

## Gaucher disease in Colombia: Mutation identification and comparison to other hispanic populations

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### Abstract

Gaucher disease is the most common of the lysosomal storage disorders, affecting all ethnic groups. The pathology of this recessively inherited disease arises from the accumulation of glucocerebroside in tissues due to deficient activity of the enzyme glucocerebrosidase (E.C. 3.2.1.45). The glucocerebrosidase (*GBA*) gene spans a 7.2 kb fragment located on locus 1q21, consisting of 11 exons and 10 introns. Located 16 kb downstream is a highly homologous pseudogene sequence [M. Horowitz, S. Wilder, Z. Horowitz, O. Reiner, T. Gelbart, E. Beutler, The Human Glucocerebrosidase gene and pseudogene: structure and evolution. *Genomics* 4 (1) (1989) 87–96.]. Fourteen fragments comprising 11 exons of the *GBA* gene were analyzed in DNA samples from 25 Colombian patients using denaturing High Pressure Liquid Chromatography (DHPLC). Sequencing of abnormal findings led to the discovery of three novel mutations (c.595\_596delCT, c.898delG and c.1255G > C [p.D419H] in exons 6, 7, and 9 of the *GBA* gene) with high prevalence among Colombian patients. We have also found the presence of a double mutation p.L483P + p.E355K (L444P + E326K, traditional nomenclature) in two different families classified as Gaucher type 1. This mutation was previously reported in one patient with Gaucher type 2. We have found DHPLC to be a reliable and sensitive method for the detection of mutations and allelic variation in Gaucher patients.

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**Keywords:** Gaucher disease; *GBA*; Mutation; Colombian patients; Glucocerebrosidase deficiency

### Introduction

Gaucher disease (MIM# 230800) is the most common of the lysosomal storage disorders. It is characterized by the abnormal accumulation of glucocerebroside in tissues and organs throughout the body, primarily in the cells of the reticuloendothelial system. This autosomal recessive disease has been observed in all ethnic groups studied so far [2], including the Colombian population.

The first type I Gaucher disease patient in Colombia was diagnosed in 1982, and began receiving enzyme replacement therapy (ERT) in 1996 with satisfactory results. Since then, about 60 patients have been identified and confirmed by enzyme analysis, with 12 patients receiving ERT. We were interested in defining the molecular aspects of this patient population and compare our findings to those of other groups, especially those of Hispanic ancestry.

Three types of Gaucher disease have been described. Clinical features of type I Gaucher disease include anemia, thrombocytopenia, leucopenia, hepatosplenomegaly [3], osteopenia, and osteoporosis associated with pathological fractures [4]. Neuronopathic features are

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present in patients with type 2 (acute neuronopathic) and type 3 (chronic neuronopathic). Type 2 Gaucher disease (MIM# 230900) (or acute neuronopathic) presents severe hepatosplenomegaly associated to profound neurological impairment where these patients generally die before 2 years of age. Patients with type 3 Gaucher disease (MIM# 231000) (chronic neuronopathic) have a heterogeneous presentations with variable neurologic and visceral involvement. [5]. Chronic neuronopathic patients have been subdivided on the basis of neurological involvement into subtypes a and b. Type 3a patients develop progressive neurological deficits characterized by cognitive impairment, myoclonic, and generalized tonic-clonic seizures, and supranuclear gaze palsy [6]. Patients with type 3b Gaucher disease exhibit subtle neurological deficiencies such as supranuclear gaze palsy as the sole neurological manifestation, associated with severe visceral manifestations. This broad heterogeneity makes this an artificial division, suggesting that the different forms of neuronopathic Gaucher disease are part of a phenotypic spectrum rather than separate entities [7].

Despite this, a few genotype-phenotype correlations have been established. The p.N409S (N370S, traditional nomenclature) allele has been associated with absence of neurological involvement. To date, it has always been present either in homozygous or heterozygous state in patients with type 1 disease where homozygotes exhibit a less severe phenotype [8,9]. A high correlation has been found between p.L483P (L444P) homozygosity and Gaucher type 3 in Swedish (Norrbottnian) patients [10,11], associating this allele to the neuronopathic form of Gaucher disease. Similar phenotypes have been described in Japanese patients homozygous for the p.L483P allele [12].

