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New analytical tools and epidemiological data for the identification of HbA_2 borderline subjects in the screening for beta-thalassemia

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ABSTRACT

The increase of HbA_2 is the most important feature in the identification of beta-thalassemia carriers. However, some carriers are difficult to identify, because the level of HbA_2 is not in the typical range. Few data are available concerning the prevalence of such unusual phenotypes, and knowing their expected prevalence could be helpful in detecting systematic drifts in the analytical systems for HbA_2 quantification.
In this study we report a retrospective investigation in two centres with high prevalence of beta-thalassemia. The prevalence of borderline subjects was found to be 2.2 and 3.0%, respectively. The genotypes of a subgroup of these subjects were then analyzed and in about 25% of cases a mutation in the globin genes was identified. We conclude that the occurrence of HbA_2 borderline phenotypes is not a rare event.
In order to obtain more accurate HbA_2 measurements the development of an international reference measurement system for HbA_2, based on quantitative peptide mapping, has been recently started. We believe that the innovative approach of our method could also be used as a model to develop accurate quantitative methods for other red cell proteins relevant to the biodynamic properties and the surface electrochemistry of erythrocytes.

1. Introduction

The quantitative determination of HbA_2 is an essential test in screening for beta-thalassemia carriers [1]. To this purpose, highly accurate and precise measurements are needed, since the difference between normal and pathological HbA_2 values is small. Moreover, due to the large variability in phenotypes, some cases of slightly increased or borderline HbA_2 levels (defined as between 3.3 and 3.7% of total hemoglobin) can be found, and it is essential not to miss these cases in order to avoid an incorrect diagnosis. Cases of borderline HbA_2 levels, with reduced MCV and MCH, could occur as a consequence of a mild beta$^+$-thalassemia mutation, co-inherited delta and beta-thalassemia or coexisting pathological conditions such as iron deficiency [2,3]. Cases with normal MCV and MCH may be simply an artefact due to an inaccurate calibration of the measuring system, or can be part of the normal distribution of non-carriers, or even the effect of an unknown genetic determinant able to increase the HbA_2 level [4].

From an analytical point of view, commonly used quantitative methods include microchromatographic and HPLC techniques, although new tests based on immunochemistry or capillary electrophoresis have also been proposed [5,6]. Anion exchange chromatography on microcolumns has long been proposed as the reference method for HbA_2 measurement [7], but it is a manual technique with low specificity and high imprecision. Considerable advantages in terms of precise quantification, savings in time and full automation are provided by HPLC methods over the labour intensive conventional techniques, such as elution of Hb bands from cellulose acetate electrophoresis or anion exchange microcolumn chromatography [8].

Few data are available in the literature on the occurrence of HbA_2 borderline subjects in laboratory routine, and little information is available about the accuracy of the present methods for measuring this minor hemoglobin. With this work we therefore aimed to explore this topic by making a retrospective investigation on the occurrence of HbA_2 borderline phenotypes in areas with a high prevalence of thalassemic syndromes. We also want to briefly illustrate a candidate

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Table 1
Characteristics of patients analyzed in the retrospective investigations in Cagliari and Palermo

Study	Subjects	Sex		Age, years mean±SD	MCV		HbA_2 borderline	
		F, %	M, %		<80 fL	≥80 fL	MCV<80 fL	MCV≥80 fL
CA-Adults	8520	59.9	40.1	31±7	29.7%	70.3%	1.2%	2.9%
CA-5B	1825	51.3	48.7	13±1	36.6%	63.4%	0.3%	0.9%
CA-Ogliastra	6151	57.4	42.6	32±6	17.9%	82.1%	0.2%	3.7%
PA	1848	64.7	35.3	42±18	35.5%	64.5%	3.2%	7.1%

reference system for HbA_2 which will improve the standardization of the measurements in the near future.

