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# High frequency of mutation G377S in Brazilian type 3 Gaucher disease patients

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## Abstract

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Gaucher disease (GD), the most prevalent lysosome storage disorder, presents an autosomal recessive mode of inheritance. It is a paradigm for therapeutic intervention in medical genetics due to the existence of effective enzyme replacement therapy. We report here the analysis of GD in 262 unrelated Brazilian patients, carried out in order to establish the frequency of the most common mutations and to provide prognostic information based on genotype-phenotype correlations. Among 247 type 1 GD patients, mutation N370S was detected in 47% of all the alleles, but N370S/N370S homozygosity was found in only 10% of the patients, a much lower frequency than expected, suggesting that most individuals presenting this genotype may not receive medical attention. Recombinant alleles were detected at a high frequency: 44% of the chromosomes bearing mutation L444P had other mutations derived from the pseudogene sequence, present in 25% of patients. Three neuronopathic type 2 patients were homozygous for L444P, all presenting additional mutations (E326K or recombinant alleles) that probably lead to the more severe phenotypes. Six children, classified as type 1 GD patients, had a L444P/L444P genotype, showing that neuronopathic symptoms may only manifest later in life. This would indicate the need for a higher treatment dose during enzyme replacement therapy. Finally, mutation G377S was present in 4 homozygous type 1 patients and also in compound heterozygosity in 5 (42%) type 3 patients. These findings indicate that G377S cannot be unambiguously classified as mild and suggest an allele-dose effect for this mutation.

### Key words

- Gaucher disease
- GBA gene
- Allele dose-effect

## Introduction

Gaucher disease (GD), the most prevalent lysosome storage disorder, presents an autosomal recessive mode of inheritance.

Clinically, it is a multisystemic disease with variable expression, generally characterized by hepatosplenomegaly, anemia and thrombocytopenia. Bone and pulmonary disease may also be present. The presence and sever-

ity of neurologic symptoms define the three types of GD. Approximately 95% of patients present type 1 GD (MIM# 230800), without neurologic involvement and with onset during childhood or adulthood (1). Neuroopathic variants are type 2 GD (MIM# 230900), characterized by early onset and survival of up to 2 years of age, and type 3 GD (MIM# 231000) of infantile or juvenile onset and with a less ominous course with survival into adulthood.

GD is caused by mutations in the GBA gene (1q21). This is a 7-kb gene containing 11 exons, which codes for the acid  $\beta$ -glucosidase enzyme, also known as glucocerebrosidase (2). The absence of this enzyme leads to the accumulation of glucocerebroside in the cells of the reticuloendothelial system and ultimately to the clinical manifestation of GD. More than 200 GD-causing mutations have been identified so far and the most frequent ones are [c.1226A>G;N370S] and [c.1448T>C;L444P], herein called N370S and L444P, respectively. The clinical diagnosis of GD may be confirmed by the biochemical assay of acid  $\beta$ -glucosidase activity. Unlike enzymatic diagnosis, mutation analysis has a considerable, although not absolute, predictive value with respect to disease prognosis (1). Opportunely, an extensive genotype-phenotype correlation is present for the most frequent mutations. Generally, GD-causing mutations are classified as mild, severe or null alleles (3). The presence of a mild mutation leads to type 1 GD even in combination with a second severe mutation or a null allele. The neuroopathic variants are due to homozygosity of severe mutations, or the combination of a severe mutation and a null allele. Homozygosity of null alleles is not found, suggesting that this genotype is incompatible with life. Despite the genotype-phenotype correlation, a variable expression of clinical symptoms is present in patients with the same genotype, indicating the existence of other factors, genetic and/or environmental, influ-

encing the phenotype (4).

