Abstract

HbA₁c was discovered in the late 1960s and its use as marker of glycemic control has gradually increased over the course of the last four decades. Recognized as the gold standard of diabetic survey, this parameter was successfully implemented in clinical practice in the 1970s and 1980s and internationally standardized in the 1990s and 2000s. The use of standardized and well-controlled methods, with well-defined performance criteria, has recently opened new directions for HbA₁c use in patient care, e.g., for diabetes diagnosis. Many reports devoted to HbA₁c have been published in *Clinical Chemistry and Laboratory Medicine* (CCLM) journal. This review reminds the major steps of HbA₁c history, with a special emphasis on the contribution of CCLM in this field.

Keywords: *Clinical Chemistry and Laboratory Medicine*; diabetes mellitus; glycation; HbA₁c; history.

Introduction

The discovery of glycated hemoglobins, especially of its major component HbA₁c, in the late 1960s, and the implementation of their evaluation in clinical practice in the 1970s, have constituted milestones in the follow-up and treatment of patients with diabetes mellitus. HbA₁c measurement is probably one of the most significant advances made in laboratory medicine for patient care during the course of the last four decades. This review will provide an overview of the history of HbA₁c, mainly through the contribution of the *Clinical Chemistry and Laboratory Medicine* (CCLM) journal. Even though in the early years no publications were made in the journal because the topic was more relevant to basic research, it was possible, as soon as HbA₁c entered the field of laboratory medicine, to follow the major steps of its history through CCLM.

The times of pioneers and discoveries

Following older works of lesser impact, the heterogeneity of human hemoglobin (Hb) has been clearly demonstrated for the first time by Allen et al. in 1958 using cation exchange chromatography [1]. Other groups confirmed this heterogeneity and established the identity between Hb fractions separated by chromatography and by electrophoresis [2, 3]. These early experiments showed the unexpected elution of minor Hb peaks (called “fast hemoglobins”, or HbA₁) before the major HbA fraction (eventually called HbA₀). These peaks were designed as HbA₁a to HbA₁e on the basis of their chromatographic elution order [1–4]. They were primarily considered genetically determined Hb fractions, although their formation from HbA had been suggested since 1966 [5]. It eventually turned out that these Hb fractions resulted from the binding of various adducts to HbA, leading to changes in physicochemical properties of the molecules (e.g., electric charge) which allowed their separation. After many investigations aimed at identifying the bound components [6], the structures of these different fractions have been progressively discovered, and it was shown in the late 1970s that various sugars or sugar phosphates were able to form these minor hemoglobins, which were incidently glycated hemoglobins [7]. Glucose was unequivocally identified to generate the most abundant HbA₁c fraction HbA₁c, which was shown to be an Amadori product formed by the irreversible binding of glucose to the β-N-terminal valine residues of globin chains, rearranged into 1-deoxy-1-N-valyl-fructose [8–12] and present in the ring form [13]. This process was
demonstrated to occur during the 120-day lifespan of the erythrocytes [14], glycated Hb content being higher in older than in younger red blood cells [15, 16]. Although mentioned in 1966 [6], the intermediary formation of a labile Schiff base (labile HbA\textsubscript{1c}, Hb pre-A\textsubscript{1c}) preceding the Amadori rearrangement and the formation of the characteristic keto-amine linkage was formally described in 1981 only [17].

The structures of the other minor components have been discovered in the following years. If reasonable evidences suggested that HbA\textsubscript{1a}, and HbA\textsubscript{1b}, were characterized by the binding of fructose-1,6-bisphosphate and glucose-6-phosphate to the \(\beta\) globin N-terminal extremities, respectively [18, 19], HbA\textsubscript{1c} structure was described in 1991 only, pyruvate being identified as the characteristic adduct [20].

Simultaneously, it was demonstrated that \(\beta\)-N-terminal valine residues although being the preferential sites of glycation in vivo as well as in vitro [21] were not the sole modified amino acid residues and that a significant percentage of HbA\textsubscript{c} was glycated on side chains of lysine residues [21–23]. More than that, it was shown that the other normal hemoglobin species HbA\textsubscript{2} and HbF [24, 25], but also variants [26–28], were glycated like HbA, although the glycation kinetics could be different [29].