To date, more than 160 mutations have been registered in the human mutation database at Cardiff, UK (<http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html>). Previous studies of Argentinean Gaucher patients have been shown there to be to at least five European countries contributing to the ancestry backgrounds of these patients [13,14]. We embarked on a study of the Colombian Gaucher patients to determine whether the mutations, allele frequencies, and clinical features were similar to those of other Latin American populations. A preliminary study using six allele specific oligonucleotide (ASO) probes for the most prevalent mutations in the Ashkenazi Jewish communities (c.114+1G>A, c.84\_85insG, p.N409S (N370S), p.V433L (V394L), p.D448H (D409H), and p.L483P (L444P)) (Cabrera-Salazar et al., unpublished observation) revealed a mutation profile of p.N409S (38%) and p.L483P (24%) in 22 patients. The remaining alleles were not detected using this screening approach, prompting us to explore the use of other methodologies.

Previous studies of the *GBA* gene in Argentineans and Spaniards used single strand conformation polymor-

phism (SSCP) and subsequent DNA sequence analysis to identify mutations in Gaucher patients [13,14]. It has been suggested that the higher sensitivity and reproducibility of denaturing high-performance liquid chromatography (DHPLC) when compared to SSCP, allows for an improved detection of abnormalities with a semi-automated process [15]. DHPLC is a sensitive method for the detection of DNA sequence variations based on the separation of heteroduplexed DNA amplicons using the melting profiles of the DNA. This technique is useful in the detection of point mutations, insertions and deletions in the coding regions of different genes. DHPLC has been used successfully to study the *TSC 1* and 2 genes [15], the *CFTR* gene [16,17], the  $\alpha$ -galactosidase (*a-GAL*) [18], as well as the hereditary non-polyposis colon cancer (HNPCC) genes *hMLH1* and *hMSH2* [19] with outstanding results. This automated technology has been shown to be sensitive enough to detect mutations in fragments ranging from 100 to 1500 bp. It is for this reason that we chose to employ this technology in our search for mutations in these patients.

## Materials and methods

### Patients

Whole blood samples were collected from 25 patients, two of which were siblings (#'s 7 and 8, 18 and 19) (23 unique probands) previously diagnosed as type 1 based on clinical presentation and glucocerebrosidase enzymatic assay, which was performed according to previously published techniques [20]. Informed consent was granted either by patients or by their legal guardians under a protocol approved by the Ethics committee of the Pontificia Universidad Javeriana. Genomic DNA extraction was performed using the Wizard genomic DNA purification system (Promega, USA) according to the manufacturer's instructions. The samples were adjusted to a concentration of 150 ng/ $\mu$ L to perform the corresponding assays. An S&S 903 dried blood spot card (Schleicher & Schuell Bioscience, USA) was collected from 104 healthy unrelated Colombian individuals and used as normal controls. Genra Generation DNA isolation from dried blood spot reagents (Genra Systems, USA) were used to isolate DNA from each dried blood spot using a 3 mm disc punch from the card.

### PCR amplification

Gene specific (GenBank Accession No. J03059) [1] PCR primers (Table 1) similar to the ones used by Cormand et al. [13] were designed to avoid cross amplification of the pseudogene sequence (GenBank Accession No. J03060) and flank, where possible and noted in Table 1, each exon with some intronic sequence.

