1,5-Anhydroglucitol in Saliva Is a Noninvasive Marker of Short-Term Glycemic Control

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Context: In most ethnicities at least a quarter of all cases with diabetes is assumed to be undiagnosed. Screening for diabetes using saliva has been suggested as an effective approach to identify affected individuals.

Objective: The objective of the study was to identify a noninvasive metabolic marker of type 2 diabetes in saliva.

Design and Setting: In a case-control study of type 2 diabetes, we used a clinical metabolomics discovery study to screen for diabetes-relevant metabolic readouts in saliva, using blood and urine as a reference. With a combination of three metabolomics platforms based on nontargeted mass spectrometry, we examined 2178 metabolites in saliva, blood plasma, and urine samples from 188 subjects with type 2 diabetes and 181 controls of Arab and Asian ethnicities.

Results: We found a strong association of type 2 diabetes with 1,5-anhydroglucitol (1,5-AG) in saliva ($P = 3.6 \times 10^{-13}$). Levels of 1,5-AG in saliva highly correlated with 1,5-AG levels in blood and inversely correlated with blood glucose and glycosylated hemoglobin levels. These findings were robust across three different non-Caucasian ethnicities (Arabs, South Asians, and Filipinos), irrespective of body mass index, age, and gender.

Conclusions: Clinical studies have already established 1,5-AG in blood as a reliable marker of short-term glycemic control. Our study suggests that 1,5-AG in saliva can be used in national screening programs for undiagnosed diabetes, which are of particular interest for Middle Eastern countries with young populations and exceptionally high diabetes rates. **(*J Clin Endocrinol Metab* 99: E479–E483, 2014)**

Early diagnosis of type 2 diabetes (T2D) can alleviate health consequences of the disease. However, epidemiological studies show that at least a quarter of the cases with T2D may be undiagnosed (1, 2). Saliva has been proposed as an emerging resource for noninvasive diagnostics (3). In many countries dentists and oral hygienists

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Abbreviations: 1,5-AG, 1,5-anhydroglucitol; BMI, body mass index; HbA1c, glycosylated hemoglobin; HMC, Hamad Medical Corporation; T2D, type 2 diabetes.

see their patients for regular annual or semiannual checkups, which would be ideal for systematic T2D screenings (4). Recently metabolomic studies have been focusing on the identification of new biomarkers for T2D in blood and urine (5–8), using modern mass spectroscopy and nuclear magnetic resonance technologies that allow for the quantification of hundreds of endogenous and exogenous small molecules (metabolites) in a minimal amount of sample (9). As intermediates of human metabolism, disease-associated changes in metabolite levels represent functional readouts of disturbed biological processes. Here we report a metabolomic biomarker discovery study that unites collection of saliva, blood plasma, and urine samples in a large clinical study on patients with T2D and healthy controls. Using a combination of three nontargeted mass spectrometry-based metabolomic platforms, we detected more than 2000 individual metabolites in samples collected from 369 individuals.

Materials and Methods

Study design

This study was embedded in the Qatar Metabolomics Study on Diabetes, a cross-sectional case-control study with 374 participants. The study was realized by collaboration between the Dermatology Department of Hamad Medical Corporation (HMC) and Weill Cornell Medical College-Qatar. The study was approved by the Institutional Review Boards of HMC and Weill Cornell Medical College-Qatar (Research Protocol number 11131/11). Written informed consent was obtained from all participants. Study participants were enrolled between February and June 2012 at the Dermatology Department of HMC in Doha, Qatar. Inclusion criteria were a primary form of T2D (cases) and absence of major systemic disorders (controls). Data from five participants were excluded from the analysis due to incomplete records, leaving 176 cases and 193 controls. Of initially 193 enrolled controls, 12 individuals had a glycosylated hemoglobin (HbA1c) greater than 6.5% and were subsequently classified as cases, resulting in 188 cases and 181 controls. Studies have shown that matching gives similar results as after adjustment for covariates but diminishes statistical power (10, 11). Therefore, no matching was performed between cases and controls for age, gender, or ethnicity.

Table 2. Sample Characteristics

Samples	Subjects, n	Metabolites, n
Saliva	328	581
Plasma	359	720
Urine	356	877

The total number of samples was 1043. The total number of analyzed metabolites was 2178.

