A specific and sensitive assay for blood levels of glycated CD59: A novel biomarker for diabetes

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Increasing evidence links the complement system with complications of human diabetes. The complement regulatory protein CD59, an inhibitor of formation of membrane attack complex (MAC), is inhibited by hyperglycemia-induced glycation fostering increased deposition of MAC, a major effector of complement-mediated tissue damage. CD59, an ubiquitous GPI-anchored membrane protein, is shed from cell membranes by phospholipases generating a soluble form present in blood and urine. We established an enzyme-linked immunosorbent assay (ELISA) to measure serum/plasma glycated human CD59 (hCD59) (GCD59) and evaluated its potential as a diabetes biomarker. We used a synthetic peptide strategy to generate (a) a mouse monoclonal antibody to capture hCD59, (b) a rabbit monoclonal antibody to detect GCD59, and (c) a GCD59 surrogate for assay standardization. ELISA conditions were optimized for precision, reproducibility, and clinical sensitivity. The clinical utility of the assay was initially evaluated in 24 subjects with or without diabetes and further validated in a study that included 100 subjects with and 90 subjects without a diagnosis of diabetes. GCD59 (a) was significantly higher in individuals with than in individual without diabetes, (b) was independently associated with HbA1c, and (c) identified individuals with diabetes with high specificity and sensitivity. We report the development and standardization of a novel, sensitive, and specific ELISA for measuring GCD59 in blood. The assay distinguished individuals with diabetes from those without, and showed strong correlation between GCD59 and HbA1c. Because GCD59 likely contributes to the pathogenesis of diabetes complications, measurement of blood levels of GCD59 may be useful in the diagnosis and management of diabetes. Am. J. Hematol. 00:000–000, 2013. © 2013 Wiley Periodicals, Inc.

Introduction

Prior evidence supports a strong link between the complement system, activity of the complement regulatory protein CD59, and the pathogenesis of vascular complications of diabetes [1–6]. Complement activation ultimately leads to formation of the cytotoxic, pore-forming membrane attack complex (MAC), the main effector of complement-mediated tissue damage. Insertion of the MAC into cell membranes induces the release of cytokines and growth factors that promote inflammation, thrombosis, and cell proliferation, as characteristically seen in target organs of diabetic complications [1,7–9].

CD59 is a complement regulatory protein ubiquitously expressed on mammalian cell surfaces; it specifically inhibits MAC formation and thereby protects “self” cells from complement-mediated damage [10,11]. We and others have reported that the complement regulatory function of human CD59 (hCD59) is reduced in diabetes [1,3,12]; this is because (1) the non-enzymatic glycation of a Lys41 residue within the active site of hCD59 results in the formation of functionally inactive glycated CD59 (GCD59) [1,12], and (2) there is decreased CD59 expression [3].

The co-localization of MAC deposits and GCD59 in kidneys, nerves, and vasculature of patients with diabetes further support glycation-inactivation of hCD59 as one potential mechanism contributing to the hyperglycemia-associated tissue damage responsible for the major vascular complications of human diabetes [8]. Though hCD59 is a cell membrane glycoprophatidylinositol(GPI)-bound protein, soluble hCD59 shed off from cell membranes by action of phospholipases is present in human blood (serum/plasma concentration ~ 100 ng/ml) [13] and urine (< 4 ng/ml) [14]. We hypothesized that blood levels of GCD59 may represent a novel pathogenically relevant biomarker that reflects integrated blood glucose levels over time. We report here the development, optimization and validation of an enzyme-linked immunosorbent assay (ELISA) for serum/plasma GCD59. Our novel methodology offers a prototypical example that can serve as a general solution to the challenging problem of assay standardization for post-translationally modified proteins, particularly those that, like GCD59, are in low abundance, heterogeneous in nature, and difficult to purify. In addition, we evaluated the clinical utility of this novel assay in 24 subjects.
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with and without diabetes. The availability of a sensitive and specific assay to measure GCD59 will foster the conduction of large human studies to assess the potential clinical utility of GCD59 in the management of diabetes and the role of complement in the pathogenesis of the diabetes complications, a major cause of morbidity and mortality in the adult population.

