Standardization of hemoglobin A2 and hemoglobin F: achievements and perspectives

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Research Paper

Standardization of hemoglobin A_2 and hemoglobin F: Achievements and perspectives

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ABSTRACT

The establishment of reference systems for the standardization of hemoglobin A_2 (HbA_2) and fetal hemoglobin (HbF), both critical for improving diagnostic accuracy in conditions such as β-thalassemia and sickle cell disease, are described. Efforts were led by the IFCC and other groups to address and reduce the variability in laboratory measurements of these hemoglobins. This document outlines the production of certified reference materials (CRMs) for HbA_2 and the development of a reference measurement procedure using isotope dilution mass spectrometry. Similarly, standardizing HbF is essential for supporting diagnostic and therapeutic strategies, particularly in managing sickle cell disease. HbF levels can predict disease outcomes and guide treatment plans. Significant challenges remain in achieving consistent measurement across laboratories, and the process for standardization for this minor hemoglobin has just begun. We are confident that the implementation of these reference systems will provide improved accuracy and traceability in the future.

1. Introduction

Improving the quality of Laboratory Medicine is an important part of the strategic plan promoted by the IFCC for the 2024–2026 biennium [1]. Outlined among the strategic goals is the promotion of global standardization of laboratory methods, the provision of information on reference materials, reference methods, and globally available services. As part of this strategy, the IFCC promoted the establishment of a working group for hemoglobin A_2 (HbA_2) standardization some years ago. Subsequently, the creation of a working group for the standardization of fetal hemoglobin (HbF) was also promoted.

The motivation behind the creation of these two working groups was the improvement of laboratory services in relation to the diagnosis and treatment of thalassemia syndromes (especially β-thalassemia), which affect a large number of people in the world [2].

In this manuscript we report the activities carried out by the two working groups, which have led to the completion of the terms of reference for HbA_2, and the start of activities for the HbF working group.

1.1. Why standardize HbA_2 and HbF

Measurement of hemoglobin A_2 is an important parameter for beta thalassemia diagnosis and carrier screening [3,4]. In noncarriers, HbA_2 values are typically between 2 and 3 %, while in carriers they are generally above 4 %. There is a zone of intermediate values (borderline values) that complicate carrier identification and require further molecular investigation [5], it is therefore important that the measurement be accurate. Furthermore, it has been highlighted that HbA_2 may represent an innovative therapeutic tool for the treatment of sickle cell anemia [6]. Indeed, unlike fetal hemoglobin, hemoglobin A_2 has

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functional properties very similar to those of hemoglobin A and has a pancellular distribution. Preliminary experiments in mice have proven that induction with a hybrid transcription factor allowed the expression of nearly 20 % hemoglobin A_2, with beneficial effects toward sickle cell polymerization [7].

The working group has defined analytical goals for HbA_2, based on both biological variability data and expert opinion [8]. Unfortunately, evaluation of the current methods, using both external quality assessment data [9] and inter-laboratory comparisons [10] assessed against the analytical goals demonstrated unacceptable variability. This justifies the effort to establish the production of at least one reference measurement procedure and certified reference materials.

The measurement of Hb F in red cells is clinically useful in the diagnosis of δβ thalassemia, because the amount of Hb F is raised in this condition [11]. Hb F is also slightly elevated (1–5 %) in pregnancy, in hereditary persistence of fetal hemoglobin (HPFH) and sometimes in β thalassemia trait, as well as in non-deletional HPFH (most often above 20 %). The determination of HbF is also useful in the diagnosis and management of sickle cell disease as well as in monitoring hydroxycarbamide therapy. Moreover, HbF can be raised in various other acquired conditions [4], and it has been proposed as a predictor of better outcome in some myelodisplastic and leukemic syndromes [12]. HbF measurements are also used for monitoring feto-maternal hemorrhage and for neonatal screening. If the time of gestation is taken into account, the ratio between HbF and HbA could be indicative of β-thalassemia trait. Finally, recent evidence from innovative clinical trials for sickle cell disease (SCD) and β thalassemia based on genome editing support the design of innovative therapies based on fetal hemoglobin production [13,14]. This is very relevant, since the burden of sickle cell disease is expected to continue to rise in the next decades [15]. Unfortunately, currently available measurements are poor predominantly at lower concentrations, as evidenced from various EQAS exercises [16]. Typically, inter-laboratories CVs are around 11 % at HbF near 4 %, and increase up to 50 % for HbF < 1 %. The reason is that there is no reference system available, nor reference measurement procedures or certified reference materials (CRMs).

