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A proposal to consider: sensitivity, specificity and normal range values of the enzymatic deficiency of lysosomal enzymes

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Abstract.

Background. Lysosomal storage diseases (LSD) are a group of more than 50 genetic disorders and demonstrating deficiency of enzyme activity (EA) should be the first step in its diagnosis. Referral for molecular studies when an LSD is suspected is mainly based in the measurement of the percent relative enzyme activity; nevertheless, the great variability observed in healthy subjects can make the decision unreliable, especially when the patient has some degree of residual activity. *Objectives*. To evaluate the sensitivity and specificity of enzymatic diagnosis in leukocytes and describe normal values of EA in amniocytes and leukocytes. *Design and Methods*. 16 lysosomal enzymes from leukocytes were studied in 151 LSD patients and 869 apparently healthy subjects. Enzyme activities for 13 LSDs were studied in 72 amniocyte cultures at 13 and 18 days culture (12 suspected LSDs and 60 controls). *Results.* The EA for lysosomal enzymes were significantly lower in LSD patients than in controls; the sensitivity and specificity of EA for diagnosis of LSD were mainly above 95% in the majority of the diseases. The normal lysosomal EA in amniocytes was reported; 12 prenatal diagnoses assayed resulted negative for LSD. *Conclusions.*The high sensitivity and specificity of the enzymatic diagnosis of LSDs suggests its utility to identify subjects with the suspicion of LSD. The description of the normal range of 16 lysosomal enzymes in leukocytes and 13 enzymes in amniocytes could be an important source of information for further studies.

Introduction.

Lysosomal storage diseases (LSDs) are a group of more than 50 genetic disorders. Clinical symptoms are caused by the deficiency of specific enzyme(s) function and resultant substrate accumulation in the lysosomes, which leads to impaired cellular function and progressive tissue and organ dysfunction (Yu et al, 2011). LSDs are mainly classified according to the accumulated substrate in sphingolipidosis, glucoproteinosis, mucolipidoses, mucopolysaccharidosis (MPS), and others.Measurements of lysosomal enzyme activities play an important role forthe clinical diagnosis of LSDs, because most of them result from a deficiency of one of these enzymes. In a few cases, non-enzymatic lysosomal proteins or non-lysosomal proteins involved in lysosomal biogenesis are deficient (Filocamo and Morrone, 2011).

Almost all LSDs show a broad clinical spectrum with respect to the severity of symptoms, age of onset and progression, which is considered to be related with the amount of accumulated

substrates and the different levels of residual enzyme activity (Wenger et al, 2002; Wenger et al, 2003;Wilcox, 2004). The demonstration of enzyme activity deficiency should be the first stepin the diagnosis of lysosomal disease (Winchester, 2005), which should be subsequently confirmed by identifying the genetic mutation (Menéndez, 2012).

The definitive enzyme diagnosis ofl ysosomal storage diseases is demonstrate the deficiency based on the function of aspecific protein,usually enzyme activity, which results in the accumulation or excretion of a particular product storage(Winchester, 2005).

Enzymatic diagnosis can be pre and postnatal, but the main drawback is the wide variability of enzymatic activities in affected patients (or fetuses), possibly due to residual enzyme activity in some cases associated with other factors (Gieselmann, 2005). Additionally, the activities reported for lysosomal enzymes are also very variable in presumably healthy populations, ranging from high specific activities -as is expected in apparently healthy subjects- to lower values, near those reported in patients (Wenger and Louie, 1991; Krasnopolskayaet al, 1993).

Very frequently, the decision on a specific clinical diagnosis for referral to molecular studies is based on the measurement of the percent relative activity(percentage of the specific activity of the patient with respect to that of a control subject assayed in parallel). Nevertheless, in our opinion, the great variability observed in healthy subjects can make the decision unreliable, especially when the patient has some degree of residual activity.The aim of this work was to evaluate the sensitivity and specificity of enzymatic diagnosis of LSDs based on the specific activity of the patients, considering the range of normal values in our laboratory for these enzymes in leukocytes and amniocytes.

Materials and Methods.

