

# Lysosomal storage disorders: Molecular basis and laboratory testing

Mirella Filocamo<sup>1\*</sup> and Amelia Morrone<sup>2</sup>

<sup>1</sup>S.S.D. Lab. Diagnosi Pre-Postnatale Malattie Metaboliche, Dipartimento di Neuroscienze, IRCCS G. Gaslini, Largo G. Gaslini 5, Genova, Italy

<sup>2</sup>Metabolic and Muscular Unit, Clinic of Pediatric Neurology, Department of Sciences for Woman and Child's Health, University of Florence, Meyer Children's Hospital, Viale Pieraccini n. 24, 501329 Florence, Italy

\*Correspondence to: Tel: +39 (0)10 5636792; Fax: +39 (0)10 383983; E-mail: mirellafilocamo@ospedale-gaslini.ge.it

Date received (in revised form): 7th January 2011

## Abstract

Lysosomal storage disorders (LSDs) are a large group of more than 50 different inherited metabolic diseases which, in the great majority of cases, result from the defective function of specific lysosomal enzymes and, in few cases, of non-enzymatic lysosomal proteins or non-lysosomal proteins involved in lysosomal biogenesis. The progressive lysosomal accumulation of undegraded metabolites results in generalised cell and tissue dysfunction, and, therefore, multi-systemic pathology. 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. The diagnosis of most LSDs—after accurate clinical/paraclinical evaluation, including the analysis of some urinary metabolites—is based mainly on the detection of a specific enzymatic deficiency. In these cases, molecular genetic testing (MGT) can refine the enzymatic diagnosis. Once the genotype of an individual LSD patient has been ascertained, genetic counselling should include prediction of the possible phenotype and the identification of carriers in the family at risk. MGT is essential for the identification of genetic disorders resulting from non-enzymatic lysosomal protein defects and is complementary to biochemical genetic testing (BGT) in complex situations, such as in cases of enzymatic pseudodeficiencies. Prenatal diagnosis is performed on the most appropriate samples, which include fresh or cultured chorionic villus sampling or cultured amniotic fluid. The choice of the test—enzymatic and/or molecular—is based on the characteristics of the defect to be investigated. For prenatal MGT, the genotype of the family index case must be known. The availability of both tests, enzymatic and molecular, enormously increases the reliability of the entire prenatal diagnostic procedure. To conclude, BGT and MGT are mostly complementary for post- and prenatal diagnosis of LSDs. Whenever genotype/phenotype correlations are available, they can be helpful in predicting prognosis and in making decisions about therapy.

## Keywords:

## Introduction

Although the first clinical descriptions of patients with lysosomal storage disorders (LSDs) were reported at the end of the nineteenth century by Warren Tay (1881)<sup>1</sup> and Bernard Sachs (1887; Tay–Sachs disease),<sup>2</sup> and by Phillippe Gaucher (1882) (Gaucher disease),<sup>3</sup> the biochemical nature of the accumulated products was only elucidated some 50 years later

(1934) in the latter, as glucocerebroside.<sup>4</sup> Considerably more time was then required for the demonstration by Hers (1963) that there was a link between an enzyme deficiency and a storage disorder (Pompe disease).<sup>5</sup> In the following years, the elucidation of several enzyme defects led to the initial classification of the various types of LSDs according to their clinical pictures, pathological manifestations and the biochemical nature of the undegraded substrates. Although part of

this classification is still maintained, it is continually updated on the basis of newly acquired knowledge on the underlying molecular pathology.

At present, more than 50 LSDs are known. The majority of these result from a deficiency of specific lysosomal enzymes. In a few cases, non-enzymatic lysosomal proteins or non-lysosomal proteins involved in lysosomal biogenesis are deficient.

The common biochemical hallmark of these diseases is the accumulation of undigested metabolites in the lysosome. This can arise through several mechanisms as a result of defects in any aspect of lysosomal biology that hampers the catabolism of molecules in the lysosome, or the egress of naturally occurring molecules from the lysosome. Lysosomal accumulation activates a variety of pathogenetic cascades that result in complex clinical pictures characterised by multi-systemic involvement.<sup>6–10</sup> Phenotypic expression is extremely variable, as it depends on the specific macromolecule accumulated, the site of production and degradation of the specific metabolites, the residual enzymatic expression and the general genetic background of the patient. Many LSDs have phenotypes that have been recognised as infantile, juvenile and adult.<sup>7</sup>

Table 1 summarises the various defective proteins, the type(s) of main accumulated metabolites and the distinct genes responsible for each specific LSD type/subtype. It also reports screening and diagnostic tests available for each disease.

## The endo-lysosomal system

The original concept that the lysosome is only one component of a series of unconnected intracellular organelles of the endo-lysosomal system<sup>12</sup> has been widely modified by recent studies. Lysosomal function is now considered in the larger context of the endosomal/lysosomal system.<sup>13</sup> In this highly dynamic system, which mediates the internalisation, recycling, transport and breakdown of cellular/extracellular components and facilitates dissociation of receptors from their ligands, the lysosome represents the greater degradative compartment of endocytic, phagocytic and autophagic pathways.