2. Establishing a reference system for HbA_2

Fig. 1 shows the traceability chain proposed for the determination of HbA_2, developed by the WG, adapted from a previous publication [17]. At the top of the chain the definition of the measurand is declared, in terms of the amount-of-substance fraction of HbA_2 versus total hemoglobin. This enables HbA_2 to be reported in terms of percentage to total hemoglobin, instead of mmol/mol, thus simplifying the implementation of the reference system for the end users.

As calibrants, recombinant HbA_0 and HbA_2 ($\alpha_2\beta_2$ and $\alpha_2\delta_2$), unlabeled as well as in ^{15}N-labeled form (99 % isotopic enrichment), were obtained by custom synthesis from Trenzyme GmbH (Konstanz, DE). Production was based on co-expression of N-terminally His-tagged α- and untagged β- (or δ-, resp.) subunits in *E. coli*. Purification was performed by Nickel affinity chromatography according to standard protocols. The purity assessment for the HbA_2 is described in [18] and the purity uncertainty was estimated at 0.04 % (k = 2). For the HbA_0 calibrant, the purity after the affinity chromatography was determined at 99.5 % with an uncertainty of 0.06 % (k = 2) assuming a rectangular distribution. The calibrants for HbA_2 and HbA_0 were value assigned by amino acid analysis performed by two laboratories according to previous papers [19,20]. The results of the purity assessments for the calibrants are reported in Table 1. As both calibrants were used for the value assignment of the CRMs, a combined uncertainty $u_{cal,\ rel}$ was estimated by combining the two uncertainties from the two calibration solutions described above so $u_{cal,\ rel} = 0.747$ %.

With regard to the Reference Measurement Procedure (RMP), the laboratories participating in the certification of the CRMs used the same measurement procedure for the amount-of-substance fraction of HbA_2 versus total hemoglobin, previously described [21]. The method uses isotope dilution mass spectrometry (ID-HPLC-MS/MS). The whole sample is subjected to tryptic digestion. After the digestion, signature peptides for the α- and δ-chains are quantified. Two peptides are quantified for the δ-chain and three peptides are quantified for the α-chain. The RMP has been submitted to the JCTLM, in order to be listed in the JCTLM database.

The CRMs were prepared from donations of pre-filtered erythrocytes

Table 1
Uncertainty budget for purified HbA_0 and HbA_2 molalities (reproduced with permission from Ref. [23]).

Analytes	p	Mean molality	S (nmol/g)	$U_{char,\ cal\ rel}$ (%)	$U_{pur,\ rel}$ (%)	$U_{calibrant,\ rel}$ (%)
HbA_0	2	1.611	0.238	0.688	0.145	0.703
HbA_2	2	0.426	0.030	0.252	0.020	0.253

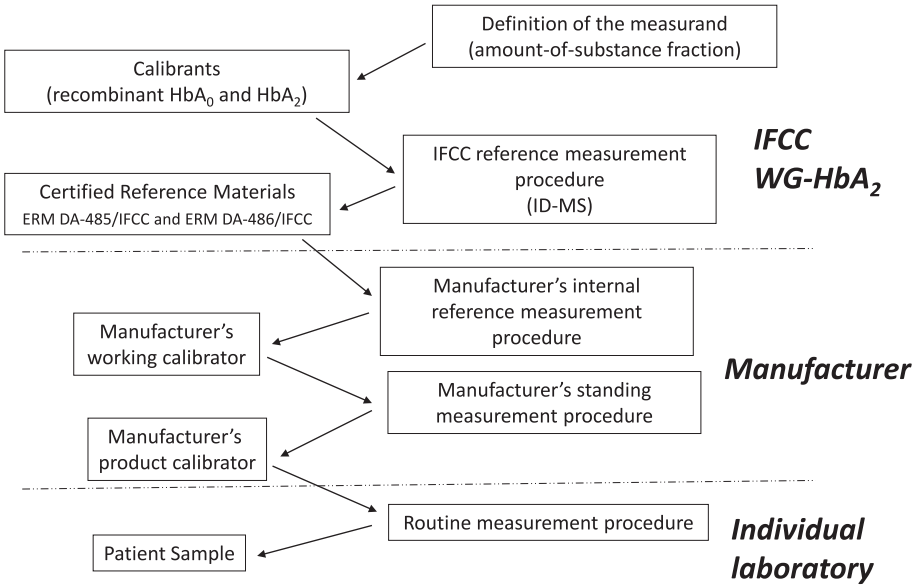

Fig. 1. Traceability chain for the determination of HbA_2 (adapted from Ref. [17]).

