

# Translating the A1C Assay

In the clinical management of diabetes, the A1C assay has become indispensable. Used worldwide to monitor chronic glycemia, the assay is an essential tool to determine whether a patient has achieved the core goal of therapy for diabetes: a marked and sustained reduction in plasma glucose to achieve as close to a normal level as can be safely attained. With the publication of the A1c-Derived Average Glucose (ADAG) study in this issue of *Diabetes Care* (1), the evolution of the A1C assay continues and an important milestone has been reached. To better appreciate this recent report, a brief and admittedly incomplete historical perspective may be useful.

It was 60 years ago that Allen et al. (2) showed that hemoglobin A (which makes up about 97% of total hemoglobin) contains three minor components, designated HbA<sub>1a</sub>, HbA<sub>1b</sub>, and HbA<sub>1c</sub> (A1C). In the decades that followed, we learned that a hexose molecule is attached to these components (3) and that hemoglobin A actually has two more minor glycosylated derivatives. The five altogether comprise ~5–7% of the HbA molecule (4).

In the early course of the biochemical dissection of hemoglobin, Huisman and Dozy (5) noted, virtually in passing, that the level of glycosylated hemoglobin components was increased in a few individuals they studied who happened to have diabetes. It took 4 more years, however, for Rahbar and colleagues (6,7) to document that diabetes is clearly associated with an elevation in glycosylated hemoglobin. The Rahbar reports stimulated other investigators to confirm these initial findings and to seek an explanation for how glucose binds to hemoglobin. It was not for another few years, in 1972, that Bunn et al. (8) elegantly showed that the cause of the increased glycosylated hemoglobin in diabetes, which was predominantly the A1C component, was a result of excess nonenzymatic glycation that occurred throughout the lifespan of red cells and in an essentially irreversible process.

The A1C-diabetes story then shifted from clinical chemistry to clinical medicine. Koenig et al. (9) were the first to show that A1C levels correlated well with fasting blood glucose, and they concluded that A1C levels “probably reflect

... the mean daily blood glucose concentration ... and may provide a better index of control of the diabetic patient.” Indeed, soon after their report, many other investigators confirmed a strong association between A1C and glycemic control and that the measurement had clinical utility (10–15), clearly surpassing in utility what was then the conventional assessment of metabolic control over time (e.g., signs, symptoms, urine, and blood glucose levels) (15).

The thorough biochemical experiments performed in the 1970s and 1980s, most notably by Mortensen and Christophersen (16), demonstrated that the fraction of A1C in a sample depends on the glucose levels over a previous period, along with red cell turnover, reaching a steady state sometime between 4 and 12 weeks. Such kinetics were supported by many clinical studies in both type 1 and type 2 diabetic patients where the A1C level was found to correlate well with glucose regulation (17) or the mean blood glucose derived over time from multiple fingersticks (9,15,18–24).

As the use of the A1C test gained traction, dozens of different analytical methods based on different assay principles (e.g., ion-exchange chromatography, affinity chromatography, immunoassay, and electrophoresis) were used to measure glycosylated hemoglobin. Without a common reference method and in the absence of a standardized assay, results varied considerably when the same sample was tested by different laboratories or methods or even when the same sample was tested repeatedly by one methodology. It was quite common, for example, to have values ranging from 4.0 to 8.1% on the same blood sample (25). In addition, the assays used then (and even now) in clinical medicine not only measured A1C itself but also more or lesser amounts of the other glycosylated hemoglobin components, and results were reported as A1C, HbA<sub>1</sub>, or total glycosylated hemoglobin. The results were also influenced by other interfering substances in the sample.

The Diabetes Control and Complications Trial (DCCT) Study Group, recognizing these problems, centralized the measurement of A1C from the onset of the study so as to avoid confounding re-

sults if such a key analyte were to be measured at many sites (26). Also, in anticipation of the DCCT results, the American Association for Clinical Chemistry (AACC) established, in 1993, an A1C standardization workgroup to bring consistency to the measurement of A1C and to facilitate the traceability of results back to the DCCT such that these results could be directly related to the risk or progression of diabetes complications.

After the standardization protocol was developed, the American Association for Clinical Chemistry group was dissolved and the National Glycohemoglobin Standardization Program (NGSP) began in 1996 (27). Briefly, in the NGSP, the reference method is the measurement of A1C by ion-exchange high-performance liquid chromatography, as was used in the DCCT. Manufacturers of testing equipment can receive NGSP certification if their instruments are calibrated to match the results obtained by the NGSP. Laboratories can also be certified by the same protocol and thereby document optimal performance in their setting.

All this has led to a dramatic reduction in interlaboratory variability and a marked improvement in the precision and comparability of values (28). In 2007, ~99% of all A1C test results in the U.S. were traceable to those obtained in the DCCT, with similar percentages in test results throughout the U.K. and in Canada (D. Sacks, personal communication). Although comparable data are not readily available from other countries, it appears that much of the world's A1C testing is traceable to the DCCT numbers.

Still, issues remain. First, the high-performance liquid chromatography reference method used by the NGSP is somewhat nonspecific in that the methodology, like many others, measures more than just A1C in a sample. Although this problem is obviated by the consistent use of one reference method, in the world of clinical chemistry, this situation is “metrologically unsound.” Second, although most methods used worldwide are NGSP certified, there are other standardization programs, most notably in Japan (29) and in Sweden (30). Thus,

there is no truly international standardization program.

