Alpha-Galactosidase A Levels in Colombian Males with End-Stage Renal Disease: Ten Years of Selective Screening in Dried Blood Spots

Journal of Inborn Errors of Metabolism & Screening
2022, Volume 10: e20210033
DOI: https://doi.org/10.1590/2326-4594-JIEMS-2021-0033

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Abstract

Fabry disease is a metabolic alteration linked to an enzymatic deficiency of Alpha-Galactosidase A, this disorder compromises the sphingolipid metabolism, leading to an accumulation of lysosomal globotriaosylceramide and is inherited in an X-linked recessive way. The diagnostic of this disease, in general, requires the confirmation of below-normal levels of Alpha-Galactosidase A obtained from dried blood spot (DBS) samples, followed by an assessment of the enzyme in leukocytes. We aimed to report the Alpha-Galactosidase A values obtained in Colombian males with end-stage renal disease (ESRD) screened using dried blood spot samples during ten years. This screening was performed with samples sent to the analysis center from 6156 patients between 2006- 2016. All patients with low levels in enzyme activity (compared to the control population) were sent to confirmation through enzyme analysis in isolated leukocytes. 26 males (0.42%) with low levels of Alpha-Galactosidase A were identified (Range 0.0 – 1.14 nmol/ml/hour, cut-off: 1.15), 22 patients were subsequently measured in isolated leukocytes having a confirmation of Fabry disease in 5 patients (0.08% of total male population) (Range: 0.3 –4.7 nmol/mg prot/h). These results are similar to those reported in studies with comparable characteristics being this the first reporting frequency of Fabry disease among Colombian males with end-stage renal disease.

Keywords

Lysosomal disease, Dried blood spots, Acid Alpha-galactosidase, Acid Hydrolases, Fabry disease, End-stage renal disease (ESRD), Hemodialysis, Screening, X-linked recessive inheritance.

Introduction

Fabry disease (McKusick 301500) is part of a group of more than 60 hereditary diseases affecting lysosomal metabolism. It is considered one of the most frequent disorders worldwide after Gaucher disease and has a pan-ethnic distribution with an estimated incidence of 1:40000 to 1:60000. A frequency of 1:117000 has been reported in carriers [1,2]. This metabolic disorder is related to a deficiency of the Acid Hydrolase Alpha-Galactosidase A (AGAL, EC3.2.1.22) and it is considered a metabolic sphingolipid X-linked disorder. The enzymatic deficiency causes chronic storage of globotriaosylceramide in the lysosome, producing cellular dysfunction in the vascular endothelium, cardiomyocytes of the heart, podocytes in the kidneys, Meissner and Auerbach neurons in the gastrointestinal tract, as well as some cells in other organs [3,4].

Mutations associated with the disease are all distributed within the seven exons composing the GAL gene (Xq22) and more than 600 alterations involving missense, nonsense, deletion, and insertion mutations, mostly related to a single familiar gene (private mutations) have been identified [1,5,6].

The classic phenotype of Fabry disease is observed in affected males and some females who due to the effect of lyonization and the sort of mutation, can display even more severe clinical characteristics than males. However, due to the non-specificity of the symptoms, it is common that without an affected proband in the family, most of these patients are diagnosed during

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Received October 07, 2021. Accepted for publication January 13, 2022.

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adulthood (mean 29 years), having an advanced stage disease. For carrier women, a wide spectrum of phenotypic expressions related to random inactivation of the X chromosome has been described [7].

Clinical manifestations of Fabry Disease mainly include frequent pain, achroparestesyas, systemic or localized angiokeratomas, hypohidrosis, and verticillate cornea. With disease progression, patients develop renal, cardiac, gastrointestinal, and cerebrovascular dysfunction leading all of them to an early death [4,7–9].

The diagnostic process for Fabry disease starts with clinical suspicion in symptomatic patients as well as with screening programs in targeted populations. These patients are first screened with DBS to obtain levels of the enzyme Alpha Galactosidase A. The enzyme values are notably decreased or even undetectable on classic Fabry male patients, thus, enzymatic activity testing alone can be used to diagnosis for male patients. Genetic confirmation of the disease-causing GLA mutation is important to help rule out benign polymorphisms that cause reduced levels of Alpha Galactosidase A activity in otherwise healthy males [10]. In carriers, enzymatic assays are not very reliable, because the activity values can overlap those from healthy controls [11,12].

