Gaucher disease: Biochemical and molecular findings in 141 patients diagnosed in Greece

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ABSTRACT

Gaucher disease (GD) is characterized by a marked phenotypic and genetic diversity. It is caused by the functional deficiency of the lysosomal enzyme β-glucocerebrosidase (GCase), which in most instances results from mutations in the GBA1 gene and over 500 different disease causing mutations have been described. We present the biochemical and molecular findings in 141 GD cases (14 were siblings) with the three types of the disorder diagnosed in Greece over the last 35 years. 111/141 (78%) GD patients were of Greek origin. The remaining patients were Albanian (24/141; 17%), Syrian (2/141; 1.4%), Egyptian (2/141; 1.4%), Italian (1/141; 0.7%) and Polish (1/141; 0.7%). Mutation analysis identified 28 different mutations and 37 different genotypes. Seven of the mutations were not previously reported (T231I, D283N, L175P, F81L, Y135S and T482K). The most frequent mutations were N370S, D409H;H255Q and L444P. Mutation D409H;H255Q was only identified in Greek and Albanian patients. Sixteen mutations, including the novel ones, were identified only in one allele. Although the N370S mutation was identified only in type 1 patients, not all of type 1 patients carried this mutation. Our results highlight the heterogeneity of Gaucher disease and support the Balkan origin of the double mutant allele D409H;H255Q.

1. Introduction

Gaucher disease (GD) is a rare autosomal recessive disorder that belongs to the group of lysosomal storage diseases. It results from the functional deficiency of the lysosomal enzyme β-glucocerebrosidase (GCase; also called glucosylceramidase or acid β-glucosidase, E.C. 3.2.1.45) which, in most instances, is associated with mutations in the GBA1 gene located on chromosome 1 (1q21) (MIM#606463, Gen Bank accession no J030591). The deficient enzyme activity leads to the accumulation of its substrate glucosylceramide, mainly in tissue macrophages, transforming them into the characteristic Gaucher cells. On the basis of the presence and rate of progression of neurological involvement, GD has traditionally been subdivided into three phenotypes. GD type 1 is the non-neuronopathic (MIM#230800), type 2 is the acute neuronopathic with early onset and rapid neurological deterioration (MIM#230900), whereas type 3 is the chronic neuronopathic phenotype with slower progressing neurological features [1]. However, it has become evident that this distinction is often blurred and the disease would be more correctly characterized as a continuum of phenotypes ranging from asymptomatic adult patients at the mild end to perinatal lethal disease at the severe end of the spectrum [2–5]. The GBA1 gene is located on the long arm of chromosome 1 (1q21) with a highly homologous pseudogene (GBAP) located 16 kb downstream. The high degree of sequence identity and proximity of GBA1 and GBAP contributes to recombination events between the two loci [6,7].

Up to date more than 500 mutations in the GBA1 gene have been described in GD patients (HGMD professional 2019.4) highlighting the molecular heterogeneity of the disorder. Some of these mutations, such as N370S, L444P, c.84dup, IVS2 + 1 G > A and RecNcII, are more common. Differences in the frequency of different mutations is observed in different populations. For instance, mutations N370S, L444P, c.84dupG and c.115 + 1G > A account for 90% of the mutant alleles in Ashkenazi Jewish patients, whereas they account for < 75% of the mutant alleles in other populations [7–9]. Although genotype - phenotype correlations are not straightforward, it is possible to draw some predictions. For example, the N370S mutation is only found in association with type 1 GD whereas

Abbreviations: GBA1, Glucocerebrosidase gene; GD, Gaucher disease; GCase, β-Glucocerebrosidase

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The same mutation and even in identical twins [10]. Manifestations and severity can be observed in patients homozygous for neurological involvement. Yet, considerable heterogeneity in disease homozygosity for mutation L444P is generally associated with some neurological involvement. Yet, considerable heterogeneity in disease manifestations and severity can be observed in patients homozygous for the same mutation and even in identical twins [10].

We present the biochemical and molecular findings in 141 (14 were siblings) cases of GD diagnosed in Greece the last 35 years. 111/141 (11 were siblings) cases of GD diagnosed in Greece the last 35 years. 111/141 (11 were siblings) were of Greek origin and 27/125 were of other ethnic origins, mainly Albanians, living in Greece. We also describe 7 novel mutations identified in our cohort of GD patients.