Phenotyping

Information regarding age, gender, ethnicity, and a history of T2D was obtained through questionnaires. Based on the country of birth of their parents and grandparents, subjects were divided into three major ethnicity groups: Arabs, South Asians, and Filipinos (Table 1). Using standardized protocols, trained researchers determined weight lightly clothed to the nearest decimal with an electronic scale (SECA Scale 813) and height without shoes and to the nearest decimal using a stadiometer (SECA Mobile Stadiometer 217). Body mass index (BMI) was calculated (kilograms per square meter). HbA1c levels were determined at the Department of Laboratory Medicine and Pathology of HMC (Cobas 6000; Roche Diagnostics).

Metabolomics measurements

Nonfasting saliva, plasma, and urine specimens were collected and processed using standardized protocols (Table 2). Saliva was obtained using the Salivette system following the manufacturer's recommendations. After collection the samples were stored on ice for transportation. Within 6 hours after sample collection, all samples were centrifuged at 2500 × *g* for 10 minutes, aliquoted, and stored at −80°C. Metabolic profiling was achieved using ultrahigh-performance liquid-phase chromatography and gas chromatography separation, coupled with tandem mass spectrometry at Metabolon Inc using established procedures (12). In total, 1568 different metabolites were detected. Osmolality in saliva and urine was measured for normalization purposes. Median process variability, determined by repeated measurements of pooled samples, was 15.3% in saliva, 15.8% in

Table 1. Subject and Sample Characteristics

Subjects	T2D (n = 188)	Controls (n = 181)	*P* Value
Age, y	53.8 (35.0–70.7)	38.5 (23.6–62.3)	<.001
Gender, % female	81 (43.1%)	99 (54.7%)	.03
Ethnicity			
Arab, %	93 (43.1%)	113 (62.4%)	
South Asian, %	74 (39.4%)	39 (21.5%)	.002
Filipino, %	14 (7.4%)	22 (12.2%)	
Other or mix, %	7 (3.7%)	7 (3.9%)	
BMI, kg/m^2	29.5 (21.6–42.6)	27.6 (21.7–39.1)	.004
HbA1c, %	7.8 (5.6–11.5)	5.5 (4.7–6.2)	<.001
HbA1c, mmol/mol	62 (38–102)	37 (28–44)	<.001
Glucosuria, %	67 (36.2%)	2 (1.1%)	<.001

Values represent median (90% range) or number of subjects (percentage). *P* values are based on Mann-Whitney *U* or χ^2 test.

plasma, and 9.8% in urine. In the initial sample set of 374 subjects, 147 metabolites were detected in saliva, plasma, and urine, 391 were detected in two sample types, and 1030 were detected in one sample type. Thus, a total of 2253 metabolites were measured in the three biofluids (603 in saliva, 759 in plasma, and 891 in urine). After excluding metabolites with fewer than 50 valid detections (many of which were xenobiotics related to medication), 2178 metabolites were used for the analyses. Details on the metabolic panel together with quality control data based on technical replicates are reported in Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org.

Statistical analysis

Metabolite levels were scaled by run-day medians, normalized using osmolality (saliva and urine data only), log transformed, and z-scored. Metabolites with more than 20% missing data points were imputed to the smallest detected value because it can be assumed that they are likely below the detection limit of the method. Values for metabolites greater than 4 SD from the mean were excluded from the analyses. Multivariate linear regression, adjusting for age, gender, ethnicity, and BMI, was used to assess the statistical significance of the association of metabolites with T2D as previously described (13). A stringent Bonferroni level of significance of 2.3×10^{-5} (= 0.05/2178) was used to infer association. All statistical analyses were performed using the SPSS version 20 (IBM) and R version 2.14 (R-project).

Results

After adjusting for covariates and multiple testing, 94 (of 2178) metabolites were significantly associated (after Bonferroni correction, $P < 2.3\times10^{-5}$) with T2D (Supplemental Table 1). Of these metabolites, three were detected in saliva, namely 1,5-anhydroglucitol (1,5-AG; $P = 3.6\times10^{-13}$), X-11315 (a metabolite of unknown biochemical identity; $P = 1.5\times10^{-5}$), and metformin ($P = 1.9\times10^{-6}$). The remaining significantly associated metabolites were found in plasma (n = 28) and/or urine (n = 63), most of which have previously been reported in association with T2D (5–8, 13). Subjects with T2D had lower 1,5-AG levels than controls in saliva and plasma but not in urine (Figure 1A). The covariates age, gender, BMI, and ethnicity had no significant effect on 1,5-AG in the linear model. The 1,5-AG saliva association with T2D was observed equally in Arabs, South Asians, and Filipinos ($P <$ .005, for each group) (Figure 1B). 1,5-AG in saliva correlates with 1,5-AG in blood (R = 0.74, Figure 1D) and anticorrelates with blood glucose (R = −0.51, Figure 1E) and HbA1c (R= −0.59, Figure 1F). Subjects with T2D also had lower levels of the unidentified metabolite X-11315 in saliva, and X-11315 was also detected in

