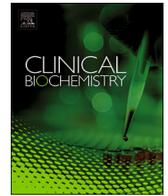




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## Case Report

Hb Vanvitelli: A new unstable  $\alpha$ -globin chain variant causes undiagnosed chronic haemolytic anaemia when co-inherited with deletion –  $\alpha^{3.7}$ .Maddalena Casale<sup>a,\*</sup>, Flora Cozzolino<sup>b,c,1</sup>, Saverio Scianguetta<sup>a</sup>, Piero Pucci<sup>b</sup>, Vittoria Monaco<sup>b</sup>, Gianmaria Sanchez<sup>a</sup>, Claudia Santoro<sup>a</sup>, Roberta Rubino<sup>a</sup>, Monica Cannata<sup>d</sup>, Silverio Perrotta<sup>a</sup><sup>a</sup> Dipartimento della Donna, del Bambino e di Chirurgia Generale e Specialistica, Università degli Studi della Campania Luigi Vanvitelli, Naples, Italy<sup>b</sup> Ceinge Biotecnologie Avanzate, Naples, Italy<sup>c</sup> Dipartimento di Scienze Chimiche, Università Federico II, Naples, Italy<sup>d</sup> Laboratorio per la Diagnosi Molecolare di Malattie Rare, Villa Sofia-Cervello Hospital, Palermo, Italy

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## ABSTRACT

Hb variants are structurally abnormal haemoglobins which can originate a wide range of phenotypes from clinically silent conditions to very severe disorders. In many cases, diagnosis is very difficult due to the instability of Hb mutants or the occurrence of misleading symptoms, such as cyanosis or hypoxia. Here we report the case of a young female with undiagnosed chronic haemolytic anaemia and low oxygen saturation in the absence of respiratory distress. High performance liquid chromatography showed the occurrence of an abnormal peak in the HbA2 region, which disappeared few days after blood sampling. Genetic analysis of both  $\alpha$  genes revealed the – $\alpha^{3.7}$  deletion in heterozygous state and a novel mutation c.130 T > C leading to the substitution of Phenylalanine at codon 43 with Leucine in the  $\alpha 1$  gene. This substitution originated a new Hb variant, named Hb Vanvitelli, with a molecular mass of  $15,092.2 \pm 0.4$  Da. Biochemical and laboratory tests described a hyper unstable Hb variant with altered oxygen affinity that was clinically significant only when co-inherited with genetic defects affecting the  $\alpha 2$  locus. This case highlights the genetic complexity and diagnostic pitfalls of Hb variants, defined “experiments of nature” which can generate severe clinical conditions.

## Background

Defects in synthesis and function of hemoglobin (Hb) are extremely widespread, affecting about 6% of global population and millions of people worldwide. Because of its functional role and genetic complexity, Hb disorders severely impact on human health with significant morbidity and mortality in affected patients [1]. Hb variants are structurally abnormal haemoglobins determining a wide range of clinical effects from silent to very severe disorders [2]. More than one thousand of Hb variants have already been described with about half of them being originated by defects in  $\alpha$  genes [3]. Identification of Hb variants is still challenging as often they are unstable and not detectable by the most common diagnostic procedures (electrophoresis or chromatography). Patients may then undergo several and expensive laboratory and instrumental tests before a correct diagnosis can be formulated [2]. Moreover, the presence of  $\alpha$  chain variants might result in a wide range of clinical features, from silent to severe phenotypes,

making the assessment of a correct diagnosis mandatory to allow genetic counselling and yield new insight in haematology practice.

Here we report the case of a hyper unstable Hb variant with altered oxygen affinity due to a novel mutation in the  $\alpha 1$  globin gene, causing chronic haemolytic anaemia when co-inherited with the – $\alpha^{3.7}$  deletion. Diagnostic pitfalls and clinical significance are also discussed.