Table 1  
PCR primers used for amplification of the *GBA* gene

Exon(s) amplified	Primer sequences (5'-3')	Size (bp) <sup>c</sup>
Exon 1	F ATCCTCTGGGATTTAGGAGC <sup>a</sup> R CTGTGACAATGCTGATTGGG <sup>a,b</sup>	471
Exon 2	F GAATGTCCCAAGCCTTTGA R AAGCTGAAGCAAGAGAATCG	406
Exon 3	F CAGACCTCACTCTGCTTGT <sup>a</sup> R GGAGGACCCAGCCTGGCCCA <sup>a</sup>	320
Exon 4	F GGGTACTGATACCTTATT <sup>a</sup> R TCAATGGCTCTATGTCATCT <sup>a</sup>	271
Exon 5–6	5F ACCCAGGAGCCCAAGTTCCCTTT 6R ACAGATCAGCATGGCTAAAT <sup>a</sup>	749
Exon 7	F CTCGGCTTCCCAAAGTGCTG <sup>a</sup> R ATAGTTGGGTAGAGAAATCG	482
Exon 8	F TGTGCAAGGTCCAGGATCAG R TTTGCAGGAAGGGAGACTGG	341
Exon 9a	F CACAGGGCTGACCTACCCAC <sup>b</sup> R GATGGGACTGTCGACAAAGT <sup>a</sup>	187
Exon 9b	F ACTGGAACCTTGCCCTGAAC <sup>a</sup> R GCTCCCTCGTGGTGTAGAGT <sup>b</sup>	268
Exons 10–11	10F GAGAGCCAGGGCAGAGCCTC <sup>a</sup> 11R CTCCTTAGTCACAGACAGCG <sup>b</sup>	569
Universal F-Tag	F CGCCAGGGTTTTCCAGTCACGAA	
Universal R-Tag	R GAGCCACTATCGACTACGCGATCA	

The primers were designed using the Oligo 6.0 program and are *GBA* gene specific, except where noted. “F” and “R” refer to forward and reverse primer, respectively.

<sup>a</sup> Primers noted according to Cormand et al. [13].

<sup>b</sup> Primers which are present in the Pseudogene sequence. Nucleotide highlighted in bold underlined type represent the only nucleotide difference between the *GBA* gene and the pseudogene sequence.

<sup>c</sup> Includes the addition of the F&R universal sequencing tags (48 bp total) to the PCR product.

Each primer was also synthesized with a 5' universal sequencing tag to simplify the downstream sequencing of variant positive samples.

PCR amplification of exons 1, 2, 4, 5–6, 7, and 9a (notation of exon as per Cormand et al. [13]), and 9b through exon 11 was performed in a mixture containing 5 µL of 10× Platinum *Taq* PCR Buffer (Invitrogen, USA), 0.5 µL of 20 mM dNTP mix (Pharmacia, USA); 1.5 µL of 50 mM MgCl<sub>2</sub> (Invitrogen, USA), 35 µL dH<sub>2</sub>O, 20 pmol of each primer, 400 ng of genomic DNA and 2 U of Platinum *Taq* DNA polymerase (Invitrogen, USA). Amplification took place in an MJ Research DNA Engine Thermocycler, using two profiles. The first was used for exons 2, 5–6, and 7 This consisted of an initial denaturation at 95 °C for 2 min, and then 35 cycles of; 94 °C 30 s, 65 °C for 30 s, and 72 °C for 30 s followed by an extension step at 74 °C for 7 min. The other profile was used for the other exons and consisted of an initial denaturation at 96 °C for 2 min, and then 33 cycles of; 96 °C 30 s, 55 °C for 30 s, and 74 °C for 1 min followed by an extension step at 74 °C for 5 min.

PCR of exons 3, 6, and 8 included the addition of 20 µL of 5 M betaine (Sigma, USA) to the reaction mix

to improve the quality of the PCR reaction [21,22]. The dH<sub>2</sub>O volume was adjusted to 15 µL for each reaction and amplified by using the second cycling profile described. Primary PCR to amplify exons 9b through 11 generates a 951 bp gene specific product, which was purified with a Microcon YM100 size exclusion column (Millipore) and 2 µL was used as input into secondary PCRs to amplify exons 10 and 11 for DHPLC analysis reducing the likelihood of pseudogene amplification.

PCR products were processed for heteroduplex formation according to the recommendations of the DHPLC manufacturer (Transgenomic, USA), by denaturing at 95 °C for 5 min and allowing for controlled cooling by decreasing 0.1 °C every 50 s using a thermocycler, until a final temperature of 50 °C was reached.