2. Patients and methods

2.1. Patients

The present report includes 141, 111 of Greek origin and 30 of other ethnic origins, GD patients of which 14 were siblings. They were referred for diagnosis to the Institute of Child Health on the basis of their clinical evaluation and/or laboratory studies.

A brief description of the patients is shown in Table 1.

The laboratory investigations performed include assaying of chitotriosidase activity in plasma, assaying of GCase in white blood cells and/or skin fibroblast cultures and DNA analysis. The study was approved by the Ethics Committee of the Institute of Child Health, Athens.

2.2. Methods

2.2.1. Enzyme studies

Chitotriosidase activity was assayed in plasma using the 4-methylumbelliferyl β-D-N, N’-transaminases, Leucopenia, Anemia, Member of patient family

| Type 2 | Birth – 8 months | Hepatosplenomegaly |
|--------|------------------|--------------------|
| n = 13 | Median 1 month   | Hydrops Fetalis, Collidion Baby |
| (9F-4M)|                  | Ascites             |
|        |                   | Bilkirrhinemia, ↑ transaminases |
|        |                   | Cholestasis, Hyperemia, Opisthotonus |
|        |                   | Irritability, Oculomotor Apraxia |

| Type 3 | 10 months – 8 years | Hepatosplenomegaly |
|--------|-------------------|--------------------|
| n = 8  | Median 2.5 years  | Supranuclear paralysis, Psychomotor retardation |
| (3F-5M)|                  |                    |

F: Female, M: Male

Table 1

Age of diagnosis and clinical characteristics of the Gaucher disease patients diagnosed in Greece.

| GD patients | Age of diagnosis | Reasons for referral |
|-------------|------------------|----------------------|
| Type 1      | 3–77 years       | Splenomegaly         |
| n = 120     | Median 28 years  | Hepatosplenomegaly   |
| (59F-61M)   | Bone Involvement | Cholestasis          |
|             | Thrombocytopenia |                      |
|             | Pancytopenia     |                      |
|             | Leucopenia       |                      |
|             | Anemia           |                      |
|             | Member of patient family |

Type 2

| n = 13 | Birth – 8 months | Hepatosplenomegaly |
|--------|------------------|--------------------|
| (9F-4M)|                  | Hydrops Fetalis, Collidion Baby |
|        |                   | Ascites             |
|        |                   | Bilkirrhinemia, ↑ transaminases |
|        |                   | Cholestasis, Hyperemia, Opisthotonus |
|        |                   | Irritability, Oculomotor Apraxia |

Type 3

| n = 8  | 8 years          | Hepatosplenomegaly |
|--------|-----------------|--------------------|
| (3F-5M)| Median 2.5 years|                    |
|        | Supranuclear paralysis, Psychomotor retardation |

| F: Female, M: Male |

2.2.2. Molecular analysis

Mutations N370S, D409H, H255Q, L444P, R120W, IVS10-1G > A, IVS5-2A > G and Y108C were investigated by PCR and restriction enzyme analysis. Automated sequencing of all exons and flanking regions of the GBA1 gene was applied for the identification of the rest of the mutations. Automated sequencing was also used, when appropriate, to study the presence of Rec alleles and discriminate between L444P and L444R.

The prediction tools used for investigating the pathogenicity of novel mutations were PolyPhen-2 HumDiv and Hum Var; SIFT Human Protein and SIFT Blink; Mutation Taster.

3. Results

The demographics of all the patients diagnosed, 111 of Greek origin and 30 of other ethnic origins, are shown in Table 1. Depending on the presence and severity of neurological findings they were classified as Type 1 (n = 120), Type 2 (n = 13) and Type 3 (n = 8). Age of diagnosis for the type 1 patients ranged from 3 to 77 years, for the type 2 cases from birth to 8 months and for type 3 from 10 months – 8 years.

