Hemoglobin A$_{1c}$ Measurements over Nearly Two Decades: Sustaining Comparable Values throughout the Diabetes Control and Complications Trial and the Epidemiology of Diabetes Interventions and Complications Study

Michael Steffes,[1*] Patricia Cleary,[2] David Goldstein,[3] Randie Little,[3] Hsiao-Mei Wiedmeyer,[3] Curt Rohlfing,[3] Jack England,[3] Jean Bucksa,[4] Maren Nowicki,[4] and the DCCT/EDIC Research Group

Background: **Clinical trials require assays that provide consistent results during the course of a study. The hemoglobin A$_{1c}$ (HbA$_{1c}$) assay, a measure of chronic glycemia, is critical to the study of diabetes control and complications.**

Methods: **The Diabetes Control and Complications Trial (DCCT) and its follow-up study, the Epidemiology of Diabetes Interventions and Complications (EDIC), required 20 years of consistent HbA$_{1c}$ results, measured by three different ion-exchange HPLC procedures. To maintain and document consistent HbA$_{1c}$ results measured in the DCCT and EDIC Central Biochemistry Laboratory, a backup laboratory used frozen hemolysates as long-term calibrators and a HPLC method with a single lot of Bio-Rex 70 resin.**

Results: **Over 20 years, long-term quality-control values have remained constant. Four studies of nondiabetic ranges produced nearly identical values [mean (SD), 5.1 (0.5)%, 4.9 (0.3)%, 5.0 (0.4)%, and 5.0 (0.3)%].**

Conclusion: **The overall consistency of the HbA$_{1c}$ assays during the 20-year course of the DCCT and EDIC has been critical in establishing the benefits of intensive therapy and in understanding the relationship between long-term glycemia and the development and progression of the complications of diabetes.**

© 2005 American Association for Clinical Chemistry

The Diabetes Control and Complications Trial (DCCT)[5] demonstrated the benefits of intensive glycemic control on the long-term microvascular and neurologic complications of type 1 diabetes mellitus (*1–5*). Essential to this decade-long study was the availability of a consistent, reliable hemoglobin A$_{1c}$ (HbA$_{1c}$) assay (*6*). In addition, the follow-up study of the DCCT, the Epidemiology of Diabetes Interventions and Complications (EDIC), now in its 10th year, required similarly consistent HbA$_{1c}$ results that could be compared with the DCCT results (*7, 8*). With three changes in the methodology for HbA$_{1c}$, sustaining comparable values continuously during the 20 years of the DCCT and EDIC has presented a major challenge. In this communication, we describe the measures taken to assure consistency of the HbA$_{1c}$ results over two decades of study.

[1] Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN.

[2] Biostatistics Center, the George Washington University, Rockville, MD.

[3] Departments of Pathology and Anatomical Sciences and Child Health, University of Missouri, Columbia, MO.

[4] Fairview University Medical Center, Minneapolis, MN.

*Address correspondence to this author at: University of Minnesota, Department of Laboratory Medicine and Pathology, MMC 609, 420 Delaware St. S.E., Minneapolis, MN 55454. Fax 612-273-3489; e-mail steff001@umn.edu.

Address for reprints: The DCCT/EDIC Research Group, Box NDIC/DCCT, Bethesda, MD 20892.

[5] Nonstandard abbreviations: DCCT, Diabetes Control and Complications Trial; HbA$_{1c}$, hemoglobin A$_{1c}$; EDIC, Epidemiology of Diabetes Interventions and Complications; CHL, Central Hemoglobin Laboratory; BHL, Backup Hemoglobin Laboratory; QC, quality control; NGSP, National Glycohemoglobin Standardization Program; and UKPDS, United Kingdom Prospective Diabetes Study.