For many years, the non-enzymatic character of the glycation reaction was not clearly demonstrated. This is probably the reason why the wrong terms of glycosylation, glucosylation or glycosylated hemoglobins were primarily used instead of glycation or glycated hemoglobins for describing this process and the compounds formed [30, 31]. We had hypothesized in our laboratory that a part of HbA\textsubscript{1c} could be enzymatically formed by reaction of hemoglobin with erythrocyte membrane glycoconjugates [32, 33], but we have not had the opportunity to confirm this proposition with more specific and modern methods.

It was progressively proven that glycation was not restricted to hemoglobin, but was a general process affecting all the proteins in the organism, intra- or extracellular, circulating or tissular [34–36]. In the early 1980s, glycation was demonstrated to progress continuously, especially in proteins with long half-lives (e.g., skin collagen [37]), and to form, mainly by oxidative processes (the general process being referred to as glycoxidation), complex products called Advanced Glycation End Products (AGEs), suspected to participate in degenerative long-term complications of diabetes mellitus and other chronic diseases [38]. The structure of many of these components, their pathophysiological roles and their use as biomarkers have been established [39]. They corresponded to the Maillard products, described in 1912 in food industry when reducing sugars were heated with amino acids or proteins during sterilization processes [40]. In an evolutionary perspective, the ancient demonstration by Bunn and Higgins that glucose was the least reactive among aldohexoses was very interesting, suggesting that this ose had been chosen by the organism as the most important metabolic fuel because of its lower ability to induce glycoxidation-dependent damages [41].

The times of clinical enthusiasm

As early as 1962, Huisman and Dozy had shown the increase of the HbA\textsubscript{1} fraction in red blood cells of diabetic patients, raising the hypothesis of a reaction of HbA\textsubscript{1c} with “components other than glutathione”, but not especially with glucose [15]. The interest for HbA\textsubscript{1c} increased rapidly when Rahbar described in 1968 the elevated percentage of this fraction to total hemoglobin in diabetic patients [42, 43]. One of the first systematic demonstration of HbA\textsubscript{1c} increase in diabetic patients was made by Trivelli et al. in 1971 [44].

Following these founding works, a tremendous number of diabetology units appropriated this parameter despite the lack of reliable and validated assay methods, and many clinical studies were published describing the close relationship between HbA\textsubscript{1c} values and diabetes control. Thus, it was suggested in the mid and late 1970s to perform a periodic monitoring of HbA\textsubscript{1c} in routine practice for retrospectively documenting glycemic control [45, 46].

Whereas reservations were made on the significance of the parameter and on the necessity of technological improvements to reach the necessary quality in patient care [47], many reports further confirmed or discussed the huge potential of this new parameter in monitoring diabetes mellitus [48–50]. However, an indisputable scientific demonstration of the semiological value of HbA\textsubscript{1c} measurement was still lacking. In this respect, the most important advances were made by the large-scale epidemiologic studies of the Diabetes Control and Complications Trial (DCCT) in type 1 diabetes [51] and the United Kingdom Prospective Diabetes Study (UKPDS) group in type 2 diabetes [52, 53]. They clearly demonstrated the link between HbA\textsubscript{1c} values and degenerative long-term complications, underlining the need for an optimal control of glycemic balance using the HbA\textsubscript{1c} marker.

During this period, a number of methods based on various principles (i.e., ion-exchange chromatography, affinity chromatography, electrophoresis, colorimetric assay, immunoassay) and methodologies (i.e., micro- and minicolumns of chromatography, low pressure liquid
chromatography (LPLC), high pressure liquid chromatography (HPLC), manual assays, automated assays] were proposed for assaying HbA\textsubscript{1c}, evaluated and compared [54–56]. Besides, other tests were designed for measuring total (fructosamine assay) or specific (e.g., albumin or low density lipoproteins) glycated plasma proteins, as complementary tools for assessing diabetic control when HbA\textsubscript{1c} was not fully informative, especially in the case of shortened red blood cell lifespan or in the presence of a hemoglobin variant [57–64]. Various studies were also published in the journal, demonstrating the biological consequences of glycation and glycoxidation on protein functions [65, 66] especially in the case of lipoproteins [67–69]. Besides, the link between serum protein glycation and urinary albumin excretion (incorrectly referred to as “microalbuminuria”), another newly discovered marker of diabetic complications, was also underlined [70, 71].

