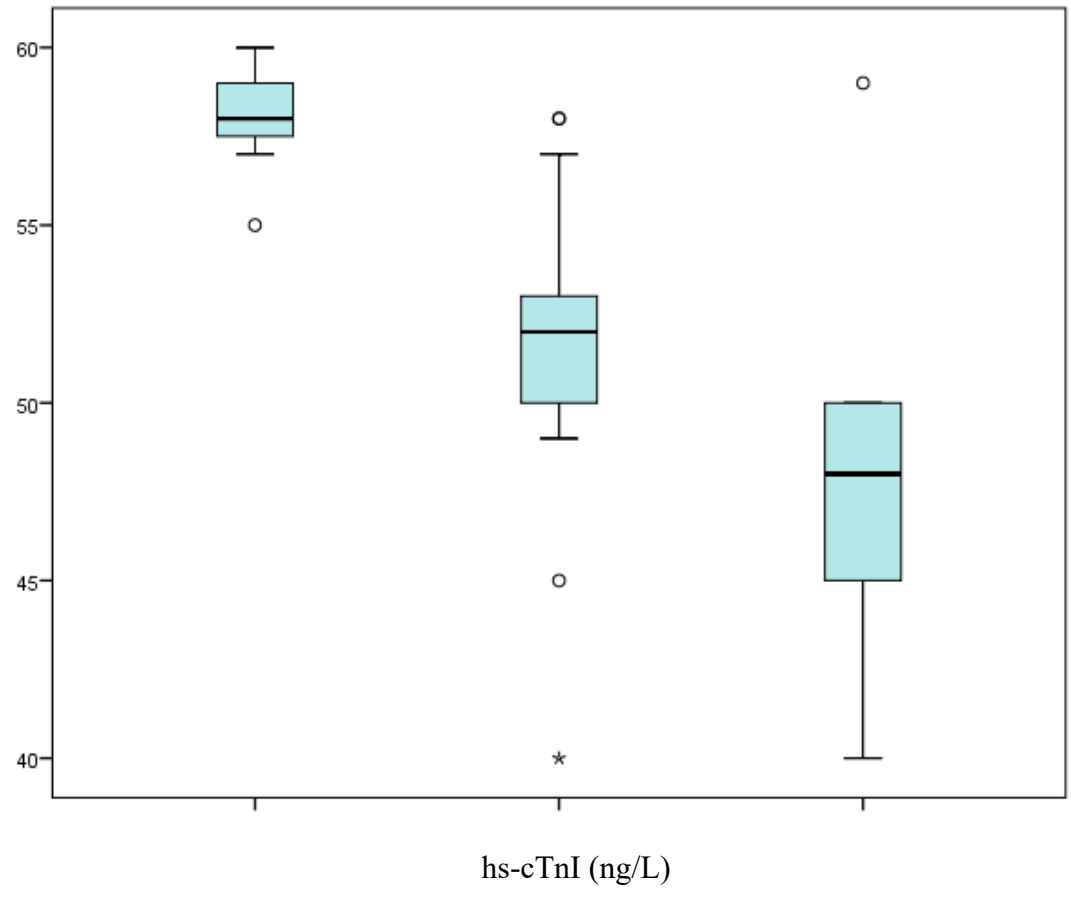
Table 8. hs-cTnI and LVEF data

	LVEF ≥ 55% *N=11*	LVEF < 55% *N=19*
Pathological hs-cTnI, *n(%)*	3 (27.3%)	11 (57.9%)
Quantifiable hs-cTnI, *n(%)*	6 (54.5%)	16 (84%)

Graph 4 Distribution of hs-cTnI (y-axis; mean, ng/L) throughout the groups



Graph 5. Correlation of hs-cTnI (x-axis, mean, ng/L) with Cardiac Function (LVEF, y-axis)



5 Discussion

DMD is the most prevalent and severe type of muscular dystrophy. The current management of DMD has been influenced by the accessibility of increasingly sensitive diagnostic techniques, new therapeutic strategies, and the earlier application of therapeutic interventions, offering the potential to enhance both the duration and quality of patients' lives. Heart and respiratory function involvement is a classic hallmark of DMD, and it has a profound impact on the prognosis [58, 94], as cardiorespiratory complications traditionally represent the leading causes of death in DMD. Cardiac complications continue to be a critical concern that affects overall survival, as cardiomyopathy is now recognized as the leading cause of death in DMD. Consequently, ensuring the preservation of cardiac function is critical to extend the survival of individuals with DMD.

This is the first study analyzing the cardiac progression in a large cohort of DMD patients followed at the Gaslini Institute. The primary objective of the study was to investigate the progression of cardiac damage in this patient population through a comprehensive assessment of their cardiac function, employing a longitudinal analysis of echocardiographic data collected over the years. The secondary objective was to identify potential early biomarkers.

Overall, the study showes that cardiac function in patients with DMD progressively deteriorates with age, regardless of the ongoing therapies, in agreement with the literature data [40]. In our cohort, this impairment first became evident on echocardiographic examination from the first half of the second decade of life; more specifically, the average age of onset of LVEF decline was 14 years, approximately two years after the average age of loss of ambulation. In our patient cohort, more than half exhibited compromised cardiac function at the last cardiological visit, with varying degrees of severity.

