

## Prevalence Rate of Thalassemia Carriers among Individuals with Microcytosis or Hypochromia in Portugal

## Prevalência de Portadores de Talassémia em Indivíduos com Microcitose ou Hipocromia em Portugal

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### ABSTRACT

**Introduction:** Microcytosis and hypochromia result from deficient hemoglobin synthesis in red blood cells and are easily detected in a complete blood count test. These conditions are mainly due to iron nutritional deficiency, but may also result from some genetic diseases, such as thalassemia. The aim of this study was to determine the contribution of  $\beta$ - and  $\alpha$ -thalassemia to these abnormal hematological phenotypes in a representative sample of adult individuals living in Portugal who participated in the first Portuguese National Health Examination Survey (INSEF).

**Methods:** Among the 4808 INSEF participants, 204 had microcytosis, hypochromia or both. The corresponding 204 DNAs were screened for changes in the  $\beta$ -globin gene by next-generation sequencing and Sanger sequencing. In addition,  $\alpha$ -thalassemia deletions within the  $\alpha$ -globin cluster were investigated by Gap-PCR and multiplex ligation-dependent probe amplification.

**Results:** In this selected subgroup of INSEF participants, 54 had  $\alpha$ -thalassemia (26%), predominantly caused by the  $-\alpha^{3.7kb}$  deletion, and 22 were  $\beta$ -thalassemia carriers (11%) mainly due to point mutations in the  $\beta$ -globin gene previously known in Portugal.

**Conclusion:** Thalassemia trait is a frequent cause of microcytosis or hypochromia in Portugal since this genetic condition was found in 37% of the investigated cases.

**Keywords:** Erythrocytes; Erythrocyte Indices; Hematologic Tests; Portugal; Thalassemia/diagnosis; Thalassemia/genetics

### RESUMO

**Introdução:** A microcitose e a hipocromia são alterações nos glóbulos vermelhos resultantes de um défice de síntese da hemoglobina e são facilmente identificáveis aquando da realização de um hemograma. Estas condições são, em grande maioria, devidas a um défice nutricional em ferro, contudo podem ser consequência de algumas doenças genéticas, como por exemplo a talassémia. Neste trabalho, pretendemos determinar a contribuição da  $\beta$ - e da  $\alpha$ -talassémia para a ocorrência destes fenótipos hematológicos anómalos, numa amostragem representativa de indivíduos adultos residentes em Portugal e que participaram no primeiro Inquérito Nacional de Saúde com Exame Físico (INSEF).

**Métodos:** De entre os 4808 participantes no estudo INSEF, 204 apresentavam microcitose, hipocromia ou ambas. Os 204 ADNs correspondentes a estes indivíduos foram usados para pesquisa de alterações no gene da  $\beta$ -globina por sequenciação de nova geração e por sequenciação de Sanger. Para além disso, foram pesquisadas deleções  $\alpha$ -talassémicas no agrupamento génico da  $\alpha$ -globina por Gap-PCR e *multiplex ligation-dependent probe amplification*.

**Resultados:** Neste subgrupo selecionado de participantes no estudo INSEF, 54 tinham  $\alpha$ -talassémia (26%), predominantemente devida à deleção  $-\alpha^{3.7kb}$ , e 22 eram portadores de  $\beta$ -talassémia (11%) devido à presença de mutações pontuais no gene da  $\beta$ -globina na sua grande maioria já anteriormente observadas em Portugal.

**Conclusão:** Este estudo revelou que o traço talassémico é uma causa frequente de microcitose ou hipocromia em Portugal, uma vez que foi detetado em 37% dos casos investigados.

**Palavras-chave:** Eritrócitos; Índices de Eritrócitos; Portugal; Talassémia/diagnóstico; Talassémia/genética; Testes Hematológicos

### INTRODUCTION

Microcytosis and hypochromia result from deficient hemoglobin synthesis in precursors of erythroid cells, causing a reduction in both mean corpuscular volume and mean corpuscular hemoglobin of mature red blood cells, which are easily detected when performing a complete blood count in a hematology analyzer. These changes may be associated with anemia and are a consequence of genetic or acquired conditions, such as iron deficiency,  $\alpha$ -thalassemia or  $\beta$ -thalassemia traits. The abnormal morphological findings do not allow, by themselves, to differentiate the pos-

sible causes. Therefore, in order to find their origin, a battery of diagnostic tests is required, including measurement of serum ferritin, transferrin saturation or total iron binding capacity (to diagnose iron deficiency), hemoglobin A2 level estimation or screening for mutations in the  $\beta$ -globin gene (to diagnose  $\beta$ -thalassemia trait), and screening for molecular lesions in the  $\alpha$ -globin genes by molecular techniques (to diagnose  $\alpha$ -thalassemia).

