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## BIOMARKERS IN DIABETES: HEMOGLOBIN A1c, VASCULAR AND TISSUE MARKERS

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### Abstract

Biomarkers are conventionally defined as ‘biological molecules that represent health and disease states.’ They typically are measured in readily available body fluids (blood or urine), lie outside the causal pathway, are able to detect sub-clinical disease, and are used to monitor clinical and sub-clinical disease burden and response to treatments. Biomarkers can be “direct” endpoints of the disease itself, or “indirect” or surrogate endpoints. New technologies (such as metabolomics, proteomics, genomics) bring a wealth of opportunity to develop new biomarkers. Other new technologies enable the development of non-molecular, functional or bio-physical tissue-based biomarkers. Diabetes mellitus is a complex disease affecting almost every tissue and organ system, with metabolic ramifications extending far beyond impaired glucose metabolism. Biomarkers may reflect the presence and severity of hyperglycemia (i.e. diabetes itself), or the presence and severity of the vascular complications of diabetes. Illustrative examples are considered in this brief review. In blood, hemoglobin A1c (HbA1c) may be considered as a biomarker for the presence and severity of hyperglycemia, implying diabetes or pre-diabetes, or, over time, as a “biomarker for a risk factor”, i.e. hyperglycemia as a risk factor for diabetic retinopathy, nephropathy, and other vascular complications of diabetes. In tissues, glycation and oxidative stress resulting from hyperglycemia and dyslipidemia lead to widespread modification of biomolecules by advanced glycation end products (AGEs). Some of these altered species may serve as biomarkers, whereas others may lie in the causal pathway for vascular damage. New non-invasive technologies can detect tissue damage mediated by AGE formation: these include indirect measures such as pulse wave analysis (a marker of vascular dysfunction) and more direct markers such as skin autofluorescence (a marker of long-term accumulation of AGEs). In the future, we can be optimistic that new blood and tissue-based biomarkers will enable the detection, prevention, and treatment of diabetes and its complications long before overt disease develops.

### Keywords

Biomarkers; Hemoglobin A1c; Carbonyl stress; Pulse wave analysis; Autofluorescence; Retina

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## DEFINITIONS: BIOMARKERS AND RISK FACTORS

The term “biomarker”, alternatively called “molecular marker” or a “signature molecule” requires clear definition and must be distinguished from “risk factor.” A biomarker has been defined as “*a biological molecule found in blood, other bodily fluids, or tissue which represents a sign of a normal or abnormal process or of a condition or disease. A biomarker may be used to see how well the body responds to a treatment for a disease or condition*”<sup>[1, 2]</sup>. Examples by this definition include prostate specific antigen (PSA), a biomarker for prostate cancer (whose utility has become controversial of late),<sup>[3]</sup> or, in the context of diabetes or pre-diabetes, hemoglobin A1c (HbA1C), which reflects hyperglycemia over several weeks preceding the test.<sup>[4]</sup> In contrast, a risk factor may be defined as something that increases the chance of developing a disease. Risk factors typically have a causal element, and may be classified as unmodifiable (e.g. age, gender, or genotype), or modifiable (e.g. LDL cholesterol, a risk factor for atherosclerotic disease),<sup>[5]</sup> smoking (a risk factor for lung cancer),<sup>[6]</sup> or chronic hyperglycemia (a risk factor for the microvascular complications of diabetes).<sup>[7]</sup>