Several studies have reported an estimated frequency of the disease about 1 in 40000 to 1 in 117000. Recent evidence from newborn screening studies has reported higher frequencies from 1 in 7800 newborn males [13] to 1 in 1368 newborn males [14]. However, these increased frequencies can be due to misinterpretations that are frequently made with non-disease-causing polymorphisms, to different unknown significance variants, to different classes of non-disease related mutations, as well as, in some cases, the identification of more attenuated and later-onset forms of the disease [15].

The accumulation of substrate in the renal endothelial cells, the tubular cells, the mesangial cells, and the podocytes in patients with Fabry disease led to chronic renal disease.[16] Thus, a significant number of Fabry disease patients are identified at End-Stage Renal Disease patient programs. Data reported from specific screening studies, suggests a higher prevalence for this "high-risk population" with values between 0.04% and 1-7% [17]. In a systematic review conducted in 2010, a proportion of 0.33% (95% CI; 0.20% a 0.47 %) for Fabry disease was estimated evaluating ten included studies [18].

Due to the absence of reports and lack of proper diagnostic tools, diseases like Fabry were considered non-existent in Colombia for many years. Today, thanks to several case reports and case series from academic and clinical groups, it is known that Fabry is a present disease and that a lack of data on its impact on the country's population still exists [19].

The purpose of the present report is to show the results from a nationwide screening on Colombian ESRD males. Furthermore, an attempt to estimate the frequency of Fabry disease for this high-risk population in the country was done by performing leukocyte enzymatic analysis as a confirmatory method, in samples obtained from the DBS-positive screened patients.

Materials and Methods

Control and Study Patient's Sample Collection

We analyzed 6156 DBS samples collected from male patients with End-Stage Renal Disease, referred to our laboratory between July 1, 2006, and July 31, 2016. 2337 control samples from healthy subjects were analyzed during the same time. Participants in this study signed an informed consent containing the benefits and risks of participating and documenting their voluntary participation. These consents were approved by the Ethics Committee of the Universidad de Los Andes before sampling. Additionally, all the procedures in the experimental protocol were approved by the same ethics committee and followed the regulations stated in the Helsinki Declaration (1993), the regulations for health research stated on resolution 8430 of 1993 from Colombian Ministry of Health, and other relevant international guidelines and regulations.

DBS were collected using 903 Schleicher and Schuell filter paper from Whatman, Reference: 10534612, Manufacturer: GE Healthcare Bio-Sciences Corporation, USA. Samples were collected from the patients on the day of their programmed hemodialysis before being attached to the dialysis circuit. Once impregnated, they were dried to room temperature for 8 hours or longer. The time between sampling and arrival to our lab was 5 days on average. Once the samples arrived, these were stored at 4°C until analysis. The time for analysis was within 40 days from sampling.

As a confirmation technique, we used enzymatic assay in isolated leukocytes, following Shapira et al 1989 protocol [20], for all patients with low enzymatic DBS levels. Likewise, control samples from 1010 healthy subjects were analyzed using this technique.

Reagents and Lab Equipment

Substrates and calibrator: 4-methylumbelliferyl-Alpha-D-galactoside was used to evaluate Alpha-galactosidase A. Beta-galactosidase was used as a control enzyme for all samples using 4-methylumbelliferyl-Beta-D-galactoside as a substrate. Samples with low levels of Alpha-galactosidase A were additionally measured for total hexosaminidase with 4- Methylumbelliferyl-2-Acetamide-2-Deoxi-D- Glucopyranoside substrate as a double-check enzyme control. Fluorescence values were compared to a calibration curve prepared using 4-methylumbelliferone (4-MU). All previously listed reagents were provided by Sigma (St. Louis, MO, USA).

Buffers: Citrate-Phosphate buffer 0.15 M. pH: 4.4 was used to evaluate Alpha -galactosidase A, Citrate-Phosphate buffer 0.1 M. pH: 4.4, 0.45% NaCl was used to evaluate Beta-galactosidase. Glycine-Carbonate buffer 0.17 M. pH: 10 was used to stop enzyme reactions. Assays were done using black, 96-hole polypropylene microplates. Aluminum paper thermo- sealed laminates provided by Corning (Lowell, USA) were used to avoid evaporation. Samples were shaken and vibrated during the elution and

incubation process using the Titramax 1000 plate shaker and the Unimax 1010 incubator/shaker from the Heidolph group (Schwabach, Germany). M2 Spectramax from Molecular Devices Corp. was used to measure fluorescence (excitation 360 nm, emission 460nm).

For the subsequent confirmation of isolated leukocytes enzyme activity, we follow Shapira et al protocol [20] using the same previously described substrates.

Finally, for both DBS and isolated leukocyte samples, N-Acetyl-D-galactosamine 0.25 M was used as an inhibitor for α-galactosidase B. This isoform is not implied in Fabry disease, but it could interfere by degrading the substrate used in the study. This inhibitor was also provided by Sigma (St. Louis, MO, USA).