Iron deficiency is the leading cause of the microcytic hypochromic anemia worldwide.<sup>1</sup> In developed countries,

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iron deficiency is frequently caused by insufficient dietary iron intake or by conditions that cause either iron loss or decreased iron absorption.<sup>2</sup> In our country, a recent study that analysed adults aged over 18 years old living in mainland Portugal estimated a prevalence rate of iron deficiency anemia of 10.9% when considering participants with both anemia and ferritin below 30 ng/mL, and a prevalence rate of iron deficiency of 31.9% when considering participants without anemia but with ferritin below 30 ng/mL.<sup>3</sup>

The hemoglobinopathies encompass all genetic diseases of hemoglobin. They fall into two main groups: thalassemias and structural hemoglobin variants. Thalassemias are among the most common human genetic diseases worldwide and are caused by reduced production of the  $\alpha$ - or  $\beta$ -globin chains of hemoglobin, resulting in  $\alpha$ - or  $\beta$ -thalassemia, respectively.<sup>4</sup> The worldwide distribution of  $\alpha$ -thalassemia is quite similar to that of  $\beta$ -thalassemia, extending from sub-Saharan Africa, throughout the Mediterranean region and Middle East, to the Indian sub-continent and East and Southeast Asia. Through recent migration and mobility flows, thalassemias are widely prevalent across the world, including the American continent, Australia, Western Europe and, in more recent years, Northern Europe.<sup>5,6</sup>

The prevalence rate of hemoglobinopathies in Portugal was estimated by epidemiological studies in the nineties. The  $\alpha$ -thalassemia trait was observed in 10% of the newborns.<sup>7</sup> Moreover, a countrywide adult male based study revealed a prevalence rate of  $\beta$ -thalassemia trait of 0.45% in mainland Portugal.<sup>8</sup> However, an uneven distribution throughout the country with some regional prevalence rates as high as 5% were observed.<sup>8-10</sup> These results supported the implementation of a Nationwide program (Programa Nacional de Controlo das Hemoglobinopatias - PNCH) as well as the publication of a national guideline on hemoglobinopathies by the Directorate General of Health (Direção-Geral da Saúde) in 2004.<sup>11</sup> As far as we know, no large epidemiological studies about this subject and no monitoring of the application of the guideline have been conducted in Portugal since then.

In this study, we aimed to estimate the contribution of  $\alpha$ - and  $\beta$ -thalassemia to the presence of microcytosis or hypochromia phenotypes in a representative sample of adult individuals living in mainland Portugal, and in the autonomous regions of Madeira and Azores, who participated in the first Portuguese National Health Examination Survey (INSEF).<sup>12</sup> In addition, we intended to identify the molecular basis underlying the  $\alpha$ - and  $\beta$ -thalassemia cases and to evaluate their corresponding hematological phenotypes. Moreover, other aims of this study were to analyse the demographic characteristics of thalassemia carriers in order to assess if they were followed-up by a general practitioner/family physician and to understand their self-perception

about their health status.

## METHODS

### Study design and population

This is a sub-study of the first Portuguese National Health Examination Survey (INSEF),<sup>12</sup> a cross-sectional population-based survey previously performed by INSA in 2015. The survey included three components: physical examination, blood collection and health interview, targeting non-institutionalized individuals, aged between 25 and 74 years old, living in Portugal for more than 12 months, as described elsewhere.<sup>12</sup> The INSEF sample was selected using a two-stage probabilistic cluster design, stratified by region and degree of urbanization (rural/urban). Of the total selected individuals, 4911 effectively participated. However, the existence of a chronic disease preventing blood collection, or a known severe anemia were considered exclusion criteria for blood collection. In addition, some cases were excluded due to unavailable data regarding the subsequent blood tests or unsuccessful DNA extraction. Therefore, in the current study, the sample was restricted to 4808 participants.

### Blood samples and data collection

After obtaining the participants' written informed consent, trained healthcare professionals conducted all the INSEF procedures in primary health care centers. The information regarding the demographic and health status of participants was collected using a computer-assisted personal interview software. For this study, the following variables were considered: age, sex, nationality, region of residence and degree of urbanization (rural/urban). Self-perception of health status was assessed with the question "In general, how do you rate your health? (very good/good/fair/poor/very poor)". Participants were also inquired if they were followed by a family physician (yes/no). In addition, participants were asked if they were aware of having anemia, diagnosed by a medical doctor, (yes/no). Pregnancy and tobacco smoking status were also self-reported.

