HEMOGLOBINOPATHIES

From Diagnosis to Specialized Consultancy

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Other books by the same authors published by the same publisher:

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Emoglobinopatie. Dalla diagnosi alle consulenze specialistiche (Italian Edition). 2020

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The content of this book is designed primarily for discussion, understanding of the genetic mechanisms of the defects represented in the actual clinical reported cases, as well as for the interpretation of the associated hematologic phenotypes. The conclusions or hypotheses reported should not be considered as unique tools for the diagnosis of sometimes apparently similar clinical situations or as suggestions for the treatment of specific patients by physicians.

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To our respective parents,
families and collaborators

Foreword to the English Edition

This book summarizes the results obtained by capillary electrophoresis (CE) using Capillarys 3 (Sebia) during phenotype studies of the different hemoglobin (Hb) disorders. Historically, studies of Hbs by liquid electrophoresis started in 1949 with Pauling revealing that the mobility of Hb S differed from that of Hb A. At this time only a very limited number of electrophoresis equipment, U-tubes type, was available and several hours were required to perform each analysis, a special optical device (Schlieren) was necessary to follow the electrophoretic separation of the Hb components and no quantitative measure was possible. This was only, and hardly, an analytical method.

In the following years, quantitative evaluation of the Hb fractions was obtained by open chromatography methods on ion-exchange columns such as AE- or DEAE-Sephadex. High throughput techniques were further developed allowing real clinical applications. Two types of technologies were successfully used, the first one was high performance liquid chromatography on ion exchange columns (IE-HPLC) and the second, more recently introduced, was capillary electrophoresis. At the end of the 1980's, capillary electrophoresis instruments became commercially available. These equipments are now fully automated leading to rapid separation with high resolution and accurate quantification of the various Hb fractions.

According to the HbVar database, the actual number of Hb entries with structural modifications is of around 1400 and that of thalassemias of around 540. Most of these abnormalities may be detected (or suspected) by capillary electrophoresis. Structural abnormalities result often by difference in the retention times of the peaks, while thalassemias are usually suggested by the presence of abnormal levels of Hb A_2 or Hb F or of an unusual proportion of the various Hb components.

Up to now, a book summarizing the observed phenotypes was not available. This is now achieved by the work of Giuseppina Barberio and Giovanni Ivaldi, initially published in Italian (Piccin Nuova Libraria, Padova). In a first section of this book, structure, function and biosynthesis of the various Hbs are thoroughly described. Near to 200 cases of abnormal profiles obtained by capillary electrophoresis analysis are reported as examples. They are discussed according to the proportion of the various Hb components observed. For each example, this book presents the ethnical origin of the patients, the hematological data, the iron status, the main features of the clinical presentation and the results obtained by genetic molecular studies. Many possible associations of these various disorders are considered. A large part of this book details the profiles obtained on babies less than two years old. Another part discusses, in adult patients, the different cases according to the levels of Hb A_2 and Hb F and the characteristics of the electrophoretic profiles. Rare variants with specific electropherograms, are reported.

It is now obvious that capillary electrophoresis (CE) became one of the analytical methods required in the characterization of any Hb variant. The CE migration position is a reproducible value that can be used as an aid in the identification of Hb variants. The units used to determine the migra-

tion position do not correspond to migration time; they are arbitrary units defined by the manufacturer that appear on the x-axis below the electropherogram, from 0 to 300. The migration position for each Hb is normalized relative to the standardized position of Hb A (position 150) and Hb A_2 (position 243). If Hb A and/or Hb A_2 is present, the electrophoretic profile is divided in 15 migration zones, presumptive identification of any abnormal fraction is obtained according to the migration zone in which the abnormal fraction migrates. This division in 15 zones allows discrimination between the most common Hb variants: Hb S, Hb C, Hb D-Punjab, and Hb E. This method also allows the measurement of Hb A_2 in the presence of Hb E. In the absence of Hb A and/or HbA_2, the migration position is imprecise. Thus, the manufacturer recommends mixing the sample with a normal control in order to introduce Hb A and Hb A_2 in the sample and therefore get the normalization of the migration positions. The migration position of more than 400 Hb variants has now been determined.

