Am J Blood Res 2025;15(3):47-56
www.AJBlood.us /ISSN:2160-1992/AJBR0164460

Original Article

Structural and clinical characterization of Hb Móstoles (HBA2:c.176A>G; p.His59Arg): a new unstable alpha-globin variant with thalassemic features

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Received March 11, 2025; Accepted June 3, 2025; Epub June 15, 2025; Published June 30, 2025

Abstract: We report a novel α-globin gene variant, hemoglobin (Hb) Móstoles, characterized by a single nucleotide substitution in the HBA2 gene [α2 58(E7) His > Arg; HBA2:c.176A>G], associated with a 3.7-kb deletion in the homologous chromosome. This variant was identified in a Moroccan family living in Spain. The proband, a four-year-old girl, presented with microcytosis and hypochromia. The abnormal Hb was maternally inherited and detected in two of the proband's four siblings, while the 3.7-kb deletion was paternally inherited. Hb analysis using high-performance liquid chromatography and capillary electrophoresis revealed an abnormal peak in the Hb S region, with a concentration of approximately 3%. Hematological parameter assessment of the four carriers demonstrated that, despite being a structural hemoglobinopathy, Hb Móstoles is associated with an alpha-thalassemia phenotype and may exacerbate clinical manifestations when coexisting with other *HBA1* and *HBA2* mutations.

Keywords: Thalassemic hemoglobinopathies, alpha globin variants, hypochromia

Introduction

Thalassemic hemoglobinopathies are a rare subset of hemoglobin (Hb) disorders, characterized by alterations in the synthesis and stability of globin chains, leading to anemia of varying severity. Although significantly less prevalent compared with classical alpha- and beta-thalassemia, their identification is crucial owing to their specific clinical implications and diagnostic challenges [1].

Structural hemoglobinopathies with a thalassemic phenotype are caused by mutations in globin genes that affect Hb stability, production, or assembly. These alterations can generate protein instability, alpha and beta chain synthesis imbalance, messenger RNA (mRNA) processing defects, or abnormal interactions between globins, leading to ineffective erythropoiesis and hemolysis. Consequently, the clinical manifestations are analogous to those of thalassemias, including anemia, microcytosis, and hypochromia. Currently, over 1,400 Hb variants have been reported in primary genetic databases, including ITHANET and HbVar, with a significant proportion classified as unstable Hbs. These variants result from point mutations that affect Hb structure and stability, generating forms prone to intracellular precipitation, Heinz body formation, and reduced erythrocyte lifespan [2-4].

Most reported thalassemic variants involve beta chain alterations (58%), while alpha chain variants are less common (42%) but equally significant. Alpha chain variants pose a unique diagnostic challenge, as they are often present with mild or asymptomatic phenotypes, requiring specialized testing for detection [5, 6].

This study identifies a novel mutation responsible for an unstable Hb variant associated with a thalassemic phenotype.

https://doi.org/10.62347/PSSD1758

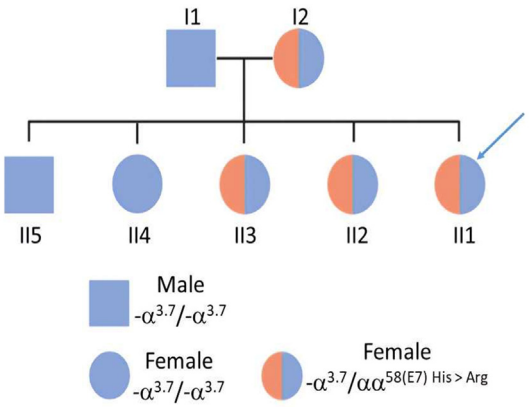


Figure 1. Pedigrees of the family. The proband is indicated by an arrow.

Material and methods

The proband was a four-year-old girl who presented with microcytosis and hypochromia. The quantification of HbA2 and HbF by high-performance liquid chromatography (HPLC) revealed an abnormal peak of 3% in the region corresponding to HbS. Her family underwent further evaluation at the Hemoglobinopathies Reference Laboratory, Hospital Clínico San Carlos, Madrid (Spain).

