Clinical and Molecular Study on Duchenne Muscular Dystrophy

Gesmar Volga Haddad Herdy¹, Roberta Duarte Bezerra Pinto², Guilherme de Almeida Costa¹, Ana Flavia Malheiros Torbey¹, Vivianne Galante Ramos³, Marcio Moacyr Vasconcelos¹

¹Universidade Federal Fluminense – Serviço de Pediatria – Niterói, RJ – Brazil
²Universidade Federal Fluminense – Programa de Residência em Clínica Médica – Niterói, RJ – Brazil
³Fundação Oswaldo Cruz – Rio de Janeiro, RJ – Brazil

Abstract

Background: Duchenne Dystrophy is the most common and severe form of muscular dystrophy. It has an X chromosome-linked recessive inheritance and affects boys’ striated muscles and myocardium. It is caused by mutations in the dystrophin gene, the largest human gene, composed of 79 exons.

Objectives: To check the early cardiac changes in pediatric patients with Duchenne muscular dystrophy (DMD) and carry out the molecular study of changes in the dystrophin gene.

Methods: Prospective study involving pediatric patients with DMD, with clinical assessment, measurement of serum levels of creatine phosphokinase, electrocardiogram, Doppler echocardiography and dynamic electrocardiography and DNA genotyping, with amplification of the 18 most affected exons.

Results: A group of 11 boys aged 6-14 years was studied. Clinical cardiological examination did not reveal any major changes. An increase in creatinine phosphokinase was detected in all patients. Electrocardiogram showed early changes, with high R waves in V1 (n=7) right bundle branch block (n=2), delta waves and short PR interval (n=1), and signs of disturbance of ventricular repolarization (n=1). Echocardiogram showed signs of systolic dysfunction. Dynamic electrocardiogram (Holter) showed changes in 4 patients: with many extrasystoles (n=3) and Wolff-Parkinson-White syndrome (n=1). All children received corticosteroid therapy. There was no significant correlation between exon 52 deletion and arrhythmias (p=0.43). The molecular study revealed an exon 52 deletion in 4 patients with dilated cardiomyopathy, of which 2 had concomitant deletion of exons 1 and 50, respectively. Other 7 patients had deletions of exons 48, 51, 52 and 57.

Conclusions: Electrocardiogram showed the first changes in pediatric patients with DMD. In cases with dilated cardiomyopathy and arrhythmia, the deletion of exon 52 was detected.

Keywords: Duchenne muscular dystrophy; Dystrophin; Child
and cardiac muscles, which are replaced with fibrotic tissues\(^2\). Molecular research helps differentiate the main forms of dystrophy. Dystrophin messenger RNA is predominantly expressed in the skeletal, cardiac and smooth muscles, with low levels in the brain\(^3\). Clinical features and cardiovascular manifestations of the disease vary\(^1\). Most patients have cardiomyopathy, but symptoms can be masked by muscle weakness.

This study aims to check the early cardiac changes in pediatric patients with Duchenne muscular dystrophy (DMD) and carry out the molecular study of changes in the dystrophin gene.

### Methods

A prospective study, involving pediatric patients with DMD, with clinical assessment, measurement of serum levels of creatine phosphokinase, electrocardiogram, Doppler echocardiography and dynamic electrocardiography and DNA genotyping, with amplification of the 18 most affected exons.

This research project was approved by the Research Ethics Committee at Faculdade de Medicina da Universidade Federal Fluminense, under no. CAAE-0163.02.58.203/10.

Patients diagnosed with progressive muscular dystrophy (Duchenne type) were referred to the pediatric cardiology outpatient facility. In order to detect early cardiac changes in pre-clinical stage, patients older than six years underwent conventional electrocardiogram (Dixtal-Biomedical, Manaus, Brazil), two-dimensional echocardiography Doppler flowmetry (Vivid 3-GE, New Jersey, USA) and dynamic electrocardiography (Holter 24 hours), using the CardioSmart software (Cardios, Cardiolight recorder - São Paulo, Brazil).