2. Materials and methods

2.1. Subjects and design of the study

The study was conducted retrospectively in two centres specialized for the prevention of thalassemic syndromes: the Thalassemia Unit in Cagliari and the Azienda Ospedaliera A. Cervello in Palermo. In the Cagliari centre we separately analyzed the data obtained during 14 months 2003–2004 in 4 different studies: a) Adults (CA-Adults); b) adolescents between 12 and 14 years (CA-5B); c) subjects from a restricted area (CA-Ogliastra) of the Sardinia island. The data relative to all the measurements performed in 2002–2003 were collected in the Palermo centre.

2.2. Laboratory analysis

Complete whole blood cell count was collected on all subjects. HbA_2 was measured by two HPLC systems from the same manufacturer (Bio-Rad Laboratories, Segrate, Milano), Variant I (PA) and II (CA). Bio-Rad Variant analyzers are fully automated HPLC which use buffers and conditions specifically designed for hemoglobin analysis. The separation of the different Hb fractions is obtained by a cation exchange column and an increasing ionic strength elution gradient, performed with two dual piston pumps. Double wavelength detection (415 and 690 nm) is used to monitor the eluted Hb components. Two-level calibration of the instrument and sample analysis were carried out according to the manufacturer's recommendations.

Each laboratory was also asked to collect data on internal quality control, in order to check the stability of their analytical HPLC systems over time. This was typically performed by analyzing two control materials, provided by the same manufacturer, for every analytical run.

A subset of specimen from subjects with borderline HbA_2 values was further investigated by reverse dot blot [9] and gene sequencing in order to search for mutations in the β-globin gene, and by GAP-PCR to search for the $\alpha\alpha\alpha^{anti3.7}$ condition, which is sometimes associated with borderline HbA_2 [2]. The molecular analysis has been also applied to the study of α and δ-globin genes [10,11].

3. Results

The internal data of the Quality Control procedures of both centres proved to have good reproducibility and stability of both instrumentations during the whole period of the study. Typical between-run CVs were in the range of 4.8% at the normal HbA_2 concentration and 2.4% at the pathological level. A poorer reproducibility (CV up to 10%) was noted for HbF, especially when present in very low amounts (1% or lower). At more relevant HbF concentrations, an improvement in imprecision was found with CVs ranging from 1.9 to 5.0%.

Table 1 reports the basic descriptive statistics of the cases analyzed in our studies. There were significant differences in the dimensions of the cohorts, but all were pertinent to areas where there is quite a high prevalence of beta-thalassemia. Indeed in these regions the prevalence of carriers of beta-thalassemia is between 6.0% (Sicily) and