in citrate–phosphate-dextrose and saline-adenine-glucose-mannitol. The individual donations were collected between 17/01/2020 and 27/07/2020, following informed consent, at Policlinico San Matteo (Pavia, IT). Each portion of donated blood used for the production of both materials was tested for the presence of HBs antigen, HCV antibodies and for HIV1/HIV2 antibodies and found to be negative. Processing was performed as described in a previous publication [22]. The influence of oxidation was minimized by storing the material in containers filled with nitrogen with an extra level of protection by closing the sachets in an argon atmosphere, which reduces the presence of oxygen. Each vial produced contained nearly 50 mg of lyophilized hemolysate. Before assignment of the HbA_2 title by the RMP, the CRMs were assessed with regard to homogeneity, transport stability, storage stability in the short- and long-term, and commutability. Details of the procedures followed are available in the final certification report [23].

The homogeneity studies showed no outlying unit means or trends in the filling sequence for ERM-DA486/IFCC. For ERM-DA485/IFCC a trend in the filling sequence was noted and, therefore, an additional rectangular uncertainty was added to the homogeneity uncertainty contribution. The stability studies were carried out using an isochronous design [24]. In this approach, units were stored for a particular length of time in different temperature conditions. Afterwards, the units were moved to conditions where further degradation can be assumed negligible (reference conditions). At the end of the isochronous storage, the samples were analyzed simultaneously under repeatability/intermediate precision conditions.

The results of the transport stability study proved that the CRMs are stable at 4 weeks when transported at −20 °C. The results of the storage stability studies proved that the material is stable up to one year at −20 °C. An uncertainty for 1 year at this temperature has been included in the final uncertainty budget. The materials are included in the JRC's regular stability monitoring programme, to control their further stability.

The commutability study was carried out according to the guidelines of the IFCC working group on Commutability using the 'difference in bias analysis' [25] and the 'calibration effectiveness analysis'. In the first part of the study, the commutability of the two CRMs was evaluated based on their calibration effectiveness for nine IVD measurement procedures (MP). In total, forty-two clinical samples (CS) were measured with each of the nine IVD MP. The obtained measurement results were used to evaluate the MP to MP variability before the mathematical recalibration. The variability was calculated as the inter-measurement procedure bias range (IMPBR) as described in the IFCC guidelines [26]. Afterwards a mathematical recalibration of the measurement results of each MP was done based the measurement results obtained of the two CRMs. These results show that the mathematical recalibration of the 9 IVD MPs to the certified values of the 2 CRMs led to a significant improvement of the between-MP variability (i.e. from 17.1 % to 10.9 %). However, they also indicate the presence of a non-commutability bias for one method. When this MP is excluded from the analysis the IMPBR over the eight remaining IVD-MPs lowered from 17.1 % to 5.8 %. This IMPBR is considered to be appropriate for the intended use of the CRMs.

In the second part of the study, the difference in bias analysis approach was used to assure that there was no significant non-commutability bias between the RMP and the individual IVD MPs. For this, twenty of the CS used in part 1 of the commutability study were also measured with the RMP. Due to the labour-intensive nature of the RMP it was not feasible to measure all forty two CS. The commutability of the CRMs was assessed for each combination of the reference method with one routine IVD method using the difference in bias approach and a commutability criterion was set at 7.5 %. The results of the study show the absence of a significant non-commutability bias for the 2 CRMs between the reference method and eight of the IVD MPs. For one IVD MP, ERM-DA485/IFCC showed a significant non-commutability bias while ERM-DA486/IFCC was commutable. This observation is in line with the outcome of the first part of the study and, at the moment, it is not clear if the non-commutability bias is real or if it was caused by a measurement error or technical mistake when the ERM-DA485/IFCC was measured with the specific IVD-MP. ERM-DA485/IFCC was shown to be commutable for combinations of the reference method with Bio-Rad D10, BioRad Variant II beta-thal, BioRad Variant II dual kit, Tosoh G11, Menarini HbNext, Arkray HA-8180 T, Trinity Premier HR and Sebia Capillarys3 Tera. ERM-DA486/IFCC was shown to be commutable for combinations of the reference method with BioRad D10, BioRad Variant II beta-thal, BioRad Variant II dual kit, Tosoh G11, Menarini HbNext, Arkray HA-8180 T, Trinity Premier HR, Sebia Capillarys3 Tera and Helena V8 ultrascreen.

