Assessing the suitability of non-molecular methods for screening beta-thalassemia carriers: clinical insights from laboratory data

Morena Sortino[1], Mladen Trbos[2], Rossella Tomaiuolo[1,3], Michela Tassara[4], Alessandra Di Cesare[2], Chiara Di Resta[1], Giuseppe Banfi[1,3], Massimo Locatelli[2]

[1]Vita-Salute San Raffaele University, Milan, Italy
[2]Laboratory Medicine Service, IRCCS Ospedale San Raffaele, Milan, Italy
[3]IRCCS Ospedale Galeazzi Sant'Ambrogio, Milan, Italy
[4]Immunohematology and Transfusion Medicine, IRCCS Ospedale San Raffaele, Milan, Italy

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ABSTRACT

Introduction: this study evaluates non-molecular methods for detecting beta-thalassemia carriers using data collected over six months at IRCCS Ospedale San Raffaele. Given the prevalence of beta-thalassemia in regions with significant migratory flows, the study emphasizes the need for continuous epidemiological monitoring and appropriate genetic testing.
Methods: hemoglobin patterns were analyzed using capillary electrophoresis in 1684 patients and compared with complete blood counts and biochemical tests.
Results: the study revealed pathological hemoglobin patterns in 13% of cases. The aim was to determine the prevalence of various hemoglobinopathies, specifically beta-thalassemia and the beta-thalassemic trait.
Discussion and Conclusion: while non-molecular methods are cost-effective for initial screening, they require supplementation with detailed clinical assessments and molecular tests to improve diagnostic accuracy. This approach is crucial for regions with high prevalence and migratory influences, emphasizing the need for updated national health registries and standardized international screening practices.

Key Words**:** *hemoglobinopathies, thalassemia, hematology*

INTRODUCTION

The term "hemoglobinopathies" refers to a group of hereditary disorders affecting red blood cells caused by gene mutations encoding the synthesis of the globin chains of hemoglobin (1). They can be categorized into thalassemias and hemoglobin variants, giving rise to clinical conditions ranging from mild hypochromic anemia to severe transfusion-dependent anemia (2). Structural hemoglobin variants result from amino acid substitutions within the globin chains, whereas thalassemias are characterized by a reduced or absent synthesis of these chains (3). Globally, 5% to 7% of population is estimated to carry a hemoglobin defect (4).

Among hemoglobinopathies, sickle cell anemia is prevalent, with a carrier rate of 2.1% approximately (5) and a higher distribution in Central America, South America, the Arabian Peninsula, the Middle East, India, and the Eastern Mediterranean (6).

Thalassemia carriers represent about 1.5% of the global population, mainly concentrated in North Africa, the Mediterranean region, India, and Southeast Asia (7,8).

In Italy, the interaction of high native prevalence rates, particularly in the southern regions, islands, and the Po Delta (9), with ongoing substantial migratory flows from Africa, the Balkans, and the Middle East (10), complicates epidemiological tracking. According to the latest estimates presented in 2019 by the National Registry of Thalassemia and other Hemoglobinopathies, established

Corresponding author: Morena Sortino, Vita-Salute San Raffaele University, Via Olgettina, 58, Milan, Italy - Email: sortino.morena@hsr.it

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at the National Blood Center, approximately 6337 cases of thalassemia and 2023 cases of hemoglobinopathy were identified in Italy, including 1275 with sickle cell anemia (11).

As a result, the scarcity of updated and reliable epidemiological data makes it more challenging to allocate resources for the management and follow-up of patients (6).

First-level methods, including complete blood count, iron status assessment, and hemoglobin pattern analysis, are cost-effective and widely accessible for diagnosing hemoglobinopathies. Second-line genetic testing may be reserved for selected individuals since these conditions are among the few genetic diseases where carrier screening can be predominantly conducted using low-cost laboratory methods (12).

