Identification of Amadori-Modified Plasma Proteins in Type 2 Diabetes and the Effect of Short-Term Intensive Insulin Treatment

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OBJECTIVE — Growing evidence supports that nonenzymatic glycation products may cause hyperglycemia-induced diabetes complications. Amadori-modified proteins are the intermediates of nonenzymatic glycation and constitute the forms of glycated proteins in diabetes. The objective of the current study was to utilize two-dimensional gel electrophoresis, Western blot, and mass spectrometry to identify Amadori-modified plasma proteins in type 2 diabetic patients with poor glycemic control and assess the impact of short-term insulin treatment on the glycation of these proteins.

RESEARCH DESIGN AND METHODS — We compared eight type 2 diabetic subjects (aged 56 ± 3 years and BMI 29.7 ± 0.9 kg/m²) with an average diabetes duration of 8.5 years (range 3–19) with equal numbers of weight-matched (aged 56 ± 2 years and BMI 30.1 ± 10.0 kg/m²) and lean (aged 58 ± 2 years and BMI 25 ± 0.5 kg/m²) nondiabetic subjects who have no first-degree relatives with diabetes. Two separate blood samples were collected from the type 2 diabetic subjects, one following 2 weeks of withdrawal of all antidiabetic medications (T2D−) and another following 10 days of intensive insulin treatment (T2D+). Plasma proteins were separated using single- and two-dimensional gel electrophoresis. Western blot analysis was performed, and several proteins, which reacted with the Amadori-antibody (1-deoxyfructosyl lysine), were identified by tandem mass spectrometry.

RESULTS — No significant differences in the glycation of proteins between the obese and lean groups were noted, but type 2 diabetic patients had several proteins with higher glycation than the control groups. We identified 12 plasma proteins with reduced reaction to the anti-Amadori antibody upon intensive insulin treatment. A significant (P < 0.03) difference in Amadori modification was observed between the T2D− and control subjects for all these proteins except the Ig light chain. Insulin treatment reduced Amadori modification of albumin (23.2%, P < 0.02), fibrinogen (46.4%, P < 0.001), Ig heavy chain constant region (20.7%, P < 0.05), transferrin (25.4%, P < 0.04), and Ig light chain (13.3%, P < 0.02). In addition, Western blot analysis of two-dimensional gel electrophoresis identified α-fibrinogen precursor, β-fibrinogen precursor, fibrinogen γ-B chain precursor, hemopexin, vitamin D binding protein, and serine protease inhibitor as proteins with a reduced reaction to anti-Amadori antibody upon intensive insulin treatment.

CONCLUSIONS — The current approach offers the opportunity to identify Amadori modification of many proteins that may cause functional alterations and offers the potential for monitoring short-term glycemic control in diabetic patients.

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Hyperglycemia-induced advanced glycation end products (AGEs) are implicated as one of the main underlying mechanisms of chronic complications in diabetes (1–9). Nonenzymatic glycation reactions between extracellular proteins and glucose are one of the leading pathways for the formation of AGEs. This involves the condensation reaction of the carbonyl group of sugar aldehydes with the free amino groups or NH₂ terminus of proteins, resulting in the formation of a Schiff base. This condensation product undergoes rearrangement through reversible acid-based catalysis to intermediate Amadori adducts. AGEs are the complex end products of the irreversible chemical reactions of the Amadori adduct.

Recent reviews (8,10,11), based on several published results, support the relationship of AGEs with complications of diabetes, and they are specifically implicated in retinopathy (12,13), nephropathy (14–17), neuropathy (18,19), immunodeficiency (20), and generalized vasculopathy (21,22) in diabetes. AGE formation may modify the functional group of proteins, thus producing abnormal interactions between molecules, resulting in their altered functions. Studies involving humans (23,24), animals (25,26), and cell culture (27,28) demonstrate a strong relationship between Amadori-albumin and diabetes-specific complications. Several markers have been identified for the screening, diagnosis, and monitoring of the disease. The measurement of HbA₁c in diabetic patients reflects glycemic status over the previous 4–6 weeks and is routinely used as an index of long-term glycemic control. Hemoglobin has a relatively long life in blood, with the average life span of red cells being 120 days. The decay of HbA₁c is also slow, with an average half-life of 29 days (29). In addition to HbA₁c, quantification of various AGEs is suggested as a long-term assessment of hyperglycemia mainly because of the property of AGEs to
Amadori-modified plasma proteins in diabetes