Thus, HbA\textsubscript{1c} had become an essential parameter for patient care in diabetes mellitus in the 1980s [72], although many methods relying on different principles and without standardized procedures were used [73].

**The times of standardization**

It rapidly turned out in the 1990s that HbA\textsubscript{1c} results were not comparable from one laboratory to another and from one country to another. This was first due to the use of methods exhibiting very different analytical performances [73, 74] and second due to the lack of standardization at the international level, although standardization schemes had been implemented and evaluated in different countries like the US, Japan and Sweden [75–77], as extensively reviewed in reference [78]. Especially, in the US, the National Glycohemoglobin Standardization Program (NGSP) had implemented an efficient network of reference laboratories, monitored by a steering committee [79], which had ensured the certification of many routine methods. Besides, HbA\textsubscript{1c} values reported in the key clinical studies of DCCT [51] and UKPDS [52] validating the use of this parameter in clinical practice and in research were based on NGSP derived numbers [77].

Unfortunately, the reference system supporting the NGSP standardization, based on a cation-exchange chromatography method using the Bio-Rex 70 resin, was not specific enough to robustly support the long-term international standardization of the assay [80]. For that reason, a working group (WG) on HbA\textsubscript{1c} was created in the mid 1990s under the auspices of the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) in order to address this problem. The ultimate mission of this IFCC-WG was to design a reference method and to produce adapted reference materials. Taking advantage of previous publications reporting the preparation and evaluation of various control [81, 82] or reference materials [83] for HbA\textsubscript{1c}, this WG proposed in 1998 a candidate primary reference material, made of purified β-N-terminal glycated (HbA\textsubscript{1c}) and non-glycated (HbA\textsubscript{0}) HbA [84], which was used for establishing the internationally approved IFCC reference method for HbA\textsubscript{1c}. This method relied on the separation and quantification by HPLC coupled to mass spectrometry (LC/MS) or capillary electrophoresis (LC/CE) of HbA\textsubscript{0} and HbA\textsubscript{1c} N-terminal hexapeptides obtained by enzymatic digestion, and was published in 2002 in CCLM [85].

The implementation of the new IFCC reference system and of HbA\textsubscript{1c} results traceable to this method was managed by the IFCC-WG on HbA\textsubscript{1c} [86], as detailed in the journal in 2007. Indications were given regarding the standardization process, the change of units and the terminology used [87, 88]. Indeed, the adoption of the new IFCC reference method implied different changes in result management. The most important issue was related to the units used. Owing to the different specificities of the methods, the results of HbA\textsubscript{1c} expressed in percentages of total hemoglobin were different, being approximately lower by 2% in absolute value with the IFCC system compared to the NGSP system. The same held true for the relationship with the other national standardization schemes. As such a change would have been unsuitable for clinical purpose, it was recommended to express the results as a molar ratio of HbA\textsubscript{1c} to HbA\textsubscript{0} in mmol/mol (SI units, or “IFCC units”) in place of percentage of HbA\textsubscript{1c} to total Hb (“NGSP units”) [89]. Master equations were calculated to correlate IFCC values with values obtained by the other methods, in order to unequivocally establish the correspondence between the different systems [90]. This decision was an important change and as a consequence could lead to confusion in clinician and patient populations. It was claimed that a significant modification of numeric results could lead to inappropriate therapeutic decisions and even reduce metabolic control [91].