Methods

To establish an ELISA to measure GCD59, we (1) developed a method to separate GCD59 from non-glycosylated CD59 (NGCD59), (2) synthesized hCD59-derived peptide antigens based on the amino acid sequence of hCD59 and used them to develop two monoclonal antibodies, one for capture of hCD59 (termed MABtotCD59 and recognizing both NGCD59 and GCD59) or a sandwich ELISA (termed MABGlyCD59), and (3) synthesized a surrogate GCD59 that was used as a standard enabling reproducible quantification of GCD59 in large cohorts of individuals across a broad spectrum of glycemic control. Once the assay was established and optimized, we assessed its clinical utility by measuring plasma GCD59 in two groups of individuals, one with diabetes and the other without diabetes.

Purification of NGCD59 and GCD59. NGCD59 and GCD59 needed for antibody validation and assay development were separated by affinity chromatography using the anti-CD59 mouse monoclonal antibody BRIC-229 (IBGRL, UK). Like other activity neutralizing anti-CD59 antibodies, BRIC-229 preferentially binds to and retains NGCD59, separating it from GCD59 that flows through the BRIC-229 affinity chromatography column. Since urine is a rich source of CD59 [1,15], 50 ml of a urine pool obtained from two poorly controlled diabetic individuals was run twice through a 3 ml column in which biotinylated BRIC-229 was immobilized on streptavidin-sepharose beads (GE Healthcare, Sweden). After washes with 30 ml phosphate buffer saline (PBS)/0.05% Tween-20/0.05% sodium azide, NGCD59 was eluted from the BRIC-229 column with 10 ml 0.1 M glycine-HCl buffer, pH 3 and neutralized with 100 mM Tris-HCl buffer, pH 8. The eluate was dialyzed 48 hrs at 4 °C, lyophilized and reconstituted in PBS, pH 7.4. GCD59 was purified from the flow-through for GCD59 by using a sandwich ELISA (termed MABGlyCD59), and (3) synthesized a surrogate GCD59 that was used as a standard enabling reproducible quantification of GCD59 in large cohorts of individuals across a broad spectrum of glycemic control. Once the assay was established and optimized, we assessed its clinical utility by measuring plasma GCD59 in two groups of individuals, one with diabetes and the other without diabetes.

ELISA for GCD59. Capture MABtotCD59 mouse monoclonal antibody (0.3 µg/ml in 50 mM carbonate-bicarbonate buffer, pH 9.6 at 25°C) was coated on the wells of ELISA plates (overnight at 4°C) and neutralized with 0.25 µl protein-free blocking buffer (Thermo Scientific; 1 hr at room temperature (RT)). Human plasma or serum samples (50 µl) were reduced by incubation with 2.5 µl of 1 M freshly prepared NaBH4 (final concentration: 50 mM, at RT for 1 hr), the reaction was quenched with 1% acetic acid (1 ml per 50 µl of sample) and then added to the plates at the desired dilution (1% protein free blocking buffer/10 mM EDTA/1% NP40), and incubated for 1 hr at RT. GCD59 was detected with MABGlyCD59 rabbit monoclonal primary antibody (100 µl/well of a 2.5 µg/ml antibody solution; for RT for 2 hrs), followed by goat anti-rabbit IgG-HRP (Bethyl Laboratories) secondary antibody (100 µl/well of a 14 ng/ml antibody solution; RT for 1 hr) using as substrate 3,3',5,5'-tetramethylenediamine (TMB) (Thermo Scientific). Plates were washed between steps with 1X PBS 0.05%-Tween-20.

Assay standardization and calibration. Since limited availability of purified GCD59 precluded its extensive use as an assay calibrator, we synthesized and validated a surrogate GCD59 standard. To this end, both antigenic peptides (hCD59[44–66]) and (K41(N-glucitol)hCD59[37–50]), respectively used to raise MABtotCD59 and MABGlyCD59 antibodies, were coupled by a PEG linker using the same functional groups on the antigens and preserving the same orientation that was used in the KLH-based immunogens described above. On one end of the PEG linker a succinimidyl ester was coupled to the e- NH2 of the side-chain of the antigen peptide hCD59[44–66]. A maleimido moiety on the other end of the PEG linker was reacted with a thiol function on the side-chain of the C-terminal cysteiny moiety of the antigen peptide [K41(N-glucitol)]hCD59[37–50].