Enzyme Activity Assay

DBS assays for Alpha-galactosidase A, Beta-galactosidase, and Total Hexosaminidase, were done using a protocol based on previously described by Chamoles et al. [12] and Uribe & Giugliani [21], using 1.2 mm diameter disks (~0.52 ul of blood). Patients and controls were analyzed in duplicate with one blank for each sample. Fluorescence readings were compared against a 10-point 4-MU calibration curve (concentration range 5.7 to 0.011 nm/100ul), and activity values were expressed in nmol/ml/hour.

Leukocyte enzyme assays were done, as mentioned before using a modified protocol based on a previous description by Shapira et al. [20]. Catalysis activity obtained was expressed in nanomoles of hydrolyzed substrate/hour/mg of protein. The protein contained in each sample was measured in duplicate following the Lowry et al. 1951 method [22] and BCA Protein Assay commercial method provided by Thermo Scientific (Rockford, USA) (Supplementary Table S1).

Statistics

Descriptive statistics and graphic representation were made using IBM statistic package SPSS Statistic v.23 provided by SPSS Inc.

Results

Patients

During the 10-year study period, 6156 samples were received in the analysis lab from males with end-stage renal disease screened at different hemodialysis centers throughout the country. The age range was 18 to 93 years, mean: 57 years, SD: +/- 15.74.

Enzymatic Values for Alpha-Galactosidase A

All referred DBS samples were analyzed simultaneously for Alpha-galactosidase A, beta-galactosidase, and hexosaminidase, following the previously described process. The screening detected 26 (0.42%) males with low enzymatic levels for Alpha-galactosidase (cut-off ≤1.15 nmol/ml/hour, previously reported in 2013 by Uribe & Giugliani) [21]. 41 samples had highly overexpressed enzymatic activity (<20 upper limit for normal controls) and 6089 samples exhibited normal values compared with normal population controls.

From the 26 low enzymatic activity samples, 5 had control enzymes altered, too, and were valued as poor-quality samples (Table 1). Following the screening process, the confirmation protocol in isolated leukocyte samples was performed for the 21-low enzymatic-level screened patients. Two patients were lost in the follow-up. The results for the 19 leukocyte samples were: 14 normal enzymatic levels, and 5 confirmed Alpha galactosidase A deficient patients (0.08%) (95% CI 0.02–0.19) (Figure 1). The confidence intervals were calculated using the Poisson distribution.

Table 1. DBS enzymatic activity for Alpha-Galactosidase A and Beta-Galactosidase in males.

	Alpha-Galactosidase A (nmol/ml/hour) Range/Mean/SD	Beta-Galactosidase (nmol/ml/hour) Range
Normal controls		
3 mo - 88 y (n= 2337)	R: 2.0 – 21.8 M: 7.4 SD:3.5	R: 19 – 99*
Patients with ESRD		
Normal enzyme levels (n= 6089)	R: 1.16 ** – 25.8	R: 26.3 – 81.1
Age: 18 – 93 y		
Low enzyme levels (n=26) Age: 18 - 76 y	R: 0.0 – 1.14	R: 5.27 – 94.9
Over-expressed enzyme levels (n=41) Age: 18 - 79 y	R: 27.0 – 77.2	R: 26.4 – 199.1

* Colombian controls Beta-Galactosidase range activity
** Cut-Off ≤ 1.15 as reported by Uribe & Giugliani., 2013 [21]

