The diagnostic value of vacuolar characterization in muscular biopsy in Pompe's Disease

O valor diagnóstico da caracterização vacuolar em biópsia muscular na Doença de Pompe

El valor diagnóstico de la caracterización vacuolar en biopsia muscular en la Enfermedad de Pompe

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Resumo
Introdução. Através da biópsia muscular podemos observar a formação de vacúolos que alteram a estrutura das células e tecidos na Doença de Pompe. A presença desses vacúolos varia à medida que a doença progride relacionando-se com o amplo espectro clínico apresentado pela doença. Objetivos. Após a confirmação da doença, examinar a possibilidade de diagnosticar ou excluir o diagnóstico da doença de Pompe através das características vacuolares apresentadas. Método. Análise do material da biópsia muscular de pacientes selecionados no ambulatório de Investigação de Doenças Neuromusculares da Universidade Federal de São Paulo. Através de técnicas de coloração e histoquímica foi realizado um estudo comparativo das características histológicas encontradas. Resultados. Trinta e três biópsias tiveram o diagnóstico de Doença de Pompe confirmado, sendo 13 mulheres e 20 homens. Deste grupo, 23 receberam o diagnóstico com 18 anos ou mais, e 10 receberam o diagnóstico com idade inferior a 18 anos. Membrana delimitante e localização subsarcolemal foi a principal característica vacuolar encontrada, manifestando-se em 86,6% dos casos estudados. Foi observado integração entre as membranas dos vacúolos em 62,5% dos casos. Encontramos também necrose, substituição de tecido muscular por conjuntivo ou adiposo, aumento de atividade mitocondrial e ausência de predominância em um tipo de fibra. Conclusão. A biópsia muscular permite analisar uma série de peculiaridades apresentadas pelos vacúolos na Doença de Pompe e assim, demonstra ser uma técnica certeira, permitindo chegar a uma rápida conclusão e identificar fatores determinantes para a condução clínica e da manutenção da qualidade de vida do paciente com Doença de Pompe.

Unitermos. Doença de Pompe; Doenças Neuromusculares; Biópsia; Histologia; Citopatologia

Abstract
Introduction. Through muscle biopsy we can observe the formation of vacuoles that alter the structure of cells and tissues in Pompe's disease. The presence of these vacuoles varies as the disease progresses, relating to the broad clinical spectrum presented by the disease. Objectives. After confirming the disease, examine the possibility of diagnosing or excluding the diagnosis of Pompe's disease through the vacuolar characteristics presented. Method. Analysis of the muscle biopsy material of selected patients at the Neuromuscular Diseases Investigation Clinic at the Federal University of São Paulo. Through staining and histochemical techniques, a comparative study of the histological characteristics found was performed. Results. Thirty-three biopsies had the diagnosis of Pompe's disease confirmed, being 13 women and 20 men. Of this group, 23 received the diagnosis when they were 18 years old or more, and 10 received the diagnosis under the age of 18 years. Delimiting membrane and subsarcolemal location were the main vacuolar characteristic found, manifesting in 86.6% of the studied cases. Integration between the vacuole membranes was observed in 62.5% of the
cases. We also found necrosis, replacement of muscle tissue by connective or adipose tissue, increased mitochondrial activity and absence of predominance in one type of fiber.

**Conclusion.** Muscle biopsy allows to analyze a series of peculiarities presented by vacuoles in Pompe's Disease and, thus, it proves to be a sure technique, allowing to reach a quick conclusion and to identify determining factors for the clinical conduct and maintenance of quality of life of the patient with Pompe’s disease.