## Case report.

A 14-year-old female from a little town near Naples, in Southern Italy, was admitted to our outpatient clinic for evaluation of her prolonged and undiagnosed anaemia. The girl was the second child of non-consanguineous parents. No issues were reported during pregnancy and delivery. There was no family history of hereditary haemolytic anaemia or any genetic disease. Personal history revealed recurrent abdominal pain and exertional dyspnoea. Clinical and laboratory evaluation following syncope at the age of 13 years revealed haemolytic anaemia (Hb 102 g/L, MCV 87.9 fL, Reticulocytes 9.45% =  $351,000 \times 10^6/\mu\text{L}$ , Total Bilirubin 44.29  $\mu\text{mol/L}$ , Indirect Bilirubin 38.13  $\mu\text{mol/L}$ , Haptoglobin

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0 g/L, LDH 659 U/L, Serum ferritin 352.78 pmol/L, Transferrin saturation 48%). Hepatosplenomegaly (longitudinal diameter of liver 130 mm, spleen 145 mm) was detected at abdomen ultrasound. Blood smear showed intense anisopoikilocytosis with normal white cells and platelets. Common tests for diagnosis of haemolytic anaemia, including direct and indirect antiglobulin test, cold agglutinins, osmotic fragility, high performance liquid chromatography (HPLC), antinuclear antibody (ANA), complement component 3 (C3) resulted normal. Infection due to hepatitis B and C virus and human immunodeficiency virus, red cell enzyme deficiency and paroxysmal cold haemoglobinuria were ruled out.

#### Laboratory Analyses: HPLC, Genetic Analysis, Hb variant detection.

Laboratory tests performed in our lab confirmed the haemolytic anaemia with hepatosplenomegaly, and low oxygen saturation (SpO<sub>2</sub>) at the pulse oximetry (86–88%). Oxygen saturation at co-oximetry on arterial blood (SaO<sub>2</sub>) was 78.9% (normal: > 95%) and partial pressure of oxygen (PaO<sub>2</sub>) was 42.6 mmHg (normal range: 80–100 mmHg) in the absence of respiratory distress. A new HPLC investigation was then performed, suspecting the occurrence of a Hb variant. The HPLC chromatogram showed HbF and HbA<sub>2</sub> values of 1.9% and 3.7% respectively and the presence of an abnormal peak coeluting in the HbA<sub>2</sub> region (Fig. 1). Venous P50 (oxygen tension when hemoglobin is 50% saturated with oxygen measured by venous gas parameters according to a mathematical formula [4,5]) was 30.37 mmHg (normal value 22–26 mmHg).

When the HPLC examination was again performed a few days after blood sampling, the abnormal peak in the HbA<sub>2</sub> area disappeared, confirming the occurrence of an unstable Hb variant. Following informed consent, a detailed investigation of the alpha and beta globin genes was carried out.

Genomic DNA was extracted from peripheral blood leukocytes using the Flexigene DNA Kit (Qiagen GmbH, Hilden, Germany). Complete coding and intronic sequences of HBA1 and HBA2 genes were amplified by polymerase chain reaction (PCR) as two long amplicons and nested primers were used to sequence both genes. Detailed methods and primer sequences are available on request. The PCR products were sequenced using the ABI 310 DNA Sequencer and the ABI PRISM Dye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems, Milan, Italy), according to the manufacturer's instructions.

No defects were observed in the analysis of the beta gene, whereas genetic analysis of the  $\alpha$  genes revealed the occurrence of a  $-\alpha^{3,7}$  deletion in heterozygous state and a novel mutation at codon 130 in the  $\alpha 1$  gene (c.130 T > C) originating the substitution of Phe43 with Leucine (Fig. 2). Genetic investigation of the other members of the family showed that the proband's mother was carrier of the  $-\alpha^{3,7}$  deletion in heterozygous state, while the father was heterozygous carrier of the new mutation c.130 T > C in the  $\alpha 1$  gene. Both brothers did not

display any mutation in the  $\alpha$  and  $\beta$  globin genes. Family pedigree and laboratory parameters are reported in Fig. 3.