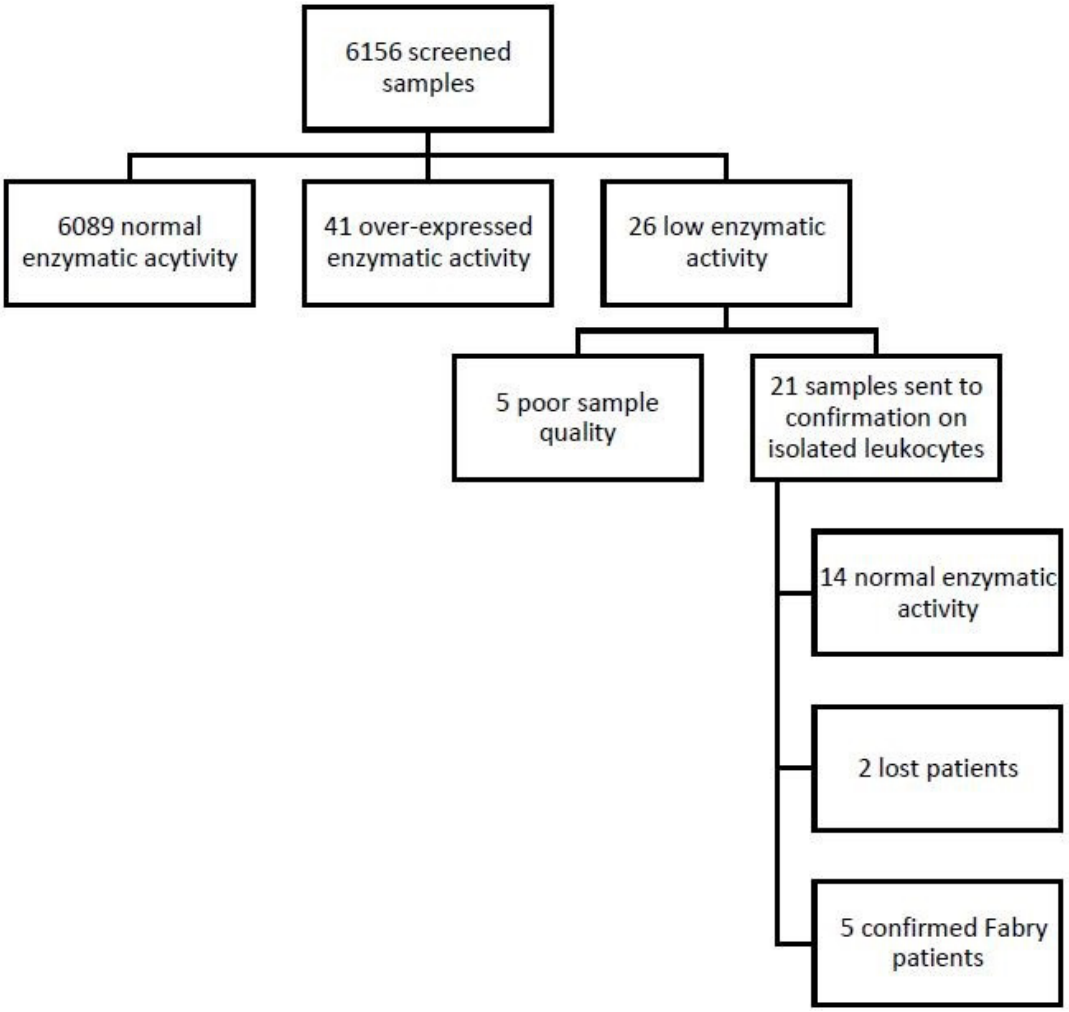


Figure 1. Screening process.

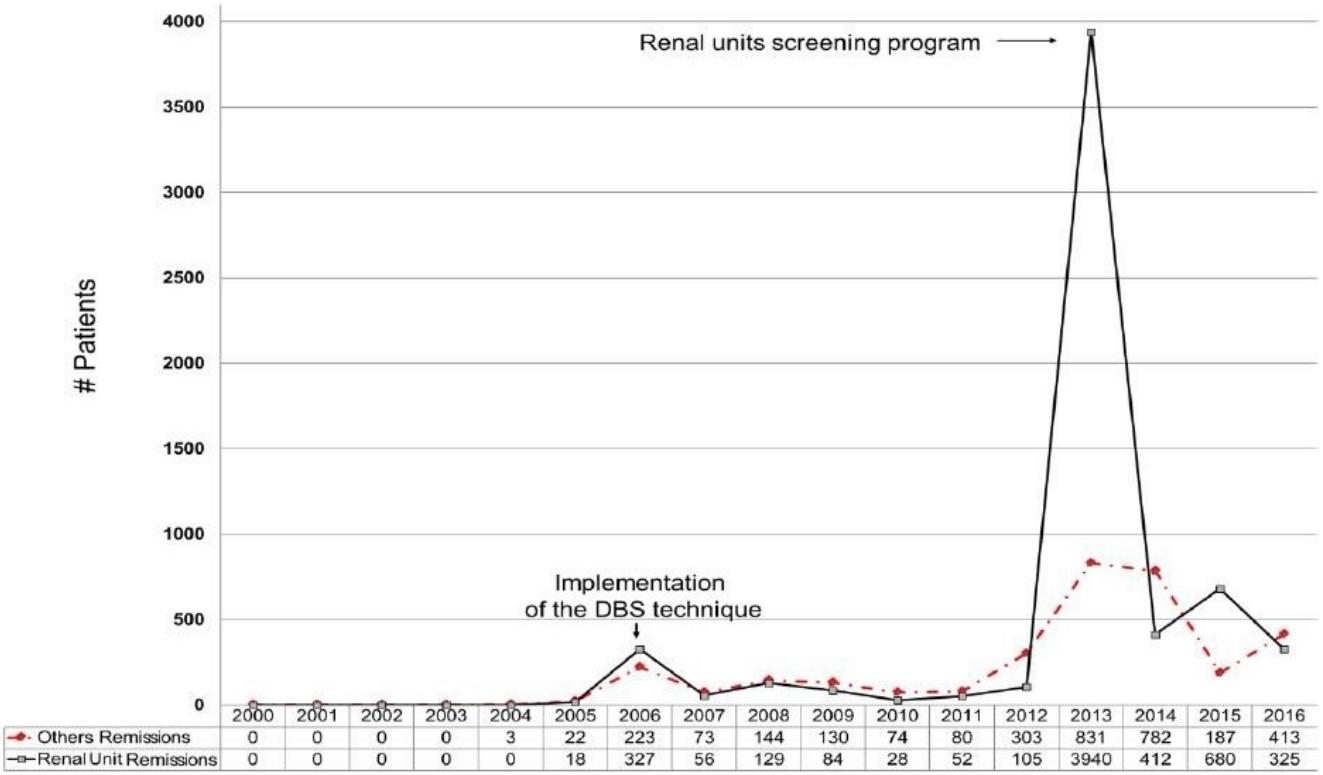
Values for Alpha-Galactosidase A enzymatic activity for male controls varied from 2.0 to 21.8 nmol/ml/h on DBS assay, and 23 to 175 nmol/ml/mg of protein for leukocytes assay.