Another issue was widely debated in this journal, related to HbA\textsubscript{1c} name and nomenclature. It was suggested by the committee on Nomenclature, Properties and Units (C-NPU) of the IFCC to use the official denomination of HbA\textsubscript{1c}, “[hemoglobin beta chain (Blood)-N-(1-deoxyfructos-1-yl)hemoglobin beta chain; substance fraction]”, instead of “[hemoglobin (Fe; Blood)-hemoglobin A1c (Fe); substance fraction]”, and to use “DOF haemoglobin fraction” or even “DOF haemoglobin” instead of HbA\textsubscript{1c} in the daily
laboratory speech [89]. The use of this term was subject to controversy [92], but it was finally agreed that neither the systematic name nor the abbreviation would be used in clinical practice, HbA1c being the preferred term [93].

As the implementation of the new IFCC system generated numerous concerns at the field level, a “summit” meeting [87] was organized in 2007 between the IFCC and three major diabetology societies, the American Diabetes Association (ADA), the European Association for the Study of Diabetes (EASD) and the International Diabetes Federation (IDF). A consensus was reached [94] which recognized the necessity of worldwide HbA1c standardization and acknowledged the new IFCC reference system as the only valid anchor to implement standardization of the measurements. Besides, it was recommended to report results both in mmol/mol (IFCC units) and percentages (derived NGSP units), using the master equation [90]. It was also suggested that glycemic goals appearing in clinical guidelines should concomitantly be expressed in “estimated average glucose” (eAG) or “A1c derived average glucose” (ADAG) calculated from HbA1c values, as proposed by Nathan et al. in an ambitious clinical study [95]. Although this study used adequate methodologies to assess the relationship between HbA1c and calculated glucose values [96], this item was no more retained in the updated 2010 consensus [97]. The recommendations have been implemented in many countries, in most cases in collaboration with diabetology societies [98, 99]. The worldwide standardization of HbA1c is still in progress, monitored by an integrated project (IP) of IFCC, the stability of the master equations being ensured by the IFCC international HbA1c network [86]. The use of IFCC-aligned method allowed the validation of specific reference intervals, e.g., in pregnancy [100].

The times of expansion

Whereas HbA1c was unanimously recognized as the gold standard of diabetic survey, limitations of its use were identified because of possible interferences in HbA1c assay encountered in various clinical situations.

First of all, renal failure was identified as a confounding situation: a) because of the decreased erythrocyte lifespan in patients with renal diseases; and b) because of the increased formation of carbamylated hemoglobin (cHb) due to the binding to N-terminal β chains of hemoglobin of isocyanic acid, a urea byproduct formed in excess because of hyperuremia [101]. Physico-chemical properties of cHb and HbA1c are close, which explains the poor separation of the two species in some charge-based separation techniques. Very early, a link between increased HbA1c and chronic renal failure was underlined and discussed, pointing out the role of shortened erythrocyte lifespan [102] without necessarily relating this finding to analytical interferences in the first reports [103–105]. Then, interference of cHb on HbA1c assay by electrophoresis or HPLC has been well-documented in this journal [106, 107]. Even though the technical advances have reduced the interferences [108], it turns out that cHb remains a potential concern for HbA1c assays [109].

A second critical situation is due to the presence of a hemoglobin variant and has been recognized for a long time as a confounding factor for HbA1c determination. First, the possible hemolytic disease resulting from the presence of the variant, especially in homozygous patients, could shorten RBC lifespan so that the HbA1c test lost its informative value. Second, the presence of a variant affected most of the assay methods based on separation principles, such as HPLC or electrophoresis, whereas affinity or immunological methods were less affected, depending on the variant type. Whereas most of the information was summarized in a very comprehensive way on the NGSP site [79], significant papers were published in CCLM on that topic demonstrating the interference of specific variants in HPLC [110–114] or with point-of-care testing devices [115]. Besides, it was underlined that the presence of a variant could alter the calculation of estimated average glucose [116]. Even though the evaluation of the most recent devices showed a real improvement which allowed the validation of HbA1c results in the presence of the most common variants, these occurrences must be identified and treated with caution. Still, the differences of kinetics of glycation between HbA1c and variants remain an unsolved question conditioning the clinical interpretation.

