

# Glycation and biomarkers of vascular complications of diabetes

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**Abstract** Propensity to diabetic nephropathy (DN), retinopathy (DR), and cardiovascular disease (CVD) varies between individuals. Current biomarkers such as indicators of glycemia (HbA1c), retinal examinations, and albuminuria, cannot detect early tissue damage. HbA1c also doesn't reflect most glycative and oxidative chemical pathways that cause complications, and studies of new biomarkers to measure their end-products are needed. This review proposes the study of advanced glycation end products (AGEs) and oxidation end-products (OPs) in long-term diabetes outcome studies. AGEs integrate the activity of glycation pathways that form dicarbonyls, while OPs reflect superoxides, hydroxyl radicals, and peroxides. We discuss using these biomarkers to predict risk of development and progression of DN, DR, and CVD, and to determine if they confer risk independently of the level of HbA1c. We also discuss methods and guidelines to document sample quality in such studies. These studies have the potential to validate unique biomarkers during the early stages of diabetes in those who are at high risk of diabetic complications. Information on basic mechanisms responsible for complications could also stimulate development of therapeutic approaches to delay or arrest them. The ultimate goal is to predict those requiring aggressive therapies during the earliest stages, when prevention or reversal of complications is still possible.

**Keywords** Biomarkers · Diabetic complications · Advanced glycation end-products · Oxidative end-products · Clinical trials

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## The problem of diabetic complications

The long-term vascular complications of diabetes are major health problems in the US and in the developed world and involve all ethnic populations. It is redundant to repeat the staggering figures showing that over 20 million people in the US alone have diabetes, and it is estimated that 285 million people are afflicted with diabetes worldwide in 2010 (Shaw et al. 2010). Diabetes is it now is the leading cause of new cases of blindness in individuals between the ages of 30 and 70 years (Frank 2004), accounts for 50% of new cases of kidney failure in the US (Association 2007) with similar trends of diabetes related renal failure multi-nationally (Williams 2010), and is a major cause of cardiovascular disease (CVD) (Hunt et al. 2007). Based on the current incidence of diabetes and demographics, it has been projected that the number of Americans with diabetic retinopathy will triple to 16 million by 2050 (Saaddine et al. 2008) and the major cause of the dramatic expansion in rates of end-stage renal disease is due to new cases of diabetic nephropathy. People with diabetes also have a dramatic increase in the risk of heart attack and stroke. It was primarily treatment of these devastating complications that drove the cost of caring for diabetes to \$173 billion in the US alone in 2007.

## Variable susceptibility to diabetic complications

It is now widely recognized that variable propensity to diabetic complications occurs among individuals with diabetes (DCCT Research Group 1997; Fioretto et al. 1999; Seaquist et al. 1989). Accelerated diabetic nephropathy (DN) and retinopathy (DR) occur in some individuals with modest hyperglycemia, while others never progress in spite

of poor glycemic control over many years (Krolewski et al. 1987; Andersen et al. 1983). This is illustrated by the fact that a sub-group with type 1 diabetes develops DN despite good HbA1c's, while two-thirds avoid DN in spite of exposure to supra-physiologic glucose levels over time. To understand these observations, it is proposed that a genetic basis for susceptibility (or resistance) to development of complications, in addition to glycemia, can play an important role (Borch-Johnsen et al. 1992; Krolewski et al. 1987). Family studies show clustering of the risk of DN (Prager et al. 1981; Pettitt et al. 1990; Seaquist et al. 1989; DCCT Research Group 1997), as well as concordance for the severity and patterns of diabetic glomerular structural lesions among type 1 diabetic sibling pairs (Fioretto et al. 1999). Further support for a variable response of the kidney to hyperglycemia comes from work showing that other less well-defined variables, of equal or greater importance to glycemia, strongly influence nephropathy risk (Caramori et al. 2002).

### Current biomarkers for diabetic complications

Although several large-scale studies have clearly demonstrated that intensive glucose control plays a role in preventing or delaying the incidence of diabetic complications, effective biomarkers for early detection of clinically silent eye, kidney, or cardiovascular damage have not been established. As a consequence intensive therapies are less likely to be instituted during the early stages when complications are potentially preventable or reversible. Consequently, we frequently wait until frank clinically observable signs or symptoms occur to do so.

It has become increasingly clear that our current biomarkers of hyperglycemia (HbA1c) for identifying the "high-risk" subgroup have significant limitations. The landmark diabetic control and complications trial (DCCT) showed that intensive therapy will reduce the risk of complications, but that: "other features of diabetic glucose control are not reflected by HbA1c, and may add to, or modify, this risk" (DCCT Research Group 1995). For example, they found that the levels of HbA1c alone explained only about 15% of the total variation in risk of progression of retinopathy, the primary DCCT outcome (DCCT Research Group 1995). Likewise, the "Natural History of Diabetic Nephropathy Study" (Drummond et al. 2002, 2003) has shown that only 9% of the risk of progressive glomerular basement membrane (GBM) thickening in type 1 diabetes is accounted for by the baseline HbA1c level (M. Mauer, personal communication). For diabetes related cardiovascular disease, glycemia (reflected by HbA1c) is an even poorer predictor of the degree of vascular damage, even though CV risk is dramatically

