Determination of Reference Values for Alpha-N-Acetylglucosaminidase Activities in Patients with Sanfilippo Type B Disease and Control Population in Colombia

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Abstract
Sanfilippo B is a lysosomal disorder characterized by the pathological accumulation of heparan sulfate. It is caused by mutations in the NAGLU gene that codes for the alpha-N-acetylglucosaminidase enzyme. The objective of this study was to determine the reference values and frequency of Sanfilippo B in Colombia through an enzyme analysis of leukocytes extracts. We aim to inform the community and the health system so that they can work in a preventive way, providing an early diagnosis of patients and thus providing an appropriate management of the symptoms. We carried out an endpoint assay that indirectly quantifies NAGLU activity through the cleavage of 4-methylumbelliferone from the 4-methylumbelliferyl-2-acetamido-2-deoxy-α-D-glucopyranoside substrate. The activity of 463 healthy volunteers (Range: 0.6 - 4 nmol/mg/h, Median: 1.69 +/- 0.73) as well as 462 patients referred for clinical suspicion, was calculated. From the last group, 7 cases turned out to be positive (Range: 0 - 0.24 nmol/mg/h, Median: 0.13 +/- 0.09). The cut-off point according to ROC analysis between affected patients and controls was 0.42 nmol/mg/h. To our knowledge, this study is the first in Colombia where an estimated frequency of Sanfilippo type B is calculated by providing enzyme activity ranges and a cut-off point.

Keywords
Mucopolysaccharidosis III, heparan sulfate, Sanfilippo syndrome B, NAGLU deficiency, fluorometric endpoint assay.

Introduction
Mucopolysaccharidosis (MPS) are a group of lysosomal disorders where the abnormal and pathological accumulation of a partially degraded glycosaminoglycan (GAG) is observed. In consequence, the subtype of MPS and the corresponding clinical manifestations are determined by the enzyme that is affected or absent in the degradation pathway of a certain GAG. [1] Currently, a total of 7 subtypes of MPS are known, which are caused by deficiencies of 11 enzymes. It is also known that each type of GAG tends to accumulate to a greater extent in certain organs, and thus, gives rise to a particular set of symptoms. For example, heparan sulfate (HS) accumulation produces mostly neurological symptoms and is involved in MPS I, II, III and VII[2]; keratan sulfate is related to corneal opacity and skeletal alterations observed in MPS IV[2]; chondroitin sulfate is the common GAG found in MPS IVA, VI[3] and VII[4,5]; and dermatan sulfate, observed in MPS I, II and VI[2,6], has been shown to be responsible for cardiopathies[2], abnormal cartilage matrix homeostasis[7] and impaired elastic fiber assembly.[8]

MPS III, also called Sanfilippo disease, is one of the MPS disorders. It stands out among the other MPS disorders because the central nervous system (CNS) is highly affected, while the somatic component is minimal. It is inherited in an autosomal recessive manner[9] and one of its indicators is the accumulation of HS. When the HS degradation is affected, it begins to accumulate in lysosomes and therefore within cells, affecting multiple organs and systems, and causing its excessive excretion in the urine. The first manifestations of this disease involve the appearance of mildly dysmorphic facial features, developmental

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delay and speech impairment.[9] These symptoms are usually followed by severe behavioral problems, which occur between 3 and 5 years of age, and include hyperactivity, anxiety, and destructive or aggressive behaviors. As the MPS III progresses, the cognitive decline accelerates until the patient is bedridden or even in a vegetative state, which significantly reduces the life expectancy and quality of life.[10]

Sanfilippo disease is divided into 4 subtypes (A, B, C, and D), each one characterized by the deficiency of a different lysosomal enzyme, but all of them being involved in the catabolism of the HS. More specifically, Sanfilippo type B or MPS III B (OMIM # 252920) is caused by mutations in the NAGLU enzyme which codes for the enzyme alpha-N-acetylglucosaminidase[11] or also called NAGLU (EC 3.2.1.50). This gene is known to reside at position 21.2 of the long arm of chromosome 17 and to be approximately 8.2kb in length.[12] According to an update in the NCBI database in October 2019, it has been reported that NAGLU (gene ID: 4669) has 7 exons instead of 6. More than 170 mutations have been reported, and the majority are located in the last exon[13,14], which is known to have the greatest length (1210 bp) compared to the others (382 bp, 56 bp, 147 bp, 146 bp, 85 bp, and 256 bp respectively).