Finally, other clinical situations able to interfere with HbA1c results have been discussed in the journal. This is the case of troubles of iron metabolism [117, 118], e.g., in HIV infection [119] even in non-diabetic subjects [120]. In all occurrences, the importance of taking into account within-subject biological variations in both type 1 [121] and type 2 [122] diabetic patients and in non-diabetic subjects [123] for clinical interpretation was underlined.

After the conditions of a correct interpretation of HbA1c results in the monitoring of patients with diabetes mellitus have been established, other possibilities of use of HbA1c have been explored, especially for diagnosis. The idea to measure HbA1c for screening and diagnosis of diabetes mellitus had been suggested for many years. Already in the 1970s and early 1980s, HbA1c and/or total HbA1c were proposed as indicators of glucose intolerance [124, 125] to be used in diabetes detection programs [126]. Adaptations
of values were even suggested to adapt the criteria to elderly people [127] or to pregnant women with gestational diabetes [128]. However, other reports suggested that additional long-term studies were needed to assess the interest of this parameter [129]. Many informative but inconclusive reports were published during these decades. Eventually, in 2009, ADA proposed to use the HbA1c assay for diabetes diagnosis [130]. This proposition, which was made possible by the improved quality of most of the methods used in clinical laboratories, was considered very appealing for many reasons, such as lower preanalytical sensitivity especially for fasting, lower biological variability and easier sampling. However, this approach had several limitations, especially with reference to inter-ethnic variations and pitfalls related to RBC turnover in the presence of a Hb variant [131]. This strategy was further discussed in CCLM with special reference to population specificities, e.g., to rule out patients at high risk of developing type 2 diabetes in Spain [132] or for detecting newly diagnosed diabetes and pre-diabetes in China [133].

Simultaneously with clinical advances, major methodological improvements have been made in the recent years. Many new devices introduced on the market using different principles of HbA1c measurement were scientifically evaluated: HPLC [134, 135], immunoassay [136], enzymatic assay [137] and even capillary electrophoresis [138] which was previously considered unsuitable for routine HbA1c assay [139]. Most of these methods showed improved performances in terms of precision and specificity. This is a crucial point because desirable performances must be reached for ensuring the optimal use of HbA1c in patient care management [140]. Indeed, the necessity of an appropriate use of the test was underlined, as well for patient outcome as for control of healthcare expenses [141]. For that purpose, appropriate tools and models must be used to quantify the quality of the clinical outcome [142]. New modalities of sampling were also tested. For example, HbA1c assay from dried blood spot, which had been already proposed more than a decade ago [143], proved an acceptable, easy and inexpensive alternative for blood collection when strict preanalytical procedures were respected [144].

The times of new challenges

The increased prevalence of diabetes mellitus worldwide, which makes it a non-infectious epidemic disease, and the extended use of HbA1c in screening and diagnosis ensure a glorious future to this parameter. New evidence of clinical utility of HbA1c in various situations, e.g., in predicting cardiovascular risk even in non-diabetic patients [145], opens new possibilities for its use in patient care. Besides, other promising fields of investigation have arisen. They could help understand still unexplained variations of HbA1c in specific clinical presentations. For example, the genetic features related to deglycating or protecting enzymes, such as fructosamine-3-kinase, could explain the inter-individual variations of the glycation rates and of the severity of long-term diabetic complications [146]. Further evidence must be determined to explain the link between genetic traits and the poorly understood “glycation gap”, which refers to observed differences between HbA1c and other indicators of glycemic control like glycated proteins [147]. Along with this theoretical enigma, additional interesting fields have to be explored in laboratory medicine practice, in order to better define the input of new markers, such as glycated albumin [148] and AGEs [38]. Clinical Chemistry and Laboratory Medicine will naturally continue to be the right place [149, 150] for summarizing the lessons from the past and for facing the challenges of the future.

Conflict of interest statement

Author’s conflict of interest disclosure: The author stated that there are no conflict of interest regarding the publication of this article.
Research funding: None declared.
Employment or leadership: None declared.
Honorarium: None declared.

Received August 27, 2012; accepted August 28, 2012; previously published online September 19, 2012

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