ORIGINAL ARTICLE

Hemoglobinopathies: A Longitudinal Study Over Four Decades

Elisabeth Kohne, Enno Kleihauer

SUMMARY

Background: Hemoglobinopathies are among the most common hereditary diseases worldwide, with high prevalence in the Mediterranean basin, Africa, and Asia. Although they are rare in the indigenous central European population, they have become much more common in Germany recently through the immigration of millions of people from endemic regions.

Methods: In a long-term study (1971–2007), 100 621 hemoglobin analyses were performed and retrospectively evaluated. Basic clinical and hematological information were provided by the participating physicians. The hemoglobin defects were characterized with hematological and biochemical methods, as well as by DNA analysis in selected cases (from the mid-1980's onward). 73% of the analyses were performed in patients with an immigration background, 27% in patients of German ethnic origin.

Results: 34 228 persons, or 34% of those studied, were found to have a hemoglobinopathy. Most cases involved thalassemia syndromes (25 798 cases, 25.6%); the second most common type was a structural abnormality of hemoglobin (8 430 cases, 8.4%). This study provides the first broad overview of the occurrence, spectrum, and geographical distribution of hemoglobinopathies in Germany.

Conclusions: These data show that hemoglobinopathies are a relevant health problem in the population of Germany today. This is not an epidemiological study, and thus it is unknown to what extent these data are representative. Because hemoglobin defects are of widely diverse genetic and clinical types, specialized laboratory analysis is needed to diagnose them correctly and provide a basis for proper therapeutic decisions.

Hemoglobinopathies are among the most common hereditary diseases of the world's population. About 4.5% of all human beings carry a gene for a thalassemia or hemoglobin anomaly (1–3, e6). The areas in which such abnormalities were originally most common extend from Africa over the Mediterranean basin and the Near and Middle East to Southeast Asia and the Indian subcontinent. Global migration in the modern period has led to a continual spread of these anomalies to all regions of the world, with the result that they are rapidly becoming more common in the industrialized regions of Northern and Central Europe as well (4, 5, 7–12, e6).

Amid the changing, multiethnic population of today's Germany, there are 15 million persons with an immigrant background (13). More than 9 million of the immigrants now living in Germany come from risk countries for hemoglobinopathies (as defined by the relevant WHO criteria), in which the prevalence of heterozygous carriers ranges from 5% to over 30%, depending on the region (1, 3). It follows from a simple calculation, therefore, that perhaps 400 000 persons now living in Germany carry hemoglobinopathy genes.

No precise epidemiological data are available at present, because there have been no systematic studies to count the number of carriers or patients with overt disease.

The diseases arising from a disturbance in the formation of hemoglobin (Hb) are divided into two categories:

- Thalassemias
- Hemoglobinopathies in the narrower sense, i.e., hemoglobin structural variants: these are commonly designated Hb anomalies (4, 16, 17).

Physicians in Germany have devoted an increasing amount of attention to Hb anomaly diseases in recent years and have found them to be an important issue in patient care. It is thus useful for clinicians to have an overview, complete with numerical data, concerning the occurrence, spectrum, and geographical distribution of Hb defects.

Methods

The present analysis is based on a retrospective evaluation of laboratory diagnoses made from 1971 to 2007 (inclusive) at the hemoglobin laboratory of the Department of Child and Adolescent Medicine at the University of Ulm, Germany. Blood samples preserved with

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Klinik für Kinder und Jugendmedizin, Hämoglobinlabor Universitätsklinikum
 Ulm: Prof. Dr. med. Kohne, em. Prof. Dr. med. Kleihauer

BOX 1

Indications for hemoglobin analysis

- Anemia, erythrocyte hypochromia and/or microcytosis, after the exclusion of iron deficiency
- Chronic hemolytic anemia
- Drug-induced anemia
- Vascular occlusion crises of as yet undetermined cause in patients from endemic areas for HbS and/or HbC
- Cyanosis of hematological origin
- Polyglobulia/erythrocytosis of as yet undetermined cause
- Fetal and neonatal anemias
- Prevention issues:
 - Screening of pregnant women
 - Partner diagnosis
 - Prenatal diagnosis
 - Family testing

EDTA were sent to the laboratory by physicians in hospitals and private practices from the entire Federal Republic of Germany. The examinations were mainly ordered by general practitioners, pediatricians, internists, laboratory physicians, human geneticists, and gynecologists. The tested material was derived from patients with different types of hematological findings that led to varying differential diagnoses of possible hemoglobin-related pathology.

The patients' immigrant background, country of origin, ethnic heritage, age, and sex were determined from the accompanying written information, as were the baseline hematological and clinical data. In some cases, when the country of origin was not specified, it was determined from the patient's surname, if such a determination seemed plausible.

