INTRODUCTION

The twin epidemics of obesity and diabetes threaten to overwhelm healthcare systems in the U.S. and worldwide (1). A major challenge in dealing with the burden of diabetes is our inability to easily and conveniently detect diabetes and prediabetes in at-risk populations due to the lack of a non-invasive, point-of-care test. As discussed below, body fluids such as saliva and urine constitute a rich source of potential non-invasive biomarkers that may have both scientific and economic advantages over current serum-based diagnostic tests. The successful development of simple, non-invasive testing for diabetes will have a significant impact on the effective management of this critical public health crisis, and will be of particular benefit in developing countries.

THE EVOLVING CLASSIFICATION OF DIABETES

Classical type-1 diabetes mellitus (T1DM), also known as juvenile, autoimmune, or insulin-dependent diabetes (IDDM), is characterized by an early age of onset and the progressive loss of insulin secretory capacity due to β cell destruction. This form of diabetes is generally distinguished from classical type-2 (T2DM), also known as adult-onset or non-insulin-dependent diabetes (NIDDM). T2DM is characterized by progressive hyperinsulinemia, peripheral insulin resistance, and β-cell insufficiency. The principal molecular basis for T1DM is generally thought to be islet destruction caused by an autoimmune response, although a specific precipitating antigen has yet to be identified (2). T2DM, on the other hand, is a potentially more complex disorder that involves both insulin resistance and β-cell dysfunction. It is currently felt that clinically apparent T2DM is manifested only when β cell secretory capacity is insufficient to maintain an adequate level of hyperinsulinemia to overcome peripheral insulin resistance (3). The incidence of both T1DM and T2DM is increasing. In fact, an increasing proportion of pediatric patients are now presenting with features typical of T2 rather than T1DM, reflecting the mixed nature of many cases of diabetes, as discussed below. While much attention has recently focused on the obesity epidemic and its connection to the metabolic syndrome and T2DM, it is important to remember that T1DM, while comprising only 5-10% of all diabetes, is increasing and is also thought to be associated with obesity (4, 5). Thus, the environmental factors driving the increase in T2DM may also be contributing to a parallel increase in T1DM.

It is not generally appreciated that the current classification scheme for diabetes is a relatively recent phenomenon. Prior to the 1970-80’s, diabetes was considered a single pathologic entity that exhibited a spectrum of symptoms. The subsequent adoption of guidelines from the American Diabetes Association and the World Health Organization defining two types of diabetes based on the extremes of clinical presentation (i.e., young, thin, insulin-sensitive, and insulinopenic vs older, often overweight/obese, insulin-resistant, and hyperinsulinemic) obscured the true continuum of the disease. As a result, new intermediate classifications such as type 1.5, double diabetes, and latent autoimmune diabetes of adulthood (LADA) have proliferated to encompass the actual range of symptoms seen in patients (6-8). Several commentators have argued that it is now appropriate to return to the more nuanced concept of diabetes as a complex disease entity that may have a wide range of presentations (9, 10). The ramifications of this for accurate diagnosis include the presence of autoantibodies in putative T2DM patients and aspects of insulin resistance in putative T1DM patients. The application of novel biomarkers to more accurately stage at-risk individuals in the progression of pre-diabetes to frank disease and to characterize the nature of existing disease in diagnosed patients will greatly facilitate the management of this health crisis, the extent of which is detailed in the next section.