To emphasize the importance of continuous epidemiological monitoring and the appropriateness of requests for genetic and molecular testing, this study aims to assess the diagnostic effectiveness of non-molecular methodologies in detecting beta-thalassemia and beta-thalassemic trait (BTT) carriers over six months at the IRCCS Ospedale San Raffaele (Milan) (Figure 1). By analyzing hemoglobin patterns using capillary electrophoresis and correlating these findings with comprehensive blood counts and biochemical tests, this study seeks to identify the prevalence of different types of hemoglobinopathies and evaluate the appropriateness of these methods in routine clinical practice. This approach could contribute to more informed and effective screening and management strategies in populations at risk.

METHODS

The population consisted of 1186 women and 498 men, categorized into three age groups: ≤14 years, 15-49 years (fertile age according to WHO), and ≥50 years.

The average age was 38 years. The subpopulation aged ≤14 years consisted of 77 subjects, including 40 females and 37 males; subjects aged between 15 and 49 years were 1444, with 1076 females and 368 males; finally, the population aged ≥50 years included 163 subjects (70 females and 93 males).

The data were retrospectively collected using the consultation function on DNLab, the Laboratory Information System (LIS) of IRCCS Ospedale San Raffaele. To select samples with a request of a hemoglobin pattern analysis, the "acceptance date" filter was applied; it corresponds to the day when the request was taken into consideration. Six months were chosen from January 01, 2022, to June 30, 2022. The search yielded 1687 results. Two entries were excluded because the examination was not performed: a further collection was requested due to a technical issue, or the sample did not reach the laboratory. A third entry, corresponding to an External Quality Assessment (EQA), was omitted. Therefore, the total number of considered samples was 1684.

The analysis was performed on the Capyllaris 2 Flex Piercing system (Sebia, France), a capillary electrophoresis. Values outside the reference ranges (HbA 96-99%, HbA_2 2-3.2%, and HbF 0-1%) were considered pathological. Furthermore, for women of childbearing age with high levels of HbF, the presence of pregnancy was verified through research in electronic medical records.

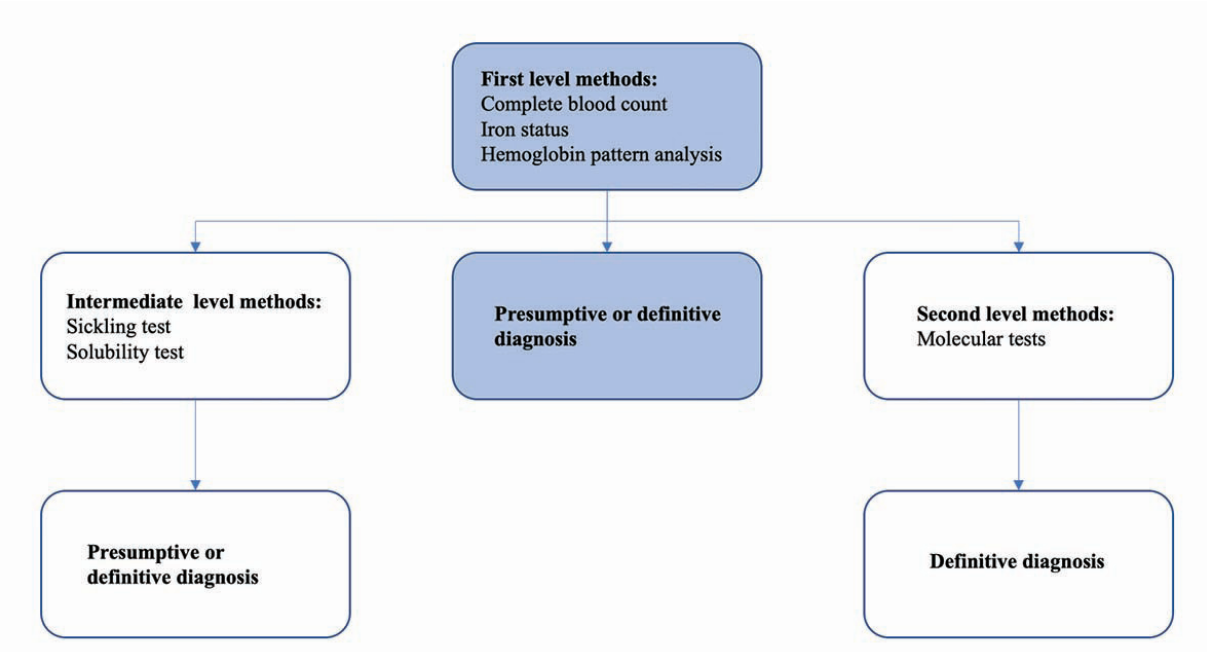

Figure 1
Diagnostic workflow for hemoglobinopathies (modified from reference 13). This study focuses on the first analysis level, highlighted in blue.