All of the test results were documented, counted, and tabulated year by year through retrospective evaluation of the archived patient data and the meticulously kept laboratory notebooks. Incomplete data were filled in by telephone inquiries and by written solicitation of further information from the referring physicians.

Basic age and sex data were not evaluated in this study.

The distinction between immigrants and the native German population was drawn by classifying patients as immigrants if they had come from one of the countries named in the tables of "Migration Status" that are published by the German Federal Statistical Office (13).

The main indications for referral for hemoglobin analysis were hypochromic, microcytic anemia after the exclusion of iron deficiency, and hemolysis of as

yet undetermined cause. A complete list of indications is given in *Box 1*.

The analytical methods used in the laboratory were drawn from a large and regularly updated repertoire of specialized hematological, protein-chemical (electrophoresis, chromatography), functional-analytical (e.g., O₂ affinity), and molecular genetic testing procedures. Detailed descriptions of the testing methods can be found in multiple previous publications (14–16) and are also given in a *Supplement* to this paper.

The laboratory had developed a full range of test methods by the 1980's. Further modifications came through modernization of equipment and simplification of procedures. There were no major changes of sensitivity or specificity.

Quality assurance was based on long-term collaboration with American and British hemoglobinopathy centers (the ICSH Expert Panel on Abnormal Hemoglobins, University of Texas, Medical Branch at Galveston, and Center for Disease Control, Atlanta, Georgia, USA [1975]; the Study Group on Basic Laboratory Methods of Hemoglobinopathy Detection, Dept. of Cellular and Molecular Biology, Augusta, Georgia, USA [1990]; the ICSH project "Estimations of fetal haemoglobin," Royal Postgraduate Medical School, London, U.K.[1991]) and with participation in the German round-robin tests of Instand e.V.

Many of the thalassemia syndromes and Hb anomalies that were diagnosed in the authors' laboratory were further confirmed in the framework of scientific research projects by the demonstration of the underlying genetic defects (20, 21).

Results

The main results of the longitudinal study are presented in *Table 1*. A total of 100 621 blood samples were studied. About three-quarters of them were from adults, and one-quarter were from children of all ages.

Hb analysis yielded pathological findings in 34 228 patients (34.0%). As expected, most of these patients (29.6% of total) had an immigration background. The thalassemia syndromes were the largest group (25.6%); anomalous Hb variants, mainly the sickle-cell syndromes, were found in 8.4% of patients. Among persons of native German background, the frequency of positive diagnoses was 4.4% in this pre-selected patient collective.

The available data do not allow any conclusion with regard to the comprehensiveness of testing. We cannot exclude the possibility that some of the persons we took to be of native German origin actually had an immigrant background, e.g., persons whose families immigrated to Germany long ago.

The patients' countries of origin and their geographic distribution in Germany are shown in *eFigures 1 and 2*.

Thalassemia syndromes

Beta-thalassemias: the immigrant population

Among the various types of thalassemia, beta-thalassemias are the types most commonly seen in

Central Europe (*Table 1*). beta-thalassemias were found in 19 637 cases (26.7%) among persons with an immigrant background undergoing evaluation for anemia. Most of the patients came from the Mediterranean countries of southern Europe or from Turkey. The most common type of beta-thalassemia is classic heterozygous beta-thalassemia (=beta-thalassemia minor), of which there were 18 101 cases (92.2%). Moreover, the diagnosed beta-thalassemias were highly diverse, with a wide spectrum of different types. The second most common type was beta-thalassemia major (858 cases), which is recognized as one of the clinically most severe types of hemoglobinopathy (4, 16, 23).

Beta-thalassemias: the native German population

Among patients of native German origin, beta-thalassemia minor was still the most common type (*Table 2*), present in 3693 (13.7%) of the patients studied. Two patients of German origin were also found to have variant types of beta-thalassemia intermedia.

An anomalous hemoglobin of the Lepore type was found in 207 German persons with the hematological features of a thalassemia minor. 22 were found to have a delta beta-thalassemia, which was originally detected by family testing and has been confirmed in the last 10 years by genetic analysis.

Alpha-thalassemias: the immigrant population

Until now, publications concerning the presence of alpha-thalassemias in Germany have been limited to reports of single persons and/or families. Up to the mid-1990's, our laboratory had identified 350 patients with confirmed alpha-thalassemia (11, 16). In the 10 years up to the end of 2007, the number of diagnostic cases jumped to 2468 as a result of the increased immigration of persons from Asia, mainly Southeast Asia. Most carriers of alpha-thalassemia had a heterozygous alpha-thalassemia. The authors found 348 cases of HbH disease and 9 of Hb Constant Spring. Three neonates had very severe, lethal homozygous alpha⁰-thalassemia, which is known by the clinical designation "Hb Bart's hydrops fetalis syndrome."