The diagnosis of this disease begins with the medical observation of the first clinical symptoms, which occurs when the parents of the patient start to identify those signs and decide to see a doctor. However, this first attempt is usually hampered since some of the symptoms can go unnoticed or be confused with others belonging to more common diseases.[10] Following the medical examination, it is recommended to quantify and analyze the presence of GAGs in the urine. This process can be carried out by the implementation of low sensitivity methods, such as: turbidity tests, which rely on the interaction between GAGs and a cationic detergent or albumin;[15] the berry test, in which dried urine spots are commonly stained with toluidine blue to detect metachromatic staining of urinary acid mucopolysaccharides;[16] and the precipitation of uronic acids with carbazole.[17] However, the first two techniques have caused false-negative results in Sanfilippo and morquio diseases.[18] Also, there are more sensitive and commonly used techniques such as dimethylmethylene blue staining[19] and even spectrophotometry. However, these tests require a considerable volume of urine and the results are highly dependent on the urine’s pH and concentration.[20,21]

A definitive diagnosis requires the quantification of the enzyme activity in cell extracts such as fibroblasts or leukocytes. This method is the gold standard, because it allows the type of MPS (III, for example) and also the corresponding subtype (A, B, C, or D) to be identified, since it assesses the specific enzyme involved in each case. To do so, it is necessary to use an artificial substrate labeled with a fluorogenic compound, such as 4-methyl-umbelliferone.[10] A labeled substrate allows for the determination of the enzyme’s activity through cleavage of the substrate liberating the fluorophore that can then be measured.[10]

The diagnosis of MPS in Colombia starts with medical assessment and continues with the performance of medical tests that evaluate the biochemical profiles of urine, cell extracts, and dried blood spots (DBS).[22,23] These analyses give information about GAGs storage and enzyme activity, but they need to be carried out in specialized laboratories, which are not commonly found across the country. The first screening study for mucopolysaccharide metabolism disorders in Colombia was not carried out until 2002 by our laboratory, and it involved 140 individuals with clinical suspicion of MPS.[24]

To date we have not found confirmatory enzyme studies for Sanfilippo type B disease in Colombia. This is a problem since there is not much information about the frequency of the disease within the Colombian population and therefore it is an underestimated condition. The main objective of this work is to characterize the presence of Sanfilippo type B disease in Colombia through a NAGLU enzyme analysis in leukocytes extracts. We hope that by screening patients with clinical suspicion of MPS in Colombia, we can contribute to the early diagnosis of this disease and alert the community and the government about its presence. We know that if an accurate and prompt diagnosis is provided, this will benefit the health system, and most importantly, this will aid in the management of patients’ symptoms, which will improve their quality of life.

In this report, we determined the reference range for NAGLU activity, we found the percentage of positives within the analyzed sample and we established a cut-off point which will allow the medical community to differentiate between healthy volunteers (HV) and patients affected by MPS IIIB.

Methods

Sample Collection

The first study group consisted of a sample of 468 HV, comprised of individuals between 0.5 and 70 years of age, who were not clinically defined for MPS. These volunteers signed informed consent forms and agreed autonomously to provide a blood sample of 10 ml (venipuncture). The second group consisted of 462 high-risk (HR) patients, whose samples were submitted from different regions of Colombia and were referred to the Biochemistry Research Center (CIBI) due to clinical suspicion of MPS. The age range for this group was between 0.4 and 38.5 years. In both cases, the samples were collected between the years 2015 and 2019.