Moreover, for samples with suspected beta-thalassemia or BTT, a complete blood count (CBC), including hemoglobin (Hb), red blood cell (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), red cell distribution width (RDW), and reticulocytes, was investigated. However, this additional test was not available for all subjects. The CBC and reticulocytes were analyzed using flow cytometry on the Sysmex XN 9000-3000 platform (Sysmex, Germany).

Furthermore, iron, ferritin, transferrin (TRF), vitamin B12, and folate were evaluated. These parameters were analyzed with the Cobas e801 analyzer (Roche, Switzerland) using methodologies such as colorimetry for iron, electrochemiluminescence (ECLIA) for ferritin, vitamin B12, and folate, and immunoturbidimetry for transferrin.

It has been hypothesized that thalassemia carriers (or thalassemic patients) can be differentiated from non-thalassemic patients based on their respective HbA_2 values. To test this hypothesis, a clinical hematologist reviewed patients' clinical documentation and laboratory data in the electronic medical records (GALILEO-E-Health Solution, Noemalife), distinguishing likely thalassemia carriers from the other patients.

The criteria used to identify thalassemia carriers included:

- comparison of the patient's iron status and mean corpuscular volume (MCV): regular or abundant iron status with reduced or significantly reduced MCV;
- MCV <70 fL;
- absence of reactive thrombocytosis;
- persistent reduction in MCV over time, especially in patients treated with iron supplements;
- absence of anamnesis or clinical-laboratory conditions suggestive of iron deficiency anemia;
- pre-existing documentation in the anamnesis of thalassemia trait;
- positive family history;
- ethnic/geographical origin of the patients;
- presence of a confirmatory genetic test performed subsequently.

This approach aims to improve the differentiation between thalassemia carriers and non-thalassemic individuals based on the outlined hematological and clinical criteria.

The goal was to assess whether a significant difference in HbA_2 values exists between these two subpopulations.

For the differential analysis between groups, we employed Welch's t-test due to significant differences in the number of subjects per group. This approach allowed us cope with the heterogeneity of sample sizes.

RESULTS

No alterations in hemoglobin pattern analysis tests were found in 87% of cases (1463/1684); however, pathological results were observed in 13% of the studied population (221/1684). Table 1 reports the percentages of pathological hemoglobin identified.

Of the 146 (66.2%) samples presumably indicative of thalassemia, 28 showed increased HbF, 87 had only HbA_2 above the normal range, and 31 samples exhibited an increase in both proteins, with one showing a hemoglobin variant in the F zone.

In addition, it was observed that out of the 59 patients with elevated levels of HbF, six were under the age of 2 and five were women with documented pregnancies.

Among the 146 samples that exhibited abnormal levels of HbF and HbA_2, CBC and levels of iron, ferritin, transferrin, vitamin B12, folate and reticulocytes were also evaluated. Of these, 79 had no results for the considered parameters, and 67 showed only some of these results (Tables 2 and 3). In particular, 62 CBC, 21 iron and ferritin assays, 18 transferrin assays, 15 reticulocyte counts, 12 folate assays, 10 vitamin B12 determinations were requested.

From the evaluation of the clinical records, it emerged that 17 patients had a positive history of thalassemia, while no information was reported for the remaining 50 subjects. The mean (SD) HbA_2 value for the group with confirmed thalassemic trait was 4.49 (0.89), whereas the mean for subjects without reported anamnestic data was 4.46 (1.26). Consequently, no statistically significant difference was detected between the two groups (p=0.46). Despite individual variations, the lack of a substantial difference between the two groups suggests that the HbA_2 level alone may not be sufficiently discriminatory to determine the thalassemia carrier status without additional contextual information or supporting tests. The closeness of the mean values confirms that the HbA_2 test can be used as an initial screening tool but should be interpreted cautiously and in conjunction with further clinical and anamnestic evaluations for an accurate diagnosis.