Alpha-thalassemias: the native German population

Alpha-thalassemia is rare, but not unknown, among native Germans (16). The authors have seen a total of 214 cases to date (*Table 1*); an Hb disease was present in 14.

Anomalous hemoglobins

Hb anomalies that are common around the world: the immigrant population

The most common type of Hb anomaly, which the authors found in 6225 (6.2%) of all patients studied, was sickle-cell hemoglobin. The sickle-cell syndromes have become much more common in Germany over the past few decades as the result of increased immigration, particularly from Turkey, Africa, the Arab countries, and Asia.

TABLE 1

Hemoglobinopathies in the Ulm Longitudinal Study: overview

Tests performed	Total number (percent)	Immigrant population	Native German population
Total number of persons tested	100 621 (100)	73 615 (73.2)	27 006 (26.8)
Hemoglobinopathies overall	34 228 (34.0)	29 784 (29.6)	4444 (4.4)
Types of hemoglobinopathy			
Thalassemia syndromes	25 798 (25.6)	21 891 (29.7)	3907 (14.5)
Beta-thalassemias	23 330 (23.2)	19 637 (26.7)	3693 (13.7)
Alpha-thalassemias	2468 (2.4)	2254 (3.1)	214 (0.8)
Hemoglobin structural anomalies (Hb anomalies)	8430 (8.4)	7893 (10.7)	537 (2.0)
Anomalies that are common around the world (HbS anomalies)	6225 (6.2)	6225 (8.5)	0 (0)
HbE, HbC, HbD	1465 (1.5)	1331 (1.8)	134 (0.5)
Anomalies that are rare around the world	740 (0.7)	337 (0.5)	403 (1.5)

Hb, hemoglobin; HbS, sickle-cell hemoglobin

Nearly half (3085) of the HbS patients (*Table 3*) suffered from severe sickle-cell disease with variable genetic and clinical manifestations including vascular occlusive crises and hemolysis. The other half (3140 patients) were asymptomatic heterozygous carriers of HbS.

The second most common category of anomalous hemoglobin among immigrants was HbE, which was seen in 613 patients (*Table 3*). One-third of these persons were homozygous for HbE. HbE causes a clinically mild hypochromic-microcytic anemia that can become hemolytic when the patient takes certain medications or has a viral infection. A heterozygous HbE anomaly becomes clinically manifest through mild anemia and variable hypochromia. HbE combinations, particularly with alpha- and beta-thalassemias, were just as common. The affected patients were mainly from Indonesia, India, Thailand, Burma, and Malaysia, although some of them were from countries of the former Soviet Union such as Tadjikistan and Turkmenistan.

The third most common category of anomalous hemoglobin was HbC, which was seen in 525 patients. Heterozygosity for HbC is particularly common in West Africa; HbC has spread from West Africa all over the world. Homozygous HbC disease is characterized by a mild to moderate hemolytic anemia. Patients who are heterozygous for HbC have no hematological abnormalities.

Combined forms of various hemoglobinopathies with one another, e.g., HbSC, and with thalassemias,

TABLE 2

Hemoglobinopathies in the Ulm Longitudinal Study: thalassemia syndromes

Type of thalassemia syndrome	Total number (percent)	Immigrant population	Native German population
Beta-thalassemias	23 330 (100)	19 637 (84.2)	3693 (15.8)
Beta-thalassemia minor	21 555 (92.4)	18 101 (92.2)	3454 (93.5)
Beta-thalassemia major	861 (3.7)	858 (4.4)	3 (0.08)
Beta-thalassemia intermedia	96 (0.4)	94 (0.5)	2 (0.05)
Delta beta-thalassemias	255 (1.1)	233 (1.2)	22 (0.6)
Hb Lepore syndromes	518 (2.2)	311 (1.6)	207 (5.6)
Hereditary HbF persistence	45 (0.2)	40 (0.2)	5 (0.1)
	Total number (percent)	Immigrant population	Native German population
Alpha-thalassemias	2468 (100)	2254 (91.3)	214 (8.7)
Alpha-thalassemia minor	2094 (84.8)	1894 (84.0)	200 (93.5)
HbH disease	362 (14.7)	348 (15.4)	14 (6.5)
Hb Constant Spring	9 (0.4)	9 (0.4)	0 (0.0)
Hb Bart's hydrops fetalis syndrome	3 (0.1)	3 (0.1)	0 (0.0)

Hb, hemoglobin; HbF, fetal hemoglobin

e.g., HbE/alpha-thalassemia, are listed in *Table 3*, with particular attention to HbS and HbE anomalies. These forms make themselves evident as primary, genetically determined multiple illnesses with diverse manifestations; they are relatively common, because many of the Hb defects are seen in the same population and are often passed down together to the descendants of affected persons.

