INTEGRATIVE ALGORITHMS IN THE DIAGNOSTICS OF LYSOSONAL STORAGE DISEASES

INTEGRATIVNI ALGORITMI U DIJAGNOSTICI POREMEĆAJA U SKLADIŠTENJU LIZOZOMA

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Summary
Lysosomal storage disorders (LSDs) are a clinically heterogeneous group of more than 50 disorders which still represent a challenge in modern medicine. The efficacy of many current and proposed therapies relies heavily upon early detection and treatment prior to the onset of irreversible pathology. Although there are multiple paths and algorithms to a final diagnosis, the diagnostic strategy for LSDs still mostly relies on initial clinical suspicion followed by adequate specialist and laboratory management – a selective screening approach. Despite the fact that the technology of tandem mass spectrometry enables newborn screening, such screening is generally acceptable only for a population at high risk for a certain LSD. As diagnostic testing for these disorders may be difficult, communication between the clinician who has established clinical suspicion and laboratory personnel will help complete this process.

Keywords: lysosomal storage disorders, diagnostic strategy, a selective screening approach

Introduction
Lysosomal storage disorders (LSDs) are a clinically heterogeneous group of more than 50 inborn errors of metabolism. Although the first clinical descriptions of patients with some LSDs were reported at the end of the nineteenth century, the biochemical nature of the diseases was elucidated some 50 years later. Finally, in 1963 Hers recognized the link between lysosomal enzyme deficiency and storage in Pompe patients. Since then, new advances in the pathophysiology of these complex disorders have been continuously made (1).

LSDs are caused by genetic defects causing a lack or severe deficiency in the activity of the following: acid lysosomal hydrolases involved in degradation of various macromolecules, proteins involved in lysosomal transportation, proteins required to deliver enzymes into lysosomes, or activators of lysosomal enzymes. The progressive lysosomal accumulation of undegraded or partially degraded metabolites acti-
mates a variety of pathogenic mechanisms that result in dysfunction of cells, tissues and organs. Storage may begin during early embryonic development and the clinical presentation for LSDs can vary from an early and severe phenotype to late-onset mild disease.

To date, the mammalian lysosome has been shown to contain about 60 soluble luminal proteins and about 25 transmembrane proteins. Recent proteomic studies suggest that there may be even many more proteins within this organelle. Discovery of such proteins has important implications for understanding the function of the lysosome but can also lead the way toward discovery of the genetic basis of human diseases with hitherto unknown etiology (2). Most LSDs are inherited in an autosomal recessive manner, except Fabry disease and Hunter syndrome which are X-linked recessive disorders, and Danon disease which is an X-linked dominant disorder.

Individually, LSDs are very rare, but this group as a whole has an estimated prevalence of around 1:5000–1:8000 live births (3). There have been a number of reports on the prevalence of particular LSDs in selected populations; thus, Gaucher disease and Tay-Sachs disease occur in the Ashkenazi Jewish population in the ratio of 1:855 births and 1:3900 births, respectively (4).

LSDs can be classified on the basis of the nature of accumulated substrates (e.g., mucopolysaccharidoses, lipid storage disorders, oligosaccharidoses) or, more recently, by understanding the molecular defect. According to the latter, the following disorders may be distinguished: non-enzymatic lysosomal protein defects; transmembrane protein defects; lysosomal enzyme protein defects; posttranslational processing defects of lysosomal enzymes; trafficking defects in lysosomal enzymes; polypeptide degradation defects; neuronal ceroid lipofuscinoses (5).

Despite some similarities in the clinical phenotype of different LSDs, there are no two disorders with identical pathophysiology. In some patients, the presentation may be in utero or during the neonatal period, whereas in others disease onset may occur in late adulthood. Although the disorders are generalized, one organ or body system may be affected more than others. The most frequent symptoms are the following: dysmorphic features; bony abnormalities (dysostosis multiplex); organomegaly; central nervous system involvement; neurological symptoms (developmental delay, hypotonia, ataxia); ophthalmologic signs (corneal clouding or macular cherry-red spot); heart and cutaneous abnormalities; renal diseases. The common characteristic of all these symptoms is that they are progressive and the clinical course of disease should be understood as a dynamic process (1).