Materials and Methods

OVERVIEW

The DCCT enrolled 1441 individuals with type 1 diabetes mellitus, beginning in 1983 (9). Although the DCCT ended in 1993 (1), EDIC, the follow-up study, continues to the present. To sustain the consistency of the HbA$_{1c}$ values over the combined duration of these studies, the assay systems used to measure HbA$_{1c}$ have been calibrated and monitored against a single reference procedure (6). Together these assays defined a consistent nondiabetic range (4.0–6.05%) over the course of the DCCT (1) and EDIC. Furthermore, the outcomes of these studies set the basis of the HbA$_{1c}$ treatment goal (7%) recommended by the American Diabetes Association (10).

LABORATORIES

The Central Hemoglobin Laboratory (CHL) and Backup Hemoglobin Laboratory (BHL) were established in 1983. The CHLs have analyzed all routine HbA$_{1c}$ specimens for the DCCT and EDIC. The BHL has provided external quality control (QC) and backup laboratory services for the CHL. The CHL was initially located at the Joslin Diabetes Center (Boston, MA); the BHL has been continuously located at the University of Missouri-Columbia School of Medicine (6). In 1986, all measurements of HbA$_{1c}$ were temporarily moved to the BHL for 6 months, until the CHL was moved to the University of Minnesota (Minneapolis, MN). New instruments with automated capacity [initially Diamat HPLC (Bio-Rad Laboratories) and subsequently Tosoh A1c 2.2 Plus HPLC (Tosoh Medics, Inc.)] were introduced in Minnesota. For all CHL assays, calibrators were assigned values based on comparisons between the CHL and the BHL (see below). Separate QC aliquots were assayed in the CHLs to determine acceptance of each assay run.

REFERENCE METHOD AT THE BHL

The BHL has performed the reference method for HbA$_{1c}$ during the entire course of the DCCT and EDIC, using a HPLC assay with Bio-Rex 70 ion-exchange resin, as described in detail previously (6). Whole blood was transported at 4 °C on wet ice by overnight courier from clinical centers to the BHL. The labile glycated fraction was removed by incubation of washed, packed erythrocytes in saline at 37 °C for at least 5 h, with subsequent lysis of the erythrocytes in water. Cellular debris was removed by mixing with CCl$_4$ followed by centrifugation; the hemolysate sample was injected on the HPLC column. The HbA$_{1c}$ fraction eluted after HbA$_{1a}$ and HbA$_{1b}$ and before the HbA$_0$ fractions. Percentage HbA$_{1c}$ was calculated by dividing the area under the HbA$_{1c}$ peak by the total area under all peaks.

Four lots of calibrators have been prepared at the BHL (for the BHL method only) since 1978 with blood from single nondiabetic donors according to preparation methods similar to those for patient samples. Aliquots of calibrator hemolysates for single use were stored at −70 °C or in liquid nitrogen until the day of analysis. A long-term QC sample was first prepared at the Joslin Diabetes Center laboratory (Boston, MA) in 1983, by use of pooled blood collections from patients with diabetes mellitus (6). The hemolysate preparation was similar to that prepared at the BHL. In addition to the long-term (QC) sample, the Joslin CHL also prepared low, medium, and high pooled controls, which were used at both Joslin and the BHL between 1983 and 1987. The BHL then prepared additional control materials, using single diabetic and nondiabetic donors; these control materials were used from 1987 to present.

FIRST CHL METHOD

The first CHL, based at the Joslin Diabetes Center, used a HPLC method identical to the method used at the BHL until early 1986, with calibration and internal QC procedures as described previously (6).

SECOND CHL METHOD

At the University of Minnesota (Minneapolis, MN), a Diamat HPLC dedicated ion-exchange system was used from November 1986 until April 2002 (1). The Diamat instrument was calibrated daily by use of a single calibrator provided by the manufacturer (with a value of 8–9%) standardized to the BHL reference system. Nominal values for this calibrator were adjusted based on comparisons between the CHL and BHL for the samples assayed in both the CHL and BHL. With each new lot of calibrator, values were checked and adjusted based on analysis with the previous calibrator. Occasionally (e.g., with changes in columns at the CHL), in-house whole-blood materials were exchanged between the CHL and BHL to verify CHL calibration. These comparisons were in addition to the QC specimens used by the CHL to monitor the assay with each run and to accept or reject the run. During the DCCT and EDIC, different lots of columns and reagents were provided by Bio-Rad, some with apparently different performance characteristics; these are noted when appropriate.