Enzyme Activity Test

The test was based on the protocol proposed by Shapira et al.[25] and consisted of a fluorometric endpoint assay, which indirectly evaluated NAGLU activity through the release of 4-methylumbelliferone. To carry out the analysis of the enzyme of interest, 4-methylumbelliferyl-2-acetamido-2-deoxy-alpha-glucopyranoside was used as the substrate. Likewise,
4-methylumbelliferyl-D-Galactoside was used for the analysis of the control enzyme β-galactosidase (see Table 1). As a fluorescence calibrator, 4-methylumbelliferone (4-MU) was used. All substrates and reagents were obtained from Sigma (St. Louis, MO, USA) and are listed in Table 1.

The assays were carried out in 96-well black polypropylene microplates sealed with aluminum foil sheets, to avoid evaporation of the samples during incubation (Corning Lowell, USA). Both the elution and the incubation required the use of an orbital shaker (Titramax 1000 or Unimax 1010, Heidolph group, Schwabach, Germany). A Spectramax M2 (Molecular Devices Corp) was used to read fluorescence at the end of each assay.

Following the guidelines in Table 1, the NAGLU activities of 468 HV and 462 HR patients, were determined in duplicate. The amount of protein in each sample was also determined in duplicate once with the method of Lowry et al.[26] and the other with the BCA Protein Assay (Thermo Scientific, Rockford, USA). Based on the calibration curve for 4-MU and the protein quantification of each sample, the enzymatic activity was expressed in nanomoles of degraded substrate per milligram of protein per hour (nmol/mg/h).

**Statistics**

The statistical analysis, was carried out using Minitab 17, Microsoft Excel, and the IBM SPSS Statistic 19 statistical package provided by the Statistical Package for the Social Sciences software IBM SPSS Statistic Inc. SPSS19 (Chicago, Illinois, USA). The Shapiro-Wilk test was used to determine whether the data follow a normal distribution, and the Mann-Whitney test was applied to check if there was a significant difference between the study groups. The graphs were made with GraphPad Prism software, and the calculation of the cut-off point (to discriminate HV from the affected patients) was calculated using the Receiver Operating Characteristics (ROC) curve analysis, also from SPSS software.

**Results and Discussion**

The enzymatic analysis of both groups (HV and HR) yielded the activity values and the distributions in Figure 1. In accordance with this figure, the Shapiro-Wilk normality test showed that, despite the size of the samples (463 and 462 individuals), no set of data follows a normal distribution; on the contrary, they show a certain degree of positive asymmetry. After performing an outlier test, 5 values were discarded from the HV group, while the outliers for the HR group were not eliminated (see orange arrow in Figure 1), because the overexpression of NAGLU may be a risk indicator of other conditions. Even though these patients do not suffer from Sanfilippo type B, they could be overexpressing NAGLU as a compensatory mechanism if they

| Enzyme | Reaction buffer | Substrate | Incubation time / °C | Stop buffer |
|--------|----------------|-----------|----------------------|-------------|
| alpha-N-acetylglucosaminidase | Citrate phosphate 0.2 M, pH 4.3 | 4-Methylumbelliferyl-2-acetamido-2-deoxy-alpha-glucopyranoside | 2 h / 37 °C | Glycine carbonate 0.17 M, pH 10 |
| β-Galactosidase | Citrate Phosphate 0.1 M, pH 4.4 | 4-Methylumbellifer -D-Galactoside, 0.8 mM | 20 min / 37 °C | Glycine carbonate 0.17 M, pH 10 |

**Table 1.** Reagents and conditions used to estimate NAGLU activity.[25]

**Figure 1.** On the left, in green, the distribution of NAGLU activity in healthy volunteers is shown. On the right, in blue, the distribution of NAGLU activity for patients remitted for clinical suspicion of MPS. The grey arrow highlights the 7 patients found to be affected by Sanfilippo disease, while orange arrow represents patients with overexpression of NAGLU.
have a deficiency of another lysosomal enzyme or, they may suffer from another disease/condition. For example, lysosomal acid lipase has been shown to be overexpressed in atherosclerosis[27] and β-hexosaminidase has been detected at a twofold increase in conditioned media containing overexpressed pro-IGF II.[28]

It may also be possible that some patients overexpress NAGLU because HS is being abnormally accumulated in the body, due to other pathologies.[29,30]

To discard experimental error as a cause of the elevated values, we assessed the quality of these samples according to the activity obtained for the control enzyme, β-Galactosidase. We found that all data was within the range of reference (80.1 to 557 nmol/mL/h) established by Uribe et al.[31] in a previous report on this enzyme in Colombia, so this possibility was discarded.