One hundred and fifty one patients withenzymatic diagnosisofLSDs and869apparently healthy individualswith no family historyof presentingany type ofLSD(Menéndez, 2012)were included. Seventy-twoamniocyte culturesat 13 and18 days were studied (12-suspected LSDs and 60 controls). The control group was conformed by healthypregnant women - with no family history of LSDs and agestational age between16-20 weeks as described by Vermaet al (1995) andMenéndez et al (2008). Enzyme activity in amniocytes for 13 diseases was measured in 12 pregnant women with gestational age between16-20 weeks, who requested prenatal diagnosis because of a previous child with LSD.

Sixteenlysosomal enzymes from leukocyteswere studiedin patientsand controlsforthe quantificationof enzymedeficiency bycolorimetricandfluorimetrictechniques as detailed previously (Menéndez, 2012). Normal specific enzyme activity was calculated for each enzyme in leukocytes and amniocytes. The sixteen lysosomal enzymes were: α-L-iduronidase(MIM#252800) (EC 3.2.1.76), n-acetylglucosaminidase(MIM#609701) (EC 3.2.1.50), Arilsulfatase B (MIM#611542) (EC 3.1.6.12), β-glucuronidase(MIM#611494) (EC 3.2.1.31), α-mannosidase(MIM#609458) (EC3.2.1.24), α-L-fucosidase(MIM#612280) (EC3.2.1.51), β-galactosidase(MIM # 611458) (EC3.2.1.2.3), Hexosaminidase A (MIM#606869) (EC3.2.1.52), Hexosaminidase A (MIM#606869) yB(MIM#606873) (EC3.2.1.52), Arylsulfatase A(MIM#607574) (EC3.1.6.8), β- glucosidase(MIM #606463) (EC 3.2.1.45), α-galactosidase(MIM #300644) (EC 3.2.1.22), α-1-4-glucosidase(MIM #606800) (EC 3.2.1.3), Arylsulfatases A,B,C(MIM#607939) (EC 3.1.6), Sphingomyelinase(MIM #257200) (EC 3.1.4.12), Acid Esterase (MIM #278000)(EC 3.1.1.13).

Ethical Procedure.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 2010. Informed consent was obtained from all patients for being included in the study. Proof that informed consent was obtained must be available upon request.

The integrity of theparticipantswas respected;protecting theirprivacy and the confidentialityof their information, in order to minimizethe impact ontheir physical andmental integrity.Genetic counselingwas offeredto all pregnant womenincluded in theamniocentesisstudy, through the Maternal and ChildCare Program.

Statistical analysis.

Normality andhomogeneity ofvariance of the enzyme activities were evaluated in normal subjects and patients. Medians of control and patient groups were compared through Mann-Whitney´s U test. ROCcurve analysiswasperformedto determinethe sensitivity and specificityof enzyme activityfor diagnosingthe enzyme deficiency. All testswere considered significantat $p<0.05$. Analyses were performedwithStatisticav8.0andSPSSv18.0programs.

Results.

Specific enzyme activity in leukocytes.

In table 1 the enzymatic activity of 16 lysosomal enzymes in patients and controls are shown. All enzyme activities were significantly lower in patients with respect to controls. No patients were diagnosed with the following LSDs: MPS IIIB, β-glucuronidase, Sandhoff ́s disease and MSD; while only one patient was diagnosed with Fabry ́s disease and Wolman ́s disease respectively.

Table 1. Lysosomal enzyme activities in leukocytes of patients and controls.