The new Hb variant was characterised by a combination of HPLC chromatography and mass spectrometry. A red cell lysate from the patient was diluted 1:10 with 0.2% TFA and fractionated by reversed phase HPLC producing the chromatogram shown in Fig. 4. Individual globin peaks were collected and their accurate molecular weight was determined by ES-MS on a Q-TOF hybrid instrument (Waters, Milford, MA, USA) showing the occurrence of a normal  $\beta$ -globin (molecular mass of 15,867.5  $\pm$  0.7 Da) and the presence of two components in the  $\alpha$  globin peak, a normal  $\alpha$ -globin (molecular mass of 15,125.9  $\pm$  0.9 Da) and an anomalous alpha chain displaying a molecular mass of 15,092.2  $\pm$  0.4 Da, about 34 Da lower than the normal  $\alpha$  globin, indicating the occurrence of an  $\alpha$  chain variant. The alpha globin peak was digested with trypsin and an aliquot of the resulting peptide mixture was directly analyzed by MALDI-MS (ABI Sciex, Framingham, MA, USA) (Fig. 5). All the mass signals in the spectrum were mapped onto the anticipated sequence of the normal  $\alpha$  chain on the basis of their mass values. An anomalous mass signal occurred at  $m/z$  1799.9 and was tentatively assigned to the abnormal peptide 41–56 with a molecular mass decreased by 34 Da. The abnormal mass signal was then isolated in the mass spectrometer and submitted to tandem MALDI-MS-MS analysis producing the fragmentation spectrum shown in Fig. 6. The amino acid sequence of the abnormal peptide was determined by manual inspection of the spectrum as Thr-Tyr-Leu-Pro-His-Phe-Asp-Leu-Ser-His-Gly-Ser-Ala-Gln-Val-Lys, corresponding to the varied 41–56 peptide containing a Leucine residue at position 43 replacing the normally occurring Phenylalanine. The new abnormal Hb was then defined as c.130 T > C (p.Phe43Leu) variant and named Hb Vanvitelli. This variant shows the same amino acid replacement of Hb Hirosaki but detected in the  $\alpha 1$  gene.

#### Discussion

In case of chronic haemolytic anaemia, the occurrence of Hb variants should be suspected when the most common reasons for haemolysis have been ruled out [2]. Here we report the observation of a novel mutation in the  $\alpha 1$  globin gene, leading to the Phe43 - > Leu substitution, co-inherited with the most common deletion  $-\alpha^{3,7}$ . The combined defects determined chronic haemolytic anaemia and low SpO<sub>2</sub> at the pulse oximetry while the novel mutation was essentially asymptomatic in the heterozygous state showing only mild reticulocytosis and normal Hb value.

On the basis of biochemical and laboratory findings, this new Hb variant was described as a hyper unstable mutant. Hb stability is impaired by different recognized mechanisms, such as globin chain elongations, amino acid deletions, alterations of secondary structure, amino acid substitutions within the hydrophobic interior or within the heme pocket [6]. However, mutations involving heme pocket have been associated to multiple biochemical effects, including reduced cooperativity, high rate of haem loss, autooxidation and unfolding of the globin mutants [7]. Phenylalanine at codon 43 (at the CD1 helical region in the heme pocket) is one of the invariable residues within all alpha chains and plays a critical role in maintaining the heme in the proper position for interaction with the globin chains [8]. Substitution of this Phe residue results in highly unstable Hb variants with altered O<sub>2</sub> affinity [2]. The same amino acid substitution was reported for Hb Hirosaki (c.132C > G - p.Phe43Leu) [9] and Hb Sens (c.130 T > A - p.Phe43Ile) [8] both occurring in the  $\alpha 2$  gene. In Hb Torino (c.130 T > G - p.Phe43Val), Phe43 was replaced by Val but it is unclear whether the substitution occurred in the  $\alpha 1$  or  $\alpha 2$  gene [10]. Similar variants due to substitution of the Phe residue at the CD1 helical region in the heme pocket have been reported for the  $\beta$  chain in Hb Bruxelles [7,11], Hb Hammersmith [12] and Hb Buccaresti-Louisville [13,14]. In a significant proportion of these Hb variants, congenital haemolytic anaemia and cyanosis have been reported, confirming the multiple biochemical effects due to

