



Hb Belluno [β 111(G13)Val \rightarrow Gly; β 133(H11)Val \rightarrow Val (HBB: c.335T > G;402G > C)]: Incidental Detection of a New Clinically Silent β Chain Variant During Hb A_{1c} Determination by High Performance Liquid Chromatography

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ORIGINAL ARTICLE

Hb Belluno [β 111(G13)Val \rightarrow Gly; β 133(H11)Val \rightarrow Val (*HBB*: c.335T > G;402G > C)]: Incidental Detection of a New Clinically Silent β Chain Variant During Hb A_{1c} Determination by High Performance Liquid Chromatography

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Abstract

A previously unreported β chain variant, Hb Belluno [β 111(G13)Val \rightarrow Gly; β 133(H11)Val \rightarrow Val (*HBB*: c.335T > G;402G > C)], was incidentally discovered in a woman suffering from diabetes, during glycated hemoglobin (Hb A_{1c}) assay. Its presence was suspected because of a small abnormal peak with a retention time just shorter than that of normal Hb A_{1c}. Standard high performance liquid chromatography (HPLC), capillary zone electrophoresis (CZE) and agarose gel electrophoresis did not allow to separate the variant from Hb A. The reversed phase HPLC of globin chains showed the presence of a heterozygous β -globin variant amounting to approximately 43.5% of the total β chains. Later, this variant was found in five other members of the same family and DNA sequencing analysis confirmed a β -globin gene mutation. The variant is clinically silent in all patients and showed a slight instability with both heat and isopropanol tests. The other three mutations at this locus also affect stability. Hemoglobin (Hb) variants may invalidate the results of Hb A_{1c} analysis and could result in mismanagement of diabetes. A comment alerting the requesting clinician to the presence of the Hb variant must be appended to the Hb A_{1c} result. Additionally, many Hb variants can be chromatographically and/or electrophoretically silent. Therefore, when the clinician suspects a variant Hb, it is not sufficient to get a negative response from an HPLC screening test to rule it out. A dialogue with the pathologist is essential, involving exchange of information and sharing a diagnostic work-up including surveys to assess Hb stability and oxygen affinity, as much as DNA sequencing.

Introduction

In patients with diabetes, glycated hemoglobin (Hb) measured as Hb A_{1c} is used for evaluating long-term control of the disease. Glycated Hb is the result of irreversible non enzymatic glycation of both the two α and β chains of normal adult Hb A. Sixty percent of the glucose is bound to the N-terminal valines of the β chains. A small amount of glucose binding occurs in the α chain N-terminal valines. The remainder occurs at the 44 lysine side chains, 11 on each of the α and β chains. Although several methods based on different principles [high performance liquid chromatography (HPLC), immunoagglutination, boronate affinity assays and electrophoresis] have been developed, a cation exchange HPLC is the comparison method designated by the Diabetes Control and Complications Trial (DOCT) (1) for Hb A_{1c} monitoring.

Keywords

Glycated hemoglobin (Hb A_{1c}), hemoglobin (Hb) variants, β chain, valine 111

History

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As HPLC came into use for the determination of Hb A_{1c} in the 1980s, an increasing number of cases with abnormal Hbs have been detected on the basis of abnormal levels of Hb A_{1c} or abnormal elution patterns. We report a new clinically silent Hb variant, identified by the presence of an abnormal HPLC elution pattern in Hb A_{1c} assessment.

Patients, materials and methods

The new variant was first detected in a 64-year-old female (VA) of North Italian origin; she was initially investigated for a type 2 diabetes mellitus (T2DM). Two of her children, a 36-year-old male (OD) and a 25-year-old female (OR), were invited to our institution for investigation. Another two male patients, a 66-year-old (TL) and 68-year-old (VG), were also investigated for T2DM. At the time of the Hb A_{1c} assay, we did not know of any family relationship with patient VA. The proband (VM), a 44-year-old woman without diabetes, received the test for Hb A_{1c} during generic investigations; at that time, no family ties to the other patients were known.