On the contrary, using clinical criteria resulted in a different distribution of the population (37 subjects with a compatible profile with thalassemia or thalassemic trait; 30 subjects with an incompatible or doubtful profile) and a statistically significant difference in the means (SD) of the two groups, 5.09 (0.68) and 3.70 (1.21) respectively

Table 1
Distribution of pathological hemoglobin detected in the study population

Percentages (absolute frequencies)	Results
66.2% (146)	thalassemia major or β thalassemic trait
24.4% (54)	HbS
4.0% (9)	HbSC
1.8% (4)	HbC
1.3% (3)	HbE
1.3% (3)	variant in the HbF zone
0.5% (1)	J-Rovigo variant
0.5% (1)	HbH

Table 2
Complete blood count and iron status in subjects with thalassemic characteristics

CATEGORIES	PARAMETERS	HB (g/dL)	RBC (10^{12}/L)	MCV (fL)	MCH (pg)	RDW (%)	FERRITIN (ng/mL)	TRANSFERRIN (g/dL)	IRON (μg/dL)
All (37)	MEDIAN	11.2	5.6	63.8	19.8	18.4	64.5	2.47	79
	MEAN	11	5.5	64.6	20.5	18.6	205.7	2.52	85.8
	MIN	8.2	3.2	52.5	15.3	15.4	10	1.54	28
	MAX	13.3	6.9	82.7	31.1	24.9	1970	3.46	159
>2 years old (29)	MEDIAN	11.4	5.7	63.9	19.9	18.2	115	2.44	96
	MEAN	11.2	5.6	64.9	20.6	18.4	340.9	2.4	89.7
	MIN	8.2	3.4	52.5	15.3	15.4	10	1.54	28
	MAX	13.3	6.9	80.2	31.1	24.9	1970	3.34	159
Pregnant (3)	MEDIAN	9.4	4.4	67.1	21.2	18.2	NA	NA	117
	MEAN	9.8	4.5	71.3	22.3	18.5	NA	NA	117
	MIN	8.4	3.2	64	19.8	17.2	NA	NA	117
	MAX	11.7	5.9	82.7	26	20.1	NA	NA	117
≤2 years old (5)	MEDIAN	10.3	5.6	59.4	18.6	19.3	54	2.8	67.5
	MEAN	10.4	5.7	59.4	18.4	19.3	46.6	2.9	62.3
	MIN	9.6	5.3	56.2	17.1	17.7	23	2.5	35
	MAX	11.3	6.1	63.9	19.6	20.4	65	3.5	79

Table 3
Complete blood count and iron status in subjects with incompatible or doubtful profiles.

CATEGORIES	PARAMETERS	HB (g/dL)	RBC (1012/L)	MCV (fL)	MCH (pg)	RDW (%)	FERRITIN (ng/mL)	TRANSFERRIN (g/dL)	IRON (μg/dL)
All (26)	MEDIAN	12.3	5.1	77.8	23.7	16.8	88	2.4	78
	MEAN	12.1	5	77	24.9	16.7	326.4	2.5	315.9
	MIN	8.7	3.3	51.2	14.9	11.9	4	1.59	24
	MAX	15.5	6.9	95.3	32.3	25.8	1766	3.32	2323
>2 years old (20)	MEDIAN	12.4	5.2	77.8	23.7	16.8	307.5	2.12	78
	MEAN	12.4	5	77.4	25.1	16.5	515.5	2.12	459.2
	MIN	8.7	3.3	59.6	18.4	11.9	32	1.6	36
	MAX	15.5	6.9	95.3	32.3	25.8	1766	2.5	2323
Pregnant (3)	MEDIAN	12.3	4.2	88.5	29.4	14.8	40	3.1	153.5
	MEAN	12.1	4.5	83.3	27.3	14.9	104	3.1	153.5
	MIN	11.2	4.2	69.1	21.8	13	36	3.1	128
	MAX	12.8	5.1	92.4	30.8	16.9	236	3.1	179
≤2 years old (3)	MEDIAN	11	5.3	68.3	21.1	20.6	11	3.1	48.5
	MEAN	10.5	5.1	68.4	21.6	19.6	44.7	3.1	48.5
	MIN	9.3	3.8	51.2	14.9	13	4	2.8	24
	MAX	11.1	6.2	85.8	28.9	25.2	119	3.3	73

($p=6.29\text{-}^{07}$).