Although hydrolytic enzymes are present in endosomes and lysosomes, they function optimally in the lysosome, as it is the most acidic compartment.

## Lysosomal enzymes: Synthesis and trafficking

The lysosomal enzymes, which are synthesised in the rough endoplasmic reticulum (ER), move across the ER membrane to the lumen of the ER via an N-terminal signal sequence-dependent translocation. Once in the ER lumen, they are N-glycosylated and their signal sequence is cleaved. They then proceed to the Golgi compartment and, at this stage, the lysosomal enzymes, which require the mannose 6-phosphate (M6P) marker to enter the lysosome, acquire the M6P ligand by the sequential action of a phosphotransferase and a diesterase.<sup>14–16</sup> The receptor–protein complex then moves to the late endosome, where dissociation occurs; the hydrolase translocates into the lysosome and the receptor is recycled either to the Golgi apparatus or to the plasma membrane.

The final steps in the maturation of the lysosomal enzyme include proteolysis, folding and aggregation.

Not all lysosomal enzymes depend on the M6P pathway, however. Recently, it has been shown that the lysosomal integral membrane protein type 2 (LIMP-2)—a ubiquitously expressed transmembrane protein mainly found in the lysosomes and late endosomes—is a receptor for lysosomal M6P-independent targeting of glucocerebrosidase.<sup>17</sup> Figure 1 depicts a simplified scheme of M6P-dependent enzymes sorting to the lysosome.

## Epidemiology

To date, worldwide epidemiological data on LSDs are not available or are limited to distinct populations. Apart from selected populations presenting a high prevalence for specific diseases, such as the Ashkenazi Jewish population at high risk for Gaucher disease,<sup>18</sup> Tay–Sachs disease and Niemann–Pick disease;<sup>19</sup> the Finnish population with its high incidence of aspartylglucosaminuria<sup>20</sup> and infantile/

Table 1. Lysosomal storage disorders

OMIM	Disease	Defective protein	Main storage materials	Preliminary test	Gene symbol	MIM ID	Diagnostic test
<b>Mucopolysaccharidoses (MPSs)</b>							
607014 607015 607016	MPS I (Hurler, Scheie, Hurler/Scheie)	$\alpha$ -Iduronidase	Dermatan sulphate, heparan sulphate	GAGs (U)	IDUA	252800	BGT, MGT
309900	MPS II (Hunter)	Iduronate sulphatase	Dermatan sulphate, heparan sulphate	GAGs (U)	IDS	309900	BGT, MGT
252900	MPS III A (Sanfilippo A)	Heparan sulphamidase	Heparan sulphate	GAGs (U)	SGSH	605270	BGT, MGT
252920	MPS III B (Sanfilippo B)	Acetyl $\alpha$ -glucosaminidase	Heparan sulphate	GAGs (U)	NAGLU	609701	BGT, MGT
252930	MPS III C ( Sanfilippo C)	Acetyl CoA: $\alpha$ -glucosaminide N-acetyltransferase	Heparan sulphate	GAGs (U)	HGSNAT	610453	BGT, MGT
252940	MPS III D (Sanfilippo D)	N-acetyl glucosamine-6-sulphatase	Heparan sulphate	GAGs (U)	GNS	607664	BGT, MGT
253000	MPS IV A (Morquio A)	Acetyl galactosamine-6-sulphatase	Keratan sulphate, chondroitin 6-sulphate	GAGs (U)	GALNS	612222	BGT, MGT
253010	MPS IV B (Morquio B)	$\beta$ -Galactosidase	Keratan sulphate	GAGs (U)	GLBI	611458	BGT, MGT
253200	MPS VI (Maroteaux-Lamy)	Acetyl galactosamine 4-sulphatase (arylsulphatase B)	Dermatan sulphate	GAGs (U)	ARSB	611542	BGT, MGT
253220	MPS VII (Sly)	$\beta$ -Glucuronidase	Dermatan sulphate, heparan sulphate, chondroitin 6-sulphate	GAGs (U)	GUSB	611499	BGT, MGT
601492	MPS IX (Natowicz)	Hyaluronidase	Hyaluronan	–	HYALI	607071	BGT, MGT
<b>Sphingolipidoses</b>							
301500	Fabry	$\alpha$ -Galactosidase A	Globotriasy/ceramide	–	GLA	300644	BGT, MGT
228000	Farber	Acid ceramidase	Ceramide	–	ASAH1	613468	BGT, MGT
230500 230650	Gangliosidosis GM1 (Types I, II, III)	GM1- $\beta$ -galactosidase	GM1 ganglioside, Keratan sulphate, oligos, glycolipids	Oligos (U)	GLBI	611458	BGT, MGT