All blood samples were collected in EDTA-containing vacutainers. A complete blood count (CBC) was done for all

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six patients by the UniCell DxH 800 counter (Beckman-Coulter Srl, Cassina de Pecchi, Italy). Hb A_{1c} was estimated first with the Tosoh G7 and subsequently with the Tosoh HLC-723G8 (G8) Automated Analyzer (Tosoh Bioscience Srl, Rivoli, Italy) following the manufacturer's instructions and with apposite reagents, column and program.

Until mid-2013, we used an in-house method to perform the screening of Hb variants. Briefly, analysis were performed with a Perkin-Elmer series 200 liquid chromatograph equipped with a Link 900 data processor and an LC/95 spectrophotometer (Perkin Elmer Spa, Milan, Italy). A PolyCAT A column was used (LabService Analytica Srl, Anzola dell'Emilia, Italy) following instructions provided by Ou *et al.* (2) for gradient program and sample preparation. After mid-2013, screening for Hb variants was performed by the Tosoh HLC-723G8 (G8) with dedicated reagents, column and program (β -Thalassemia mode).

Capillary zone electrophoresis (CZE) was performed with the Sebia Capillarys system (Sebia Italia Srl, Bagno a Ripoli, Italy) initially with Capillarys and later with Capillarys2 Flex Piercing instrument, following the manufacturer's instructions and using programs and reagents for Hb assay. Alkaline and acid agarose gel electrophoresis were performed with the Hydrasys automated system following instructions and using kits supplied by the manufacturer (Sebia Italia Srl). Analysis of the globin chains was carried out by reversed phase HPLC using a LiChrospher 100 CH-8/2 column (Fisher Scientific Italia, Rodano, Italy) and a ternary eluent (acetonitrile-methanol-NaCl 0.155 M, pH 2.7) as described by Leone *et al.* (3).

Genomic DNA was extracted from a blood sample of the proband using the automated method Bio-Robot EZ1 (Qiagen Spa, Milan, Italy). Molecular characterization was undertaken using direct automatic sequencing performed by ABI PRISM™ 3130xl Genetic Analyzer (Applied Biosystems Italia, Monza, Italy). The β -globin gene was specifically amplified using pairs of forward and reverse primers shown in Table 1. The amplified products were sequenced by the commercial ABI PRISM™ BigDye v3.1 Cycle Sequencing kit (Applied Biosystem Italia). The obtained sequences were compared through the FASTA server reference database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Stability of Hb was evaluated by Heinz bodies staining and with the heat and isopropanol stability tests as described by Bain and Wild (4) and Bain and Swirsky (5). Venous and arterial blood gas analysis was performed using the Siemens

RAPIDPoint 405 system (Siemens Healthcare Italia, Milan, Italy). Oxygen affinity was calculated by an equation that converted venous oxygen tension and oxygen saturation to P₅₀ (6).

Results

The new Hb variant was identified for the first time in early 2013, when we were using the Tosoh G7 for the Hb A_{1c} assay and our in-house HPLC method for hemoglobinopathies screening. For patient VA, Tosoh G7 showed a small abnormal peak with a retention time just shorter than that of normal Hb A_{1c} (Figure 1a). For this reason, the presence of a Hb variant was suspected; we then proceeded to use our in-house HPLC and CZE.

The first HPLC showed a doubling of the Hb A peak (Figure 1b) but the result was not reproducible in subsequent sessions, nor with our in-house method, nor with Tosoh G8 HPLC. However, this aberrant finding strengthened our suspicion of a Hb variant, although it was not possible to properly quantify it. Neither CZE nor agarose gel electrophoresis at alkaline or acid pH allowed the separation of the suspected variant from Hb A (data not shown).

The reversed phase HPLC of separated globin chains clearly showed the presence of a heterozygous β -globin variant amounting to approximately 43.5% of the total β chains (Figure 1c) and therefore, the calculated Hb A variant was about 42% of the total Hb. The blood count was normal (Table 2), as were the bilirubin and haptoglobin levels (data not shown).

Obviously, we reported the presence of a clinically silent variant Hb to the family doctor, which made the Hb A_{1c} assay unreliable. We also invited the doctor to extend the search for Hb variants to the first and second degree relatives of the patient VA. Two of her children (OD and OR) showed the variant, confirmed by the finding of a chromatogram identical to that of the mother on the HPLC globin chain analysis. Even the son and daughter showed a normal blood count (Table 2).