The significant difference in means and variability of HbA_2 levels between the two groups underscores the importance of using robust clinical criteria to assess BTT.

In addition, the means (SD) for subjects with a profile highly suggestive of beta-thalassemia and those with a doubtful profile within the three groups (pregnant women, children under 2 years of age, and adults) are as follows: 4.87 (0.57) *versus* 2.93 (1.1); 5.34 (0.50) *versus* 2.63 (0.93); 5.07 (0.72) *versus* 3.93 (1.17) (Tables 4 and 5).

Patients with a clear and compatible clinical profile for BTT show higher and more consistent levels of HbA_2, supporting their use as diagnostic indicators in the context of BTT screening.

The variability in HbA_2 levels in the group with an uncertain or incompatible profile could complicate the diagnosis and management of these patients, highlighting the need for more detailed diagnostic approaches or further investigations to confirm or rule out BTT.

DISCUSSION

The results highlight the diversity of hemoglobinopathies within the studied population. Pathological findings were observed in 13% of cases.

For the group that exhibited HbA_2 and HbF levels beyond the reference range, hematological indices, iron status, vitamin B12, and folate levels were evaluated, along with anamnestic data, to verify their correspondence with the most characteristic thalassemic profiles (14–16). Out of the total subjects included in the study, approximately 1.2% (17 subjects) had a thalassemic/BTT profile in their clinical history. This percentage increases to 2.5% (37 subjects) when classified on the basis of both anamnestic and laboratory data. In both cases, however, the data confirm the prevalence reported globally and nationally in other studies (7,8,17).

Table 4
HbA, HbA_2, HbF percentages in subjects >2 years old, pregnant women and children ≤2 years old with thalassemic characteristics.

HB	PARAMETERS	All (37)	>2 years old (29)	Pregnant (3)	≤2 years old (5)
HBA	MEDIAN	93.9	94.2	92.6	91.8
	MEAN	92.4	92.7	91.3	91.1
	MIN	59.6	59.6	87.9	87.1
	MAX	95.4	95.4	93.4	93.6
HbA_2	MEDIAN	5.3	5.1	4.7	5.5
	MEAN	5.1	5.1	4.9	5.3
	MIN	2.9	2.9	4.4	4.5
	MAX	6.1	6.1	5.5	5.8
HBF	MEDIAN	0.6	0	1.9	2.9
	MEAN	2.5	2.2	3.8	3.5
	MIN	0	0	1.9	0.6
	MAX	37.5	37.5	7.7	8.4

Table 5
HbA, HbA_2, HbF percentages in subjects >2 years old, pregnant women and children ≤2 years old with incompatible or doubtful profiles.

HB	PARAMETERS	All (30)	>2 years old (24)	Pregnant (3)	≤2 years old (3)
HBA	MEDIAN	95	95	95.8	95.5
	MEAN	94	94.2	94.5	92.1
	MIN	84.6	86.5	91.8	84.6
	MAX	96.4	96.4	96	96.3
HbA_2	MEDIAN	4.1	4.2	2.3	2.2
	MEAN	3.7	3.9	2.9	2.6
	MIN	1.9	1.9	2.3	2
	MAX	5.7	5.7	4.2	3.7
HBF	MEDIAN	1.05	1	1.7	1.5
	MEAN	2.3	1.9	2.5	5.2
	MIN	0	0	0	0.8
	MAX	13.4	11.1	5.9	13.4

The percentage of HbA_2 is significantly higher in the group with thalassemic characteristics only when the grouping is based on hematological parameters. This is likely because most subjects in the study are outpatients for whom clinical information is lacking, making classification errors more probable. Specifically, looking at the mean HbA_2 in the thalassemia/BTT group and that in the group with an incompatible or doubtful profile, it is clear that the first group could be defined with reasonable certainty, emphasizing the need for further evaluations in the second group, as reported in the past (14,15,18).