Both of these issues led the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) in 1995 to embark on the development of a reference method that would be very specific, i.e., only measures A1C, and that could lead to worldwide standardization based on a metrologically sound international measurement system (31). Not only did the IFCC succeed (32,33) in developing such an assay but the reference method has been approved by all of their member societies, and a global network of reference laboratories has been established (33).

But progress often brings other difficulties and problems. First, the IFCC method is very complicated, requires costly equipment (a mass spectrometer), and is very expensive. Thus, as with many other reference methods, it cannot be used by a clinical laboratory to measure A1C in routine samples. That means it can only be used to calibrate laboratory instruments that measure A1C as before, i.e., by any one of a wide variety of methods. Although disappointing, this does not diminish the virtue of now having a much more robust standardization program.

Second, and much more important, since the new reference method measures A1C itself, and thus non-A1C components are no longer detected, the normal range for A1C is reduced—by about two percentage points lower than that currently reported. Moreover, the IFCC recommended (to be metrologically correct) that A1C be expressed in millimoles A1C per mole of total hemoglobin; this would result in a normal range of around 29–43 mmol A1C/mol hemoglobin (34).

A shift to lower A1C percentages would no doubt be intolerably confusing and likely lead to a deterioration in glycemic control (35), but a wholesale shift to the IFCC units would surely create mayhem. Although one could clearly program a laboratory instrument to convert the new IFCC values to DCCT-derived values, the IFCC maintained that the expression of an analyte as a percentage is not metrologically sound and thus should not be used. In response to the direction proposed by the IFCC, an American Diabetes Association/European Association for the Study of Diabetes/International Diabetes Federation workgroup was formed (including some of the IFCC leadership) to

make recommendations on how this impending crisis could be avoided (36).

What emerged was not only the recommendation that DCCT-derived numbers should be maintained if possible but also that an international study should commence to look more closely at the relationship between A1C and mean blood glucose. If the study was “successful,” at least we could adopt an A1C-derived unit (e.g., “estimated average glucose” in milligrams per deciliter or millimoles per liter) that would obviate the IFCC objection to having laboratory results expressed as a percentage. This path forward was then ratified in an official consensus statement issued by all four organizations (37).

The rationale for another study examining the relationship between mean blood glucose and A1C stemmed from the belief that the previously published reports used a variety of measures of glucose concentration, recruited only small numbers of subjects (and mostly those with type 1 diabetes), performed measurements over a relatively short time period, and, most notably, performed relatively infrequent sampling of blood glucose (mostly during the daytime). For example, the often-cited conversion table in the American Diabetes Association Standards of Medical Care in Diabetes—2007 (38) was based on very limited capillary glucose sampling in the DCCT, and the study was actually not intended to establish the relationship between average glucose and A1C. Thus, greater confidence was needed that A1C truly represents an average glucose.

The results of the international study are now reported (1) and confirm and extend previous findings. The strengths of the study are that it examined the relationship between average glucose and A1C across a wide spectrum of A1C values—from ~5% to as high as 13%—and in more people than ever before studied. Also, both normal subjects and subjects with type 1 and type 2 diabetes were enrolled in numbers sufficient to conclude that the relationship between the two variables was consistent between these subgroups and also in relation to other important variables (i.e., age, ethnicity, smoking). Finally, the study obtained ~2,700 glucose measurements in each participant, which is far greater than the number obtained in nearly all previous studies. The results clearly support the hypothesis that there is a strong linear relationship between mean blood glucose

and A1C, with a coefficient of correlation ( $R^2$ ) of 0.84.

The data from ADAG indicate that at any mean glucose or A1C level, there is some scatter (see Fig. 1 in the ADAG report), thereby conveying a less than perfect correlation. Is that primarily due to measurement error, or does it suggest that an A1C level reflects processes beyond a straightforward time and glucose concentration–dependent glycation of hemoglobin? Addressing this uncertainty would require an even larger study conducted ideally at only one site, with more diverse subjects, uninterrupted continuous glucose monitoring for months at a time, and, most important, a measurement error much lower than currently seen. The report by Nathan et al. (39) in which 24,000 glucose measurements were done on each participant resulted in an  $R^2$  (0.81) and regression equation very similar to that reported in the ADAG study, suggesting that performing more measurements will not in itself improve the correlation.

Thus, we have ~16–19% of the variation unaccounted for, but given that there is a small measurement error in the determination of A1C (perhaps 2–5%), and a larger coefficient of variation in the measurement of glucose (10–20%), the constraints imposed by methodology can explain the residual variation.

We are unable, of course, to conclude from the study that the relationship holds for all populations. That is, many populations (e.g., Asians, Pacific Islanders, children) were not studied, and it is conceivable that the physiology of glycation differs in such groups, although there is no obvious reason why that would be so. A recent study (40) that showed a relatively poor correlation between average glucose and A1C in children should not raise doubts about the translation of the ADAG study to other populations. In that report (40), it is unclear whether the A1C values were stable throughout the study and how many glucose measurements were obtained in each participant, and there are doubts regarding the precision and accuracy of the continuous glucose-monitoring system device used and other issues (1).

In the ADAG study, the differences between various ethnic groups were not statistically significant. However, the study was not adequately powered to detect such differences and, in one group, the differences came close to being significant. Although other reports have shown





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