Initially, given the complete lack of clinical signs and symptoms due to the presence of the Hb variant, we felt no need for DNA testing for definitive identification. Only more recently, when we found three other patients with the same variant and as they were all more or less close relatives, we decided to proceed with direct sequencing of the β -globin gene.

The last three patients (TL, VG and VM) showed a normal blood count (Table 2) with no history of jaundice and with normal bilirubin and haptoglobin levels. We obtained informed consent for DNA analysis from the last patient (VM) identified as a carrier of the variant Hb. Molecular analysis had shown a heterozygous variation of nucleotide sequence *HBB*: c.335T>G (corresponding to a missense change Val111Gly) and a heterozygous variation of nucleotide sequence *HBB*: c.402G>C (corresponding to a synonymous change Val133Val).

This latter mutation is recorded in the NCBI database of human short genetic variations (<http://www.ncbi.nlm.nih.gov/snp>) with the reference number rs113082294. It was defined as a benign allele and lacked functional studies and data on geographic distribution. It had been sequenced in the course

Table 1. Sequence of the forward and reverse primers used for polymerase chain reaction β -globin gene amplification.

Primers	Sequences (5' > 3')
Forward primer 1 (beta5)	GCT TAC CAA GCT GTG ATT CCA AAT ATT ACG
Reverse primer 1 (pco4)	GGT GAA CGT GGA TGA AGT TGG
Forward primer 2 (r37)	CCA ATC TAC TCC CAG GAG CA
Reverse primer 2 (r94)	GGT TAA GTT CAT GTC ATA GG
Forward primer 3 (dgge2)	AAG GAG ACC ATA GAA ACT G
Reverse primer 3 (int2r)	GCA TCA GTG TGG AAG TCT CAG G
Forward primer 4 (pe)	GTA TCA TGC CTC TTT CGA CC
Reverse primer 4 (pf)	GAA TGT GGG AGG TCA GTG C

