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Diagnosis and clinical relevance of co-inheritance of haemoglobin D-Punjab/ β + -thalassemia traits in an immigrant Afghan family

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ABSTRACT

We report on a Pashtun family affected by haemoglobin D-Punjab/ β + -thalassemia to increase the awareness of the increasing prevalence of haemoglobinopathies among primary care physicians. We highlight the diagnostic approach of these conditions and the benefits of genetic counselling.

INTRODUCTION

Inherited disorders of haemoglobin (Hb) affect approximately 7% of the global population and represent a major health problem.¹ The incidence of haemoglobinopathies in previously non-endemic countries in Europe has been increasing steadily as a result of multi-ethnic immigration.^{2,3} Structural variants are caused by point mutations (eg, Hbs S, C, E, D-Punjab, O-Arab) or by unequal crossover events between the δ -globin and β -globin genes (Hb Lepore) that result in Hb molecules of varying stability and oxygen affinity. The thalassemias result from defective synthesis of the α -globin or β -globin chains that make up the tetrameric human Hbs and are encoded on chromosome 16 and on chromosome 11, respectively.⁴ The α -thalassemias are characterised by a decrease in the amount of alpha chains. This results in an excess of β -globin molecules that precipitate, and subsequently cause increased destruction of erythrocytes.¹ The degree of clinically manifest impairment depends on the number of α -globin genes affected. The β -thalassemias are phenotypically diverse, that is, from transfusion-dependent to asymptomatic microcytic hypochromic anaemia, as a result of an imbalance in α -globin and β -globin chain production. Synthesis of β -globin chain can be partially reduced (β +) or completely silenced (β 0). Because of the wide geographic distribution of β -globin chain defects that are inherited in homozygous or compound heterozygous combinations, the β -thalassemias constitute the most important global public health problem.³ We report on a Pashtun family affected by Hb D-Punjab/ β + -thalassemia, to increase the awareness of the increasing prevalence of haemoglobinopathies as a result of migration in multi-ethnic populations, to highlight the diagnostic approach of these conditions and the benefits of genetic counselling and to characterise the clinical relevance of our findings.

HAEMATOLOGICAL ANALYSIS OF AN AFGHAN FAMILY

A 38-year-old woman presented with for severe microcytic, hypochromic anaemia during the

second trimester of her eighth pregnancy. We found abnormal haemograms in her family members' blood samples that were obtained for unrelated conditions. We therefore analysed this non-consanguineous Pashtun family living in Belgium for haemoglobinopathies (table 1). The mother (patient 2) had an anaemia related to iron deficiency, but only minor response to oral iron supplementation. Automated capillary zone electrophoresis (CZE) was performed on the Minicap Flex Piercing CZE system (Sebia; Norcross, Georgia, USA) according to manufacturer's instructions and showed a variant elution pattern of Hb haemolysates, suggesting heterozygosity for a Hb D-Punjab Hb variant (table 1 and figure 1).

The 9-year-old son (patient 6) had mild anaemia with erythrocytosis (table 1) and a blood smear showing microcytic, hypochromic red blood cells, schistocytes, target cells and spherocytes. The CZE pattern showed Hb A 3.5%, Hb A2 4.7%, Hb F <0.5% and Hb D 91.8% (figure 1B). The high amount of the Hb D variant with a low fraction of Hb A in absence of a history of red blood cell transfusions suggested compound heterozygosity for Hb D/ β + -thalassemia.⁵

The father (patient 1) was not anaemic, but a marked erythrocytosis, microcytosis and hypochromasia with normal serum ferritin levels (82 μ g/L), a high amount of Hb A2 4.3% and absence of a Hb variant suggested a β -thalassemia trait (figure 1C). The other members of the family, that is, patients 3, 5, 8, 9 had a haemogram within the reference range and a normal profile at CZE, while patients 4 and 7 had a phenotype compatible with a heterozygosity for Hb D-Punjab and a beta-thalassaemia trait, respectively. Patient 6 had a phenotype compatible with a compound heterozygosity for Hb D/ β + -thalassemia.

We evaluated the red cell osmotic fragility for the family members of whom fresh EDTA was available (figure 2), using the 'naked eye single tube red cell osmotic fragility test' (NESTROFT), a low cost screening test for β -thalassaemia trait.^{4,6} Briefly, this test is based on the reduced osmotic fragility of red cells in β -thalassaemia, that results in persistent turbidity 20 min after suspending 20 μ L of fresh EDTA blood into a tube containing 4 mL of a 0.36% hypotonic saline solution. The tube is then held against a white paper with a thin black line. If the line is not visible or blurred, the test is considered positive, that is, reduced osmotic fragility.