Biomarkers may be viewed as direct or indirect markers of the extent of disease, but regardless, they typically lie outside the causal pathway.<sup>[11]</sup> They are used to monitor the burden of clinical or subclinical disease. A ‘direct biomarker’ is directly attributable to disease itself. Elevated PSA or thyroglobulin, produced by prostate or thyroid cancer cells respectively, provide direct measures of cancer burden.<sup>[3, 8]</sup> In diabetes, elevated HbA1c is a consequence of an (often) asymptomatic underlying “disease state”, namely hyperglycemia.<sup>[4]</sup> Glycation of hemoglobin is, in itself, without functional consequence, i.e. it does not directly cause disease, and so HbA1c may most accurately be described as a direct biomarker for glycemia over 6-8 weeks before the test. Additionally, since sustained hyperglycemia is well established as a *risk factor* for microvascular complications of diabetes,<sup>[9]</sup> HbA1c may also be considered as a “biomarker for a risk factor”, i.e. a marker of risk for diabetic retinopathy, nephropathy, and other vascular complications. In this last example, the vascular complications do not cause the elevation of HbA1c, nor is glycated hemoglobin a direct participant in the causal pathway for vascular disease, so HbA1c is, in this context, an indirect biomarker. In diabetes, these matters are further complicated by (a) the question of whether hyperglycemia is viewed as the central issue, or whether the complications of diabetes are actually the greatest concern (some treatments in development hold promise to inhibit or block the vascular complications without affecting glucose levels), (b) the recent (and still controversial) decision to utilize HbA1c as a diagnostic criterion for the diabetes<sup>[10]</sup> and (c) the realization that diabetes is not just a disease of carbohydrate metabolism, but rather one that affects many other aspects of metabolism.<sup>[4, 11]</sup>

## UTILITY OF BIOMARKERS

Biomarkers provide the ability to identify people with subclinical disease before the development of overt clinical disease. They enable preventive measures to be applied at the subclinical stage, and the responses to preventive or therapeutic measures to be monitored. They facilitate studies of disease mechanisms, and they enable the assessment of new preventive and therapeutic measures by providing surrogate end points for intervention studies. In short, biomarkers enable us to monitor the burden of both subclinical and clinical disease.<sup>[12]</sup>

## IDENTIFICATION OF BIOMARKERS

### CHALLENGES

The development and characterization of an effective biomarker is arduous. In the context of diabetes, the slow development of vascular complications in humans means that very long-term studies are necessary. Biomarker utility must be confirmed in at least two, and ideally more, independent populations. In addition, while the utility of biomarkers may be high when diseases are considered at the population level, this may not apply to the individual patient: for example, the significance of PSA and HbA1c may be highly variable among individuals. [3, 4, 13] In addition, biomarkers can only be measured in tissues which are accessible, and often, such tissues are not the actual site of disease. With new, developing technologies, we need to think beyond blood (serum and plasma) and urine measures and consider ways to assess biomarkers and risk factors directly in the tissues affected by disease. In this regard, it is important to remember that the body has many microenvironments. These are found at tissue, cellular, and molecular levels, and they may not easily be reflected by markers present in the circulation. One clear example in diabetes is the retina, a highly specialized tissue that constitutes only 1/200,000 of total body weight, and as a result, any putative circulating biomarker would have to be highly specific to the retina (not to vascular disease in general). A major challenge in the field of biomarkers is to dissect cause and effect in the pathogenesis of disease, and in many cases, association has been mistaken for causation. In the area of diabetes, it is frequently stated that the Diabetes Control and Complications Trial (DCCT) showed that 'improved glycemic control' (or even 'lowering HbA1c') reduces complications [14, 15]. While the decreases in complications were indeed very likely a consequence of improved glycemia, intensive management in the DCCT addressed many other issues that could also have contributed to these effects; and, as detailed above, HbA1c is a biomarker, with no known role in mediating disease.

### OPPORTUNITIES

Studies over the past two decades have yielded sample sets (mostly blood products and urine) from large cohorts of people with both type 1 and type 2 diabetes that have been collected longitudinally over time, with detailed documentation of clinical outcomes [14-17]. Study of these stored samples in the light of new knowledge and technologies will afford opportunities to find new biomarkers identifying people with subclinical complications of diabetes. Secondly, new technologies, including proteomics, metabolomics, genomics, new imaging techniques, and functional measures will enable exploration of previously unknown territories, and will allow opportunities for the identification of biomarkers outside the conventional areas of plasma, serum, and urine studies. Recently, an excellent overview of the application of new methodologies and their potential to identify circulating biomarkers in diabetes has been published by McKillop and Flatt [18].