Our study confirms the beneficial impact of cardioprotective therapy on cardiac outcomes, both in terms of the severity of cardiomyopathy and the onset time. Indeed, our research has demonstrated that patients who had started cardioprotective therapy showed the initial signs of cardiac damage in echocardiography at a significantly older age compared to patients of the same age who started cardiac treatment at a later age and with compromised cardiac function. Furthermore, at their last visit, they exhibited significantly higher LVEF values compared to patients who had not undergone cardioprotective therapy.

In our case series, there are 3 patients who initiated cardioprotective therapy before the age of 10. Of these, one patient is still under 10 years old and has preserved LVEF. Another patient began to show compromised LVEF at 17 years, later than the average, while the third started to exhibit compromised LVEF at 10 years, which is younger than the average. However, the number of patients treated at such an early age is too limited to draw significant conclusions.

Moreover, we show a direct relationship between respiratory function, both in terms of FEV1 and FVC values, and the progression of cardiac function. This data underscores the importance of including spirometry as a standard practice in every follow-up visit for DMD patients, starting from the age when these measurements are reliable. The patients in our case series were indeed subjected to spirometry at every follow-up visit starting from the age of 5-6, in accordance with current recommendations [60].

Additionally, we observed a statistically significant inverse correlation between BMI and cardiac function. In this regard, while some studies suggest no correlation between BMI and cardiac function [95], a recent study suggests that underweight is associated with lower LVEF and a higher prevalence of cardiomyopathy compared to normal weight or overweight patients [96]. Future longitudinal study is needed to better understand how BMI affects cardiomyopathy.

We did not find a statistical correlation between genotype and cardiologic phenotype, in contrast to the association between certain deletions and the severity of DMD-related cardiomyopathy that some studies in the literature have shown[58, 59]. This is likely due to the small number of patients for each genotype.

Nevertheless, it's worth highlighting the case of a 22-year-old patient in our cohort. During their latest cardiac evaluation, this patient still exhibited preserved LVEF, which is a unique finding in their age group. This individual's age of ambulation loss, occurring at 15.7 years, exceeds the cohort's average. In this specific case, the favorable cardiac profile doesn't align with the typical age for initiating cardiac therapy, as this patient started treatment later than the cohort's average, at 17.9 years. It's important to note that this patient carries a stop mutation (R2982X). Further research is needed to investigate the potential correlation between these stop mutations and cardiac profile.

Regarding early diagnosis, as of today, there are no guidelines legitimizing the use of cardiac injury biomarkers, and scientific data suggest the importance of identifying them for better cardiac management of DMD patients.

We assessed the suitability of high-sensitivity cardiac troponin I as a potential early biomarker for cardiac damage in a small number of patients.

Despite literature providing encouragement in this regard, its application in patients with muscular dystrophy, particularly DMD, remains an understudied area.

This analysis showes a correlation between the progression of cardiac damage and hs-cTnI levels and an age distribution that increases throughout childhood and reaches a peak around the first half of the second decade of life, before declining in later years. We also identified elevated hs-cTnI in a subgroup of patients with preserved cardiac function at the time of sampling, of which 2 were under the age of 10. This particular subgroup will be the focus of the next phase of our study, where we will investigate the biomarker's progression alongside changes in echocardiography findings. The ultimate goal will be to determine how early hs-cTnI levels rise in comparison to detectable echocardiographic abnormalities, and consequently, understand the most appropriate timing for initiating cardioprotective therapies. For half of the patients who underwent blood sampling, standard TnI values were also available and measured concurrently. Patients with normal hs-cTnI levels also exhibited normal standard TnI levels. It is noteworthy that only one patient, among those with elevated hs-cTnI, showed abnormal values in standard TnI as well. Conversely, 7 patients with elevated hs-cTnI had standard troponin I within the normal range. This underlines the ability of hs-cTnI to detect early cardiac damage more effectively than standard TnI.

This study has some limitations. It was not possible to use the strain echocardiography method as an assessment parameter, as it wasn’t performed on all patients due to issues with inadequate acoustic windows in some older subjects. It was not possible to conduct the study using cardiac MRI for all patients due to

limitations stemming from need for sedation for some patients, cost considerations, and limited accessibility. Regarding the measurement of hs-cTnI, one limitation is the small number of patients who underwent blood sample collection.

In conclusion, this study has shown that in our cohort of DMD patients, cardiac dysfunction can be detected echocardiographically around 14 years of age, about two years after the mean age of loss of ambulation, and it worsens significantly with age.

Cardioprotective therapy emerges as a protective factor, both attenuating the severity of cardiomyopathy and delaying its onset.

Respiratory parameters also exhibit show a statistically significant decline with increasing age, and it is noteworthy that a significant direct correlation exists between LVEF% and spirometry values, including FVC and FEV1.

Additionally, there is a statistically significant inverse correlation between LVEF and BMI.

High-sensitivity cardiac troponin I proves to be a suitable biomarker for tracking the evolution of cardiomyopathy. The identification of detectable or clearly pathological hs-cTnI values in a subgroup of patients with preserved LVEF at the time of the study suggests its potential as an early biomarker for cardiac damage.

Further studies are indeed required to gain deeper insights into the clinical significance of hs-cTnI within the context of DMD and to determine at what stage its levels increase in relation to the detection of echocardiographic abnormalities.

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