Hb anomalies that are common around the world: the native German population

Among Hb anomalies that are common around the world, neither HbS nor HbC has been encountered to date among native Germans, or in the offspring of mixed couples with one native German and one immigrant partner. On the other hand, the authors found 84 heterozygous carriers of HbE who were of native German origin (*Table 3*).

The authors found the first HbD anomalies in Germans as early as the 1970's (11). The variants that were mainly involved were HbD Punjab and HbD Ibadan.

Hb anomalies that are rare around the world

A complete listing of the Hb anomalies that are rare around the world and were found by the authors' laboratory in persons of native German and immigrant origin can be found in *eTables 1 and 2*.

The pathological (disease-causing) Hb defects often cause serious hematological diseases with severe anemia, frequently requiring transfusions or other forms of treatment.

The following findings deserve special mention: the largest class of rare hemoglobinopathies in this study was that of the unstable hemoglobins, most commonly Hb Cologne and the disease that it causes, Hb Cologne disease (hemolytic Heinz body anemia). This disorder was found in 103 persons of German and 25 persons of immigrant background.

The second most common class of clinically relevant rare hemoglobinopathies consisted of types in which oxygen transport in the blood is impaired. These anomalies manifest themselves clinically with erythrocytosis, usually of moderate severity.

The third most common class is still that of the pathological methemoglobins (HbM anomalies). The prototype, Hb Hörlein-Weber (named after its discoverers), was the first hemoglobin anomaly ever described. It was identified in Germany in 1948 (24).

Discussion

The first Hb analyses in Germany for the detection and identification of anomalous hemoglobin variants and thalassemia syndromes were carried out as early as 1959 in Freiburg by the pediatric hematologist K. Betke and his colleague E. Kleihauer. This was the beginning of a new subspecialty within hematology (5, 16). For many years, the diseases diagnosed in this way were regarded as exotic curiosities (7, 11, 22).

This article provides the first numerically extensive overview of the presence and spectrum of hemoglobinopathies among the population currently inhabiting the Federal Republic of Germany. It must be emphasized, however, that this study does not meet the criteria for an epidemiological study and thus cannot be considered representative. Rather, it is exclusively based on the laboratory diagnoses made in a large collective of patients with hematological diseases. It may well be the case that some of the patients who were taken to be of native German origin actually had an immigrant background, and vice versa.

The identification of more than 30 000 affected persons (34% of roughly 100 000 persons tested) suffices to show that hemoglobin diseases are a matter of practical clinical relevance in the current population of Germany, which contains a large immigrant component.

Computation yields the following rough estimates with regard to the frequency of hemoglobinopathies: In Germany today, there are roughly 9 million persons whose background is in the main regions of the world where hemoglobinopathies are present, with an average carrier prevalence of 4.5% (1, 3, e6). It follows that some 400 000 persons in Germany may be carriers of hemoglobinopathy genes.

Consequences for immigrants to Germany

The frequency, distribution, and diversity of hemoglobinopathies have changed over the years. Most recently, there have been new developments as a result of the immigration of many people from African, Arab, and Asian countries, a change from the earlier pattern of immigration primarily from the Mediterranean basin. Consequently, there has been an enormous increase not just in the number, but also in the genetic and clinical hematological heterogeneity of hemoglobin defects in Germany. A noteworthy example is the large increase in HbE anomalies and in alpha-thalassemia, both of which are very common in Southeast Asia and China. There is a resulting practical need to perform previously unknown tasks in diagnosis, therapy, and preventive medicine.

The type of thalassemia or Hb variant that is present must always be specifically identified in a specialized laboratory, so that the appropriate form of treatment can be determined. Caring for the affected patients requires close collaboration of family physicians with specialists from multiple disciplines, including internists, pediatricians, diagnostically and clinically specialized hematologists, molecular and human geneticists, gynecologists, occupational health physicians, and social workers. Guidelines for patients and for adult medicine are now available <http://www.uni-duesseldorf.de/AWMF/II/025-016.htm> and <http://www.uni-duesseldorf.de/AWMF/II/025-017.htm>.

An approximate calculation yields the following order-of-magnitude estimate of the number of persons requiring chronic treatment for hemoglobin diseases: If one adds together all the cases of beta-thalassemia major, dominant thalassemic hemoglobinopathies, clinically severe types of alpha-thalassemia, sickle-cell syndromes, and HbC and HbE diseases that were diagnosed over the past two decades in the present longitudinal study alone, one arrives at a total of more than 3000 patients needing lifelong, intensive medical treatment.

Consequences for the native German population

The demonstration of thousands of cases of thalassemia and hundreds of cases of anomalous hemoglobins among persons of native German origin confirms that hemoglobin defects can regularly be found in this population, just as in other populations to which these disorders are not endemic. Nonetheless, hemoglobin defects are still rare among native Germans.