Venous non-fasting blood samples were collected in EDTA Vacutainer® tubes to perform a complete blood count (CBC) in each regional laboratory, as previously described.<sup>12</sup> CBC includes the measurement of the following hematological parameters: red blood cells (RBC), hemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), hematocrit (Ht), and red cell distribution width (RDW). Anemia was defined as Hb < 12.0 g/dL for female, < 11.0 g/dL for pregnant female, and < 13.0 g/dL for male subjects.<sup>13</sup> Hemoglobin levels were adjusted for smoking according to World Health Organization (WHO) recommendations.<sup>13</sup> No adjustment was performed for altitude, since

all collection sites were under 1000 m. Microcytosis was defined when MCV < 80 fL and hypochromia when MCH < 27 pg.<sup>14</sup>

INSEF samples were stored in the central facilities of INSA and were used for DNA extraction in a nucleic acid extractor, MagNA pure LC (Roche®, Germany). DNA quantity and quality were assessed using a spectrophotometer NanoDrop One (Thermo Fisher Scientific®, USA).

### Beta-globin gene screening for mutations by next-generation sequencing (NGS)

In order to screen for mutations in the  $\beta$ -globin gene (*HBB*), a DNA fragment of 2106 bp was amplified, from c.-159 (in 5'UTR) to c.\*474 (in 3'UTR), using the forward primer 5'-TAAGCCAGTGCCAGAAGAG-3' and the reverse primer 5'-GAGTCAAGGCTGAGAGATGCAGGA-3' in a T-Gradient Thermocycler, Biometra®. Amplicon purification was performed using the Agent AMPure XP PCR Purification kit (Beckman Coulter®, USA) followed by quantification in a Qubit 3.0 fluorometer (Life Technologies®, USA). To prepare the sequencing libraries, the Nextera XT DNA Library Prep (Illumina®, USA) was used. Libraries were sequenced in a MiSeq equipment (Illumina®, USA) using a 0.3 Gb flow cell. Data analyses of the sequencing results comprised three steps: quality control using the MultiQC® 1.6.dev0 software; mapping reads to reference genome GRCh38 using bowtie; and variant calling where base call quality values were corrected for systematic error with the software GATK®. Varying positions were filtered for variant quality and genotypes were only considered when there was a minimum read depth of 20x. Genotypes with an allelic balance below 30% and above 70% were excluded.

### Beta-globin genetic variants validation by Sanger sequencing

Validation of each type of genetic variant found by Next-generation Sequencing (NGS) was done by Sanger sequencing after PCR purification using ExoSAP-IT PCR Product Clean-up (Applied Biosystems®). Sanger sequencing was performed using the ABI Prism Big Dye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems®) in a 3500 Genetic Analyzer (Applied Biosystems®). Sequences were analysed using the software FinchTV v1.4.0 (Geospiza®). The obtained sequences were compared with the reference sequences, using Ensembl<sup>15</sup>: ENSG00000244734 for *HBB* gene sequence; ENST00000335295.4 for the corresponding transcript and the UniProtKB P68871 for the  $\beta$ -globin protein sequence.

### Deletional alpha-thalassemia screening

The common  $-\alpha^{3.7\text{kb}}$  deletion was detected by Gap-PCR as described.<sup>16</sup> DNA samples negative for this deletion were

screened for copy number variation in the  $\alpha$ -globin gene cluster by multiplex ligation-dependent probe amplification (MLPA) using the SALSA MLPA® probemix P140-C1-0415 HBA (MRC-Holland). The amplified fragments were isolated by capillary electrophoresis in a 3130xl genetic analyzer, ABI PRISM® (Applied Biosystems®) and evaluated using the Coffalyser.net software (MRC-Holland).

### Statistical analyses

The statistical analyses were performed using the SPSS® software (IBM® Corp. Released 2018. IBM® SPSS Statistics for Windows®, Version 26.0. Armonk, NY: IBM® Corp). For descriptive analysis, continuous variables were represented as mean and standard deviation, median, minimum, and maximum values; for categorical variables, absolute and relative frequencies were used.

The genotype/phenotype association study was performed comparing the hematological parameters (RBC, Hb, MCV, MCHC, MCH, Ht, and RDW) between two subgroups of the selected participants. One group included the "Thalassemia carriers", and the other group was composed of participants without thalassemia but with microcytosis, hypochromia, or both. Due to sex-related differences, RBC and Hb comparisons were stratified by sex. Group comparisons were performed using the parametric T-test. To evaluate the normality assumption within groups, the Shapiro-Wilk normality test was applied. To test the homogeneity of variance assumption we used Leven's test. For variables that did not follow the normal distribution, the Mann-Whitney test was used for comparisons. Statistical significance was defined as  $p$ -value < 0.05.