Finally, the values assignment was carried out by performing a characterization study resulting in two data sets (each of twenty-four measurements spread over two measurement series) per measurand. The obtained data were evaluated against compliance with the requested instructions and their technical validity, as follows: (a)compliance with the instructions given (sample preparations and measurements performed on four days, and the measurement sequence); (b) absence of values given as below LOD or below limit of quantification (LOQ). Based on the assessed criteria, no data sets were found to be technically invalid. The unweighted mean of the means of the accepted data sets were assigned as the certified value for each material. The values assigned to the materials, expressed as amount-of substance of HbA_2 versus total Hb, were 2.78 % (U[1] 0.18 %) and 5.86 % (U 0.20 %) for ERM-DA485/FCC and ERM-DA486/IFCC, respectively. It is worth noting that these uncertainties are significantly lower than the ones quoted when the RMP was defined (i.e. 5.2 and 5.1 %, for samples with normal and elevated HbA_2 fractions respectively) [21].

These materials were produced and certified in accordance with ISO 17034:2016 [27] and ISO 33405:2024 [28]. ERM-DA485/IFCC and ERM-DA486/IFCC were produced within the scope of ISO 17034 accreditation. The materials are intended for the calibration of value transfer methods for blood-based protein standards and control materials used for the quantification of HbA_2 by routine methods. The CRMs will be submitted to JCTLM to be listed in the JCTLM database for higher-order reference materials.

3. Establishing a reference system for HbF

A WG dedicated to the standardization of HbF was established by the IFCC in 2023. Due to the ongoing activities related to the standardization of HbA_2, which most of the members of the new WG are still engaged in, most of the work is outstanding. Nevertheless, a few points were endorsed, as follows.

With regard to the definition of the measurand, this was more complex than for HbA_2, as briefly discussed here. HbF ($\alpha_2\gamma_2$) is formed by two α- and two γ-globin chains consisting of 141 and 146 amino acids, respectively. While α-chains are the same as those contained in adult hemoglobins, i.e. HbA ($\alpha_2\beta_2$) and HbA_2 ($\alpha_2\delta_2$), γ-chains are characteristic for HbF and differ from the similar β-chain by 39 amino acids. Two types of γ-chains can be present in HbF, $^{G}\gamma$ and $^{A}\gamma$, they are functionally identical but differ at position 136 where the amino acid present is either an alanine or a glycine. Moreover, a common variant of $^{A}\gamma$ chain is $^{A}\gamma^{T}$ where there is an isoleucine to threonine substitution at position 75. $^{G}\gamma$ and $^{A}\gamma$ globin chains are encoded by two distinct genes located in the β-globin cluster on chromosome 11. The $^{G}\gamma$:$^{A}\gamma$ ratio is around 70:30 at birth and usually 40:60 in the trace amount of HbF found in the adult. Changes in this ratio have been observed in some hemoglobin disorders [29,30]. Based on these considerations, it was determined that the measurand would be defined as total HbF (i.e. the sum of the two fetal hemoglobin tetramers, both the $^{G}\gamma$ chain, the $^{A}\gamma$ chain) over total Hb.

With regard to the calibrants, recombinant HbA_0 and HbF ($\alpha_2\beta_2$ and

[1] U=expanded uncertainty with a coverage factor k=2.18 and k=2.36, for the ERM-DA485/IFCC and ERM-DA486/IFCC, respectively.

$\alpha_2\gamma_2$), unlabeled as well as in ^{15}N-labeled form ($\geq$ 99 % isotopic purity enrichment), will be obtained by custom synthesis from a biotech Company (an open call from the JRC is under preparation), as previously described for the HbA_2 reference system. These will be purified and characterized in the same way.