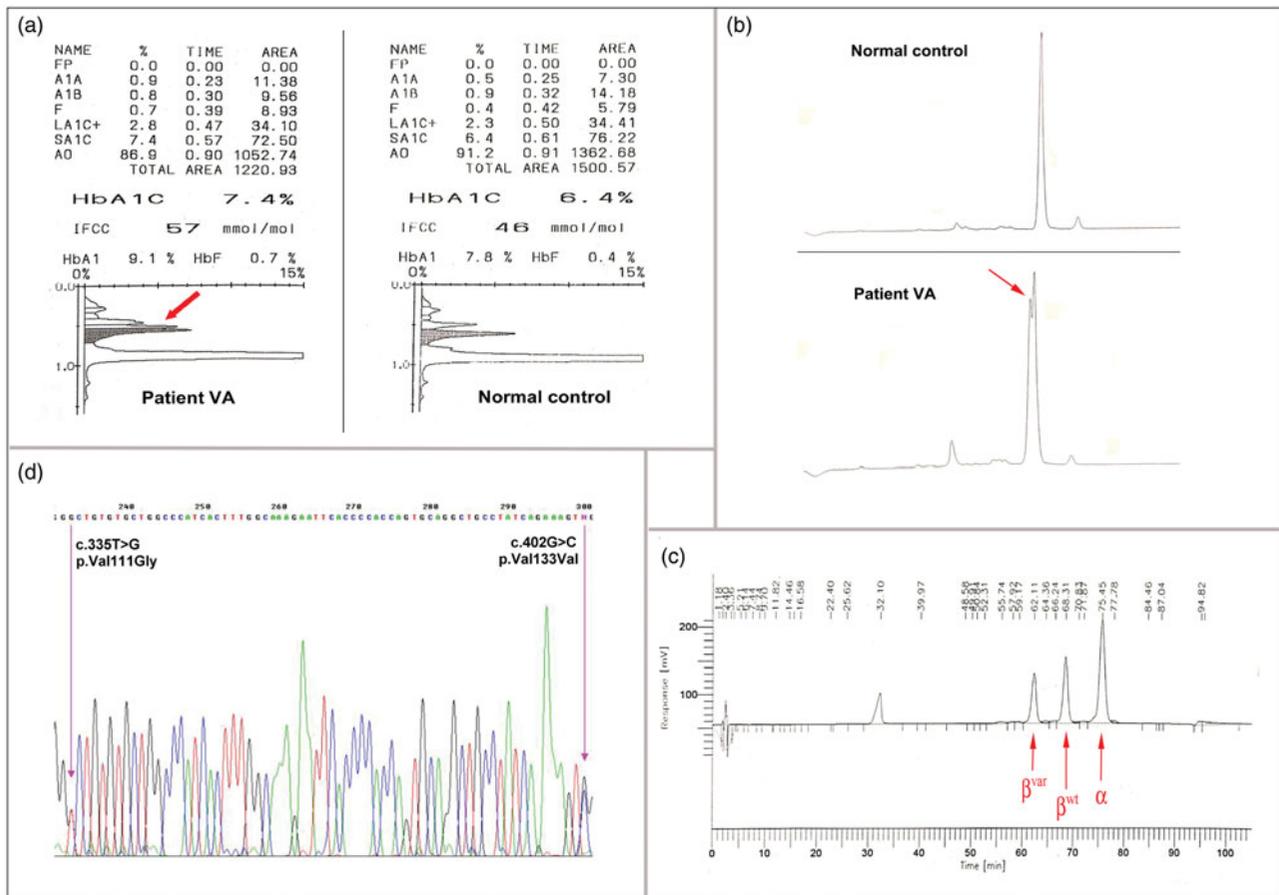


Figure 1. The significant steps that led to the identification of the new variant Hb Belluno. a) Tosoh G7 chromatograms of the patient VA (on the left) and of a normal control (on the right). The arrow indicate the anomalous peak with a retention time just shorter than that of normal Hb A_{1c}. b) The first time our in-house method showed a doubling of the Hb A peak for the patient VA (red arrow). This result was not reproduced in the subsequent sessions of the same method or with Tosoh G8 HPLC. c) Reversed phase HPLC globin chain separation of the patient VA. The X axis denotes the retention time in minutes. The β -globin variant was identified as β^{var} and the wild-type β -globin as β^{wt} . d) Direct sequencing of the proband's DNA: exon 3 of the β -globin gene.

Table 2. Complete blood count results of all of six patients at time of anomalous chromatogram peak discovered during the Hb A_{1c} assay.

Patient (sex-age)	Hb (g/dL)	RBC ($10^{12}/L$)	PCV (L/L)	MCV (fL)	MCH (pg)	MCHC (g/dL)
VA (F-64)	13.1	4.32	0.37	87.0	30.3	34.8
OD (M-36)	15.9	4.87	0.46	95.4	32.6	34.2
OR (F-25)	14.0	4.82	0.42	87.9	29.0	35.1
TL (M-66)	16.3	5.00	0.47	94.4	32.5	34.5
VG (M-68)	15.3	5.02	0.43	86.5	30.4	35.2
VM ^a (F-44)	15.9	5.18	0.47	91.2	30.6	33.6

Hb: hemoglobin; RBC: red blood cell counts; PCV: packed cell volume; MCV: mean corpuscular volume; MCH: mean corpuscular Hb; MCHC: mean corpuscular Hb concentration.

^aProband.

of the 1000 Genomes project and had a reported global minor allele frequency (MAF) $G = 0.0018/9$. Direct sequencing of about 6200 *HBB* genes performed at our Molecular Genetics Laboratory of Ferrara identified the mutation only in this patient. Thus, we can consider this variant to be extremely rare and with no clinical effects. Because of the unavailability of other family members to undergo genetic testing, we have not yet been able to determine whether the two mutations are in the same or a different allele. The sequence of the DNA

region affected by the two single nucleotide replacements (β -globin gene, exon 3) is shown in Figure 1d.