Although a significant amount of information has been published on the molecular genetics and biochemistry of LSDs, the link between storage material and disease pathogenesis has not yet been fully understood. The lysosome is no longer viewed as just an end-point degenerative compartment. We are aware that lysosomes are integrally involved in phagocytosis, autophagy, exocytosis and receptor recycling. The knowledge about the entire endosome-lysosome system will be critical in understanding the complex pathogenesis of LSDs (6). Each disorder, however, has a wide spectrum of clinical presentations depending on the effect of mutations on residual enzyme activity. Other factors, including genetic background and environmental factors, presumably play a role in disease progression.

**Results of a diagnostic algorithm for LSDs – benefits for patients**

LSDs were for a long number of years of marginal interest to clinicians and laboratory diagnostics, as these disorders were considered mostly incurable and were associated with lethal outcome. The diagnoses made were used chiefly for genetic counselling, while the therapy was symptomatic. Over the past two decades, considerable progress has been made in the treatment of LSDs and with regard to the outcome of patients.

Enzyme replacement therapy (ERT) for type 1 Gaucher disease opened an era of replacing the deficient enzyme in the lysosome with a recombinant enzyme. The results were encouraging and have transformed the lives of patients (7). Currently, approved ERT is available for Fabry disease, Pompe disease, some mucopolysaccharidoses (MPS) (MPS type I, MPS type II, and MPS type VI). Clinical trials of ERT are underway for a number of LSDs (MPS type IV A, Niemann-Pick disease type B, metachromatic leukodystrophy). However, there are some clinical limitations of ERT: it is a time consuming procedure typically given to patients every two weeks by intravenous infusions; insufficient biodistribution; does not cross the blood–brain barrier (BBB) to any significant extent so it cannot be used to treat the central nervous system. To overcome this challenge, some efforts have been made with modified recombinant enzymes able to cross the BBB with intrathecal injections or with high-dose ERT (8). Another promising therapy which has been approved for some LSDs (Gaucher disease type 1, Niemann-Pick type C) is substrate reduction therapy (SRT) with orally available small molecules of imino sugar which can cross the BBB. However, this compound has some side effects (gastrointestinal symptoms, polyneuropathy) (9).

Ever better understanding of the pathophysiology of LSDs has broadened the approach to therapy with the endosomal-autophagic-lysosomal system to
a pathogenic cascade that impacts multiple cellular systems and organelles. Progression in knowledge has resulted in the fact that there are currently several other approved treatments and experimental strategies to restore catabolic activity: bone marrow transplantation, enzyme enhanced therapy, gene therapy, stop codon read-through therapy, calcium modulation therapy, molecular chaperone reactivating defective enzyme and others (10). The therapy approach has so far been based either on reducing the amount of produced substrate that cannot be adequately degraded in the lysosome, or on intense substrate degradation. More recently, a master regulator of lysosomal biogenesis and function transcription factor (TFEB) has been proposed as a therapeutic approach (enhanced exocytosis therapy). TFEB promotes the degradation of lysosomal substrates, controls the number of lysosomes and regulates expression of lysosomal genes. Promising results of testing of the above-mentioned therapeutic strategies in tissue culture models and/or in animal models of LSDs offer hope that physicians and patients will, in the near future, have the possibility to choose the most optimal therapy for each patient (11). However, one should be aware that the efficacy of many current and proposed therapies relies heavily on early detection and treatment prior to the onset of irreversible pathology.