GD is the first lysosome storage disease for which an efficient enzyme replacement therapy (ERT) was developed (Imiglucerase) and became a paradigm for therapeutic intervention in human genetics (5). ERT is the standard treatment for type 1 and 3 GD patients, and efficiently ameliorates non-neurological symptoms. The enzyme dose infused during ERT is established for each patient according to body weight and clinical manifestations. Dose individualization is subject to medical decision including the initial dose and its reduction during the life-long treatment. The high cost of treatment dictates that ERT should be administered at the most efficient possible dose. In Brazil, the patients' therapy is subsidized by the public health system. Thus, the disease may be of low incidence but has a significant economic impact. There are no estimates of GD prevalence in Brazil, but there are approximately 400 diagnosed cases (6), the third largest patient population undergoing ERT in the world.

In this study the frequency of the most common mutations in a sample of 262 unrelated Brazilian GD patients was determined. Several genotype-phenotype correlations were identified and their implications for the diagnosis and treatment of GD are discussed.

## Patients and Methods

### Gaucher disease patients and relatives

Overall, 580 samples were received in the laboratory, from patients, relatives and cases with an unconfirmed clinical diagnosis of GD. Samples came from the different regions of Brazil during a three-year period (2000-2003). The patients with confirmed GD were diagnosed by their doctors after medical evaluation and the determination of reduced glucocerebrosidase activity measured in leukocytes (6). Only three patients were Ashkenazi Jewish. Mouth mucus was

collected by physicians or health professionals participating in the treatment of the patients and sent to the laboratory in 2-mL Eppendorf tubes. Participants signed informed consent forms and the project was approved by the Brazilian National Committee of Ethics in Research (CONEP).

Mutation screening, for statistical purposes was performed in one case from each family, for a total of 262 cases, 247 of which were classified by their physicians as type 1, 3 as type 2, and 12 as type 3 GD patients. Since not all of these patients were evaluated by one of the authors, we could not confirm the classification in every case. Most of these patients are undergoing ERT, except type 2 cases, which are not treated and have a reduced life expectancy. The genotype was analyzed separately for type 2 and 3 patients in order to avoid bias in the evaluation of allelic and genotypic frequencies.

### Molecular diagnosis

DNA was extracted from mouth mucus by the method of Richards et al. (7). Mutations N370S, L444P, G377S, c.84insG (84insG), IVS2+1G>A, c.1263\_1317del (55del), V460V, D409H, and E326K were analyzed on the basis of restriction fragment length polymorphism. The enzymatic effect of these mutations, their screening strategy, the primers (Invitrogen, Carlsbad, CA, USA), and restriction endonucleases (New England Biolabs, Ipswich, MA, USA or from Fermentas, Vilnius, Lithuania) used for detection and the reference method, when previously established, are described in Table 1. After endonuclease digestion, fragments were submitted to 12% polyacrylamide gel electrophoresis (mutations N370S, G377S, 84insG, and IVS2+1G>A) or 2% agarose gel electrophoresis (mutations L444P, V460V, D409H, 55del, and E326K).

All patients were screened for mutations N370S and L444P, the most frequent mutations worldwide. Mutation G377S is the third

most frequent mutation in Portugal and was therefore also included in the initial analysis. In the absence of these three mutations in one of the alleles, the first one hundred patients were screened for mutations 84insG and IVS2+1G>A. Patients homozygous for N370S, G377S or L444P were investigated for the presence of mutation 55del, present in the pseudogene (psGBA) contiguous to the GBA gene. This deletion, if present in GBA, would be localized at the annealing site of primers used for detecting mutations N370S, G377S and L444P (8).

Because mutation L444P may be a part of a recombinant allele (REC), with DNA sequence derived from the highly homologous psGBA, all patients presenting this mutation were tested for V460V (9). When V460V was detected, indicating the presence of a REC, mutations 55del and D409H were screened in order to determine the extent of the recombination sequence. Moreover, when a REC was detected, the patient's parents were tested, when possible, to determine if the mutations were in the same allele. The most frequent RECs are RecNciI (L444P, A456P and V460V) and RecTL (D409H, L444P, A456P, and V460V). Due to the absence of analysis of A456P, we opted to present RECs by the mutations actually detected. Finally, the 15 patients classified as types 2 and 3 were also tested for 55del, D409H and E326K due to the high frequency of these mutations among them.