Internal controls at three concentrations (low, medium, and high) of GCD59 were prepared from pooled plasma samples from individuals with and without diabetes, and used to accept or reject the individual runs, according to pre-specified criteria following Westgard rules [19].

Human subjects. Partners Healthcare Institutional Review Board (IRB) approval was obtained for the human study described below. For preliminary assessment of the potential clinical utility of the GCD59 ELISA, we tested a ‘training’ set of 24 consecutive plasma samples collected by the Partners’ Crimson Biospecimen Repository [20] from subjects who had blood drawn for routine clinical indications that included an Hba1c measurement at either the Brigham and Women’s Hospital or Massachusetts General Hospital (affiliates of Partners Healthcare). In this ‘training’ set, that was part of a larger prospective investigation on subjects who were still on-going, 10 were from individuals without and 14 from individuals with diabetes. Selection criteria included: to be considered as having diabetes, a Hba1c value >6.5% [21]; to be considered as not having diabetes, a Hba1c value <6.0% and lack of any 250.XX ICD-9 code in the medical record. Additional information on subjects’ age and sex was available and used to calculate gender-specific means/standard errors from the same blood sample separated for GCD59 measurement. Collected plasma was used to measure GCD59, and the mean GCD59 for each group was compared using unpaired t-tests (data reported as means +/- standard error of means). In addition, the correlation between GCD59 and Hba1c values were quantified via Pearson correlations. The level for significance was set at α = 0.05, with all reported P-values as two-tailed.

We also tested a set of plasma samples from 190 consecutive patients, 100 with and 90 without a diagnosis of Type 2 Diabetes, prospectively collected by the Partners’ Crimson Biospecimen Repository [20] from individuals who had blood drawn for routine clinical indications that included a Hba1c measurement. Inclusion criteria for this study were expanded to: age ≥ 18–65 years, estimated glomerular filtration rate ≥ 60 ml/min and serum creatinine < 1.4 mg/dl in the last 2 years. Patients with Type 2 Diabetes had to have an ICD diagnosis of 250.XX and at least one Hba1c that was > 6.5% in the prior 2 years [21]. Patients without Type 2 Diabetes could not carry an ICD diagnosis indicating a diagnosis of diabetes and had to be considered as not having diabetes, a Hba1c value of <6.0% in the prior 2 years. Additional information on subjects’ age, gender and the Hba1c value measured in the same blood sample separated for GCD59 measurement was also available. Information regarding blood glucose was not available.

Analyses were performed using multivariable linear regression with adjustments for available co-variables: age, gender, and diabetes status (yes or no). Pearson coefficients (r) were determined for correlations

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between normally distributed variables; the level for significance was set at $\alpha = 0.05$, and reported $P$-values as two-tailed. Data analyses were performed using SAS statistical software, v9.1.

The Partners Healthcare IRB reviewed and approved the human study reported here, and all animal studies were approved by the Harvard Medical School Standing Committee on Animals (IACUC; protocol #03507).

Results

Purification of NGCD59 and GCD59 by affinity chromatography. Glycation of CD59 on amino acid K41, which is located at the core of its active site [22–24], results in a parallel loss of activity and of immune reactivity toward neutralizing antibodies such as YTH53.1 and BRIC-229 [1,12]. Preliminary experiments in our laboratory established that an affinity chromatography column that utilizes BRIC-229 binds and retains NGCD59 while GCD59 flows through the column. NGCD59 was eluted from the BRIC-229 affinity column, while GCD59 was immunoprecipitated first with goat polyclonal anti-CD59 antibody (Goat Poly Anti-CD59; red fluorescence) and then with MABGlyCD59 antibody (green fluorescence). The bar graph at the bottom of Fig. 1B depicts the ratio of green (GCD59) over red (total CD59) fluorescence (quantified using an infrared Odyssey scanner) for each individual sample. The inset shows the mean ± SEM of the ratios for the individuals with (D) and without diabetes (N).