**Keywords.** Pompe disease; Neuromuscular disease; Biopsy; Histology; Cytopathology

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**Resumen**

**Introducción.** Mediante biopsia muscular podemos observar la formación de vacuolas que alteran la estructura de células y tejidos en la Enfermedad de Pompe. La presencia de estas vacuolas varía a medida que avanza la enfermedad, en relación con el amplio espectro clínico que presenta la enfermedad. **Objetivos.** Una vez confirmada la enfermedad, examinar la posibilidad de diagnosticar o excluir el diagnóstico de la enfermedad de Pompe a través de las características vacuolares presentadas. **Método.** Análisis del material de biopsia muscular de pacientes seleccionados en la Clínica de Investigación de Enfermedades Neuromusculares de la Universidad Federal de São Paulo. Mediante técnicas de tinción e histoquímica se realizó un estudio comparativo de las características histológicas encontradas. **Resultados.** En 33 biopsias se confirmó el diagnóstico de enfermedad de Pompe, siendo 13 mujeres y 20 hombres. De este grupo, 23 recibieron el diagnóstico cuando tenían 18 años o más, y 10 recibieron el diagnóstico antes de los 18 años. La membrana límite y la localización subarcolémica fue la principal característica vacuolar encontrada, manifestándose en el 86,6% de los casos estudiados. La integración entre las membranas de las vacuolas se observó en el 62,5% de los casos. También encontramos necrosis, sustitución de tejido muscular por tejido conectivo o adiposo, aumento de la actividad mitocondrial y ausencia de predominio en un tipo de fibra. **Conclusión.** La biopsia muscular permite analizar una serie de peculiaridades que presentan las vacuolas en la Enfermedad de Pompe y, así, resulta ser una técnica segura, permitiendo llegar a una conclusión rápida e identificar factores determinantes para la conducta clínica y el mantenimiento de la calidad del paciente, de la vida con la enfermedad de Pompe.

**Palabras clave.** Enfermedad de Pompe; Enfermedades neuromusculares; Biopsia; Histología; Citopatología

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**INTRODUCTION**

Within the so-called group of inborn errors of metabolism, we can find the glycogen storage disease type II or Pompe’s disease\(^1\). This can be defined generically as a neuromuscular, genetic, metabolic, and rare disease, which leads to low or complete absence of the production of an enzyme. This enzyme deficiency causes the accumulation of glycogen inside the cells, especially in the muscles and liver,
configuring the symptoms muscle weakness, fatigue, and pain, and can manifest at any age\(^2\).

This accumulation is caused by a deficiency in the activity of the acid alpha-glucosidase lysosomal enzyme (GAA), which catalyzes the breakdown of glycogen into glucose; at low pH (4.0-5.0), through the hydrolysis of the portions to alpha-1.4 and 1.6 of the glycogen\(^3,4\). This storage leads to loss of function and lysosomal damage and cell tecidual\(^5\).

Like other rare diseases, Pompe's disease is not identified in the first instance. Patients with a slower evolution are more difficult to diagnose making the current process of disease confirmation very tiring and time-consuming\(^6\).

Pompe's disease has a large clinical spectrum as cells and tissues are compromised, so symptoms vary according to the lesions in the smooth, skeletal, and cardiac muscles mass cells, commonly leading to clinical manifestations in other systems\(^7\). Although useful for diagnosis, laboratory tests such as creatine kinase (CK) measurement, chest X-rays, echocardiogram, electrocardiogram (ECG) and electroneuromyography (ENMG) do not show specific characteristics that allow accurate diagnosis\(^8\).

On the other hand, the quantification of the activity of the GAA enzyme by techniques such as dried blood stain has been gaining ground due to its precision and speed, in addition to being a non-invasive and safe method for the patient\(^9\). However, tests like this are only applied in cases of
strong suspicion, and often with a long clinical history, and its execution is limited to some laboratories¹⁰.

Considered as a complementary test or an alternative for enzyme dosage tests and molecular tests, a muscle or skin biopsy is an important option, as it is possible to identify a disease, in some cases, through specific histopathological findings¹¹.