### Results

The sample analyzed was composed of 247 (94%) type 1, 3 (1%) type 2 and 12 (5%) type 3 GD patients. Type 2 GD patients may be under-represented since they are not treated by ERT and have a short life expectancy. Nevertheless, the distribution was similar to that of other studies in distinct populations (1).

The genotypic distribution in the 247 type 1 patients is shown in Table 2. Among

the first one hundred patients analyzed, only one allele harboring mutation 84insG and none with IVS2+1G>A were detected. Thereafter, screening for these mutations was halted and they were considered to be unidentified. Almost half of the patients were compound heterozygotes presenting the N370S/L444P genotype. Combination of N370S with a second unidentified mutation was the second most frequent genotype, present in approximately 30% of the alleles. The remaining patients had many other genotypes.

Molecular analysis of 6 children with a

clinical diagnosis of type 1 GD showed a genotype characteristic of type 3 GD (homozygous for L444P). For statistical purposes these children were considered to be type 1 patients according to their clinical characterization, although their genotypes suggest that neuropathic symptoms may be expected to occur later in life.

The mutation frequencies, independently of their genotypic distribution, are presented as the first column of Table 3. While almost half of the patients' alleles carry N370S (47%), the other half is divided between L444P (27%), G377S (2%) and an

Table 1. Screening target, primers and restriction enzymes used for the detection of some mutations that cause Gaucher disease.

Mutation (cDNA sequence)	Phenotypic effect <sup>a</sup>	Screening target	F and R primers (Ref.)	Restriction endonuclease
N370S (c.1226A>G)	Mild	All patients	F: 5'gcctttgtccttacccctcg3' R: 5'acgaaagttacgcaccaatt3' (32)	<i>XhoI</i>
G377S (c.1246G>A)	Mild/severe	All patients	F: 5'gcctttgtccttacccctcg3' R: 5'acgaaagttacgcaccaatt3'	<i>AluI</i> or <i>PvuII</i>
L444P (c.1448T>C)	Severe	All patients	F: 5'ctgaaccccaaggaggac3' R: 5'tgaatggagtagccaggtaga3' (33)	<i>NciI</i>
V460V (c.1497G>C)	<sup>b</sup>	Patients with L444P	F: 5'ctgaaccccaaggaggac3' R: 5'tgaatggagtagccaggtaga3'	<i>BcgI</i>
84insG (c.84insG)	Null allele	First 100 patients	F: 5'cactgcctgaagtagatgc3' R: 5'gaatgtccaagcctttga3'	<i>BsaBI</i>
IVS2+1G>A	Null allele	First 100 patients	F: 5'gaatgtccaagcctttga3' R: 5'aacagagtaagactctggttc3'	<i>HphI</i>
D409H (c.1342G>C)	Severe	Types 2 and 3 Patients with V460V	F: 5'aacctgattccctatctc3' R: 5'gctccctcgtggttagagt3'	<i>StyI</i>
E326K (c.1093G>A)	<sup>c</sup>	Types 2 and 3	First PCR: F: 5'acaattagctgggtgtggc3' R: 5'taagctcacactggccctgc3' Nested PCR: F: 5'tgtgcaaggtccaggatcag3' R: 5'gaggtctgcttgcaggaag3'	<i>BbsI</i> or <i>BpiI</i>
55del (c.1263_1317del)	Null allele	Types 2 and 3  Homozygotes for N370S, G377S and L444P Patients with V460V	F: 5'aacctgattccctatctc3' R: 5'gctccctcgtggttagagt3' (8)	<sup>d</sup>

<sup>a</sup>Phenotypic effect of mutations according to Ref. 3. Mutation G377S has been detected with both mild and severe effects (see text). <sup>b</sup>Mutation V460V is a silent alteration used to indicate the presence of a recombinant allele. <sup>c</sup>E326K is a modifier that may be present in combination with other Gaucher disease-causing mutations. <sup>d</sup>Identification of mutation 55del may be visualized directly after PCR.

unidentified mutation (24%).