Table 1—Subject characteristics

<table>
<thead>
<tr>
<th>Variable</th>
<th>Lean control subjects</th>
<th>Weight-matched control subjects</th>
<th>Type 2 diabetic subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>35 ± 2</td>
<td>36 ± 2</td>
<td>36 ± 2</td>
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<tr>
<td>Height (cm)</td>
<td>174 ± 4</td>
<td>170 ± 3</td>
<td>170 ± 3</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>76 ± 4</td>
<td>87 ± 4</td>
<td>86 ± 4</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25 ± 0.5</td>
<td>30.1 ± 1.0</td>
<td>29.7 ± 0.9</td>
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<tr>
<td>Fat mass (kg)</td>
<td>49.3 ± 4.5</td>
<td>51.2 ± 3.7</td>
<td>52.8 ± 4.6</td>
</tr>
<tr>
<td>Fat free mass (kg)</td>
<td>21.4 ± 1.8</td>
<td>29.6 ± 3.1</td>
<td>27.8 ± 2.4</td>
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<tr>
<td>Total abdominal fat (cm²)</td>
<td>265 ± 31</td>
<td>412 ± 51</td>
<td>417 ± 35</td>
</tr>
</tbody>
</table>

Glucose control

<table>
<thead>
<tr>
<th></th>
<th>Initial value (T₂D[−])</th>
<th>After 10 days (T₂D[+])</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mmol)</td>
<td>5.1 ± 0.1</td>
<td>12.6 ± 1.0†</td>
</tr>
<tr>
<td>Insulin (pmol)</td>
<td>28 ± 4</td>
<td>29 ± 4</td>
</tr>
<tr>
<td>Insulin sensitivity (× 10⁻⁵/pmol/l)</td>
<td>7.64 ± 1.54</td>
<td>2.03 ± 0.38†</td>
</tr>
<tr>
<td>Serum fructosamine (μmol/l)</td>
<td>191 ± 6.6</td>
<td>289.4 ± 17.0†</td>
</tr>
</tbody>
</table>

Data are means ± SE for eight subjects (four women and four men) per group. Fat mass and fat free mass were determined by dual-energy X-ray absorptiometry. Abdominal fat was determined from single-slice computed tomography scans of the abdomen. *Different from control groups, †Different from T₂D[+].

remain irreversibly attached to proteins and continue to accumulate over the entire life span of the proteins (30). The fructosamine assay provides an index of short-term glycemic status over the previous 7–14 days. The fructosamine assay reflects glycation of total serum proteins and has an average decaying half-life of 16.5 days (29). The major glycosylated protein measured by the fructosamine assay is albumin, with a half-life of ~19 days. Additional plasma glycated proteins with shorter half-lives would allow clinicians to achieve tight glycemic control and to closely monitor the effect of treatment in diabetic patients. Identification of additional proteins may also help to further our understanding about the pathogenesis of diabetes complications.

The aim of the current study was to examine the extent of glycation of plasma proteins in type 2 diabetic patients with poor glycemic control and the effect of short-term insulin treatment on glycation of these proteins. Those proteins that have measurable glycation products may be used as indexes of diabetes control during a short treatment period. A monoclonal antibody raised against glucose-derived Amadori-modified lysine residues (1-deoxy fructosyl lysine) was used to investigate the extent of glycation, and mass spectrometry was used to identify these proteins.

RESEARCH DESIGN AND METHODS — Eight type 2 diabetic patients with an average diabetes duration of 8.5 years (range 3–19) were studied and compared with eight weight-matched and eight lean nondiabetic subjects without any first-degree relatives with diabetes. The subject data were previously reported in another aspect of this study (31) and are summarized in Table 1. The study protocol was approved by the institutional review board of the Mayo Clinic and Foundation.