THIRD CHL METHOD

In April 2002, a Tosoh A1c 2.2 Plus dedicated ion-exchange HPLC system replaced the Diamat system at the CHL. The Tosoh 2.2 Plus was calibrated weekly by use of two calibrators provided by the manufacturer (usually with values of ~5% and 11% for each calibrator), with values adjusted based on comparisons with the BHL and with consistency checked as described above for the Diamat. All data reduction was performed automatically; analysis time was 3 min. The transition from the Diamat to the Tosoh instrument was monitored with 178 samples, showing consistency ($r^2 = 0.992$; $y = 1.036x − 0.236$, where x is the Diamat and y is the Tosoh 2.2 Plus) between the instruments.

EXTERNAL QC FOR CHL ANALYSES

For external QC throughout the DCCT, ~10% of the specimens were prepared as split duplicates at the clinical centers; samples were sent masked to the BHL and CHL (1). On a quarterly basis, some centers shipped additional split-duplicate specimens to both the CHL and BHL. After 1993, 10 samples were exchanged monthly between the CHL and BHL. Beginning in May 1996, this monthly 10-sample exchange became part of the National Glyco-hemoglobin Standardization Program (NGSP) network monitoring program. Differences between the BHL and CHL were used as needed to recalibrate the assays in the CHL, as described above. In addition, 5–10% of specimens were split and sent as duplicates, to check on reliability of the system from collection through shipping, analysis in the laboratory, and reporting to the Biostatistics Center at George Washington University.

REPRODUCIBILITY OF THE NONDIABETIC RANGE

As an additional check to ensure that the HbA_{1c} assay did not drift over the course of the DCCT and EDIC studies, four nondiabetic range studies were performed with the assumption that the true nondiabetic range should not change over time (1). The first study was performed in 1983 at the onset of the DCCT; the second study in 1988 after the CHL changed from Joslin to Minnesota; the third study in 1992 at the end of the DCCT; and the fourth study in 2000, during EDIC. For each study in 1983, 1985, and 1992, nondiabetic individuals were enrolled, selected to match the age and gender distributions of the DCCT/EDIC groups at baseline; in 2000, the age and gender distribution matched the contemporary age of the EDIC participants. Thus, the nondiabetic individuals in 2000 were older than the previously measured nondiabetic groups. All were recruited from all participating clinical sites in the United States and Canada. Before 2000, local glucose values were used to assure nondiabetic status. For the 2000 study, fasting glucose was measured centrally with a glucose oxidase method [on a Vitros analyzer (Ortho Clinical Diagnostics)] at the CHL to assure that no participants had impaired fasting glucose or diabetes mellitus. HbA_{1c} was analyzed by both the CHL and BHL.

STATISTICAL ANALYSES

Values are reported as the mean (SD). The coefficient of reliability was determined as: (between-specimen variance − within-specimen variance)/(between-specimen variance + within-specimen variance) (11, 12). Comparisons over time and among values were completed by use of standard parametric procedures with the SAS statistical package, Ver. 8.2, (SAS Inc.).

Results

STABILITY OF A REFERENCE METHOD FOR TWO DECADES

Yearly means for a very long term lot of frozen hemolysate used for nearly two decades as a QC specimen by the BHL are shown in Fig. 1. The overall CV for this specimen was <2% from 1984 through 2003, and results remained very close to the value originally established in 1984 [8.28 (0.10)%]. However, beginning in 2001, this lot of hemolysate demonstrated a small reduction in measured values, with clear evidence in 2003 (presumably attributable to deterioration of the material). Thus, we relied on the other control samples to demonstrate the consistency of the assay, with the overlapping lots demonstrating equally good stability over time (Fig. 1).