WaveMaker software (Transgenomic) was used to derive the melting temperature and the conditions necessary for DNA fragment analysis on the Transgenomics WAVE DNA Fragment analysis system (data not shown). The heteroduplexed products were analyzed by DHPLC and their retention times were compared to normal controls processed using the same methodology. Products with an abnormal chromatogram were DNA sequenced on either an Applied Biosystems 377 or 3100 Automated Sequencer using the ABI BigDye Ver3 chemistry according to the manufacturer's recommendations. The use of the universal sequence tags on the 5' of each gene specific primer allowed for one universal set of conditions for the sequencing reactions. Sequence analysis comparison was performed using the Sequencer V.3.3.1 Software (Gene Codes, USA).

## Results and discussion

We have studied 25 Gaucher patients from Colombia, 24 of which were classified as Gaucher type 1 and one as Gaucher type 3. Table 2 lists the mutations and the frequencies for this study group and Table 3 lists the genotypes for each patient as well as clinical and demographic information. Mutation nomenclature follows the suggestions set forth by the Human Genome Variation Society (<http://www.hgvs.org/mutnomen>); the traditional nomenclature which removes the first 39 amino acids of the preprotein is included in parenthesis ( ) for ease of comparison to previous publications (for review see [23]).

### Missense mutations

Abnormalities of the chromatographic patterns of fragment 9a were observed in 26 alleles. Further sequencing of this fragment revealed that the nature of this change in retention times was due to an A to G mutation at nucleotide 1226 which results in a change of an asparagine 409 to serine; p.N409S (N370S) in the

Table 2  
Summary of mutations in *GBA* and frequencies in Colombian patients

Mutation	Number of occurrences	Mutation frequency
p.N409S (N370S)	24 <sup>a</sup>	52.2
p.L483P (L444P)	5 <sup>a</sup>	10.9
c.595_596delCT	4	8.7
p.D419H (D380H)	3	6.5
p.K237E (K198E)	3	6.5
p.Y352H (Y313H)	2	4.3
p.L483P + p.E355K (L444P + E326K)	2 <sup>a</sup>	4.3
p.G234W (G195W)	1	2.2
c.898delG (RecNciI)	1	2.2
	1	2.2

Total chromosomes studied = 46. Mutations are named according to the recommendations of the HGVS (see text for details). Reference sequence (NCBI: M16328). Numbering of the cDNA uses the A of ATG translation initiation codon as +1. The more traditional nomenclature, which removes the first 39 amino acids of the preprotein, is included in parentheses ( ).

<sup>a</sup> In two families in which there were two affected members, only one person was taken into account for frequency calculation.

heterozygous state (Fig. 1A). Two patients (Patient #6 and 17, Table 3) were homozygous for the mutations p.N409S mutation which has a chromatographic pattern reflecting the homozygous state (data not shown). Patients #18 and 19 are brother and sister, respectively. The remaining missense alleles were distributed as follows: four had the mutation p.L483P (Fig. 1B), three

(#11, 12, and 22) the p.K237E (K198E) (Fig. 1C), and one (#1) had the p.G234W mutation (Fig. 1C).

Only one patient (#2) in this study was found to carry the (RecNciI) mutation. In contrast, the study by Cormand et al. [13] of Argentinean Gaucher patients, reported the (Rec NciI) allele with a 21% prevalence and a relatively low frequency of the p.L483P (non-recombinant) allele (approximately 6.4%). However, Giraldo [24] found a high prevalence of the p.L483P allele (18.5%) and a low prevalence (3%) of the (Rec NciI) recombinant allele in the Spanish Gaucher patients. The low frequency of this recombinant allele in Colombian patients could suggest a greater proportion of Hispanic ancestry between this group when compared to the Argentinean population, whose ancestry includes a greater admixture from several countries within Europe.

Three patients (#7, 8, and 15) had a double mutation p.L483P + p.E355K in one allele, and p.N409S on the other allele. Patients 7 and 8 are sister and brother, respectively, whereas 15 is unrelated. The same p.L483P + p.E355K double mutation was reported in two patients one with Gaucher type 2 phenotype in which a genotype of p.E355X + p.L483P/p.E272X (E233X) was present [25] and one mild type 1 with a genotype p.K196Q (K157Q)/p.D179H (D140H) + p.E355K [26]. The p.E272X mutation has been associated with a severe phenotype [26]. In our patients, the presence of the p.N409S mutation appears to determine