Other conditions that can cause increased HbA_2 and HbF include inherited causes such as other hemoglobinopathies and thalassemias, various forms of anemia (e.g., dyserythropoietic anemia, aplastic anemia), and acquired causes such as thyrotoxicosis, HIV infection, certain medications, hepatocellular carcinoma, different types of leukemia, and other conditions (19,20).

One of the study's limitations is represented by the non-exclusion, during the population selection phase, of children under two years of age and pregnant women, as, in their case, the increase in HbF can be explained as a physiological phenomenon (19). However, since the subjects in the study were comprehensively evaluated, not only focusing on laboratory data related to the hemoglobin profile, it was still possible, in cases where sufficient information was available, to determine the group to which individual subjects belonged.

Even when considering pregnant women and children as separate subgroups, the difference in HbA_2 values remains statistically significant in the compared groups.

The majority of the subjects under examination are females in their childbearing age, and the average age of the study population is 38 years, possibly influenced by the fact that in Italy, screening, although not systematic, is conducted during the preconception or pregnancy phase,

as well as during prenatal and neonatal periods (13).

The study underscores the complexity of diagnosing hemoglobinopathies, particularly recognizing the carrier status of thalassemia. Integrating anamnestic data with clinical and laboratory criteria is crucial for an accurate assessment.

The results reaffirm that in a population like the Italian one, where there are significant migratory flows from areas with a high prevalence of hemoglobinopathies (6), it is essential to adhere correctly to screening programs that include the use of first-level tests such as the determination of hemoglobin fractions and variants, erythrocyte indices, and iron status (13), to address the challenges posed by these genetic diseases effectively. Moreover, given the migratory patterns and the diverse origins of the population studied, our findings underscore the necessity for international collaboration in health surveillance systems, which could facilitate better understanding and management of hemoglobinopathies across borders. This could lead to standardized screening practices sensitive to the genetic diversity seen in migrant populations. Improving screening programs by incorporating routine genetic counseling and molecular diagnostic tests could significantly enhance the detection rates of hemoglobinopathies, particularly in regions with high prevalence rates. Such initiatives should be backed by policy changes that support comprehensive healthcare coverage for genetic disorders.

Future research should focus on longitudinal studies that can track the progression of hemoglobinopathies in high-risk populations, particularly in areas with high immigration rates. This would allow for the development of targeted intervention strategies that can be implemented at community and healthcare system levels to manage these conditions better.

CONCLUSIONS

This study has provided valuable insights into the prevalence and complexity of diagnosing hemoglobinopathies, particularly beta-thalassemia, within a diverse Italian population influenced by substantial migratory flows. Our findings confirm the critical role of integrating clinical, anamnestic, and laboratory data in identifying thalassemia carriers, which aligns with the variations in hemoglobin patterns observed across different subpopulations.

Our data highlighted that non-molecular diagnostic methods, such as CBC and hemoglobin fraction analysis, are essential yet insufficient for a definitive diagnosis. Detailed clinical assessments must complement these methods and, when necessary, molecular techniques to enhance diagnostic accuracy. The diversity of hemoglobinopathies identified in this study highlights the necessity for a broad range of diagnostic tools tailored to the genetic backgrounds of the population served.

Moreover, the study has underscored the importance of updating and maintaining national health registries. Such registries are invaluable in tracking the prevalence and evolution of hemoglobinopathies, which is crucial for allocating healthcare resources appropriately and designing public health policies that reflect the current demographic and genetic landscapes.

In conclusion, the availability of first-level methodologies provides a foundation for diagnosing hemoglobinopathies. In conjunction with an evaluation by expert physicians in the field, these methods offer cost-effective means in clinical practice for carrier screening, especially in populations with high prevalence, reserving the use of invaluable tools, such as genetic and molecular methods, for preconception diagnostics and specific cases (12). This integrated approach enhances the accuracy of diagnoses and ensures that genetic screening is accessible and practical for widespread application.

CONFLICT OF INTEREST

None

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