Continued

Table 1. Continued

OMIM	Disease	Defective protein	Main storage materials	Preliminary test	Gene symbol	MIM ID	Diagnostic test
272800	Gangliosidosis GM2, Tay-Sachs	$\beta$ -Hexosaminidase A	GM2 ganglioside, oligos, glycolipids	–	HEXA	606869	BGT, MGT
268800	Gangliosidosis GM2, Sandhoff	$\beta$ -Hexosaminidase A + B	GM2 ganglioside, oligos	–	HEXAB	606873	BGT, MGT
230800 230900 231000	Gaucher (Types I, II, III)	Glucosylceramidase	Glucosylceramide	Chito <sup>+</sup> (S)	GBA	606463	BGT, MGT
245200	Krabbe	$\beta$ -Galactosylceramidase	Galactosylceramide	–	GALC	606890	BGT, MGT
250100	Metachromatic leucodystrophy	Arylsulphatase A	Sulphatides	Sulphatides (U)	ARSA	607574	BGT, MGT
257200 607616	Niemann–Pick (type A, type B)	Sphingomyelinase	Sphingomyelin	–	SMPDI	607608	BGT, MGT
<b>Oligosaccharidoses (glycoproteinoses)</b>							
208400	Aspartylglucosaminuria	Glycosylasparaginase	Aspartylglucosamine	Oligos (U)	AGA	613228	BGT, MGT
230000	Fucosidosis	$\alpha$ -Fucosidase	Glycoproteins, glycolipids, Fucoside-rich oligos	Oligos (U)	FUCA1	612280	BGT, MGT
248500	$\alpha$ -Mannosidosis	$\alpha$ -Mannosidase	Mannose-rich oligos	Oligos (U)	MAN2B1	609458	BGT, MGT
248510	$\beta$ -Mannosidosis	$\beta$ -Mannosidase	Man( $\beta$ 1 $\rightarrow$ 4)GlnNAc	Oligos (U)	MANBA	609489	BGT, MGT
609241	Schindler	N-acetylgalactosaminidase	Sialylated/asialoglycopeptides, glycolipids	Oligos (U)	NAGA	104170	BGT, MGT
256550	Sialidosis	Neuraminidase	Oligos, glycopeptides	Bound SA (U), Oligos (U)	NEU1	608272	BGT, MGT
<b>Glycogenoses</b>							
232300	Glycogenosis II/ Pompe	$\alpha$ 1,4-glucosidase (acid maltase)	Glycogen	CK (S)	GAA	606800	BGT, MGT
<b>Lipidoses</b>							
278000	Wolman/CESD	Acid lipase	Cholesterol esters	–	LIPA	613497	BGT, MGT

Continued

Table 1. Continued

OMIM	Disease	Defective protein	Main storage materials	Preliminary test	Gene symbol	MIM ID	Diagnostic test
<b>Non-enzymatic lysosomal protein defect</b>							
272750	Gangliosidosis GM2, activator defect	GM2 activator protein	GM2 ganglioside, oligos	–	GM2A	613109	MGT
249900	Metachromatic leucodystrophy	Saposin B	Sulphatides	Sulphatides (U)	PSAP	176801	MGT
611722	Krabbe	Saposin A	Galactosylceramide	–	PSAP	176801	MGT
610539	Gaucher	Saposin C	Glucosylceramide	–	PSAP	176801	MGT
<b>Transmembrane protein defect</b>							
Transporters							
269920 604369	Sialic acid storage disease; infantile form (ISSD) and adult form (Salla)	Sialin	Sialic acid	Free SA (U)	SLC17A5	604322	MGT
219800	Cystinosis	Cystinosin	Cystine	–	CTNS	606272	MGT
257220	Niemann–Pick Type C1	Niemann–Pick type 1 (NPC1)	Cholesterol and sphingolipids	Chito <sup>+</sup> (S)	NPC1	607623	Filipin test, MGT
607625	Niemann–Pick, Type C2	Niemann–Pick type 2 (NPC2)	Cholesterol and sphingolipids	Chito <sup>+</sup> (S)	NPC2	601015	Filipin test, MGT
Structural Proteins							
300257	Danon	Lysosome-associated membrane protein 2	Cytoplasmatic debris and glycogen	–	LAMP2	309060	MGT
252650	Mucopolidosis IV	Mucopolin	Lipids	–	MCOLN1	605248	MGT
<b>Lysosomal enzyme protection defect</b>							
256540	Galactosialidosis	Protective protein cathepsin A (PPCA)	Sialyloligosaccharides	Bound SA (U), Oligos (U)	CTSA	613111	BGT <sup>a</sup> , MGT
<b>Post-translational processing defect</b>							
272200	Multiple sulphatase deficiency	Multiple sulphatase	Sulphatides, glycolipids, GAGs	Sulphatides (U), GAGs (U)	SUMF1	607939	BGT <sup>b</sup> , MGT