The highest value for a low enzymatic level on DBS was: 1.14 (Cut-Off: 1.15 nm/ml/h). (Table 1). The highest value for a confirmed Fabry patient (low level on isolated leukocyte assay) was: 4.7 nmol/mg prot/h. (Range: 0.3-4.7 nmol/mg prot/h)

Discussion

Before 2005, except for a few isolated published reports from Argentina and Brazil in 2002-2003 [23], and a few other case reports from other Latin American countries [19], the presence of Fabry disease in the region was virtually unknown. The further development of specific treatments for the disease generated a growing interest and facilitated the diagnosis and access to therapy for these patients. However, it was not until the useful implementation of the DBS analysis methods that initiation of screening programs, creation of awareness, and facilitation of diagnosis for Fabry patients was possible. In our experience, for Colombia, the standardization of DBS analysis at the beginning of this study enabled high-risk population screening in communities located geographically distant from the research center. As shown in Figure 2, the number of samples forwarded to our lab was significantly increased when the DBS method was developed and validated, and further increased when screening programs focused their efforts on ESRD males in hemodialysis centers. These screenings led to the detection of new Fabry diagnosed patients.

The capability for Alpha-galactosidase A activity alone as a diagnostic test for male patients has been widely discussed. Overall, very low enzymatic levels are highly suggestive for diagnosis of Fabry disease in males (homozygotes) [10], it has been suggested that it should be confirmed by obtaining a second measurement of low enzymatic activity in leukocytes. However, with the increased availability of genetic testing, looking for disease-causing mutations has become a standard to establish disease phenotype and to look for benign polymorphisms, and in females the definitive way to establish the diagnosis. Additionally, the recent use of globotriaosylsphingosine (lysoGb3) as a biomarker, has become critical to provide additional diagnostic information [16].



	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016
Others Remissions	0	0	0	0	3	22	223	73	144	130	74	80	303	831	782	187	413
Renal Unit Remissions	0	0	0	0	0	18	327	56	129	84	28	52	105	3940	412	680	325

Figure 2. Increase in the number of patients referred for clinical suspicion of Fabry disease due to DBS screening methods in a high-risk population. Data for referrals from the Renal Unit are shown in black and all other referrals in red.

In our outcomes, comparative values for Alpha-Galactosidase A in DBS between normal controls and chronic ESRD patients did not exhibit significant differences as shown in Figure 3 (left). Twenty-six DBS samples showed a range value from 0.0 to 1.14 (below cutoff of 1.15 previously reported). Five out of these 26 had also low levels in the control enzymes, reason for which they were discarded as poor samples. For the other 21, a second assay using isolated leukocytes was requested. Two of these patients were lost on the follow-up, so the total number of analyzed leukocyte samples was 19. A confirmation of very low levels (Range: 0.3-4.7 nmol/mg prot/h) for 5 out of these 19 patients was obtained, making possible a diagnosis of Fabry disease. The estimated prevalence for our study population was 0.08% (95% CI 0.02–0.19), meaning 1 case by 1232 ESRD male patients. The estimated false-positive rate for the DBS screening technique was 0.28% (n=14). We know that these false-positive results on DBS can be due to poor collection, low sample quality, leucopenia, or pancytopenia. That is the reason for which is imperative that DBS screenings must be followed by a confirmation technique as has been previously addressed in other reports. In our case, we obtained it by performing enzymatic leukocytes analysis, but as DNA tests, LysoGb3, and further diagnostic techniques become more available, we hope a gold standard analysis method will be soon harmonized.