Biomarkers to monitor diabetes and associated micro- and macrovascular complications may be broadly classified as follows: genomic (single-nucleotide polymorphisms), transcriptomic (mRNA), proteomic (proteins and glycoproteins), metabolites (lipids, sugars, amino acids), markers of subclinical disease (arterial function, aortic plaque burden) and metabolic end-products (urinary proteins) [12, 13, 18]. In this review, we will focus principally on HbA1c and biomarkers identified by new technologies that may enable non-invasive studies of tissues that are the targets (or surrogate targets) of the complications of diabetes. As examples, we will consider emerging biomarkers related to vascular function (pulse wave analysis) and of tissue modification/damage (skin autofluorescence and retinal leakage) in diabetes. Abnormalities of vascular function may detect early abnormalities of blood vessels before the onset of structural changes and manifest disease. Noninvasive studies of connective tissues such as skin may provide markers to reflect generalized

connective tissue damage throughout the body, including for example, critical tissues such as vascular basement membranes. Finally, we will consider future possibilities for biomarker development for the retina – a microcirculation that is unique in many ways, including the fact that can be viewed directly. Interestingly, these tissue-specific biomarkers each relate to, and depend upon, an important underlying mechanism for the complications of diabetes, the complex chemistry that relates to glycation and oxidation of critical biomolecules. Furthermore, these biomarkers extend the definition given above: they are functional and physic-chemical measures, not specific molecules.

## HEMOGLOBIN A1c (HbA1c) AS A BIOMARKER IN DIABETES

The use of HbA1c as a diagnostic criterion for diabetes (> 6.5%) and pre-diabetes (5.7-6.4%) was recently added to the standards of care by the American Diabetes Association (ADA) based on the recommendations of the International Expert Committee [10, 19]. The consensus recognized several advantages of HbA1c in comparison to fasting plasma glucose levels or glucose level 2-h post-75g oral glucose load. Specifically, HbA1c was viewed as a better standardized assay than glucose, a better index of overall glycemic exposure, and as less subject to biological variability, pre-analytic instability, prandial status, and acute stress [10]. On the other hand, the costs of providing the assay in certain parts of the world could preclude its routine use, in which case, the International Expert Committee recommends using previously recommended glucose criteria to diagnose diabetes. The epidemiological evidence on the role of age, race, genetics, and physiology that are biological determinants of the HbA1c-blood glucose relationship further limits the assumption that HbA1c is a consistent measure of mean blood glucose [20]. The HbA1c-blood glucose relationship has been best described by the HbA1c-derived average glucose study [21]. This international study included a total of 507 individuals (268 patients with type 1 diabetes, 159 with type 2 diabetes, and 80 non-diabetic controls) for whom HbA1c data were obtained at baseline and at three months, measured in a central laboratory. Average glucose was calculated from at least two days of continuous glucose monitoring using seven-point daily self-monitoring of capillary glucose performed at least three days per week. Results showed a significant correlation between HbA1c and estimated average glucose (eAG) which did not differ by age, sex, diabetes type, race, or smoking status. Though these findings are clinically significant, the study had some notable limitations, including under-representation of ethnic groups (especially Asians and Africans), the average glucose estimates were based on two methods (continuous interstitial glucose monitoring (CGM) and intermittent self-monitoring of capillary glucose), and exclusion of children, pregnant women, and diabetic patients with erythrocyte disorders that might affect red cell survival time (which the estimate assumes is a constant). These factors limit the direct application of these results to the general population [21]. The inter-individual heterogeneity in red blood cell (RBC) life span and in glucose gradient across RBC membranes have also been shown to be strong determinants of hemoglobin glycation in diabetic and non-diabetic subjects [22, 23]. In an observational study with a small sample size (n=12, diabetic=6, non-diabetic=6), the mean age of circulating RBCs ranged from 39 to 56 days in diabetic subjects, and 38 to 60 days in non-diabetic controls, with significant variations in measured HbA1c when corrected for mean RBC age [22]. In another study (n=26; 21 diabetic and 5 non-diabetic), inter-individual variations in glucose gradient of RBCs, as measured by ratio between plasma glucose and RBC glucose ( $C_i$ -to- $C_o$ ) were observed, and these may contribute to variations in hemoglobin glycation and the interpretation of HbA1c as a biomarker for diabetes, and its values in predicting diabetes complications [23]. The role of race/ethnicity as a significant determinant of hemoglobin glycation has been reported by several large observational studies [24-26] and these findings seriously question the validity of a single set of HbA1c criteria for all diabetic patients.