Study protocol and sample collection

Type 2 diabetic patients were studied on two separate occasions, one after the withdrawal of all antidiabetes treatments for 2 weeks (T₂D−) and another occasion following 10 days of four daily injections of regular insulin with an aim to maintain premeal blood glucose values of 80–100 mg/dl (T₂D+). The subjects were randomly assigned to the order of the treatment regimen, with a washout period of 8–10 weeks between regimens. The subjects were admitted to the general clinical research center for studies described elsewhere (31). During the T₂D− phase, the subjects were given an overnight intravenous saline infusion, while during the T₂D+ phase, they were infused with insulin intravenously overnight to maintain their blood glucose between 80 and 100 mg/dl. While on either insulin or saline infusion, blood samples were drawn at 7:00 A.M. after an overnight fast.

Blood glucose, insulin, and serum fructosamine measurement

Glucose was measured using a Beckman Glucose Analyzer 2 (Beckman Instruments, Fullerton, CA). The plasma concentration of insulin was measured using a two-site immunoenzymatic assay (Linco Research, St. Louis, MO). The serum fructosamine was measured at the central clinical laboratory of the Mayo Clinic using a Hitachi 911 fructosamine calorimetric rate reaction. The intravenous glucose tolerance test was performed for the determination of insulin sensitivity and acute response to glucose (32,33).

SDS-PAGE and Western blot

A total of 0.25 µl of plasma from each subject was loaded onto 10% SDS-PAGE and run under reducing conditions in a Criterion gel apparatus (Bio-Rad Laboratories, Hercules, CA) at 100 V constant until the dye front reached the bottom of the gel. The gels were stained with Coomassie blue for proteomic analysis. For the Western blot, the plasma proteins in the gel were transferred to a polyvinylidene fluoride membrane using a semidry blot (Bio-Rad Laboratories) at 17 V constant for 70
min. After the transfer, the membrane was blocked with 5% defatted milk. The membrane was incubated in a 1:10 dilution culture filtrate of monoclonal IgM antibody (HB-9644; American Type Culture Collection [ATCC], Manassas, VA) raised against glucose-derived Amadori-modified lysine residues (1-deoxyfructosyl lysine). The membrane was subsequently incubated with a rabbit anti-mouse peroxidase-conjugated secondary antibody at a concentration of 1:10,000. Detection was accomplished using an enhanced chemoluminescence plus reagent.

All incubations were performed for 60 min on a rocker in 0.05M Tris-buffered saline pH 7.5 with 0.05% Tween 20.

**Albumin and globulin depletion from plasma**

Plasma samples from the T2D− and T2D+ phases of a type 2 diabetic subject were subjected to depletion of albumin and globulin, respectively, using SwellGel Blue and NAb Protein-A Spin Chromatography kits (both from Pierce Biotechnology, Rockford, IL). All steps were performed according to the manufacturer's instructions at equal volumes and conditions.

**Two-dimensional gel electrophoresis**

Protein samples of 0.5 μl of total plasma and 10 μl of depleted plasma from the T2D− and T2D+ phases of a diabetic patient were dissolved in 125 μl immobilized pH gradient (IPG) rehydration buffer containing 9M urea, 4% CHAPS (3-[3-cholamidopropyl]dimethylammonio]-1-propane sulfonate), 65 mMol/l diethiothreitol, 0.2% 3–10 pH Biolyte (Ampholyte solution from Biorad), and a trace of Bromophenol blue. IPG strips that were 7 cm and 3–10 pH and 4–7 pH were allowed to rehydrate with the above sample mixture for 12–16 h at room temperature in a rehydration/reswelling tray. The rehydrated IPG strips were subjected to isoelectric focusing in a protein isoelectrofocusing (IEF) cell (Biorad Laboratories, Hercules, CA) using a three-step protocol with a maximum of 4,000 V increased from 300 V for 3 h in the second step and a third step continuing at 4,000 V for 24,000 V hr. The cell temperature was kept at 20°C, with a maximum current of 50 μA/strip. The IEF-completed IPG strips were equilibrated for the SDS-PAGE in a two-step equilibration using 2.5 ml of equilibration buffer (6M urea, 2% SDS, 0.375 mol/l Tris-HCl pH 8.8, and 20% glycerol), with 130 mmol/l diethiothreitol in the first step and 135 mmol/l iodoacetamide in the second step. The equilibration steps were done in a rehydration tray for 10 min each on a rotary shaker at room temperature. The