We also performed further tests on the proband VM to assess *in vitro* stability and oxygen affinity of the variant Hb. The search for Heinz bodies was negative, while the isopropanol and heat stability tests were slightly positive (Figure 2). The blood gas analysis of the arterial and venous blood was normal; in particular, the P_{50} was 23.6 mm/Hg (normal range 22.6 to 29.4 mm/Hg). Figure 3 shows a partial reconstruction of the family pedigree of the proband and the other carriers of the missense mutation responsible for the Hb phenotype.

Discussion

The structural variants are mainly composed of missense mutations that cause single amino acid substitutions in the globin protein, resulting in an abnormal, or 'variant' Hb tetramer. Less commonly, Hb variants are associated with deletions, multiple amino acid substitutions, antitermination mutations, and altered posttranslational processing. Naturally occurring Hb mutations cause a range of biochemical abnormalities, some of which produce clinically significant symptoms.

The most common and medically important Hb variants include Hb S (*HBB*: c.20A>T), Hb C (*HBB*: c.19G>A), Hb

Figure 2. Results of the isopropanol stability test (a) and the heat stability test (b) of the patient VM (tubes on the right) and a normal control (tubes on the left). The positivity of both tests is shown as turbidity of the patient's Hb solution.

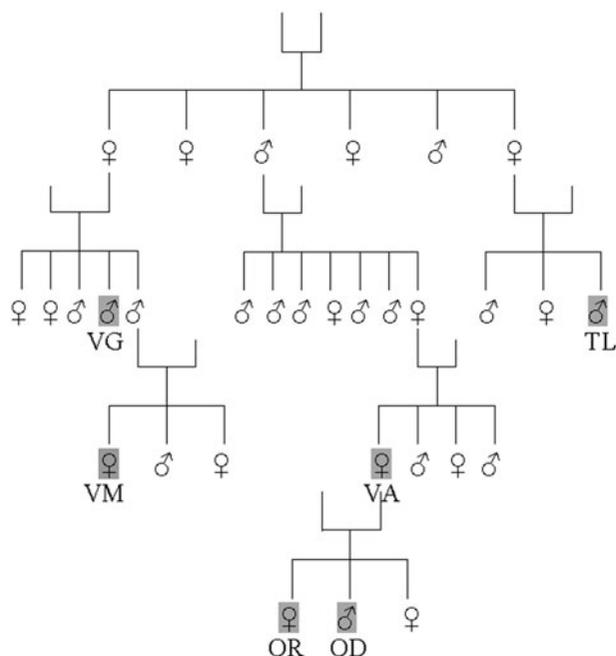
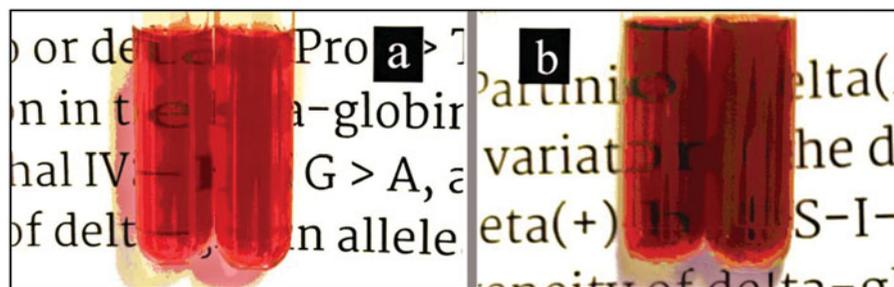


Figure 3. Partial reconstruction of the pedigree, indicating the family ties between the proband VM and the other five patients identified as carriers of the Hb Belluno variant.

E (*HBB*: c.79G > A) and some thalassemias (e.g. ‘thalassemic hemoglobinopathies’), all of which are under positive genetic selection because they confer survival advantages in areas where malaria is endemic. In addition to these prevalent mutant proteins, there are also other well known naturally occurring Hb variants that are rare individually but common collectively. By convention, most of these variants are named after the geographic origin of the affected individual. Although many Hb variants are clinically silent, some produce clinical manifestations of varying severity. Analyses of these variants, which can be considered to be ‘experiments of nature’ (7), have generated valuable insights into structure-function relationships within the Hb molecule, with interesting and important clinical consequences.