Continued

Table 1. Continued

OMIM	Disease	Defective protein	Main storage materials	Preliminary test	Gene symbol	MIM ID	Diagnostic test
<b>Trafficking defect in lysosomal enzymes</b>							
252500	Mucopolipidosis II $\alpha$ / $\beta$ ,	GlcNAc-1-P transferase	Oligos, GAGs, lipids	Oligos (U)	GNPTAB	607840	BGT <sup>c</sup> , MGT
252600	III $\alpha$ / $\beta$						
232605	Mucopolipidosis III $\gamma$	GlcNAc-1-P transferase	Oligos, GAGs, lipids	Oligos (U)	GNPTG	607838	BGT <sup>c</sup> , MGT
<b>Polypeptide degradation defect</b>							
265800	Pycnodysostosis	Cathepsin K	Bone proteins	X-ray	CTSK	601105	MGT
<b>Neuronal ceroid lipofuscinoses (NCLs)</b>							
256730	NCL 1	Palmitoyl protein thioesterase (PPT1)	Saposins A and D	Ultrastructure	PPT1	600722	BGT, MGT
204500	NCL 2	Tripeptidyl peptidase I (TPP I)	Subunit c of ATP synthase	Ultrastructure	TPP I	607998	BGT, MGT
204200	NCL 3	CLN3, lysosomal transmembrane protein	Subunit c of ATP synthase	Ultrastructure	CLN3	607042	MGT
256731	NCL 5	CLN5, soluble lysosomal protein	Subunit c of ATP synthase	Ultrastructure	CLN5	608102	MGT
601780	NCL 6	CLN6, transmembrane protein of ER	Subunit c of ATP synthase	Ultrastructure	CLN6	606725	MGT
610951	NCL 7	CLC7, lysosomal chloride channel	Subunit c of ATP synthase	Ultrastructure	MFSD8	611124	MGT
600143	NCL 8	CLN8, transmembrane protein of endoplasmic reticulum	Subunit c of ATP synthase	Ultrastructure	CLN8	607837	MGT
610127	NCL 10	Cathepsin D	Saposins A and D	Ultrastructure	CTSD	116840	MGT

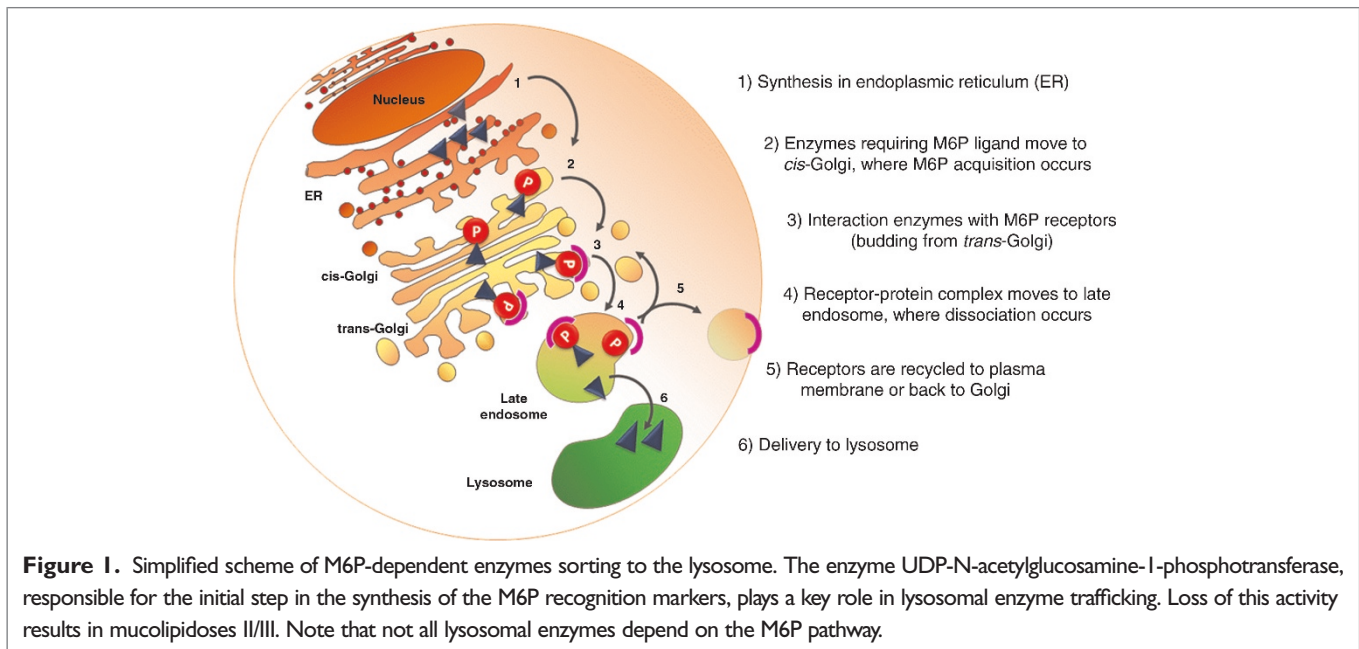
Abbreviations: CK, creatine kinase; CLN, with expansion; GAGs, glycosaminoglycans; GLcNAc-1-P, with expansion; Oligos, oligosaccharides; S, serum; SA, sialic acid; U, urine; Chito, chitotriosidase

<sup>a</sup>Defect of  $\beta$ -galactosidase and neuraminidase and/or cathepsin A

<sup>b</sup>Decrease in some lysosomal and non-lysosomal sulphatases

<sup>c</sup>Some lysosomal hydrolase activities increased in plasma and decreased in cultured fibroblasts