Anti-glycated hCD59 rabbit monoclonal antibody (MABGlyCD59). The glucose-mediated glycation of proteins, a major mechanism of tissue damage in diabetes, involves the spontaneous reaction of glucose with amino groups, mostly $\varepsilon$-amino groups that are located at glycation motifs within specific proteins [25,26]. The reaction is initiated by the formation of Lys(1-N-glucopyranosyl), the Schiff base, followed by a slow rearrangement to the more stable

Figure 1. Specificity of the anti-glycated hCD59 rabbit monoclonal antibody (MABGlyCD59). A. GCD59 (G) and NGCD59 (NG) affinity purified from a urine pool of individuals with poorly controlled diabetes, separated by SDS-PAGE and immuno-blotted with goat polyclonal anti-hCD59 antibody before (non-reduced) and after (reduced) treatment with Na-borohydride (left panels in the reduced and non-reduced frames depicting red fluorescent bands). The GCD59 band is also recognized by the MABGlyCD59 antibody but only after Na-borohydride reduction (green fluorescent band in the G lane of the Reduced frame). The full-length blotted membranes used to create this figure are shown in Supporting Information Fig. S1. B. Affinity purified hCD59 from a group of individuals with and without diabetes separated by SDS-PAGE and immunoblotted first with goat polyclonal anti-CD59 antibody (Goat Poly Anti-CD59; red fluorescence) and then with MABGlyCD59 antibody (green fluorescence). The bar graph at the bottom of Fig. 1B depicts the ratio of green (GCD59) over red (total CD59) fluorescence (quantified using an infrared Odyssey scanner) for each individual sample. The inset shows the mean ± SEM of the ratios for the individuals with (D) and without diabetes (N).
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Lys(N′-1-deoxy-fructos-1-yl), also called Amadori product [27–29]. In vitro, when the Amadori product is exposed to a reducing agent such as sodium borohydride, it is reduced to the open ring Lys(N′-1-glucitol) residue [30,31]. In the synthesis of the antigenic peptide used to raise an antiglycated hCD59 rabbit monoclonal antibody, we introduced a pre-formed protected Lys(N′-1-glucitol) residue replacing the original amino acid K41. For this reason, recognition of GCD59 by the MABGlyCD59 antibody was expected to occur only after reduction of GCD59 with sodium borohydride. This reduction converts both Schiff base and Amadori product, into the corresponding Lys(N′-1-glucitol) form.

For the final round of hybridoma screening, we used WB to select hybridomas whose supernatants recognized purified GCD59 only after reduction with sodium borohydride. This method permitted exclusion of hybridomas whose supernatants reacted with NGCD59.

One of the selected and repeatedly recloned hybridomas (designated as MABGlyCD59), produced an IgG kappa 1 monoclonal antibody that recognized only the sodium borohydride reduced form of purified urine GCD59 (Fig. 1A, right panels labeled with green fluorescence). Immunoblotting of the same membrane with goat anti-CD59 specific polyclonal antibody revealed that (1) the protein band detected by MABGlyCD59 was CD59 (Fig. 1A, left panels under reduced and non-reduced conditions), and (2) significantly higher ratios of green (MABGlyCD59) over red (goat polyclonal anti-CD59) fluorescence were detected in samples of purified urine GCD59 from individuals with diabetes as compared to individuals without diabetes (Fig. 1B).

Mouse anti-hCD59 monoclonal antibody (MABTotCD59). Based on Bodian’s et al. mapping of antibody binding sites in hCD59 [22], we designed and synthesized a series of peptide antigens corresponding to hCD59 epitopes located away from the glycation site, and used them to raise mouse monoclonal antibodies. One of these antibodies, termed MABTotCD59, showed comparable affinity toward both NGCD59 and GCD59 in WB analysis of purified protein (not shown). The specificity of the antibody for CD59 was demonstrated by WB analysis of unprocessed human urine. Urine proteins (from non-diabetic individuals) were resolved by SDS-PAGE and immunoblotted with MABTotCD59 and not the Amadori-modified GCD59 (designated as MABGlyCD59) fluorescence were detected in samples of purified urine GCD59 from individuals with diabetes as compared to individuals without diabetes (Fig. 1B).

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MABTotCD59, which utilizes MABTotCD59 for capture and MABGlyCD59 for detection is highly specific for GCD59.