Table 3  
Clinical and demographic characteristics of subjects in this study

Patient #	Age	Ethnicity	Genotype	Type of GD	Clinical features	Geographic region of origin
1	25	Hispanic	p.N409S/p.G234W	1	SX, SBD,	Antioquia
2	17	Hispanic	p.N409S/(RecNciI)	1	S,H, MBD	Cundinamarca
3	50	Eur/Mest.	p.N409S/p.D419H	1	SX, SBD, AN, BC	Cundinamarca
4	28	Hispanic	p.N409S/c.595-596delCT	1	SBD, BC,S	Quindio
5	36	Hispanic	p.N409S/p.D419H	1	SX, SBD, BC, FX	Cundinamarca
6	43	Mestizo	p.N409S/p.N409S	1	S,H,A, T	Cundinamarca
7	22	Hispanic	p.N409S/p.L483P + p.E355K	1	SX,H,SBD, AN,BC	Cundinamarca
8	14	Hispanic	p.N409S/p.L483P + p.E355K	1	S,H,A, T	Cundinamarca
9	11	Mestizo	p.N409S/c.595-596delCT	1	S,A,T	Cundinamarca
10	9	Mest-Amer.	p.N409S/c.595-596delCT	1	S,H,A,T	Tolima
11	7	Hispanic	p.N409S/p.K237E	1	A,T	Cundinamarca
12	12	Hispanic	p.N409S/p.K237E	1	S,H,A,T	Cundinamarca
13	7	Hispanic	p.L483P/p.L483P	3b	S,H,A,T, MBD	Atlántico
14	20	Hispanic	p.N409S/c.595_596delCT	1	S H A T	Cundinamarca
15	3	Hispanic	p.N409S/p.L483P + p.E355K	1	S H T	Valle del Cauca
16		Hispanic	p.N409S/p.D419H	1	SBD, A ,T, AN, BC	Casanare
17		NA	p.N409S/p.N409S	1	NA	NA
18	23	Hispanic	p.N409S/p.L483P	1	A,T,BC,AN,SBD	Cundinamarca
19	26	Hispanic	p.N409S/p.L483P	1	A,T,BC,AN,SBD	Cundinamarca
20		Hispanic	p.N409S/p.Y352H	1	A,T BC	Cundinamarca
21		Hispanic	p.N409S/c.898delG	1	NA	NA
22	30	Hispanic	p.N409S/p.K237E	1	A, T,MBD	Tolima
23		Hispanic	p.N409S/p.L483P	1	NA	NA
24		Hispanic	p.N409S/p.L483P	1	NA	NA
25		Hispanic	p.N409S/p.Y352H	1	S, H, A, T, MBD	Cundinamarca

SX, splenectomy; S, splenomegaly; H, hepatomegaly; A, anemia; T, thrombocytopenia; MBD, mild bone disease; SBD, severe bone disease; BC, bone crises; AN, avascular necrosis; FX, fractures; and NA, not available.