Since mutation L444P may be present alone or as part of a REC, this possibility was always examined. Among type 1 GD patients, 46% (62/134) of alleles with L444P were found to be RECs with L444P+V460V. Testing for D409H followed the detection of V460V and only 2% (3/134) of alleles with L444P were positive, disclosing a REC with L444P+V460V+D409H. Mutation 55del was not detected in any of the RECs, suggesting that they probably had pseudogene-derived mutations restricted to exons 9 and 10. However, other recombination events upstream from 55del may be present. When including type 2 and 3 patients in the statistical analysis, RECs were identified in 44% (66/150) of the chromosomes bearing mutation L444P and in 25% (66/262) of all GD patients.

The 15 patients with type 2 and 3 GD were screened for mutations N370S, G377S, L444P, V460V, 55del, D409H, and E326K. Table 4 presents the genotypes found. We detected a homozygote patient whose parents are first cousins presenting E326K in combination with L444P in both alleles with a type 2 clinical phenotype. The only compound heterozygote presenting L444P in combination with a REC also had type 2 GD. Among the type 3 patients, the presence of mutations N370S in one case and G377S in 5 cases was unexpected since these are generally considered to be mild mutations (3).

## Discussion

### Frequency of the most common mutations

Although 47% of all GD-causing mutations are N370S (Table 3), only ~10% of the patients are N370S/N370S homozygotes (Table 2), a frequency much lower than the 22% expected ( $0.47^2$ ). These data probably reflect the fact that many N370S homozygotes are not obtaining medical attention due to the absence or mildness of symptoms. The 24 homozygotes herein detected indicate a penetrance of 45% for this genotype ( $0.10/0.22$ ), a value that is close to that described for the Ashkenazi Jewish population (10) and for the Portuguese population (11). These cases indi-

Table 2. Genotypic frequency observed in 247 Brazilian patients with type 1 Gaucher disease.

Genotype	Number of patients (frequency)
N370S/L444P	112 (45.3%)
N370S/?	70 (28.3%)
N370S/N370S	24 (9.7%)
??	19 (7.7%)
L444P/?	9 (3.6%)
L444P/L444P	6 (2.4%)
G377S/G377S	4 (1.6%)
G377S/N370S	2 (0.8%)
G377S/L444P	1 (0.4%)
Total	247 (100%)

? = unidentified mutation.

Table 3. Frequency of the most common mutations in Brazilian, Portuguese and non-Jewish North American type 1 Gaucher disease patients.

Mutation	Brazil	United States of America	Portugal
	Present study N (%)	Zhao and Grabowski, 2002 (12) N (%)	Amaral et al., 1996 (13) N (%)
N370S	232 (47.0%)	183 (43.7%)	29 (53.7%)
L444P	134 (27.1%)	107 (25.5%)	7 (13.0%)
G377S	11 (2.2%)	Not determined	4 (7.4%)
Others	117 (23.7%)	129 (30.8%)	14 (25.9%)
Total	494 alleles (100%)	419 alleles (100%)	54 alleles (100%)

Statistical analysis comparing the present study with the North American sample considered G377S to belong to the "others" category.

cate that despite its Mendelian mode of inheritance, GD presents complex phenotypes.

The frequency of the two most common GD-causing mutations among Brazilian patients is similar to that described for other populations, where N370S and L444P represent roughly half and one quarter of the alleles, respectively (1). Mutations 84insG and IVS2+1G>A were found to be too rare. In fact, these mutations have a high frequency among Jewish patients but not in the general population of most countries (1). The low detection rate of the 84insG and IVS2+1G>A in the present study may reflect the low frequency of the Jewish population (<0.1%) in Brazil.

From Table 3 it is possible to observe that the frequency of the most common mutations in Brazilian type 1 GD patients detected in the present study is similar to that found in a sample of non-Jewish North Americans (12) ( $P = 0.263$ ). However, it is

significantly different ( $P = 0.028$ ) when compared to that of Portuguese cases (13), according to chi-square analysis. The genetic background of the Brazilian population includes important African and Indigenous components, in addition to the Portuguese ancestry, that may explain the greater similarity to the North American sample.