Sodium cyanoborohydride is a less potent and more selective reducing agent than sodium borohydride; it converts Lys(N′-1-glucitol) into only the more labile Schiff base and not the Amadori product [32]. The relative contribution of Schiff base- and Amadori-modified GCD59 to the signal detected in the GCD59 ELISA was determined by pre-incubation of plasma samples from diabetic individuals with either sodium borohydride (50 mM) or sodium cyanoborohydride (75 or 100 mM; 1 hr, RT). The results demonstrated that the ELISA signal elicited by GCD59 derives almost exclusively from Amadori-modified GCD59 (not shown).

Assay standardization. The heterogeneity and low abundance of CD59 combined with the complexity and low yield of GCD59 purification prohibits the use of purified GCD59 as an ELISA calibrator for large human studies. As of today, expression of post-translationally modified proteins as well as their ab initio total synthesis is unaccomplished missions. To circumvent the challenge of GCD59 assay standardization, we synthesized a surrogate of GCD59 and used it as an assay standard and calibrator. In this surrogate, hCD59[44–66] and [K41(N′-1-glucitol)]hCD59[37–50], the two antigenic peptides, respectively used to raise the capture (MABTotCD59) and the detection (MABGlyCD59) antibodies, were linked through a PEG linker (Fig. 3A).

The detailed structure of GCD59 surrogate is depicted in Supporting Information Fig. S3. This GCD59 surrogate was recognized by both cognate antibodies in either WB or direct ELISA (not shown) and was used to calibrate the assay. A representative calibration curve of the previously described sandwich ELISA that utilizes MABTotCD59 for capture, MABGlyCD59 for detection (primary) and GCD59 surrogate calibrator concentrations from 0.5 to 25 ng/ml is shown in Fig. 3B. We observed a linear GCD59 surrogate calibration-dependent absorbance at 450 nm between 0 and 6 ng/ml (inset to Fig. 3B). Levels of GCD59 in serum or plasma samples tested with the standardized GCD59 ELISA are expressed in Standard Peptide Units (SPU): one SPU is the OD reading corresponding to 1 ng/ml concentration of the surrogate GCD59 hybrid peptide in the
Figure 3. Synthetic Surrogate GCD59 for assay standardization. A. Diagram of synthetic surrogate standard in which both antigen peptides, hCD59[44–66] and (K41(N-gluco-5)hCD59[37–50]), respectively were used to raise the capture mouse monoclonal antibody MABTotCD59 and the detection MABGlyCD59 antibodies, were coupled by a PEG linker. 

B. Characteristic surrogate GCD59 standard peptide dose-response curve in the here described sandwich ELISA that utilizes MABTotCD59 as capture and MABGlyCD59 as detection antibodies.

with simultaneous values of random glucose and HbA1c. The GCD59 values were strongly associated with HbA1c ($r = 0.77$, Supporting Information Fig. S4A). The test showed a high sensitivity (93%) and specificity (100%) to discriminate diabetic from non-diabetic individuals, and generated a Receiver Operating Characteristic (ROC) curve with an area under the curve = 0.98 (Supporting Information Fig. S4B).

In the validation human study, 100 subjects were diagnosed with Type 2 Diabetes (HbA1c Mean ± SEM: 7.1 ± 0.21%, age (y) Mean ± SEM: 51.8 ± 1). Samples for this cohort were collected without any reference as to duration of or specific treatments for diabetes. Another cohort of 90 subjects did not meet criteria for Type 2 Diabetes (HbA1c Mean ± SEM: 5.5 ± 0.05%, age (y) Mean ± SEM: 46 ± 1). The results showed that GCD59 was significantly higher in individuals with as compared with individuals without diabetes (Mean ± SEM: no diabetes: 0.35 ± 0.03 SPU; diabetes: 1.49 ± 0.11 SPU, $P < 0.00001$). The area under the curve of a ROC curve generated from the GCD59 values and a binary diagnosis of with or without diabetes was 0.88. In adjusted linear regression models, GCD59 concentrations were independently and positively associated with HbA1c in the entire study population ($\beta = 0.90$, $P < 0.0001$). This independent positive association between GCD59 and HbA1c was apparent when evaluated in just the subgroup of individuals with diabetes ($\beta = 0.88$, $P < 0.0001$), or just the subgroup without diabetes ($\beta = 0.75$, $P < 0.001$).