Blood was collected, kept in refrigeration for a few hours and sent to the Laboratório de Genética Humana of Fundação Getúlio Vargas (Fiocruz) and Universidade do Grande Rio (Unigranrio) for molecular research, which was carried out by one of the researchers. The genomic DNA extraction was performed using the technique described by Miller et al.\(^7\) from a layer of 5-mL aliquot of peripheral blood leukocytes.

### Genotyping for mutations in the dystrophin gene

The genotyping for each patient was carried out using multiplex polymerase chain reaction (PCR) following the already established protocol for detecting, with specific probes, the different mutations previously described in the human dystrophin gene\(^8\).

### Molecular investigation of the dystrophin gene

For molecular investigation of dystrophin gene to be conducted, 18 pairs of oligonucleotides (primers) were used, which allow for differential amplification of regions comprising the 18 exons that are the most common targets of DMD mutation.

For each amplification reaction, a 50-ml solution is prepared, containing: 250 ng genomic DNA; 200 mM dNTPs; 1 mM of each primer; 1X PCR buffer (Perkin Elmer, Tucson, USA); 2.5 mM MgCl\(_2\) (Perkin Elmer, Tucson, USA); and 0.3 U of AmpliTaq Gold (Perkin Elmer, Tucson, USA). We used the PTC-100 Programmable Thermal Controller thermo cycler (Memphis, USA) (Peltier-effect cycling, MJ Research, San Diego, USA) programmed for an initial denaturation for 7 minutes at 94°C, followed by 25 cycles for 30 seconds at 94°C, for 4 minutes at 65°C, for 10 minutes at 72°C and a final 4-minute cycle at 72°C. 10 μL of PCR products were mixed with 2 μL loading dye and, then, subjected to agarose gel electrophoresis on 2.5%. Electrophoresis occurred in a horizontal vessel, using TBE 1X as a loading buffer. After the electrophoresis loading, the gel was removed from the vessel and immersed in ethidium bromide solution for 5 minutes. After staining, the gel was placed under a transilluminator, which allows visualization of the fragments obtained by means of the PCR. The gels were photographed by the ImageMaster VDS system (Bufallo, USA)\(^9,10\). For deletion of exon 57 to be detected, the Multiplex Ligation-dependent Probe Amplification (MLPA)\(^9\) method was used.

Through amplification an electrophoretic loading was performed to check exons 4, 8, 12, 17, 19, 44, 45, 48 and 51 (Chamberlain et al.\(^9\)) and exons 1, 3, 6, 13, 43, 47, 50, 52 and 60 (Beggs et al.\(^11\)).

Patients were monitored in pediatric cardiology outpatient facilities, with heart tests repeated every six months, in addition to other outpatient facilities (neurology, physiotherapy and orthopedics).
Statistical analysis was performed to check the association between the presence of arrhythmias and exon deletion using the Fisher test.

Results

A group of 11 male patients, aged 6-14, was studied. In the first cardiac assessment, 6 patients had postural changes in gait and 5 were already wheelchair-bound. Their family history showed that 5 of them had relatives with the same disease (brother, uncle and cousins) and 6 were unaware of affected relatives. All reported having felt the onset of symptoms and muscle weakness between 4-6 years of age. Gowers sign has been present since they were 6 years old. The main neurological examination data were: reduced or absent deep tendon reflexes and difficulty walking, which usually began as an anserine gait, gradually evolving into difficulty climbing steps to the pronounced weakness with eventual confinement to a wheelchair.

The early cardiovascular clinical examination showed no significant signs. All patients showed to have normal blood pressure, pulse and palpation of the precordium. An innocent-type systolic murmur on the left sternal border was listened in two patients. In one patient, the systolic murmur was harsh, radiating to the right side, and the second heart sound was more intense with unchanged unfolding. No change in heart rhythm was clinically observed, in any of the cases.

In all patients, the enzymatic markers, especially creatine phosphokinase (CPK), were greatly increased, as were the CPK-MB fraction and lactic dehydrogenase (LDH).

