Update of the spectrum of mucopolysaccharidoses type III in Tunisia: identification of three novel mutations and *in silico* structural analysis of the missense mutations

Souad Ouesleti, Maria Francisca Coutinho, Isaura Ribeiro, Abdehedi Miled, Dalila Saidane Mosbahi, Sandra Alves

Porto, Portugal

Original article

Background: **Mucopolysaccharidoses type III (MPS III) are a group of autosomal recessive lysosomal storage diseases, caused by mutations in genes that code for enzymes involved in the lysosomal degradation of heparan sulphate: heparan sulfate sulfamidase (*SGSH*), *α*-N-acetylglucosaminidase (*NAGLU*), heparan sulfate acetyl-CoA: *α*-glucosaminide N-acetyltransferase (*HGSNAT*), and N-acetylglucosamine-6-sulfatase (*GNS*).**

Methods: **In this study, we have performed the molecular analysis of the *SGSH*, *NAGLU* and *HGSNAT* genes in 10 patients from 6 different MPS III Tunisian families.**

Results: **In the *SGSH* gene, two mutations were identified: one novel (p.D477N) and one already described (p.Q365X). In the *NAGLU* gene, two novel mutations were discovered (p.L550P and p.E153X). For the novel missense mutations found in these two genes we performed an *in silico* structural analysis and the results were consistent with the clinical course of the patients harboring those mutations. Finally, in *HGSNAT* gene, we found the splice-site mutation c.234+1G>A that had already been reported as relatively frequent in MPS IIIC patients from countries surrounding the basin of the Mediterranean sea. Its presence in two Tunisian MPS IIIC families points to the hypothesis of its peri Mediterranean origin. With the exception of the c.234+1G>A mutation, that was identified in two unrelated MPS IIIC families, the other identified mutations were family-specific and were always found in homozygosity in the patients studied, thus reflecting the existence of consanguinity in MPS III Tunisian families.**

Conclusions: **Three novel mutations are reported here, further contributing to the knowledge of the molecular basis of these diseases. The results of this study will allow carrier detection in affected families and prenatal molecular diagnosis, leading to an improvement in genetic counseling.**

World J Pediatr 2017;13(4):374-380

Key words: **molecular characterization; mucopolysaccharidosis III mutation**

Introduction

The mucopolysaccharidoses (MPSs) are a group of lysosomal storage disorders, caused by deficiency of enzymes catalyzing the stepwise degradation of glycosaminoglycans (GAGs) and characterized by intralysosomal accumulation and increased excretion in urine of partially degraded GAGs, which ultimately results in cell, tissue, and organ dysfunction. In general, MPSs are transmitted in an autosomal recessive fashion, except for MPS II, which is X-linked.[1] The Sanfilippo syndrome, or MPS III, is caused by impaired degradation of heparan sulfate[1] and includes four subtypes, each due to the deficiency of a different enzyme: heparan sulfate sulfamidase (MPS III type A; OMIM*252900), *α*-N-acetylglucosaminidase (MPS III type B; OMIM*252920), heparan sulfate acetyl-CoA: *α*-glucosaminide N-acetyltransferase (MPS III type C; OMIM*252930), and N-acetylglucosamine-6-sulfatase (MPS III type D; OMIM*252940). At the clinical level, all subtypes are quite similar, with a characteristic severe central nervous system degeneration associated with mild somatic disease. Onset of clinical features usually occurs between

Author Affiliations: Biochemical Service, CHU Farhat Hached, 4000, Sousse, Tunisia (Ouesleti S, Miled A); Research and Development Unit, Department of Human Genetics, National Institute of Health Dr. Ricardo Jorge, Porto, Portugal (Coutinho MF, Alves S); Biochemical Genetics Unit, Medical Genetics Center Dr. Jacinto de Magalhães, Porto Hospital Centre, Porto, Portugal (Ribeiro I); Laboratory of Analysis, Treatment and Valorization of Pollutants of the Environment and Products, Faculty of Pharmacy, University of Monastir, 5000, Tunisia (Mosbahi DS)

Corresponding Author: Sandra Alves, Research and Development Unit, Department of Human Genetics, INSA Rua Alexandre Herculano, 321,4000-055 Porto, Portugal (Tel: +351 223 401 100; Fax: +351223 401 109; Email: sandra.alves@insa.min-saude.pt, alvessandra@hotmail.com)

doi: 10.1007/s12519-017-0005-x
Online First January 2017

2 and 6 years, severe neurologic degeneration occurs in most patients between 6 and 10 years of age, and death occurs typically during the second or third decade of life. Type A has been reported to be the most severe, with earlier onset, rapid progression of symptoms, and shorter survival.[2-4]