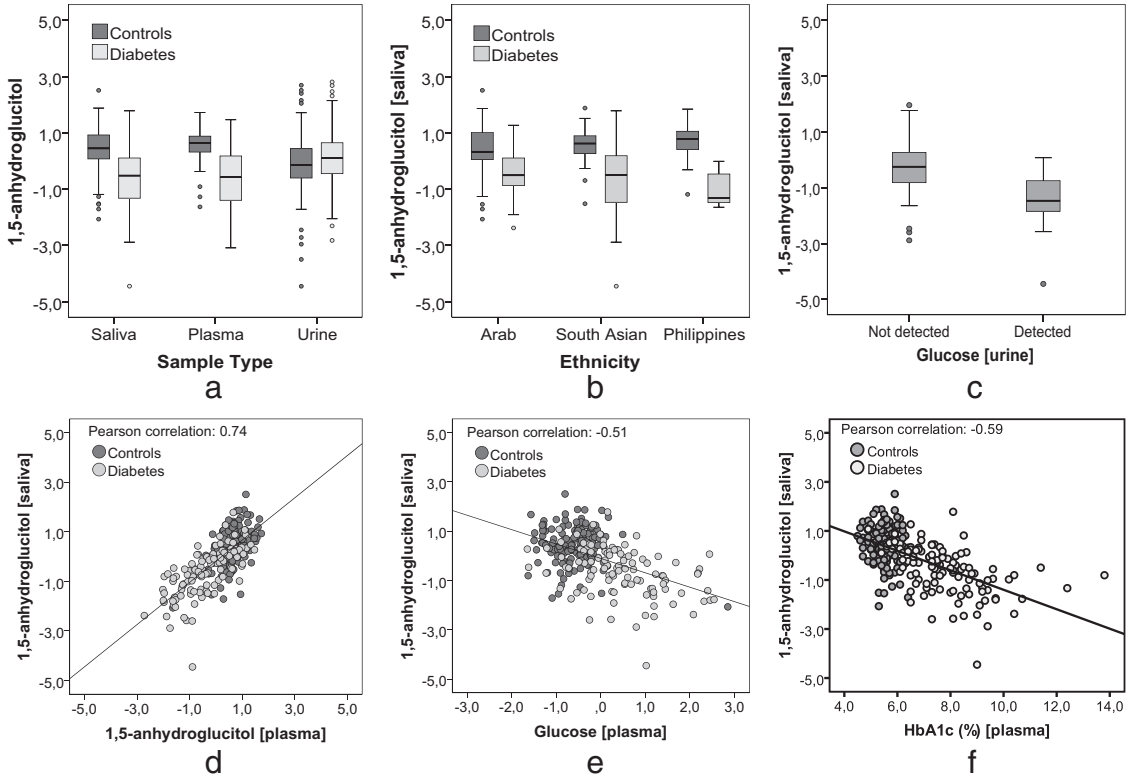

Figure 1. 1,5-AG levels in saliva, blood, and urine and by ethnicity. A, Boxplots of 1,5-AG levels in saliva, plasma, and urine. B, Boxplots of 1,5-AG levels in saliva by ethnicity. C, Boxplot of 1,5-AG levels in saliva in patients with T2D with and without glucosuria. The line in the middle of the box represents the median, the borders are the interquartile range, and the lower and upper boundary is the 90% range. D, Scatterplot of 1,5-AG levels in saliva with 1,5-AG in blood plasma. E, Scatterplot of 1,5-AG levels in saliva with glucose in plasma. F, Scatterplot of 1,5-AG levels in saliva with HbA1c (percentage) in plasma; 1,5-AG and glucose data were log transformed and Z-scored.

blood and highly correlated with the 1,5-AG levels in plasma (R = 0.54, $P < .001$). In patients with T2D, the 1,5-AG levels in saliva were lower in subjects with glucosuria ($P < .001$) (Figure 1C). In total there were 31 subjects with glucosuria, of which 28 subjects were detectable with a Z-score cutoff of −0.42 SD or lower for 1,5-AG in saliva (sensitivity 90.3%). Using this same cutoff, the specificity for glucosuria was 78.4%.