Hematological parameters were assessed using a DxH 900 automated hematology analyzer (Beckman Coulter, USA). Hb variants were identified and quantified using both HPLC (VARIANT II™, Bio-Rad Laboratories) and capillary electrophoresis (CEs) (Minicap Flex-Piercing, Sebia, Norcross, GA) following the manufacturers' instructions as previously described [7].

DNA was extracted using an automated Qiagen EZ1 system (Qiagen GmbH, Hilden, Germany) and quantified with an Invitrogen Qubit 3 fluorometer (Thermo Fisher Scientific, Massachusetts, USA).

Screening for common mutations in the alpha-globin gene cluster was performed using the α-Globin StripAssay kit (ViennaLab Diagnostic GmbH, Vienna, Austria) and multiplex ligation-dependent probe amplification (MLPA) (MRC Holland, Amsterdam, The Netherlands) following the manufacturer's instructions.

The variants were further characterized by Sanger sequencing of the *HBA1*, *HBA2*, and *HBB* genes, as previously described [8, 9]. Targeted next-generation sequencing (NGS) libraries for thalassemia were constructed using the Devyser Thalassemia NGS assay kit (Devyser, Bränningevägen, Sweden). This assay specifically identifies sequence variants in *HBA1*, *HBA2*, and *HBB*. It also identifies exonic copy number variations (CNVs), small or large insertions and deletions (indels), and single nucleotide variants (SNVs) within the alpha-globin and beta-globin gene clusters. The resulting amplicons were sequenced using a MiSeq (Illumina Inc., San Diego, USA), and data analysis was conducted using the Devyser Amplicon Suit v3.7.

Furthermore, brilliant cresyl blue staining was performed for all family members to detect characteristic inclusions.

To investigate the structural impact of the identified mutation, a three-dimensional model of the protein was generated using Swiss-Model Protein and assessed based on the alignment quality provided by the tool [10].

An *in silico* analysis of the mutation was conducted using computer tools to predict its functional impact and pathogenicity according to the American College of Medical Genetics and Genomics (ACMG) guidelines. Databases such as Genome Aggregation Database (gnomAD) and prediction algorithms such as Sorting Intolerant From Tolerant (SIFT), Polymorphism Phenotyping v2 (PolyPhen-2), MutationTaster, Functional Analysis Through Hidden Markov Models (FATHMM), Deleterious Annotation of genetic variants using Neural Networks (DANN), and MetaLR were used. Additionally, genomic evolutionary rate profiling (GERP) was used to assess evolutionary conservation, while SpliceAI and database of splicing consensus single nucleotide variants (dbscSNV) predicted potential slicing effects. The results were interpreted according to the current ACMG criteria [11].

Family analyses included the proband (II-1), her parents (I-1 and I-2), three sisters (II-2, II-3, and II-4), and one brother (II-5) (**Figure 1**).

All hematological and clinical evaluations were conducted with the prior informed consent of the patients, in compliance with the Declaration of Helsinki.

Table 1. Hematological data

	Age	Sex	Hb (g/dL)	MCV (fL)	MCH (pg)	CMCH (g/dL)	RDW (%)	Reticulocytes (%)	Genotype
I1	47	M	14.8	70.8	22.5	31.8	14.6	1.06	$-\alpha^{3.7}/-\alpha^{3.7}$
I2	43	F	10.9	69.3	22.8	32.9	14.7	2.48	$-\alpha^{3.7}/\alpha\alpha^{58(E7)\ His > Arg}$
II1	4	F	10.2	62.3	19.5	31.3	17	0.21	$-\alpha^{3.7}/\alpha\alpha^{58(E7)\ His > Arg}$
II2	12	F	12.2	68.3	21.6	31.7	16.1	1.50	$-\alpha^{3.7}/\alpha\alpha^{58(E7)\ His > Arg}$
II3	14	F	10.6	70.2	21.8	31.0	17.0	1.28	$-\alpha^{3.7}/\alpha\alpha^{58(E7)\ His > Arg}$
II4	18	F	11.3	67.0	20.7	30.9	16.7	0.73	$-\alpha^{3.7}/-\alpha^{3.7}$
II5	24	M	14.7	69.3	22.4	32.3	14.8	0.71	$-\alpha^{3.7}/-\alpha^{3.7}$

MCV: Mean Corpuscular Volume, MCH: Mean Corpuscular Hb, MCHC: Mean Corpuscular Hb Concentration, and RDW: Red Cell Distribution Witdth.