**Figure 1—** Western blot analysis of representative plasma samples from different study groups: lean control, weight-matched control, T2D−, and T2D+ from the same subject. A total of 0.25 μl of each sample was run under reducing conditions in a 10% SDS-PAGE, and the proteins were transferred to polyvinylidene fluoride membrane. A 1:10 dilution of culture filtrate (primary antibody) of clone HB-9644 and a 1:10,000 dilution of rabbit anti-mouse peroxidase-conjugated secondary antibody were used for the blot. Detection was accomplished by an enhanced chemoluminescence plus reagent.

**Figure 2—** Western blot analysis of plasma proteins from two-dimensional gel electrophoresis. Plasma samples from a representative diabetic subject were chosen for performing two-dimensional gel electrophoresis followed by Western blot. Western blot was performed on two-dimensional gel electrophoresis of equal amounts of globulin-depleted plasma (top panels) as well as albumin and globulin-depleted plasma (lower panels) from T2D− (left panels) and T2D+ (right panels) samples. The Western blot showed increased glycation for several plasma proteins from the samples of T2D+. The corresponding gel spots were excised from the Coomassie blue-stained two-dimensional gels of respective samples and analyzed for protein identification by tandem mass spectrometry. All of the identified proteins showed either a reduction or absence of glycation after the insulin treatment (T2D+). The data are from three reproducible experiments done separately on the same samples.
second-dimension separation by subunit molecular weight was performed by vertical SDS-PAGE. We used the mini-PROTEAN 3 cells and 10% two-dimensional prep precast gels (both from BioRad) for the second dimension of the 7-cm IGP strips. The strips were loaded onto the IGP well of the gel with the help of molten agarose and run at 100 V constant until the dye front reached the bottom of the gel. The gels were either stained with Coomassie blue for proteomic analysis or subjected to Western blot analysis, as described previously.

**Mass spectrometry**
The gel bands and spots that reacted with the antibody HB-9644 were excised for mass spectrometry analysis. The gel pieces were destained, subjected to reduction and alkylation, and trypsin digested, and the resulting peptides were extracted. The peptides were analyzed by nano-LC/MS/MS (liquid chromatography/mass spectrometry/mass spectrometry) using an ultimate cap-LC system (LC Packings, San Francisco, CA) directly coupled with an API QSTAR PulsarTM hybrid quadrupole time-of-flight LC/MS/MS mass spectrometer (Applied Biosystems, MDS Sciex, Foster City, CA). Data were processed with the software program Pro ID (Applied Biosystems) for protein identification. Pro ID operates by first preparing a formatted database from a protein or DNA FASTA file. At run time, the precursor molecular weight (MW) and fragment masses from each experimental MS/MS spectrum were compared with the database fragment masses for all peptides with the same precursor MW. For this study, the SWISS-PROT database was searched.

**Statistics**
Values are expressed as means ± SE. A two-tailed unpaired t test was performed for comparisons among the groups. A paired t test was performed to determine the effect of insulin treatment on nonenzymatic glycation of plasma proteins in people with type 2 diabetes.

**RESULTS** — The lean control subjects had lower BMIs and total abdominal fat than the type 2 diabetic patients and the weight-matched nondiabetic control subjects, whereas the latter two groups had similar body composition and were similar in all other aspects (Table 1).

**Plasma glucose, insulin, and serum fructosamine levels**
The plasma glucose levels were higher in the T₁D⁻ (Table 1) than T₂D+ group as well as the control groups. Both nondiabetic control groups had similar glucose values. Plasma insulin was significantly higher in the T₁D⁻ group than in the T₂D⁻ group and the two control groups. The weight-matched nondiabetic control subjects tended to have higher insulin levels than the lean control and T₂D⁻ subjects, but the differences did not reach statistical significance. The plasma insulin levels in the T₁D⁻ and lean control groups were similar. The insulin sensitivity of the control groups was significantly higher than the T₂D⁻ group, as previously reported (31). The serum fructosamine levels were significantly higher in the T₂D⁻ than the control groups and showed a significant reduction (13%) after 10 days of insulin treatment (Table 1).