<sup>d</sup>Note that 5–7 per cent of the population have a recessively inherited defect in the chitotriosidase gene, which leads to false-negative values.<sup>11</sup>



juvenile neuronal ceroid lipofuscinosis,<sup>21</sup> as far as we know, prevalence data on LSDs, as a group, have only been reported in Greece,<sup>22</sup> the Netherlands,<sup>23</sup> Australia,<sup>24</sup> Portugal<sup>25</sup> and the Czech Republic.<sup>26</sup> As a group, overall incidence of LSDs is estimated at around 1:5,000–1:8,000.<sup>24</sup>

### Classification: From the nature of the primary stored material to the type of molecular defect

LSDs can be grouped according to various classifications. While, in the past, they were classified on the basis of the nature of the accumulated substrate(s), more recently they have tended to be classified by the molecular defect (Table 1). A classic example of LSDs grouped by storage is the group of mucopolysaccharidoses, resulting from a deficiency of any one of 11 lysosomal enzymes that are involved in the sequential degradation of glycosaminoglycans (or mucopolysaccharides). In the group of sphingolipidoses, undegraded sphingolipids accumulate due to an enzyme deficiency or to an activator protein defect (the latter is classified in Table 1 as a group according to the molecular defect). Among the oligosaccharidoses (also known as glycoproteinoses), a single lysosomal hydrolase

deficiency causes storage of oligosaccharides. In some cases, a deficiency in a single enzyme can result in the accumulation of different substrates. For example, GM1 gangliosidosis and Morquio-B disease are both caused by an acid  $\beta$ -galactosidase activity defect, yet results in GM1 ganglioside and keratan sulphate accumulation, respectively.

Table 1 also reports the emerging classification of diseases based on the recent understanding of the molecular basis LSDs. This subset includes groups of disorders due to: (i) non-enzymatic lysosomal protein defects; (ii) transmembrane protein defects (transporters and structural proteins); (iii) lysosomal enzyme protection defects; (iv) post-translational processing defects of lysosomal enzymes; (v) trafficking defects in lysosomal enzymes; and (vi) polypeptide degradation defects. Finally, another group includes the neuronal ceroid lipofuscinoses (NCLs), which are considered to be lysosomal disorders, even though distinct characteristics exist. While, in the classic LSDs, the deficiency or dysfunction of an enzyme or transporter leads to lysosomal accumulation of specific undegraded substrates or metabolites, accumulating material in NCLs is not a disease-specific substrate but the subunit c of mitochondrial ATP synthase or sphingolipid activator proteins A and D.<sup>27</sup>

Multiple sulphatase deficiency (MSD) is also worth mentioning. It has been shown that MSD results from a post-translational processing defect due to the failure of the C $\alpha$ -formylglycine-generating enzyme to convert a specific cysteine residue, at the catalytic centre of all sulphatases, to a C $\alpha$ -formylglycine residue.<sup>28,29</sup> Another rare LSD, galactosialidosis, is associated with the defective activity of two enzymes,  $\beta$ -galactosidase and sialidase. In these diseases—classified as ‘lysosomal enzyme protection defects’—a multi-enzyme complex between the two lysosomal enzymes and the protective protein, cathepsin A (PPCA), forms improperly.<sup>30</sup>

The breakdown of certain glycosphingolipids by their respective hydrolases requires the presence of activator proteins, known as sphingolipid activator proteins or saposins, encoded by two different genes. The defective function of the GM2 activator protein results in the AB variant of GM2 gangliosidosis.<sup>31</sup> The prosaposin is processed to four homologous saposins (Sap A, Sap B, Sap C and Sap D).<sup>32</sup> Deficiency of Sap A, Sap B and Sap C results in variant forms of (i) Krabbe disease, involving abnormal storage of galactosylceramide;<sup>33</sup> (ii) metachromatic leucodystrophy (MLD), associated with sulphatide storage;<sup>34</sup> and (iii) Gaucher’s disease, involving glucosylceramide storage,<sup>35</sup> respectively. Rarely, a total deficiency of prosaposin has been reported, resulting in a very severe phenotype.<sup>36</sup>

Mucopolidoses result from defects in the enzyme UDP-N-acetylglucosamine-1-phosphotransferase, which plays a key role in lysosomal enzyme trafficking.<sup>37</sup> This enzyme is responsible for the initial step in the synthesis of the M6P recognition markers essential for receptor-mediated transport of newly synthesised lysosomal enzymes to the endosomal/prelysosomal compartment (Figure 1). Failure to attach this recognition signal leads to the mistargeting of all lysosomal enzymes that require the M6P marker to enter the lysosome.

## Laboratory diagnosis

Like other metabolic diseases, LSDs show remarkably varied clinical signs and symptoms, which may occur from the *in utero* period to late adulthood,

depending on the complexity of the storage products and differences in their tissue distribution. Indeed, the recognition of LSD clinical features requires clinical expertise, as most of them are not specific and can be caused by defects in other metabolic pathways (mitochondrial and peroxisomal), or by environmental factors. Even in the presence of typical clinical signs and symptoms, samples and diagnostic tests are different for each group of lysosomal disorders and often are specific to a given disease.