MPS IIIA is caused by mutations in the heparan sulfate sulfamidase gene (*SGSH*);[5] MPS IIIB results from defects in the α-N-acetylglucosaminidase gene (*NAGLU*);[6] MPS IIIC is originated from mutations in the heparan sulfate acetyl-CoA: α-glucosaminide N-acetyltransferase gene (*HGSNAT*);[7,8] and MPS IIID, which is extremely rare, is caused by mutations in the N-acetylglucosamine-6-sulfatase gene.[9]

Previous molecular studies conducted in Tunisian MPS III patients[10] reported mutation heterogeneity and a high rate of homozygous patients, reflecting the effects of endogamous marriages in this population that is characterized by family-specific mutations and presence of various homozygous patients in the affected families. In fact, according to the Tunisian Office for Family and Population Affairs data, consanguinity remained relatively high during 1991 to 2001 with rates of close and unknown consanguineous unions representing, respectively, 21% and 19% of all marriages in the country.[11] When the mode of inheritance of a disease is autosomal recessive the majority of the patients (91.87%) have both parents from the same regional locality and consanguinity is present in 83% of the MPS Tunisian families.[11]

Here, we add new data to the Tunisian MPS III mutational spectrum by characterizing another 6 MPS III families (2 MPS IIIA, 2 MPS IIIB and 2 MPS IIIC). The patients were clinically characterized and the subsequent molecular analysis of the 3 corresponding genes revealed 5 different mutations in *SGSH*, *NAGLU* and *HGSNAT* genes (three of them are novel). Causal mutations were always found in homozygosity, an observation which reinforces previous reports on the presence of consanguinity in the MPS III affected families of Tunisia. It was also possible to establish a strong genotype-phenotype correlation, which is particularly helpful for genetic counseling of affected families.

Methods

Clinical diagnosis

Our sample included 10 Tunisian MPS III patients from 6 unrelated families. All the families were consanguineous. In families 1, 3 and 4 it was possible to analyse both parents. For families 2, 5 and 6, we didn´t have access to parents' samples. In those cases, although very unlikely due to the consanguinity of the families, the presence a deletion of the corresponding gene in one of the alleles cannot be completely ruled out. The diagnosis of each MPS III subtype was established according to the clinical symptoms (Table 1). All samples were obtained under informed consent and procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration.

Molecular diagnosis

Genomic DNA was isolated from whole blood according to the standard procedures. All exons and adjacent intronic regions of the *SGSH*, *NAGLU* and *HGSNAT* genes were amplified by polymerase chain reaction (PCR) (primers sequences and PCR amplification conditions available on request). Fragments were purified with ExoSAP-IT (GE

Table 1. Clinical information of the patients under study

Family and patient ID	MPS IIIA			MPS IIIB			MPS IIIC			
	Family 1 Patient 1	Family 1 Patient 2	Family 2 Patient 1	Family 3 Patient 1	Family 3 Patient 2	Family 4 Patient 1	Family 5 Patient 1	Family 5 Patient 2	Family 6 Patient 1	Family 6 Patient 2
Region	Sousse (East)	Sousse (East)	Siliana (Center)	Kairouan (Center)	Kairouan (Center)	Gabes (South)	Kairouan (Center)	Kairouan (Center)	Kélibia (Cap Bon)	Kélibia (Cap Bon)
Consanguinity	+	+	+	+	+	+	+	+	+	+
Age at diagnosis (y)	5	3	2	8	6	4	1	4	2	4
Present age (y)	6	4	8	11 (deceased)	9	8	11	14 (deceased)	13	15 (deceased)
Dysmorphic features	++	+	++	+	++	+	+	++	+	++
Dysostosis multiplex	++	+	+	++	++	+	+	++	+	++
Mental retardation	+	+	++	++	++	+	++	++	+	++
Deafness	+	+	+	+	ND	+	+	++	ND	ND
Hepatomegaly/ splenomegaly	+/+	+	+/+	+/+	+/ND	ND/+	+/+	+/+	+/+	+/+
Heart failure	ND	ND	+	+	+	+	ND	+	+	+
Behavioral disorders	+	ND	+	++	++	++	+	++	++	++
Other	Progressive loss of language Repeated lung infection	Delayed language	Macro-cephaly Repeated ear infections Hyper-telorism	Sleep disorder Hyper-trichosis	Epilepsy	Fragile and incomplete dentition Macro-glossy	Agitation	Seizures Tetraplegia	Tetraplegia Recurrent lung infections	Epilepsy Seizures Tetraplegia

The clinical characteristics listed in this table were reported in last medical visit. ND: not determinated; MPS: mucopolysaccharidoses; "+": present; "++": present with an increased intensity.