From clinical suspicion to final diagnosis – selective screening

In general terms, there is no simple diagnostic screening test which can detect all LSDs. As these diseases may present with a broad range of phenotypes, with a variable age of onset, symptom severity and degree of central nervous system involvement, a generally accepted strategy of laboratory diagnostics is implemented in the form of the so-called selective screening. Such a diagnostic approach is based primarily on establishing clinical suspicion, appropriate specialist management and final confirmation of the diagnosis by laboratory tests. In addition to the above-mentioned most common symptoms, physicians are often, when establishing a clinical suspicion, faced with the problem of patients presenting with isolated symptoms that occur more frequently in other non-lysosomal disorders (e.g., neurological symptoms, behavioural disturbance). Besides, the attitude that involves consideration of LSDs only after all other possible diseases have been excluded is still present to a significant extent.

Establishment of clinical suspicion is in the diagnostic algorithm followed by appropriate specialist management (neurological, radiological, ophthalmological, etc.) and laboratory diagnostics in specialist metabolic laboratories for selective screening. Organization and test range in metabolic laboratories differ in individual countries. Regardless of that, however, it is – before considering specific tests – appropriate to consider an unspecific test that may also aid in the diagnostic process. For example: continuously elevated activity of creatine kinase (Pompe disease?); unexplained proteinuria (Fabry disease?); vacuolated lymphocytes (different LSDs); specific cells in bone marrow aspirates (Gaucher cells, Niemann-Pick foam cells). All relevant clinical, morphological and biochemical patient data should be forwarded (in an appropriate form) together with the biological material to a metabolic laboratory for selective screening and aid in selecting adequate tests and interpretation of obtained results.

If clinical suspicion is undetermined, the diagnostic process usually proceeds with a preliminary screening test. In most cases, this includes glycosaminoglycans (GAGs) and oligosaccharide excretion pattern in urine. Adequate sample quality is essential for such urinary screening. For oligosaccharide analysis, the necessary sample is first morning urine, while a 24 h urine sample is preferred for GAGs analysis. If MPS diagnosis has been made after established clinical suspicion and initial laboratory management, running a qualitative analysis of GAGs simultaneously with quantitative analysis is strongly recommended to reduce false negative GAG results.

The urinary excretion of GAGs is high in infants and young children and decreases with age, when normalized to creatinine (12). Thus, in the case of some MPS, the excretion of total GAGs can be borderline or even below the reference range for age. Such an approach is especially important if MPS IVA is considered a possibility, particularly for patients with a mild form of disease who do not present with symptoms until adolescence. Some spot tests and turbidity tests are no longer recommended because of very poor specificity and sensitivity. Currently, quantitative analysis of GAGs in most laboratories is usually performed by spectrophotometric analysis with dimethylmethene blue (13). For qualitative GAG analysis, laboratory can use either multiple-step thin layer chromatography (TLC) or multiple-step electrophoresis and TLC for oligosaccharide separation. Despite the available methods, interpretation of results is still subjective and can be challenging. Therefore, participation of the laboratory in external proficiency testing is strongly recommended, e.g., the EQA scheme provided by the European Research Network for Evaluation and Improvement of Screening, Diagnosis and Treatment of Inherited Disorders of Metabolism (ERNDIM). Recently, liquid chromatography – tandem mass spectrometry (LC-MS-MS) has also become available for detection of urinary GAGs and oligosaccharide analysis (14). The results obtained by such screening may narrow diagnostic suspicion to only a few possible LSDs (for instance, a report of isolated heparan sulfate fraction indicates MPSs type III) or exclude a considerable number of LSDs. Besides GAGs and oligosaccharides, there are also more specific preliminary tests in urine such as the quantitative assess-
ment of urinary sulfatide which can be useful in case of discrimination between metachromatic leukodystrophy and the pseudodeficiency or increased urinary excretion of free sialic acid in sialic acid storage diseases.

Plasma chitotriosidase (an enzymatic marker of macrophage activation) measurement could be helpful as initial screening primarily when Gaucher or Niemann-Pick disease is suspected. When interpreting the obtained concentrations, we should take into account that a patient might have a 24-base pair duplication in the chitotriosidase gene which prevents the formation of chitotriosidase protein (possibility of a false negative result), and that moderately elevated chitotriosidase activity may be expected also in other LSDs (GM1-gangliosidosis, Krabbe disease, alpha-mannosidosis, Wolman diseases, etc.), as well as in non-lysosomal diseases (sarcoidosis, glycogenosis type IV, beta thalassemia, etc).