Despite these concerns, HbA1c is now a routinely used biomarker for determination of chronic glycemia in diabetes, and several large studies have shown HbA1c to respond to lifestyle (diet, weight loss) and pharmacological therapy in diabetic patients. In the Diabetes Prevention Program Outcomes Study, 10-year follow-up of diabetes incidence and weight loss showed lower HbA1c and fasting glucose concentrations in the metformin (n=924) and intensive lifestyle intervention (n=910) groups than the placebo group (n=932) [27]. Mean HbA1c values were lower in the intensive treatment group (n=711) versus conventional treatment group (n=730) of the Diabetes Control and Complications Trial (DCCT) [28]. As a biomarker in the diagnosis and progression of the disease, HbA1c has been shown to be decreased by exercise and weight loss [29, 30] and by pharmacological (exenatide) therapy [31], and increased in response to diets high in fats [32] in patients with diabetes.

## METABOLIC BIOMARKERS IN DIABETES: GLYCATION, OXIDATION, AND CARBONYL STRESS

Consideration of HbA1c serves as an introduction to a much wider field of actual and potential biomarkers for diabetes and its complications. The discovery of HbA1c, and its identification as a glycated form of hemoglobin, [33] opened a field of diabetes research that has since been burgeoning for over forty years: the chemistry of glycation (non-enzymatic glycosylation) and its consequences, including the role of advanced glycation end-products (AGEs), free-radical damage mediated by reactive oxygen species, and lipoxidation. From today's vantage point, it is perhaps surprising that the importance of these processes in biology was not realized earlier. It is now fully 100 years since the first detailed descriptions of the browning reactions between amino acids and simple carbohydrates were defined by Louis Camille Maillard (1912) working in Paris, [34] but his work focused on food preservation and the maintenance of its nutritional value and sensory appeal, and the link to living systems was not made for almost sixty years. [33] Modification of many types of biomolecules (not only proteins, but also phospholipids and nucleic acids in both intra- and extra-cellular locations), is mediated by reactive intermediate products formed as a result of carbohydrate and lipid oxidation. In diabetes, increased substrate for these oxidative reactions (resulting from hyperglycemia and dyslipidemia) and increased oxidative stress accelerate the molecular damage, yielding altered species that may serve as both markers and mechanisms of disease. [35, 36] The binding of AGEs with cellular receptors activates pathways that have been implicated in the pathogenesis of vascular complications in diabetes. [37] Serum levels of soluble receptors of AGE products (RAGE) have been proposed as predictive biomarkers of risks of coronary heart disease in type 2 diabetic patients [38], and of carotid intima-media thickness in patients with type 1 diabetes [39].

Fifteen years ago, we introduced the term "carbonyl stress" to encompass the combined 'glycooxidative' and 'lipoxidative' stresses imposed on biomolecules with advancing age but greatly enhanced by diabetes. [40] The term reflected the fact that most glyoxidation or lipoxidation intermediates, whether derived from carbohydrate or lipid oxidation, contain a reactive carbonyl moiety. Carbonyl stress is thus imposed by free radical oxidation acting on biological substrates, which include carbohydrates, such as glucose, and lipids, such as polyunsaturated fatty acids, to yield intermediate reactive products which possess carbonyl moieties. The reactive carbonyls in turn modify macromolecules, including proteins, nucleic acids, and phospholipids to yield advanced glycation end-products (AGEs) or advanced lipoxidation end-products (ALEs). The net effect is altered structures of proteins (potentially affecting the function of enzymes, receptor-ligand interactions etc.), lipids, and genetic material, that may eventually contribute to the development of disease. Carbonyl stress acts widely throughout the body, yielding some products that mediate disease, and others that are "bystanders", with the potential to serve as biomarkers [41, 42].

Included in the definition of “biomarker”, as stated above, are markers found in tissues, not just in bodily fluids such as blood or urine. In diabetes, as in many conditions, the most important sites of disease are in tissues (especially blood vessels, and specifically those in certain locations), not in the blood or urine. To identify biomarkers, we tend to rely on measurements from blood or urine, for obvious reasons of convenience and accessibility, but new technologies will enhance our ability to measure biomarkers in tissues. Furthermore, these technologies will enable us to use biomarkers that are not molecular, but rather functional in nature. Two examples of tissue-based biomarkers in the context of diabetes are (a) measures of vascular dysfunction, which in turn leads to vascular injury [43], and (b) measurement of autofluorescence of skin connective tissue proteins [44]. In both, carbonyl stress may be implicated: in the first case by causing endothelial dysfunction (i.e. causing disease), and in the second case as a surrogate measure or marker of long-term, whole-body glycoxidative damage.