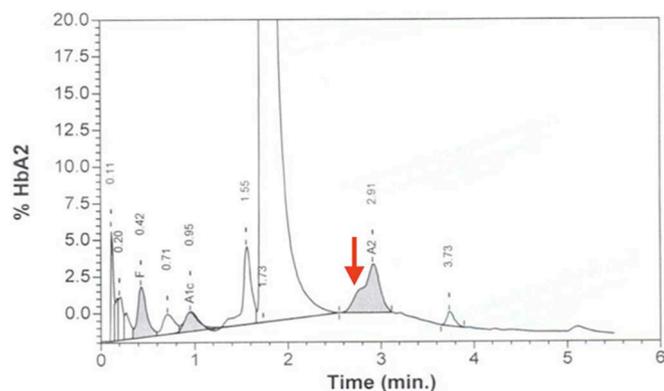


Fig. 1. HPLC of the patient's blood sample. The arrow indicates the abnormal peak.

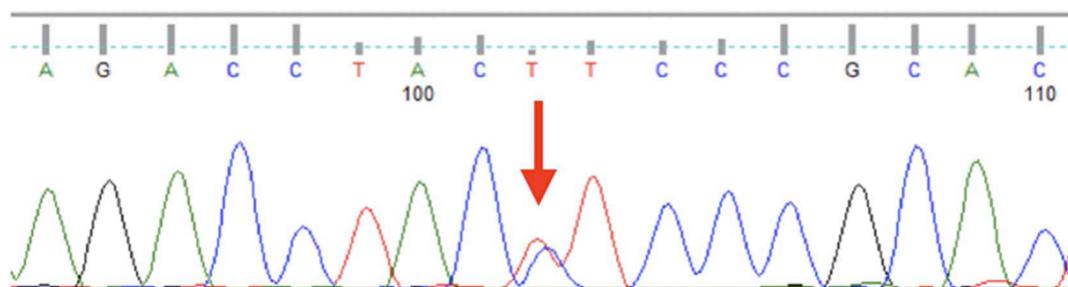


Fig. 2. DNA sequencing chromatogram showing the novel mutation in the  $\alpha 1$  gene.

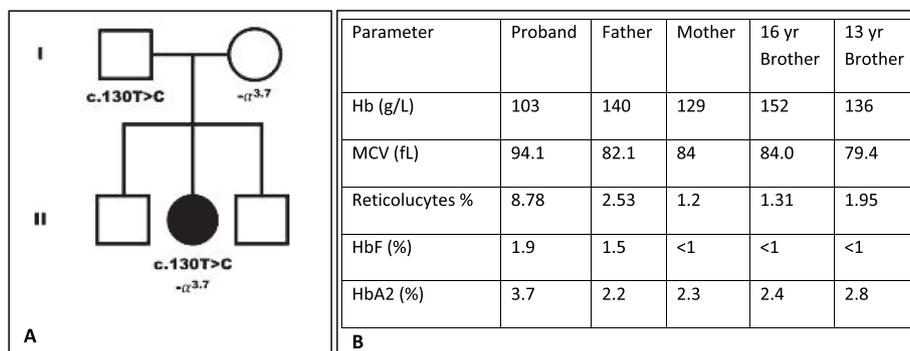


Fig. 3. Family Pedigree (A) and Blood Parameters (B).