## RESULTS

### Hematological phenotype of the selected participants and their demographic characteristics

The overall sample included in this study comprised 4808 individuals, 2573 (53.5%) female and 2235 (46.5%) male (ratio female/male of 1.2). Following blood tests, there was microcytosis, hypochromia, or both in 204 (4.2%) participants (Table 1), who were selected for  $\beta$ - and  $\alpha$ -thalassemia molecular screening. Within this selected group, 157 were female (77%) and 47 male (23%), presenting a mean age of  $49.3 \pm 11.8$  years old, and a ratio female/male of 3.3. Hypochromia was observed in 201 individuals (98.5%), while microcytosis was observed in 112 individuals (54.9%). The two phenotypes were detected simultaneously in 109 individuals (53.4%). Regarding the Hb level, 88 out of the 204 individuals (43.1%) had anemia, which was observed in 14 of the 47 male (29.8%) and in 74 of the 157 female subjects (47.1%).

Most of the 204 selected participants were Portuguese ( $n = 172$ ; 84.3%), and 32 (15.7%) had another nationality:

**Table 1** – Hematological parameters of the selected 204 individuals presenting microcytosis or hypochromia in the first Portuguese National Health Examination Survey

Hematological parameters	Mean	SD	Median	Min.	Max.
RBC (x10 <sup>12</sup> /L)_Male	5.67	0.08	5.71	4.30	6.70
RBC (x10 <sup>12</sup> /L)_Female	4.78	0.04	4.77	3.75	7.30
Hemoglobin (g/dL)_Male	13.9	0.3	14.2	9.8	16.7
Hemoglobin (g/dL)_Female	11.9	0.1	12.0	6.5	15.3
MCV (fL)	77.7	0.4	79.5	56.9	86.9
MCH (pg)	24.8	0.2	25.6	16.2	28.1
MCHC (g/dL)	31.9	0.1	31.9	28.3	36.1
Hematocrit (%)	38.6	0.3	38.1	23.0	52.0
RDW (%)	15.4	0.1	15.2	12.1	22.3

SD: standard deviation; Min.: minimum; Max.: maximum; RBC: red blood cells; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; RDW: red cell distribution width

Angola, Brazil, Cape Verde, France, South Africa, Bulgaria, Canada, China, India, Mozambique, Moldova, Pakistan, and Syria.

The 204 selected participants with microcytosis or hypochromia were found distributed by all the seven Regional Health Administration (ARS) areas: in mainland Portugal - North (n = 27), Centre (n = 17), Lisbon and Tagus Valley (n = 29), Alentejo (n = 35), and Algarve (n = 35), and in the autonomous regions of Madeira (n = 28) and Azores (n = 33). Forty-nine (24%) were rural residents and 155 (76%) were living in urban areas.

### Alpha-thalassemia diagnosis

The results regarding  $\alpha$ -thalassemia, obtained by Gap-PCR and MLPA methodologies,<sup>16-18</sup> are shown in Table 2. Of the 204 participants, 54 had  $\alpha$ -thalassemia (26.5%). The 3.7kb deletion was found in 52 participants with the heterozygous genotype ( $-\alpha^{3.7kb}/\alpha\alpha$ ) and in one with the homozygous genotype ( $-\alpha^{3.7kb}/-\alpha^{3.7kb}$ ). The less common  $\alpha$ -thalassemia deletion of 4.2kb was found in one participant ( $-\alpha^{4.2kb}/\alpha\alpha$ ). Another change in the *HBA* cluster was found in two participants: the triple  $\alpha$ -globin gene ( $\alpha\alpha\alpha^{anti-3.7kb}/\alpha\alpha$ ).

### Beta-thalassemia diagnosis

The screening for changes in the *HBB* gene, performed

by NGS, revealed 29 different genetic variants. However, 19 of them were considered benign, probably not affecting gene expression because they were located outside of the gene-coding regions or occurred within introns (12 were deep-intronic and 7 were located in the 3'UTR). Therefore, these genetic variants will not be presented in this study because they are unlikely to have clinical relevance. The other 10 different genetic variants, which were classified as pathogenic or probably pathogenic by ClinVar, were validated by Sanger sequencing, and are shown in Table 3. Twenty-two participants (10.8%) were classified as  $\beta$ -thalassemia carriers, and other three were detected as carriers of a hemoglobin structural variant (Hb S, Hb C, and Hb D-Portugal).