Age of diagnosis and clinical characteristics of the Gaucher disease patients diagnosed in Greece.

| Patients | Chitotriosidase Activity (nmols/ml/h) | β-Glucosidase Activity WBC (nmols/mg Protein/h) | β-Glucosidase Activity Fibroblasts (nmols/mg Protein/h) |
|----------|--------------------------------------|-----------------------------------------------|---------------------------------------------------|
| Type 1   | 426–35,825a                          | 0.25–4.6                                      | 0–8.0                                             |
|          | mean: 12545                          | mean: 1.89                                    | Mean: 3.47                                        |
|          | n = 111                              | n = 97                                        | n = 23                                            |
|          |                                     |                                              |                                                   |
| Type 2   | 720–72,366                           | 0.25–3.5                                      | 0.34–4.0                                          |
|          | mean: 2948                           | mean: 1.3                                     | Mean: 2.0                                         |
|          | n = 11                               | n = 12                                       | n = 8                                            |
|          |                                     |                                              |                                                   |
| Type 3   | 8332–35,000                          | 1.9–2.5                                       | 0–1.6                                            |
|          | mean: 15232                          | mean: 2.18                                    | Mean: 0.8                                        |
|          | n = 8                                | n = 6                                        | n = 2                                            |
|          |                                     |                                              |                                                   |
|          | Normal Range 0–150                   | 6–23                                          | 19–113                                           |

a Two patients had zero chitotriosidase activity being homozygotes for the 24 bp duplication.

Table 2

Biochemical and molecular findings in the Gaucher disease patients diagnosed in Greece.

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Allele distribution in our cohort of patients (n = 125).

| cDNAa | Protein (Traditional in GD) | Protein (as recommended by HGVSa) | No. of alleles |
|-------|-----------------------------|-----------------------------------|---------------|
| c.1226A > G | N730S | p.N409S | 121 (49.2%) |
| c.1342G > Cc.882 T > G | D409H | p.D448H;H294Q | 6 (4.8%) |
| c.1448 T > G | L444P | p.L483P | 25 (10.2%) |
| c.475C > T | R120W | p.R159Q | 8 (3.3%) |
| c.1505G > A | Y116H | p.Y155H | 1 (0.4%) |
| c.762-2A > G | IVS2 + 1G | p.G241R | 3 (1.2%) |
| c.374C > A | L175P | p.L214P | 2 (0.8%) |
| c.115 + 1 G > A | RecNcil | 5 (2.0%) |
| c.1603C > T | R359X | p.R398* | 1 (0.4%) |
| c.657 T > C | G202R | p.G241R | 3 (1.2%) |
| c.846 C > T | R257X | p.R296* | 2 (0.8%) |
| c.260G > A | R48Q | p.R87Q | 1 (0.4%) |
| c.440A > G | Y108C | p.Y147C | 1 (0.4%) |
| c.463 T > C | R47X | p.R86* | 2 (0.8%) |
| c.721G > A | G202R | p.G241R | 3 (1.2%) |

No. of alleles (10.2%). It accounted for 17/192 (8.9%) and 8/54 (16.7%) identifications.

Mutations in the same codon have been described for three of these codons and are data in favor of the pathogenicity of these novel mutations. The effect of mutation T231I on the enzyme activity was evaluated by in vitro expression. According to our findings, the mutation reduces the enzyme activity to 12% of wild type.

Their pathogenicity was evaluated using different prediction tools. The results are shown in Table 7. Overall, mutations T231I, N462Y, L175P, F81L, D283N and Y135S were classified as disease causing whereas T482K was evaluated as damaging with low confidence with the SIFT Human Protein and SIFT Blink tool and as benign with the other tools used. The fact that some of the novel mutations affect the same codons of previously described mutations confirms the relevance of these codons and are data in favor of the pathogenicity of these novel mutations. The effect of mutation F81L on the enzyme activity was evaluated by in vitro expression. According to our findings, the mutation reduces the enzyme activity to 12% of wild type.

**Table 4**

Genotypes and clinical subtypes of all diagnosed Gaucher disease patients.