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Table 1 Red blood cell analysis and ferritin levels of the family affected with Hb D and β^+ -thalassemia trait

Patient	Gender	Hb (g/L) (132–173)	RBC ($\times 10^{12}/L$) (3.80–5.10)	Ht (L/L)	MCH (pg) (27–31)	MCV (fL) (81–100)	RDW (%)	Reticulo (‰)	PLT	Ferritin ($\mu\text{g/L}$) (6–137)
1	M	134	7.08	0.429	19	60.5	13	17.32	402	82
2	F	77	3.97	0.249	19.4	62.8	16.4	34.76	358	8
3	F	130	4.57	0.383	28.3	83.8	12.4	16.22	349	13
4	F	139	5.31	0.406	26.2	76.5	12	12.19	367	8
5	M	139	5.17	0.418	26.8	80.9	12.9	11.81	318	32
6	M	115	6.98	359	16.5	51.4	12	16.65	410	28
7	F	112	6.6	0.369	17	55.8	11.9	18.12	363	27
8	M	125	4.86	0.367	25.7	75.6	11.3	8.74	445	ND
9	F	110	4.65	0.336	23.8	72.2	13.3	13.29	449	ND

F, female; Hb, haemoglobin; Ht, haematocrit; M, male; MCH, mean corpuscular haemoglobin; MCV, mean corpuscular volume; ND, not done; PLT, platelet count; RBC, red blood cells; RDW, red cell distribution width; reticulo, reticulocytes.

If the line is clearly visible through the contents of the tubes, the test is negative. Reduced osmotic fragility was detected in patients 1, 6 and 7.

The hemoglobinopathies that affect this family were characterised at a molecular level by massive parallel sequencing of the whole coding sequence of the HBB genes in the parental blood samples. We identified a heterozygous mutation HBB:c.364G>C (p.Glu122Gln), known as Hb D-Punjab in the mother. The father was found to be heterozygous for HBB:c.92+5G>C, a β^+ type mutation known as IVSI-5 (G>C). By extrapolation, we assigned the β -chain genotypes and Hb chain designations to the other members of the family (table 2).

DISCUSSION

Hb D-Punjab (also known as Hb D-Los Angeles) was first described in 1951.⁷ It is prevalent in Punjab region, North-west India (estimated frequency 2.0%), but it has also been reported in Italy, Belgium, Austria, Turkey, China and Brazil.⁸ Hb D is a stable Hb variant, with only mildly reduced oxygen affinity compared with Hb A.⁹ In classic electrophoresis of the Hb fractions, Hb D-Punjab migrates with Hb S under alkaline pH, and with Hb A under acidic pH.⁴ Modern automated high-throughput methods, such as high-performance liquid chromatography and Hb CZE identify clinically relevant Hb variants with approximately 100% sensitivity and specificity greater than 90%.⁴ Hb D-Punjab can be inherited in at least four different states: heterozygous, homozygous Hb D disease, or compound heterozygous states like Hb D-thalassemia (Hb D/ β^0 or β^+) and co-inheritance with other Hb variants. The more severe

co-inheritance is the association between Hb D-Punjab and Hb S that leads to similar clinical and haematological manifestations as in sickle cell anaemia. A couple at risk should be referred for counselling. Because Hb D-Punjab trait is clinically silent, we consider the CZE detection of the mother's Hb D-carrier status an incidental finding. As indicated by the low serum ferritin level, her microcytic anaemia was explained by iron depletion that showed only mild improvement in response to oral iron supplementation treatment during pregnancy.

The remarkably high Hb D fraction (91.8%) in patient 6 could be mistaken for homozygous Hb D disease, a condition that is not associated with haematological or clinical manifestations. A recent study suggested that a Hb D fraction greater than 92% can be used to discriminate homozygotes from Hb D-Punjab/ β -thalassemia double heterozygotes.¹⁰ However, the finding of hypochromic, microcytic red cell morphology with erythrocytosis in the presence of Hb A and an elevated Hb A2 fraction alerted us to the possible coinheritance of a β -thalassemia trait in our patient. The presence of a β^+ -thalassemia trait was confirmed by genetic testing of the father.