## BIOMARKERS OF VASCULAR FUNCTION IN DIABETES

Endothelial activation leading to vascular dysfunction has been regarded as an early, preclinical component in the development of vascular diseases [45]. The endothelium is a monolayer of cells lining blood vessels throughout the body; it may be regarded as an organ weighing in total over 1 kg [46]. It has numerous functions, acting as a structural barrier between the circulation and the tissue, as a source of growth factors and angiogenic and anti-angiogenic factors, controlling thrombosis and fibrinolysis, mediating inflammation, and mediating vascular tone [46]. In diabetes, all of these functions are perturbed: basement membranes are thickened, permeability is altered, angiogenic/antiangiogenic balance is disturbed (with increases in either cell proliferation or cell death), thrombosis and platelet activation are increased, inflammation is enhanced, vascular tone is altered, and there is injury mediated by accumulation of lipids in the subendothelial space [47]. These functional effects may precede measurable structural changes, providing opportunities for functional biomarker development. One means by which endothelial function can be assessed is through pulse wave analysis, which can now be accomplished using a variety of instruments. Pulse wave analysis becomes abnormal early in the development of hypertension, diabetes, kidney disease and connective tissue disorders, and identifiable abnormalities may be reversible at these early stages [48, 49]. One measure of vascular function obtained with pulse wave analysis is ‘small artery elasticity’. In our own studies, we have found that small artery elasticity in patients with type 1 diabetes, compared with healthy, non-diabetic control subjects, is significantly decreased in the presence of microvascular complications of diabetes, and slightly decreased in the absence of complications. Furthermore, decreased small artery elasticity was independently associated with elevated levels of anti-angiogenic Pigment Epithelial Derived Factor (PEDF), which is thought to play an important role in promotion of vascular complications [50]. Several intervention studies in healthy individuals and in patients with type 2 diabetes or metabolic syndrome have reported a significant change in pulse wave velocity as a biomarker of arterial stiffness or vascular dysfunction. Supplementation of omega-3-fatty acids and serum lycopene concentrations have been shown to decrease and be inversely associated with pulse wave velocity, respectively [51-53]. Pharmacological therapy (atorvastatin and thiazolidinediones) or a short-term aerobic exercise regimen have also been shown to reduce pulse wave velocity, a biomarker of arterial stiffness measured non-invasively in adults with type 2 diabetes and/or hypertension and hypercholesterolemia [54-56].

## TISSUE BIOMARKERS IN DIABETES: SKIN AND RETINA

The glycation of skin collagen and the accumulation of advanced glycation end products (AGEs) has been strongly correlated with long-term diabetes complications, even after

adjusting for HbA1c [14]. A newly-described noninvasive method to assess tissue AGEs involves skin autofluorescence. This method is based on the specific fluorescence characteristics of AGEs and has been employed in both skin and lens [57, 58]. Studies by our group and others have shown that autofluorescence is increased in the presence of diabetes and may serve as a biomarker in identifying risks for microvascular complications in type 2 diabetic patients [14, 59]. In (unpublished) preliminary studies, we have found negative associations between small artery elasticity and tissue autofluorescence in patients with diabetes, i.e. low small artery elasticity may be associated with increased autofluorescence after correction for age.