The four more common  $\beta$ -thalassemia point mutations detected in this study were the same already described as prevalent in Portugal: codon 39 (C>T), IVS-I-1 (G>A), IVS-I-6 (T>C) and IVS-I-110 (G>A).<sup>19</sup> The less common mutations, codon 15 (G>A) and codon 6 (-A), have already been described as occurring less frequently in Portugal.<sup>19</sup> The  $\beta$ -thalassemia deletion, codon 41/42 (-CTTT), was detected in this study for the first time in Portugal, but was found in an individual born in a foreign country.

One  $\beta$ -thalassemia carrier (mutation IVS-I-6 T>C) was also diagnosed with  $\alpha$ -thalassemia ( $-\alpha^{3.7kb}/\alpha\alpha$ ), and consequently is considered double heterozygous. The same was observed for the HbS carrier, who also has the

**Table 2** – Pathogenic deletions and insertions affecting the alpha-globin genes in the 204 studied individuals with microcytosis or hypochromia

Molecular lesion	Genotypes	Consequences	Number of cases	
			(n)	(%)
3.7 kb deletion	$-\alpha^{3.7kb}/\alpha\alpha$	$\alpha$ -thalassemia	52	25.5
3.7 kb deletion	$-\alpha^{3.7kb}/-\alpha^{3.7kb}$	$\alpha$ -thalassemia	1	0.5
4.2 kb deletion	$-\alpha^{4.2kb}/\alpha\alpha$	$\alpha$ -thalassemia	1	0.5
3.7 kb insertion	$\alpha\alpha/\alpha\alpha^{anti3.7kb}$	-	2	0.9
none	$\alpha\alpha/\alpha\alpha$	-	148	72.6

Table 3 – Pathogenic changes detected in the beta-globin gene in the 204 studied individuals with microcytosis or hypochromia

	Genomic position*	rs identification	HGVS nomenclature**	Common nomenclature	Consequence	Number of cases	
						(n)	(%)
Pathogenic variants detected in <i>HBB</i> gene	g.5226774	rs11549407	c.118C>T	Cd39 (C>T)	$\beta$ -thalassemia	8	3.9
	g.5226924	rs35724775	c.92+6T>C	IVS-I-6 (T>C)	$\beta$ -thalassemia	4	1.9
	g.5226929	rs33971440	c.92+1G>A	IVS-I-1 (G>A)	$\beta$ -thalassemia	3	1.5
	g.5226820	rs35004220	c.93-21G>A	IVS-I-110 (G>A)	$\beta$ -thalassemia	3	1.5
	g.5226974	rs34716011	c.48G>A	Cd15 (G>A)	$\beta$ -thalassemia	2	0.9
	g.5227001	rs63749819	c.20del	Cd6 (-A)	$\beta$ -thalassemia	1	0.5
	g.5226762	rs80356821	c.126_129del	Cd41/42 (-CTTT)	$\beta$ -thalassemia	1	0.5
	g.5227002	rs334	c.20A>T	Hb S	Hemoglobin variant	1	0.5
	g.5227003	rs33930165	c.19G>A	Hb C	Hemoglobin variant	1	0.5
	g.5225678	rs33946267	c.364G>C	Hb D-Portugal	Hemoglobin variant	1	0.5
Without alterations in <i>HBB</i> gene						179	87.5

\*: genomic coordinates according to (GRCh38.p12); \*\*: reference sequence based on a protein coding mRNA (NM\_000518.5)

$\alpha$ -thalassemia allele ( $-\alpha^{3.7kb}/\alpha\alpha$ ).

### Association of thalassemia with the hematological phenotype

After the molecular characterization, the population presenting microcytosis or hypochromia was divided in two groups: the group of "Thalassemia carriers" (n = 75), which includes the  $\alpha$ -thalassemia carriers, the  $\beta$ -thalassemia carriers, the  $\beta$ - and  $\alpha$ -double heterozygous, and the homozygous for the  $-\alpha^{3.7kb}$  allele; and the group of "Individuals without thalassemia" (n = 129). The comparison of the hematological parameters between these two groups is presented in Table 4. The group of thalassemia carriers had a higher number of RBCs and a higher level of Hb in female subjects ( $p < 0.001$ , and  $p = 0.007$ , respectively). No differences in male subjects were observed for these hematological parameters. In addition, thalassemia carriers had a higher Ht ( $p < 0.001$ ) and a lower RDW ( $p < 0.001$ ) than the group without thalassemia.