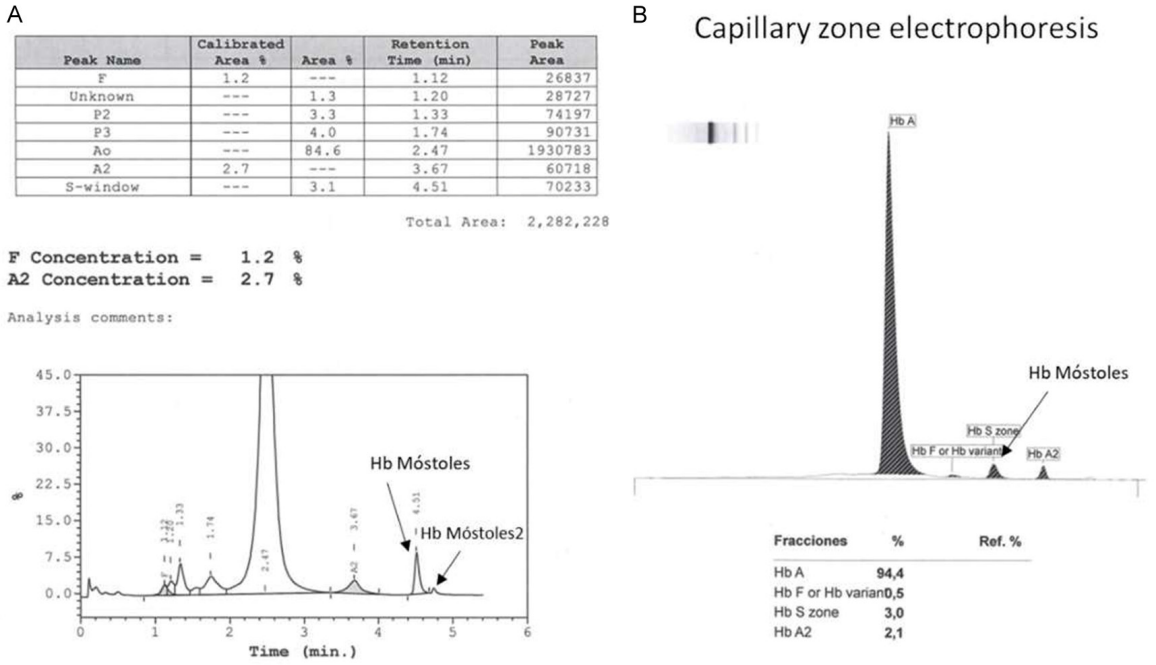


Figure 2. Analysis of Hb by HPLC and ECs. A. HPLC chromatogram showing the different hemoglobin peaks, with the presence of Hb Móstoles and Hb Móstoles 2 identified at specific retention times. B. ECs confirming the presence of the abnormal Hb Móstoles fraction along with the normal fractions of Hb A, Hb A2, and Hb F.

Results

The family study revealed that all members had microcytosis and hypochromia, as detailed in **Table 1**.

HPLC and CZE identified an abnormal Hb peak in the proband, her mother (I-2), and two sisters (II-1 and II-2), representing 2-3% of the total Hb. HPLC detected a peak with a retention time (RT) of 4.51 min within the HbS window, while CZE localized the peak in the HbS zone. Additionally, HPLC revealed a smaller adjacent peak, which could not be quantified but likely represents the HbX2 fraction of the variant chain (**Figure 2**; **Table 2**).

Initial testing using the α-Globin StripAssay kit detected a 3.7-kb deletion in homozygosity in three family members (I-1, II-4, and II-5) and in heterozygosity in the remaining four (I-2, II-1, II-2, and II-3). Analysis by MLPA and NGS confirmed the results (**Figure 3**).