Healthcare, Waukesha, WI) and sequenced using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA). Results were analysed with FINCH TV 1.3.1 (Geospiza, Seattle, WA).

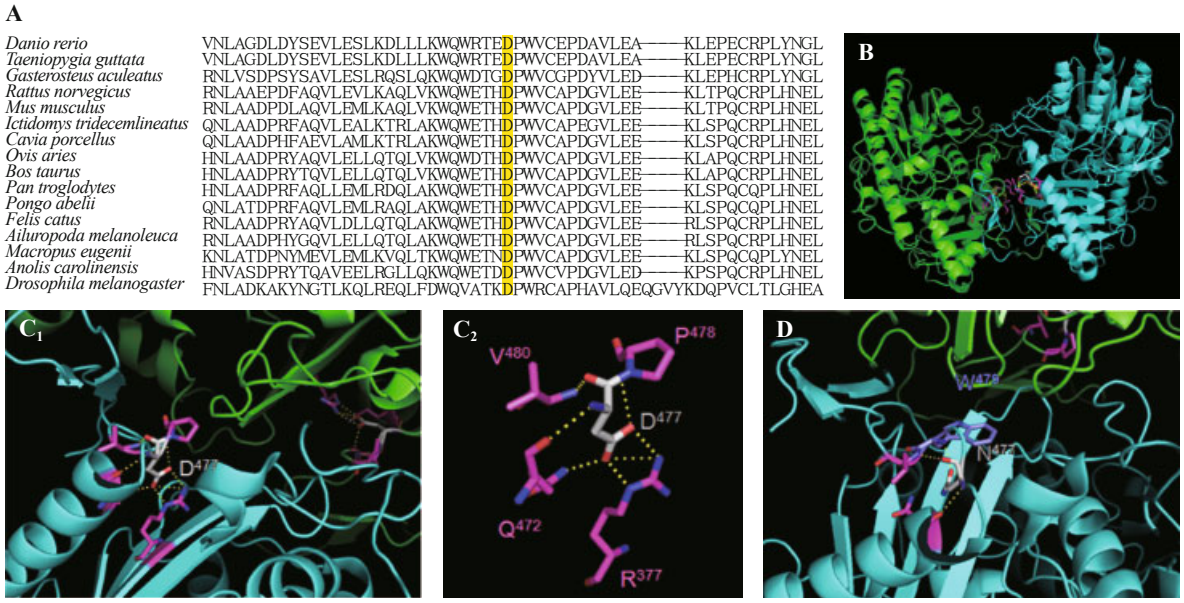

Fig. 1. Missense *SGSH* mutation detected in the MPS IIIA patients from family 2 (c.1429G>A; p.D477N). **A**: Alignment of SGSH protein around amino acid residue D477 (highlighted in yellow) from several species; **B**: Stereo figure showing three-dimensional structure of the SGSH dimmer with the amino acid position 477 (wild type: D477) highlighted in both chains. Relevant side chains that interact with the residue of interest are depicted and labeled. β-strands, helices and coils indicate the secondary structure elements that form the scaffold for the interacting residues. The three-dimensional structure analysed refers to the mature protein, which has 20 amino acids fewer than the coded SGSH (signal peptide); **C**: Zoomed in view of the wild-type D477 residue (in grey) and its various polar contacts and salt bridges with surrounding aminoacids: R377, Q472, P478 and V480 (in pink): 1) embedded in the protein's three-dimensional structure; 2) isolated (for easier analysis); **D**: Steric clash caused by the presence of a mutant N residue at position 477, with loss of two polar contacts (R377 and P478) and gain of an additional one (W479, in dark blue). SGSH: N-sulfoglucosamine sulfohydrolase; MPS: mucopolysaccharidoses.