amplified when diabetes is added to dyslipidemia and hypertension (Haffner et al. 1998; Action to Control Cardiovascular Risk in Diabetes Study Group et al. 2008). Our inability to detect this risk leads to a situation where many individuals with diabetes already have significant disease at the time of diagnosis after many years of silent evolution (Dluhy and McMahon 2008; Cefalu 2008). Some factors responsible for the shortcomings of HbA1c as a tool to predict diabetic complications could include its relative insensitivity to glycemic fluctuations (Monnier and Colette 2006; Beisswenger et al. 2001; Ceriello et al. 2004; Rohlfing et al. 2002), or to the inability of this early chemical reaction product (Amadori product) to detect variable production of more complex glycation/oxidation products that can induce vascular damage.

Our other current biomarkers for progression of DR and DN, including retinal morphological change or the appearance of albuminuria on regular examinations, are unable to identify those at greatest risk during the long 10–20 years "silent phase" when evolving or incipient damage to the kidney, eyes, and CV system is not clinically apparent (Fioretto et al. 1999). By the time these markers become positive, substantial pericyte drop-out and avascular capillaries are frequently present in the retina (Ejaz et al. 2008), while substantial irreversible glomerular damage can be present by the time microalbuminuria occurs (Fioretto et al. 1999). It is even more difficult to identify the diabetic sub-group who develop progressive renal impairment in the absence of significant albuminuria (Perkins and Krolewski 2009). It is also widely recognized, that CV disease associated with the diabetes may remain silent for many years, in spite of the gradual accumulation of serious and life-threatening lesions (Haffner et al. 1998; Turner et al. 1998). Although prognosis for retaining vision is improved by pan-retinal photocoagulation, waiting to treat proliferative retinal changes by laser, rather than preventing it in a susceptible individual, results only in slowing of progression, and leads to significant retinal damage and visual loss secondary to multiple laser burns (Fong et al. 1999; ETDRS 1991). In addition, more aggressive treatment for DN with angiotensin converting enzyme inhibitor (ACEI) and angiotensin receptor blockers (ARBs), instituted when albuminuria is detected, is unable to slow progression of structural glomerular lesions, as recently shown by the Renin-Angiotensin System Study (RASS) (Mauer et al. 2009), suggesting that prevention in a highly susceptible individual is a far superior approach. Based on the limitations of the currently used biomarkers, our clinical treatment decisions have to be made on the premise that all diabetic patients are equally susceptible to complications when we attempt to initiate early aggressive goals for treatment of glycemia.

Our lack of good biomarkers for diabetic complications also makes clinical trials for new therapies impractical or very expensive, since large numbers of patients must be followed for the long periods of time to achieve hard clinical endpoints such as diabetes related organ failure, or death. We also lack good biomarkers that reflect the potential value of reducing glycemic fluctuations, leading to incomplete validation as well as frequent and cumbersome sampling schedules in clinical studies. Because of these considerations, important clinical trials to develop new drugs and therapies for diabetes may not be done, due to a lack of widely accepted and clearly defined clinical endpoints that are detectable within a realistic timeframe. New biomarkers for diabetic complications that can be readily obtained from biologic samples would reduce the need to achieve only traditional endpoints in clinical trials, and would therefore lower costs by reducing the number of patients and the time required for completion.

### The role of glycation and oxidation in the pathogenesis of diabetic complications

It is recognized that high glucose levels in diabetes can lead to dysfunction of key vascular cells by increasing the levels of glycation and oxidative damage to cellular and plasma proteins. One of the most important processes associated with hyperglycemia is non-enzymatic glycation, which has gained increasing acceptance as one of the significant mechanisms contributing to the tissue damage seen in diabetes (Bucala and Cerami 1992; Brownlee 1992; Monnier 1989; Bunn and Higgins 1981). This immediate process involves spontaneous reactions between reducing sugars and amines on arginine, lysine, or N-terminal amino acids, and its extent depends only on the nature and concentration of the reactants (sugars and amines). It involves a complex series of chemical reactions that lead to the formation of early glycation products (EGPs), including Amadori products (fructosyl-lysine (FL), N-terminal Fructosyl compounds and their reactive Schiff's bases). Other important reactive products include highly reactive  $\alpha$  dicarbonyl compounds (methylglyoxal (MG), 3-deoxyglucosine (3DG), and glyoxal (G)), which can be formed by glycolytic intermediates, lipid peroxidation, and the degradation of glycated proteins. Studies in cell culture and animal models have shown that EGPs and dicarbonyls are directly toxic to tissues, are precursors of advanced glycation end products (AGEs) (Thornalley 1999; Thornalley et al. 2003), and activate multiple biochemical pathways (Nishikawa et al. 2000; Brownlee 2001) that further increase glycation and in turn, activate pathways that lead to additional cellular dysfunction (Du et al. 2003; Soriano et al. 2001; Hammes

et al. 2002; Huang et al. 2002; Hirata et al. 1997). The effects of AGEs are also amplified by interaction with cellular receptors, which further increase their toxicity (Kalea et al. 2009; Yan et al. 2009).