Sanger sequencing of the *HBA2* and *HBA1* genes identified a missense mutation in codon 58 of the second exon of *HBA2* (c.58G>C), resulting in the substitution of histidine (His) with arginine (Arg) at position 59 of the alpha-globin chain (His59Arg) (**Figure 4A**). This variant has not been previously described in databases or in scientific literature. Reverse

Table 2. Hb analysis by HPLC, ECs and genotype

	HPLC				ECs				Genotype
	HbA0 (%)	HbA2 (%)	HbF (%)	HbX (%)	HbA0 (%)	HbA2 (%)	HbF (%)	HbX (%)	
I1	85.0	2.7	0.7	N.D	97.6	2.4		N.D	$-\alpha^{3.7}/-\alpha^{3.7}$
I2	84.6	2.7	1.2	3.1	94.4	2.0	0.5	3.0	$-\alpha^{3.7}/\alpha\alpha^{58(E7)\ His > Arg}$
II1	84.1	2.6	2.1	1.7	94.5	2.0	1.4	1.9	$-\alpha^{3.7}/\alpha\alpha^{58(E7)\ His > Arg}$
II2	84.6	2.6	1.4	3.1	94.1	2.0	0.7	3.2	$-\alpha^{3.7}/\alpha\alpha^{58(E7)\ His > Arg}$
II3	85.6	2.6	1.1	2.9	94.7	2.0	0.4	3.0	$-\alpha^{3.7}/\alpha\alpha^{58(E7)\ His > Arg}$
II4	87.4	2.8	0.4	N.D	97.8	2.2		N.D	$-\alpha^{3.7}/-\alpha^{3.7}$
II5	87.9	2.8	0.2	N.D	97.7	2.3		N.D	$-\alpha^{3.7}/-\alpha^{3.7}$

N.D: Not detected.

strand sequencing confirmed the mutation (**Figure 4B**), detecting it in homozygosity. Given that only one *HBA2* gene was amplified in the affected individuals, while the other allele contained the 3.7-kb deletion, preventing its amplification as the target sequence of the reverse primer was not present. However, NGS analysis reported a 30% mutation rate (**Figure 4C**), likely owing to the limitations of the assay. The NGS analysis involved 300-bp amplicons, making it difficult to differentiate between *HBA1*, *HBA2*, and the hybrid *HBA1-HBA2,* given their high homology [12]. The carriers of the 3.7-kb deletion have three functional alpha-globin genes (*HBA1*, *HBA2*, and *HBA1-HBA2*), which explains the observed mutation rate. Given that the mutation is in an amplicon common to all three genes, determining its precise location among the genes is infeasible. However, Sanger sequencing confirmed that the genetic alteration is specifically located in *HBA2*.

The three-dimensional model generated with Swiss-Model Protein revealed structural alterations owing to the substitution of His by Arg. Modifications in the stability of the region near residue 59 were found due to differences in size. This alteration can explain the formation of Heinz bodies, as confirmed by brilliant cresyl blue staining.

The images generated by Swiss-Model Protein revealed significant structural differences between the normal and the mutated protein (**Figure 5A** and **5B**), demonstrating the functional impact of the new variant.

In silico analysis using the gnomAD database (PM2), along with MutationTaster, FATHMM, DANN, and MetaLR, showed that the mutation was potentially pathogenic (PP3). However, SIFT classified the mutation as of uncertain significance. Evolutionary conservation assessed by GERP indicated an uncertain value (4.15), while GenoCanyon and fitCons found a possible moderate deleterious effect. Overall, these findings suggest that the mutation can have a significant functional impact.

This newly identified Hb variant was named Móstoles, after the city where the family lived.

Discussion

This study identifies a novel point mutation (c.58G>C) in the *HBA2* gene, which causes a His to Arg substitution at position 59 of the alpha-globin chain. This His is located in the E helix, a highly conserved area among vertebrates. The mutation generates a new Hb variant (Hb Móstoles), which appears as an abnormal peak of approximately 3% in the HbS region on HPLS and CZE. This low percentage may result from the formation of a hyperunstable chain that can barely form a functional Hb tetramer or from rapid denaturation of the tetramer after incorporating the abnormal chain [13].

