The spectrum of β-thalassemia mutations in Gaza Strip, Palestine

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ABSTRACT

Background: β-Thalassemia is a disorder caused by mutations at the hemoglobin β-gene (HBB) locus. Its most important manifestation, the major form, is characterized by severe hypochromic and hemolytic anemia and is inherited in an autosomal recessive mode. In Gaza Strip, Palestine 0.02% of the population has been identified as β-thalassemia major.

Design and methods: An assessment of mutations was performed in 49 transfusion dependent patients with β-thalassemia major and in 176 β-thalassemia carriers diagnosed with a mean erythrocyte cell volume (MCV) <80 fl and a proportion of HbA2 >3.5%. In addition 39 individuals suspicious for β-thalassemia carrier status due to a reduced MCV (<80 fl) but a normal HbA2 were screened.

Results: By screening with three hybridization assays a proportion of 80% of the thalassemic chromosomes from patients and carriers was identified to carry five different mutations of the hemoglobin (Hb) β-gene. Subsequent DNA sequencing confirmed these and revealed further 9% of the chromosomes to be affected by other mutations. In addition six chromosomes from suspicious carriers were detected to carry β-thalassemia mutations. Of the 15 different HBB mutations identified the variant IVS-I-110 G>A was the most frequent mutation identified in 34% of the thalassemic chromosomes, followed by IVS-I-1 G>A, IVS-I-6 T>C, Codon 39 C>T, and Codon 37 G>A. Three novel HBB variants were discovered by direct sequencing of the gene: 5' UTR-50 (--G), 5' UTR-43 C>T, and IVS-II-26 T>G.

Conclusions: The spectrum of HBB mutations described is of the Mediterranean type whereby the allele frequencies of the most common mutations differ from those, which were previously described for the population of the Gaza Strip and other Palestinian populations. The data presented may promote the introduction of molecular testing to the Palestinian premarital screening program for β-thalassemia in Gaza Strip, which will improve the screening protocol and genetic counseling in the future.

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Introduction

The thalassemias are hereditary anemias caused by mutations, which quantitatively affect the synthesis of human hemoglobin. They have been found in ethnicities originating from nearly every geographic location in the world; however, they are most common in those originating from tropical and subtropical regions of Africa and Asia and from the Mediterranean basin [1,2]. Thalassemias are classified according to which particular globin chain(s) is produced in a reduced amount. The main types which have now been defined are the α, β、γ and γβ-thalassemias. While α and β-thalassemia are the most common types of thalassemia however, β-thalassemia major and intermedia are the clinically most important types causing severe transfusion dependent anemia with reduced life expectancy in the homozygous and compound heterozygous states [3–5]. Up-to-date, more than 400 different mutations have been reported and identified in the β-globin gene, which are responsible for the development of the β-thalassemia (database HbVar, http://globin.cse.psu.edu/hbvar/menu.html) [6]. Moreover, a total of more than 700 genetic variants – which may or may not cause thalassemia – have been discovered in the β-globin gene region as documented by the National Center for Biotechnology Information (GenBank, http://www.ncbi.nlm.nih.gov/snp/).

In the Gaza Strip, Palestine, β-thalassemia is recognized as a major health problem. More than 320 patients diagnosed with β-thalassemia major are currently being transfused and managed in local hospitals. The spectrum of mutations causing β-thalassemia in the Gaza Strip was not addressed comprehensively so far, the only sources with molecular genetics analyses were reported by Filon et al. in 1995 and Ayesh et al. in 2005 [7,8]. The study of Filon et al. included 39 transfusion dependent patients from the Gaza strip, no carriers were included in their study. The
study of Ayesh et al. focused on prenatal diagnosis on the West Bank and Gaza Strip, however, from the 88 couples at risk for a thalassemic child only 17 (19.3%) originated from the Gaza Strip.

This study aims to describe the mutation spectrum from a larger sample of transfusion dependent \(\beta\)-thalassemia patients and from \(\beta\)-thalassemia carriers. In addition samples from microcytic and/or hypochromic subjects with borderline elevated HbA2 values were also included.