We did perform a search of the literature, looking for similar and comparable studies done in the ESRD population. In Table 2 we listed some of the studies found. In 2018, Doheny et al. [24] reported a comprehensive record of studies from 1995 to 2017. In their list, they included 67 publications, 27 of them on hemodialysis patients (males and females) but excluded those not reporting specific GLA mutations. The results reported a prevalence of 0.42% for the combined analyzed hemodialysis studies (23954 males total) before dismissing those with benign GLA variants and the final prevalence estimated after the exclusion was 0.21%. Other reports as the Saito et al. [17] in the JFAST Japan study, reported 5,408 male cases with a prevalence of 0.04%. Spada et al [25] in an Italian study, reported 1765 male cases with 0.22% prevalence, and Merta et al. [26] in the Czech study included 1521 male cases with a 0.26% prevalence. Of particular interest for us, in Latin America, there has been an increase in the reports. Coutinho et al. [27], reported a selective screening with the application of an initial algorithm to increase the suspicion of Fabry disease. In this group, of 2956 males and females highly suspicious of the disease, 1% of males were diagnosed. Similarly, Silva et al. [28] reported another Brazilian study with 2583 hemodialysis males obtaining a 0.12% prevalence for Fabry disease in this group. Moreover, Frabasil et al. [29] reported a cohort of 9604 Argentinian male cases