Glucose induced oxidative stress (OS) is also initiated in diabetes and can produce direct damage or lead to activation of the major pathways that produce diabetic complications (Brownlee 2005; Baynes and Thorpe 1999; Hink et al. 2001; Nishikawa et al. 2000). Although non-enzymatic reactions are responsible for the immediate reactions associated with glycation, enzymatic factors are known to control the production and detoxification of the major EGP and dicarbonyls. Thus, processes determined by genetic factors that vary among individuals can ultimately regulate the levels of dicarbonyl and Amadori products. Among these could be programmed mechanisms that regulate production of EGPs, dicarbonyls, or OPs, or mechanisms that are potentially protective by leading to their decomposition (Beisswenger et al. 2005).

### Translational studies supporting increased glycation and oxidation increase susceptibility to diabetic complications

Data supporting the role of glycation and oxidation pathways in the pathogenesis of DN and DR in humans

We, and others, have generated data supporting the hypothesis that genetic predisposition to DN/DR may involve differences in the capacity to produce or degrade EGPs, or regulate oxidative stress.

#### *Dicarbonyls*

We have previously shown that activation of pathways that determine levels of  $\alpha$  dicarbonyls occurs in patients that are susceptible to DN (Beisswenger et al. 2005). Variable production of MG and 3DG occurs in type 1 diabetes, and a positive relationship between levels of  $\alpha$  dicarbonyls and DN in three diabetic study populations supports this hypothesis in both type 1 and 2 diabetes (Beisswenger et al. 2005). This confirms a role for variable production of  $\alpha$  dicarbonyl compounds in determining propensity to diabetic complications, and suggests that this overproduction will result in increased levels of related AGEs. As already pointed out, the production and detoxification of these toxic AGE precursors are ultimately controlled by enzymatic mechanisms (Beisswenger et al. 1998), suggesting that genetic and environmental factors could regulate tissue glycation and potentially account for variable complication rates observed when individuals are exposed to the same degree of hyperglycemia.

## Oxidative stress

To address the relationship between oxidative stress (OS) and propensity to DN, we have just completed studies of OS in cultured skin fibroblasts from subjects with Type 1 Diabetes Mellitus (T1DM), where DN was confirmed by both clinical and biopsy characteristics (Beisswenger et al. 2008). These studies were performed in fibroblasts obtained from the upper quintile (Fast Progressors: FP;  $N = 25$ ) and lower quintile (Slow Progressors: SP;  $N = 25$ ) of biopsy proven glomerular change, in a study population of 125 subjects with T1DM, and in 25 non-diabetic controls (CON). Overall, these studies confirmed our previous *in vivo* data (Beisswenger et al. 2005), and showed for the first time in human studies that OS, as measured by the fluorescent probe 2,7-dichlorodihydrofluorescein diacetate, was consistently greater in the FP relative to the SP and controls. As proposed, but not previously documented in translational studies, we also showed that GAPDH activity, but not its mRNA message, was significantly decreased in fibroblasts from FP, relative to SP and CON. These results directly link OS to DN risk in a clinical translational study, and suggest that these changes may be secondary to genetic factors that control progression and/or metabolic memory carrying over from the prior *in vivo* milieu. These findings are supported by data confirming that cultured skin fibroblasts (SF) correlate well with genetically determined behavior of renal cells (Ceriello et al. 2000), and that they also contain pathways associated with the risk or protection from complications. It is also likely that oxidative stress in fibroblasts correlates with that in retinal pericytes or aortic vascular endothelial cells as well (Hammes et al. 2003). We, therefore, anticipate that phenotypes that predict biochemical patterns of complication susceptible and resistant patients, will correlate with the resulting biochemical profiles characteristic of DR and CVD as well as DN.

The concurrent increases in dicarbonyl stress and oxidative stress seen in these recent translational studies is consistent with Brownlee's proposed unifying hypothesis of diabetes complications (Brownlee 2005). It remains to be documented that elevated levels of biomarkers that reflect products produced by these pathways will identify those at greatest risk of diabetic complications.

## The ideal biomarker

The ideal biomarker should optimally have the following characteristics

1. be measured in a minimally invasive way
2. be useful when measured repeatedly over time

3. identify early stages of disease
4. indicate future disease prognosis
5. correlate well with progression of disease and response to therapy
6. have a strong scientific basis to justify its use.

## Biomarkers that reflect glycation and oxidative stress

We believe that strong biomarker candidates potentially capable of detecting susceptibility to complications are those that reflect the selective activation of glycation and oxidative pathways in high-risk individuals. These pathways lead to the formation of a spectrum of early glycation products (EGPs), and the chemically reactive  $\alpha$  dicarbonyl compounds, methylglyoxal, 3-deoxyglucosone, and glyoxal (Beisswenger et al. 2001, 2005). These  $\alpha$  dicarbonyls, in turn, lead to the formation of later stage chemical reactions to form AGEs, a process that is independent from HbA1c formation (Thornalley et al. 2003). Increased glucose-induced oxidative stress (OS) may also be caused by inherent differences in processes that control cellular oxidative mechanisms, which can directly and independently activate major pathways that produce diabetic complications (Baynes and Thorpe 1999; Beisswenger et al. 2005). Both dicarbonyl stress and OS have been shown to be selectively activated in those prone to diabetic complications, resulting in higher levels of glycated and oxidized protein and lipid byproducts (Brownlee 2005; Baynes and Thorpe 1999), but not directly to changes in HbA1c.