**Materials and methods**

**Subjects**

The study included 264 unrelated Palestinian individuals living in the Gaza Strip. They were categorized into three groups: group (i) included 49 transfusion dependent \(\beta\)-thalassemia patients, which were diagnosed using case definitions given by Weatherall and Clegg [4], group (ii) included 176 \(\beta\)-thalassemia confirmed carriers (MCV < 80 fL and/or MCH < 26 pg, HbA2 > 3.5%), and group (iii) included 39 microcytic and/or hypochromic subjects (MCH < 80 fL or MCH < 26 pg) whose HbA2 was below the confirmation of \(\beta\)-thalassemia carrier state (HbA2 ≤ 3.5%), with normal body iron level indicated by a serum ferritin level of > 15 μg/l [9]. The individuals of groups (i) and (ii) were identified through a program of obligatory premarital testing for \(\beta\)-thalassemia in the Gaza Strip [10].

Ethical approval was obtained from the scientific and ethical committees at the Thalassemia and Hemophilia Centre, Palestine Avenir Foundation. The study protocol is in accordance with the ethical standards laid down in the 1964 and 1975 Declarations of Helsinki including the latest modifications of 2008 (http://www.wma.net/en/10home/index.html). Written informed consent was obtained from subjects prior to drawing blood samples in accordance with the guidelines of the Thalassemia and Hemophilia Centre, Palestine Avenir Foundation.

**Blood collection and hematological laboratory analysis**

Venous blood samples of 2.5 ml volume were drawn from the study subjects and were collected in K2-EDTA anticoagulant containers (Meus, Piove Di Sacco, Italy). For subjects classified into group (i) blood withdrawals were performed a few minutes before the regular blood transfusion. Fractions of the blood samples which were intended to be used for later DNA isolation were spotted in 100 μl aliquots to 5 mm dots on cards of Alphostar 2206 grade newborn screening filter paper (ID Biological Systems, Greenville, SC, USA). After an overnight drying at room temperature, the cards were placed between sheets of glassine weighing paper (Schleicher & Schuell, Germany) in order to inhibit cross contamination of samples and were packaged with silica gel desiccant packages to absorb humidly.

The complete blood counts including counts of white blood cells (WBC), red blood cells (RBC), platelets (PLT) and the measurement of hemoglobin (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and red cell distribution width (RDW) were performed using a Cell Dyne 1700 electronic counter (Sequioa-Turner Corporation, California, USA).

HbA2 values were quantified using ion exchange HbS-free interference micro column chromatography (Biosystem, Barcelona, Spain) while the serum ferritin levels were determined using an IMX system (Abbott, Abbott Park, II, USA).

**DNA isolation**

DNA was extracted from the filter-paper spots following a superparamagnetic-bead based DNA extraction procedure (LGC Genomix, formerly AGOWA, Germany). Isolated DNA samples were checked for quantity using the Quant-IT™ dsDNA Broad-Range Assay Kit (Quant-IT™ PicoGreen®, Invitrogen, Germany) and checked for quality by a PCR amplification of exons 1–3 of the HBB.

**Targeted screening for specific HBB mutations**

The screening comprised assays for the mutations IVS-I-1 G>A, IVS-I-6 T>C, IVS-I-110 G>A, Codon 37 G>A, Codon 39 C>T and Codons 106/107 +/−G, which were previously reported to be frequent in the Arabic and/or Mediterranean populations [7,8,11–16]. Four assays, each of which comprised a PCR reaction and subsequent hybridization were established for type in these 6 mutations (Table 1). The analysis of hybridization was based on the dynamic allele specific hybridization (DASH) technique with fluorescence energy transfer of fluorophores for the readout of hybridization intensity [17].

A PCR machine (Primus 96, Peqlab, Germany) and a LightCycler® 480® system (Roche Diagnostics, Germany) were used to perform the combined PCR and hybridization assays. The reactions were carried out in a final volume of 10 μl each. Buffers and reagents were used as reported elsewhere [17]. The primers and the fluorophore-coupled probes were synthesized by a manufacturer (Biomers, Germany). The PCR working protocol cycle consisted of a 3-minute initial denaturation step at 94 °C, followed by a 45-cycle program, each of which included denaturation at 94 °C for 20 s, annealing for 30 s at 58 °C and 55 °C for reactions 1, 2, 4 and 3, respectively, and elongation at 72 °C for 30 s. Assay specific melting temperatures of the hybridization of probes (Tm) with the wild type alleles and mutations were measured (Table 1). The allele specific Tm values enabled the identification of homozygous, heterozygous and compound heterozygous genotypes. The genotypes were confirmed by Sanger sequencing. Samples found with deviating melting temperatures, which did not comply with those defined in Table 1, were subjected to sequencing.