In regions where thalassemias are frequent, a large variety of molecular disorders are often observed and may coexist in a single patient. Several types of α-thalassemias with various degrees of severity, depending on the number of α genes affected, and of the type of mutation are observed. This modifies the relative proportion of the different Hbs found on the electropherogram. In addition, different types of β-thalassemias may coexist, resulting in a total absence of the biosynthesis of a β chain (β^0) or to a small or moderate defect (β^+ or β^{++}). An α gene triplication behaves as a β^+-thalassemia. Hereditary persistence of Hb F is another usual defect. Hb E is a thalassemic Hb variant frequently observed in populations of S-E Asia. Anemia and microcytosis may result from iron deficiency which is a frequent disorder observed in these populations. Thus the profile of Hb percentage needs to be carefully discussed taking into accounts all these factors.

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From the Preface to the Italian Edition

This manual follows the one published in 2017 (G. Barberio, G. Ivaldi. *Talassemie e Varianti dell'Emoglobina in Elettroforesi Capillare: Casi Clinici.* Piccin Nuova Libraria) proposing a greater number of cases. The authors' goal was once again to describe how, by reasoning on the clinical-anamnestic information available and on laboratory findings, it is possible to arrive at consistent diagnostic solutions or hypotheses. The diagnostic paths, developed starting from the first level examinations, reach conclusions that sometimes require confirmation or further investigation through molecular examinations. DNA analysis, when necessary or useful, is suggested on a case-by-case basis. Each patient examined gave adequate consent and was subsequently informed about the genetic characteristics found. 61 cards have also been included in the text in which many globin defects are illustrated, variously combined, with the production of different phenotypes in the family environment. With these cards we wanted to provide useful elements for genetic counseling mainly dedicated to the combination of rare hemoglobin defects and heterozygous compounds observed or hypothesized even at birth. The interpretations and information reported can integrate the knowledge of the clinical geneticist and hematologist who always and in any case remain the reference figures in communicating with the patient. The laboratory professional who works in the field of hemoglobinopathies has the task of making use of increasingly advanced tools and methods, to better discriminate the numerous phenotypes produced by the different causes and genetic combinations. Knowledge of the theoretical aspects of hemoglobinopathies and observation of the phenotypes produced by the varied series of daily diagnostics can ultimately improve the appropriateness of the analytical approach. We hope that our work will be useful to all those involved in the prevention, diagnosis and management of hemoglobinopathies, in all areas ranging from diagnosis to specialized consultancy.

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Notes for Readers

1. Cases Subdivision Criteria

Clinical cases are divided according to patient's age:

- Newborns and children younger than 2 years
- Adults (> 2 years)

Cases described in the first age category include both thalassemias and variants.

Adult cases are divided according to Hb A_2 percentage (thalassemias) or according to the migration zone of the principal hemoglobin fraction detected. Other fractions are described case-by-case.

2. General Content

Cases descriptions contain clinical information, red blood cells indices, iron status and the clinical query that leads to the test. Sometimes some information is not available because not all cases come from the same laboratory. We decided to proceed with an interpretation and to obtain a diagnosis (definitive or presumptive) because often in real life we must handle cases where clinical pre-test information and hematological and iron data are missing. In newborn cases hematological and iron indices are often not available; however, at birth, these data have less impact on the global electrophoresis evaluation.

3. Cases Choice Criteria

Sometimes the single observation of the hemoglobin distribution didn't raise diagnostic doubts and didn't suggest other investigations. However, a diagnostic study was performed because we knew about a different picture obtained with an alternative method. The doubt could derive from the Hb A_{1c} quantification or following a functional test performed to detect the presence of polycythemia or hemolytic anemias, or simply following the observation of abnormal hematological parameters in subjects with a normal iron balance.

4. Interpretation of Symbols Used in Cases Description

Abnormal data are reported in red. Among hematological parameters, the abnormal data also present an arrow (up or down directed) describing their increase or decrease. Regarding this, the normal ranges for both hematological and iron parameters are reported in appendix 3 and 4.

In the Table *"hemoglobin distribution"* the arrows ↑↓ refer, for the different fractions and according to patients age, to the references reported at the end of this chapter: for newborns (1), for children younger than 2 years (2), and for adults (3). In case of detection of additional fractions

(normally not present), the indication reported on the table is an asterisk. Hb A doesn't report the indication ↓↑ in adult patients while this indication is present if the test is performed at birth for the clear importance that this quantification has from the birth up to six months. The reported fractions are labeled according to the automated detection performed by the Phoresis software. If the identification was made afterwards (e.g. after mix with control blood as per manufacturer procedure), in the table the correct identification will be reported on brackets. If iron parameters are lacking in the table this will be reported as "not available" otherwise the table will report the iron status parameters. In some cases we were not able to report a colored picture of the electrophoresis because of the impossibility to obtain its electronic version.