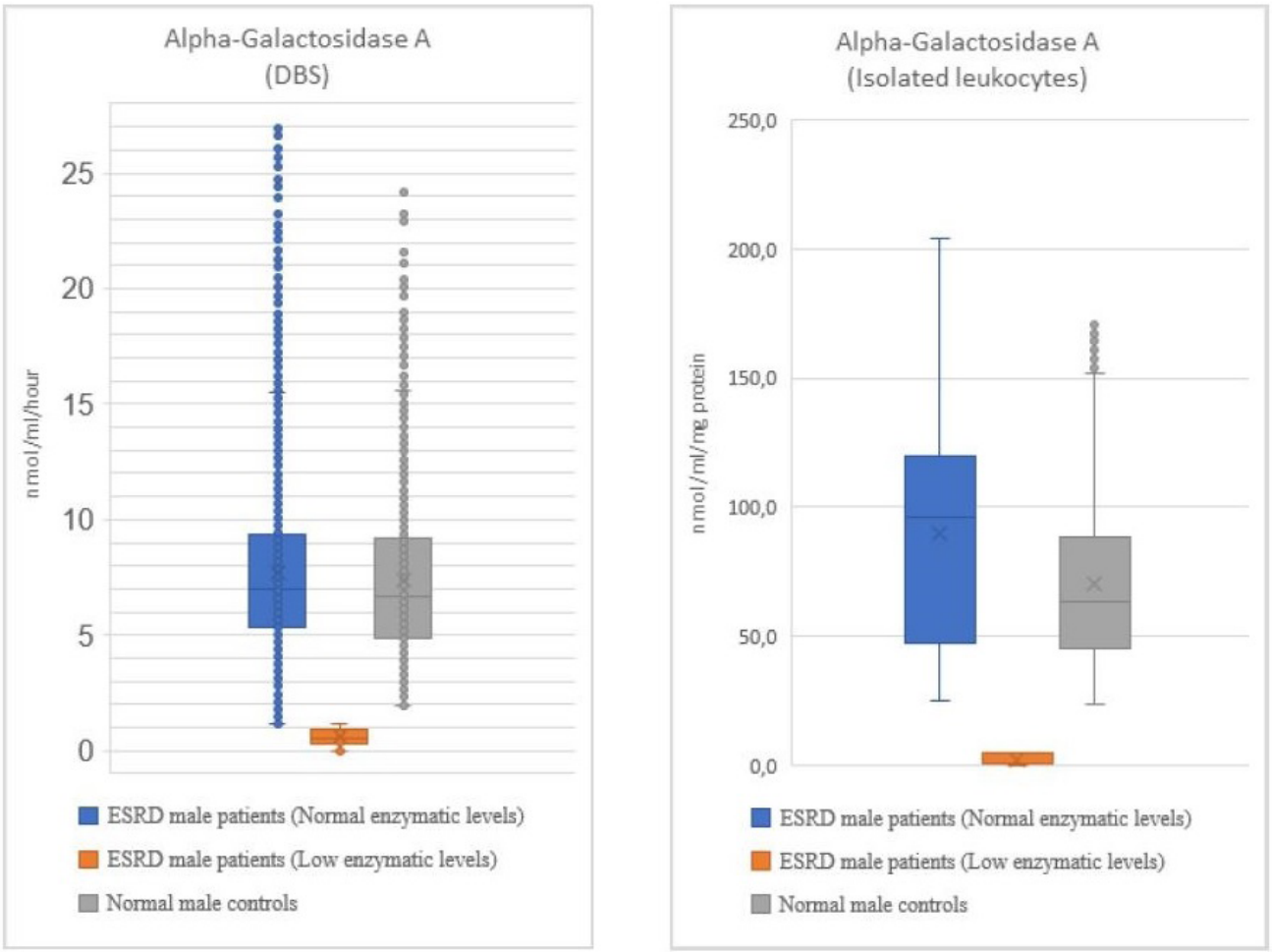


Figure 3. Alpha-galactosidase A levels in ESRD and control male populations studied in DBS (left) and leukocyte extracts (right). In blue is shown the activity of patients with normal enzyme levels, in orange the activity with low enzyme levels and in gray the activity of control males.

with a prevalence of 0.23%. Our study is the first report for the frequency of Fabry disease in Colombian ESRD male patients. The calculated prevalence of 0.08% for this population although is in the lower rank of these reported prevalences, it is still within the generally estimated occurrence (up to 1.2%, or ranges between 0.00 and 1.69%) [17, 18, 24–49] for hemodialysis males.

We hypothesize these differences could be due to a series of different factors including the unspecific selection of the patient cohorts in the different referring chronic ESRD centers, the different screening methods, the differences between sampling size, ethnicity, and other demographic variations, and even the different genetic presentations of the disease (associated to different mutations). Our highest similarity was with 2 Japanese studies.First, Maruyama et al. [30] that reported 1 case of Fabry in 1453 males with ESRD (0.07%) and reported 33 false-positive patients for the initial screening (this was performed by measuring Alpha-galactosidase A in plasma); Second, Saito et al. study [17], in which the prevalence rate was 0.04%, with two confirmed Fabry patients from 5408 males with ESRD.

On a final note, compared with similar studies, false-positive results on DBS techniques are notably less than those reported for plasma techniques [31]. This has been hypothesized to do with the better stability of dried phase samples instead of liquid phase samples, and the requirements of strict control on temperature and other variables for plasma samples that in many cases could not be solved. The DBS technique, as commented, has advantages in avoiding these problems.

The absence of confirmation for GLA mutations can be considered a limitation of this study. However, our primary interest was to show the results obtained in alpha-galactosidase A activity in DBS and the possibility of having confirmation by measuring it in leukocytes. In the case of males, due to the homozygous nature of this genetic disease and the shown outcomes in Figure 3 (right), we are confident that the diagnosis for the 5 patients was correct. Other limitations include the absence of information on the causes of ESRD diagnosis in our cohort, possible selection biases in the patients to screen, and the absence of a more selective approach to classifying the hemodialysis population (as the one made by Coutinho et al.[27]). With these considerations, it is possible that the numbers for this specific population and consequently the frequency, could vary and be higher than those reported.

Table 2. Comparative values for Fabry in males with ESRD on similar studies.