**Sequencing**

Sequencing was performed on an ABI 310 Genetic Analyzer (Applied Biosystems, Germany) using the BigDye® Terminator v3.1 Cycle Sequencing Kit and conditions suggested by the manufacturer. The HBB gene was partially amplified with two PCR reactions using Qiagen® Taq DNA Polymerase Kit (Qiagen, Germany). The PCR conditions were applied as described in Rund et al. [18], but primers and temperature were modified as given in Table 2. A first reaction amplified 146 bp of the 5′ untranslated region (UTR), exon 1, intron 1, exon 2, and 117 bp of the intron 2 region neighboring exon 2. A second reaction amplified 234 bp of the intron 2 region neighboring exon 3 and 302 bp of the 3′ untranslated region. This region was omitted a segment of 499 bp of intron 2 in between the two reactions, which does not frequently contain HBB mutations (database HbVar). The amplification was followed by a digestion step with recombinant shrimp alkaline phosphatase (USB Amersharm, Germany) and exonuclease 1 (New England Biolabs, Germany) to remove remaining dNTPs and primers prior to running 10 μl BigDye reactions according to the manufacturer’s protocol. The sequencing was performed bi-directional. Sequences were analyzed with SeqScape® v2.1.1 software (ABI).

**Results**

Within the three groups of (i) 49 transfusion dependent \(\beta\)-thalassemia patients, of (ii) 176 \(\beta\)-thalassemia carriers, and of (iii) 39 microcytic and/or hypochromic individuals whose HbA2 was below the confirmation of \(\beta\)-thalassemia carrier state 15 different HBB mutations were found, which were previously reported to cause \(\beta\)-thalassemia (Table 3). In addition, two genetic variants without disease association, the polymorphisms IVS-II-16 and IVS-II-74, and three novel variants...
with unknown disease association, −50 (−G), −43 (C>T), IVS-II-26 (T>G), were identified (Table 3).

When calculating the frequency of known causal mutations, which were identified in 274 chromosomes from 49 patients (98 thalassaemic chromosomes assumed, 94 [96%] identified) and 176 carriers (176 thalassaemic chromosomes assumed, 151 [86%] identified) a detection rate of 89% was achieved for the combined screening with hybridization assays and consecutive Sanger sequencing in case of discordant genotype–phenotype conditions.

The initial screening was performed with 4 PCR-based hybridization assays. The assays (I–IV) were designed to detect (I) IVS-I-1 and IVS-I-6, (II) IVS-I-110, (III) Codon 37 and Codon 39, and (IV) Codon 106/107. The mutation at Codon 106/107 was not identified in our sample. The common polymorphisms [IVS-II-16 (G>C) & IVS-II-74 (T>G)], which are known not to cause β-thalassemia patients a proportion of 65% was homozygous, 27% was compound heterozygous while in 8% only one causal mutation was identified within the sequenced regions of the HBB. The common polymorphisms [IVS-II-16 (G>C) & IVS-II-74 (T>G)] which are known not to cause β-thalassemia were identified as well (Table 4).

The initial screening was performed with 4 PCR-based hybridization assays. The assays (I–IV) were designed to detect (I) IVS-I-1 and IVS-I-6, (II) IVS-I-110, (III) Codon 37 and Codon 39, and (IV) Codon 106/107. The mutation at Codon 106/107 was not identified in our sample. The application of the assays I–III to both groups of (i) the 49 transfusion dependent β-thalassemia patients and of (ii) the 176 β-thalassemia carriers resulted in the identification of causal mutations in 80% of the 274 chromosomes assumed to be thalassemic.