Structural hemoglobin variants can have a significant clinical impact depending on how they alter hemoglobin function, stability, and erythrocyte integrity. These mutations may lead to hemoglobin instability, increased susceptibility to oxidation, and precipitation of denatured globin chains, which are associated with hemolysis and ineffective erythropoiesis. Clinically, patients may present with anemia, jaundice, splenomegaly, or features mimicking thalassemia syndromes. For instance, unstable

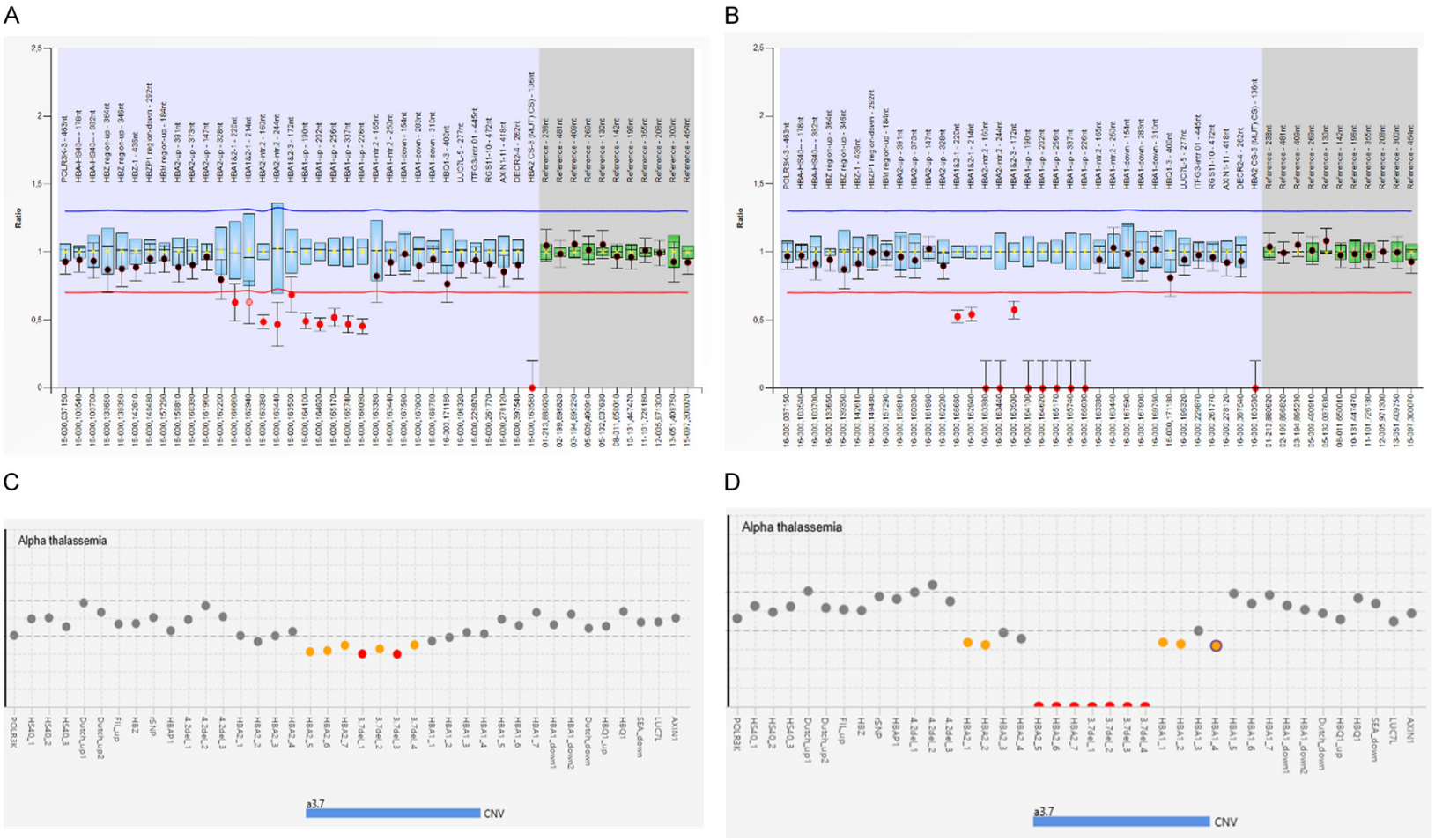
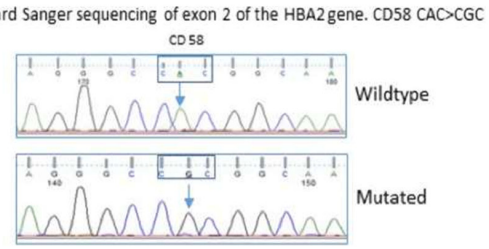
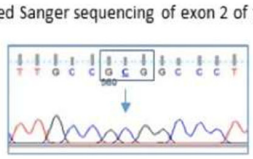