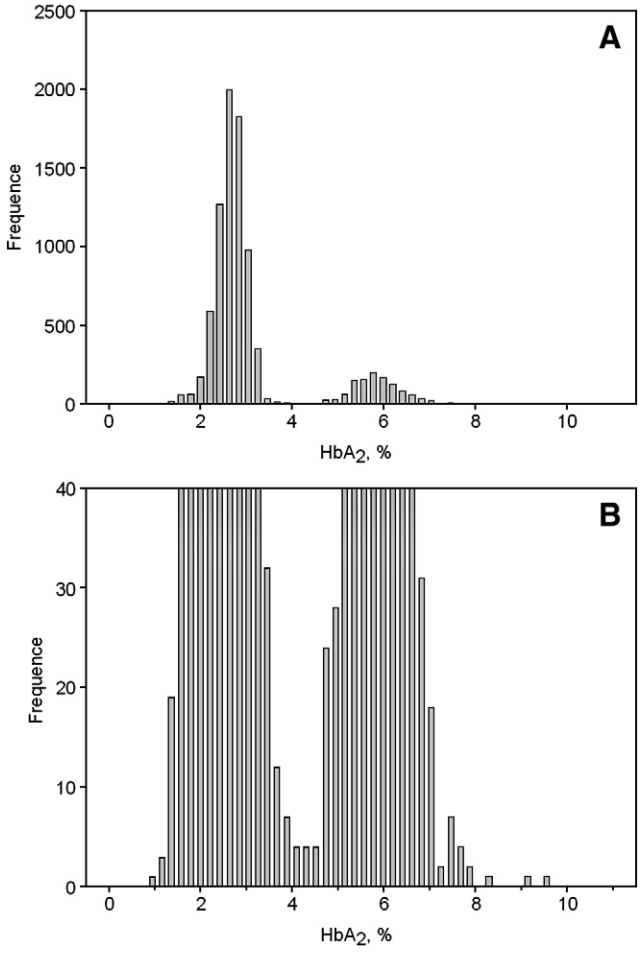

Fig. 1. Distributions of all HbA_2 measurements performed in Cagliari on adult subjects (study 1: CA-Adults) during 2003–2004. The plot in B is an enlargement of the same data of plot A.

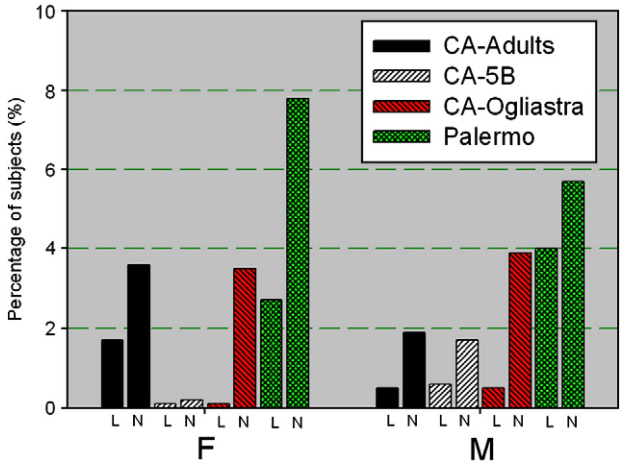

Fig. 2. Distribution of HbA_2 borderline subjects in the four studies, separated by sex (F, M) and by MCV (L: 80 fL; N: ≥80 fL).

Table 2
Genotype of 234 (over 1734) subjects with HbA_2 borderline

NEG/-α3.7	2
NEG/IVS 1 nt 6	20
β[a]+δCd27	7
NEG/αααα[anti3,7]	10
Hb Variants[b]	3
Cap+1570	1
β prom. (-101; -92)	10

[a] β-thal mutations: β 039, IVS I nt 1, IVS I nt 110.
[b] Hb Variants: Hb Acharnes (cd 53 GCT>ACT); Hb Kokomo (cd 74 GGC>AGC), Hb Ernz (cd 123 ACC>AAC).

10.3% (Sardegna) [12]. The overall incidence of subjects with reduced mean cell volume (MCV), between 16.1% and 40.1% is in close agreement with the prevalence data, and is of course higher because among the subjects with low MCV, those with iron deficiency are also included. The incidence of subjects with reduced MCV was indeed lower among the Ogliastra cohort, compared to those calculated on the cohorts of subjects attending both Hospitals.

Fig. 1 reports the typical distribution of the whole data of HbA_2 measurements performed in Cagliari (study 1: CA-Adults) over the period of the study. The distributions of the other studies were essentially similar and are not reported in detail. Essentially, two sub-populations were identified, the left one including most of the non beta-thal carriers together with some alpha-thal carrier, and the right one including the vast majority of carriers of beta-thalassemia. As is clearly evident from the enlargement in the bottom portion, analysis of the so-called borderline subjects, i.e. HbA_2 between 3.3 and 3.7%, is focused to the right shoulder of the left distribution, i.e. that typical of normal healthy subjects.