THE PUBLIC HEALTH STATUS OF DIABETES

Over the last several years, the number of persons in the US diagnosed with diabetes (predominantly T2DM) has reached almost epidemic proportions, with about 18 million diagnosed diabetics in the US alone, at a cost of $174 billion for 2007 (11). The true magnitude of the problem is much greater based upon the latest estimates that 40% of diabetes is undiagnosed (12); thus, the true number of diabetics in the US is approaching 29 million, and an additional 68 million are projected to be prediabetic. As depicted
in Figure 1, these trends are not unique to the US, but are also characteristic of much of the developing world (13, 14). Improved detection techniques and biomarkers are urgently needed across the entire spectrum of diabetes initiation and progression. Since 70% of individuals with prediabetes will progress to frank diabetes (15), and 7% of newly diagnosed T2DM patients in the US have been diabetic for approximately 4 to 7 years before diagnosis (16), the ability to ascertain those individuals at risk for the development of clinically apparent diabetes is critical to effectively focus potentially limited clinical resources. In particular, it is desirable to screen and start treating glucose-intolerant individuals as early as possible, since, even before the onset of diabetes, vascular lesions gradually develop with deterioration of glucose tolerance. Data from the recent Whitehall II study demonstrate that changes in the rate of change of glycemia and insulin sensitivity and secretion are evident from 3-6 years before diagnosed T2DM (17); thus, other biomarkers may provide robust assessment of prediabetes and T2DM risk. Additionally, since β-cell function is already compromised by the time that overt alterations in glucose homeostasis such as impaired glucose tolerance (IGT) or impaired fasting glucose (IFG) are evident, timely intervention is important to maintain residual insulin secretory capacity. The effectiveness of early intervention and lifestyle modification or medication has been demonstrated by the DPP and the UKPDS and verified by subsequent analyses (18-21).

ISSUES WITH CURRENT GUIDELINES FOR DIAGNOSIS.

The current ADA guidelines for diagnosis of diabetes and prediabetes (22) are based largely upon the notion that there is a threshold fasting plasma glucose concentration above which the risk of diabetes-specific microvascular complications (specifically retinopathy) is significantly increased. In other words, the strong chance of developing retinopathy is equated with the presence of clinically defined T2DM. It has been recently noted that there were significant problems with the population-based studies upon which the current diagnostic criteria are based (23), including small sample size, inadequate knowledge of the glycemic history of the participants, and the inherently poor reproducibility of OGTTs. A more recent study, based upon more carefully controlled analyses, has raised serious issues about the inadequacy of the retinopathy assessments in these earlier studies, and, by relying on the newer population studies, has concluded that there is no clear inflection point in the relationship between fasting plasma glucose and risk of retinopathy (24).

By extension, these considerations also affect the utility of IFG and IGT as classifications for prediabetes. In other words, if there is no clear transition from prediabetes to diabetes in the spectrum of fasting plasma glucose levels, then what are the parameters that can accurately define prediabetes or, perhaps more correctly, risk for developing diabetes as defined by a particular diabetic complication? This problem is illustrated by the recent report that patients with a diagnosis of isolated IFG, considered to be a category with normal muscle insulin sensitivity, in fact exhibit a wide range of insulin resistance (25); thus, currently accepted classifications of prediabetes are potentially much less definitive and, therefore, less useful, than previously thought.

The definition of prediabetes based upon IGT assessed by an OGTT was introduced many years ago. Subsequently, the classification of IFG was introduced to provide a less complicated and expensive parameter. IFG and IGT are now thought to actually reflect different aspects of the development of insulin resistance in T2DM, with differing underlying pathologies (26-28). Specifically, IGT is associated with peripheral insulin resistance and loss of first- and second-phase insulin secretion, while IFG is associated with hepatic insulin resistance and absence of first-phase insulin secretion. These distinctions in location of insulin resistance and extent of beta-cell dysfunction are consistently seen in Native American (29), Mexican-American (30), and adult (31) and adolescent (32) Caucasian populations. The combination of poor correlation between plasma glucose and diabetic complications, poor reproducibility of OGTTs, and the potentially distinct pathologies of IGF and IFG raise serious questions about the adequacy and relevance of current diagnostic
criterias for diabetes and prediabetes. A separate, but related issue is the consistency of plasma glucose measurements themselves (33). Thus, the prediabetic state is clearly more complex than previously appreciated, while the means of assessing prediabetes as well as overt diabetes are fraught with both technical and patient-variability issues. Resolution of this situation will be facilitated by the discovery of new biomarkers for diabetes risk, progression, and monitoring.