The nondiabetic range studies, postulated to demonstrate no changes in mean values in a healthy nondiabetic population over time, also confirmed the stability of the assay and the frozen hemolysates used for QC. The values of HbA_{1c} and selected characteristics of the nondiabetic populations over the course of the DCCT and EDIC are shown in Table 1. There were no appreciable differences over the time span evaluated, with mean HbA_{1c} values
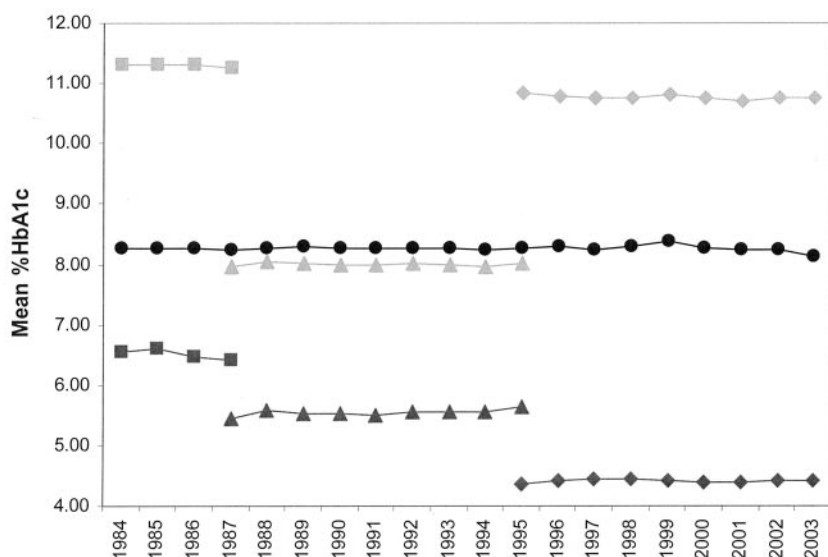

Fig. 1. Mean HbA_{1c} measured by the BHL for one very long term QC specimen (●) and six additional QC specimens measured for shorter periods to demonstrate consistency throughout the DCCT and EDIC.

Each point represents the mean of 34–653 measurements during each year of use. For each point the CV was <3%.

Table 1. HbA$_{1c}$ by age in nondiabetic study participants, with approximately equal numbers of men and women in each group or subgroup.[a]

	1983	1988	1992	2000
Mean (SD) age, years	24.9 (8.4)	25.8 (8.3)	25.2 (9.2)	39.6 (11.0)
n	119	150	151	197
Mean (SD) HbA$_{1c}$, %				
All participants	5.1 (0.5)[b]	4.9 (0.3)[c]	5.0 (0.4)[b]	5.0 (0.3)[b]
n	119	150	151	197
Under 20 years	5.1 (0.5)[b]	4.9 (0.3)[c]	5.0 (0.3)[b,c]	
n	40	44	53	
20–29 years	5.1 (0.6)[b]	4.8 (0.3)[c]	5.0 (0.4)[b]	4.9 (0.3)[b,c]
n	35	52	45	49
30–39 years	5.0 (0.4)[b]	5.0 (0.4)[b]	5.0 (0.4)[b]	5.0 (0.3)[b]
n	39	54	48	45
40–49 years				5.0 (0.3)
n				47
Over 50 years				5.2 (0.3)
n				51

[a] The data designated as years of age represent subsets (for which we have at least 30 participants) of the overall data presented as "All participants".

[b,c] For comparisons within each age group (reading horizontally), HbA$_{1c}$ values with different letters were significantly different ($P < 0.05$), whereas values sharing the same letter were not significantly different.

from 1983, 1992, and 2000 not statistically significantly different from one another. The 1988 mean was slightly but statistically significantly lower than in 1983 ($P < 0.01$), in 1992 ($P < 0.02$), and in 2000 ($P < 0.01$). Also shown in Table 1 are comparisons within different age groups (reading horizontally). The values across the different years were nearly identical, with the values from 1988 slightly lower for the <20 year and 20–29 year groups (Table 1), mirroring the results of the whole populations sampled (Table 1). HbA$_{1c}$ results from all years studied for the 30–39 year group were not significantly different.