Electrocardiogram showed signs of high R waves in V1 and narrow Q waves in left precordial leads in 7 patients (Chart 1; Figure 1); 2 patients showed to have right ventricular hypertrophy, and 1 patient showed to have diffuse disorder of ventricular repolarization. A child who manifested a paroxysmal tachycardia crisis had a short PR interval with delta wave.

Echocardiography revealed signs of decreased systolic function in 4 patients: cases 1, 5, 10 and 11 (Chart 1). These patients had an ejection fraction <46% and manifested mild dilation of the left ventricular cavity. One patient had small ventricular septal defect (case 2), and examination of 6 showed to be normal.

The dynamic electrocardiogram was considered normal in 6 children, aged 6-13. Three of which had bimorphic supraventricular and ventricular extrasystoles. Case # 4, an 8-year old child, showed a shorten PR interval, widened QRS and delta waves, whose baseline electrocardiogram already showed characteristics of Wolff-Parkinson-White syndrome. The ECG tracing of case # 5, a 14-year old child, showed major changes (Chart 1; Figure 2): tachycardia with bigeminy and trigeminy, and more than 190 ventricular extrasystoles.

In addition to receiving physical therapy, all patients were using corticosteroids as treatment. The four patients with ventricular dysfunction received enalapril, and the one whose electrocardiogram (Holter) showed arrhythmias also received carvedilol. The patient with Wolff-Parkinson-White syndrome was treated with propafenone.

In subsequent reassessments, the most serious case showed improvement both in LV systolic function and in arrhythmia record, with decreased supraventricular and ventricular extrasystoles for 24 hours after drugs were administered. Two patients showed improvement in systolic function after treatment with vasodilators. The other patients have not shown significant cardiac abnormalities to date.

Cases # 10 and 11 had a common history of an elder brother who had died two years earlier from heart failure, whose diagnosis was too late.

The molecular study showed deletion of the following exons: 48 (n=2), 51 (n=1), 57 (n=2), 52 (n=6); all the four cases, with impaired LV systolic function, had deletion of exon 52. Cases # 1 and 3 also showed deletion of exon 1, besides that of exon 52; in cases #10 and 11, the defect showed in exons 50-52. Therefore, there was deletion of exon 52 in 6 (54.5%) patients, and 2 of them also showed deletion of another exon (Chart 2). A Fisher’s exact test was conducted to evaluate a possible association between this deletion and arrhythmia revealed on the electrocardiogram, which was not confirmed (p=0.43).
<table>
<thead>
<tr>
<th>Case</th>
<th>Onset age (in years)</th>
<th>DMD family history</th>
<th>Physical examination</th>
<th>ECHO</th>
<th>ECG</th>
<th>Holter</th>
<th>Enzymes</th>
<th>Medication</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>1 cousin</td>
<td>Difficulty walking</td>
<td>Deficit of LV systolic and diastolic functions; (EF=42%)</td>
<td>R in V1. Negative T in anteroseptal derivations</td>
<td>Normal</td>
<td>CK total=15400; CKMB=805; LDH</td>
<td>Prednisone, Enalapril</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>NA</td>
<td>Digitigrade walking, lumbar lordosis</td>
<td>Perimembranous IVC</td>
<td>rsR' V1, peaked P waves, right ventricular overload (RVO)</td>
<td>Normal</td>
<td>CK=13205 CKMB=609</td>
<td>Prednisone</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>1 uncle and 3 cousins</td>
<td>Slightly anserine gait</td>
<td>Normal</td>
<td>Suggestive of septal hypertrophy</td>
<td>XT Rare SVXT, Idioatrial rhythm</td>
<td>CK total = 15811; CK = 415.1</td>
<td>Deflazacort</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>NA</td>
<td>Difficulty walking</td>
<td>Normal</td>
<td>Shorten PR interval; Delta wave</td>
<td>PR↓ widened QRS with delta wave</td>
<td>CK=4783; CKMB=148; LDH</td>
<td>Deflazacort, Propafenone</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>NA</td>
<td>Wheelchair user</td>
<td>Moderate LV contractile dysfunction (EF=44%).</td>
<td>Unspecific changes in ventricular repolarization</td>
<td>Tachycardia with bigeminy and trigeminy, 1903 XT vent</td>
<td>CK=3643; CKMB=119; LDH</td>
<td>Deflazacort, Carvedilol, Enalapril</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>Several cousins</td>
<td>DTR ↓↓, absence of Achilles' reflex, Anserine gait</td>
<td>Normal</td>
<td>Sinus tachycardia</td>
<td>Rs in V1, R in V2, Q in D1, V4, V5, V6</td>
<td>Normal</td>
<td>CK=22554</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>NA</td>
<td>Wheelchair user since the age of 10</td>
<td>Normal</td>
<td>rSR' in V1, RS in V2 and V3, RBBB</td>
<td>Supra XT, Rare, bimorphic vent XT</td>
<td>CPK=6482</td>
<td>Prednisone</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>NA</td>
<td>Hypertrophy of the calves, Gowers sign, wheelchair</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>CPK=18200</td>
<td>Prednisone</td>
</tr>
<tr>
<td>9</td>
<td>1</td>
<td>NA</td>
<td>Wheelchair user since the age of 9</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>CPK=22240</td>
<td>Homeopathic</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>Two brothers (1 dead)</td>
<td>Wheelchair user</td>
<td>Mild LV dysfunction and LV dilatation</td>
<td>rsR' V1, peaked P waves, right ventricular overload (RVO)</td>
<td>Isolated XT</td>
<td>CPK=13250</td>
<td>Prednisone, Enalapril</td>
</tr>
<tr>
<td>11</td>
<td>8</td>
<td>Two brothers (1 dead)</td>
<td>Wheelchair user since the age of 8</td>
<td>Mild LV dysfunction and LV dilatation</td>
<td>RBBB</td>
<td>Normal</td>
<td>CPK=3314, CKMB=177, LDH=915</td>
<td>Prednisone, Enalapril</td>
</tr>
</tbody>
</table>