Concerning the accumulation of advanced glycation end products in skin tissues, we have hypothesized that individual variations in oxidative stress may modulate susceptibility to the complications of diabetes. This hypothesis is an attempt to explain differing susceptibilities among individuals with similar glycemic exposure over many years to the development of complications. It suggests that a given degree of chronic hyperglycemia may have different consequences depending on oxidative stress and/or antioxidant defenses. This hypothesis was strengthened by our recent study of 96 type 1 diabetic patients, of whom 54 were participants in the Diabetes Control and Complication Trial [60]. All the patients in this study had duration of diabetes longer than 10 years. They were characterized as either prone or resistant to complications: those who were prone had severe retinopathy, microalbuminuria, or a cardiovascular event, while those who were resistant had none of these complications. Control (non-diabetic) subjects were also studied. Skin biopsies were taken and measures of early glycation, advanced glycoxidation or lipoxidation, and 'pure' oxidative damage (oxidation of methionine residues yielding methionine sulfoxide (MetSO) and not involving carbohydrates or lipids) were determined. As expected, this study confirmed that in non-diabetic patients, early collagen glycation is relatively constant, increasing only slightly throughout life, whereas advanced glycation products accumulate in a linear fashion with advancing age. It also confirmed that healthy, non-diabetic individuals vary greatly (up to two fold) in the rate at which they accumulate these products. MetSO also increases with advancing age in non-diabetic people, again with significant inter-individual variation. In diabetic patients, the rate of accumulation of glycoxidation and lipoxidation products is increased, as would be expected. Of great interest, however, MetSO increased at the normal rate, i.e. the same as non-diabetic controls, with advancing age in diabetic patients who were complication-resistant. In contrast, in diabetic patients who were complication-prone, the rate of accumulation of methionine sulfoxide was significantly increased. In summary, the study showed that after controlling for glycemia, glycoxidation and oxidation products accumulated more rapidly in diabetic patients who are prone to complications than in those who were resistant to complications. For the glycoxidation products studied (after accounting for long-term glycemia), and for MetSO (regardless of glycemia), accumulation rates in complication-resistant diabetic patients were similar to the average rates in non-diabetic controls. These findings are consistent with the hypothesis that variations in oxidative stress and/or antioxidant defenses mediate susceptibility to the development of vascular complications [60]. This implies that any biomarker which could assess changes in oxidative stress would be of potential utility in defining risks for complications.

Recently, noninvasive means to measure fluorescence of skin collagen, the surrogate measure of cumulative glycoxidative damage in skin, have been developed [57, 59]. This technique, which obviates the need for skin biopsy, is under intensive evaluation as a means to screen for the presence of undiagnosed diabetes, and as a means to determine susceptibility for complications of diabetes. It represents a new form of biomarker which detects tissue damage independent of changes in short-lived plasma proteins or urine. Combined with serial HbA1c measurements to enable chronic exposure to hyperglycemia to be estimated, the non-invasive measurement of skin fluorescence provides an opportunity

for a biomarker to monitor susceptibility to oxidative damage – since effects in skin are likely to reflect long-term stresses throughout the body.

Intensive treatment of hyperglycemia (~ 5 years) has been shown to slow the accumulation of advanced glycation products in skin collagen and to inhibit the development of abnormal physicochemical characteristics of skin collagen in the DCCT cohort [14]. On the other hand, a 4-month period of intensive glucose lowering therapy in patients with type 1 diabetes caused a significant decrease in initial glycation product of skin collagen (fructoselysine, [FL]), while no changes were observed in complex products of browning and oxidation (pentosidine, N<sup>3</sup>-(carboxymethyl)lysine [CML]), and skin fluorescence [61]. These findings suggest that skin autofluorescence serves as a biomarker of tissue damage in diabetes, and is a useful non-invasive marker of cumulative damage, that may be slowed or perhaps reversed by long-term intensive therapy for hyperglycemia. Skin autofluorescence has also been shown to increase post-prandially in type 2 diabetic patients and in healthy controls following a meal containing AGEs [62], and as discussed in the review by Goh & Cooper (2008), a low AGE-diet might be a therapeutic strategy for reducing exogenous AGE exposure in diabetes [63]. Several pre-clinical studies in diabetic animal models have shown reversal of skin damage by specific biomolecules, such as Vitamin A, L-carnitine, and alpha-lipoic acid [64-66]. However, controlled clinical studies are needed to identify optimal therapeutic interventions to lower skin AGEs, quantified by skin autofluorescence in early and advanced stages of diabetes.