For both immigrants and native Germans, the diagnostic evaluation should always be based on clear indications and clearly stated questions to be answered, both in general and specifically with regard to the particular procedures to be used.

Routine hemoglobin analysis or even DNA analysis is now increasingly performed, without any specific indication, for all patients with anemia. This type of "scattershot" diagnostic testing is unjustified and economically wasteful. Before any hemoglobin analysis is performed, patients with hypochromic anemia should

TABLE 3

Hemoglobinopathies in the Ulm Longitudinal Study: Hb anomalies

Type of Hb anomaly	Total number (percent)	Immigrant population	Native German population
Hb anomalies that are common around the world			
HbS overall	6225 (73.8)	6225 (78.9)	0 (0)
HbAS	3140 (37.2)	3140 (39.8)	0 (0)
Sickle-cell disease HbSS, HbSC, HbSO, HbSE, HbS Lepore, HbS/β-thal., HbS/α-thal., HbS/HbH	3085 (36.6)	3085 (39.1)	0 (0)
HbE HbEE, HbAE, HbE/β-thal., HbE/α-thal., HbE/HbH	697 (8.3)	613 (7.8)	84 (15.6)
HbC HbCC, HbAC, HbC/β-thal., HbC/α-thal.	525 (6.2)	525 (6.7)	0 (0)
HbD Punjab, Iran, Ibadan, Ouled Rabah, Los Angeles, Camperdown, Neath	243 (2.9)	193 (2.4)	50 (9.3)
	Total number (percent)	Immigrant population	Native German population
Hb anomalies that are rare around the world			
Unstable hemoglobins	194 (2.3)	51 (0.6)	143 (26.6)
HbM variants	54 (0.6)	11 (0.1)	43 (8.0)
Hb variants with erythrocytosis (↑ O ₂ -affinity)	47 (0.5)	5 (0.06)	42 (7.8)
Hb variants with falsely elevated HbA _{1c} (Hb Okayama etc.)	35 (0.4)	1 (0.01)	34 (6.3)
Other variants	410 (4.9)	269 (3.7)	141 (26.3)

HbS, sickle-cell hemoglobin; thal., thalassemias; Hb, hemoglobin

BOX 2

Hemoglobinopathy screening in gynecology

Persons to be tested

- All pregnant women from populations at risk; in case of a positive test, the partner should be tested as well
- All partners with a positive family history

Tests to be performed

- Blood count, iron status
- Hemoglobin analysis in positive cases:
 - DNA testing of both partners → genetic counseling
 - Prenatal diagnosis with fetal DNA analysis from chorionic villi

have a complete blood count and a determination of iron status, while patients with hemolytic anemia should undergo a basic diagnostic assessment for hemolysis. A simple rule for patients of native German origin is that an unstable hemoglobin such as Hb Cologne should only be considered in the differential diagnosis of patients with an otherwise unexplained hemolytic anemia. In every case of erythrocytosis of clearly "hematological" origin, after polycythemia vera has been excluded, a pathological hemoglobin with elevated oxygen affinity should be considered. Thalassemia should be considered in cases of hypochromic anemia that are not traceable to iron deficiency.

Conclusion

Without a doubt, the scientific and practical clinical knowledge of hemoglobinopathies has improved, in Germany as elsewhere. Nonetheless, further improvement is needed in many areas. The diagnostic assessment of these disorders should be more strongly oriented toward published guidelines for the hematological and molecular genetic testing (14, 15, 17–19) of affected patients and heterozygous carriers. The genetic counseling of patients and their families could also be better. Particularly in gynecology, the large number of asymptomatic female carriers presents a major challenge (for recommendations, see *Box 2*). We refer readers to the guidelines mentioned above and to a few more recent publications (12, 22, 25) for help in therapeutic decision-making.

This article aims to improve knowledge of hemoglobin disorders among physicians in active clinical practice. Further aims are to inform readers of the numerous and diverse hemoglobin defects that have become "naturalized" in today's multiethnic German population and to motivate an effort to deal with the problems that these diseases cause.

Dedication

The authors dedicate this article to Prof. Dr. med. Dr. h.c. K. Betke on the occasion of his 95th birthday.

Conflict of interest statement

The authors declare that they have no conflict of interest as defined by the guidelines of the International Committee of Medical Journal Editors.