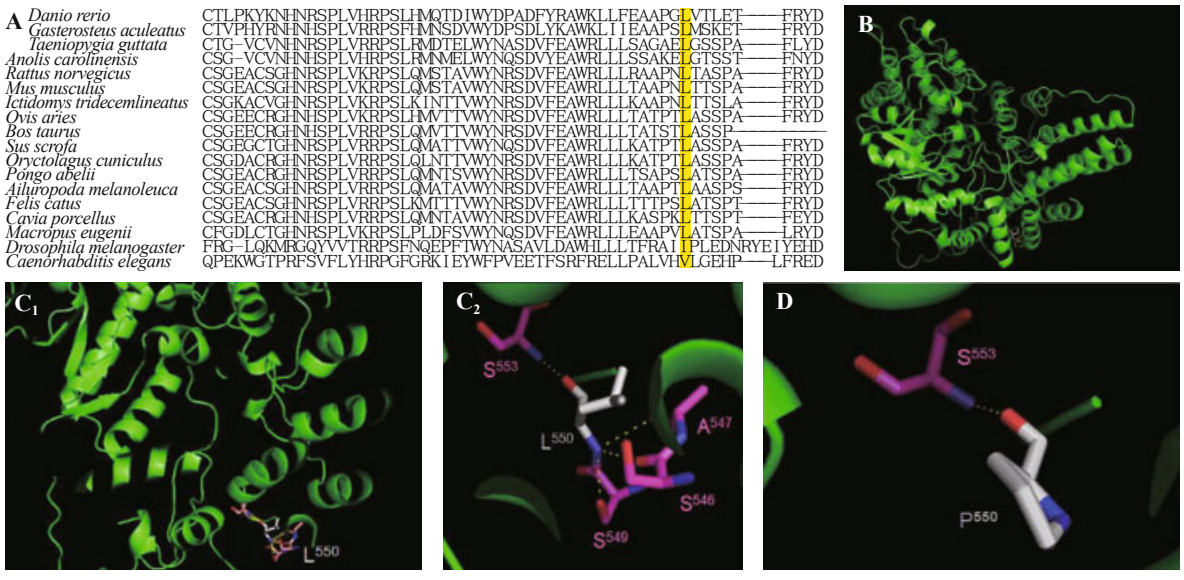

Fig. 2. Missense *NAGLU* mutation detected in the MPS IIIB patients from family 3 (c.1649T>C; p.L550P). **A**: Alignment of NAGLU protein around amino acid residue L550 (highlighted in yellow) from several species; **B**: Stereo figure showing three-dimensional structure of the NAGLU monomer with the amino acid position 550 (wild type: L550) highlighted. Relevant side chains that interact with the residue of interest are depicted and labeled. β-strands, helices and coils indicate the secondary structure elements that form the scaffold for the interacting residues. The three-dimensional structure analysed refers to the mature protein, which has 23 amino acids fewer than the coded NAGLU (signal peptide); **C**: Zoomed in view of the wild-type L550 residue (in grey) and its various polar contacts and salt bridges with surrounding aminoacids: S546, A547, S549 and S553 (in pink): 1) embedded in the protein's three-dimensional structure; 2) isolated (for easier analysis); **D**: Dramatic steric clash caused by the presence of a mutant P residue at position 550, with loss of three of the original polar contacts (S546; A547 and S549). NAGLU: N-acetyl-α-glucosaminidase; MPS: mucopolysaccharidoses.

The presence of each mutation was always confirmed in two independent amplifications. In addition, 50 healthy donors were screened for the newly identified mutations through gDNA sequencing.

Other analyses

To evaluate the potential effect of missense mutations on protein function, three bioinformatics tools were used: polymorphism phenotyping (PolyPhen) (*http://coot.embl.de/PolyPhen/*),[12] sorting intolerant from tolerant (SIFT) (*http://blocks.fhcrc.org/sift/SIFT.html*)[13] and protein variation effect analyzer (PROVEAN) (*http://provean.jcvi.org/index.php*).[14] Amino acid sequences of several species (Figs. 1 and 2) were retrieved from Ensembl (*http://www.ensembl.org/index.html*). Multiple alignments of the *SGSH* and *NAGLU* amino acid sequences were performed with the ClustalW program.

Structural analysis

Modelling of *NAGLU* tertiary structure was performed using the structure of the CpGH89 from *Clostridium perfringens* (a close bacterial homolog of *NAGLU*; Protein Data Bank file: 2VCC.pdb; *http://www.rcsb.org/pdb/explore.do?structureId=2VCC*) as template, as already proposed by Ficko-Blean et al.[15] Modelling was carried out by Swiss Model Server (*http://swissmodel.expasy.org/*).