RBBB – right bundle branch block; IVC – interventricular communication; CKMB – fraction of CPK enzyme derived from myocardium; CPK – creatinine phosphokinase; DMD – Duchenne muscular dystrophy; ECG – electrocardiogram; ECHO – echocardiography; LDH – lactate dehydrogenase; NA – not available; DTR – deep tendon reflexes; RV – right ventricle; LV – left ventricle; XT – extrasystoles; LV – left ventricle; EF – ejection fraction
Figure 1
Case 1 electrocardiogram showing high R waves in V1 and narrow q waves in V5 and V6.

Figure 2
Traçado Dynamic ECG (Holter) tracing, showing a ventricular tachycardia interval.
Discussion

Five patients had a family history of DMD affecting siblings, uncles or cousins. The other 6 who had no known affected relatives probably suffered new mutations, which may be present in 30.0% of the cases.

The electrocardiogram detected the DMD changes in 7 patients, mainly R waves in V1-V3 and Q waves in D1, aVL and V6. The proportion observed of electrocardiographic abnormalities (64.0%) is the same as that of a series of cases studied in a Brazilian university, and it approaches the rates described in the literature. These abnormalities represent loss of posteriorly-oriented muscle strength due to scar tissue of the posterior portion of the left ventricle, sometimes extending laterally, common in the dystrophic myocardium. One case had tachycardia crisis, and during the investigation, there were electrocardiographic signs of Wolff-Parkinson-White syndrome, even without changes on the echocardiogram.

Fayssoil et al. also described a case of Wolff-Parkinson-White syndrome associated with Duchenne muscular dystrophy in an adult patient. This association generally occurs when a cardiomyopathy already exists, and is more frequent in Becker disease. As mentioned above, electrocardiographic changes occurred earlier than the echocardiographic changes and may be the only cardiac manifestation.

Echocardiographic assessment revealed only 4 patients with impaired ventricular function. The asymptomatic cardiac involvement occurs around the age of 6 (25.0% of cases) with changes revealed on the ECG; the echocardiographic signs of cardiomyopathy may appear as early as the age of 10, and may progressively lead to heart failure. Cardiac involvement is present in two forms. In Becker muscular dystrophy, its progress is slow, beginning after the age of 5, while in Duchenne muscular dystrophy, heart failure progress is quick.