5. Most Frequent Variants

Regarding HbS and other variants (C-E-Lepore), different reporting ways can be found. This is due to the fact that only some (few) first or intermediate-level laboratories can report as "certain" these variants without a molecular method. This refers to the possibility to confirm with supplementary tests (sickling, Carrel, etc.) or with alternative methods (CE vs. HPLC) the presence of the mentioned hemoglobin variants (see Recommendation SITE and SIBioC.)

6. Diagnostic Conclusion

The diagnostic conclusion can be definitive or presumptive; in this last case, the first level laboratory can ask for a second level laboratory evaluation in order to conclude the diagnosis. In this case the blood sample and all obtained data will be provided to the second level laboratory.

7. Molecular Tests

The 184 documented clinical cases have often been concluded by suggesting a second level diagnostic path. The result of the DNA analysis, when available, was reported in the text. In other situations, a table has been inserted with suggestions relating to the globin genes to be studied and the most appropriate methods to use or the most available and cited in Table 16.

Hypothetically implicated globin genes and related diagnostic methods were indicated with a red symbol when molecular analysis was considered useful or necessary. While a yellow symbol was used to indicate that DNA analysis was considered optional or otherwise not necessary for immediate prevention. At times, such tests or others might be considered useful in the perspective of a more complete characterization to support genetic counseling.

8. Molecular Characterization Result

The molecular analysis result is reported for many of the documented cases if consent was obtained. In some cases is only reported the opportunity and the modality to proceed with a molecular test based on the indications provided from the hemoglobin distribution, the erythrocyte indices, the iron

status, and the diagnostic query. The result, when available, is reported using the traditional nomenclature and the indications of the Human Gene Variation Society (HGVS) reported on the *Hb Var* Database. For every characterized case the progressive identification number, as per *Hb Var*, was reported

9. References

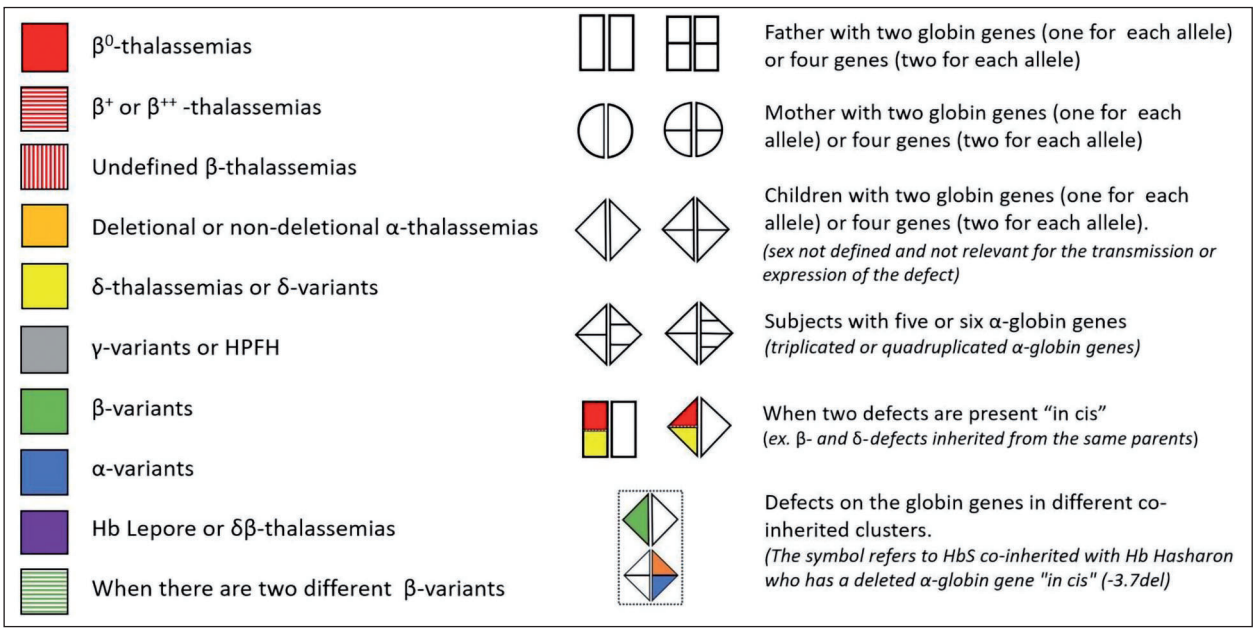
Every chapter reports a bibliography that has the primary purpose of deepening the study of what is reported on every topic. Moreover for every case concluded with molecular characterization, a specific reference was provided. These references have been selected based on the hematological, functional, and clinical characteristics of the case.