The X-ray structure of the enzyme SGSH has already been determined,[16] and is available at the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB file: 4MIV.pdb; *http://www.rcsb.org/pdb/explore/explore.do?structureId=4MIV*).

For both proteins, *in silico* mutagenesis visualization and analysis were performed with PyMOL (*http://pymol.sourceforge.net*).

Results

Identification and characterization of mutations

In this study, we have analysed the entire coding regions of *SGSH*, *NAGLU* and *HGSNAT* in 6 Tunisian MPS III families and we were able to successfully characterize the changes in all patients (Table 2). Each newly detected variant was found not to be present in a minimum of 50 healthy individuals (100 control alleles), strongly suggesting that none of them was a polymorphic variation.

SGSH gene

In the two families with MPS IIIA, two different mutations, p.Q365X and p.D477N, were found in the *SGSH* gene (Table 2). In family 1, the affected child was found to be homozygous for the nonsense mutation p.Q365X[17] that had already been described in one MPS III Tunisian family.[10] Both parents were heterozygous for the same mutation. This mutation is due to a C to T transition at nucleotide 1093, generating a stop codon at position 365. Its presence reduces the encoded protein that lacks the last 138 residues, which would result in a total absence of enzyme function.

In the other MPS IIIA family, we detected the presence of a novel missense mutation in the two affected siblings. This mutation is caused by a G to A substitution at nucleotide 1429 and originates the replacement of a residue of aspartic acid, an acidic and charged amino acid, by asparagine, which is a non-charged amino acid, at position 477 (p.D477N). When submitted to the predictive methods implemented in PolyPhen, SIFT and PROVEAN this mutation was scored as potentially damaging to the protein function with a high probability of functional impairment (Table 2). The amino acid substitution affects a residue which is highly conserved among the species analysed and the three-dimensional modeling of this missense mutation clearly indicates that it is deleterious to molecular protein function (Fig. 1) since it causes a steric clash by the loss of two polar contacts (R377 and P478), further altering the structure through the gain of an additional one (W479).

NAGLU gene

Regarding the MPS IIIB families, two novel mutations, p.L550P and p.E153X, were found in the *NAGLU* gene.

The p.L550P missense mutation was found in homozygosity in the two affected siblings of family

Table 2. Mutations identified in MPS III Tunisian patients

Family	Pathology	Gene	Mutation (cDNA)	Mutation (protein)	Exon/intron affected	Prevision of the effect of the mutation in protein function			Reference
						Polyphen	SIFT	PROVEAN	
1	MPS IIIA	*SGSH*	1093C>T/1093C>T	Q365X	e8	NA	NA	NA	18
2	MPS IIIA	*SGSH*	1429G>A/1429G>A	D477N	e9	Probably deleterious	P<0.05 not tolerated	Deleterious	This study
3	MPS IIIB	*NAGLU*	1649T>C/1649T>C	L550P	e6	Probably deleterious	P<0.05 not tolerated	Deleterious	This study
4	MPS IIIB	*NAGLU*	457G>T/457G>T	E153X	e2	NA	NA	NA	This study
5 and 6	MPS IIIC	*HGSNAT*	234+1G>A/234+1G>A	D68VfsX19	i2	NA	NA	NA	8

NA: not applicable; MPS: mucopolysaccharidoses; *SGSH*: N-sulfoglucosamine sulfohydrolase gene; *NAGLU*: N-acetyl-α-glucosaminidase gene; *HGSNAT*: heparan α-glucosaminide N-acetyltransferase gene; Polyphen: polymorphism phenotyping; SIFT: sorting intolerant from tolerant; PROVEAN: protein variation effect analyzer.

3, and in heterozygosity in both parents. It is caused by a T to C transition at nucleotide 1649 that leads at the protein level to the replacement of leucine, which is a non polar highly hydrophobic amino acid, by proline, a basic and less hydrophobic amino acid. All bioinformatic tools predict this amino acid substitution to be pathogenic (Table 2). The affected residue (L550) is highly conserved among species and the three-dimensional modeling of this missense mutation clearly indicated that its alteration to a proline residue is deleterious to the protein function (Fig. 2) since it leads to the complete loss of three of the original polar contacts established by the original leucine residue (S546; A547 and S549). The nonsense mutation, p.E153X was found in homozygosity in the affected child of the family 4 and in heterozygosity in both parents.