All patients had either difficulty walking or were already wheelchair-bound. The disease manifests itself more seriously in the legs, making it difficult to walk, run and climb stairs, and leading to frequent falls. The loss of walking ability occurred at about the age of 10 in the cases described by Herdy et al.

In the pre-clinical stage, the muscles affected already have abnormal histological findings, which have been confirmed in muscle tissues of fetuses with DMD. CPK levels may be increased right after birth, up to 10-100 times the normal level, although clinical symptoms appear later. These diseases cause alternating areas with myocyte hypertrophy, necrosis, myocardial fibrosis or myocardium replacement with connective tissue and fat. The posterior epicardium becomes thinner, causing dilation of the left ventricle (LV) cavity. Concurrently, there is a general muscle weakness.

The severity and the onset of the cardiomyopathy are not directly related to muscular deficiency. In Becker muscular dystrophy, due to partial loss of dystrophin, cardiomyopathy may be the initial manifestation in spite of a few changes in skeletal muscles.

Among the patients who reported a family history of deaths from DMD, the latter two patients (Chart 1) had an affected brother who died from cardiomyopathy and heart failure. Cardiac conduction disorders, ventricular arrhythmias and sudden death are frequent. Therefore, the cardiac abnormalities are a major cause of death.

The molecular study conducted in this series of cases (Chart 2) showed that all patients with cardiomyopathy...
had deletion of exon 52. However, Jefferies et al. showed that patients with Duchenne/Becker dystrophy, with deletion of exons 51 and 52, have a lower risk of developing cardiomyopathies. Nigro et al. showed association between cardiomyopathy and deletion of exons 48-49. Other authors reported that patients with deletions of exons 2-9 showed them at an earlier age.

The association between the type of deletion and the onset or the severity of the cardiomyopathy is, therefore, undefined, which would be useful for possible interventions or future therapies. In agreement with the literature, 9 (82.0%) out of the 11 described cases had deletions detected in the so-called hotspot region of the DMD, between exons 45 and 53, which are affected by most of the reported deletions.

For the treatment of cardiomyopathy, angiotensin converting enzyme (ACE) inhibitors are used, when there are signs of systolic LV dysfunction. It was observed that the association of ACE inhibitors with aldosterone inhibitors (spironolactone) attenuates fibrosis of myocardium and skeletal muscle. In an experimental study with rats, an early association of lisinopril and spironolactone was used and better performance was obtained with less muscle wasting in general.

Spurney et al. reviewed 174 cases of Duchenne muscular dystrophy, assessing cardiac function, of which 27.0% showed signs of cardiomyopathy. For some authors, starting ACE Inhibitors is valid even before cardiomyopathy signs appear, which is not recommended in the guidelines. Two patients studied here had improved cardiac function with use of enalapril, and in case of most severe arrhythmia, there was a decrease in supraventricular and ventricular extrasystoles with use of a combination of enalapril and carvedilol.

Corticosteroids also seem to have protective effect on the myocardium.

In a recent analysis, when reviewing recent randomized studies, Angelini mentioned that monotherapy using piridopril can slow cardiac dysfunction, while another study showed that esplerone association with cardioprotective drugs leads to decreased circumference of LV.

During the evolution, these patients are subjected to various multidisciplinary procedures (physiotherapy, neurology, orthopedics, cardiology, psychology) at a loss in formal learning. These deficiencies are described by some authors, but little is known about the etiopathogenic aspects of these manifestations. In some of the described patients, the cognitive assessments are still ongoing.

It is concluded that the electrocardiogram showed the first changes in most of the affected children. Some showed signs of arrhythmia and dilated cardiomyopathy on the echocardiogram, the exon 52 deletion of which was revealed on the molecular study. There was no statistically significant correlation between exon 52 deletion and arrhythmias.

Potential Conflicts of Interest
No relevant conflicts of interest.

Sources of Funding
This study was partially funded by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

Academic Association
This study is not associated to any graduate programs.

References