| Genotypes | TYPE 1 | TYPE 2 | TYPE 3 | TOTAL |
|-----------|--------|--------|--------|-------|
| N7/10S | D409H | H255Q | 29 | 29 (23.4%) |
| N7/10S | N7/10S | 17 | 17 (13.7%) |
| N7/10S | L444P | 14 | 14 (11.3%) |
| N7/10S | IVS6-2A > G | 6 | 6 (4.8%) |
| N7/10S | R120W | 6 | 6 (4.8%) |
| N7/10S | IVS510-1G > A | 5 | 5 (4%) |
| N7/10S | 7 | 3 | 3 (2.4%) |
| N7/10S | A309V | 3 | 3 (2.4%) |
| N7/10S | RecNcil | 3 | 3 (2.4%) |
| N7/10S | R257X | 2 | 2 (1.6%) |
| N7/10S | R47X | 2 | 2 (1.6%) |
| N7/10S | R48Q | 2 | 2 (1.6%) |
| N7/10S | T231I | 1 | 1 (0.8%) |
| N7/10S | N462Y | 1 | 1 (0.8%) |
| N7/10S | L175P | 1 | 1 (0.8%) |
| N7/10S | R496C | 1 | 1 (0.8%) |
| N7/10S | F81L | 1 | 1 (0.8%) |
| N7/10S | IVS6-2A-G | 1 | 1 (0.8%) |
| N7/10S | IVS510-1G | 1 | 1 (0.8%) |
| N7/10S | N370S | 1 | 1 (0.8%) |
| N7/10S | T231I | 1 | 1 (0.8%) |
| N7/10S | T482K | 1 | 1 (0.8%) |
| N7/10S | L444P | 1 | 1 (0.8%) |
| N7/10S | Y135S | 1 | 1 (0.8%) |
| N7/10S | G202R | 1 | 1 (0.8%) |
| N7/10S | T482K | 1 | 1 (0.8%) |
| N7/10S | L444P | 1 | 1 (0.8%) |
| N7/10S | Y116H | 1 | 1 (0.8%) |
| N7/10S | D409H | H255Q | 5 | 5 (4%) |
| N7/10S | D409H | H255Q | 2 | 2 (1.6%) |
| N7/10S | IVS510-1G > A | 1 | 1 (0.8%) |
| N7/10S | RecNcil | 1 | 1 (0.8%) |
| N7/10S | G202R | 1 | 1 (0.8%) |
| N7/10S | IVS2 + 1G | 1 | 1 (0.8%) |
| N7/10S | RecNcil | 1 | 1 (0.8%) |
| N7/10S | L444P | 3 | 3 (2.4%) |
| N7/10S | D409H | H255Q | 2 | 2 (1.6%) |
| N7/10S | IVS6-2A-G | 1 | 1 (0.8%) |
| N7/10S | IVS510-1G | 1 | 1 (0.8%) |

them (T231I, p.T270I; D283N (p.D322N); N462Y, p.N501Y; F81L, p.F120L; Y135S, p.Y174S; T482K, p.T521K) were identified in Greek type 1 patients and one (L175P, p.L214P) in an Albanian type 1 patient. Mutations in the same codon have been described for three of the above. These were mutations T231R [13], N462K [14], N462S [15], Y135C [16], Y135X [17] and were identified in GD patients and/or patients with Parkinson’s disease.

The most frequent mutation was L444P, found in 25/246 identified alleles (10.2%). It accounted for 17/192 (8.9%) and 8/54 (14.8%) of the alleles identified in Greek and non-Greek patients, respectively. The mutation was identified in heterozygosity in type 1 and type 3, and in homozygosity in type 3 patients.

Of the remaining mutations, 16 (T231I, D283N, N462Y, L175P, R496C, F213L, H311R, W184R, R359X, F81L, Y135S, T482K, Y108C, Y116H, D409H, IVS2 + 1G → A) were identified only in single alleles in both Greek and non-Greek patients. Mutations IVS10-1G > A, IVS6-2A > G and G202R were only identified in Greek patients, accounting for 7/192 (3.6%), 6/192 (3.1%) and 3/192 (1.6%), respectively, of the identified alleles. IVS10-1G > A was identified in heterozygosity in type 1 and type 2 patients whereas IVS6-2A > G was identified in heterozygosity only in type 1 patients. G202R was identified in heterozygosity in type 1 and in homozygosity in type 2 patients. R120W was identified in heterozygosity in both type 1 and type 2 Greek patients 7/192, 3.6% of the identified alleles) and in heterozygosity in one type 1 Albanian patient (1/54, 1.9% of the identified alleles).