Over the past few years, elucidation of the chemical structure of complex AGEs and oxidation end products that reflect these precursor pathways, and the evolution of specific and quantitative LC-MS/MS methods to measure these products, have set the stage for their use in large-scale outcome studies. Our understanding that each represents end products of distinct precursor pathways, also allows us to use these products to assess and integrate the impact of increased EGP and a dicarbonyl production on complications over time. New biosynthetic methods have also allowed the production of highly purified normal and heavy stable isotopic analytical standards, allowing accurate quantification in biological samples. A key factor in this expanded analytical capability is the triple quadrupole LC-MS/MS that has made the proposed studies possible. These systems allow unprecedented analytical sensitivity and we have further improved sample throughput by developing new, single column analytical methods, which can quantify multiple AGEs and OPs on shortened runs.



Measurement of potential biomarker assays by liquid chromatography/triple quadrupole mass spectroscopy

A battery of unique AGEs and oxidation biomarkers can be measured in plasma and urine samples by LC–MS/MS. As previously discussed, these products reflect and integrate the activity of glycation pathways that form early glycation products (EGPs), including Amadori products and  $\alpha$  dicarbonyl compounds, as well as oxidative stress pathways productive of superoxides, free hydroxyl radicals, and peroxides.

Utilizing these methods, the following potential profile of unique biomarkers can be measured utilizing internal standardization by stable isotope substituted. We have modified the methods developed for the concurrent quantitative measurement of biomarkers indicative of protein glycation, oxidation, and nitrosative damage (Ahmed et al. 2002) by employing a single  $2.0 \times 250$  mm Synergy 4micron 80A column (Phenomenex, USA) with a mobile phase of Methanol/H<sub>2</sub>O gradient with 0.29% heptafluorbutyric acid, with a total analysis time of 60 min.

The following specific products have potential as biomarkers for glycative and oxidative stress (See Fig. 1 to observe the structural formulae)

#### *Arginine-derived AGEs*

These biomarkers include the quantitatively important hydroimidazolones (HI); which are AGEs derived from arginine residues modified by glyoxal, MG, and 3-DG and include G-H1 (glyoxal hydroimidazolone), MG-H1 (methylglyoxal hydroimidazolone), and 3DG-H (3-deoxyglucosone hydroimidazolone), respectively.

#### *Lysine-derived AGEs*

Other important AGEs that can be measured are lysine-based and include glyoxal-derived N<sub>ε</sub>-carboxymethyl-lysine (CML), and MG-derived N<sub>ε</sub>-carboxyethyl-lysine (CEL) (Thornalley et al. 2003). Other AGEs that can be measured include the more traditional product, pentosidine, which is measured by HPLC and fluorescence detection (Sell et al. 1991).

*Quantitative markers of oxidative damage to proteins* can also be measured and include methionine sulfoxide (MetSO), formed by the oxidation of the sulfhydryl group on Methionine (Yu et al. 2006). The tyrosine cross-link, dityrosine, as well as a widely studied marker of combined oxidative/nitration damage to proteins, 3-nitrotyrosine (3-NT), can also be measured (Geibauf et al. 1996). To

amplify the information obtained on the role of oxidative stress in the development of diabetic complications, another unique oxidative product, 2-aminoadipic acid, a product resulting from metal catalyzed oxidation of lysyl residues (Sell et al. 2007), can also be measured.

#### *Fructoselysine and HbA1c*

Early-stage reactions, or early glycation products (EGPs) that can be measured include fructosyl-lysine (FL) and N-terminal fructosyl-amino acids. The commonly used N-terminal fructosyl adduct, HbA1c, can be measured by high-performance liquid chromatography (HPLC) (Diamat method; Bio-Rad Irvine, CA), and determines the percentage of hemoglobin with a fructosyl-valine residue on the N-terminus of the  $\beta$ -chain, which in turn accounts typically for 60% of fructosamine residues, the remaining 40% being FL residues. Total FL residues in serum proteins can be determined by GC/MS (Miyata et al. 1998), as can FL free adducts.