The following step in the diagnostic algorithm of LSDs should include lysosomal enzyme activity analysis. Lysosomal enzymes are present in almost all tissues and biological samples. However, serum is useful chiefly in the case of suspicion of mucolipidoses. Leukocytes isolated from whole blood are the most useful material for most lysosomal enzymes. Efforts should be made, however, to perform leukocyte isolation 24 h post-draw at the latest. Lymphocytes isolated from whole blood are necessary to establish the diagnosis of Pompe disease because of possible interference between isoenzymes in leukocytes. For adequate lymphocyte isolation, blood should be in the laboratory 4 h post-draw at the latest.

Sample deterioration may also become an issue when the shipping of whole blood to laboratory takes too long and/or is done at inappropriate temperature. Besides, only after the procedure of leukocyte/lymphocyte isolation is it possible to establish if sample quantity was sufficient for enzyme activity determination. Fibroblast samples are recommended for enzyme activity analysis of all LSDs, or even necessary in some cases (e.g., sialidosis, mucolipidosis types II and III). However, skin punch biopsies are invasive and approximately 3–6 weeks are required prior to the analysis of culture cells. In the last 5 years, dried blood spot (DBS) samples have become very popular and practical for measurement of lysosomal enzyme activities. Such sampling has a lot of advantages: easy to collect and store, only small blood volumes are required (i.e., 60 µL), samples can be shipped via regular mail at room temperature. Besides these advantages, there are some issues for DBS assays (influence of hematocrit, hemoglobin, leucocytosis; absence of quality control schemes; laboratories have different cut-off values and use different units). A prerequisite for adequate interpretation of measured values is adherence to the general guidelines for DBS sample preparation, handling and storage (15). DBS methods are still considered as screening methods. Confirming positive DBS results by enzyme activity analysis from second samples (e.g., leukocytes, fibroblasts, DNA) is strongly recommended.

Lysosomal enzyme assays of the above-mentioned samples are usually performed using synthetic (fluorimetric or colorimetric) substrates. The radiolabelled substrates are used for a very limited number of proteins (e.g., sphingolipid activator proteins). In addition to these currently used methods, MS-MS based methods have been developed and successfully introduced into routine metabolic laboratory. The advantage of this method is its ability to quantitate multiple reaction products from a single incubation and injection from a single 3.2-mm DBS (16).

Regardless of the methods used, an additional lysosomal enzyme (control enzyme) should be measured for each enzyme assay to control sample integrity. Further, enzyme activity in the late-onset form of LSDs can be near normal in any enzyme assay. It should be kept in mind that there are also individuals who show greatly reduced enzyme activity but remain clinically healthy (=pseudodeficiency). Conversely, there are circumstances in which affected individuals with clinical pictures containing some glycosphingolipidoses show normal activity of the relevant lysosomal enzyme. These patients should be investigated for a potential defect of an activator protein or saposins (e.g., a clinically suspected metachromatic leukodystrophy patient with normal arylsulphatase A activity and abnormal patterns of urinary sulphatides should be referred for a molecular genetic analysis of the prosaposin gene). Molecular analysis can confirm the enzymatic diagnosis of an LSD and clarify the type of genetic variation. In cases with known genotype-phenotype correlation, this information is crucial for anticipating the course of disease and for evaluating treatment options such as pharmacological chaperones or substrate reduction therapy. Besides conventional molecular analysis techniques, in some cases, additional molecular studies are needed to detect deletions/insertions, gross rearrangements and potential transcription defects (e.g., patients affected by X-linked Fabry disease in which no mutations have been identified by a traditional molecular genetic technique). When a novel mutation is identified, further investigation may be needed to determine whether or not it is pathogenic in nature. Moreover, genotyping individual LSD patients is important for genetic counselling and also allows identification of carriers in a family. In general, the interpretation of molecular results should be careful in order to avoid misinterpreting a disease-causing mutation as a polymorphism, and vice versa.