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KEY MESSAGES

- Hemoglobinopathies have become markedly more common in Germany and are still on the rise. About 4.8% of all persons currently living in Germany carry hemoglobinopathy genes.
- The thalassemia syndromes are the most common type of hemoglobinopathy; among them, the beta-thalassemias are most common. Increased immigration from Asian countries has also led to a major rise in the frequency of alpha-thalassemias in recent years. Diagnostic testing for congenital anemias reveals thalassemia in as many as 29.7% of immigrants.
- Hemoglobin structural anomalies (Hb anomalies) occupy second place in terms of frequency. The most common ones are the sickle-cell diseases and HbC and HbE anomalies.
- The number of immigrants who are seriously ill and require chronic treatment because of a hemoglobinopathy is currently above 3000.
- Hemoglobin defects are regularly detected even in patients from the native German population, though such cases are still very rare.
- A typical feature of the multiethnic population of Germany today is the extraordinarily diverse group of hemoglobinopathies within it, occupying a broad spectrum of variable disease states and genetic defects. Competent, specialized diagnostic testing is necessary to provide the basis for optimal therapeutic decision-making.

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Corresponding author

Prof. Dr. med. Elisabeth Kohne
Hämoglobinlabor Universitätsklinikum Ulm
Klinik für Kinder und Jugendmedizin
Eythstr. 24
89075 Ulm, Germany
elisabeth.kohne@uniklinik-ulm.de

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ORIGINAL ARTICLE

Hemoglobinopathies: A Longitudinal Study Over Four Decades

Analytical methods

Elisabeth Kohne, Enno Kleihauer

Among the methods used in the course of the entire longitudinal study (1971–2007), some are currently only of historical interest (14–17, 19, e17, e23) and some do not correspond to the testing methods currently in use in the authors' laboratory (see the main article for further information).

1. Baseline hematological testing

In every case, complete blood count data were related to us by the physicians sending in the sample or were measured in the authors' laboratory with an automatic hematological analyzer.

Blood smear studies and reticulocyte counts were performed for many patients as supplementary studies and were later taken into consideration for the evaluation of disease states.

Further elements of baseline hematological diagnostic testing included iron status parameters (iron, transferrin, ferritin) and hemolysis parameters (bilirubin, LDH, haptoglobin). Data of this type were made available in the accompanying written information.

2. Electrophoresis

Hemoglobin electrophoresis was used to detect and quantify normal hemoglobin fractions and anomalous hemoglobins. The following electrophoresis techniques (at pH 8.6 and 6.2) were used in this study:

- Starch block electrophoresis (e1)
- Microzone electrophoresis on cellulose acetate sheets, agarose sheets, or citrate agar (e2, e3)
- In some tests, isoelectric focusing in polyacrylamide gels was used to separate variants that are difficult to detect with standard electrophoresis techniques (e4).

3. Chromatography

The chromatographic methods used in the early years of the study were based on DEAE-Sephadex columns (e5). These methods were replaced by the now common HPLC techniques, which were used to quantify normal and pathological hemoglobins and to detect and distinguish anomalous hemoglobin variants (e6, e7).

Note on the identification of Hb anomalies with electrophoretic and HPLC methods: Whenever an anomalous hemoglobin was detected, its identity was tested with a combination of different separating techniques. These studies were further supplemented

with other test procedures (see: chemical methods, cytological testing).

4. Chemical methods

- In order to distinguish HbS from other variants with the same electrophoretic migration characteristics, the so-called solubility test was used (e8).
- The quantitative determination of HbF was performed with classic alkali denaturation (e9).
- Special denaturation tests for the simple detection of unstable hemoglobins included heat denaturation at 69.5°C and the isopropanol test (e10).

5. Cytological testing

- The demonstration of HbF cells on the object carrier was performed by the acid elution method (e11).
- Intra-erythrocytic inclusion bodies (Heinz bodies) were detected by the incubation of 0.1 mL of blood with brilliant cresyl blue solution followed by the preparation of smears as in reticulocyte counting (e12).
- For sickle-cell testing, erythrocytes were deprived of oxygen and then inspected under a microscope (e13).

6. Functional testing

The function of normal and pathological hemoglobins was tested as follows:

- Methemoglobin determination and MetHb spectral analysis (e14).
- Determination of the oxygen affinity of the blood by automated drawing of oxygen binding curves (16).

7. Identification of rare variants

In the first few years of the study, the structural defects of the rare Hb anomalies were investigated with the methods of protein chemistry. From the mid-1980's onward, molecular biological analyses were performed additionally or exclusively, as indicated.

Protein-chemical structural analyses (e7)

These analyses were performed with the following individual techniques, depending on the particular anomaly being studied (e7):

- Separation and quantification of normal and abnormal fractions
- When necessary (if the anomalous polypeptide chains were insufficiently separated from the normal ones), preliminary isolation of anomalous hemoglobins with various chromatographic techniques (e7)
- Separation of polypeptide chains (e15)
- Peptide mapping of tryptic, amino-ethylated peptides with the fingerprint technique (e16)
- Amino acid sequencing with the automated Edman degradation method (e7).