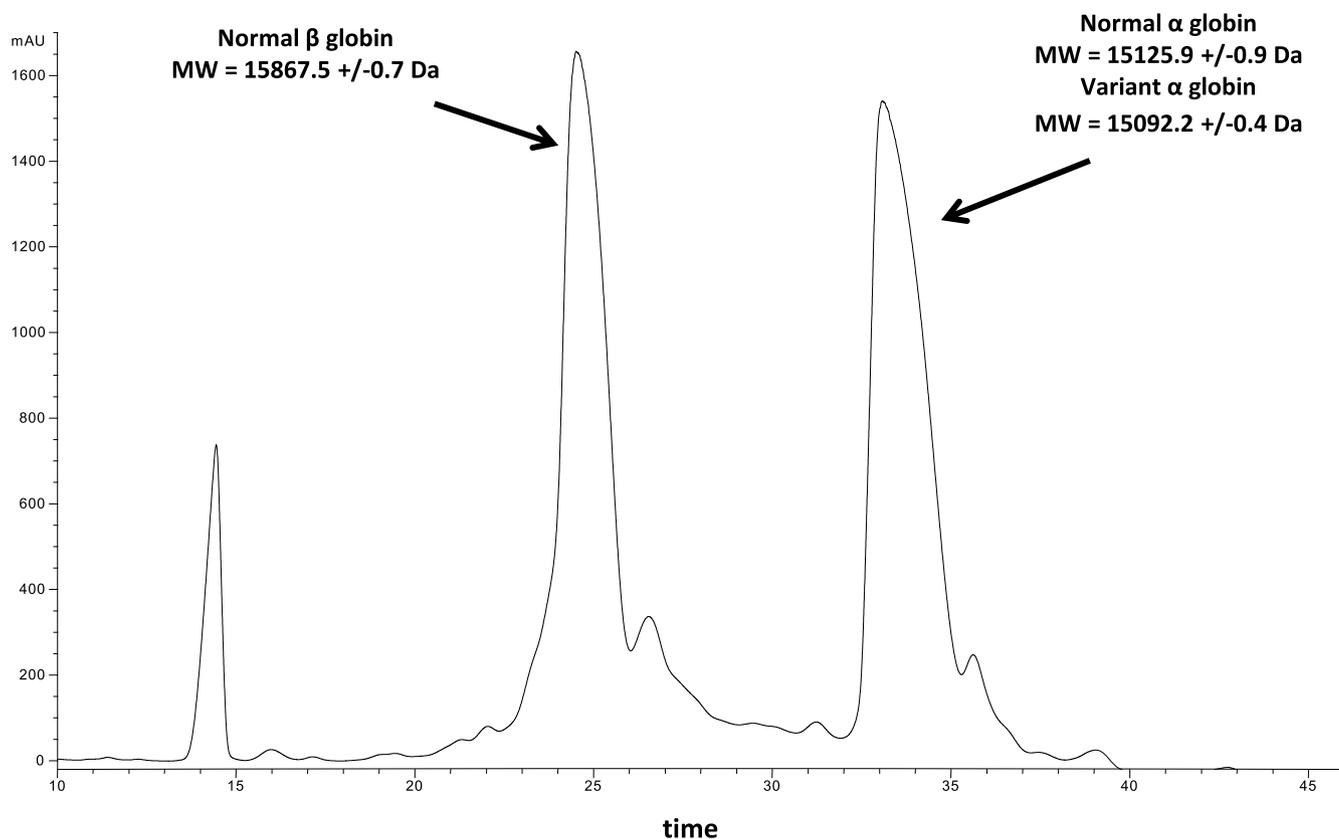


Fig. 4. Total ion current (TIC) profile of the LC-MS analysis of the proband's sample. The anomalous globin chain coeluted with the normal  $\alpha$  globin. The molecular mass of all the globins are indicated.

mutations involving the heme pocket [2].

The proband showed lip cyanosis and slate gray color of the skin, that are confusing and misleading symptoms of low O<sub>2</sub> affinity Hb variants [15]. The pulse oximetry revealed a low SpO<sub>2</sub> without

respiratory distress, suggesting the possible presence of a Hb variant, although the previously performed HPLC was normal. To date, 21 different Hb variants have been associated to low SpO<sub>2</sub> and a few of them had concordantly reduced SaO<sub>2</sub> and/or PaO<sub>2</sub> [16]. Pulse oximetry is a

