

## Molecular Heterogeneity of Glucose-6-Phosphate Dehydrogenase (G6PD) Deficiency in the Portuguese Population

### Heterogeneidade Molecular da Deficiência em Glicose-6-Fosfato Desidrogenase (G6PD) na População Portuguesa

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#### ABSTRACT

**Introduction:** Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common enzyme defect in the world, affecting more than 500 million people. In Portugal, the average frequency of G6PD deficiency in males was estimated at about 0.5% and since the year 2000 several G6PD-deficient variants have been identified. The main goal of this study was to improve the knowledge on the molecular heterogeneity of G6PD deficiency in the Portuguese population.

**Material and Methods:** A retrospective analysis of the mutational profile of 138 unrelated Portuguese individuals (101 males; 37 females), with no known sub-Saharan ancestry, who had been diagnosed with G6PD deficiency between 1994 and 2020 at the Molecular Hematology Unit of Centro Hospitalar e Universitário de Coimbra. The molecular study was done by direct Sanger sequencing or PCR-RFLP analysis.

**Results:** Twenty-one different pathogenic mutations were found. Among them, 20 were missense, causing the amino acid change, and one was an in-frame deletion in exon 10. The three most frequent mutations belong to the *G6PD* c.376A>G African background haplotype, namely the G6PD variants: A- (c.202G>A; p.68Val>Met) (58.6%), Betica (c.968T>C; p.323Leu>Pro) (12.1%) and Santamaria (c.542A>T; p.181Asp>Val) (4.3%).

**Conclusion:** There is a wide molecular heterogeneity of G6PD deficiency in the Portuguese population.

**Keywords:** Anemia, Hemolytic; Glucosephosphate Dehydrogenase Deficiency/epidemiology; Mutation; Portugal

#### RESUMO

**Introdução:** A deficiência de glicose-6-fosfato desidrogenase (G6PD) é o defeito enzimático mais comum no mundo, afetando mais de 500 milhões de pessoas. Em Portugal, a frequência populacional da deficiência de G6PD no sexo masculino foi estimada em cerca de 0,5%, e desde o ano 2000 têm vindo a ser descritas diversas variantes G6PD causadoras da deficiência. O principal objetivo deste estudo foi melhorar o conhecimento sobre a heterogeneidade molecular da deficiência de G6PD na população portuguesa.

**Material e Métodos:** Análise retrospectiva do perfil mutacional de 138 indivíduos não aparentados de naturalidade portuguesa (101 homens e 37 mulheres), sem ascendência subsaariana conhecida, diagnosticados com deficiência de G6PD entre 1994 e 2020 na Unidade de Hematologia Molecular do Centro Hospitalar e Universitário de Coimbra (CHUC). O estudo molecular foi feito por sequenciação direta de Sanger ou análise por PCR-RFLP.

**Resultados:** Identificaram-se 21 mutações patogénicas diferentes. Destas, 20 são mutações *missense*, que levam à troca de aminoácido, e uma é uma deleção *in-frame* de 18 nucleótidos no exão 10. As três mutações mais frequentes pertencem ao haplótipo subsaariano *G6PD* c.376A>G, nomeadamente as variantes G6PD: A- (c.202G>A; p.68Val>Met) (58,6%), Betica (c.968T>C; p.323Leu>Pro) (12,1%) e Santamaria (c.542A>T; p.181Asp>Val) (4,3%).

**Conclusão:** Existe uma elevada heterogeneidade molecular da deficiência de G6PD em Portugal.

**Palavras-chave:** Anemia Hemolítica; Deficiência de Glucosefosfato Desidrogenase /epidemiologia; Mutação; Portugal

#### INTRODUCTION

Since Glucose-6-phosphate dehydrogenase (G6PD) deficiency (OMIM#300908) is the most common enzyme defect in human populations, affecting more than 500 million people.<sup>1</sup> The prevalence of G6PD deficiency is highly variable with a particular high incidence in tropical Africa and Asia, Middle East, Southern Europe and Mediterranean countries, reflecting mainly the association with the worldwide distribution of malaria, but also the impact of human migration and resettlement events.<sup>2</sup>

The G6PD enzyme (E.C. 1.1.1.49) is responsible for the first step of the pentose phosphate pathway, in which NADPH, the reducing power required to protect red blood cell against oxidative stress, is produced.<sup>3-5</sup> Most of G6PD deficient individuals are asymptomatic, only experiencing

episodic acute haemolytic anaemia (AHA) in the contexts of oxidative stress when exposed to infection, certain drugs or fava beans ingestion (classes II, III and to IV of G6PD variants).<sup>5,6</sup> Only a few rare class I variants, cause chronic non-spherocytic hemolytic anemia (CNSHA).<sup>5,7,8</sup>