Mutation G377S is the third most frequent among GD patients in Portugal and Spain (13). In Brazil, it also appeared at a considerable frequency (2.2%), indicating that it is important in preliminary screening. A correspondence in mutations causing another lysosome storage disease has also been described for Tay-Sachs disease and points to a common origin of the mutated alleles prior to the Portuguese colonization of Brazil (14).

Among the alleles with L444P, 44% (66/150) were shown to be RECs, present in 25.2% (66/262) of patients, corresponding to 12.6% (66/524) of the alleles studied. Our approach was limited by the lack of analysis of the A456P mutation, present in the RECs. Therefore, we have opted to present RECs by the mutations actually diagnosed. Since some patients may have A456P and lack the silent mutation in V460V, the frequency of RECs in this sample may be even higher. The high frequency of RECs was also described in other countries (9,15). It is generally observed that the RECs have a more severe impact on clinical manifestation than L444P alone (9,16-18). In agreement with this observation the three homozygotes for L444P additionally carrying E326K or RECs had type 2 GD, while those with genotype L444P/L444P but lacking complex alleles had milder phenotypes. It is possible that these patients' more severe phenotypes reflect a modifier effect of E326K and RECs leading to a lower residual enzyme activity, although other loci may also have influenced the severity of the phenotype (4,19). The type 2 patient with the L444P+E326K/L444P+E326K genotype did not present the severe perinatal-lethal phenotype, downplaying this genotype-phenotype correlation (20).

Table 4. Genotypes of 15 neuronopathic Brazilian patients with Gaucher disease.

Genotype	Phenotype
L444P+E326K/L444P+E326K <sup>a,b</sup>	Type 2
L444P+V460V/L444P	Type 2
L444P+E326K/L444P <sup>b</sup>	Type 2
L444P/L444P	Type 3
L444P/D409H	Type 3
L444P/D409H	Type 3
L444P+V460V/?	Type 3
L444P+V460V/?	Type 3
55del/?	Type 3
L444P+E326K/G377S	Type 3 <sup>d</sup> /ME
L444P+E326K/G377S <sup>b</sup>	Type 3 <sup>d</sup>
L444P/G377S/E326K <sup>c</sup>	Type 3 <sup>d</sup>
G377S/?	Type 3 <sup>d</sup>
G377S/?	Type 3 <sup>d</sup>
L444P+V460V/N370S	Type 3 <sup>d</sup> /ME

ME = myoclonic epilepsy. <sup>a</sup>Parents are first cousins. <sup>b</sup>These patients have siblings not included in this study, presenting the same genotype and phenotype. <sup>c</sup>E326K phase was not determined, i.e., it was cis or trans to L444P. <sup>d</sup>Neuronopathic patients with a clinical diagnosis of type 3 but carrying mutations considered to be mild. The patient carrying N370S did not have a detailed oculomotor evaluation.

### Type 1 pre-neuronopathic affected children

Among type 3 GD patients, neurological manifestations appear after 10 years of age in 50% of the cases (1). Therefore, it is always possible that children under this age, diagnosed as type 1, may actually be type 3 patients that are in a pre-neuronopathic phase. Molecular diagnosis may help to identify these patients and this was the case for 6 children in this study, aged 5 to 6 years, in which mutation L444P was detected in homozygosity. It is possible that other children clinically classified as type 1 patients, with unidentified mutations and without a mild mutation, are in the same situation. Although only ~5% of GD patients are type 3 cases, the onset of GD is generally during infancy, which makes the possibility of a pre-neuronopathic patient a frequent doubt. The anticipation of the diagnosis of a neuronopathic GD variant by DNA testing is due to the need to inform the family, since these patients are likely to manifest developmental and language problems (4,21). More important is the impact on ERT. One possibility is that a high-dose treatment may impact the neuronopathic symptoms (21,22) but conclusive evidence is still lacking for GD. The European consensus for the treatment of neuronopathic GD has established that the ERT of type 3 patients, including the pre-neuronopathic ones identified by molecular diagnosis, is to be performed at a higher dose that should not be reduced even after symptoms ameliorate (23).