With regard to the RMP, this will be designed and validated using the same approach as for HbA_2 (isotopic LC-MS/MS after tryptic digestion), in three different laboratories. In this new WG a manufacturer (Arkray Inc. Japan) will also participate to the development of the RMP, which will enlarge the network of the reference laboratories.

Finally, with regard to the CRMs, these will be produced starting from donations of pre-filtered erythrocytes, as well as from placental blood in order to be able to produce mixtures of hemolysates covering high levels of HbF (nearly 20 %). The certification of these CRMs for HbF will follow the design previously illustrated for the release of the ERM-DA485/IFCC and ERM-DA486/IFCC.

4. Monitoring the implementation of the standardization

A roadmap for the standardization of HbA_2 was prepared by our group recently [31], briefly outlining progress so far, and what is required in the next few years to reach global standardization. In our opinion, several stakeholders need to be involved, including manufacturers, metrology institutions, External Quality Assurances Scheme (EQAS) providers, Reference Material producers, laboratory professionals, scientific societies and public health institutions. Several actions could be implemented to assess the degree of standardization obtained for example, inter-comparison studies using split samples distributed to a selected group of laboratories. Another approach is the evaluation of HbA_2 results obtained from current methods using commutable materials submitted for EQAS exercises, from the same centers over a number of years. This has been previously performed with results obtained from 15 Italian laboratories, using five different analytical methods [32] and shown to be a reliable process to evaluate the implementation of the standardization.

Given that the Reference system for HbA_2 has now been established, there is a requirement for the manufacturers to produce and release IFCC traceable reagents before such exercises to evaluate the impact can be initiated. It is estimated that this may require 6 to 12 months, depending on the manufacturer and on their regulatory bodies. The same approach will be followed for the implementation of HbF, standardization once the Reference System is established.

5. Conclusions

As per bottom-up proteomics protocols, the Reference Measurement Procedure for HbA_2 has been designed in such a way that intact tetrameric HbA_2 and HbA in the native and labeled form are used for calibration rather than synthetic signature peptides, in order to mimic the measurand in clinical samples. This allows a potentially incomplete tryptic digestion of Hb to be accounted for and preservation of the traceability of the measurand over the whole measurement process. Additionally, the use of an intact protein calibrator enables the analysis of several signature peptides of one protein, thus providing a statistically sound result for the target protein. In the literature, similar protocols have been designed to establish RMP for other measurands, such as growth hormone, procalcitonin and tau-protein [33–35], and we believe this is the best way to define RMP for proteins. We envision that the same principle will allow the development of a similarly robust RMP for HbF in the near future.

Finally, with regard to the uncertainty of the measurements performed in the laboratory, we believe that the relatively small uncertainty calculated for the Certified Reference Materials for HbA_2 will allow the laboratory professional to measure HbA_2 with more confidence. This can be demonstrated by examples following the standardization of HbA_{1c} which show that a final contained uncertainty (either on a normal or on a diabetic sample) can be attained at laboratory level [36]. If the same level of uncertainty is obtained for HbA_2, we may be confident that the laboratory can easily meet the minimum quality level of total error for HbA_2 (i.e. 4.5 %) established on the basis of biological variability studies [6].

C.A. declares no conflict of interest.
P.K. declares no conflict of interest.
K.H. declares no conflict of interest.
Y.D. declares no conflict of interest.
C.A. is employed by ARKRAY Inc., Kyoto.
A. Murakami is employed by ARKRAY Inc., Kyoto.
G.A. declares no conflict of interest.

CRediT authorship contribution statement

Andrea Mosca: Writing – review & editing, Supervision, Project administration, Investigation, Conceptualization. **Cristian Arsene:** Methodology, Investigation. **Renata Paleari:** Methodology, Investigation. **Patricia Kaiser:** Methodology, Investigation. **Kees Harteveld:** Supervision. **Yvonne Daniel:** Writing – review & editing, Supervision. **Chie Amano:** Methodology. **Atsushi Murakami:** Methodology. **Guy Auclair:** Supervision, Methodology, Investigation, Funding acquisition.

Declaration of competing interest

A. Mosca and R.P. are components of the University of Milano, carrying out researches on clinical studies, methods development and validation for diabetes and hemoglobin related disorders, sometimes sponsored by various Companies. Both have received speakers' honoraria or consulting fees from Menarini Diagnostics, Sebia, and Tosoh Bioscience.

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Data availability

Data will be made available on request.

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