The enzyme deficiency results from mutations in the *G6PD* gene (OMIM#305900) located in the telomeric region of chromosome Xq28.<sup>9</sup> Therefore, the inheritance of G6PD deficiency shows a typical X-linked pattern: hemizygous males, as well as homozygous females for *G6PD* mutations, will have the disorder; heterozygous females are just carriers even though they may have haemolytic episodes, specially at an advanced age, due to an imbalanced lyonisation (X-inactivation) favouring the mutated allele.

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The *G6PD* gene spans over 18 kb and is composed of 13 exons and 12 introns.<sup>10</sup> More than 200 G6PD variants are reported, and most of them are due to single nucleotide substitutions that lead to changes of aminoacids (missense mutations).<sup>11,12</sup> In sub-Saharan Africa, the G6PD A- variant (c.376A>G / c.202G>A), with about 12% of normal activity, is the most common and can be present with a frequency of up to 25%.<sup>7</sup> In the Mediterranean region, the Middle East and India, the most common mutation, c.563C>T (Mediterranean variant), characterized by less than 10% of normal enzyme activity, is present at a frequency of 2% - 20% in different populations.<sup>7</sup> Patients with CNSHA have the Class I variants, with mutations more frequently located in exon 10, which encode aminoacids positioned at the dimer interface, and are thus essential for protein stability.<sup>1,5</sup>

In Portugal, the average frequency of G6PD deficiency in males was estimated to be 0.51%<sup>13</sup> and 0.39%<sup>14</sup> in different studies, and not uniformly distributed throughout the country. The aim of the present study is to describe the molecular heterogeneity of G6PD deficiency in the Portuguese population, based on the molecular studies performed at the Molecular Hematology Unit of Centro Hospitalar e Universitário de Coimbra (CHUC) between 1994 and 2020. *G6PD* mutations found in other Portuguese individuals previously described in the literature were also included for analysis.

## MATERIAL AND METHODS

### Population

This retrospective study assessed the mutational profile of 138 Portuguese individuals with G6PD deficiency (101 males and 37 females; aged between 2 and 74 years), diagnosed between 1994 and 2020, based on recent data and results obtained in past periods, some of which have been published.<sup>15,16</sup> All individuals, unrelated and with no known sub-Saharan ancestry, were mostly from central Portugal (about 65%), but also from the southern (about 15%) and northern (about 8%) regions, and the Azores archipelago (about 2%). In about 10% of the cases, it was not possible to identify the place of birth.

The study was conducted in accordance with the Declaration of Helsinki. All data were anonymized and analyzed together, never individually. Being a retrospective study, with anonymized data, analyzing results obtained between 1994 and 2020, some of them already published, it was not considered mandatory the submission to the ethics committee approval, and it was not possible to obtain now the informed consent.

### Hematological and biochemical studies

The diagnosis of G6PD deficiency was made based on the clinical history, routine hematological parameters quantified in a Sysmex XN-1500™ analyzer (Sysmex Eu-

rope GMBH, Norderstedt, Germany) and demonstration of a reduced erythrocyte G6PD activity apart from the hemolytic episode. Enzyme quantification was accomplished by quantitative spectrophotometric enzymatic assay according to recommendations of the International Committee for Standardization in Hematology (ICSH).<sup>17</sup> The percentage of enzymatic activity compared to normal levels was calculated using as a control, in each case, a healthy age-matched male without G6PD deficiency.

### Molecular studies

Genomic DNA was extracted from EDTA peripheral blood samples, using standard methodologies. *G6PD* exons 2 to 13 were amplified by the polymerase chain reaction (PCR) using appropriate oligonucleotide primers, as previously described.<sup>18-20</sup> Molecular studies of the *G6PD* gene were performed by direct Sanger sequencing of all the exons and adjacent intronic regions and 3'UTR regions. The three most common mutations were analyzed by PCR-RFLP. Mutation c.376A>G was verified with *FokI*, and the alleles with this nucleotide change (G6PD A variants), were tested for mutation c.202G>A with the restriction enzyme *NlaIII*; alleles with both mutations c.376G/c.202A were classified as G6PD A-. Alleles classified as A but not as A- were then tested for mutation c.968T>C (G6PD Betica) with *MspI*, or for mutation c.542A>T (G6PD Santamaria) with *SfaNI*. Sanger sequencing was performed using the ABI Prism BigDye® Terminator V 1.1 Cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and the ABI 3130 genetic analyzer (Applied Biosystems).