Figure 3. Comparison of results obtained by MLPA and NGS CNV in the detection of deletions in the alpha-globin cluster. Graphs (A and B) represent MLPA analyses for the -$\alpha^{3.7}$/αα and -$\alpha^{3.7}$/-$\alpha^{3.7}$ genotypes, respectively. Graphs (C and D) show the results obtained with NGS CNV for the same genotypes. A concordance between both techniques is observed in the identification of deletions in the alpha-globin cluster. In the MLPA analyses (A and B), the reduction in probe intensity ratios allows for the detection of specific deletions. Similarly, in the NGS CNV analyses (C and D), a decrease in coverage in the affected region confirms the presence of the same deletions detected by MLPA. This demonstrates the utility of NGS CNV as a reliable alternative for alpha-thalassemia diagnosis, complementing traditional methods such as MLPA.

A



B



C

Analysis identified from the DEVYSER Software. *CNV* analysis for amplicons in the *HBA* gene complex

Position	Class	VCC	Report	Type	Transcript	c.HGVS	p.HGVS	VAF%	Gene	Amplicon
chr16:g.227008A>G	4	H		s_MV	NM_000558.4	c.176A>G	p.His59Arg	33.75	HBA1	HBA1_4
chr16:g.223204A>G	4	H		s_MV	NM_000517.4	c.176A>G	p.His59Arg	35.67	HBA2	HBA2_4

Figure 4. Detection of the CD58 CAC>CGC mutation in the *HBA2* gene by Sanger sequencing and bioinformatics analysis using DEVYSER software. A. Sanger sequencing of exon 2 of the *HBA2* gene in the forward direction, showing the difference between the wild-type and mutated sequences. B. Sanger sequencing in the reverse direction confirming the mutation. C. CNV analysis and detected variants in the *HBA* gene cluster using DEVYSER, identifying the c.176A>G mutation in *HBA1* and *HBA2*.

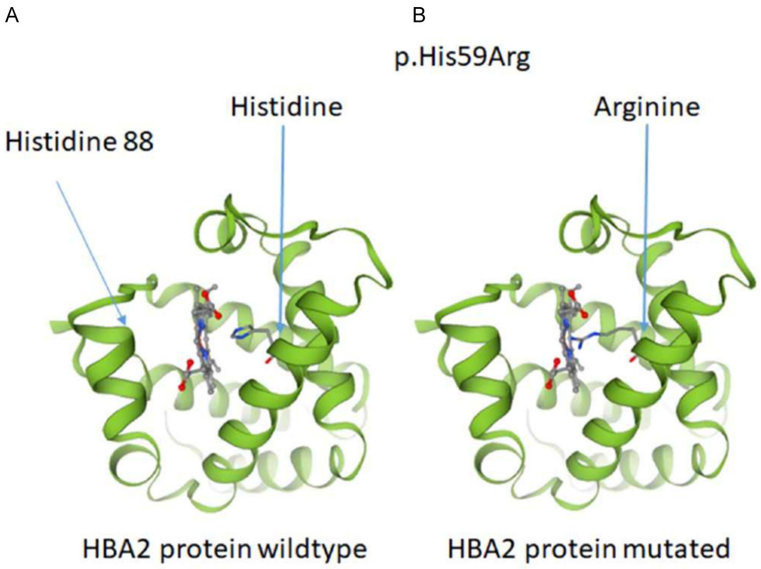


Figure 5. Three-dimensional model of the HBA2 protein using Swiss-Model Protein. A. In the three-dimensional structure, His59 interacts with nearby residues through hydrogen bonds. Its orientation within the heme cavity stabilizes the heme group and contributes to maintaining the tertiary structure of the globin. Its interaction with His88 influences oxygen binding to heme, optimizing oxygen transport function. B. The substitution of Arg59 introduces an additional positive charge in the heme cavity. Due to its longer side chain, it may establish new electrostatic interactions with nearby residues, causing structural displacement in the region surrounding the heme group. This could interfere with heme stability and affect protein functionality. This structural alteration may also expose the heme group, promoting Heinz body formation due to oxidative destabilization.

hemoglobins such as Hb Zurich and Hb M-Boston, which affect the same distal histidine residue as Hb Móstoles, have been associated with hemolytic anemia and inclusion body formation due to structural destabilization of the heme pocket [14-17]. Wajcman et al. also emphasized that many unstable alpha-globin variants contribute to Hb H disease or thalassemia intermedia phenotypes, particularly when co-inherited with alpha gene deletions [5]. Therefore, identifying and characterizing these variants is essential for appropriate clinical management, genetic counseling, and avoiding misdiagnosis with other microcytic anemias.