Figure 2 demonstrates the high rate of outliers for the high-risk patients compared to the healthy population. This fact suggests that, although these patients are not positive for MPS III B, we highly recommend the analyses of the activities of other enzymes involved in the HS degradation pathway, or the activities of enzymes deficient in pathologies with similar symptoms. Figure 2 also shows that there is no significant difference in terms of median and standard deviation (HV: 1.69 +/- 0.73 nmol/mg/h vs HR: 1.70 +/- 0.91 nmol/mg/h) between control and high-risk groups. This result was supported by the Mann Whitney test in which the P-value of 0.91 indicated that these two samples are not significantly different.

Continuing with the statistical analysis, we found that 7 of the 462 patients referred for clinical suspicion of MPS III B were positive for this disease. The enzyme activity values for these patients can be seen on the left side of the distribution, corresponding to the lowest values compared with the rest of high-risk patients. The maximum value found in affected individuals was 0.24 nmol/mg/h (see gray arrow in Figure 1).

Similar values have been obtained in affected patients by Marsh[32] who reported 0.32 nmol/mg/h in leukocytes, and by Meijer[33] who described a median activity of 0.17 nmol/mg/h and 0.27 nmol/mg/h for the severe phenotype and less severe phenotype, respectively, in fibroblasts.

Based on the values obtained, we established that the range of activity for the affected population is between 0 and 0.24 nmol/mg/h (Median: 0.13 +/- 0.09 nmol/mg/h), while the range of the healthy group is between 0.6 and 4 nmol/mg/h (Median: 1.69 +/- 0.73 nmol/mg/h). The median for the last group is also similar to that reported by Marsh[32] which was 1.63 nmol/mg/h in leukocytes.

We found that 1.52% of referred patients were actually positive. This fact explains why the medians of the HR and HV groups are so similar since the majority of the population is not affected (and therefore their enzymatic activities are within the normal range).

Comparing HV and affected, we observed that 100% of the positive patients’ activities were below 0.24 nmol/mg/h and that only 5% of the healthy volunteers’ activities were below 0.8 nmol/mg/h (with a minimum value 0.6 nmol/mg/h). In patients in the HR population who were MPS III B negative, 0.47 nmol/mg/h was found to be the closest value to those from affected patients. These comparisons made it possible to identify an area where there is no overlap of enzymatic activity between the study groups and, above all, they provide evidence that the enzymatic activity of those affected is quantitatively separated from the rest of the population.

A P-value <0.0001 was obtained from a Mann-Whitney test between control and affected patients, (see Table 2). The results indicate that the activities between these study groups are significantly different and, primarily, that the implemented methodology correctly represents the patient’s condition. This fact was corroborated with a Kruskal-Wallis multiple comparison analysis between affected vs. HV (p-value <0.0001) and affected vs. HR (p-value <0.0001) (Table 2).

Figure 3 shows the cut-off point obtained after successfully performing the ROC curve using the SPSS software. Based on this analysis of the Colombian population, the cut-off point when testing NAGLU deficiency in leukocyte extracts was established to be 0.42 nmol of degraded substrate per milligram of protein per hour (99% confidence, 100% sensitivity, and 100% specificity). This value is similar to the one obtained by Meijer[33] which was 0.58 nmol/mg/h in fibroblast, and to the one reported by Civallero[34] which was 0.48 nmol/ml/h in dried blood filter paper samples. In our case, all enzyme activity values found to be equal to or less than 0.42, were considered positive and the result was confirmed by performing the Gold standard test in cell extracts a second time.