The Belluno province is almost entirely mountainous (Dolomites) and has an extremely low incidence of thalassemia and hemoglobinopathies endemic in other Italian regions or in the world (Hb S, Hb C, Hb E). In a preceding unpublished study regarding an unusually high incidence of Hb D-Los Angeles [$\beta 121(\text{GH}4)\text{Glu} \rightarrow \text{Gln}$; *HBB*: c.364G > C], in a limited geographical area of the Belluno Province, we evaluated the global prevalence of structural hemoglobinopathies incidentally discovered during Hb A_{1c} assay.

Between January 2010 and December 2013, we performed 63,448 Hb A_{1c} assays for 19,948 patients and identified 107 carriers of a Hb variant, corresponding to a prevalence of 0.54%. Our data are consistent with those of other investigators, published for regions with a relatively low incidence of endemic hemoglobinopathies [*i.e.* 0.40% in northern Italy (8), 0.14% in Ireland (9)] and significantly lower than those regions with a higher incidence [*i.e.* 2.30% in Tunisia (10) and Singapore (11)].

The rate of incidentally detected Hb variants has increased in all laboratories using HPLC to measure Hb A_{1c}. The presence of variants may invalidate the results of Hb A_{1c} analysis and could result in a missed diagnosis or a misdiagnosis of diabetes or mismanagement of a patient with diabetes. It is, therefore, imperative that a comment alerting the requesting clinician to the presence of the Hb variant is appended to the Hb A_{1c} result. We have correctly adopted this policy for the last 5 years, also for variants of very little significance such those of the δ -globin chain.

Two types of $\alpha\beta$ contact are defined in the Hb molecule. One is the $\alpha 1\beta 1$ (or $\alpha 2\beta 2$) contact involving B, G and H helices and the GH corner, and the other is the $\alpha 1\beta 2$ (or $\alpha 2\beta 1$) contact involving mainly helices C and G and the FG corner (12,13). When Hb A goes from the deoxy to the oxy configuration, the $\alpha 1\beta 2$ and $\alpha 2\beta 1$ contacts undergo the principal changes associated with cooperative oxygen binding, and these are named the sliding contacts. At the $\alpha 1\beta 1$ and $\alpha 2\beta 2$ interfaces, on the other hand, negligible changes are found insofar as the crystal structure has been examined. Consequently, these are called simply the packing contacts, and their role in Hb function was not clear for a very long period of time. More recently, many investigators have emphasized the key role of the $\alpha 1\beta 1$ (and $\alpha 2\beta 2$) interface(s) in stabilizing oxygen bound to Hb tetramer and identified a number of interactions that can play a role in establishing the communication of the dimers (14–17).

In their review, Sugawara *et al.* (17) effectively described the dual role of $\alpha 1\beta 1$ (and $\alpha 2\beta 2$) interface(s): one for stabilizing the HbO₂ tetramer against acidic autoxidation and the other for controlling the fate (removal) of its own erythrocyte from the blood circulation. The $\alpha 1\beta 1$ interface produces a conformational constraint in the constructed chains of the molecule *via* tilting of the distal (E7) histidine residues [$\alpha 58(\text{E}7)\text{His}$ in the α chain and $\beta 63(\text{E}7)\text{His}$ in the β chain]. In the latter role, it was shown that the $\alpha 1\beta 1$ (and $\alpha 2\beta 2$) interface(s) may exert delicate control of the tilting capability of the distal (E7) histidine residues leading to degradation of the Hb molecule to hemichrome, and subsequent Heinz-body clustering within the erythrocyte. In the

spleen, rigid intra-erythrocytic hemichrome inclusions (Heinz bodies) are known to act as 'sticking points,' hence Heinz body-containing red cells become trapped and undergo hemolysis.

Figure 4 shows the residues of α (31, 34–36, 105–123) and β -globin (30–40, 51, 110–128, 131) that are recognized to play an important role in maintaining the stability of the HbO₂ tetramer. To date, the Database of Human Hemoglobin Variants and Thalassemias (HbVar) has reported entries for a total of 1253 Hb structural variants, 870 of which involved the β -globin gene (18). From a search of the above mentioned database, it emerged that the variant we identified is so far unknown. We decided to name this new variant Hb Belluno for the place of residence of the carriers.