Molecular-genetic testing

These analyses were performed on the basis of certain criteria:

- **Criteria in the setting of thalassemia evaluation:**
genetic typing of beta-thalassemia major, molecular diagnosis of beta-thalassemia intermedia; with respect to genetic questions: relatives, partners (in case of genetic risk), prenatal diagnosis, combinations of different types of thalassemia and of thalassemias with hemoglobinopathies
- **Criteria in the setting of evaluation of Hb anomalies:**
identification of newly detected abnormal hemoglobins and rare anomalies; clearer characterization of Hb variants that cannot be separated either electrophoretically or chromatographically (e.g., in erythrocytosis with elevated blood oxygen affinity, or in Heinz body anemias); combinations of different types of hemoglobinopathy or of anomalous hemoglobins with thalassemia syndromes;

with respect to genetic questions: relatives partners (in case of genetic risk), prenatal diagnosis.

For DNA analyses, polymerase chain reaction (PCR)-amplified complete sequencing was performed, either of the beta-chain genes in beta anomalies or of the alpha-chain genes in alpha anomalies. The PCR products were studied as single-strand DNA in comparison to normal control samples, and the type of anomaly was determined by comparison of the nucleic acid sequence of the altered DNA with that of normal DNA (e15).

8. Laboratory diagnostic testing of alpha-thalassemias

The following methods were used to study alpha-thalassemias in the laboratory:

- Demonstration of HbH cells by incubation of 0.1 mL EDTA blood at 37°C with brilliant cresyl blue solution and preparation of smears
- Detection of HbH with electrophoretic and/or HPLC techniques
- DNA analyses:
 - screening test for the detection of the 6 most common deletional alpha-thalassemias with a PCR-based so-called single-tube multiplex screen (e23)
 - demonstration of all alpha-thalassemia deletions with the MLPA and MAPH technique (e24)
 - demonstration of non-deletional alpha-thalassemic mutations of the alpha1 and alpha2 genes after specific PCR amplification and sequencing of single-strand-amplified DNA products with single-strand sequencing (e1).

eTABLE 1

Rare anomalous hemoglobins: native German population

Designation of Hb anomaly	Manifestations	Number of patients
Hb Cologne	chronic hemolytic anemia	103
HbE	mild hypochromic anemia; hemolysis induced by medications, viral infections, oxidative damage	84
HbD Ibadan, Iran, Los Angeles, Neath	normal	50
HbM Milwaukee, Iwate, Saskatoon, Boston	cyanosis, methemoglobinemia, erythrocytosis	45
Hb Okayama	Falsely elevated HbA _{1C} , hemat. and clinically healthy	39
HbJ Meerut, Iran, Chicago, Camagüey, Baltimore, Auckland, Paris, Cambridge, Amiens, Wenchang Wuming, Bangkok	normal	22
Hb Hammersmith	severe hemolytic anemia	1
HbA ₂ anomalies	normal	17
Hb Andrew Minneapolis	erythrocytosis	17
Hb York	erythrocytosis	10
Hb Cheverly	chronic hemolytic anemia	9
Hb Camperdown	normal	8
Hb Little Rock	erythrocytosis	7
Hb Presbyterian	normal	7
Hb Vanderbilt	severe erythrocytosis	6
Hb Johnstown	mild erythrocytosis	6
HbI Interlaken, Philadelphia	normal	5
Hb Freiburg	cyanosis, hemolytic anemia	5
Hb Ohio	erythrocytosis	5
HbN Baltimore, Seattle, Timone	normal	4
Hb Nottingham	severe hemolytic anemia	3
Hb Saint Mandé	cyanosis	3
Hb Shepherds Bush	hemolytic anemia	3
Hb Zurich	Episodic drug-induced hemolytic anemia	3
Hb San Diego	erythrocytosis	3
Hb Hasharon	hemolytic anemia on exposure to toxic substances	2
Hb Malmö	erythrocytosis	2
Hb Tübingen	episodic hemolytic anemia, cyanosis, methemoglobinemia	2
Hb Athens GA	normal to mild erythrocytosis	4
Hb Nagoya	chronic hemolytic anemia	2
Hb Palmerstone North	erythrocytosis	1
Hb Savannah	hemolytic anemia	1
Hb Pyrgos	mild erythrocytosis	2
Hb Olympia	erythrocytosis	1