Enzymedeficiency (Disease)	Enzyme activity (nmoles*hour*mg protein).				Mann-W U test Z/p
	Patients	n	Controls	n	
α- L- iduronidase MPS I (MIM #607014)	4.8 (4.5-6.4)	27	8.6 (5.1-13.2)	284	2.26/0.0241
n-acetylglucosaminidase MPS IIIB (MIM #252920)	-	-	32.3 (22.1-70.3)	49	-
Arilsulfatase B MPS VI (MIM#253200)	50.4 (22.2-79.7)	9	185.8 (117.7-325.0)	222	5.08/0.0000
β-glucuronidase MPS VII (MIM#253220)	-	-	32.1 (15.4-55.9)	236	-
α-mannosidase α-Mannosidosis (MIM#609458)	11.8 (3.4-33.0)	15	43.5 (25.7-68.3)	246	5.33/0.0000
α-L-fucosidase Fucosidosis (MIM #230000)	3.3 (0.33-9.3)	15	19.6 (12.1-38.7)	272	6.44/0.0000
β-galactosidase GM_1Gangliosidosis (MIM#230500)	3.7 (1.9-10.0)	11	32.7 (17.2-51.4)	244	5.55/0.0000
Hexosaminidase A Tay-Sachs(MIM #272800)	39.1 (5.6-91.4)	8	212.8 (106-358)	117	4.72/0.0000
Hexosaminidases A y B Sandhoff(MIM #268800)	-	-	116.0 (58.2-208.7)	163	-
Arylsulfatase A MLD (MIM #250100)	9.7 (3.8-21.6)	9	41.6 (24.5-64.4)	254	5.06/0.0000
β- glucosidase Gaucher I (MIM # 230800)	0.9 (0.6-6.2)	11	12.1 (6.1-25.1)	150	4.67/0.0000
α-galactosidase Fabry(MIM #301500)	3.3	1	12.6 (4.9-23.0)	67	-
α-1-4-glucosidase Glycogenoses II (MIM #232300)	6.7 (3.9-9.9)	11	13.8 (6.3-22.0)	259	3.64/0.0003
Arylsulfatases A,B,C MSD (MIM #272200)	-	-	8.4 (5.4-18.6)	145	-

Enzymedeficiency (Disease)	Enzyme activity (nmoles*hour*mg protein).				Mann-W U test Z/p
	Patients	n	Controls	n	
Sphingomyelinase Niemann-Pick A (MIM # 257200) y B (MIM #607616)	22.3 (15.9-24.0)	3	62.1 (39.8-98.3)	135	2.86/0.0042
Acid Esterase Wolman ́s disease (MIM #27800)	4.5	1	45.5 (23.3-77.4)	20	-
Total determinations		**121**		**2863**	

Median (10-90 percentils)

EA: Enzimatic activity (nmoles*hour*mg protein).

n: Number of patients and controls.

MLD: metachromatic leukodystrophy

MSD: multiple sulfatase deficiency

Table 2 showsthe sensitivityand specificity, and theproposedcutoff values for 10 lysosomal enzymes. There was practically no overlap between patients and controls, and the sensitivity and specificity for enzymes corresponding to 8 diseases (80%) was very high (> 95%).β-glucosidase (Gaucher disease) and α-1-4-glucosidase (Glycogenoses II) had somewhat lower sensitivity and specificity (82.6% and 88% respectively).

Table 2.Sensitivityand specificity of specific lysosomalenzyme activities for the diagnosis of LSDs.

Lysosomal enzyme	n (controls)	Cutoff	Sensitivity	Specificity	p	AUC and 95% Confidence Interval
α- L- iduronidase	284	3.5	99.3%	88.0%	0.000	0.98 (0.96-1.01)
n-acetylglucosaminidase	222	95.6	100%	100%	0.000	1.00 (1.0-1.0)
Arylsulfatase B	246	22.5	100%	88.0%	0.001	0.89 (0.69-1.09)
β-glucuronidase	272	13.7	95.7%	100%	0.000	0.99 (0.98-1.01)
β-galactosidase	244	14.9	95.7%	100%	0.000	0.99 (0.98-1.01)
Hexosaminidase A	117	96.4	100%	100%	0.000	1.00 (1.0-1.0)
Arylsulfatase A	254	21.9	100%	100%	0.000	1.00 (1.0-1.0)
β- glucosidase	150	8.7	82.6%	88.0%	0.000	0.93 (0.83-1.03)
α-1-4-glucosidase	259	8.6	82.6%	88.0%	0.009	0.82 (0.63-1.01)
Sphingomyelinase	135	24.3	98.3%	100%	0.004	0.99 (0.96-1.01)

Median (10-90 percentils)

MLD: metachromatic leukodystrophy

AUC: area under de curve

Specific enzyme activity in amniocytes

Amniocyte cultures were evaluated for prenatal diagnosis of: MPS I, MPS VI, Fucosidosis, GM1 gangliosidosisGaucher´s disease, TaySach´s disease and Niemann Pick A/B.The decision on prenatal LSD diagnosis for the 12 amniocyte cultures from pregnancies with LSD suspicion had been previously established based on relative enzyme activity and published (Menéndez et al, 2008). All relative activities were above 50% and the prenatal diagnosis was considered negative for LSD. Only one prenatal diagnosis (for fucosidosis) displayed a lower relative activity (27.4%), and was referred for molecular diagnosis. Polymerase Chain Reaction analysis in this case revealed a double band (540 and 370 bp), corresponding with a heterozygous for the Q422X mutation. The specific activities for all amniocyte cultures with suspected LSD were well within the normal ranges reported for the respective enzyme, including the fucosidosis heterozygous fetus (10.1nmoles*hour*mg protein).It should be noted that the control amniocyte culture assessed in parallel with this patient displayed a very high specific activity (36.8 nmoles*hour*mg protein), outside the normal range presented in Table 3.