If we compare only the  $\beta$ -thalassemia carriers (n = 22) with the group without thalassemia, they had higher values of RBC [mean  $6.08 \pm 0.41 \times 10^{12}/L$  for males ( $p = 0.013$ ) and  $5.64 \pm 0.54 \times 10^{12}/L$  for females ( $p < 0.001$ )], and a marked microcytosis [MCV, mean  $66.3 \pm 6.0$  fL ( $p < 0.001$ )] and hypochromia [MCH, mean  $20.8 \pm 2.1$  pg ( $p < 0.001$ )]. Moreover, more than half of the  $\beta$ -thalassemia carriers (54.5%) also presented with anemia.

On the other hand, if we compare the  $\alpha$ -thalassemia carriers (n = 54) with the group without thalassemia, they were characterized by mild microcytosis or normocytosis [MCV, mean  $81.2 \pm 3.2$  fL ( $p < 0.001$ )] but all of them had hypo-

chromia [MCH, mean  $25.9 \pm 1.0$  pg ( $p = 0.005$ )] and only nine (17%) had anemia.

The only individual (a male) with a double heterozygosity for  $\beta$ - and  $\alpha$ -thalassemia, revealed a very mild phenotype, with no anemia or microcytosis (Hb = 14.5 g/dL, MCV = 82.3 fL). He only presented with hypochromia (HGM = 24.4 pg).

### Demographic and health characteristics of the thalassemia carriers

The group positive for thalassemia (n = 75) is comprised of 44 females and 31 males. They come from all the aforementioned seven regions of Portugal. Eighteen are rural residents and 57 are living in urban areas. Almost all  $\beta$ -thalassemia carriers had Portuguese nationality (99%). On the other hand, a wider range of nationalities was found in  $\alpha$ -thalassemia carriers: 36 from Portugal and 17 from Brazil, Cape Verde, Angola, France, India, and Pakistan.

Although 88 out of the 204 selected participants had anemia, according to their self-reported health, anemia was only self-reported by four female participants, one of them having a  $\beta$ -thalassemia trait. There was no male self-reported anemia. One woman with  $\alpha$ -thalassemia self-reported pregnancy but did not report anemia, which turned out to be true.

Most thalassemia carriers reported their health as fair (n = 44, 58.7%) or good (n = 25, 33.3%). Most thalassemia carriers had a family physician (n = 56, 74.7%). Considering only the  $\beta$ -thalassemia carriers, 15/22 (68%) had a family physician.

**Table 4** – Hematological parameters in the studied population (n = 204 from the first National Health Examination Survey) with microcytosis or hypochromia – comparison between two subgroups. “Thalassemia carriers” versus other “Without thalassemia”.

Hematological parameters	Individuals without thalassemia (n = 129)				Thalassemia carriers* (n = 75)				p-value
	Mean	SD	Median	Min. Max.	Mean	SD	Median	Min. Max.	
<b>RBC (x10<sup>12</sup>/L)_Male</b>	5.45	0.62	5.38	4.45 6.70	5.77	0.53	5.80	4.30 6.60	0.073 <sup>a</sup>
<b>RBC (x10<sup>12</sup>/L)_Female</b>	4.67	0.46	4.70	3.75 7.30	5.08	0.52	5.08	4.29 6.40	<b>&lt; 0.001<sup>b</sup></b>
<b>Hemoglobin (g/dL)_ Male</b>	13.4	2.32	14.0	9.8 16.7	13.2	1.3	13.3	11.4 14.8	0.291 <sup>b</sup>
<b>Hemoglobin (g/dL)_ Female</b>	11.7	1.3	11.8	6.5 15.3	12.2	1.1	12.4	9.0 14.8	<b>0.007<sup>b</sup></b>
<b>MCV (fL)</b>	78.3	5.2	78.8	57.2 85.9	76.8	8.0	80.9	56.9 86.9	0.969 <sup>b</sup>
<b>MCH (pg)</b>	25.0	2.0	25.6	16.2 28.1	24.4	2.7	27.7	17.2 26.9	0.521 <sup>b</sup>
<b>MCHC (g/dL)</b>	31.9	1.1	31.9	28.3 36.1	31.8	0.9	31.8	28.7 33.5	0.603 <sup>b</sup>
<b>Hematocrit (%)</b>	37.2	4.4	37.0	23.0 52.0	41.0	4.4	40.7	31.4 51.2	<b>&lt; 0.001<sup>a</sup></b>
<b>RDW (%)</b>	15.8	1.9	15.6	12.3 22.3	14.7	1.5	14.5	12.1 19.1	<b>&lt; 0.001<sup>b</sup></b>

\*The group named “Thalassemia Carriers” includes 73 beta-thalassemia heterozygous, one homozygous for the 3.7 kb alpha-thalassemia deletion, and one double heterozygous for beta-thalassemia and alpha-thalassemia alleles. SD: standard deviation; Min.: minimum; Max.: maximum; RBC: red blood cells; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; RDW: red cell distribution width.