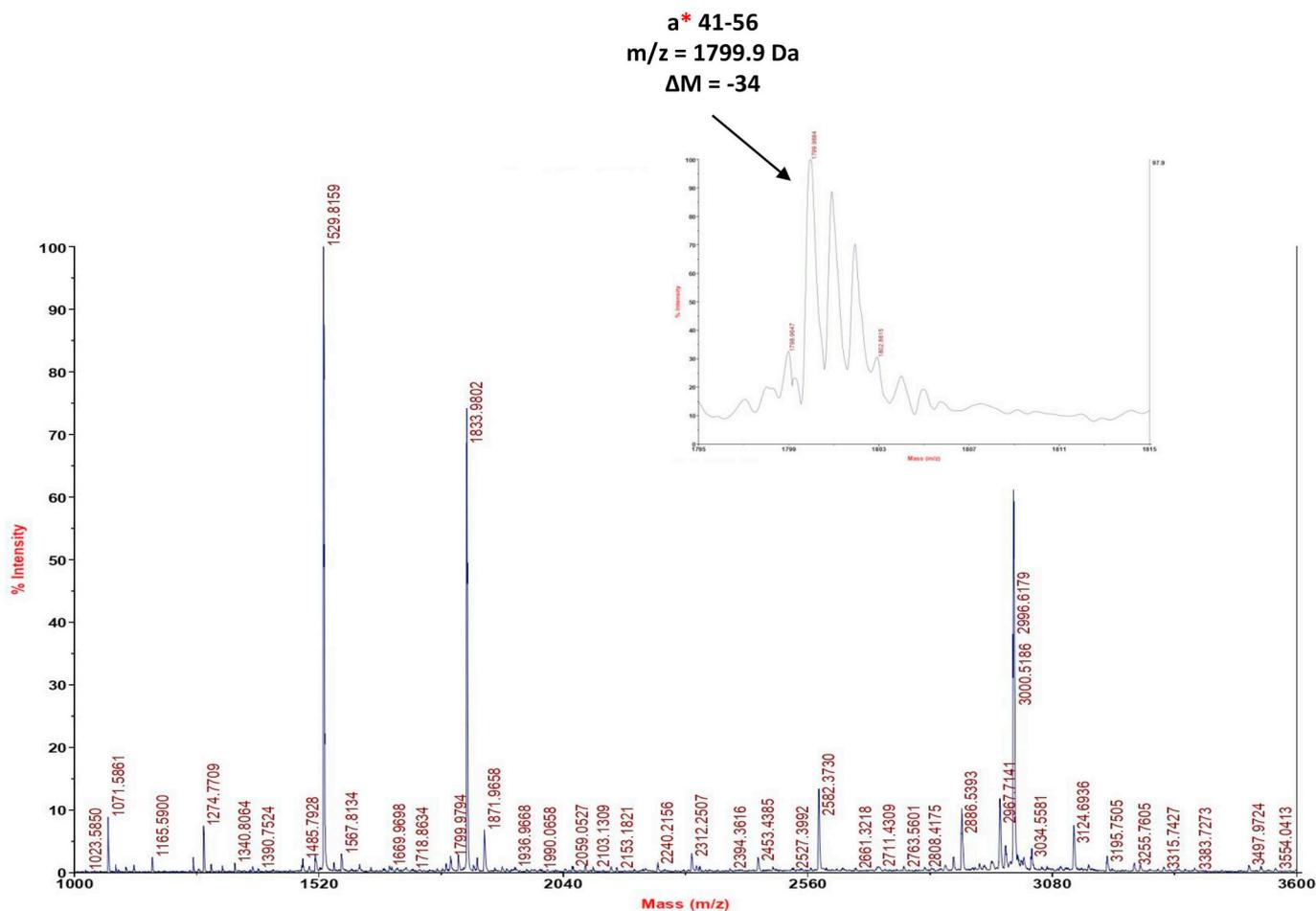


Fig. 5. MALDI-MS spectrum of the tryptic digest from the variant globin chain. The mass signal corresponding to the anomalous peptide is indicated in the insert.

non invasive spectrophotometric analysis to measure the percentage of oxy- and deoxyhemoglobin, based on their different light absorbance properties. Some Hb variants have a specific and unique absorption spectrum, closer to the light absorbance of deoxyhemoglobin, leading to the erroneous conclusion of increased deoxyHb [17–19]. Also arterial blood gas analysis (SaO<sub>2</sub> and PaO<sub>2</sub>) can be affected by the presence of low O<sub>2</sub> affinity Hb variants, as they alter the oxyhemoglobin dissociation curve and so invalidate the empirical calculation algorithm [16]. A correct diagnosis of Hb variants associated to low pulse oximetry measurement is convenient to avoid extensive cardio-pulmonary evaluation and hospital admissions to assess supposed hypoxemia and to allow a suitable monitoring of oxygenation during anesthesia and surgery [18,20]. However, in many cases, patients only received diagnosis after several and expensive diagnostic tests to rule out the causes of hypoxia [16].