The definitive diagnosis of LSDs therefore requires close collaboration between laboratory specialists and clinicians. For laboratory diagnosis, the clinician must select the appropriate test to be performed on the basis of a comprehensive evaluation that includes not only a physical assessment of the patient but also paraclinical test results (peripheral blood smears, radiological/neurophysiological findings etc). Additionally, each sample that is sent for testing should be accompanied by a detailed patient case history and family history, to allow the laboratory specialist to make a reliable evaluation of the results that might include indications for other potential investigations.

Before considering specific analyses (enzymatic and/or molecular), preliminary screening tests should be performed (Table 1). Increased urinary excretion of glycosaminoglycans is mainly found in the mucopolysaccharidoses group, while abnormal urinary oligosaccharide excretion patterns mostly characterise the oligosaccharidoses (glycoproteinoses). There are also more specific preliminary tests, such as the qualitative assessment of urinary sulphatide storage, which can give indications for MLD (due to arylsulphatase A enzyme deficiency or saposin B activator defect); increased urinary excretion of free sialic acid is suggestive of the sialic acid storage disorders (the severe infantile form [ISSD] or the slowly progressive adult form [Salla]). Abnormal serum levels of metabolites/proteins can be used as ancillary tests in some LSDs. Serum creatine kinase (CK) concentrations can be elevated in Pompe disease, while high levels of chitotriosidase can indicate Gaucher disease and, to a lesser extent, other lipidoses, such as Niemann–Pick C



(NPC). All of these preliminary (urine and serum) tests carry the risk of producing false positives/negatives, however, and need to be followed up with specific enzymatic and/or molecular analyses performed on suitable samples, leucocytes and/or cell lines (fibroblasts and/or lymphoblasts).

As about 75 per cent of LSDs are due to a deficiency in lysosomal hydrolase activity, the demonstration of reduced/absent lysosomal hydrolase activity by a specific enzyme assay is an effective and reliable method of diagnosis. In these cases, molecular analysis can refine the enzymatic diagnosis. The remaining LSDs, resulting from non-enzymatic protein defects, require molecular analysis to be performed on the specific gene for a conclusive diagnosis (Table 1).

Generally, an inherited deficiency of a lysosomal enzyme is associated with an LSD. There are, however, individuals who show greatly reduced enzyme activity but remain clinically healthy. This condition, termed as enzymatic 'pseudodeficiency' (Pd), is known in some lysosomal hydrolases. Conversely, there are circumstances in which affected individuals with a clinical/paraclinical picture resembling some glycosphingolipidoses show normal activity of the relevant lysosomal enzyme. These patients should be investigated for a potential defect of an activator protein involved in glycosphingolipid breakdown.

### Pseudodeficiency

To date, Pds due to polymorphic genetic variants, have been reported for at least nine lysosomal enzymes, including: arylsulphatase A (*ARSA* gene),<sup>38</sup>  $\beta$ -hexosaminidase (*HEXA* gene),<sup>39</sup>  $\alpha$ -iduronidase (*IDUA* gene),<sup>40</sup>  $\alpha$ -glucosidase (*GAA* gene),<sup>41</sup>  $\alpha$ -galactosidase (*GLA* gene),<sup>42,43</sup>  $\beta$ -galactosidase (*GLB1* gene),<sup>44</sup>  $\alpha$ -fucosidase (*FUCA1* gene)<sup>45</sup> and  $\beta$ -glucuronidase (*GUSB* gene).<sup>46,47</sup>

While some of these genetic conditions are rare, the arylsulphatase A Pd has been estimated to have a frequency of 7.3–15 per cent.<sup>48–50</sup> Since the Pd allele is more frequent than the alleles causing MLD (estimated to be 0.5 per cent), individuals presenting with neurological symptoms and homozygous for arylsulphatase A Pd are likely to be

misdiagnosed as MLD.<sup>51</sup> Additionally, it should be noted that Pd polymorphisms can occur on the same gene as MLD-causing mutations. It is therefore necessary to perform a combination of enzymatic and molecular analyses to determine the actual genetic make-up of MLD patients and their family members, in order to distinguish individuals carrying Pd alleles from those carrying MLD alleles.

### Activator proteins

Another complication that can potentially lead to missed diagnoses is represented by defects of those cofactors (mentioned above) required for the function of certain lysosomal enzymes involved in glycosphingolipid breakdown. Variant forms of GM2 gangliosidosis, Krabbe disease, MLD and Gaucher disease can result not only from a deficiency of an enzymatic activity, but also from defects of sphingolipid activator proteins or saposins.<sup>52</sup> In these cases, conclusive diagnosis requires a comprehensive evaluation based on a range of diagnostic procedures, including neuroradiological, neurophysiological, biochemical/enzymatic and molecular tests.<sup>53</sup>

### Biochemical genetic testing

Biochemical genetic testing (BGT), including the assay of enzymatic proteins, is feasible for most LSDs and is essential for the diagnosis of primary lysosomal enzyme deficiency.