Discussion

Here we describe the development and validation of a novel assay to measure circulating GCD59. The assay, which uses two specific monoclonal antibodies in a sandwich ELISA format, is highly reproducible and measures serum and/or plasma GCD59 in a highly specific and sensitive manner. Indeed, assuming a basal level of CD59 glycation of $\approx 5\%$ (based on the basal glycation level of HbA1c in normoglycemic individuals), and that the concentration of CD59 in human serum, is $\approx 100–150$ ng/ml [13], the reproducible detection of GCD59 in individuals without diabetes implies that the assay can detect GCD59 in the low picomolar range.

In the development of the GCD59 ELISA reported here, we designed a surrogate GCD59 strategy to generate a synthetic standard that fully recapitulates the behavior of the endogenous biomarker in an ELISA. This GCD59 surrogate can be synthesized reproducibly in large quantities allowing the performance of almost unlimited number of assays. This strategy is novel, of general applicability and provides a solution to the challenge of standardization of post-translationally modified proteins that are heterogenous, in low abundance and, therefore, not accessible through purification, ab initio total synthesis or recombinant in vitro expression. Indeed, despite the very high abundance of hemoglobin in human blood ($\approx 6$ orders of magnitude higher than blood levels of CD59), a reference method for HbA1c measurements was achieved by the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) only after many years of clinical use of HbA1c, and this reference method is “technically demanding, time consuming, very expensive and is not designed for routine analysis of patient samples” [34].

The clinical validation proof of concept human studies reported here showed that individuals with diabetes have significantly higher mean plasma GCD59 when compared to individuals without diabetes, that GCD59 is strongly
and independently associated with HbA1c, and that GCD59 values may be very sensitive and specific for identifying individuals with diabetes and hyperglycemia. Together, these results indicate that blood levels of GCD59 may represent a novel biomarker for glucose handling that can complement tests currently used in the diagnosis and management of diabetes, particularly HbA1c. HbA1c reflects the time-averaged glucose concentrations over a period of 6–8 weeks, the normal life span of the red blood cells that encase hemoglobin, but does not provide information about the glucose level excursions (i.e., the patient’s brittleness) during that period. Even though the turnover rate of membrane-bound CD59 has not been measured in humans, it is conceivably much shorter than that of hemoglobin. This assumption is supported by preliminary data from ongoing human studies showing that upon treatment to normalize pre-prandial glucose, GCD59 parallels average weekly glucose declining to close to normal values in about 2 weeks (manuscript in preparation). If this is confirmed, levels of GCD59 may reflect more acute changes in glucose handling and help physicians and patients better control glucose peaks and valleys that contribute to the development of diabetic complications. A new marker such as GCD59 could also have clinical utility in instances where HbA1c is not applicable or does not perform well, including conditions that alter red cell turnover rates, some hemoglobin variants, chronic renal failure and gestational diabetes mellitus.

As a GPI-anchored protein ubiquitously attached to the external surface of circulating and non-circulating cells, hCD59 is exposed to the glucose levels in the interstitial fluid, which at steady state are reportedly similar to glucose levels in venous plasma [35]. Soluble forms of CD59 present in blood and urine derive from bodily cell membranes from where GPI-linked CD59 is enzymatically shed [14,36,37]. Therefore, blood levels of soluble GCD59 likely reflect the extent of the glycation process at the cellular/tissue level. As such, GCD59 has the potential of becoming a biomarker informing on the pathogenesis of diabetes complications [1,8]. The reasons discussed above highlight why measurements of blood levels of GCD59 made possible by the assay reported here would complement the currently available clinical tools providing meaningful information for the diagnosis, monitoring and risk stratification of patients with diabetes.

In summary, the novel GCD59 ELISA reported here permits the reproducible measurement of blood GCD59 with significant discrimination between individuals with diabetes and normoglycemia. Development of this assay was a necessary condition prior to planning and conducting human studies to investigate comprehensively the clinical utility of GCD59 as a biomarker for glucose handling. Future human
studies are needed to establish whether measurement of blood GCD59 may simplify the diagnosis of diabetes or impaired glucose handling, help with the assessment of integrated blood glucose values over shorter periods of time than currently available tests such as HbA1c, and the stratification of patients at risk of vascular complications of diabetes.

Reference