### **Potential methods for sample preparation**

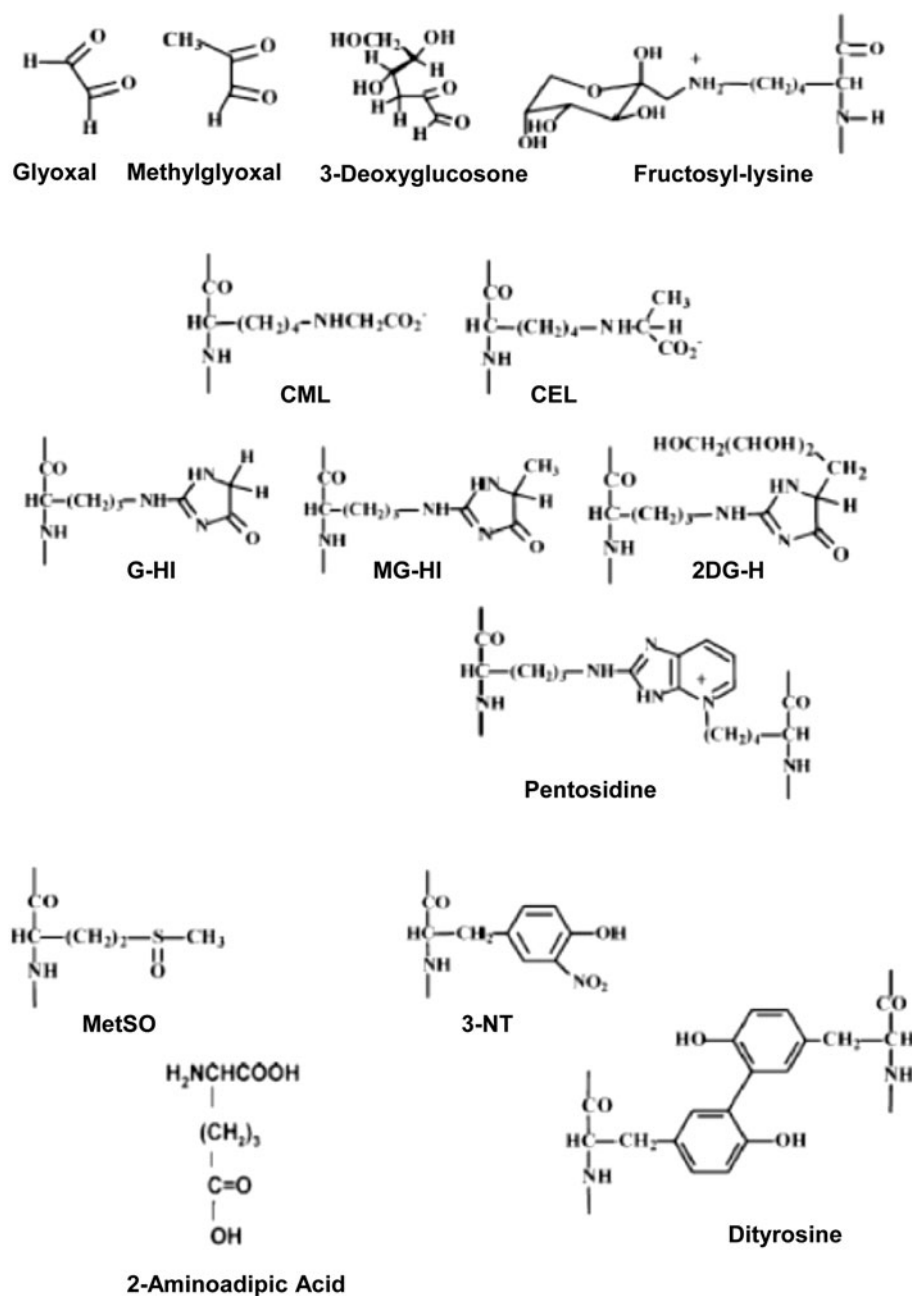
#### *Plasma sample preparation*

##### *Ultrafiltrates (free adducts)*

The rational for measuring this fraction is based on the knowledge that cells maintain the quality and functional integrity of proteins by degradation and replacement of proteins damages by oxidation and glycation (Thornalley et al. 2003; Goldberg 2003). This occurs by proteolysis, liberating the oxidized, glycated, and nitrated, amino acids as free adducts, which in turn are released into blood plasma and excreted in urine (Thornalley et al. 2003). Since these free adducts are released into blood plasma as tissue breakdown of AGEs occurs, changes in plasma concentrations reflect tissue damage in diabetes, while providing new markers indicative of the damaging effects of hyperglycemia. This “free fraction” can be prepared by centrifugation at 4°C through microspin filters (10,000 MW filter cut-off, 50  $\mu$ l aliquot).

##### *Adduct residues chemically bound to plasma proteins*

Since some of the products are acid labile, chemically bound products are determined after exhaustive sequential enzymatic digests with pepsin, pronase E, and aminopeptidase/prolidase (50  $\mu$ g protein equivalent) under nitrogen as described previously described, with controls for protease autolysis (Ahmed et al. 2002).

**Fig. 1** Chemical structure of chemical biomarkers

#### Preparation of urine samples to measure excretion of adducts

For determination of the AGE and OP biomarker profile in urine, we have used the filtrate prepared by centrifugation at 4°C through microspin filters (10,000 MW filter cut-off) as previously described (Ahmed et al. 2005a). Urine creatinine levels should also be determined to provide a uniform expression of product/creatinine urine analyte content. The urinary and serum “free fraction” determinations will also allow the calculation of renal clearance rates of each analyte.