Lysosomal enzymes are present in almost all tissues and biological samples. The choice of the sample type to be analysed is based on (i) the level of an enzyme's activity in a specific tissue, (ii) the sample stability during its transfer to the referring laboratory and (iii) the time of diagnosis.

Although enzyme activity can be assayed in some biological fluids, such as plasma, serum and urine, several enzymatic Pds have been reported in serum or plasma, so their use can lead to pitfalls in diagnosis.<sup>43,54</sup>

Leucocytes are often appropriate biological samples, although possible interference between

isoenzymes should be taken into consideration. Fibroblast samples represent the gold standard in diagnosis, since they express the optimum enzyme activity; however, they require an invasive skin biopsy and culturing. Epstein–Barr virus-transformed B-lymphoblast culture obtained from a non-invasive blood sampling can be useful, bearing in mind, however, that lymphoblasts do not express some enzymatic activities, such as arylsulphatase A. Lysosomal enzyme assays are usually performed using synthetic (fluorimetric or colorimetric) substrates showing undetectable or very low enzyme activity in cell lines of affected individuals.

The complete absence of lysosomal enzyme activity generally confirms diagnosis. Conversely, the presence of normal lysosomal enzyme activity cannot exclude a specific diagnosis if it is accompanied by suggestive clinical symptoms and/or the abnormal presence of metabolites in the urine and/or storage in peripheral smear and/or tissue biopsy. For example, a patient who presents with a clinical profile resembling Gaucher disease, with high levels of chitotriosidase activity and increased concentrations of glucosylceramide in plasma and normal  $\beta$ -glucosidase activity in skin fibroblasts, should be referred for a molecular genetic study of the prosaposin gene (*PSAP*), which codes for the cofactor Sap C required for the function of  $\beta$ -glucosidase.<sup>55</sup> Findings of normal arylsulphatase A activity and abnormal patterns of urinary sulphatides in a suspected MLD patient do not exclude the disease and should be followed up by the molecular analysis of *PSAP*.<sup>52,53</sup> The detection of residual lysosomal enzyme activity should be carefully evaluated, together with clinical and instrumental findings. Molecular genetic testing (MGT) can reveal polymorphisms that potentially lead to an enzymatic Pd.

## Molecular genetic testing

MGT performed on DNA and/or RNA comprises a range of different molecular approaches for investigating the entire gene-coding regions and exon–intron boundaries, as well as 5′- and 3′-untranslated regions (UTRs). It can confirm the enzymatic

diagnosis of an LSD, and is essential for the definitive diagnosis of LSDs resulting from non-enzymatic lysosomal proteins (Table 1) and in post-mortem diagnoses when the only suitable specimens available are DNA samples. MGT can also contribute to elucidating the findings of high biochemical residual enzyme activity in affected patients and very low enzyme activities in unaffected patients (enzymatic Pds).<sup>38–47</sup> Moreover, it is useful in genotype–phenotype correlation studies for some diseases and for indentifying at-risk family members.

MGT can clarify the type of genetic variation and its impact on the protein and on the presence of residual enzyme activity. This information is crucial in evaluating treatment options, such as enzyme replacement therapy (ERT), to date only available for some disorders, and alternative treatments such as pharmacological chaperones or substrate reduction therapy (SRT), for which clinical trials are still in progress.<sup>56,57</sup>

Particular care should be taken when interpreting genotype–phenotype correlations, even in the context of a recurrent mutation, as some patients carrying the same lesion may present with different clinical phenotypes, suggesting that other factors, such as polymorphic variants, genetic modifiers or RNA editing-like mechanisms,<sup>58</sup> can lead to changes in protein function which could influence the clinical phenotype.

In general, the interpretation of a molecular result should depend on a comprehensive evaluation that includes related clinical, paraclinical and biochemical data. For instance, additional molecular studies are needed in the case of an ascertained enzymatic deficiency that is not supported by the detection of the underlying genetic lesion in a patient with a picture suggestive of an LSD. Expression gene profiling and RNA and/or protein analyses can be helpful in revealing deletions/insertions, gross rearrangements and potential transcription defects. This could be the case in patients affected by X-linked Fabry diseases in which no mutations have been identified by traditional MGT on DNA samples. RNA analysis and real-time polymerase chain reaction have revealed an

unbalanced  $\alpha$ -galactosidase A mRNAs ratio of two unexpected alternatively spliced mRNAs, which resulted in the successive identification of a new intronic lesion affecting transcription and new pathogenetic mechanisms of Fabry disease.<sup>59,60</sup>