Another example of an opportunity for new biomarker development is the retina. Diabetic retinopathy is the most frequent cause of new-onset blindness in working age adults in the developed world. The retinas weigh approximately 300 mg (wet weight for both eyes) [67]. The retina has a unique microcirculation, but its small size in relation to total body mass means that biomarkers in plasma or urine can only be useful if they are very tissue-specific. To obviate this problem, advantage may in the future be taken of the fact that the retinal circulation is readily visualized. We have hypothesized that leakage of retinal capillaries leads to extravasation of lipoproteins. Subsequently, in the diabetic environment, these lipoproteins become further modified by oxidative stress and glycation, and then mediate injury to a wide variety of different retinal cell types. Under normal circumstances, this extravasation of lipoproteins is rigorously prevented by the inner and outer blood retinal barriers, but in diabetes, the barriers undergo chronic injury, and eventually leakage of plasma ensues. In studies published in 2008, we showed evidence of leakage of LDL into the retina to an extent which correlates with the severity of complications of diabetes [68]. New techniques for retinal imaging may take advantage of these observations, detecting retinal leakage noninvasively, thereby proving new biomarkers to detect development of retinopathy at the preclinical stage. In this regard, the Retinal Laser Doppler Velocimetry Study has shown reduced retinal blood flow in type 2 diabetic patients with mild non-proliferative diabetic retinopathy to be negatively correlated with serum LDL, possibly due to constriction of pericytes in the retinal capillaries by elevated LDL [69]. Thus, the retinal microcirculation and associated measures of serum lipoproteins may be used as biomarkers in the treatment of early-stage diabetic retinopathy.

New technologies to assess retinal hemodynamics have also been employed. In one study of people with Type 1 diabetes (well-controlled; duration of diabetes ~ 9 years), no significant abnormalities were found in laser Doppler flowmetry [70]. However, in another, where postural change was used as a challenge, people with type 1 diabetes (duration ~12 years) showed abnormal responses in arterial diameter, suggesting an early risk marker of retinopathy [71]. Thus, these new non-invasive technologies, such as laser Doppler blood flowmetry [71], which measures arterial blood column diameter and blood speed in retinal vessels, provide opportunities to detect risks or progression of diabetic retinopathy which



otherwise may not be detectable by analytes in the systemic circulation. Histopathological changes in the retina have been shown to improve in response to lifestyle intervention in adults with impaired glucose tolerance [9], or management of dyslipidemia in type 2 diabetic cohorts [72, 73].

Risk for the development of complications of diabetes involves many different factors. These include hyperglycemia, oxidative stress, dyslipidemia, insulin-resistance, carbonyl stress, abnormalities of thrombosis, altered mitochondrial function, inflammation, reactive oxygen species, reactive nitrogen species, formation of advanced glycation end products, alterations in the receptor for advanced glycation end products, and the presence of infection. Each of these stresses may be operative and have different effects in specific tissue, cellular, and molecular microenvironments. New technologies that enable measurements of these processes noninvasively at the tissue level are likely to provide a wealth of new biomarkers for the complications of diabetes.

## CONCLUSIONS

In summary, biomarkers for diabetes and its vascular complications will, in future, be sought more widely, and in affected tissues, not only in serum, plasma, or urine. Not all of the new biomarkers will be “biomolecules”: some will depend upon functional measures and some on new imaging techniques. The tissue-specific markers are especially needed in diabetes, since the disease may affect different organs (e.g. heart, eye, kidney, nerve) to different extents within the same individual: the retina is a specific example; it is highly specialized, critical, but very small tissue, with the unique attribute that its microvasculature can be visualized. The tissue-specific biomarkers, together with an anticipated wealth of data from the “omics”, using blood and urine samples from well characterized cohorts, are likely to yield a plethora of new biomarkers in the near future.

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## ABBREVIATIONS

<b>AGEs</b>	Advanced glycation-end products
<b>ALEs</b>	Advanced lipoxidation-end products
<b>CML</b>	N <sup>ε</sup> -(carboxymethyl) lysine
<b>FL</b>	fructoselysine
<b>HbA1c</b>	Hemoglobin A1c
<b>MetSO</b>	methionine sulfoxide
<b>PSA</b>	Prostate specific antigen
<b>PEDF</b>	Pigment Epithelial Derived Factor
<b>RAGE</b>	Receptors for advanced glycation-end products
<b>RBC</b>	Red blood cell