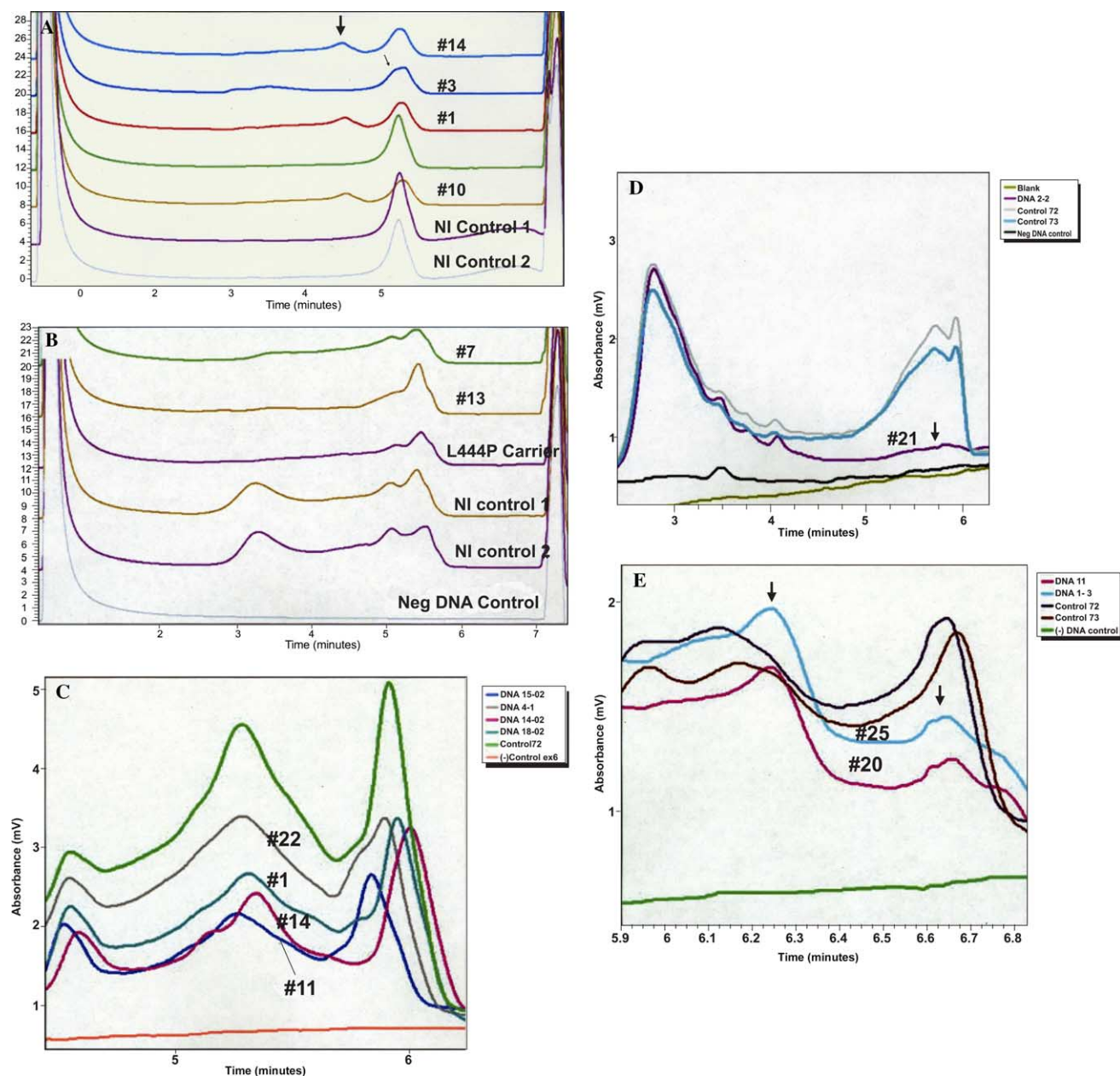


Fig. 1. DHPLC chromatograms of PCR products from Gaucher patients and normal control individuals. (A) Fragment 9a of the *GBA* gene comparing normal controls (NI control 1 and 2) to patients described in the paper. The large arrowhead indicates a shift generated by the p.N409S mutation. The small arrow in #3 is the contribution of the p.D419H mutation. (B) Exon 10, #7 was found to be a carrier of the p.L483P mutation (noted as L444P in the figure). Compare with p.L483P carrier control and normals, #13 was found to be homozygous for p.L483P. (C) Exon 6, magnification of the chromatogram peaks revealing subtle differences between patients with different mutations in the same exon. (D) Exon 7 magnification of chromatogram peaks showing the loss of peaks compared to normal (large arrowhead) in patient #21. (E) Exon 8, detailed magnification of chromatogram peaks showing a shift and alteration of the shape of the peaks (large arrowheads) in patients #25 and #20, both who carry the p.Y352H mutation. (–) DNA control—a control PCR for which no DNA was added. Blank, an injection of dH<sub>2</sub>O to establish baseline for each run series.

the type 1 presentation of the disease, which agrees with previous published observations [8]. The finding of the p.N409S/p.L483P+p.E355K genotype in two affected siblings and an unrelated patient constitutes another indicator of diversity within this population.

The p.K237E (K198E) mutation was previously reported by Orvisky [27] in a homozygous state in a type 2 patient of Colombian descent. The genotype of our

patients (#11 and 12) had the p.K237E on one allele and the p.N409S on the other. These patients did not present any neurological abnormality and therefore were classified as type 1.