Along with the previously published mutations, 7 novel mutations were identified all in heterozygosity in our cohort of patients. Six of
Genotypes, Clinical subtypes and origin of non-Greek Gaucher disease patients.

| Genotypes               | TYPE 1 | TYPE 2 | TYPE 3 | TOTAL |
|-------------------------|--------|--------|--------|-------|
| N370S/D409H/H255Q       | 19     |        |        | 19    |
| N370S/N370S             | 14     |        |        | 14    |
| N370S/L444P             | 9      |        |        | 9     |
| N370S/L444P/255-2A → G  | 6      |        |        | 6     |
| N370S/R120W             | 5      |        |        | 5     |
| N370S/IVS10-1G → A      | 5      |        |        | 5     |
| N370S/Y                 | 3      |        |        | 3     |
| N370S/A390V             | 3      |        |        | 3     |
| N370S/RecNciI           | 3      |        |        | 3     |
| N370S/R120W             | 2      |        |        | 2     |
| N370S/R48Q              | 2      |        |        | 2     |
| N370S/R47X              | 1      |        |        | 1     |
| N370S/T231I             | 1      |        |        | 1     |
| N370S/N462Y             | 1      |        |        | 1     |
| N370/T213L              | 1      |        |        | 1     |
| N370/H311R              | 1      |        |        | 1     |
| N370/R184R              | 1      |        |        | 1     |
| N370S/R359X             | 1      |        |        | 1     |
| L444P/F81L              | 1      |        |        | 1     |
| N370S/Y135S             | 1      |        |        | 1     |
| N370S/N135S             | 1      |        |        | 1     |
| N370S/G202R             | 1      |        |        | 1     |
| N370S/D283N             | 1      |        |        | 1     |
| N370S/T462K             | 1      |        |        | 1     |
| L444P/Y116H             | 1      |        |        | 1     |
| D409H/H255Q/D409H/H255Q | 2      |        |        | 2     |
| D409H/H255Q/R120W       | 2      |        |        | 2     |
| IVS101G → A/            | 1      |        |        | 1     |
| D409H/H255Q/RecNciI     | 1      |        |        | 1     |
| G202R/G202R             | 1      |        |        | 1     |
| IVS2 + 1G → A/         | 1      |        |        | 1     |
| RecNciI/IVS10-1G → A    | 1      |        |        | 1     |
| L444P/L444P             | 2      |        |        | 2     |
| L444P/D409H/H255Q       | 1      |        |        | 1     |
| D409H/H255Q/Y108C       | 1      |        |        | 1     |
| L444P/D409H             | 1      |        |        | 1     |

4. Discussion

GD, the most prevalent lysosomal storage disease, is a rare panethenic disorder and this is illustrated in our cohort of 141 patients, which includes patients of six different national origins. Its estimated prevalence in the general population ranges from 1/40,000 to 1/60,000 births but it can be as high as 1/800 births in the Ashkenazi Jewish population [17–19]. Assuming a birth rate of 100,000/year, a rough incidence estimate for GD in the Greek population would be 2.8/100,000 births. However, this is most likely an underestimate since the vast clinical variability of the disease along with the existence of patients which lack overt clinical symptoms can lead to its under-diagnosis. In fact, in a previous study of the genomic DNA of 1933 Guthrie cards, we showed that the frequency of the N370S mutation in our population was 0.0046 with 95% limits between 0.0025 and 0.0068 and thus the expected number of type 1 GD patients homozygous for the N370S mutation would be 238 [20]. However, up to date only 17 Greek GD type 1 patients with this genotype have been diagnosed in our lab, which is the only center in Greece providing the diagnosis of lysosomal storage diseases. Similar findings were reported in studies of the Ashkenazi and Portuguese populations [21,22]. Despite its apparent under-diagnosis, GD is still the most frequently diagnosed lysosomal storage disease in Greece. The cases presented here account for 27.4% (141/514) of the total number of patients diagnosed with a lysosomal storage disorder in our center. The Greek patients of our cohort originated from all parts of Greece, however clusters of the disease were identified in Central and Northern Greece, the Peloponnese and the greater area of Athens where more than half of the population resides. The majority of the patients from countries other than Greece were Albanians that immigrated to Greece since 1990 when the borders between the two countries opened. The disorder was also diagnosed in refugees that arrived in Greece in recent years.

GD is a highly heterogeneous disorder both clinically and genetically and this is also observed in our cohort of patients. Clinically our patients covered the whole spectrum of phenotypes associated with GD. Overall and on the basis of their clinical picture, 85.1% of the patients were classified as type 1, 9.2% as type 2 and 5.7% as type 3. At the severe end of the spectrum we identified patients with severe perinatal disease, one of whom had the collodion phenotype and was previously described in detail [23]. At the mild end of the spectrum we identified two asymptomatic patients that were diagnosed at the age of 8 and 19 years following the diagnosis of GD in their mothers. The genotype of the former was N370S/R120W, whereas the latter was homozygous for the N370S, carrying a genotype that is often associated with very mild disease.