THE RATIONALE FOR IDENTIFICATION AND APPLICATION OF NOVEL BIOMARKERS FOR NON-INVASIVE DIABETES SCREENING, DIAGNOSIS, AND DISEASE MONITORING.

As detailed in the previous sections, the rapid increase in the rates of individuals susceptible to or with diagnosed diabetes worldwide has not been matched by a similar improvement in the tools for assessment of risk or disease status. Although there have been recent developments in the design of algorithms employing collections of standard markers to predict T2DM (34), these approaches continue to rely on serum samples and require central laboratory analyses, which are not optimal in many situations in less-developed countries. The ability to employ simple analysis of body fluids for rapid, non-invasive testing of validated novel analytes will represent a substantial contribution to the management of the global diabetes epidemic. We will describe below two opportunities for such an approach based on our recent studies in saliva and urine.

SPECIFIC FACTORS RELATING SALIVA AND DIABETES

T2DM and periodontal disease are both chronic inflammatory illnesses (35-37), and there is a growing consensus that these two disorders are interrelated (38). Specifically, diabetes is a risk factor for severe gingivitis and periodontitis, and periodontitis is a risk factor for poor glycemic control and, possibly, diabetic complications (39-41). The effect of glycemic control on gingivitis and periodontitis (42, 43), may, in part, reflect the fact that the periodontium is a highly vascularized end organ subject to the same micro- and macrovascular complications as the retina and glomerulus. Thus, the oral cavity may be an early target of altered glucose homeostasis. In light of the issues recently brought to light with retinopathy-based diagnostic parameters discussed above, as well as the realization that IFG, IGT, or the combination may reflect distinct states of prediabetes, salivary biomarkers may well prove to be superior for identifying prediabetes and assessing disease progression.

ADVANTAGES OF SALIVARY BIOMARKERS.

Saliva has a number of distinct advantages as a diagnostic fluid. These include being non-invasive, feasible without special training or equipment, especially advantageous for pediatric or elderly populations, and amenable to large-scale population studies. For these reasons, there has been a significant increase in interest in saliva biomarkers (44-46).

THE SALIVARY PROTEOME

The salivary proteome is derived from a number of sources, including major and minor salivary gland secretions, oral bacterial products, and gingival crevicular fluid (GCF; 47). Although the majority of salivary protein by amount is comprised of the major classes of salivary protein families such as the acidic and basic proline-rich proteins, amylase, and various mucins, the salivary proteome as a whole is dynamic and complex (48). Of particular interest is GCF, which is considered a transudate or ultrafiltrate of serum. Although the proportional contribution of GCF to saliva is small, it allows saliva to exhibit levels of serum-derived proteins that may reflect their circulating levels. For example, it is possible to measure proteins such as insulin, GIP, and GH in saliva (49, 50), and GCF has been employed to measure changes in glucose in diabetics (51). Previous studies have described the salivary proteome per se (52-55), while other studies have described alterations in salivary dynamics (56, 57) or the differential abundance in saliva of single factors, such as MMP-8 (58) and EGF (59) in T2DM.

NEW SALIVARY BIOMARKERS FOR DIABETES.

In order to characterize the human salivary proteome in diabetes, we have recently analyzed whole saliva from control and T2DM individuals by multidimensional liquid chromatography/tandem mass spectrometry (2D-LC-MS/MS), and identified a total of 487 unique proteins with high confidence (60). One-third of the proteins had not been previously reported in human saliva. The functional annotation of the proteins identified is shown in Figure 2. Label-free quantification was used to identify differentially abundant protein biomarkers, which were then independently validated in saliva from control, diabetic, and prediabetic subjects by Western immunoblotting and ELISA. A total of 65 proteins demonstrated a greater than 2-fold difference in abundance.
between control and T2DM saliva. Not unexpectedly, a significant proportion of the differentially abundant proteins have functions in metabolism and immune response. Independent validation of a subset of potential biomarkers utilizing immunodetection confirmed their differential expression in T2DM. The differential abundance of a subset of these salivary biomarkers in saliva from prediabetic individuals suggests that they may be useful for risk assessment in addition to diagnosis of frank disease.