The detection of a known mutation should also be supported by a comparison of the patient's biochemical and clinical data with those available in the literature. For instance, a genotype–phenotype miscorrelation could signal incorrect genotyping. Reports of an additional nucleotide change in *cis* on a mutated allele, which potentially modifies the phenotype, are not infrequent. A somatic mosaicism was reported to be the underlying molecular mechanism for an unexpectedly severe form of Gaucher disease (type 2) in a patient in whom the beneficial effect of the mild p.N409S (traditionally named as N370S) mutation was experimentally demonstrated to be reversed by the *in cis* presence of the severe p.L483P (traditionally named as L444P) mutation.<sup>61</sup> A modulating action was also reported for a novel polymorphism (p.L436F), identified in *cis* with the known p.R201C mutation, in a patient affected by the juvenile form of GM1 gangliosidosis with a severe outcome. *In vitro* expression studies and Western blot analysis showed that the novel polymorphism dramatically abrogated the residual enzyme activity predicted to be associated with the common p.R201C mutation, explaining the severe outcome.<sup>62</sup>

Conventional MGT techniques have also been reported to be responsible for accidental misgenotyping of patients in cases of genomic lesions such as insertion/deletions, complex rearrangements and uniparental disomy. In particular, additional techniques were necessary to ascertain various gene–pseudogene rearrangements in Hunter syndrome patients which had been missed by conventional methods.<sup>63</sup> Partial/total gene deletions and various gene–pseudogene rearrangements led to incorrect genotyping in Gaucher disease during routine diagnostic mutation analysis.<sup>64,65</sup>

Finally, it is important to underline that MGT results should be interpreted with caution, even in the presence of a change previously reported as a disease-causing mutation. For years, the c.1151G >

A (p.S384N) mutation was considered to be disease-causing in patients with Maroteaux–Lamy syndrome but, recently, segregation studies in a family at risk for the syndrome conclusively revealed c.1151G > A (p.S384N) to be a polymorphism.<sup>66</sup>

## Screening tests on dried blood spot specimens

The availability of analyses of acylcarnitines and amino acids using liquid chromatography–tandem mass spectrometry technology on dried blood spot specimens (DBS) led to screening for treatable inborn errors of metabolisms (IEM) in newborns. At present, the expanded newborn screening based on DBS identifies more than 30 IEM and represents an important step forward.<sup>67</sup>

A few years ago, screening tests for several LSDs by BGT on DBS using fluorescent methods<sup>68–70</sup> were reported. Subsequently, multiplex assays of lysosomal enzymes on DBS by tandem mass spectrometry have been described.<sup>71–73</sup>

The availability of multiplex technology has facilitated the technical aspects of testing, making it easier to identify LSDs and to introduce newborn screening programmes for treatable LSDs.<sup>74,75</sup> A DBS control quality for these tests has recently been developed.<sup>76</sup> Attempts to widen screening programmes to include other LSDs are essential for patients in whom an early and presymptomatic diagnosis can provide better outcomes by reducing clinically significant disabilities.<sup>77</sup>

Obviously, reduced residual enzyme activity detected in a presymptomatic patient at newborn screening also should be investigated by standard laboratory diagnostic procedures.

## Genetic counselling

All LSDs are inherited as autosomal recessive traits, except for Fabry disease, Hunter syndrome (or mucopolysaccharidosis II) and Danon disease. These are X-linked disorders.

Once the laboratory diagnosis (enzymatic and/or molecular) of an LSD patient is ascertained, genetic counselling for at-risk couples includes prenatal

testing on chorionic villi (at 11–12 weeks) or amniocytes (at 16 weeks). Genotyping individual LSD patients also allows carriers in the family to be identified and can sometimes predict phenotypes in the patients.

## Biobanking

Sample and clinical/instrumental data banking is important for all rare genetic diseases—including LSDs—which often lead to death at an early age. Since it is likely that our understanding of genes, diseases and testing methodology will improve in the future, consideration should be given to biobanking appropriate biological material from patients affected or suspected to be affected by LSDs, as well as from their parents and other first-degree relatives, for future diagnostic and research purposes.

Biological material such as urine, whole blood, plasma, serum, leucocytes, DNA and cell lines (fibroblasts from skin biopsy and/or lymphoblasts from blood) should be stored. Effective interaction between clinicians and biobank staff is essential, since future results rely not only on the availability of appropriate biological samples, but also on the accurate recording of associated clinical/paraclinical data.

Hydrops foetalis, an extreme presentation of many LSDs, represents the best example of a complex case in which diagnosis can be achieved only if appropriate samples are stored. Indeed, hydrops foetalis can be associated with a wide spectrum of phenotypes, including mucopolysaccharidosis VII and IVA, Gaucher disease, sialidosis, GM1 gangliosidosis, galactosialidosis, ISSD, Niemann–Pick disease type C (and A), mucopolipidosis II (I-cell disease), Wolman disease and disseminated lipogranulomatosis (Farber disease).<sup>78</sup> Frequently, these LSDs are only recognised after the recurrence of hydrops foetalis in several pregnancies.<sup>79,80</sup> In order to arrive at a conclusive diagnosis and to optimise the storage of the most appropriate biological material in such complex cases, many skilled experts, including clinicians, genetics, biochemists, molecular biologists etc, must cooperate.

## Conclusions

In conclusion, BGT and MGT must be considered as complementary analyses for the diagnosis of most LSDs, for genotype–phenotype correlations and for prenatal diagnosis. MGT is essential for carrier detection, and can sometimes predict prognosis and support therapeutic choices, including the application of new therapeutic approaches.

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