Only three other variants involving replacements at β 111(G13)Val have been described: Hb Stanmore [β 111(G13)Val→Ala, *HBB*: c.335T>C] (19,20), Hb Peterborough [β 111(G13)Val→Phe, *HBB*: c.334G>C] (21,22) and Hb Fannin-Lubbock II [β 111(G13)Val→Leu; β 119(GH2)Gly→Asp, *HBB*: c.[334G>C;359G>A] (23,24). Interestingly, as for Hb Belluno, even for the above-mentioned variants there is a variable degree of instability

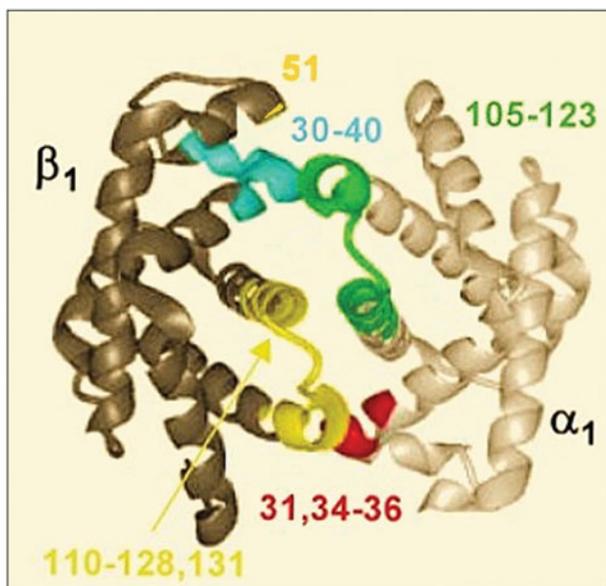


Figure 4. Schematic representation of a Hb $\alpha\beta$ dimer indicating the residues α 31, 34–36 (red), 105–123 (green) and β 30–40 (cyan), 51, 110–128, 131 (yellow). These residues are all located at the α 1 β 1 (or α 2 β 2) interface and are involved in stabilizing the Hb oxygen tetramer against acidic autoxidation [modified from Xu *et al.* (16)].

in vitro but with no clinical manifestations related with this anomaly (Table 3).

Indeed, the case of Hb Stanmore, initially described by Como *et al.* (19) was associated with thalassemia and was slightly anemic; later, Miyazaki *et al.* (20) reported one case of Hb Stanmore in a Japanese patient not suffering from thalassemia and without anemia. Hb Peterborough shows no significant clinical abnormalities in the cases described by King *et al.* (21) and by Nakanishi *et al.* (22). The Hb Fannin-Lubbock II variant was described by Qin *et al.* (23), according to whom it appears likely that the Val→Leu replacement at β 111, rather than the Gly→Asp replacement at β 119, is the cause of the instability of the variant, although in the original description by Schneider *et al.* (25), the Hb Fannin-Lubbock I [β 119(GH2)Gly→Asp, *HBB*: c.359G>A] was described as slightly unstable. The *in vitro* instability shared by all four β 111(G13)Val substitutions realized the importance that this amino acid takes in α 1 β 1 (and α 2 β 2) contacts and then in maintaining a stable form of oxygenated Hb tetramer.

For β -globin amino acid residues involved in the α 1 β 1 (and α 2 β 2) contact, only one other Val→Gly substitution is known, namely, Hb Dhonburi [β 126(H4)Val→Gly, *HBB*: c.380T>A], also known as Hb Neapolis and described in Italy by Pagano *et al.* (26) and in Thailand by Bardakdjian-Michau *et al.* (27). This mutation is electrophoretically and chromatographically silent and is responsible for a β -thalassemic phenotype. Even this mutation determines a Hb instability that appears to be due to the peculiar properties of glycine. Pagano *et al.* (26) explained that due to its small size and the absence of a large side chain, glycine is less hydrophobic than valine. The same investigators stated that the substitution of a good helix-former (valine) with a poor helix-former (glycine) cause a negative effect on the α helix formation and that factor weakens the internal bonding forces and increases molecular disorder, such as heat and organic solvents (*i.e.* isopropanol) can produce dramatic changes in the mutated molecule.