#### Existing data on blood and urine levels of AGEs and OPs in diabetes

Following our initial studies of  $\alpha$  dicarbonyls (Beisswenger et al. 1998, 1999, 2001, 2005), we have collaborated with Rabbani and Thornalley in assessing blood and urine levels of selected AGEs and OPs in preparation for larger outcome studies. These have allowed us to accrue preliminary data on the degree of between patient variability of these products, and the magnitude of differences between diabetic and control populations. In these studies, we found

that the quantitatively highest in vivo AGEs in type 1 diabetes are hydroimidazolones derived from arginine residues modified by methylglyoxal, 3-deoxyglucosone, and glyoxal (Ahmed et al. 2005a, b) produced in turn from their individual synthetic pathways. Other quantifiable AGEs in these studies include glyoxal-lysine-derived carboxymethyl (CML), and the MG-lysine product, carboxyethyl-lysine (CEL) (Thornalley et al. 2003). We have also measured quantitative markers of oxidation, including the oxidation of MetSO, formed by the oxidation of methionine, as well as other markers of nitrosive damage to proteins leading to production of 3-nitrotyrosine (Gaut et al. 2002).

These studies showed that the concentrations of some of the hydroimidazolones are remarkably high in vivo in diabetes, with up to 26% of cellular proteins having such a modification, particularly for the AGE (MG-HI) which increased up to ninefold in plasma, and 15-fold in urine. We have also shown that urinary and plasma levels of the key oxidative product (MetSO) increases fivefold in diabetes relative to controls (Ahmed et al. 2005a, b). These unprecedented increases observed in AGEs and OPs in diabetes, and the substantial differences observed between individuals, suggest that these biomarker levels, will markedly sharpen the ability to differentiate subjects at very high versus very low risk of complications. The key AGEs and OPs proposed to be potentially most predictive of complications, including hydroimidazolones and MetSO, also showed no correlation with HbA1c in these studies (Ahmed et al. 2005b), suggesting that they are respond to the level of glycemia based on individual patient characteristics.

The application of these unique chemical end products to large-scale outcome studies will be based on the concept that they reflect the activity chemical pathways that lead to complication. These proposed studies are predicated on the concept that these biomarkers represent slowly turning over end-products of glycative and oxidative pathways whose short-lived chemical precursors would not reflect long-term overproduction when checked in blood and urine samples.

Studies to date suggest that these long-lived AGEs and oxidation products have the potential to be excellent biomarkers for the inherent propensity for the development of DN/DR and perhaps CVD (Beisswenger et al. 2005). These products are unique since the magnitude of their increase in blood and urine in diabetes are unprecedented for clinical biomarkers. Their relatively slow turnover, however, makes them potentially major determinants of “metabolic memory”, a phenomenon that was so dramatically apparent in the DCCT/epidemiology of diabetes interventions and complications (EDIC) outcomes (Nathan et al. 2005). The development of liquid chromatography-triple quadrupole mass spectrometric (LC–MS/MS) methods to measure specific AGEs and oxidation products provides us for the

first time with powerful new tools to assess the efficacy of these biomarkers in predicting diabetic complications (Thornalley et al. 2003).

### Existing data on the relationship between glycation/oxidation products and diabetic complications

It has previously been proposed that AGEs and oxidation products may be risk factors for diabetic complications. Until recently, however, our knowledge of these products has been limited to the early glycation products (EGPs), several oxidation end products, and a few AGEs. Most prior studies have measured limited numbers of AGEs (Monnier et al. 2008; Beisswenger et al. 1993; Dyer et al. 1993; Monnier et al. 2005; Yu et al. 2006), particularly pentosidine and carboxymethyllysine, or have focused on a few end-products that reflect oxidative stress (Baynes and Thorpe 2000; Yu et al. 2006). A substantial number of these analyses have also been performed as semi quantitative immunoassays, which have generally not been validated against quantitative chemical analyses. Although some of these studies have shown correlations between blood levels of these products and complications (Monnier et al. 2005), none have validated their predictive value in large-scale controlled diabetes outcome studies.

### Other potential biomarkers of diabetic complication

Many biomarkers have been proposed to detect risk of progression to diabetic complications, but none have replaced our current limited long-standing markers of glycemia (e.g. HbA1c) in the clinical setting. Studies using proteomic markers have also been performed, but many have lacked proper patient populations, have produced very few useful markers, and have generally not been hypothesis driven (Niwa 2008). Proteomic studies still have substantial potential to lead to useful biomarkers of diabetic complications, but optimal outcomes are more likely to be achieved if the following guidelines are followed:

### Criteria to consider in evaluation of proteomic approaches to biomarker discovery

Personal communications from Jay Heinecke-University of Washington.

1. Preliminary data, showing strong candidate biomarkers, should demonstrate the feasibility of the approach.
2. A plausible hypothesis should link biomarkers with disease pathogenesis.

3. There should be documented expertise of the investigators with mass spectrometry, statistics, and management of large quantities of complex data.
4. The potential clinical utility of assays based on blood or urine should be documented.
5. The study design should facilitate biomarker discovery and validation early in disease pathogenesis.
6. It is important to demonstrate large differences in levels of candidate markers (e.g., odds ratios >6–10: because smaller differences are unlikely to yield biomarkers that will have a significant impact on disease diagnosis and management.
7. Strong candidate biomarkers should be initially be validated using high throughput methods in large numbers of apparently healthy diabetic subjects that subsequently develop the outcome to be studied.
8. When using mass spectroscopy, mass accuracy, and quantification of peaks of material by SELDI-TOF-MS and MALDI-TOF-MS should be documented since a major issue in analysis of serum and plasma is the complexity of these proteomes. This is also important since it is likely that biologically relevant biomarkers are likely to be present at low concentrations, making them difficult to detect and identify.
9. Documentation should be performed in a second, independent cohort of subjects, thus greatly increasing the likelihood that candidate biomarkers are of broad clinical significance.