Pitfalls in the diagnosis of hyper unstable Hb mutants have been reported, as they tend to precipitate shortly after synthesis and are not incorporated into Hb tetramers [2]. These ephemeral proteins are difficult to isolate making DNA sequencing a critical diagnostic test. Hb Hirosaki (c.132C > G - p.Phe43Leu) was discovered in a family with haemolytic anaemia [21,22] and DNA sequencing was used to characterize the mutation as several tests failed to identify a soluble variant Hb within erythrocytes.

The occurrence of an unstable variant in our proband was suspect by the abnormal shape of the HbA<sub>2</sub> peak in the HPLC analysis of a fresh blood sample; this peak disappeared when the analysis was performed few days after the sampling. We then recommend that HPLC analysis and tests for the assessment Hb variants should be performed soon after blood sampling, in order to increase the possibility to detect unstable

Hb variants. When separation of the globins was carried out, the alpha variant coeluted with the normal globin and could only be detected because of the high sensitivity of mass spectrometry. Assessment of the amino acid substitution was then performed by tandem mass spectrometry on the abnormal 41–56 peptide.

Hb Vanvitelli does not have dominant effect, as reported for Hb Torino, Hb Hirosaki and Hb Sens, but displays a pathological phenotype only when associated with other mutations in either of the two  $\alpha$  genes. This observation might be related to the different transcription level of the  $\alpha 1$  and  $\alpha 2$  globin genes. In physiological conditions, the  $\alpha 2$  mRNA level is higher than  $\alpha 1$  mRNA by approximately 3:1 during development, probably as a result of differences in gene transcription regulation. The  $\alpha 2$  gene encodes two- to threefold more protein than  $\alpha 1$  suggesting a more severe phenotype associated with  $\alpha$  globin mutations affecting the  $\alpha 2$  rather than the  $\alpha 1$  locus [23]. Hb Vanvitelli, affecting the  $\alpha 1$  globin gene, causes silent phenotype in the heterozygous state but relevant haemolytic anaemia when co-inherited with genetic defects occurring at the  $\alpha 2$  locus.

Highly variable clinical symptoms were reported among the members of the same family carrying Hb Hirosaki, ranging from silent carriers to severely anemic patients, without clear explanation of this discrepancy [22]. A detailed investigation of further defects occurring in  $\alpha$  genes in these patients might reveal detrimental effects due to possible additional mutations, as occurred in Hb Vanvitelli.

Little is known about possible therapeutic approaches to unstable Hb variants. Our patient is pale, jaundiced and complains recurrent abdominal pain due to bile sludge. She missed one year of school because of profound asthenia and exertional dyspnoea. Although the effect of splenectomy is known in different form of anemias [24–29], this