**URINE BIOMARKERS FOR DIABETES.**

Although the levels of urinary glucose are unreliable in assessing diabetic status, the evaluation of increased levels of protein in urine as well as detection of specific urinary proteins has been proven to be useful for diagnosis of diabetic nephropathy and other diseases. Urine proteomic analysis, like salivary proteome characterization, can produce new biomarkers for clinical use (61). Proteomic analysis of urine by 2D-LC-MS/MS (62) and by surface-enhanced laser desorption-ionization mass spectrometry (SELDI-MS; 63) has identified new biomarkers for diabetic nephropathy, and additional proteomic analyses of urine are expanding the list of possible urinary biomarkers of diabetes and diabetic complications (64, 65). Glycoproteins constitute the largest fraction of the urine proteome characterized to date (66, 67). The urinary N-linked glycoprotein subproteome has been characterized by Concanavalin A lectin (Con A) column purification and proteomic analyses (68).

Glycoprotein biomarkers from cells and biological fluids such as serum and urine have also been characterized by analysis of their specific carbohydrate moieties. This approach has demonstrated that the glycosylation of prostate-specific antigen differs between cells lines and sera from prostate cancer patients. Tabares et al. used Sambucus nigra lectin (SNA), Aleuria aurantia lectin (AAL), and Erythrina cristagalli lectin (ECL) to detect differential glycosylation of prostate specific antigen (69). Prion strains can be also distinguished by their glycan composition. Using post-mortem brain tissue from affected individuals, prion protein was captured on solid phase by monoclonal antibodies, and the glycans of the captured protein probed with biotinylated plant lectins (70) using an ELISA format. A similar approach has been used to identify glycan modifications on fibronectin in rheumatoid synovial fluid compared to healthy individuals (671, and prolonged pregnancies compared to normal term pregnancy (70). Patwa et al. coupled glycoprotein enrichment and isolation with an array-based approach using biotinylated plant lectins as glycan probes to identify novel glycoproteins and glycan markers in sera from subjects with pancreatic cancer (72).

**NOVEL URINARY BIOMARKERS FOR DIABETES.**

Using a combination of 2D-DIGE and LC-MS/MS, we have previously characterized urinary profiles in diagnosed T2DM patients with and without micro and macroalbuminuria, and identified specific urine proteins that were differentially abundant in T2DM alone and which exhibited additional changes in abundance with increasing albuminuria (62). The biomarker set characterized in this study was comprised primarily of glycoproteins. In a subsequent study, we have analyzed urinary glycoprotein profiles in a prospective observational cohort of 154 subjects consisting of controls, subjects with IFG or IGT, and patients with diagnosed T2DM (74). Glycoprotein levels were determined using direct and antibody-based lectin immunoassays. In the first approach, total urine proteome reactivity with a specific lectin was analyzed to quantify the proportion of the proteome containing the specific glycosylation motifs recognized by the specific lectin coupled to a solid support in an ELISA framework. This assay principle is illustrated in Figure 3. In this analysis, urine protein reaction with several lectins was demonstrated to distinguish between control, prediabetic, and diabetic groups. In the second approach, specific urine glycoproteins were captured on an ELISA plate and then probed with biotinylated lectins to assess the extent of distinct glycosylation patterns on a single protein. This analysis revealed that the type and extent of glycosylation also varied with disease status. The rationale for these approaches is illustrated in Figure 4. As illustrated in Figure 5, the glycoproteome of serum and saliva is also distinguishable between control and prediabetic and diabetic samples. These studies support the notion that specific proteins as well as their glycosylation patterns and glycoprotein


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