Figure 2. NAGLU activity in leukocyte extracts. Green represents healthy volunteers and blue represents high-risk patients who were remitted for clinical suspicion of MPS.
Table 2. Reference values for the enzymatic activity of controls and affected groups using the standardized technique on leukocyte extracts for MPS III B.

| NAGLU       | n* | Age (years) | Range of activity (nmol/mg/h) | Median +/- SD | P-value* | Cutoff point* |
|-------------|----|-------------|-----------------------------|--------------|----------|--------------|
| Normal controls | 463 | 0.5 - 70    | 0.60 - 4.00                | 1.69 +/- 0.73 | <0.0001  | 0.42 nmol/mg/h |
| Affected     | 7  | 2.9 - 16.7  | 0.00 - 0.24                | 0.13 +/- 0.09 | Mann-Whitney test |              |

* The results for the Shapiro-Wilk normality test with the indicated sample sizes were: Normal Controls p <0.0001 and Affected p <0.0001 (95% confidence). Neither group follows a normal distribution.
* P-value corresponding to the non-parametric Mann-Whitney test comparing the enzyme activity values of normal controls and affected patients.
* Obtained through ROC, based on the comparison between affected and normal controls (99% confidence, 100% sensitivity, and 100% specificity).

Conclusions

To our knowledge, this is the first study in Colombia that reports reference values for NAGLU enzyme activity in leukocyte extracts, while giving information about an estimated frequency of Sanfilippo type B disease. By assessing both study groups (463 healthy volunteers and 462 patients referred for clinical suspicion of MPS III B), it was possible to make a contribution to the definitive diagnosis test of Sanfilippo type B in our country. We found that approximately 1.52% of the analyzed samples were positive, and this gave rise to an estimated frequency of 0.15 per 70,000 live births.

Using an end-point fluorometric assay, which indirectly measures NAGLU activity, we found that there is indeed a significant difference between these values in controls and affected patients. It was also possible to calculate a cut-off point of 0.42 nmol/mg/h for NAGLU deficiency in leukocyte extracts through the ROC analysis (99% confidence, 100% sensitivity, and 100% specificity). In the case of obtaining a value equal to or lower than this cut-off point, it is assumed that the patient is affected by Sanfilippo type B disease, and the result must be confirmed by performing the same test again, with a new sample.

Finally, the results proved that the assay can be widely used in our country or within the group of high-risk patients since it correctly discriminated between both study groups and proved to be reproducible. We hope to increase the rate of Sanfilippo type B diagnosis, as well as the information about its incidence in Colombia, so we expect that this technique and the study itself, will be helpful to our health system, fellow researchers and patients.

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Declaration of Conflicting Interests

The authors declare that they have no conflicts of interest.
References

1. Muenzer J. Overview of the mucopolysaccharidoses. *Rheumatology*. 2011;50(Suppl 5):v4–v12. doi:10.1093/rheumatology/ker394

2. Colmenares-Bonilla D, Colin-Gonzalez C, Gonzalez-Segoviano A, Esquivel Garcia E, Vela-Huerta M, Lopez-Gomez F. Diagnosis of Mucopolysaccharidosis based on history and clinical features: Evidence from the Bajo Region of Mexico. *Cureus*. 2018;10(11):e3617. doi:10.7759/cureus.3617

3. Wraith JE. Mucopolysaccharidoses. In: Rimoin D, Pyeritz R, Korf B, eds. *Emery and Rimoin’s principles and practice of medical genetics*. Elsevier; 2013: chap 101. doi:10.1016/B978-0-12-383834-6.00107-5

4. Vairo F, Federhen A, Baldo G, et al. Diagnostic and treatment strategies in mucopolysaccharidosis VI. *Appl Clin Genet*. 2015;8:245–255. doi:10.2147/acg.s68650