### Statistical analysis

Hematological, biochemical and molecular data from 138 Portuguese individuals with G6PD deficiency were collected and anonymized. Frequencies of mutated alleles were calculated as the number of mutated alleles (one in hemizygous males, one in heterozygous females, and two in homozygous females) divided by the total number of mutated alleles. The levels of G6PD enzymatic activity, estimated in IU/g Hb, were compared between the two groups, hemizygous males versus heterozygous females, using the T test for equality of means. Data normality in both groups was verified using the Kolmogorov-Smirnov test. The continuous variable was described as the mean ± standard deviation (SD). Statistical analysis and graphics were performed using IBM SPSS software, version 24 (IBM, NY, USA). A *p*-value lower than 0.05 was considered statistically significant.

## RESULTS

### Mutational spectrum

The pathogenic *G6PD* mutations identified in a total of

138 Portuguese individuals with the enzyme deficiency, including 101 hemizygous males, 35 heterozygous females and two homozygous females, are detailed in Table 1. Twenty-one different pathogenic mutations were found in a total of 140 mutated chromosomes: 20 missense mutations, which led to an amino acid change, and an in-frame deletion of 18 nucleotides in exon 10 (G6PD Tondela), identified in an elderly female with CNSHA.<sup>16</sup>

The most frequent *G6PD* mutations that were identified belong to the African background haplotype c.376G (*G6PD* A allele). The most frequent variant is G6PD A- (c.202G>A; p.68Val>Met), found in 57 hemizygous males, 23 heterozygous females and 1 homozygous female (82/140 chr; 58.6%) (Table 1). The variant G6PD Betica (c.968T>C; p.323Leu>Pro) is the second most common, and was found in 16 hemizygous males and one heterozygous female (17/140 chr; 12.1%). The variant G6PD Santamaria (c.542A>T; p.181Asp>Val) was found in four hemizygous

males and two heterozygous females (6/140 chr; 4.3%).

Eighteen rare variants of *G6PD* were also found (Table 1). The *G6PD* mutation underlying variants known as Vanua Lava (c.383T>C), Taipei (c.493A>G), Shinshu (c.527A>G), Chatham (c.1003G>A), Mira d'Aire (c.1048G>C), Tondela (c.1076-c.1093del), Anadia (c.1193A>G), Covão do Lobo (c.1205C>A), Canton (c.1376G>T), Kamiube (c.1387C>T) and Flores (c.1387C>A) were found in just one chromosome; mutation underlying variants known as Azores (c.595A>G), Mexico City (c.680G>A), Figueira da Foz (c.1366G>C) and Kaiping (c.1388G>A) were found in two chromosomes; and mutation Mediterranean (c.563C>T), Coimbra (c.592C>T) and Seattle (c.844G>C) were found in three chromosomes (Table 1).

### Genotype-phenotype correlation

From the subjects with the common G6PD A- variant, we obtained information on G6PD enzyme activity for 22

**Table 1** – *G6PD* mutations found in 138 individuals (101 males and 37 females), studied at the Molecular Hematology Unit of CHUC