MONITORING OF HbA$_{1c}$ VALUES IN THE CENTRAL AND REFERENCE LABORATORIES OVER TIME

The split duplicate samples (i.e., aliquots of the same collection) sent separately to the CHLs showed consistency, with a coefficient of reliability >0.99 spanning the 20 years of the DCCT/EDIC. Overall HbA$_{1c}$ values in the CHL vs the BHL differed slightly throughout the DCCT and for most of EDIC. However, despite efforts to sustain similar values, there were small differences between the CHL Diamat and the BHL, especially in the high HbA$_{1c}$ concentration range (>10% HbA$_{1c}$), which increased beginning in 1995 until the CHL Diamat was replaced by the Tosoh 2.2 Plus in 2002. We addressed the timing (and the possible causes) for the differences between the methods that occurred during EDIC by examining the differences over time and within each lot of columns and reagents supplied by Bio-Rad, believing that most of the differences occurred with changes in columns and reagents. In doing so we found that the performance of column Lot M (which was used in the laboratory March 1993 to July 1996) deteriorated over the last half of its life (data not shown). To determine the relative magnitude of the differences post-DCCT, we used the mean values of the

differences from 1987 to mid-1993 (covering the performance of the Diamat during the DCCT) as the baseline. We then subtracted the 1987–1993 values from each of the mean values in EDIC, with the results presented in Fig. 2. Only for HbA$_{1c}$ values >10% were the absolute differences in mean values >0.2%.

In April 2002, the Tosoh A1c 2.2 Plus replaced the Bio-Rad Diamat in the CHL. The performance of the method was similar to the performance of the Diamat method during the DCCT (1987–1993) at HbA$_{1c}$ concentrations <10% and was only slightly higher (0.2%) in the >10% HbA$_{1c}$ range (Fig. 2).

Discussion

The DCCT/EDIC Study and the United Kingdom Prospective Diabetes Study (UKPDS) have shown that intensive treatment of diabetes mellitus, aiming for normoglycemia, decreases the development and/or progression of long-term complications in type 1 and type 2 diabetes mellitus, respectively (1, 8, 13–15). Both the DCCT and UKPDS have also shown a strong correlation between HbA$_{1c}$ concentrations and complications (16, 17). The conclusions of these studies rest on long-term consistency of the HbA$_{1c}$ methods; i.e., the assays delineated in this report. Many other studies and clinical trials have used HbA$_{1c}$ to measure long-term glycemic control [see Krishnamurti and Steffes (18) for a review of these studies]. In the UKPDS, Cull et al. (19) developed protocols to maintain consistency of their HbA$_{1c}$ assay, similar to the DCCT/EDIC experience. The UKPDS used a Diamat assay (19) identical to that used for the greater part of the DCCT and EDIC.

By maintaining the same assay method over time at the BHL, we were able to monitor and achieve consistent HbA$_{1c}$ results throughout the DCCT and EDIC. Calibra-
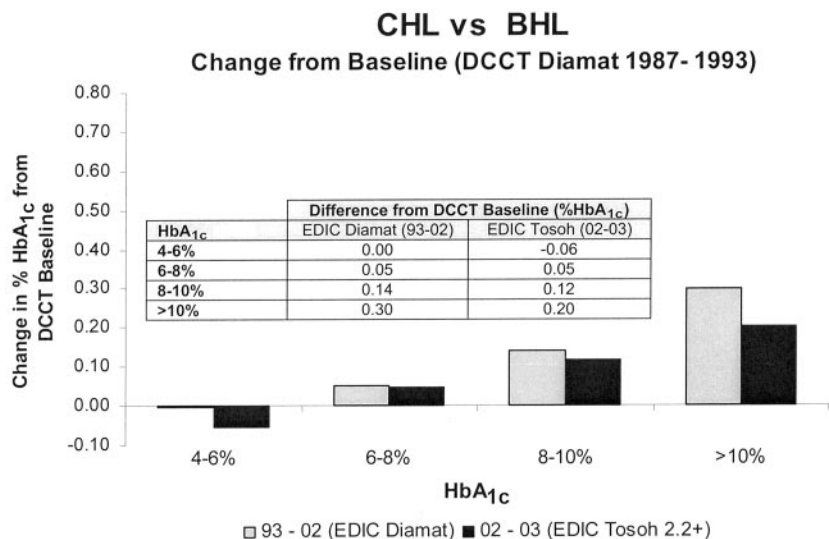
CHL vs BHL
Change from Baseline (DCCT Diamat 1987- 1993)