A histopathological finding found are the vacuoles. They are cellular structures, contained in the cytoplasm of the cell, with spherical or oval shape. They are structures that correspond to expansions of the endoplasmic reticulum or the Golgi complex, storing a content wrapped by a closed membrane that isolates something from the rest of the cytoplasm¹².

This abnormal formation of spaces or cavities that alter the structure of the organization of muscle fibers can be morphologically classified according to their size (large or small), quantity (single or multiple), position (peripheral or central), shape (free or marginal), presence of membranes or not. Such structures can be observed by at least one staining method, and it is also possible to observe whether there is a deposit of material inside these vacuoles by histochemical methods, an important characteristic for determining the cellular structure involved¹³.

In glycogenoses, empty vacuoles can be found, but when stained using the Schiff’s periodic acid technique, they present positive material. However, it should be emphasized that the vacuole is not present in the muscle biopsy of all
patients with Pompe’s disease, as well as vacuoles with positive PAS material that are not exclusive to this disease⁸.

Through the analysis of the anatomopathological findings and the characteristics of the vacuoles, we want to assess the possibility of diagnosing Pompe’s disease. This method would imply an advance in the disease diagnosis process, making it faster, less tiring, and time-consuming, it would help to distinguish the disease from others with similar clinical aspects and could affect the treatment and the evolution of the disease.

**METHOD**

This study was approved by the Research Ethics Committee of the Federal University of São Paulo (CEP/UNIFESP protocol nº 1420/2017) and by the Teaching and Research Coordination of Hospital São Paulo-University Hospital of the Federal University of São Paulo (CoEPHSP protocol nº513/2017).

We considered 2167 biopsies from the Neuromuscular Diseases Research Sector of the Department of Neurology at the Universidade Federal de São Paulo (UNIFESP), to identify patients with biopsies with characteristics of vacuolar myopathy, between the years 1984 and 2015.

**Procedure**

Dividing them into metabolic and non-metabolic diseases, 1048 were considered as metabolic diseases and 1119 as non-metabolic diseases. Within the group of
metabolic diseases, we found 621 cases of Mitochondrial disease, 193 cases of Lipidosis, 89 cases of Glycogenosis, 1 case of Mucopolysaccharidosis and 6 cases of Amyloidosis.

Cases referred to Non-Metabolic Diseases, we had 352 cases of Polymyositis/Dermatomyositis, 400 cases of Limb-Girdle Muscular Dystrophy, 206 cases of Inflammatory Myopathy, 35 cases of Inclusion Bodies Myositis, 20 cases of Oculopharyngeal Myopathy, 10 cases of Periodic Paralysis and 6 Distal Myopathy.

Within the 89 cases of Glycogenosis’ group, 29 were diagnosed as type V glycogenosis or McArdle's disease, 1 as type I glycogenosis or von Gierke's disease and 59 were further investigated to define the glycogenosis subtype. Of these cases, 33 were confirmed with DNA examination as Pompe's Disease. According to the inclusion and exclusion criteria established for this study, 228 biopsies were excluded, 138 in the Metabolic Diseases group and 90 cases in the Non-Metabolic Diseases group (Figure 1).

The fragments were collected on biopsy, mostly from the deltoid muscle (Deltoideus), after skin antisepsis with Riohex® 2% degermante (2% chlorhexidine diglytonate), followed by Riohex® 0.2% aqueous antiseptic solution. Patients received local anesthesia with 0.2% xylocaine, without vasoconstrictor, only on the skin and subcutaneous tissue without infiltrating the muscle.
Three to four fragments of the muscle, measuring approximately 8 mm, were removed, placed on sterile gauze, and immediately forwarded to the laboratory. The time between biopsy and freezing on a cork support with adraganth gum, covered with powdered talc did not exceed 10 minutes. The blocks were submerged in liquid nitrogen (−180°C) for approximately 25 seconds. They were properly identified and stored in a −80°C freezer until cut. The cuts
were made in Leica CM1850 cryostat at -25°C, with a thickness of 6 microns, being collected in coverslips of 22 x 22mm.