Variant	Mutation	Consequence	n chr (%)	Exon	Class	Hemi/Het/Hom	Reference
A-	c.376A>G / c.202G>A	p.126Asn>Asp / p.68Val>Met	82 (58.6%)	5/4	III	57/23/1	14,15
Betica	c.376A>G / c.968T>C	p.126Asn>Asp / p.323Leu>Pro	17 (12.1%)	5/9	III	16/1/0	14,15
Santamaria	c.376A>G / c.542A>T	p.126Asn>Asp / p.181Asp>Val	6 (4.3%)	5/6	II	4/2/0	14,15
Vanua Lava	c.383T>C	p.128Leu>Pro	1	5	II	0/1/0	This study
Taipei	c.493A>G	p.165Asn>Asp	1	6	II	1/0/0	This study
Shinshu	c.527A>G	p.176Asp>Gly	1	6	I	1/0/0	This study
Mediterranean	c.563C>T	p.188Ser>Phe	3	6	II	1/2/0	14
Coimbra	c.592C>T	p.198Arg>Cys	3	6	II	1/2/0	15,26
Azores	c.595A>G	p.199Ile>Val	2	6	II	0/2/0	15
Mexico City	c.680G>A	p.227Arg>Gln	2	7	III	2/0/0	This study
Seattle	c.844G>C	p.282Asp>His	3	8	III	1/0/1	14,15
Chatham	c.1003G>A	p.335Ala>Thr	1	9	II	1/0/0	15
Mira d'Aire	c.1048G>C	p.350Asp>His	1	9	III	1/0/0	15
Tondela	c.1076-c.1093del	p.362-367del	1	10	I	0/1/0	16
Anadia	c.1193A>G	p.398Glu>Gly	1	10	II	0/1/0	15
Covão do Lobo	c.1205C>A	p.402Thr>Asn	1	10	I	1/0/0	15
-	c.1311C>T	p.437Tyr=	5	11	III	5/0/0	This study
Figueira da Foz	c.1366G>C	p.456Asp>His	2	12	I	2/0/0	15
Canton	c.1376G>T	p.459Arg>Leu	1	12	II	1/0/0	15
Kamiube	c.1387C>T	p.463Arg>Cys	1	12	III	1/0/0	15
Flores	c.1387C>A	p.463Arg>Ser	1	12	II	0/1/0	14
Kaiping	c.1388G>A	p.463Arg>His	2	12	II	2/0/0	This study

n chr: number of mutated chromosomes; Hemi: hemizygous males; Het: heterozygous females; Hom: homozygous females. A total of 140 mutated alleles were found.

References are from published cases of Portuguese origin.

hemizygous males and 14 heterozygous females. The hemizygous males showed a mean G6PD activity of  $1.46 \pm 0.95$  IU/g Hb and the heterozygous females a mean enzyme activity of  $3.01 \pm 1.94$  IU/g Hb, approximately twice than that of G6PD deficient males, as expected (a statistically significant difference,  $p = 0.013$ ). The mean value of G6PD activity in 62 normal individuals (all males) was  $9.01 \pm 1.69$  IU/g Hb, ranging from 6 to 13.1 IU/g Hb (Fig. 1). Considering all the available G6PD deficient males with information on enzyme activity, most variants can be classified according to the WHO classification: class I and II, activity < 10% of normal; class III, activity 10% - 60% of normal (Table 2). Only four variants, G6PD Covão do Lobo (c.1205C>A), G6PD Figueira da Foz (c.1366G>C), G6PD Tondela (c.11076-c.1094del) and G6PD Shinshu (c.527A>G), were associated with chronic hemolytic anemia (class I variants) (Table 1).

#### The c.1311C>T polymorphism

Five hemizygous individuals with G6PD deficiency (enzymatic activity at about 75% according to G6PD class III variants) had the silent mutation c.1311C>T (rs2230037) (p.437Tyr=), in combination with the IVS11+93T>C (rs2071429) polymorphism. No other pathogenic mutations were found within the coding region and adjacent regions of the *G6PD* gene of these individuals.

#### DISCUSSION

In this retrospective study, we described the *G6PD* gene mutations found in 138 Portuguese individuals with G6PD deficiency, including 101 hemizygous males, 35 heterozygous females and two homozygous females. Twenty-one different *G6PD* pathogenic mutations were found in a total of 140 mutated alleles. The most common was the variant G6PD A- (c.202G>A) (58.6%), followed by variants G6PD Betica (c.968T>C) (12.1%) and G6PD Santamaria (c.542A>T) (4.3%) (Table 1). Despite being found on the sub-Saharan X-chromosome haplotype c.376G (variant G6PD A), which is similar to previous studies in African populations,<sup>21-24</sup> these three pathogenic *G6PD* mutations were identified in Portuguese (Caucasian) individuals with no known African ancestry. However, the possibility of African origin for these mutations cannot be excluded.