Discussion

We identified a strong negative association of 1,5-AG in saliva with T2D. Levels of 1,5-AG in saliva were positively correlated to 1,5-AG levels in blood and negatively correlated to glucose and HbA1c levels in blood. Furthermore, we found that low 1,5-AG levels in saliva were a good predictor of acute glucosuria.

In the human body, 1,5-AG is a major polyol and has been widely associated with T2D (14). Its main source is from diet (especially dairy products), although minor amounts are also produced in situ. It is well absorbed in the intestine, excretion in the feces is negligible, and no major metabolic transformations of 1,5-AG in the human body have been reported (15). The transport of 1,5-AG in the kidney is regulated by SLC5A9 (SGLT4), which is also a low-affinity-type transporter of mannose, fructose, and glucose (16). The turnover time in the healthy human body for 1,5-AG is about 2–3 weeks (17). In a situation of glucosuria, when blood glucose levels exceed the kidney reuptake limit (>180 mg/dL), 1,5-AG reuptake is inhibited by competition with glucose excretion. This is in agreement with the observed absence of an association of 1,5-AG in urine. The high correlation between 1,5-AG in saliva and blood plasma further suggests that 1,5-AG in saliva originates from blood.

Serum levels of 1,5-AG are indicative of blood glucose levels from approximately 5–7 days prior to testing, providing a valuable and intermediate readout of glycemic control that lies between HbA1c and a fasting blood glucose or a urine glucose test. The clinical use of 1,5-AG in monitoring glycemic control in T2D, for example after initiating insulin therapy, has been demonstrated previously (18), and 1,5-AG has been cleared by the Food and Drug Administration as a marker to monitor glycemic control. For example, during pregnancy 1,5-AG has been demonstrated to represent a better marker of glycemic control than HbA1c (19).

Our study group represents a typical diabetic case-control population, in which cases and controls were collected at the same center under identical conditions. In addition, the HbA1c distribution was comparable with that observed in other study groups. All study participants were enrolled at the Department of Dermatology. The subjects were therefore in most cases not under treatment for acute clinical diabetes deregulations. The metabolic state of our case group is thus most likely representative for the average patient with T2D on a day-to-day basis. There was a difference in BMI between cases and controls. However, adjusting for BMI did not materially change our effect estimates, which indicates that obesity is not likely a driving factor in determining 1,5-AG levels. Similarly, there were some differences in age, gender, and ethnicity between the case and control groups, but these were corrected for in the linear model and had no major impact on the association. Another important issue is that our subjects were in a nonfasting state at the time of sample collection: specimens were taken generally in the afternoon between 1:00 and 3:00 PM. The fact that we could detect a strong association signal under these conditions indicates that a saliva-based test can be expected to be robust and administrable under nonfasting conditions, which would be essential when recruiting patients in a screening setting. Finally, in the statistical analyses of the metabolomics data set, we applied the most conservative method of multiple testing corrections. Our findings were robust across three different non-Caucasian ethnicities (Arabs, South Asians, and Filipinos), irrespective of BMI, age, and gender. We regard the replication of associations in blood plasma and urine of many previously described metabolic markers of T2D as an external quality control of the metabolomics data.

Taken together, our data suggest that 1,5-AG in saliva constitutes a noninvasive marker for deregulated short-term glycemic control, which can be especially useful as a screening tool for undetected T2D. It may also be applicable in monitoring of glycemic control in patients who are adverse to blood sampling, such as children. We believe that 1,5-AG in saliva has the potential to be developed into a biomarker for impaired glycemic control that is applicable at a national screening program or, for example, in a dental setting. An enzyme-based test kit is presently commercialized under the name Glycomark (GlycoMark, Inc., http://www.glycomark.com/) for use with blood specimen but could be easily adjusted for use with saliva. Such a diabetes screening program is of particular interest for Middle Eastern countries with young populations and exceptionally high T2D rates (20).

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