The slides were stained with Hematoxylin-Eosin (HE), Gomori's Trichrome (GO) and Oil Red-O (ORO) and with the histochemical techniques Shiff's Periodic Acid (PAS), myofibrillar adenosine triphosphatase after pre-incubation in alkaline (pH9.4) and acidic (pH 4.65), Nicotinamide Adenine Dinucleotide Tetrazolium Reductase (NADH), Succinate dehydrogenase (SDH) and acid phosphatase (FAc), to demonstrate and evaluate specific anatomopathological aspects such as fiber sizes; distribution of muscle fibers and their types; disposition pattern; presence of vacuoles; presence of mitochondria; lipid and glycogen accumulation.

The acid phosphatase histochemical technique has no confirmatory diagnostic value for glycogenoses, as it only indicates the origin of the vacuoles and is not determinant for GAA deficiency, must only be considered capable of indicating a possible lysosomal deposit pathology.

The analysis was performed by light microscopy, on the images of the biopsy slides of the patients selected at the outpatient clinic of this sector. The images were photographed using the Nikon® Eclipse 50i microscope and Nikon® DS-Fi1 camera and NIS - Elements F3.0 software and analyzed with ImageJ® software (2014).

The descriptive analysis of the data was made on the number of diseases with vacuolar changes; predominance of Pompe's disease among cases of vacuolar myopathy; and
morphological differences, such as size, predominance, and distribution of vacuoles.

RESULTS

From the biopsies diagnosed with Pompe's Disease, it was concluded that 23 received the diagnosis aged 18 years or over, with a mean age of 43.1 years and 10 received the diagnosis under 18 years, with average age equal to 8.2 years. Thirteen cases were female and 20 cases were male.

Observing only the histological finding vacuole, we can see that the most common feature is the presence of delimiting membrane. This characteristic is found in most vacuoles, being absent only in myopathies due to lipid deposits, Periodic Paralysis and in some myopathies due to intoxication.

Its location proved to be an important factor in determining the disease. In glycogenoses and Mitochondrial Myopathies, we can observe that the vacuoles are located peripherally in the subsarcolemal region. In the other myopathies, it was possible to observe them more centralized in the muscle fiber.

Both characteristics, delimiting membrane and subsarcolemal location, could be observed in 86.6% of the studied cases. The type of vacuole most commonly observed is the rimmed vacuole type, which is an important characteristic as a classificatory factor. An important characteristic for the definition of the disease would be the
quantity of vacuoles, as well as its size, however these characteristics proved to be difficult to define because they vary according to the degree of patient affection and his age.

In glycogenesis, regardless of the form of the disease, as it progresses, there is an increase in the accumulation of glycogen within the lysosomes, which expand and may even merge with each other, giving rise to vacuoles whose length can occupy more than half the size of the fiber muscle, generating change in the internal structure, which reflects clinically with decreased muscle strength, decreased mobility and, consequently, progressive loss of muscle function. In our population, we found 62.5% of cases with fuse between the vacuole membranes, of which 81.8% were diagnosed with the late form.

We found necrosis in 25% of cases. Another memorable finding, unlike inflammatory diseases, is the infrequent encounter of an inflammatory process in glycogenoses, however with the replacement of muscle tissue by connective or adipose tissue in 81.25% of the analyzed cases.

Although studies suggest that there is no predilection for the formation of vacuoles in a specific type of muscle fiber, defining whether there is a predominance of one type is important for enzyme replacement therapy because recent studies indicate differences in the numbers of mannose-6-phosphate receptors between the types of fibers. Research has also shown that type II fibers are more resistant to TRE$^{14}$. 
Usually, there are no signs of muscle fiber atrophy, change on the fiber diameter or predilection for some type of muscle fiber in glycogenoses. In cases with Pompe's Disease, we found two cases with type II atrophy and one case with predominance of type II fibers.