**a:** T-test **b:** Mann-Whitney test; significant p-value < 0.05 are shown in bold.

## DISCUSSION

Although iron deficiency and iron deficiency anemia are, in fact, the most likely cause for the presence of an abnormal hematological phenotype of microcytosis or hypochromia,<sup>2,3,20</sup> our study reinforces the role played by thalassemia as a possible cause for that. Our results suggest that 26.5% of those cases are due to  $\alpha$ -thalassemia and 10.8% are due to  $\beta$ -thalassemia, which is a considerable high prevalence rate in both cases. The differentiation of thalassemia trait from iron deficiency and iron deficiency anemia is important for two main reasons. First, misdiagnosed  $\beta$ -thalassemia carriers will be deprived of genetic counselling and may be at risk of having seriously affected offspring. Moreover, in this case, if unnecessary iron is prescribed, individuals will not improve their hematological phenotype and it could even lead to excessive accumulation of iron deposits in the tissues, known as hemosiderosis,<sup>21</sup> in addition to having unnecessary expenses concerning medicines.

We observed a female/male ratio of 1.2 in general participants of INSEF, but after applying the selection criteria of “to have microcytosis or hypochromia” this ratio increased to 3.3, with a great predominance of affected female individuals. One reason for this may be because iron deficiency is more prevalent in women than in men.<sup>3,20</sup> Nevertheless, after the diagnosis of thalassemia, the ratio of female/male returned to 1.4 in these carriers, which is similar to the original ratio in INSEF participants. This is because thalassemia is a group of autosomal genetic diseases due to affected globin genes, which are located in chromosomes 11 and 16. Consequently, thalassemia affects females and males equally.

Our results show that almost all  $\beta$ -thalassemia carriers have Portuguese nationality and present the same spectrum of common  $\beta$ -thalassemia mutations that was previously described in Portugal.<sup>19</sup> Only one different lesion, the Cd41/42 (-CTTT) deletion, was for the first time diagnosed here. On the contrary, the common 3.7kb  $\alpha$ -thalassemia deletion was found in individuals with a larger range of nationalities. Heterozygous individuals for this condition are clinically asymptomatic, and present with slight hypochromia and eventually microcytosis or may have normal hematological findings. Even if inherited in the homozygous state,  $\alpha^+$ -thalassemia is not considered significant in the screening programs and policies designed to detect couples at risk of hydrops fetalis.<sup>22</sup> However, although heterozygous and homozygous  $\alpha^+$ -thalassemia individuals are clinically normal, and therefore do not require treatment, it is important to recognize the condition in order to elucidate the cause of microcytosis or hypochromia and not to confuse with and treat these cases as an iron deficiency condition.

We have observed that all the 54 individuals with  $\alpha$ -thalassemia have hypochromia (100%) but only 29.6% have microcytosis. Therefore, we can conclude that the first parameter is more frequently associated with  $\alpha$ -thalassemia than the latter. On the other hand, we have found that 21 out of the 22 carriers of  $\beta$ -thalassemia revealed microcytosis and hypochromia

simultaneously. It is known that these two parameters, along with an elevation of HbA2 (not measured in this study), are the typical hematological phenotype of the  $\beta$ -thalassemia carriers.<sup>23</sup> The only exception found in this study (1/22) was a man with  $\beta$ -thalassemia presenting with a very mild phenotype, without anemia or microcytosis (Hb = 14.5 g/dL, MCV = 82.3 fL), in whom just hypochromia was observed (HGM = 24.4 pg). However, this participant was a double heterozygote for  $\beta$ - and  $\alpha$ -thalassemia. It is known that the co-inheritance of  $\beta$ - and  $\alpha$ -thalassemia improves the hematological parameters, since it attenuates the disequilibrium between  $\beta$ - and  $\alpha$ -globin chains, and consequently it may be a factor for misdiagnosis of  $\beta$ -thalassemia carriers. On the contrary, if a  $\beta$ -thalassemia carrier co-inherited a triple  $\alpha$ -globin gene, the imbalance between  $\beta$ - and  $\alpha$ -globin chains is increased and consequently the phenotype worsens and could even reach a thalassemia intermedia condition.<sup>24</sup>