Author (Country)	Male Subjects (#)	Screening method	Confirmation	Age Range	Fabry Patients	Frequency (%): #/Total(%)
Bekri (France)[32]	59	WBC α-Gal A	DNA Mutation Analysis	23 to 89	1	1.69
Terryn (Belgium)[33]	180	DBS α-Gal A	WBC α-Gal A	18 to 60	1	0.56
De Schoenmakere (Belgium)[34]	278	DBS α-Gal A	DNA Mutation Analysis	18 to No limit	1	0.35
Tanaka (Japan)[35]	401	Plasma α-Gal A	WBC α-Gal A	19 to 95	4	1
Utsumi (Japan)[36]	440	Plasma α-Gal A	WBC α-Gal A	NR	2	0.45
Ichinose (Japan)[37]	450	Plasma α-Gal A	WBC α-Gal A and DNA mutation analysis	26 to 89	1	0.22
Linthorst (Netherlands)[38]	508	Whole Blood α-Gal A	DNA Mutation Analysis	>18	1	0.2
Nakao (Japan)[39]	514	Plasma α-Gal A	Lymphocytes α-Gal A and DNA mutation analysis	20 to 90	6	1.17
Maslauskiene (Lithuain)[40]	536	DBS α-Gal A	No other method reported	NR	0	0
Gaspar (Spain)[41]	543	DBS α-Gal A	DNA mutation analysis	20.7 to 91.1	3	0.55
Nishino (Japan)[42]	557	DBS α-Gal A	DBS α-Gal A	NR	1	0.17
Kikumoto (Japan)[43]	545	DBS α-Gal A	No other method reported	NR	1	0.18
Porsch (Brazil)[44]	558	DBS α-Gal A	Plasma α-Gal A	NR	2	0.36
Okur (Turkey)[45]	615	DBS α-Gal A	WBC α-Gal A and DNA mutation analysis	18 to 90	2	0.32
Fujii (Japan)[46]	625	DBS α-Gal A	Plasma α-Gal A	19 to 91	1	0.16
Ucar (Turkey)[31]	808	Plasma α-Gal A	DNA Mutation Analysis	23 to 81	2	0.24
Lv et al (China)[47]	876	DBS α-Gal A	WBC α-Gal A	18 to 87	2	0.23
Doi (Japan)[48]	1080	Plasma α-Gal A	WBC α-Gal A	NR	2	0.19
Maruyama (Japan)[30]	1453	Plasma α-Gal A	Lyso-Gb3	25.3 to 95.0	1	0.07
Kotanko (Austria)[49]	1516	DBS α-Gal A	WBC α-Gal A and DNA mutation analysis	NR	4	0.26
Merta (Czech)[26]	1521	DBS α-Gal A	Plasma α-Gal A/WBC α-Gal A	NR	4	0.26
Spada (Italy)[25]	1765	DBS α-Gal A	Plasma α-Gal A	NR	4	0.22
Saito (Japan)[17]	5408	Plasma α-Gal A	WBC α-Gal A	5 to 98	2	0.04
Silva (Brazil)[28]	2583	DBS α-Gal A	DNA Mutation analysis	18 to 91	3	0,12
Coutinho (Brazil)[27]	2956	DBS α-Gal A	DNA Mutation analysis	NR	31	1
Uribe & Gamba (Colombia)	**6156**	**DBS α-Gal A**	**WBC α-Gal A**	**18 to 93**	**5**	**0.08**
Frabasil (Argentina)[29]	9604	DBS α-Gal A	DBS α-Gal A	25 to 67	22	0.23
All Mean	**0.38**					
SD	**0.39**					
SE	**0.07**					

DBS α-Gal A: Alpha Galactosidase A in Dried Blood Spots; WBC α-Gal A: Alpha Galactosidase A in White Blood cells (leukocytes); Plasma α-Gal A: Alpha Galactosidase A in Plasma; NR: Not reported.

Conclusions

This study offers data for the first time on the frequency of Fabry disease in Colombian ESRD male patients from all over the country in a selective screening in 10 years. These findings highlight the importance of considering the diagnosis of Fabry disease in patients within this population.

Simultaneously, this study offers parallel reference values for Alpha Galactosidase A, within a control Colombian population and chronic ESRD Colombian patients, collected on DBS. These results suggest there is no difference between the groups. It is important to consider that even though leukocyte counts may affect the enzymatic activity values, as well as that white blood cell counts, are frequently altered on this patient population, there was a non-significant difference for this study.

Furthermore, we emphasize the importance of the introduction of the DBS technique in screening programs for this and similar diseases. The broad coverage to remote populations gained with the DBS facilitates the sample referral to analysis centers with easy handling and avoids refrigeration or freezing techniques that make shipment difficult.

Finally, compared with other studies, this study exhibited a low prevalence of Fabry disease in the Colombian ESRD male population. This could be related to specific conditions for ESRD male patients in Colombia, to the selection criteria applied in the referring centers, or to the different techniques used for screening and confirmation of the disease. Further studies are required to evaluate these differences.

Acknowledgments

The authors would like to thank Sanofi-Genzyme for the support given to this research and Dr. Luis Felipe Orozco and BSc Johana Ramírez-Borda, who helped with the style review of the manuscript.

Funding

The authors received no financial support for the research, authorship, and/or publication of this article.

Authors' Contributions

Both authors took part in writing and correcting the manuscript.

Declaration of Conflicting Interests

John Gamba is an employee and holds stocks from Biogen, Inc. Alfredo Uribe has received honoraria as a speaker for Sanofi Genzyme. No others to declare.

Supplementary Material

The following online material is available for this article:

Table S1 – Analytical conditions for Alpha-galactosidase A and Beta-galactosidase assessment.

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