In most cases, there is no mitochondrial proliferation with RRF-type cells, as occurs in Mitochondrial Myopathies, but there may be an increase in mitochondrial activity showing some type of metabolic compensation. This increase was observed in 37.5% of the cases of glycogenoses, and in all cases the patients were older than 18 years.

As it is a sarcoplasmic enzyme, we can observe changes in the internal architecture in the fiber, which is more evident with the NADH enzymatic reaction, where it is possible to visualize the sarcoplasmic grid in dark blue in a disorganized way and with flaws, indicating a pattern of linearization of the sarcoplasmic membranes (Figure 2).

The linearization pattern is not related to the type of fiber. We can see that this finding affects some fibers, making it possible to make a comparison with standard fibers in the same biopsy. This disorganization of the sarcoplasmic grid was observed in 83.3% of biopsies diagnosed with Pompe's disease. A case of lipid deposit myopathy has a similar pattern.
The linearization process of the sarcoplasmic grid can generate small rips or cracks in the muscle fiber, in different directions and sizes, with no specific predominance to the type of muscle fiber and unrelated to the position of the vacuoles.

However, it is possible to notice that such fissures are more prevalent in muscle fibers with apparent vacuoles. Here it is worth mentioning that as the cuts are transversal, it is not possible to observe the entire length of the muscle fiber, so a fiber that does not have vacuoles is not necessarily exempt from it. Fact that explains the presence of cracks in fibers with no vacuoles (Figure 3).
As already noted, the number of vacuoles and their size are important characteristics, but difficult to define because of the individual factors of each case. However, we can notice a difference in the number of vacuoles between the two types of glycogenoses. In type V glycogenoses, or McArdle's disease, we see that there are some vacuoles positioned more centrally in muscle fibers compared to Pompe's disease.

Another important point to note is the increase in mitochondrial activity in McArdle's disease and the increase in endomysial fibroblasts, signs that are reduced in Pompe's disease. In both Mitochondrial Myopathies and McArdle's Disease, the oxidative phosphorylation is compromised through different mechanisms.
In our study, we observed only one case of McArdle's disease that showed no change in mitochondrial activity in both GO staining and SDH histochemical technique. In the cases of Pompe's disease, three cases had changes in mitochondrial activity, all of which were of the adult form of the disease.

The mitochondrial involvement in McArdle's disease is distinct. However, the appearance of mitochondrial activity in Pompe’s disease is vague. According to the relevant literature, this finding can be concluded as caused by alteration in the metabolic cycles and in the fiber's internal architecture\textsuperscript{15,16} (Figure 4).

**DISCUSSION**

Through careful observation of the muscle tissue, we can obtain information about the morphological, biochemical, and metabolic properties of the fibers, so that we can detect the absence of enzymes, the excess of a certain substrate, and even structural changes in the muscle fiber. Such conclusions are only possible through the muscle biopsy technique with histochemical study.

Even with the absence of these techniques, we analyzed several muscle biopsies and were able to observe that certain vacuolar characteristics, which alongside other findings, strongly favor the diagnosis of this disease.
Figure 4. Muscle biopsy stained with GO and SDH indicating mitochondrial activity.

The vacuolar characteristics in Pompe's disease are striking. They are subsarcolemal, multiple, tend to fuse, have a limiting membrane, are empty, and with specific techniques we can evidence the presence of lipofuscin and the inside of the vacuoles appear stained with PAS.
These characteristics were also noted by Raben (2007)\textsuperscript{17} in a study of the autophagic pathway in nine late-onset patients.

These vacuolar characteristics, when associated with little or no necrosis, proliferation of connective tissue, linearization pattern and low mitochondrial activity, proved to be strong evidence for the diagnosis of Pompe's disease.