Taking advantage of the small differences in the red cell indices between thalassemia trait and iron deficiency observed in CBC tests, several studies have developed mathematical formulas with the aim of discriminating those conditions.<sup>25</sup> Among them, the Red Cell Distribution Width Index (RDWI) = (MCV x RDW)/RBC, provides valuable help.<sup>26,27</sup> When its value is higher than its cut-off (> 220) it is suggestive of iron deficiency. In our study, the group of thalassemia carriers had a RDWI mean of 212.6 while the group without thalassemia had a RDWI mean of 261.2, suggesting the presence of iron deficiency in most of the participants of this latter group. To validate this hypothesis, further research should be carried out, including, for example, the measurement of serum ferritin and transferrin saturation.

Another limitation of this study consisted in the incapacity to detect the clinically relevant hemoglobin variants (namely HbS) in the general participants of INSEF. In fact, this study was not designed to detect hemoglobin structural variants since it is known that most of them are not associated with the hematological changes of microcytosis or hypochromia. Nevertheless, we have detected three cases of hemoglobin variants. Among them, one is a carrier of Hb S (*HBB*:c.20A>T) who has co-inherited an  $\alpha$ -thalassemia allele ( $-\alpha^{3.7kb}$ ). Consequently, this is the reason why this participant had a hypochromic anemia (Hb = 11.1 g/dL, MCH = 25.9 pg). Another participant was diagnosed as a carrier of Hb C (*HBB*:c.19G>A). This participant had no anemia or hypochromia but presented with mild microcytosis (MCV = 72.5 fL), which is in agreement with what is described for Hb C carriers in public databases.<sup>28</sup> The third hemoglobin variant was detected in a male, who revealed a microcytic anemia (Hb = 11.1 g/dL, MCV = 75.8 fL). He is a carrier of Hb D-Portugal (*HBB*:c.364G>C) but his hematological phenotype is worse than what is described in public databases for carriers of this variant.<sup>29</sup> This fact may be explained by

a possible co-existence of iron deficiency anemia since his RDWI value was 256 (> 220). Therefore, in order to increase the knowledge about the prevalence rate of the clinically relevant structural variants in Portugal (especially of Hb S) further studies should be carried out, covering a larger sample of the population and including the biochemical characterization of the hemoglobin fractions present in fresh blood aliquots.

Our results also revealed that the thalassemia carriers are living in all the regions of Portugal, including both the mainland and the islands. Most of them self-reported their health as being fair or good, as expected for a clinically asymptomatic thalassemia carrier. However, and considering the  $\beta$ -thalassemia carriers (who have the typical hematological phenotype of microcytosis and hypochromia, and more than half also have anemia), only one of them self-reported anemia. We would like to draw attention to this fact, as it is suggestive that, probably, many of them are not aware of their genetic condition.

According to the guideline on hemoglobinopathies of the Directorate General of Health,<sup>11</sup> we would like to emphasize the role of primary health care services in identifying and managing the hereditary implications related to these genetic conditions. Furthermore, in addition to the availability of diagnostic genetic testing, we reinforce that for prevention and control of clinically severe disease cases, such as homozygosity or compound heterozygosity for  $\beta$ -thalassemia, prenatal molecular diagnosis with genetic counselling is available in Portugal.

## CONCLUSION

The present study allowed us to conclude that  $\alpha$ - and  $\beta$ -thalassemia traits are a considerable cause of microcytosis or hypochromia, with or without anemia, in individuals living in Portugal, since these genetic conditions were detected in 37% of the investigated cases. This information has clinical relevance because microcytosis and hypochromia are often interpreted as indicators of iron deficiency and, consequently, these individuals may be mistreated with oral iron therapy, and may end up not being diagnosed as thalassemia carriers. The latter may have serious consequences, particularly for  $\beta$ -thalassemia carriers, and their families, because they will be deprived of genetic counselling and may be at risk of having seriously affected offspring. We would like to draw attention and alert healthcare professionals, particularly family physicians, to this important matter.

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## AUTHOR CONTRIBUTIONS

DS: Molecular diagnosis, conception of the work, statistical analysis and critical review the manuscript.

MB: Data acquisition, DNA extraction, conception of the work and critical review the manuscript.

IK: Database management, statistical analysis and critical review the manuscript.

JM, MPM: NGS experiments, bioinformatics analysis and critical review the manuscript.

PL: Molecular diagnosis and analysis of the results.

CMD: Conception of the work and critical review the manuscript.

PF: Conception of the work, analysis of the results, draft the manuscript and critical review the paper.

## 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

The authors have declared that no competing interests exist.

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