5. Shimada T, Tomatsu S, Yasuda E, et al. Chondroitin 6-Sulfate A as a Novel Biomarker for Mucopolysaccharidosis IVA and VII. *JIMD Rep*. 2014;16:15–24. doi:10.1007/8904_2014_311

6. Coutinho MF, Lacerda L, Alves S. Glycosaminoglycan storage disease, is also a tauopathy. *Neufeld E. Sanfilippo syndrome type B, a lysosomal storage disease, is also a tauopathy. Sanfilippo syndrome: results of a systematic literature review. Clin Genet*. 2008;73(3):251–256. doi:10.1111/j.1399-0004.2007.00951.x

7. Muenzer J. Overview of the mucopolysaccharidoses.

8. Hinek A, Wilson SE. Impaired elastogenesis in Hurler disease: dermatan sulfate accumulation linked to deficiency in elastin-binding protein and elastic fiber assembly. *Am J Pathol*. 2000;156(3):925–38. doi:10.1016/S0002-9440(10)64961-9

9. Zelei T, Csetneki K, Vokó Z, Siffel C. Epidemiology of Sanfilippo syndrome: results of a complete literature review. *Orphanet J Rare Dis*. 2018;13(1):53. doi:10.1186/s13023-018-0796-4

10. Valstar M, Ruijter G, van Diggelen O, Poorthuis B, Wijburg F. Sanfilippo syndrome: A mini-review. *J Inherit Metab Dis*. 2008;31(2):240–252. doi:10.1017/S1095-008-0838-5

11. Ohmi K, Kudo L, Ryazantsev S, Zhao H, Karsten S, Neufeld E. Sanfilippo syndrome type B, a lysosomal storage disease, is also a tauopathy. *Proc Natl Acad Sci U S A*. 2009;106(20):8332–8337. doi:10.1073/pnas.0903223106

12. Beesley C, Young E, Vellodi A, Winchester B. 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(11):910–914. doi:10.1136/jmg.35.11.910

13. Mohammed E, Fateen E. Identification of three novel Homozygous NAGLU mutations in Egyptian patients with Sanfilippo Syndrome B. *Meta Gene*. 2019;21:100580. doi:10.1016/j.mgene.2019.100580

14. Mangas M, Nogueira C, Prata MJ, et al. Molecular analysis of mucopolysaccharidosis type IIIB in Portugal: evidence of a single origin for a common mutation (R234C) in the Iberian Peninsula. *Clin Genet*. 2008;73(3):251–256. doi:10.1111/j.1399-0004.2007.00951.x

15. Huang K-C, Sukagawa K, Orii T. Screening test for urinary glycosaminoglycans and differentiation of various mucopolysaccharidoses. *Clin Chim Acta*. 1985;151(2):147–156. doi:10.1016/0009-8981(85)90318-3

16. Berry H. Screening for mucopolysaccharide disorders with the berry spot test. *Clin Biochem*. 1987;20(3):365–371. doi:10.1016/s0009-9120(87)80088-7

17. Dave M, Chawla P, Dherai A, Ashavaid T. Urinary G lucosaminoglycan estimation as a routine clinical service. *Indian J Clin Biochem*. 2014;30(3):293–297. doi:10.1007/s12291-014-0456-y

18. Chihi-Kuang C, Shuan-Pei L, Shyue-Jye L, Tuen-Jen W. MPS screening methods, the Berry spot and acid turbidity tests, cause a high incidence of false-negative results in sanfilippo and morquio syndromes. *J Clin Lab Anal*. 2002;16(5):253–258. doi:10.1002/jcla.10051

19. Kubaski F, Poswar FO, Michelin-Tirelli K, et al. Diagnosis of mucopolysaccharidoses. *Diagnostics (Basel)*. 2020;10(3):172. doi:10.3390/diagnostics10030172

20. Wood T, Bodamer OA, Burin MG, et al. Expert recommendations for the laboratory diagnosis of MPS VI. *Mol Genet Metab*. 2012;106(1):73–82. doi:10.1016/j.ymge.2012.02.005