Over 500 different mutations have been described in the GBA1 gene including point mutations, deletions, insertions, splicing aberrations and various rearrangements. 425 are classified as disease-causing mutations, 79 as probable/possible pathological mutations and 1 as functional polymorphism (HGMD professional 2019.4). A notable variation in the distribution of different mutations has been observed in different populations [7–9,24–27].

The N370S mutation is particularly common in the Ashkenazi Jewish population where it accounts for 75%–80% of the alleles, its frequency is much lower in other populations and is totally absent in the Jewish population where it accounts for 75%–80% of the alleles. Similar findings were reported in studies of the Ashkenazi and Portuguese populations [21,22]. Despite its apparent under-diagnosis, GD is still the most frequently diagnosed lysosomal storage disease in Greece. The cases presented here account for 27.4% (141/514) of the total number of patients diagnosed with a lysosomal storage disorder in our center. The Greek patients of our cohort originated from all parts of Greece, however clusters of the disease were identified in Central and Northern Greece, the Peloponnese and the greater area of Athens where more than half of the population resides. The majority of the patients from countries other than Greece were Albanians that immigrated to Greece since 1990 when the borders between the two countries opened. The disorder was also diagnosed in refugees that arrived in Greece in recent years.

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The N370S mutation is particularly common in the Ashkenazi Jewish population where it accounts for 75%–80% of the alleles, its frequency is much lower in other populations and is totally absent in the Japanese [7,8,13,18,24,25]. In our study, it was the most frequently identified mutation accounting for 49.2% of the alleles in our cohort of GD patients and showing similar frequencies...
between the Greek and non-Greek patients (50.0% and 46.3% respectively). The mutation is known as the ‘neuroprotective mutation’ since it is exclusively found in type 1 patients. However, not all type 1 GD patients carry this mutation [7,13,17,18,25]. In our cohort of patients, the mutation was not identified in two patients classified as type 1. The first patient was diagnosed at the age of 10 with massive hepatosplenomegaly, which had manifested 2 months earlier. In her medical history, however, it is reported that six years earlier, following a respiratory infection, she developed splenomegaly, which regressed after six months. Her growth was normal. The second, diagnosed by enzyme studies and DNA analysis at the age of 48 years, had been splenectomised in early childhood and had severe bone involvement. They both remain free of any neurological symptoms at the age 17 and 60 years (Dr Garoufi and Dr. Marinakis, personal communication) and their respective genotypes are L444P/Y116H and L444P/F81L. L444P is the major mutation in the Norbottnian type 3 GD and in homozygosity it is identified as disease causing and in vitro expression studies showed it results in severe reduction of enzyme activity. Although the patient bearing the genotype L444P/F81L did not exhibit clinical features associated with the phenotype of the patient is not clear [29]. F81L identified in the second patient is a novel mutation. It is predicted to be disease causing mutation, whereas A448T was classified as likely to be disease causing. The impact of the coexistence of the two different mutations on the phenotype of the patient was partially funded by the Spanish Ministerio de Economía y Competitividad (SAF2016-75948-R).

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**Author statement**

All authors have approved the revision of the paper and its submission. The paper has not been published previously and is not under consideration for publication elsewhere.

Individual contribution to the article.

Evangelia Dimitriou was involved in the assaying of the activity of chitotriosidase and β-glucocerebrosidase as well as the writing of the paper.

Marina Moraitou was involved in DNA preparation, the investigation of mutations using PCR and restriction enzyme analysis, as well as the writing of the paper.

Mónica Cozar, Jenny Serra-Vinardell, Lluïsa Vilageliu and Daniel Grinberg were involved in automated sequencing of *GBA*1* and site-directed mutagenesis.

Lluïsa Vilageliu and Daniel Grinberg were also involved in the writing of the paper.

Irene Mavridou was involved in fibroblast cultures as well as the writing of the paper.

Helen Michelakakis was involved in designing, coordinating the study, evaluation of the results as well as the writing of the paper.

**Declaration of Competing Interest**

None.

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