	Difference from DCCT Baseline (%HbA$_{1c}$)	
HbA$_{1c}$	EDIC Diamat (93-02)	EDIC Tosoh (02-03)
4-6%	0.00	-0.06
6-8%	0.05	0.05
8-10%	0.14	0.12
>10%	0.30	0.20

□ 93 - 02 (EDIC Diamat) ■ 02 - 03 (EDIC Tosoh 2.2+)

Fig. 2. Difference in HbA$_{1c}$ between the CHL and the BHL at different HbA$_{1c}$ concentrations, expressed as the change from baseline, as represented by values from the DCCT Diamat (1987–1993).

tion to the BHL reference method provided stable results at the CHL, despite several changes in HbA$_{1c}$ methodology. Moreover, the stability and precision of the HbA$_{1c}$ assay have enabled the DCCT/EDIC Research Group to assess HbA$_{1c}$ as a risk factor for complications for a decade after the DCCT ended (8, 15, 20).

The challenge of maintaining consistent values over two decades was compounded by the absence of a true standard for the assay. We were not able to precisely match the values from the CHL and BHL. The Diamat with single-point calibration was suboptimal in maintaining consistent values between the CHL and BHL at higher HbA$_{1c}$ concentrations in both the DCCT and EDIC. The Tosoh A1c 2.2 Plus method also demonstrated somewhat greater variance with the BHL at the highest HbA$_{1c}$ concentrations, although to a lesser degree than the Diamat, possibly because of the two-point calibration of the 2.2 Plus. Whether some of these differences may relate to properties of the Bio-Rex resin used in the BHL method and/or to the single-point calibration of the Bio-Rex assay is not clear. In addition, the values of calibrators may not have been optimal to produce accurate values in the high ranges. Thus, even with the consistent exchange of samples between the BHL and the CHL, at higher HbA$_{1c}$ concentrations, the differences between the methods increased during the last years of Diamat use. Perhaps this was attributable to the Diamat calibrators having values of ~8%, below the values where the BHL and CHL differed to the greatest extent. However, it should be noted that only 12% of DCCT patients and 7.5% of EDIC patients had HbA$_{1c}$ concentrations >10%. Thus, there was little impact on the conclusions from these studies over nearly two decades of the DCCT and EDIC, despite changes in HbA$_{1c}$ methodology.

The success of the NGSP in standardizing HbA$_{1c}$ testing over the past several years (21) should allow continued consistency in results over time in many laboratories. This will facilitate prospective studies of relationships between glycemia and complications and ensure comparability among studies. It will also ensure optimal use of HbA$_{1c}$ as a tool in patient care, especially with the American Diabetes Association stating a target value of 7% using methods traceable to DCCT values (10). New reference procedures have been proposed (22), and these may be used in conjunction with the NGSP to base the HbA$_{1c}$ assays on definitive standards and methods. In addition, methods continue to improve with regard to precision and specificity. For these reasons, we expect that variation of results over time and among laboratories will progressively diminish, thereby providing even more consistency of HbA$_{1c}$ results.