These histopathological findings in our population are consistent with the studies conducted to date as found by Werneck (2013)\textsuperscript{18}, confirming the physiological process of the disease. Although they are not exclusive to Pompe's disease, we can observe that vacuolar characteristics in other vacuolar diseases are better characterized by the literature, helping in the differential diagnosis (Table 1).

The presence of a delimiting membrane and the location of the vacuole are characteristics that are easy to observe and that are important for diagnostic differentiation. Observation of the internal content of the vacuole helps to determine its type and eliminate different vacuolar diseases from the possible diagnosis.

Therefore, when we talk about the diagnostic value of the muscle biopsy technique for glycogenoses, we must remember its importance as the only test capable of identifying a specific type. However, if we talk about Pompe's Disease, the imprecision of the physical characteristics and the innumerable variability that make up each case, testify against the value of the technique when compared with the advances in science in the field of genetic diagnosis.
Table 1. Correlation of vacuole characteristics.

<table>
<thead>
<tr>
<th>VACUOLE</th>
<th>POMPE</th>
<th>NON POMPE</th>
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<tbody>
<tr>
<td><strong>LOCALIZATION</strong></td>
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<td></td>
</tr>
<tr>
<td>SUBSARCOLEMAL</td>
<td>X</td>
<td></td>
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<tr>
<td>CENTRAL</td>
<td></td>
<td>X</td>
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<tr>
<td>ISOLATED</td>
<td></td>
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<tr>
<td>CONFLUENTS</td>
<td></td>
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<tr>
<td>FUSION</td>
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<td><strong>NUMBER</strong></td>
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<td><strong>SIZE</strong></td>
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<td>±67%</td>
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<tr>
<td><strong>ATROPHY</strong></td>
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<td>X</td>
</tr>
<tr>
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<td>DELIMITATING MEMBRANE</td>
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<td>X</td>
</tr>
<tr>
<td>EMPYT</td>
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<td></td>
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<tr>
<td><strong>PRESENCE OF MATERIAL</strong></td>
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<tr>
<td>LIPOFUSCINA</td>
<td>X</td>
<td>X</td>
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<tr>
<td>RIMMED</td>
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<td>X</td>
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<tr>
<td>TUBULAR AGGREGATE</td>
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<td><strong>NECROSIS</strong></td>
<td>X</td>
<td>X</td>
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<tr>
<td><strong>REGENERATION</strong></td>
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<td><strong>INFLAMMATION</strong></td>
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<td><strong>CONNECTIVE TISSUE</strong></td>
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<tr>
<td>ENDOMYSIAL</td>
<td>X</td>
<td>X</td>
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<td>PERIMYSIAL</td>
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<td><strong>FAT INVOLUTION</strong></td>
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<td>SDH</td>
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<tr>
<td>RAGGED RED FIBERS</td>
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<td>MITOCHONDRIAL PROLIFERATION</td>
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<td>TYPE II FIBERS</td>
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**CONCLUSION**

Based on the work of the French health professional Henry E. Sigerist, who defended the imprecise individuality of the boundary between normal and pathological throughout the individual's lifetime, indexes and patterns
can be considered numerical barriers not necessarily applicable to the variables of that individual’s organism.

Muscle biopsy proved to be a powerful tool as it is a technique capable of showing the specific moment of the disease stage in that patient, without the distinct numerical barriers.

Although genetic and enzymatic tests can indicate whether there is progression or regression of the patient's condition within criteria and indices generally established as normal, muscle biopsy can accurately indicate the organism's condition, its reaction to a drug or therapy and support the clinical approach to be followed.

Although theoretically viable, the absence of specific histochemical techniques did not prove to be an impediment criterion for diagnosis. The distinct characteristics identified in the vacuoles of Pompe’s disease, and other peculiarities presented by the biopsy, can differentiate the disease from others with a similar clinical appearance.

The analysis of certain characteristics, apparently as simple as vacuoles, provide important information for diagnosis and may open the way for a deeper analysis of the distinct properties of each case, which may specifically and innovatively influence its treatment.

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