Among the 39 microcytic and/or hypochromic suspicious carriers whose Hba2 levels were below the diagnostic level for β-thalassemia carriers (Hba2 <3.5%) 6 individuals showed HBB mutations including IVS-I-6 (T>C), Codon 39 (C>T), and 3’ UTR + 101 (G>C) (Table 3).

Discussion

The 15 different mutations, which were found in individuals from the Gaza Strip (Table 3) are known to occur in the Mediterranean area [6]. The frequencies of the five most abundant mutations, which were found in 274 chromosomes from (i) transfusion dependent β-thalassemia patients and (ii) carriers (IVS-I-110 =0.34, IVS-I-1=0.21, IVS-I-6=0.13, Codon 39 =0.10, Codon 37 =0.03) (Table 3) showed similarity to the frequencies described previously for the population of the Gaza Strip [7,8]. Their relative high frequencies, together with high consanguinity among Gaza Strip couples may contribute to an elevated homozygosity rate of 65% among the transfusion dependent patients (Table 4). In their evaluation and recommendations on obligatory premarital tests for β-thalassemia in Gaza Strip, Tarazi et al. in 2007 reported a high degree of consanguinity among couples screened for β-thalassemia carrier state and consanguineous marriages (first and second-degree) constituted more than 40% of the marriages in the Gaza Strip [10]. In four β-thalassemic patients, we identified a single causal mutation only. This could be caused by additional unidentified alleles for β-thalassemia located in those regions of HBB which were not screened or in other chromosomal regions involved the expression of HBB [4]. However, the presence of dominantly inherited β-thalassemia alleles [19] or the concomitant occurrence of β-thalassemia mutations with triplications/quadruplications at the α-globin gene locus [20,21] could not be excluded. Individual heterozygotes for β-thalassemia associated with α-globin gene multiplications manifested a more severe clinical phenotype of β-thalassemia [20–23]. The β9 Codon 39 (C>T) mutation, which was found in two of our four heterozygous thalassemic patients, was reported to cause β-thalassemia with variable severity in individuals who showed a combination with α-globin gene multiplications [21].

Our study confirmed reports of IVS-I-110 being the most frequent mutation in the Gaza Strip [7,8] which is different from other Palestinian populations originating from the mountain regions or the West Bank [15,16]. Since the mutation IVS-I-110 is widespread in populations of the Mediterranean basin the varying frequencies most likely reflect founder effects within different tribes of the Palestinian population.

Our study demonstrates the successful use of PCR-based hybridizations assays as first line screening tools for a molecular genetic analysis of HBB gene. Using a targeted screen for 5 specific mutations with 3 different assays we identified mutations in 80% of the thalassaemic chromosomes from patients and carriers. This reduced the number of individuals, which subsequently had to undergo Sanger sequencing of HBB vastly.