### Neuronopathic patients with mutations N370S and G377S

The evidence for considering G377S a mild mutation is based on its high residual enzyme activity (17.6%) according to measurement of acid  $\beta$ -glucosidase activity (24). Moreover, all reports of G377S homozygotes involve type 1 GD patients (16,25-27). Accordingly, in the present study four G377S homozygotes had a type 1 phenotype. How-

ever, five compound heterozygotes carrying one G377S allele were type 3 patients. Other reports have also detected type 3 patients carrying one copy of G377S (3,28-30). These observations suggest the possibility of an allele-dose effect, whereby two copies of G377S would lead to an enzymatic residual activity sufficient to prevent neurological involvement, but one copy of G377S with a second severe mutation would lead to type 3 GD. An allele dose-effect has already been described for mutation K79N (31). In this model, the residual activity of mutant enzymes produced by the two alleles should be considered in an additive manner. Some mutations, such as G377S, K79N, N188S, V394L, and R463C could be at the limit of residual activity that elicits neuropathic symptoms (3,16,31).

The patients reported here represent further *in vivo* evidence for a critical role of a GD-causing mutation in the development of neurological disease. However, the participation of other modifiers cannot be excluded. In this study, another Brazilian family with two siblings (aged 5 and 15 years) presenting the G377S/L444P genotype lacked any neurological involvement (Table 2). These siblings were shown not to carry the E326K mutation (data not shown), while 2 other patients with genotype G377S/L444P+E326K presented neuronopathic symptoms (Table 4). This finding reinforces a possible negative modifier effect of E326K.

The last case in Table 4 presents neuronopathic symptoms that appeared to be inconsistent with his genotype (N370S/L444P+V460V). Mutation N370S is in trans to the REC allele that was inherited from the mother. The mild effect of N370S has been well described in hundreds of type 1 GD patients reported so far and contrasts to the neuronopathic phenotype in this case. However, the patient herein described may suffer from hypoxic-ischemic encephalopathy and therefore her neuronopathic manifestation may not be a consequence of GD.

It is clear that DNA testing provides information both on the diagnosis and the prognosis of GD and new insights on genotype-phenotype correlations should be promptly integrated into medical practice. Moreover, since ERT is individualized and the treatment dose is subject to medical decision, molecular diagnosis may be critical to establish a proper dose, as in the case of the L444P homozygote children still lacking neurological symptoms. Finally, our findings indicate that an allele dose-effect may be associated with mutation G377S. This mutation should be part of the screening panel for GD patients from Iberian countries, including their former colonies. Further studies may be critical to establish its exact genotype-phenotype correlation.

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## David Rockefeller Center for Latin American Studies Harvard University

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With the support of Mr. Jorge Paulo Lemann, the *Center* recently created the “Lemann Visiting Fellowship”. This is a fellowship for a professional for the above mentioned program whose research project has Brazil as the study objective. This fellowship is awarded once each academic year (September-June) and covers the administrative expenses of the *Center* and airline tickets. The professional will also receive financial assistance of US\$15,000 (fifteen thousand American dollars, subject to income tax), regardless of the number of semesters for which he is accepted.

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#### **For additional information, contact:**

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The Alexander Von Humboldt Foundation, which exists more than 130 years and whose actual structure was established a few years after WWII (1953), is offering fellowships for post-doctorate study and for specialized research projects in all areas of knowledge. There are 500 fellowships annually, distributed without limitation of nationality, and 150 fellowships (Feodor Lynen Research Fellowships), only for German researchers and professors, to work as invited researchers of ex-fellows of all nationalities and their Universities. The Alexander Von Humboldt Foundation Fellowships are open to all professors who meet the above-mentioned requisites and who are less than 40 years of age. There is also one specialized fellowship for candidates between 40 and 45 years of age. These fellowships are for 6 to 24 months, receiving 3400 to 4200 German marks monthly.

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