HGSNAT gene

In the two MPS IIIC families studied (families 5 and 6), an already described splice-site mutation, c.234+1G>A,[8] was found.

Discussion

Genotype-phenotype correlations are not always straightforward and, for many diseases, only a small subset of mutations is known to reliably predict phenotype.[18] Nevertheless, in this study we have tried to establish a correlation between the clinical phenotype of each patient and its respective genotype. Our analysis was prompted by the knowledge that such data could be very useful for genetic counseling of the affected families.

In the *SGSH* gene, two different mutations, p.Q365X and p.D477N were found. Mutation p.Q365X had already been associated with a severe phenotype[17] and, in the two affected siblings of this family, its presence is also associated with a severe form of MPS IIIA (Table 1). This is not the first time that the D477 residue appears to be implicated on the genesis of an early-onset, severe form of MPS IIIA; another missense mutation involving this residue, p.D477E, has been recently reported.[19] Furthermore, being a charged, highly conserved amino acid, this particular aspartic acid residue is involved in several polar contacts and salt bridges, which have a major role in keeping the integrity of *SGSH* three-dimentional structure. In fact, four additional *SGSH* disease-causing mutations affecting residues that are tightly linked with D477 have already been reported: p.Q472H;[20] p.R377C;[21] p.R377H;[22] and p.R377L.[20] All of them have an effect at protein level that is quite dramatic, by the simple disruption of the interaction between the affected residues (Q472 and R377) and the aspartic acid at position 477.[16] The mutations p.R377L, p.R377H and p.R377T cause the loss of a buried salt bridge with D477, and p.Q472H causes the loss of an H bond to D477. Both p.R377L and p.R377H were described in early onset patients. In this way, it is expected that any alteration in this residue leads to similar changes in the structure of the *SGSH* and, consequently, to the protein's loss of function and ultimately to disease. The presence of this mutation was associated with an early onset phenotype with the presence of dysostosis multiplex, mental retardation, hepatomegaly, deafness and language problems.

Two novel mutations, p.L550P and p.E153X, were found in the *NAGLU* gene. Ficko-Blean et al.[15] described the structure, catalytic mechanism, and inhibition of CpGH89 from *Clostridium perfringens*, a close bacterial homolog of NAGLU, which structure enabled the generation of a homology model of the human NAGLU enzyme. The residue L550 is conserved in the *Clostridium perfringens* sequence, and located in the core of the enzyme, within a region between two beta sheets. The presence of this mutation is most probably responsible for alterations in the structure of the enzyme therefore causing the phenotype observed in both affected siblings of this family. The presence of this mutation was associated in both siblings to severe phenotype with the presence of mental retardation, dysostosis multiplex, hepatomegaly, heart failure. The mutation p.E153X creates a premature STOP codon, leading to the formation of truncated protein with 590 amino acids missing, which is most likely a non-functional product. In addition, since this nonsense mutation creates a premature termination codons (PTC), it is expected to trigger the nonsense-mediated mRNA decay (NMD) pathway. Given these predictions, it is expected that any patient harboring this mutation will present a severe form of the disease. This was in fact observed in the affected child of the family 4 who presented early onset of the disease with the presence of mental retardation, dysostosis multiplex, deafness, splenomegaly, heart failure and behavioral disorders. Other mutation involving the same codon, p.E153X was previously reported,[23] which could mean that this codon is especially prone to mutation. In fact, Sanfilippo syndrome type B shows extensive molecular heterogeneity and common mutations have not been identified in MPS IIIB patients.[2] An exception, are mutations involving codon R565. The p.R565W mutation was found in several MPS IIIB patients[2] and in the same codon, mutations p.R565Q and p.R565P,[24-26] were also described suggesting that this codon is a mutational hotspot in the *NAGLU* gene. This could also be case of codon E153.

In the *HGSNAT* gene, the mutation c.234+1G>A is relatively frequent, and it has already been identified in MPS IIIC patients from Spain, Morocco, Turkey, France, Italy[27] and Portugal (personal unpublished data). Its presence in two MPS IIIC Tunisian families, together with its previous reported geographical distribution, points to the hypothesis of a peri-Mediterranean origin and spread of this mutation. Further studies involving an haplotypic analysis to the patients with this mutation will allow to test the validity of this hypothesis. In fact, a previous haplotype analysis conducted by Canals et al.[28] only in the Spanish and Moroccan patients carrying this mutation, has already shown a common origin for the c.234+1A mutant allele.