Of the 18 rare variants identified, six were, to the best of the authors' knowledge, only found in the Portuguese population, having already been the subject of previous publications<sup>14-16</sup>: they are the G6PD variants known as Mira d'Aire (c.1048G>C), Anadia (c.1193A>G), Tondela (c.11076-c.1094del), Covão do Lobo (c.1205C>A), Figueira da Foz (c.1366G>C) and Flores (c.1387C>A) (Table 1). The variant G6PD Azores (c.595A>G) was also found in Papua New Guinea and named as G6PD Dagua.<sup>25</sup>

This study describes five rare G6PD variants previously identified in other populations around the world but

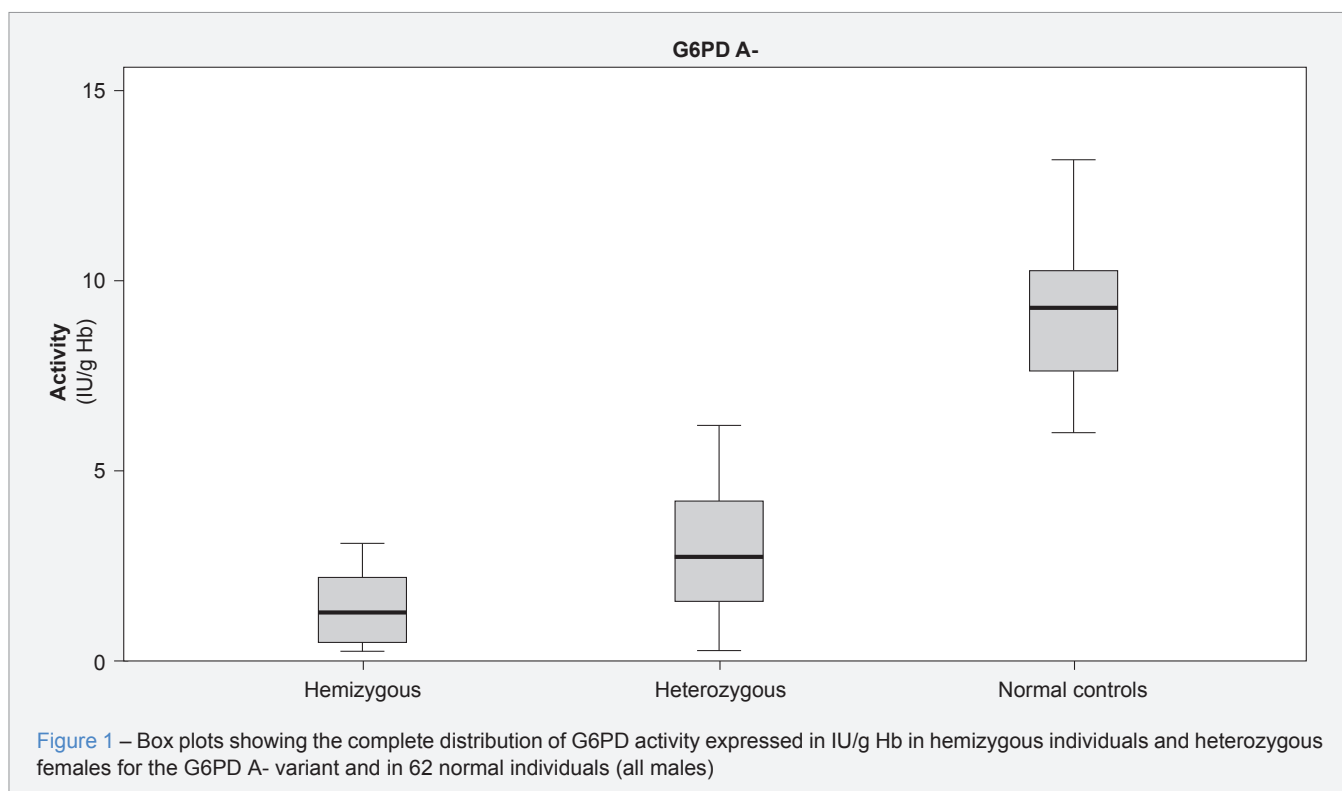


Table 2 – Enzymatic activity for 10 different G6PD variants found in 37 hemizygous males

Variant	Mutation	Class	Clinical symptoms	n	Enzymatic activity (IU/g Hb)	Enzymatic activity (% of n)
A-	c.376A>G / c.202G>A	III	AHA	22	1.46 ± 0.95	14.9
Betica	c.376A>G / c.968T>C	III	AHA	2	1.85 ± 1.91	18.5
Mexico City	c.680G>A	III	AHA	1	3.6	53.0
-	c.1311C>T	III	No*	2	5.65 ± 0.21	75.5
Santamaria	c.376A>G / c.542A>T	II	AHA	3	0.47 ± 0.38	6.0
Taipei	c.493A>G	II	AHA	1	0.5	6.0
Kaiping	c.1388G>A	II	AHA	2	1.25 ± 1.2	13.5
Covão do Lobo	c.1205C>A	I	CNSHA	1	0.1	0.9
Figueira da Foz	c.1366G>C	I	CNSHA	2	0.50 ± 0.28	4.5
Shinshu	c.527A>G	I	CNSHA	1	1.5	15.0

n: number of individuals with the mutation.