**Identification of proteins**
The proteins that showed a reduction in the reaction with the anti-Amadori antibody upon insulin treatment were identified. From the single dimension gel (Fig. 1), the identified proteins were transferrin, serum albumin, Ig heavy chain constant region, fibrin, and Ig light chains. The additional proteins identified from the two-dimensional gel (Fig. 2) were α-fibrinogen precursor, β-fibrinogen precursor, fibrinogen γ-B chain precursor, transferrin, hemopexin, vitamin D binding protein, and serine protease inhibitor (α-1 antitrypsin precursor).

The protein identification from each band or spot was based on the highest score obtained with the highest confidence and maximum number of unique peptides identified for each protein. Mass spectrometry results from the single dimension SDS-PAGE gel bands showed other proteins besides the major proteins identified from each band. For example, the Ig heavy chain band showed the presence of serum albumin as well. To confirm that it was Ig heavy chain that was glycated, we performed Western blot analysis of purified globulins from respective plasma samples (Fig. 3). The results were similar to those obtained when the Western blot was conducted on total plasma samples. The mass spectrometry results of the two-dimensional gel spots identified single proteins.

**Extent of nonenzymatic glycation in type 2 diabetic subjects and the effect of insulin treatment**
A typical immunoblot of representative samples from each group is shown in Fig. 1. An image analysis of the Western blots showed (Fig. 4) a significantly higher glycation of albumin, fibrin, transferrin, and Ig heavy chain in T₂D⁻ compared with nondiabetic control groups, though there were no significant differences in glyca-
tion of plasma proteins between weight-matched control subjects and lean control subjects. All of these proteins showed a significant reduction in glycation after 10 days of insulin treatment. Serum albumin (Fig. 4A) showed a 23.2% (P < 0.02) reduction in glycation after insulin treatment, fibrin (Fig. 4B) 34.6% (P < 0.003), Ig heavy chain constant (Fig. 4C) 20.7% (P < 0.02), transferrin (Fig. 4D) 25.4% (P < 0.04), and Ig light chain (Fig. 4E) 13.0% (P < 0.02).

Western blot analysis of the two-dimensional gel was performed only for T2D− and T2D+ samples to determine whether additional proteins would react with the HB-9644 antibody and to determine the effect of insulin treatment on glycation. We observed a remarkable reduction in glycation (antibody reaction) for β-fibrinogen precursor, hemopexin, vitamin D binding protein, and serine protease inhibitor after insulin treatment. Complete absence of antibody reaction was observed for α-fibrinogen precursor, fibrinogen γ-B chain precursor, and transferrin after 11 days of insulin treatment (Fig. 2).

**CONCLUSIONS** — The current study applied Western blot analysis using an antibody that recognizes specific Amadori formation, 1-deoxyfructosyl lysine, to determine the extent of glycation in plasma proteins of type 2 diabetic patients on poor glycemic control. We found a significant
difference in the glycation of many plasma proteins from type 2 diabetic patients following 2 weeks of poor glycemic control, when compared with weight-matched and lean control subjects. The Amadori product, the first stable product of the Maillard reactions, is quantitatively the most abundant glycated protein found in the plasma. Accumulated Amadori products may lead not only to AGE deposition in tissues but also to other pathophysiological consequences, such as activation of oxidative stress, protein aggregation and cross-linking, loss of receptor binding, and enzymatic or other physiological activities by glycated proteins (6,34). The prevention of formation of Amadori products or the removal of these modified proteins is potentially the most effective way to interrupt the glycation cascade and prevent the potential pathological consequences of glycation.

The current study also demonstrated that this glycation is significantly reduced by 10 days of short-term intensive insulin treatment and examined this approach to identify molecules to use as a short-term index of glycemia.