References

1. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group. N Engl J Med 1993;329:977–86.
2. Effect of intensive therapy on the development and progression of diabetic nephropathy in the Diabetes Control and Complications Trial. The Diabetes Control and Complications (DCCT) Research Group. Kidney Int 1995;47:1703–20.
3. Effect of intensive diabetes management on macrovascular events and risk factors in the Diabetes Control and Complications Trial. Am J Cardiol 1995;75:894–903.
4. The effect of intensive diabetes therapy on the development and progression of neuropathy. The Diabetes Control and Complications Trial Research Group. Ann Intern Med 1995;122:561–8.
5. The effect of intensive diabetes treatment on the progression of diabetic retinopathy in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial. Arch Ophthalmol 1995; 113:36–51.

6. Feasibility of centralized measurements of glycated hemoglobin in the Diabetes Control and Complications Trial: a multicenter study. The DCCT Research Group. Clin Chem 1987;33:2267–71.

7. Epidemiology of Diabetes Interventions and Complications (EDIC). Design, implementation, and preliminary results of a long-term follow-up of the Diabetes Control and Complications Trial cohort. Diabetes Care 1999;22:99–111.

8. Sustained effect of intensive treatment of type 1 diabetes mellitus on development and progression of diabetic nephropathy: the Epidemiology of Diabetes Interventions and Complications (EDIC) study. JAMA 2003;290:2159–67.

9. The Diabetes Control and Complications Trial (DCCT). Design and methodologic considerations for the feasibility phase. The DCCT Research Group. Diabetes 1986;35:530–45.

10. American Diabetes Association: clinical practice recommendations 2004. Diabetes Care 2004;27(Suppl 1):S1–150.

11. Fleiss J. The design and analysis of clinical experiments. New York: John Wiley and Sons, 1986:432pp.

12. Shrout P, Fleiss J. Intraclass correlations: uses in assessing rater reliability. Psych Bull 1979;86:420–8.

13. Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). UK Prospective Diabetes Study (UKPDS) Group. Lancet 1998;352:837–53.

14. Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). UK Prospective Diabetes Study (UKPDS) Group. Lancet 1998;352:854–65.

15. Writing Team for the Diabetes Control and Complications Trial/ Epidemiology of Diabetes Interventions and Complications Research Group. Effect of intensive therapy on the microvascular complications of type 1 diabetes mellitus. JAMA 2002;287:2563–9.

16. The relationship of glycemic exposure (HbA1c) to the risk of development and progression of retinopathy in the diabetes control and complications trial. Diabetes 1995;44:968–83.

17. Stratton IM, Adler AI, Neil HA, Matthews DR, Manley SE, Cull CA, et al. Association of glycaemia with macrovascular and microvascular complications of type 2 diabetes (UKPDS 35): prospective observational study. BMJ 2000;321:405–12.

18. Krishnamurti U, Steffes MW. Glycohemoglobin: a primary predictor of the development or reversal of complications of diabetes mellitus. Clin Chem 2001;47:1157–65.

19. Cull CA, Manley SE, Stratton IM, Neil HA, Ross IS, Holman RR, et al. Approach to maintaining comparability of biochemical data during long-term clinical trials. Clin Chem 1997;43:1913–8.

20. Nathan DM, Lachin J, Cleary P, Orchard T, Brillon DJ, Backlund JY, et al.; Diabetes Control and Complications Trial; Epidemiology of Diabetes Interventions and Complications Research Group. Intensive diabetes therapy and carotid intima-media thickness in type 1 diabetes mellitus. N Engl J Med 2003;348:2294–303.

21. Little RR, Rohlfing CL, Wiedmeyer HM, Myers GL, Sacks DB, Goldstein DE. The national glycohemoglobin standardization program: a five-year progress report. Clin Chem 2001;47:1985–92.

22. Kobold U, Jeppsson JO, Dulffer T, Finke A, Hoelzel W, Miedema K. Candidate reference methods for hemoglobin A1c based on peptide mapping. Clin Chem 1997;43:1944–51.