This splice site mutation gives rise to a truncated protein that most probably activates the NMD, a mechanism that recognizes and rapidly degrades mRNAs containing PTCs. The presence of the c.234+1G>A mutation has been associated with the classical symptoms of the disease[27] as those seen in the patients here studied who presented early onset of disease, dysostosis multiplex, mental retardation, organomegaly and behavioral disorders.

In conclusion, in this study, we have screened for the causal mutations in 2 families with MPS IIIA, 2 families with MPS IIIB and 2 families with MPS IIIC, identifying 5 different mutations. Among those, three are reported here for the first time: p.D477N in the *SGSH* gene and p.L550P and p.E153X in the *NAGLU* gene, adding new data to the worldwide mutational spectrum of each of these pathologies. For the novel missense mutations, we performed *in silico* predictions on pathogenic impact that, overall, were shown to be consistent with the clinical course of the patients harboring the mutations. The molecular diagnosis in these patients will be particularly relevant for prenatal diagnosis and for providing better genetic counseling. Such knowledge is important not only for discerning the pathophysiological mechanisms underlying the diseases, but also because it allows the implementation of family-specific surveillance strategies.

Funding: This study was partially supported by Millennium bcp Foundation (bcp/LIM/DGH/2014). Coutinho MF is grantee from the Foundation for Science and Technology Portugal (SFRH/BPD/101965/2014).

Ethical approval: All samples were obtained under informed consent and procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration.

Competing interest: None declared.

Contributors: Ouesleti S participated in the conception and design of the study, acquisition of data, revision of the article for important intellectual content. Coutinho MF participated in the acquisition, analysis and interpretation of data, and article writing Ribeiro I participated in the acquisition of data, revision of the article critically for important intellectual content. Miled A and Mosbahi DS participated in the conception and design of the study, revision of the article for important intellectual content. Alves S participated in the conception and design of the study, analysis and interpretation of data, article writing. All authors approved the final version of the manuscript to be published.

References

1 Neufeld EF, Muenzer J. The mucopolysaccharidoses. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. The metabolic and molecular bases of inherited disease, 8th ed. New York: McGrawHill, 2001: 3421-3453.
2 Yogalingam G, Hopwood JJ. Molecular genetics of mucopolysaccharidosis type IIIA and IIIB: diagnostic, clinical, and biological implications. Hum Mutat 2001;18:264-281.
3 Feldhammer M, Durand S, Mrázová L, Boucher RM, Laframboise R, Steinfeld R, et al. Sanfilippo syndrome type C: mutation spectrum in the heparan sulfate acetyl-CoA: alpha-glucosaminide N-acetyltransferase (*HGSNAT*) gene. Hum Mutat 2009;30:918-925.
4 Coutinho MF, Lacerda L, Alves S. Glycosaminoglycan storage disorders: a review. Biochem Res Int 2012;2012:471325.
5 Scott HS, Blanch L, Guo XH, Freeman C, Orsborn A, Baker E, et al. Cloning of the sulphamidase gene and identification of mutations in Sanfilippo A syndrome. Nat Genet 1995;11:465-467.
6 Zhao HG, Li HH, Bach G, Schmidtchen A, Neufeld EF. The molecular basis of Sanfilippo syndrome type B. Proc Natl Acad Sci USA 1996;93:6101-6105.
7 Fan X, Zhang H, Zhang S, Bagshaw RD, Tropak MB, Callahan JW, et al. Identification of the gene encoding the enzyme deficient in mucopolysaccharidosis IIIC (Sanfilippo disease type C). Am J Hum Genet 2006;79:738-744.
8 Hrebícek M, Mrázová L, Seyrantepe V, Durand S, Roslin NM, Nosková L, et al. Mutations in TMEM76* cause mucopolysaccharidosis IIIC (Sanfilippo C syndrome). Am J Hum Genet 2006;79:807-819.
9 Mok A, Cao H, Hegele RA. Genomic basis of mucopolysaccharidosis type IIID (MIM 252940) revealed by sequencing of *GNS* encoding N-acetylglucosamine-6-sulfatase. Genomics 2003;81:1-5.
10 Ouesleti S, Brunel V, Ben Turkia H, Dranguet H, Miled A, Miladi N, et al. Molecular characterization of MPS IIIA, MPS IIIB and MPS IIIC in Tunisian patients. Clin Chim Acta 2011;412:2326-2331.
11 Ben Halim N, Ben Alaya Bouafif N, Romdhane L, Kefi Ben Atig R, Chouchane I, Bouyacoub Y, et al. Consanguinity, endogamy, and genetic disorders in Tunisia. J Community Genet 2013;4:273-284.
12 Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, et al. A method and server for predicting damaging missense mutations. Nat Methods 2010;7:248-249.
13 Kumar P, Henikoff S, Ng PC. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. Nat Protoc 2009;4:1073-1081.
14 Choi Y, Chan AP. PROVEAN web server: a tool to predict the functional effect of amino acid substitutions and indels. Bioinformatics 2015;31:2745-2747.