### Important guidelines to follow in the performance of biomarkers studies

There are a number of important factors to consider in utilizing blood or urine samples go obtain optimal results from existing studies or planning new outcome studies:

#### Stability of adducts

In samples that have been stored for extended periods of time, it is important to document their long-term stability. In prior collaborative studies, we have investigated these issues and shown that many AGEs measured in stored plasma samples are stable over multiple years (Ahmed et al. 2002). We have also recently confirmed acceptable stability by comparing analyte levels in plasma samples stored at  $-80^{\circ}\text{C}$  for 10–15 years with those in freshly drawn plasma from diabetic subjects. Tyrosine related oxidation products have also been shown to be stable over many years (Thornalley et al. 2003).

#### Artifactual production of products

It is very important to document that products are not being artifactually produced during processing or storage. This can be a problem as illustrated by some of our recent studies of AGEs and OPs in aged serum, where presumed artifactual increase of oxidative stress, manifested as higher than expected levels of MetSO, was observed in some samples. Relative to past experience with plasma, we observed 10–20-fold increases, and wide variability in repetitive samples from the same research subjects. By documenting that MetSO was equally elevated in the same serum samples prepared by two methods (protein bound and the 10 K filtrates “free fraction”) from the same subjects, we confirmed the consistency of the process in given samples. It was also of interest that other oxidation products in these samples differed in their response to this insult. For example, pentosidine, a product that reflects both oxidative and glycative stress, showed changes similar to MetSO, while another OP, 2-aminoadipic acid, did not.

#### Plasma compared with serum

When these same products were measured in plasma samples that had previously been collected by a carefully defined protocol (collected in EDTA, immediate spinning, separating plasma from RBCs (at  $4^{\circ}$ ), followed by immediate freezing and long-term storage at  $-80^{\circ}\text{C}$ ), MetSO levels were in the expected range when compared with samples from the same subjects who showed artifactual OS in serum. None of these modifications were observed in urine samples that were collected and stored by standard protocols.

Based on these observations, it is likely that plasma is be a better choice for measurement of OP and AGEs in stored samples since it contains the chelating agent (EDTA) and is immediately spun and separated from RBCs and flash frozen after collection. Serum, on the other hand, has to undergo clotting at room temperature before separation and storage, thus exposing proteins to leukocyte myeloperoxidase and other pro-oxidant enzymes. Serum also contains no chelator of trace metals (Fe and Cu), both of which can catalyze spontaneous in vitro oxidative stress (Wolff and Dean 1987).

### Documentation of sample quality in large clinical outcome studies

Since there is always a possibility that some products may degrade or that oxidative adducts may be formed spontaneously during initial processing or with prolonged storage



of clinical samples, one can investigate these possibilities by measuring AGEs and OPs in stored plasma and urine samples from a randomly chosen sub-group of subjects from the larger study cohort. These quality control studies can utilize multiple time points over the duration of the study. The results of these initial analyses can then be used as follows:

#### Variability

To assess how each product varies, one can determine the within and between subject variability for multiple samples from a randomly chosen sub-group. The goal is to confirm that between subjects variability exceeds within subject variability for multiple timed collections.

#### Number of samples needed for acceptable quantitative estimate of biomarker

One approach is to calculate the optimal number of samples required to provide an acceptable quantitative estimate of each plasma and urinary biomarker over time as follows: A representative calculation by this approach is based on urinary pentosidine levels in 4–6 samples/diabetic subject over 5 years in one of our prior studies (ten subjects). For these measurements, it was determined that between person variance =  $7.64 \times 10^{-7}$  and within person variance =  $2.06 \times 10^{-6}$ . Thus, total variance = between person variance + within person variance =  $7.64 \times 10^{-7} + 2.06 \times 10^{-6} = 2.82 \times 10^{-6}$ . For the mean of  $N$  observations from the same person, the variance of mean would then = between person variance + within person variance/ $N$  or  $7.64 \times 10^{-7} + 2.06 \times 10^{-6}/N$ . Based on these calculations, one can then determine if the within person variance of the mean is less than the between person component. As shown in Table 1,  $N = 3$  will achieve this aim since  $2.06 \times 10^{-6}/3 = 6.87 \times 10^{-7}$  which is less than the  $1.45 \times 10^{-6}$  variance of mean value (3rd line in Table).

**Table 1** Sample variance and determination of optimal number of samples required to provide biomarker estimate

Sample $N$ per subject	Within person variance	Variance of mean	% Reduction from variance for $N = 1$
1	0.000002060	0.000002824	0.0000
2	0.000001030	0.000001794	−36.4770
3	0.000000687	0.000001450	−48.6360
4	0.000000515	0.000001279	−54.7154
5	0.000000412	0.000001176	−58.3631

Based on determination of urinary pentosidine in multiple NHS samples

Expressed in nmols/mg creatinine

Also note that in this example, for sample sizes  $>4$ , little is gained by increasing the number of analyses since each additional observation gives  $<5\%$  total reduction.