The prevalence of β -thalassemia in Afghanistan is probably high, but reliable epidemiological data are scarce. Based on a β -thalassemia syndrome prevalence of 3.8% among 369 outpatients, one study estimated a number of 1 to 1.5 million β -thalassemia carriers in Afghanistan.¹¹ The HBB:c.92+5G>C substitution is the most common β -thalassemia mutation in South Asia, with reported allelic frequencies of 64.6%, 56.3% and 36.5% in Sri Lanka, India and Pakistan, respectively.¹² An evolutionary explanation for the widespread distribution

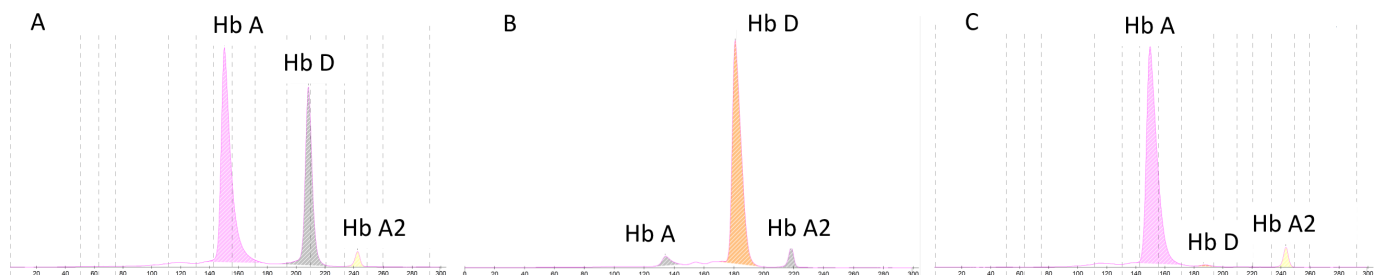


Figure 1 Elution pattern of hemolysates on the Minicap Flex Piercing CZE system (patients 2, 6 and 1). / Hemoglobin electrophoresis patterns of patient 2, patient 6 and patient 1 are shown. Normal values for the Hb-fractions on the Minicap Flex Piercing CZE system (Sebia; Norcross, GA, USA) are Hb A 96.8–97.8%; Hb A2 2.2–3.2%; and Hb F \leq 0.5%. Patient 2 (A), a Hb D carrier, has a decreased Hb A (60.8%), a normal Hb A2 (2.3%) and an additional Hb D fraction (36.9%). Patient 6 (B), a Hb D/ β^+ -thalassemia compound heterozygote, has a greatly decreased Hb A (3.5%), an increased Hb A2 (4.9%) and a greatly increased Hb D fraction (91.8%). Patient 1 (C) has a decreased Hb A (95.1%), an increased Hb A2 (4.3%) and a slightly increased for Hb F fraction (0.6%), a pattern compatible with β -thalassemia trait.



Figure 2 'naked eye single tube red cell osmotic fragility test (NESTROFT)' for patients 1, 3, and 5 through 9. / The NESTROFT is based on the reduced osmotic fragility of red cells in β -thalassaemia. Twenty microliters of fresh EDTA blood is suspended into a tube containing 4 mL of a 0.36% hypotonic saline solution. After 20 minutes the tube is held against a white paper with a thin black line. If osmotic fragility of the red cells is reduced, turbidity persists and the line is not visible or blurred (a positive test result). If the line is clearly visible through the contents of the tubes, the test is negative. Reduced osmotic fragility was detected in patients no. 1, 6 and 7. No fresh EDTA was available for patient 2 and patient 4.

of β -thalassaemia in the WHO Eastern Mediterranean Region remains elusive. Although Haldane postulated that heterozygosity for β -thalassaemia protects against severe falciparum malaria in 1949, this hypothesis remains to be evaluated in prospective clinical studies.^{13 14} However, persistence of thalassaemia genes in many populations can largely be attributed to consanguinity, a known risk factor for all recessive genetic disorders. The proportion of consanguineous marriages among populations of Afghanistan was found to be as high as 46.2%.¹⁵