Table 1

<table>
<thead>
<tr>
<th>PCR</th>
<th>Screened mutation/ variant</th>
<th>Tm</th>
<th>Amplicon</th>
<th>Primers 5’-3’</th>
<th>Probes 5’-3’ mutation position underlined</th>
<th>T, annealing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay I</td>
<td>IVS-I-1</td>
<td>44 °C</td>
<td>180 bp</td>
<td>Forward</td>
<td>TGGAGAAGCTTGAATGCCATT</td>
<td>Cy5-ACAAGACGTTTAAGGACCAATAAGG-phosphate</td>
</tr>
<tr>
<td>Wild type</td>
<td>62 °C</td>
<td>Reverse</td>
<td>CGAAGCTTGAATGCCATT</td>
<td>Sensor for IVS-I-1 G&gt;A and IVS-I-6 T&gt;C</td>
<td>GCAGTTCAGCTAACGAGT-fluorescein</td>
<td>58 °C</td>
</tr>
<tr>
<td>Assay II</td>
<td>IVS-I-110</td>
<td>54 °C</td>
<td>208 bp</td>
<td>Forward</td>
<td>AAGCTTTAAGAGACTTTTAAG</td>
<td>Cy5-ACACCTTACCTGGAACAGG-phosphate</td>
</tr>
<tr>
<td>Wild type</td>
<td>61 °C</td>
<td>Reverse</td>
<td>CTGGAAGCTTGAATGCCATT</td>
<td>Sensor for IVS-I-110 A&gt;G</td>
<td>CatCATCCAGCTTACAGT-fluorescein</td>
<td>58 °C</td>
</tr>
<tr>
<td>Assay III</td>
<td>Codon 37</td>
<td>58 °C</td>
<td>208 bp</td>
<td>Forward</td>
<td>AAGCTTTAAGAGACTTTTAAG</td>
<td>Cy5-CTTGTACCTTGAGCTGTCAG-phosphate</td>
</tr>
<tr>
<td>Wild type</td>
<td>58 °C</td>
<td>Reverse</td>
<td>CATGGTGCCTAATACCCAG</td>
<td>Sensor for IVS-I-34 A&gt;G and IVS-I-34 T&gt;C</td>
<td>CCACTATTCAGATG-fluorescein</td>
<td>58 °C</td>
</tr>
<tr>
<td>Assay IV</td>
<td>Codon 106/107</td>
<td>n.f.</td>
<td>241 bp</td>
<td>Forward</td>
<td>AAGCTTTAAGAGACTTTTAAG</td>
<td>Cy5-CTTGTACCTTGAGCTGTCAG-phosphate</td>
</tr>
<tr>
<td>Wild type</td>
<td>58 °C</td>
<td>Reverse</td>
<td>CATGGTGCCTAATACCCAG</td>
<td>Sensor for IVS-I-34 A&gt;G and IVS-I-34 T&gt;C</td>
<td>CCACTATTCAGATG-fluorescein</td>
<td>58 °C</td>
</tr>
</tbody>
</table>

Tm: specific melting temperature; Cy5: fluorophore Cy5 for fluorescence detection of emission at 670 nm wavelength; fluorescein: fluorophore used for excitation at 483 nm wavelength and transmission to Cy5; n.f.: not found, whereby a Tm of −54 °C is expected for Codon 106/107.

Table 2

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primers</th>
<th>Product length</th>
<th>Cycling conditions for 20 μl reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 &amp; 2</td>
<td>Forward</td>
<td>784 bp</td>
<td>94 °C 5 min 35×</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>72 °C 1 min</td>
<td>94 °C 5 min 35×</td>
</tr>
<tr>
<td>3</td>
<td>Forward</td>
<td>845 bp</td>
<td>94 °C 5 min 35×</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>94 °C 1 min</td>
<td>60 °C 1 min</td>
</tr>
</tbody>
</table>
Table 3  Mutation and variants identified in the HBB gene.

<table>
<thead>
<tr>
<th>HBB mutation name or variant</th>
<th>Ph</th>
<th>dbSNP</th>
<th>bp</th>
<th>HbVar ID</th>
<th>Localization at HBB (HGVS)</th>
<th>Codon variation</th>
<th>Mutation frequency&lt;sup&gt;a&lt;/sup&gt;</th>
<th>AF in patients (n = 49)</th>
<th>Count in patients (n = 49)</th>
<th>AF in carriers (n = 176)</th>
<th>Count in carriers (n = 176)</th>
<th>AF in suspected carriers (n = 39)</th>
<th>Count in suspected carriers (n = 39)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVS-I-110 (G&gt;A)</td>
<td>β+</td>
<td>rs35004220</td>
<td>5248050</td>
<td>HbVar.827</td>
<td>Intron 1</td>
<td>c.93→2G&gt;A</td>
<td>None</td>
<td>0.339</td>
<td>0.276</td>
<td>0.375</td>
<td>66</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IVS-I-1 (G&gt;A)</td>
<td>β+</td>
<td>rs33971440</td>
<td>5248159</td>
<td>HbVar.817</td>
<td>Intron 1</td>
<td>c.92→1G&gt;A</td>
<td>None</td>
<td>0.212</td>
<td>0.224</td>
<td>0.205</td>
<td>36</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Codon 39 (C&gt;T)</td>
<td>β+</td>
<td>rs11549407</td>
<td>5248004</td>
<td>HbVar.845</td>
<td>Exon 2</td>
<td>c.118C&gt;T</td>
<td>None</td>
<td>0.131</td>
<td>0.082</td>
<td>0.159</td>
<td>28</td>
<td>0.013</td>
<td>1</td>
</tr>
<tr>
<td>Codon 37 (G&gt;A)</td>
<td>β+</td>
<td>rs33974936</td>
<td>5248008</td>
<td>HbVar.841</td>
<td>Exon 2</td>
<td>c.114G&gt;A</td>
<td>CAC(Gln)→TAG</td>
<td>0.055</td>
<td>0.153</td>
<td>0.063</td>
<td>11</td>
<td>0.013</td>
<td>1</td>
</tr>
<tr>
<td>kodon 33 (C&gt;T)</td>
<td>β+</td>
<td>rs33971437</td>
<td>5248154</td>
<td>HbVar.826</td>
<td>Intron 1</td>
<td>c.92→6T&gt;C</td>
<td>None</td>
<td>0.007</td>
<td>0.011</td>
<td>0.007</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Codon 39 (C&gt;T)</td>
<td>β+</td>
<td>rs11549407</td>
<td>5248004</td>
<td>HbVar.845</td>
<td>Exon 2</td>
<td>c.118C&gt;T</td>
<td>None</td>
<td>0.131</td>
<td>0.082</td>
<td>0.159</td>
<td>28</td>
<td>0.013</td>
<td>1</td>
</tr>
<tr>
<td>Codon 37 (G&gt;A)</td>
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<td>HbVar.845</td>
<td>Exon 2</td>
<td>c.118C&gt;T</td>
<td>None</td>
<td>0.131</td>
<td>0.082</td>
<td>0.159</td>
<td>28</td>
<td>0.013</td>
<td>1</td>
</tr>
</tbody>
</table>