10. Clinical Cases

The reported cases are all real, obtained from the routine, favoring the ones that are particularly interesting in terms of diagnosis and prevention. We selected the cases able to exemplify the modalities that are useful to elaborate a diagnostic hypothesis. This is what is contained in the paragraph "Rationale" after every electrophoresis. It is important to specify that the reported conclusions are related to the most important consequence that can derive from the result of the performed tests. A complete report can, sometimes, require more diagnostic tests.

11. Cards

The cards (in the number of 61) represent a small ready-to-use manual useful to face the topic of the specialist counseling. In each one are synthetically reported some hematological, clinical, and genetic characteristics of defects that can be inherited from parents. The genealogic tree shows the most significant or unfavorable combination. In this tree the following symbols are used:



Moreover in every card is represented the real electrophoresis for the defect/defects both in adults and newborns (references are reported for all cases). In some cards we choose to report an electrophoresis scheme, considering this more explicative and more informative than the real one. A table, inserted at the end of the book, reporting the numbers of the cards (from 1 to 61) represents a useful map for the laboratory specialist or the clinician. Crossing the different defects reported on the x and y axis, it is possible to find the card referring to the studied couple. The card contains general information for the interested globin genes, but most importantly the scheme of the transmission of these defects to the offspring. This scheme can be a guide to understand the need for a more complete diagnostic prenatal or post-natal study.

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Methodological Aspects

Hemoglobin capillary electrophoresis was performed with a Capillarys 3 (software version 9.30, Sebia) instrument, an automated system able to perform, completely automatically, hemoglobin electrophoresis through a rapid and high-resolution separation. The Capillarys 3 system uses the principle of capillary electrophoresis in free phase; with this technique charged molecules are separated, in a capillary, according to their electrophoretic mobility in an alkaline buffer solution (pH = 9.4). The analysis is performed in the following way: the fresh blood sample, collected in a vial with EDTA as anticoagulant, is automatically diluted in specific segments with an appropriate hemolysing buffer, and subsequently hydrodynamically injected to the anodic extremity of the capillary through depression; the separation occurs in a specific buffer at controlled temperature by application of a high voltage at the extremities of the capillary (in molten silica); the detection occurs by reading absorption at 415 nm wavelength at the cathodic extremity of the capillary. With the alkaline pH buffer used for the analysis, the migration order of the principal hemoglobins in a normal sample is, from cathode to anode: Hb A_2, Hb F, Hb A (Hb A_2 is the hemoglobin fraction with the higher mobility which reaches the optical window first). At the end of the analysis, the Capillarys 3 system automatically performs the identification and relative quantification of each fractions (Hb A, Hb F, and Hb A_2) and the profiles can be interpreted. Hb A fraction is located at the center of the layout window and the relative electropherogram is divided into 15 different zones (from Z1 to Z15) useful to presumptively identify the different hemoglobin variants present. The ranges used, relative to every hemoglobin fraction, are the ones reported in the manufacturer's official technical manual (version 2018/12):

- HbA level is normally between 96.7 and 97.8%
- HbF: ≤ 0.5%
- HbA2: between 2.2 and 3.2%

Important characteristics of the utilized method (CE) besides the huge versatility of applications and the complete automation are: the high separative efficiency, the high sensitivity, the small amount of sample to be charged (nL), a relatively short analysis time (normally less than 30 minutes), a reduced number of solvents (few mL of buffer every day) and other materials. Capillary electrophoresis (CE), already used for the separation of serum proteins, is recognized as a valid technique also for hemoglobinopathies screening by the most important national and international recommendations/guidelines; this technique can also represent a secondary test for the diagnostic confirmation of the most common variants.

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