So, what is the clinical relevance of diagnosing the Hb D-Punjab and β^+ -thalassaemia traits? Hb D in a heterozygous or even in a homozygous state is asymptomatic. Although compound heterozygote Hb D/ β -thalassaemia cases that needed blood transfusions have been reported, Basmanj *et al* did not observe profoundly anaemic or transfusion dependent Hb D/ β -thalassaemia compound heterozygotes after screening more than 8000 β -thalassaemia carriers.¹⁶ In a recent practice guideline, compound heterozygote Hb D/ β -thalassaemia was reported as not clinically relevant, and no prenatal diagnosis is warranted.¹⁷ As outlined in the introduction, all β -thalassaemia traits can be inherited in deleterious homozygous or compound heterozygous combinations.³ Therefore, premarital or antenatal screening for these haemoglobinopathies is important. In chronic microcytic

anaemia, distinguishing β -thalassaemia trait from iron deficiency is important to avoid unnecessary and potentially harmful iron treatment. In addition, a study from Sri Lanka found that people with β -thalassaemia trait had symptoms suggestive of anaemia, including lethargy, fatigue and dizziness compared with normal subjects, and they visited their physicians more frequently.¹⁸ Surprisingly, the latter study also found that patients with β -thalassaemia trait experienced significantly more fever episodes that necessitated medical attention than controls.¹⁸

In many countries with high carrier rates, nationwide screening programmes for the hemoglobinopathies have been established.³ In 2014, a pan-European expert consensus recommended newborn screening in Europe to target only sickle cell disease, that is, Hb S.¹⁹ In previously non-endemic countries with high rates of multi-ethnic immigration antenatal screening programmes may be absent. In these countries, primary care professionals can play an important role in the identification of individuals and families at risk by detailed family medical history taking and by offering screening and genetic counselling. A high index of suspicion is required to differentiate between thalassaemias and iron deficiency anaemia (IDA), especially when microcytic anaemia is accompanied by normal iron studies. Many mathematical indices have been used to discriminate thalassaemia

Table 2 Haemoglobin fractions, β -globin genotypes and clinical phenotypes in a family affected with Hb D and β^+ -thalassaemia trait

Patient	Gender	Hb A (%)	Hb A 2 (%)	Hb F (%)	Hb D (%)	β -globin genotype	Clinical phenotype
1	M	95.1	4.3	0.6	N	c.92+5G>C/normal	β -thalassaemia trait
2	F	60.8	2.3	<0.5	36.9	Normal/c.364G>C	Hb D-carrier
3	F	97.5	2.5	<0.5	N	Normal/normal	Normal
4	F	58.4	2.7	0.5	38.9	Normal/c.364G>C*	Hb D-carrier
5	M	97.5	2.5	<0.5	N	Normal/normal	Normal
6	M	3.5	4.7	<0.5	91.8	c.92+5G>C/c.364G>C*	Hb D/ β -thalassaemia trait
7	F	96	4	<0.5	N	c.92+5G>C/normal*	β -thalassaemia trait
8	M	96.9	2.8	0.3	N	Normal/normal	Normal
9	F	97.0	2.6	0.4	N	Normal/normal	Normal

Hb fractions on the Minicap Flex Piercing CZE system, β -globin genotypes and clinical phenotypes are presented. N denotes absent.

*Extrapolated genotype.

CZE, capillary zone electrophoresis.

trait from IDA (eg, Mentzer's index that suggests mean corpuscular volume/red blood cell <13 for thalassemia and >14 for IDA).²⁰ A meta-analysis found that although these indices lack diagnostic accuracy to confirm thalassemia, they are valuable for the identification of subjects in whom further diagnostic testing is indicated, especially when combined with other factors, such as age, ethnicity or family history.²⁰ In resource limited countries with high prevalence of thalassemias, red cell osmotic fragility testing is used for population screening.⁴ For the NESTROFT test we used, Singh *et al* reported 97.7% sensitivity and 83.3% specificity for the detection of β -thalassemia trait.⁶ As stated above, modern laboratories use red cell morphology and automated high-throughput methods for the routine diagnosis of hemoglobinopathies.⁴

Molecular diagnosis of hereditary Hb disorders is only mandatory for antenatal diagnosis. It can further be employed for confirmation, prognosis or treatment of putative haemoglobinopathies that may harm the patient or his/her offspring, or as was done in this case series, for a phenotype–genotype correlation.

CONCLUSION

We described an immigrant Pashtun family affected by Hb D Punjab/ β +thalassemia. Following migratory flows, this case highlights the variety of haemoglobinopathies that general practitioners in non-endemic countries may encounter. Basic diagnostic tests such as a complete blood count, red cell indices and morphology, complemented by (automated) separation and measurement of Hb fractions assist primary care physicians in identifying common traits. For rare variants, complex cases and antenatal risk assessment, consultation and collaboration with an experienced reference laboratory specialised in molecular analysis is encouraged.

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