#### Determination of decay or rise in biomarker over time

To confirm that no significant decay or rise in the products occurs over time, it is also important to analyze the trends in the data over the duration of the study. To address this issue, we previously performed preliminary studies on repetitive measurements (4–6 samples were collected over 5 years) of urinary AGEs and OPs in ten diabetic subjects, and were unable to demonstrate significant positive or negative changes in regression coefficients of analyte levels over time.

#### Use of intraclass correlation calculation

An intra-class correlation calculation from repeated measures within subjects can also provide a summary of the reproducibility of an analyte over time. This method essentially summarizes the degree of within to between patient variation, where  $r = 1$  is indicative of no within patient variation. Using this method, one can adjust for any within subject temporal effects, if any, that may exist. An example of this approach comes from calculations from DCCT/EDIC where based on the distribution of the log of the intra-class correlation (Lachin et al. 2007), 60 random subjects with an average of 3 replicates each will allow an estimate of intraclass correlation of 0.8 or higher to within 10% with 95% confidence.

#### Assessment of stability of sample by assessing association with another biomarker

Another approach to assess the stability of the stored samples over time is to assess their association with another marker measured over the duration of a clinical outcome study. For example, one can examine the regression of the AGE “CML” on the glycation product HbA1c, since they were shown to correlate in prior studies (Ahmed et al. 2005a). Assuming that HbA1c has already been measured during the study, CML will then be measured in long-term stored plasma samples, and also in recently collected plasma samples and the results linked to the HbA1c value measured from each sample when collected. CML will be regressed on HbA1c separately using values from the long-term stored sample values, and then likewise using the newer sample values. This provides an intercept and slope of the regression of CML on HbA1c from the stored as well as the recent collections. If the CML (or any other chosen biomarker) values in the long-term samples are stable, then the slopes and the correlations from the older and newer samples should be similar.

Documentation that AGEs or oxidation products show acceptable degrees of variability, and have documented stability over time, is an important factor in deciding which products are acceptable to use as biomarkers of diabetic complications. One can then decide from this representative subgroup, which biomarkers have optimal characteristics to justify proceeding with a larger scale analysis. This decision will depend on the number of products that show acceptable variability and stability, and the specific products that are acceptable. For the latter considerations, one will give priority to obtaining acceptable variability of products that are most representative of proposed activated precursor glycation pathways (hydroimidazolones, CEL, CML) and oxidative pathways (methionine sulfoxide and 2-aminoadipic acid) and, therefore, most likely to document the study hypothesis.

### Potential benefits of developing new biomarkers for diabetic complications

Optimally, a study designed to test specific biochemical biomarkers will have a good probability of identifying a diabetic sub-group at substantially greater risk of developing complications, as well as those who are relatively protected, compared to what is provided by a history of hyperglycemia alone. As already pointed out, current biomarkers of diabetic complications have significant limitations, in that they cannot identify those at greatest risk or provide early assurance of protection from complications. Our current treatment paradigm requires that all people with diabetes be exposed to aggressive therapeutic programs to control glycemia at near normal levels, with their greater inherent risks and costs, irrespective of disease propensity. Because of the great expense and limited health care systems and providers to achieve these ends, the end result achieved is frequently inadequate glycemic levels in many subjects with diabetes.

Documentation of the predictive value of glycation/oxidation, or other biomarkers for progression of DN, DR, and CVD, would allow the identification those at high or low risk of diabetic complications during the earliest stages of diabetes. This would allow selection of risk and cost appropriate therapeutic regimens to achieve appropriate levels of glycemic control. For example, those identified as being susceptible to complications could have very stringent goals for glycemic control ( $HbA1c < 6.0\%$ ), and have intensive insulin delivery and monitoring systems initiated at the time of diabetes onset. Early initiation of medications that modify the renin-angiotensin system (Mauer et al. 2002), cholesterol and VLDL levels (Almuti et al. 2006; Degenhardt et al. 1998; Rosario and Prabhakar 2006), or specialized diets with low levels of AGEs or oxidative

products (Koschinsky et al. 1997) could also be considered. Important information provided by these studies on basic biochemical mechanisms and pathways responsible for diabetic complications could also stimulate development of novel therapeutic approaches that could modify offending toxic chemical pathways to delay or arrest DN, DR, and CVD.

Studies performed in landmark outcome studies with type 1 diabetes, may be applicable to patients with type 2 diabetes as well. It is well recognized that major clinical trials have shown a similar significant relationships between glycemic control and progression of DN and DR in both type 1 and type 2 diabetes in DCCT and UKPDS (DCCT/EDIC Complications Research G 2002; Holman et al. 2008), suggesting similarities in pathogenesis for both diabetes types. These considerations have led the ADA, AACE, and EASD to recommend similar HbA1c guidelines to prevent DR and DN in both type 1 and 2 diabetes (Nathan et al. 2008; Rodbard et al. 2008). Since the etiology of CVD in type 2 is associated with more diverse risk factors than in type 1, however, studies of type 1 should provide more focused information on the role of glycemia and its associated metabolic abnormalities on overall risk of CVD in diabetes.

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