If a clinician strongly suspects the presence of LSD but initial biochemical test results are normal, biopsies from extraneural tissues (skin or conjunctival) should be performed. Ultrastructural examination
with electron microscopy may be helpful to confirm the presence of lysosomal distension. If further investigation is necessary and disorders are not readily detected by blood or urine testing, then e.g. activator protein or saposin deficiency should be considered. Skin fibroblast culture is mandatory for diagnosis in this situation. More specialized tests on fibroblasts will be needed for transport and activator protein defects.

Due to all of the above, and depending on individual lysosomal disease and patient’s clinical data, diagnosis is usually made from the age of several weeks to several months after the established clinical suspicion. All past experience indicates that the basic prerequisite for speedy establishment of diagnosis of these disorders requires close collaboration between laboratory specialists and clinicians.

**Diagnostic strategy for LSDs – Imperative for early detection**

Due to increasing possibilities of treatment, an increasing number of reports have in the last years addressed the need for making diagnosis as early as possible (before irreversible organ and tissue damages occur) and for introducing treatable lysosomal diseases in *newborn screening programs* (17). This need has been supported by the information that lysosomal enzymes retain their activity in dried blood spots on filter paper, by development of high throughput fluorimetric methods, by increasingly available tandem mass spectrometry (MS-MS) technology, and particularly by development of the MS-MS multiplex assay. The availability of the multiplex technology has facilitated the technical aspect of testing, making it easier to identify LSDs. Pilot studies of newborn screening for specific LSDs (Fabry, Pompe, Gaucher and MPS-I) carried out so far in some countries have indicated a much greater prevalence than the prevalence estimated by clinical diagnosis (18, 19). This increase in prevalence is primarily due to recognition of later-onset forms of LSDs. Early detection of these disorders has other potential advantages. It will enable genetic counselling of parents and help avoid the prolonged and stressful process of diagnosis. Newborn screening holds the promise of early disease detection. However, presymptomatic diagnosis raises a number of ethical issues regarding the consequence of private mutations, parent–newborn relationship and patient management and treatment. Prior to introduction of such an approach to LSD diagnostics, the balance should be considered between potential harm and benefit for the patient and the family.

Due to all of the above, *high risk patient population screening* currently appears to be warranted (e.g., screening of dialysis patients and those with left ventricle hypertrophy for Fabry disease, screening of myopathic patients for Pompe disease).

**The role of biomarkers in the algorithm for LSD diagnosis and follow-up**

Biomarkers are presently routinely used in all areas of modern medicine. Introduction of ERT in treating patients with type 1 Gaucher disease has imposed the need for routine monitoring of treatment course. In addition to clinical, radiological and hematological management, determination of serum activity of the enzyme chitotriosidase – as the first biomarker in this group of disorders – began in 1994. Since then, we are actually still searching for »ideal« biomarkers for all LSDs. Plasma pulmonary and activation-regulated chemokine (PARC/CCL18) has been found particularly useful for the evaluation of those Gaucher patients who are chitotriosidase deficient. Biomarkers for some LSDs should be disease-specific and should reflect not one particular symptom but rather the total body burden of storage cells. Clinical applications of current biomarkers (primary and secondary accumulating metabolites or proteins specifically secreted by storage cells) involve aiding diagnosis, monitoring disease progression, and assessing therapeutic efficacy. Routine use of established LSD biomarkers (e.g., globotriaosylceramide-Gb3 and lyso-Gb3 (Fabry disease); glucose tetrasaccharide (Pompe disease); plasma oxysterols (Niemann-Pick type C)) is often limited by the necessary tandem mass spectrometry technology (20). A more recent proteomics approach will certainly lead to the detection of an ideal biomarker, at least for some LSDs.

It is important to state that continuous monitoring of changes in biomarker levels has one of the decisive roles in drug dose correction, which may result in cost reduction for the otherwise very expensive therapy.

**Conflict of interest statement**

The authors stated that there are no conflicts of interest regarding the publication of this article.
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