Perutz *et al.* highlighted the significance of distal His located in the E helix of the globin chain, which are critical for ligand binding to the heme

group of Hb. These His regulate and stabilize the binding of oxygen to the iron (Fe^{2+}) of the heme group [18]. The role of His in oxygen stability is attributed to their ability to form hydrogen bonds [19-21]. Other studies substituting His with glycine (Gly), alanine (Ala), leucine (Leu), or phenylalanine (Phe) demonstrated an increased rate of oxygen dissociation, confirming the strong hydrogen bonds between oxygen and His, both in alpha and beta chains [22].

According to the three-dimensional model generated by Swiss-Model Protein, substituting His (a relatively small amino acid, with an imidazole ring) with Arg (an amino acid with a larger side chain and an additional charged group) in Hb Móstoles induces a significant structural impact on the distal region of the heme group. The different size and charge of the side chain create a conformational mismatch in the structure of the Hb molecule, preventing effective Arg accommodation and distorting the protein structure. This distortion widens the heme pocket, leading to heme oxidation and destabilization of the globin chains. This mechanism can explain the formation of Heinz bodies observed in patients with this new mutation, similar to those in Hb Zurich [β63(E7) His > Arg; HBB:c.191A>G], a Hb variant characterized by the same mutation in the distal His of the beta-globin chain [14-16].

This model suggests that the structural changes caused by the substitution of His with Arg generate a mismatch in the region of interaction with the heme group, impairing oxygen binding and destabilizing the conformational structure of the globin chain. Consequently, the globin chain degrades rapidly, contributing to hemolysis. Additionally, these structural changes increase the structural tensions, reducing the overall stability of Hb and promoting protein denaturation. The Arg substitution also alters the electrostatic interactions between the alpha and the beta chains in the Hb tetramer, compromising the stable formation of Hb Móstoles and exacerbating protein instability.

Our findings are consistent with previous reports describing alpha-globin variants affecting the distal histidine (position 59) of the alpha chain. For instance, Hb Zurich [β63(E7) His > Arg], a beta chain analog of our variant, also results in significant heme instability and Heinz body formation due to disruption of heme coordination [14-16]. Similarly, other alpha-globin variants at the same residue, such as Hb Boghé, Hb Furlingen, and Hb DG-Nancheng, have been associated with microcytosis, hypochromia, and a thalassemic phenotype despite presenting as structural hemoglobinopathies. These variants share with Hb Móstoles the pathogenic mechanism of structural destabilization near the heme pocket, resulting in impaired tetramer formation and increased oxidative damage [17, 23-25]. Notably, Wajcman et al. reported that alpha-globin variants with reduced stability can phenotypically mimic deletional thalassemias and even aggravate the clinical picture when co-inherited with alpha-thalassemia deletions [5]. Our data confirm this observation, as the phenotype was more pronounced in compound heterozygotes for Hb Móstoles and the $-\alpha^{3.7}$ deletion. Taken together, these comparisons reinforce the pathogenicity of mutations in structurally critical residues and support the inclusion of such variants in the diagnostic workup of microcytic anemia.

The distal His also interacts with the chaperone alpha-Hb-stabilizing-protein (AHSP), which specifically binds to free alpha-globin chains. AHSP primarily regulates the stability, folding, and assembly of the alpha subunit of Hb [26, 27]. It is synthesized at high levels in erythroid precursors and acts by preventing the precipitation of alpha-globin chains. In the absence of AHSP, alpha-globin chains are oxidized and precipitate in erythroid precursors within the bone marrow, inducing apoptosis and ineffective erythropoiesis [28]. Conversely, when AHSP is present, iron is stabilized by a bis-histidyl coordination between His-59 and His-88 [26, 29]. In Hb Móstoles, replacement of His-59 disrupts this coordination, weakening AHSP's interaction with the alpha-globin chain. Consequently, AHSP may lose its ability to prevent the precipitation of alpha-globin chains, leading to oxidation and precipitation of alpha-globin chains in erythroid precursors, triggering apoptosis and ineffective erythropoiesis.