21. Wood TC, Harvey K, Beck M, et al. Diagnosing mucopolysaccharidosis IVA. *J Inherit Metab Dis*. 2013;36(2):293–307. doi:10.1017/s1054-013-9587-1

22. Suarez-Guerrero JL, Gómez Higuera PJI, Arias Flórez JS, Contreras-García GA. Mucopolisacaridosis: características clínicas, diagnóstico y de manejo. *Rev Chil Pediatr*. 2011;50(Suppl 5):v4–v12. doi:10.1016/j.rchp.2015.10.004

23. Puentes-Téllez MA, Lema-Barbosa PA, Garzón-Jaramillo RG, et al. A perspective on research, diagnosis, and management of lysosomal storage disorders in Colombia. *Heliyon*. 2020;6(3):e03635. doi:10.1016/j.heliyon.2020.e03635

24. Uribe A. *Biochemical studies of mucopolysaccharide metabolism disorders in Colombia*. Master’s Dissertation. Universidad de Los Andes; 2002.
25. Shapira, E. *Biochemical genetics: A laboratory manual*. Oxford University Press; 1989.

26. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem*. 1951;193(1):265-275. https://www.jbc.org/article/S0021-9258(19)52451-6/pdf

27. Zschenker O, Illies T, Ameis D. Overexpression of lysosomal acid lipase and other proteins in atherosclerosis. *J Biochem*. 2006;140(1):23-38. doi:10.1093/jb/mvj137

28. Hoeflich A, Wolf E, Braulke T, et al. Does the overexpression of pro-insulin-like growth factor-II in transfected human embryonic kidney fibroblasts increase the secretion of lysosomal enzymes? *Eur J Biochem*. 1995;232(1):172-178. doi:10.1111/j.1432-1033.1995.tb20796.x

29. Snow AD, Mar H, Nochlin D, et al. Early accumulation of heparan sulfate in neurons and in the beta-amyloid protein-containing lesions of Alzheimer’s disease and Down’s syndrome. *Am J Pathol*. 1990;137(5):1253-1270. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1877656/

30. O’Callaghan P, Sandwall E, Li J-P, et al. Heparan sulfate accumulation with Abeta deposits in Alzheimer’s disease and Tg2576 mice is contributed by glial cells. *Brain Pathol*. 2008;18(4):548-561. doi:10.1111/j.1750-3639.2008.00152.x

31. Uribe A, Ayala A, España M, Arevalo I, Pacheco N, García L. β-Galactosidase deficiency in Colombia. *J Inborn Errors Metab Screen*. 2015;3:e140011. doi:10.1177/2326409815586252

32. Marsh J, Fensom AH. 4-Methylumbelliferyl alpha-N-acetylgulosaminidase activity for diagnosis of Sanfilippo B disease. *Clin Genet*. 1985;27(3):258-262. doi:10.1111/j.1399-0004.1985.tb00217.x

33. Meijer OLM, Welling L, Valstar MJ, et al. Residual N-acetylgulosaminidase activity in fibroblasts correlates with disease severity in patients with mucopolysaccharidosis type IIIB. *J Inherit Metab Dis*. 2016;39(3):437-445. doi:10.1007/s10545-016-9916-2

34. Civallero G, Michelin K, de Mari J, et al. Twelve different enzyme assays on dried-blood filter paper samples for detection of patients with selected inherited lysosomal storage diseases. *Clin Chim Acta*. 2006;372(1-2):98-102. doi:10.1016/j.cca.2006.03.029

35. DANE – National Administrative Department of Statistics. Birth data in Colombia. Updated December, 2020. Accessed January, 2021. https://www.dane.gov.co/index.php/estadisticas-por-tema/salud/nacimientos-y-defunciones/nacimientos

36. Gomez AM, García-Robles R, Suárez-Obando F. Estimación de las frecuencias de las mucopolisacaridosis y análisis de agrupamiento espacial en los departamentos de Cundinamarca y Boyacá. *Biomedica*. 2012;32(4):602-609. doi:10.7705/biomedica.v32i4.574