The retrospective analysis of the borderline subjects among the four cohorts was then performed by separating the subjects with normal MCV (i.e. ≥80 fL), from those with reduced MCV (<80 fL). We then analyzed the occurrence of HbA_2 borderline phenotypes in these groups and the relevant information is presented in Fig. 2. The analysis evidenced a higher frequency in the borderline phenotypes among subjects with higher MCV, and significant differences among the cohorts. The biggest difference in the occurrence of HbA_2 borderline subjects was found by comparing data from Cagliari to that of Palermo, where the prevalence of borderline phenotypes was, for the individual with normal MCV, between 5 and 7%, probably due to the higher frequency of beta+ and beta++ globin gene defects present in this population [13].

In order to investigate the molecular aspects, we analyzed a representative subgroup of subjects with borderline HbA_2 values. Out of 234 subjects, a mutational defect was found in 53 subjects (22.6% of cases). The genotype of these subjects is reported in Table 2, where it can be seen that in most of the cases mild beta-thal mutations were found (such as IVS1, nt6 and some defects of the promoter of the beta globin gene), together with the triple alpha genotype. It is also worth noting, however, that some severe beta-thal mutations (such as beta 39 C→T) were found in association with delta gene deletional defects, as previously described [11].

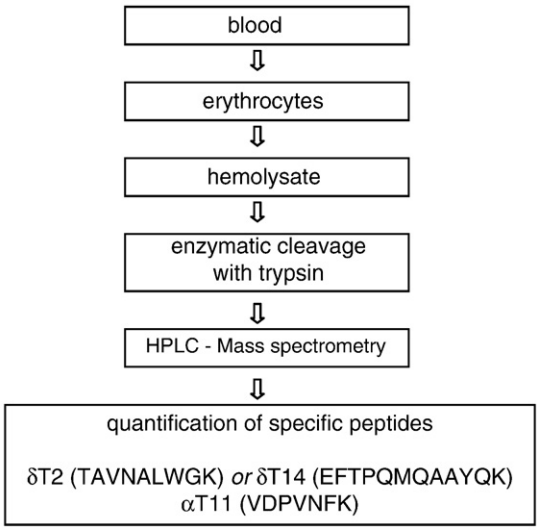

Fig. 4. Principle of the IFCC candidate reference measurement procedure for HbA_2.

In order to overcome the problems of poor alignment of routine methods for HbA_2 measurement, the International Federation of Clinical Chemistry (IFCC) has recently approved a project for the development of a complete reference system for HbA_2 and an *ad hoc* working group has been established (IFCC WG for the standardization of HbA_2). Fig. 3 depicts the IFCC reference system and the traceability chain for accuracy in HbA_2 determination.

The candidate IFCC reference measurement procedure has been developed by the IFCC WG-HbA_2 and its validation is also under development, although a standard operating procedure has already been developed. The principle of the method, which is based essentially on a quantitative peptide mapping of the peptides obtained by the digestion of red cell proteins under controlled conditions, is illustrated in Fig. 4. At present, two specific delta chain peptides (δT2 and δT14) have been selected. Work is in progress to

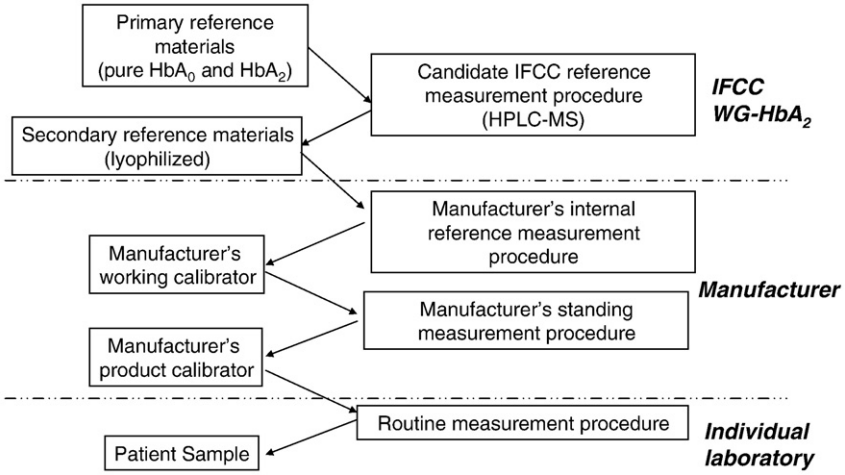

Fig. 3. IFCC Reference system and traceability chain for HbA_2 measurements.

decide which one will be the target in the final reference measurement procedure.