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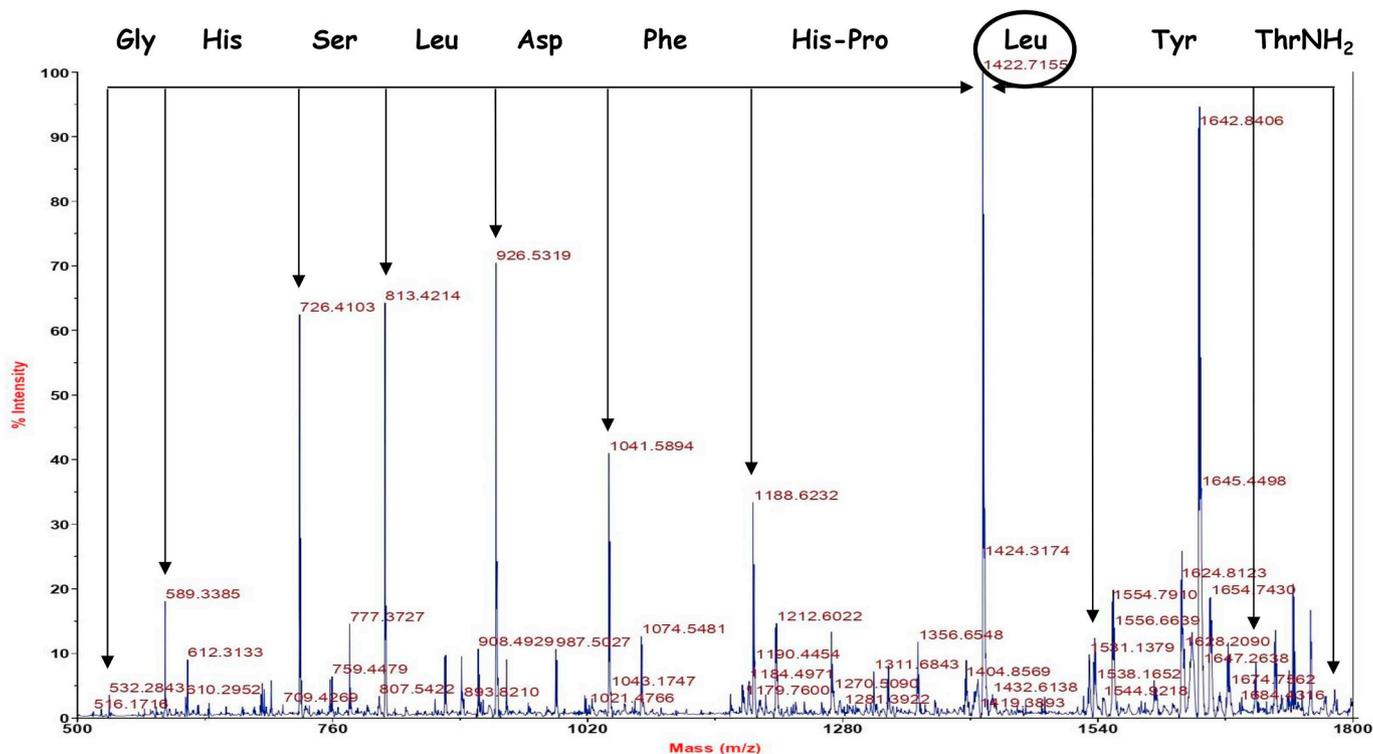


Fig. 6. Tandem MALDI-MS/MS spectrum of the mass signal at  $m/z$  1799.9 corresponding to the abnormal  $\alpha$  41–56 peptide. The interpretation of this spectrum revealed the occurrence of a Phe→Leu substitution at position 43 (circle).

procedure is reported just in very few patients affected by Hb variants with incomplete resolution of haemolysis and/or anaemia [22]. Hydroxyurea is a well-established therapeutic approach in different hemoglobinopathies, such as sickle cell disease and thalassemia syndromes [30–33], but the effect on unstable Hb variant is still unclear. Nowadays, Hb variants have widely been described in their structural features and biochemical functions, but a general and comprehensive therapeutic approach is far from being developed. Formation of an integrated network involving researchers and clinicians in charge of the day-to-day care of patients might then be essential for sharing knowledge on clinical evolution and long term management of these conditions, as observed in other haematological conditions [34].

In conclusion, we reported a new mutation in the  $\alpha$ 1 globin gene determining chronic haemolytic anaemia when inherited with the most common deletion  $-\alpha^{3.7}$ . A familiar non spherocytic anaemia should be assessed for unstable Hb variants when the most common forms of haemolysis have been ruled out. The heterogeneity of clinical symptoms among members of the same family should suggest the possible occurrence of an uncommon Hb variant co-inherited with other Hb defects. In case of unstable Hb variants, diagnostic tests should be performed soon after blood sampling to prevent variant degradation. More studies are needed to establish the impact of therapeutic procedures, such as splenectomy or hydroxyurea, on the treatment of unstable variant disorders.

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### Compliance with ethical standards

All procedures were performed in accordance with the principles of the Declaration of

Helsinki. Patient's parents signed informed consents. The Institutional Review Board considered this study dispensed from review due to the agreement of the patient's parents to publish the results by signing informed consent.

### Declaration of competing interest

The authors declare that they have no conflicts of interest.

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