HbA1c, an Amadori product, has been used extensively since the 1970s as an index of blood glucose control in clinical practice and research (35). A correlation between glycated albumin, another Amadori-modified plasma protein, and diabetic nephropathy and other complications has been recently reported (23,24). The current study demonstrates that glycated albumin is increased in type 2 diabetic patients after 2 weeks of poor glycemic control. Besides glycated albumin, we also found a significant increase in the glycation of several plasma proteins including transferrin, Ig heavy chain constant, and fibrin in type 2 diabetic patients during poor glycemic control. Previous studies have demonstrated that nonenzymatic glycation of Igs leads to impairment in immunoreactivity due to glycation of the Fc fragment of Igs (36,37). Our data are in agreement with these previous results demonstrating significant glycation of these important proteins. A recent study by MALDI/MS (matrix-assisted laser description ionization/mass spectrometry) analysis conducted on plasma samples of 60 type 2 diabetic patients shows more glycation on the Fab fragments than in the Fc portion of the Igs (20). Though our results show no significant increase in the glycation of Ig light chains in the T2D group when compared with control subjects, a significant reduction in glycation was observed after 10 days of intensive insulin treatment. It is reported that nonenzymatic glycation reduces the susceptibility of fibrin to degradation by plasmin (38). Our results show that the glycation of fibrin is significantly increased during 2 weeks of poor glycemic control and showed maximum improvement on intensive insulin treatment. It remains to be determined whether nonenzymatic glycation could result in an increased half-life of fibrin, thus contributing to the hemostatic changes that occur in metabolic syndrome (39). The current study also shows a similar increase of glycation of transferrin during poor glycemic control. An in vitro study (40) demonstrated that nonenzymatic glycation of transferrin decreases the iron binding capacity by binding the iron ions loosely on the protein and also increasing the production of free oxygen radicals including O2− and OH. The current study indicates potential pathological changes that occur in diabetes. The fructosamine assay produced results comparable to those of the results for the individual proteins. However, the percentage of the reduction of glycation observed in the fructosamine assay was much less than what is observed for the individual proteins, except Ig light chains. Fructosamine represents the glycation of the entire plasma protein. The current report shows the potential value of these newly identified plasma proteins for monitoring the effect of short-term glycomic changes in diabetic patients. In addition, the current approach will help in identifying new molecules that may be responsible for the pathogenesis of the complications of diabetes. In the current study, we performed Western blot analysis on the two-dimensional gel electrophoresis of depleted plasma samples to determine whether additional proteins could be identified as glycated during poor glycemic control in diabetes. We identified glycated allo-albumin, γ-fibrinogen precursor, α-fibrinogen precursor, hemopexin, vitamin D binding protein, β-fibrinogen precursor, and α-1 antitrypsin, all of which have specific physiological functions and may contribute to the pathogenesis of complications in diabetes. We have also shown that glycation reactions in these proteins are either absent or markedly reduced by short-term intensive insulin treatment. Our data show that a combination of Western blot analysis using specific antibodies and two-dimensional gel electrophoresis approaches yields important information on protein modification related to pathophysiology that could be useful for the treatment of diabetes. A similar proteomic approach to profile glycated proteins can be used to identify molecules that may offer additional prognostic markers other than HbA1c, specifically to detect the short-term effect of diabetes treatment. The disappearance of protein glycation depends on the in vivo half-life of individual proteins. It is also possible that nonenzymatic glycation may alter the half-lives of serum proteins. Hence, proteins with wide variability in their half-lives may be useful tools as markers of glycemic index.

The Diabetes Control and Complications Trial (41) has confirmed that intensive insulin treatment will significantly reduce the HbA1c level of type 1 diabetic patients. That trial also demonstrated conclusively that intensive insulin treatment is associated with a substantial reduction in microvascular complications, such as retinopathy and nephropathy. Others have also shown that short-term glycemic control by aggressive intensive insulin therapy significantly improves insulin action and insulin secretion in patients with type 2 diabetes with secondary failure to oral agents (42,43). The current study demonstrated the potential for utilizing many proteins as markers of glycemic status. Glycated proteins with a long half-life may continue to exert a pathogenic effect even after normoglycemia is restored. Thus, it is important to determine the glycated proteins with close correlations to the pathogenesis of diabetes.

The current study represents a novel attempt to identify and quantitate Amadori-modified plasma proteins in a group of very well-defined study subjects by using Western blot analysis in combination with two-dimensional gel electrophoresis and mass spectrometry. This approach may provide an opportunity for more focused investigations that may identify protein modifications responsible for pathogenesis of complications related to type 2 diabetes as well as to monitor insulin treatment.

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References