Designation of Hb anomaly	Manifestations	Number of patients
Hb Saitama	hemolytic anemia	1
Hb Limassol	normal	1
Hb Shaare Zedek	normal	1
Hb Yokohama	severe hemolytic anemia	1
Hb Syracuse	erythrocytosis	1
Hb Strasbourg	normal	1
Hb Atlanta	mild hemolytic anemia	1
Hb Utrecht	mild hypochromic hemolytic anemia	1
Hb Osu Christiansborg	normal	1
Hb Seattle	chronic hemolytic anemia	1
Hb Buenos Aires	hemolytic anemia	1
HbE Saskatoon	normal	1
HbF Catalonia	incidental finding	1
HbK Woolwich	hematologically normal	1
Hb Agenogi	mild anemia	1
Hb Alesa	hemolytic anemia	1
Hb Camden	normal	1
Hb Lufkin	normal	1
Hb Mainz	severe hemolytic anemia	1
Hb Radcliffe	erythrocytosis	1
Hb Regina	mild erythrocytosis	1
Hb Riverdale Bronx	hemolytic anemia	1
Hb Titusville	normal	1
Hb Rothschild	mild anemia	1
Hb Mozhaik	hemolytic anemia	2
Hb Higaschitochigi	cyanosis, methemoglobinemia	1
Hb Beth Israel	cyanosis, hematologically normal	1
Hb Vila Real	mild erythrocytosis	6
Hb Hanamaki	mild erythrocytosis	1
Hb Leiden	hemolytic anemia, episodes induced by drugs or viral infection; see HbE, above	2
Hb South Florida	normal	1
Hb Villaverde	severe erythrocytosis	1
Hb Cagliari	hemolytic hypochromic anemia	1
Hb Louisville	mild hemolytic anemia	1
Hb Mito	mild erythrocytosis	1
Hb Hoshida	normal	1

Hb, hemoglobin; HbF, fetal hemoglobin

eTABLE 2

Rare anomalous hemoglobins: immigrant population

Designation of Hb anomaly	Manifestations	Number of patients
HbD Punjab, Iran, Ibadan, Ouled Rabah, Los Angeles, Camperdown, Neath	normal	183
HbA ₂ anomalies	normal	67
HbO Arab, Padova, Indonesia	mild anemia	69
HbG Copenhagen, Coughatta, Ferrara, Honolulu, San José, Szuhu, Taipei, Philadelphia/HbC, Philadelphia, Accra	normal	40
Hb Cologne	hemolytic anemia	25
HbJ Auckland, Baltimore, Calabria, Camagüey, Havana, Iran, Paris, Sardinia	normal	18
HbI Interlaken, Philadelphia	normal	11
HbM Iwate, Milwaukee, Saskatoon	congenital methemoglobinemia	6
Hb Setif	mild hemolysis	7
HbQ Iran	normal	4
Hb Hasharon	hemolytic anemia induced by drugs, oxidative damage	3
Hb Moabit	hemolytic anemia	3
Hb Quin Hai	normal	3
HbN Baltimore	normal	3
Hb Sallanches	unknown	3
Hb Freiburg	hemolysis, cyanosis	2
Hb Stanleyville II	falsely elevated HbA _{1C}	3
Hb Hamadan	normal	2
HbK Woolwich	normal	2
HbP Nilotic	normal	1
Hb Alberta	erythrocytosis	1
Hb Agenogi	anemia	1
Hb Bethesda	erythrocytosis	1
Hb Hofu	mild anemia	1
Hb Corfu	normal	1
Hb Ohio	erythrocytosis	1
Hb Okayama	falsely elevated HbA _{1C}	3
Hb Providence	erythrocytosis	1
Hb Pyrgos	erythrocytosis	1
Hb St. Louis	hemolysis, cyanosis	1
HbO Padova	normal	1
Hb Andrew Minneapolis	erythrocytosis	2
Hb Mizushi	normal	1
Hb Hope	normal to mild anemia	1
Hb Stanleyville I	normal	1
Hb Osu Christiansborg	normal	1
Hb Beckman	chronic hemolytic anemia microcytosis	1
Hb La Lamentin	normal	1

Designation of Hb anomaly	Manifestations	Number of patients
Hb Westmead	mild anemia	1
Hb Bunbury	erythrocytosis	1
Hb Fontainebleau	normal	1
Hb Bicêtre	hemolytic anemia	1
Hb Camden	normal	1
Hb Chesapeake	erythrocytosis	2
Hb Ernzt	normal	2
Hb Handsworth	normal	1
Hb Paksé	mild anemia	1
Hb Agrinio	mild anemia, microcytosis	1
Hb Saint Etienne	hemolytic anemia	1
Hb Siríaj	normal	1
Hb Evanstone	mild anemia, microcytosis	1
Hb Pitié Salpêtrière	erythrocytosis	1
Hb Bronovo	mild microcytic anemia	1
Hb West One	normal	1
Hb Khartoum	normal	1
Hb Knossos	mild anemia, microcytosis	1
Hb Buenos Aires	normal	1
Hb Waimonolo	normal	1

Hb, hemoglobin

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eFIGURE 1

Countries of origin and percentage distribution among patients with hemoglobinopathies studied at the Ulm Hemoglobin Laboratory

eFIGURE 2

Geographical distribution of patients with hemoglobinopathies in Germany, as revealed by the Ulm longitudinal study