Using this reference procedure, values are assigned to a panel of secondary matrixed reference materials. Manufacturers will then apply these materials for calibration of their routine method, leading to traceable results of the end user's routine method. At present, primary and secondary reference materials (SRMs) are available only in the laboratory of the authors (AM and RP), but transfer of such materials to the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium) has already started. The IRMM must make a decision on the production of the SRM in September 2007.

4. Discussion

A strict analytical performance with a high degree of reproducibility and accuracy is an essential requirement for HbA_2 assay, due to the very narrow separation limit between normal and pathological HbA_2 values and to the serious consequences that can be derived from misclassification of a beta-thalassemia carrier. The data we have provided over 2 years of observation in two Italian laboratories, with a special expertise in the field of hemoglobin disorders and settled in areas with high prevalence of thalassemia syndromes, indicate that indeed, in the vast majority of cases, abnormal higher HbA_2 values can be easily recognized, since the distribution of HbA_2 in beta-thal carriers is clearly distinct from that of the non-carrier population. However, in a minority of cases, the difficult cases in which the HbA_2 is in the “gray zone”, i.e. between 3.3 and 3.7%, needs to be evaluated.

The observations we have collected clearly show that the occurrence of such cases, in areas with high endemic prevalence of beta-thalassemia, is not uncommon, occurring typically in 2–4% of the total cases analyzed over 2 years. We may then expect that the prevalence of borderline subjects could be lower in certain areas (such as Northern Europe) where thalassemia is a more rare disorder, despite the recent increase due to migratory fluxes. Personal experience with other Italian laboratories participating to External Quality Assessment Schemes on HbA_2 [14] indicates that in some cases very unusually high occurrence of borderline HbA_2 subjects (about 20% of the total analyses performed) could be due to instrumental drifts or problems. It is likely that a similar retrospective analysis could be planned in the near future in these laboratories to monitor, over a long timeline, the occurrence of borderline phenotypes and compare it to the present investigation.

Finally, the development of a new metrological system to measure this important minor hemoglobin is a big step forward, according to the most recent guidelines on standardization of laboratory tests [15]. The key elements of the system are the reference measurement procedure and reference materials. The reference measurement procedure is intended to be used to assign a certified value to a well-defined reference material. This certified reference material and the manufacturer's standing measurement procedure will then be used at the manufacturer's level to assign values to commercial calibrators. Clinical laboratories will use the routine procedures with validated calibrators to measure HbA_2 in human blood samples. In this way, the obtained values will be traceable to the reference measurement procedure and materials, and the standardization of the measurements will be reached.

We may expect that, if the project will be successfully completed, in the near future it will be possible to achieve a global world-wide harmonization of this important biochemical test. It is also important to note that the novel approach to quantitatively measure proteins by proteomics techniques such as the one illustrated in our work, could be a useful model to approach the accurate quantitative estimation of other proteins, especially when they are heterogeneous and present in complex matrices.

5. IFCC WG-HbA_2

The following are members of the IFCC Working Group for the standardization of HbA_2: Prof.ssa Donatella Caruso, Dr. Renata Paleari, Prof. Andrea Mosca (University of Milano, Italy); Dr. Christine Schaeffer, Dr. Alain Van Dorsselaer (CNRS, Strasbourg, France); Dr. Emmanuel Bissé (University Hospital, Freiburg, Germany); Dr. Barbara Wild (King's College Hospital, London, UK), Dr Brian Green (Micromass, Altrincham, Chesire, UK).

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