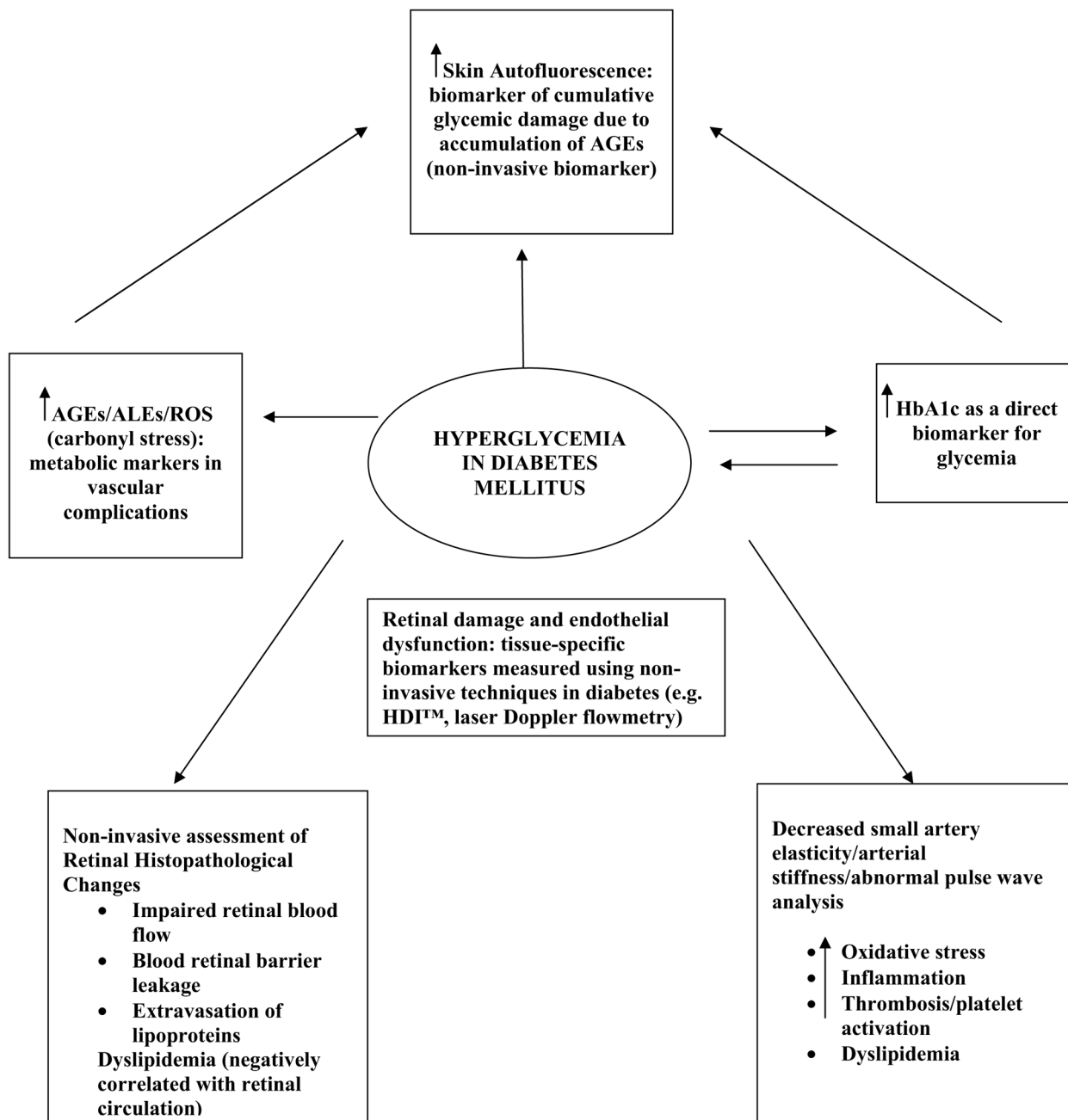
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**Fig 1. Biomarkers in diabetes: HbA1c, endothelium, skin and retina**

AGEs: advanced glycation end- products; ALEs: lipoxidation end-products; HbA1c: Hemoglobin A1c; HDI: Hypertension Diagnostics™; ROS: reactive oxygen species