The normal range for lysosomal specific enzyme activities in amniocytes at 13 and 18 days culture is shown in table 3.

Table 3. Lysosomal enzyme activity in normal amniocyte cultures.

Deficiency (Amniocytes)	Controls (13 days culture)	n	Controls (18 days culture)	n	Mann-Whitney U text Z/p
α- L-iduronidase	0.6 (0.1-2.5)	45	1.2 (0.3-3.9)	29	-2.34/0.0189
Arilsulfatase B	27.3 (22.5-56.3)	15	54.4 (12.8-127.7)	55	-2.71/0.0067
β-glucuronidase	1.2 (0.6-5.1)	31	1.5 (0.8-3.4)	24	ns
α-mannosidase	17.8 (1.1-44.9)	37	16.8 (1.7-26.1)	28	ns
α-L-fucosidase	5.8 (0.9-16.1)	46	3.8 (0.5-21.7)	29	ns
Hexosaminidases A y B	40.5 (31.0-69.4)	15	105.8 (24.4-211.9)	14	-2.62/0.0088
Arylsulfatase A	6.9 (4.5-39.3)	14	18.8 (2.7-31.6)	14	ns
β- glucosidase	2.7 (0.4-9.3)	47	2.5 (0.4-9.8)	37	ns
α-galactosidase	0.8 (0.3-3.4)	33	1.5 (0.6-6.4)	28	-2.13/0.0326
α-1-4-glucosidase	0.9 (0.5-5.1)	31	1.2 (0.7-3.2)	26	ns
ArylsulfataseA,B y C	0.9 (0.3-2.3)	33	1.7 (0.4-2.8)	40	-2.19/0.0287
β-galactosidase	6.7 (0.8-16.6)	43	4.2 (2.8-9.7)	25	ns

Deficiency (Amniocytes)	Controls (13 days culture)	n	Controls (18 days culture)	n	Mann-Whitney U text Z/p
n-acetylneuraminidase	1.2 (0.7-2.7)	10	0.6 (0.3-2.2)	11	ns
Total determinations		**400**		**360**	

Median: (10-90 percentil)

MLD: metachromatic leukodystrophy

MSD: multiple sulfatase deficiency

ns: not significative.

Discussion.

The main resultsof this work showa great variability in the specific enzyme activities for all the LSDs assayed in both patient and control groups; nevertheless, patients displayed a highly significant decrease inthe activity oflysosomalenzymes compared tocontrols, confirming the suspected diagnosis of medical specialists. Thesensitivity andspecificity of enzyme activities for LSD diagnosis wasmainly above 95% in the majority of the diseases which were evaluated.Additionally,thenormalspecific activityof lysosomal enzymes inamniocyteswas reportedat 13and 18days cultures, as well as the results obtained in 12 prenatal diagnoses, all of which resulted negative for the suspected LSD.

Specific enzyme activities- although considerably decreased - were highly variable in patients. All patients assayed in this workhad some degree ofresidualenzymatic activity, in some caseshigher or lower thanreports in theinternationalliterature(Krasnopolskayaetal, 1993). This could be explained due to the high frequency of enzyme polymorphisms reported in genetic studies, as well as other unknown genetic and epigenetic factors, which can influence the phenotype to be, developed(Gieselmann, 2005). Other authors have also reported a wide variability in specific activity values (Galjaard et al, 1975; Alkhayat et al, 1998;Hopwood et al,1990). Additionally, it should be noted that some patients with a substantial residual enzymatic activity could present attenuated forms of the disease.

Despitethisresidualactivity in patients, thesensitivity andspecificity of enzymatic activitydetectionof the 10 studied enzymes washigh, in mostcasesapproaching 100%. Only β-glucosidaseandα-1,4-glucosidase showedsensitivity and specificityvaluesbetween82-88% andthe detection ofα-L-iduronidasereported an88%specificity.