15 Ficko-Blean E, Stubbs KA, Nemirovsky O, Vocadlo DJ, Boraston AB. Structural and mechanistic insight into the basis of mucopolysaccharidosis IIIB. Proc Natl Acad Sci USA 2008;105:6560-6565.
16 Sidhu NS, Schreiber K, Pröpper K, Becker S, Usón I, Sheldrick GM, et al. Structure of sulfamidase provides insight into the molecular pathology of mucopolysaccharidosis IIIA. Acta Crystallogr D Biol Crystallogr 2014;70:1321-1335.
17 Esposito S, Balzano N, Daniele A, Villani GR, Perkins K, Weber B, et al. Heparan N-sulfatase gene: two novel mutations and transient expression of 15 defects. Biochim Biophys Acta 2000;1501:1-11.
18 Dipple KM, McCabe ER. Modifier genes convert "simple" Mendelian disorders to complex traits. Mol Genet Metab 2000;71:43-50.
19 Héron B, Mikaeloff Y, Froissart R, Caridade G, Maire I, Caillaud C, et al. Incidence and natural history of mucopolysaccharidosis type III in France and comparison with United Kingdom and Greece. Am J Med Genet A 2011;155A:58-68.
20 Pollard LM, Jones JR, Wood TC. Molecular characterization of 355 mucopolysaccharidosis patients reveals 104 novel mutations. J Inherit Metab Dis 2013;36:179-187.
21 Di Natale P, Balzano N, Esposito S, Villani GR. Identification of molecular defects in Italian Sanfilippo A patients including 13 novel mutations. Hum Mutat 1998;11:313-320.
22 Weber B, Guo XH, Wraith JE, Cooper A, Kleijer WJ, Bunge S, et al. Novel mutations in Sanfilippo A syndrome: implications for enzyme function. Hum Mol Genet 1997;6:1573-1579.
23 Schmidtchen A, Greenberg D, Zhao HG, Li HH, Huang Y, Tieu P, et al. *NAGLU* mutations underlying Sanfilippo syndrome type B. Am J Hum Genet 1998;62:64-69.
24 Beesley CE, Young EP, Vellodi A, Winchester BG. Identification of 12 novel mutations in the alpha-N-acetylglucosaminidase gene in 14 patients with Sanfilippo syndrome type B (mucopolysaccharidosis type IIIB). J Med Genet 1998;35:910-914.
25 Bunge S, Knigge A, Steglich C, Kleijer WJ, van Diggelen OP, Beck M, et al. Mucopolysaccharidosis type IIIB (Sanfilippo B): identification of 18 novel alpha-N-acetylglucosaminidase gene mutations. J Med Genet 1999;36:28-31.
26 Weber B, Guo XH, Kleijer WJ, van de Kamp JJ, Poorthuis BJ, Hopwood JJ. Sanfilippo type B syndrome (mucopolysaccharidosis III B): allelic heterogeneity corresponds to the wide spectrum of clinical phenotypes. Eur J Hum Genet 1999;7:34-44.
27 Feldhammer M, Durand S, Mrázová L, Boucher RM, Laframboise R, Steinfeld R, et al. Sanfilippo syndrome type C: mutation spectrum in the heparan sulfate acetyl-CoA: alpha-glucosaminide N-acetyltransferase (*HGSNAT*) gene. Hum Mutat 2009;30:918-925.
28 Canals I, Elalaoui SC, Pineda M, Delgadillo V, Szlago M, Jaouad IC, et al. Molecular analysis of Sanfilippo syndrome type C in Spain: seven novel *HGSNAT* mutations and characterization of the mutant alleles. Clin Genet 2011;80:367-374.

Received June 26, 2015
Accepted after revision November 25, 2015