In support of the importance of the Val→Gly substitution is the fact that all other known substitutions of β 126(H4)Val, such as Hb Molfetta [β 126(H4)Val→Leu, *HBB*: c.379G>C] (28), Hb Beirut [β 126(H4)Val→Ala, *HBB*: c.380T>C] (29,30) and Hb Hofu [β 126(H4)Val→Glu, *HBB*: c.380T>A] (31,32) are clinically silent and shown a normal stability (except Hb Hofu, described as slightly unstable). After comparing the clinical and laboratory features of Hb Belluno with those of other β -globin variants involving β 111(G13)Val and the unique variant of α 1 β 1 (and α 2 β 2) contact with the same Val→Gly substitution, it seems

Table 3. Clinical and laboratory characteristic of the hemoglobin variants with a substitution at β 111(G13)Val.

Parameters	Hb Fannin-Lubbock II	Hb Peterborough	Hb Stanmore	Hb Belluno
Clinical presentation	normal	mild anemia	normal ^a	normal
Electrophoresis	does separate	no separation	no separation	no separation
Chromatography	does separate	not reported	no separation	no separation
Oxygen affinity	not reported	decreased	decreased	normal
Stability	unstable ^b	unstable	unstable	unstable

^aThe case described by Como *et al.* (19) was anemic and associated with thalassemia, whereas the case described by Miyazaki *et al.* (20) was not associated with thalassemia and was clinically silent.

^bAccording to Qin *et al.* (22), it appears likely that the Val→Leu substitution at β 111(G13), rather than the Gly→Asp substitution at β 119(GH2) is the cause of the instability of this variant.

important to return to another topic of discussion: the incidental finding of Hb variants.

The literature is full of reports of new Hb variants not identified during investigations for hematological abnormalities but rather during Hb A_{1c} assay by HPLC (33–49). As most of these new variants are clinically silent, it is possible that any number of them is not subject to being reported in the literature. This can happen because the chromatographic pattern exhibits subtle alterations that may escape a superficial examination or because it is considered of little interest to personnel responsible of the Hb A_{1c} assay, not necessarily informed of the possible implications caused by ‘insignificant alterations.’ We want to emphasize here that it is right to point out all new Hb variants, even those of little clinical interest, because it can help the scientific community to better understand the features of this unique protein, that does not end offering surprises despite a century of research.

Paradoxically, the variant described here would have escaped the single HPLC screening tests currently adopted by our laboratory for the detection of Hb variants, while it was identified thanks to a test not dedicated to this scope, such as that for the of Hb A_{1c} assay. In fact, obviously because of different buffers used by Tosoh G8 in the VARIANT and β -Thalassemia mode, an alteration was quite evident on the Hb A_{1c} chromatogram (Figure 1) but not detectable in the β -thalassemia mode. The splitting of the chromatographic peak from patient VA, observed with our old in-house method, helped us to convince on the presence of a Hb variant and also highlighted its poor reproducibility; a run time of 30 min. for each sample certainly allowed us to obtain an excellent separation of Hb fractions, but was strongly influenced by other conditions such as composition and pH of the elution buffers and irregular performances of the column.

In conclusion, the experience gained from the observation of Hb Belluno allowed us to realize two important messages. First, when the clinician suspects a Hb variant, it is not sufficient to get a negative response from an HPLC screening test to rule it out. As always, a constructive dialogue between clinician and pathologist is essential, involving the exchange of all available information and sharing a complete diagnostic work-up, including surveys to assess stability and oxygen affinity of the suspect Hb, as much as DNA sequencing in order to have unambiguous identification of the variant. Last but not least, the pathologist must specify the method used for the detection of variant Hb and, if the screening test in HPLC was negative, should not provide a definite answer as ‘hemoglobin variants absent’ but rather as ‘hemoglobin variants not shown with the method used for its detection (HPLC)’.

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Declaration of interest

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