Thenormal rangeof lysosomalspecific enzyme activities reported in the literatureis veryvariable(Meikleet al,2004; Civallero et al, 2006).Our studyis not far from this, we foundapparently healthysubjects withlow specific activity, but which never overlapswith the valuesof patients. Two main reasons could explain thiswide variationin healthy subjects:1)the methodology ofenzymatic diagnosis (method forcell isolation, cell extraction, storage and preservationof enzymes); and 2) genotypic characteristics of the study population, considering the prevalence of the disease, its phenotypic expression and the existence of pseudodeficiencies (deGasperiet al, 2000; Li et al, 2004). Families with pseudodeficiencieshave been reported in MLD and fucosidoses (Gort, 2000).

Emory Universityhas apanelfor lysosomalspecific enzyme activities employingdiagnostic methodology similarto ours (fluorimetricmethods in leukocytes), whichreported ranges of lysosomalenzymeactivity in healthysubjects.Despite the technological similarities, only the enzyme activity ofarylsulfataseA,α-L-iduronidaseandα-D-mannosidasein healthy individualswere analogous between our groupand Emory University ́s(http://geneticslab.emory.edu). On the otherhand, thespecific enzyme activity values for α-galactosidaseα-L-fucosidase, andβ-galactosidaseobtained by us in healthysubjects were2-4times lower than thosereported bythisUniversity; whilethe range of values forβ-glucosidase, hexosaminidase A and arylsulfatase enzyme activity in our study displayed a lower variability than those reported by Emory University (http://geneticslab.emory.edu).These differences could be explained by the high level of African and European admixture inCuba, with varying proportions, which are dissimilar to NativeAmerican populations (Mao et al, 2008). In addition, the African admixture in our population is higher than in US cities (Parra et al, 1998; Cintado et al, 2009). Another factor, which could be involved, is thepossibility of having some pseudodeficients in the healthy population.

Duringthe study periodof20 yearsthere were no differencesin the variabilityof enzyme activitiesin normal subjects with time; that is, the variability was the same in samples assayed at the beginning, middle or the end of the 20 year period.Thissupports the viewthat variabilityis not subject toa specifictime or to anymethodologicalor equipmentfeature,but must be mainly related to the reasons discussed above.

Lysosomal substrate storage may begin early during embryonic development, and the clinical presentation for LSDs can vary from an early and severe phenotype to late-onset mild disease (Filocamo and Morrone, 2011). Therefore when an affectedfetus is suspected, it isimportant

to detecttheenzyme deficiencyinthe prenatal stage. Prenatal diagnosis is performed on the most appropriate samples, which includefresh or cultured chorionic villus sampling or cultured amniotic fluid.Thefluorimetricenzymetestfrom chorionic villus is recommended, as it canbe done3-4 weeksearlier than amniocentesis, which is medically and ethically more favorable. Nevertheless,the choiceof obtainingthe sample byamniocentesisorchorionic villusdepends on thegestationalage at whichthepatientwas seenfor the first time (Aboul and Fateen, 2004).

The adventof prenatal diagnosishas radically altered themanagement ofpregnancy andperinatal outcomes.Many of the molecular, genetic, functional and structuralconditions affectingthe offspringcan be detectednowin uterus, andsometimeseven treatedbefore birth.For this reason,many couplesseek counselingbefore pregnancy, especiallywhen there has been a previously affected child or when the parents are known or suspected carriers of a disease withautosomal recessivetransmission (Casagrandi et al, 2005; Menéndez et al, 2008).

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 (Filocamo and Morrone, 2011). However, reducedlysosomalenzyme activity could define a specific diagnosiswhen the specific enzymatic activity of the patient isbelow the lower limit of the normal range reported in healthy subjects or below the cut off value obtained from the ROC curve for that enzyme,as shown by the high sensitivity and specificity obtained, in spite of the great variability observed in controls.Thus,the report of lysosomal specific enzyme activity is more accurate than the report of porcentual activity, because the last depends on the value of the control employed.

The high sensitivity and specificity of the enzymatic diagnosis of LSDs described in this article demonstrate its utility to identify subjects with the suspiction of lysosomal disease.The description ofthe normal rangeof16lysosomalenzymes inleukocytesand 13enzymes incultured amnioticisan important source ofinformation for futherstudiesthat could be reported in thisissue.

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