\* Diagnostic in malaria context.

Enzyme activity values were calculated as mean ± standard deviation for two or more individuals.

Classification of mutations was performed according to WHO guidelines.

reported herein for the first time in the Portuguese population: Vanua Lava (c.383T>C), Taipei (c.493A>G), Shinshu (c.527A>G), Mexico City (c.680G>A) and Kaiping (c.1388G>A).<sup>11</sup> The G6PD variants Chatham (c.1003G>A), Canton (c.1376G>T) and Kamiube (c.1387C>T) were previously described in Portuguese individuals<sup>15</sup> and the variants Seattle (c.844G>C) and Mediterranean (c.563C>T), also found in this study, had already been previously identified in Portugal by Rodrigues *et al.*<sup>14</sup> The variant G6PD Coimbra, identified in three chromosomes, was previously found in a Portuguese patient,<sup>26</sup> and it is common in several Asian populations. Two variants previously described in the Portuguese population, G6PD Aveiro (c.806G>A)<sup>27</sup> and G6PD Gaohe (c.95A>G),<sup>14</sup> were not found in this study.

The prevalence rate of the c.1311C>T mutation observed in this study (3.6%), in combination with the IVS11+93T>C polymorphism, was similar to that found in most previous studies performed for normal or G6PD-deficient populations.<sup>28-31</sup> It was reported that these two polymorphisms in association with the 3' UTR c.\*+357A>G (rs1050757) polymorphism can be responsible for a G6PD haplotype associated with G6PD deficiency.<sup>32</sup> Interestingly, in our sample, all the subjects with the c.1311C>T allele also had the 3' UTR c.\*+357A>G polymorphism. However, it is commonly accepted that the putative functional role of the c.1311C>T polymorphism in the enzyme activity needs to be further clarified.

With this study, the authors intend to contribute to the knowledge of the mutational profile of G6PD deficiency in the Portuguese population, which, in addition to the anthropological and populational interest, may contribute towards a better understanding of the pathophysiology of

the disease and greater diagnostic suspicion, especially in the presence of chronic hemolytic anemia. In some severe class I variants, such as the Tondela variant, identification of the mutation allows for genetic counseling and prenatal diagnosis. All the pathogenic mutations identified and described to date in patients of Portuguese origin with G6PD deficiency are presented herein. While most mutations were the subject of previous publications, others, however, are described here for the first time in the Portuguese population. The lack of reliable records of the enzymatic activity of many individuals outside the acute episode does not allow a more detailed analysis of the correlation between the different mutations and G6PD activity.

## CONCLUSION

There was a wide genetic heterogeneity of G6PD deficiency in the Portuguese population. The most common variant was G6PD A- (c.202A) (58.6%), followed by the Betica (c.968C) (12.1%) and Santamaria (c.542T) (4.2%). Although these mutations are present in the sub-Saharan African background haplotype c.376G, any African ancestry in the individuals of Portuguese origin observed here with these mutations is unknown. The study also depicted a set of rare variants, previously described in several human populations, five of them reported here for the first time in the Portuguese population. It should be noted that six of these rare variants were, as far as we know, only found in the Portuguese population: they are the G6PD variants Mira d'Aire (c.1048G>C), Anadia (c.1193A>G), Tondela (c.11076-c.1094del), Covão do Lobo (c.1205C>A), Figueira da Foz (c.1366G>C) and Flores (c.1387C>A). The putative functional role of c.1311C>T polymorphism in G6PD-deficient



phenotype needs to be clarified.

## AUTHOR CONTRIBUTIONS

LM: Conception and redaction of the work, data collection.

CB: Data collection, critical review of the work.

LR: Data collection.

TM: Clinical diagnosis.

MLR: Conception and critical review of the work, clinical diagnosis.

## PROTECTION OF HUMANS AND ANIMALS

The authors declare that the procedures were followed according to the regulations established by the Clinical Research and Ethics Committee and to the Helsinki Declaration of the World Medical Association updated in 2013.

## DATA CONFIDENTIALITY

The authors declare having followed the protocols in use at their working center regarding patients' data publication.

## COMPETING INTERESTS

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MLR received funding and provision of study materials from Forum Hematológico de Coimbra.

CB, LR and TM have no conflicts of interest to disclose.

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