Three patients (#3, 5, and 16) showed a change in fragment 9a, where further sequencing established a G to C change at nucleotide 1258, which corresponds to a change of asparagine 419 to histidine; p.D419H. (Fig. 1A, #3)

Two patients (#20 and 25) were found to have abnormal shifts in exon 8 (Fig. 1E). Sequencing of this exon revealed a heterozygous T to C change at nucleotide 1054, corresponding to a change of tyrosine 352 to histidine; p.Y352H.

One patient (#13), was found to be homozygous for the p.L483P allele (Fig. 1B). This patient, initially diagnosed as having type 1 Gaucher disease, later presented with neurological symptoms despite ERT administration and was subsequently re-classified as type 3b [28]. This outcome has been observed and is in accordance with the findings of others [12,29].

#### Deletion mutations

Four patients (#4, 9, 10, and 14) showed a chromatographic abnormality in exon 6 of the gene. The chromatograph example of this is depicted in Fig. 1C. Sequencing revealed a CT deletion at nucleotide number 595–596 (c.595\_596delCT) (Fig. 1C, #11). This mutation is novel and results in a frameshift to a premature stop codon 62 amino acids from codon 199 (p.Leu199\_fs62X).

One patient (#21) was found to have a heterozygous deletion of a G at nucleotide position 898 (g.898delG) in exon 7. (Fig. 1D) This mutation is also novel and results in a frameshift to a premature stop codon four amino acids downstream (p.Ala300\_fs4X).

A clear correlation between severity of the disease and the genotype could not be established as patients with the same genotype presented different severity of the symptomatology (Table 3).

The use of DHPLC as a means for performing mutation detection for the *GBA* gene was very sensitive, leading to the identification and characterization of previously unreported mutations. Initial studies using allele specific probes (PRONTO Gaucher kit, Gamidagen, Israel) directed to the six most common *GBA* mutations in Ashkenazi Jewish individuals (p.N409S, c.84insG, p.L483P, c.114+1G>A, (RecTL), p.R535H), did not detect 38% of the alleles in our patient population. Of particular concern is the apparent discordance between the frequency of the two most common mutations (p.N409S 38%, p.L483P 24%) detected by the PRONTO kit compared to the mutations identified by DHPLC and sequencing (p.N409S 52.2%, p.L483P 10.9%). Multiple independent DNA sequencing analyses of each sample confirmed the validity of the DHPLC results and disproved initial findings from the ASO based kit. The presence of three new mutations: c.595\_596delCT, p.D419H, and c.898delG was not entirely unexpected because of the unique genetic background of the Colombian population, as has been observed in other diseases such as MPS IVA [30]. The differences in the mutation types and frequencies are not surprising when compared with Jewish, European, American, Japanese or even Argentinean populations [12,29].

When studying a particular genetic disease in a new racial or ethnic population, a sensitive scanning technology should first be employed to characterize the population. This study shows that DHPLC is a reliable and sensitive (detection of 100% of the mutant alleles) screening tool for mutation detection. Using this technology, we discovered three novel mutations (c.595\_596delCT, c.898delG and c.1255G>C [p.D419H] in exons 6, 7, and 9 of the *GBA* gene, respectively) with a relatively high prevalence among the Colombian patients studied. The deletion mutations result in the premature truncation of the protein and are presumed to be severe mutations. The p.D419H mutation has not been fully characterized though expression. However, this mutation was not detected in DNA sequence analysis of exon 9a in 104 healthy unrelated Colombian individuals (208 alleles), suggesting that this is a disease causing mutation.

We have also found the presence of a double mutation p.L483P + p.E355K (L444P + E326K) inherited with the p.N409S mutation in two different families classified as Gaucher type 1. This double mutation was previously reported in one patient with Gaucher type 2 whose other allele was p.E272X (E233X) non-sense mutation. This confirms the findings of others, where the presence of the p.N409S mutation offers a neuroprotective effect resulting in the difference between the type 1 and 2 disease [25,26]. The identification of mutations in the *GBA* gene common to Colombian population will allow for the design of specific diagnostic tests or for carrier diagnosis.

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