The low Hb Móstoles concentration in patients likely results from degradation of the mutated alpha-globin before assembling into tetramers owing to its structural instability and a possible alteration in its interaction with AHSP. If tetramers are still assembled, they remain unstable

and prone to oxidation and denaturation, leading to their rapid elimination from circulation.

This variant is the fifth to be described in this position. Except for Hb M-Boston (→Tyr; HBA2:c:175C>T), which causes cyanosis, the other variants - Hb Furlingen (→Gln; HBA2: c:177C>G), Hb Boghé (→Gln; HBA2:c:177C>A), and Hb DG-Nancheng (→Asn; HBA2:c:175C> A)-exhibit a thalassemia phenotype similar to Hb Móstoles [17, 23, 24, 2]. In Hb Móstoles, all family members showed a phenotype compatible with thalassemia, including microcytosis (MCV < 75 fL) and hypochromia (MCH < 25 pg). Three of them were caused by the loss of two alpha genes ($-\alpha^{3.7}/-\alpha^{3.7}$), while four were due to the loss of one alpha gene and the non-deletional mutation ($-\alpha^{3.7}/\alpha\alpha^{58(E7)\ His\ >\ Arg}$). Although the exact mechanism remains unclear, the formation of the Hb tetramer is deficient in this variant due to the instability of the mutated chain, leading to the thalassemia phenotype.

Phenotypic variability among carriers of structural hemoglobin variants, even within the same family, can be influenced by multiple factors. Genetic modifiers-such as coinherited alpha- or beta-thalassemia mutations, differences in the expression of alpha-hemoglobin stabilizing protein (AHSP), or polymorphisms in genes related to oxidative stress and erythropoiesis-may modulate the clinical severity. Additionally, epigenetic regulation, environmental exposures, and individual erythropoietic responses contribute to the heterogeneity observed in hematological findings. Clinically, although no curative treatment exists for unstable hemoglobinopathies, management strategies include regular hematological monitoring, folic acid supplementation, avoidance of oxidative triggers, and genetic counseling for affected families. In selected cases, splenectomy or transfusion support may be indicated. Early molecular diagnosis plays a key role in preventing misdiagnosis and guiding personalized clinical care [30].

Notably, non-deletional thalassemias are often associated with a more severe phenotype when compared with deletional thalassemias. While deletions reduce the amount of available alpha chains, point mutations can affect both HB synthesis and structural stability. The location of the mutation in *HBA2* or *HBA1* significantly influences phenotype severity, as alterations in critical regions, such as cleavage and splicing sites or residues involved in tetramer stability, can lead to more severe effects.

One such example is Hb Agrinio [α2 29(B10) Leu>Pro; HBA2:c.89T>C], a point mutation in homozygosity that can lead to the development of Hb H disease [31, 32]. Similarly, mutations in the polyadenylation signal affect the efficiency of mRNA transcription and maturation, reducing the production of alpha chains and contributing to more severe clinical manifestations [33, 34].

These findings highlight the necessity of investigating molecular mechanisms associated with globin gene expression and mutations affecting globin genes. Identifying and characterizing new molecular variants enhance the development of more rapid and accurate diagnostic tools, which are crucial for improving clinical management of hemoglobinopathies.

Conclusion

We report a novel alpha-globin variant, Hb Móstoles (HBA2:c.176A>G; p.His59Arg), associated with a thalassemic phenotype and structural instability of the hemoglobin molecule. This variant disrupts the highly conserved distal histidine in the E helix of the alpha chain, leading to impaired heme binding, protein destabilization, and features consistent with ineffective erythropoiesis. Functional modeling and hematological findings in affected family members support its classification as an unstable hemoglobin variant with clinical significance. When co-inherited with common alpha-thalassemia deletions, Hb Móstoles exacerbates the phenotype, emphasizing the need for comprehensive molecular diagnosis in microcytic anemia. This case expands the spectrum of pathogenic alpha-globin mutations and underscores the diagnostic relevance of integrating molecular, structural, and clinical data [35].

Disclosure of conflict of interest

None.

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