HBB: hemoglobin beta gene; Ph: disease phenotype according to Ref [2] and Database of Human Hemoglobin Variants and Thalassemias (HbVar: http://globin.bx.psu.edu/hbvar/menu.htm); dbSNP: reference-snp (rs) and submitted-snp (ss) accession numbers according to the database of Short Genetic Variations (GenBank, http://www.ncbi.nlm.nih.gov/snp); bp: chromosomal base pair position on chromosome 11 according to the Genome Reference Consortium Human Build 37.3, build19 (http://www.ncbi.nlm.nih.gov/projects/gencode/human/); HbVar ID: mutation nomenclature according to HbVar; HGVS: mutation according to the nomenclature of the Human Genome Variation Society (HGVS) guidelines (http://www.hgvs.org/); AF: allele frequency of genetic variants; β+: phenotype β+-thalassemia; n.n.: phenotype not known; β0: phenotype β0-thalassemia; –: variant without thalassemic phenotype; prom: promoter; n.a: not available; n.d.: not done.

<sup>a</sup> Frequency of causal mutations (with disease phenotype given) in 274 chromosomes from 49 patients and 176 carriers.

<sup>b</sup> Occurred together with IVS-I-16.

<sup>c</sup> Genotype included Codon 39 and 3' UTR +101.
The identification of causal mutations in 6 of 39 suspected carriers whose HbA2 levels were below the diagnostic level for β-thalassemia carrier state indicates the need for molecular genetic testing to improve the genetic diagnostic and counseling for β-thalassemia in the Gaza Strip where the obligatory premarital tests for β-thalassemia is performed since September 2000 and these premarital tests rely on hematological (CBC) and biochemical (HbA2 quantization) as the major tools to identify the carrier state of β-thalassemia in couples. The introduction of molecular testing for β-thalassemia carriers was suggested by Tarazi et al., in their evaluation and recommendations on obligatory premarital tests for β-thalassemia in Gaza Strip [10]. Molecular genetic testing will be increasingly important in the future because it is anticipated that the advances in the understanding of the molecular basis of the disease may lead to specific pharmacologic therapies in the future [24].

Author contributions and disclosures

M.M.S., C.T. and R.H. designed the study. M.M.S. and C.T. drafted the manuscript. I.S.T., M.S.A., and R.M.A. recruited the study participants. M.M.S. and J.S. performed assay design, genotyping and re-sequencing. I.S.T. M.S.A., R.M.A. and R.H. provided administrative support. The authors report no potential conflicts of interest.

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