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Title
Noninvasive measurement of plasma glucose from exhaled breath in healthy and type 1 diabetic subjects.

Permalink
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Journal
American journal of physiology. Endocrinology and metabolism, 300(6)

ISSN
1522-1555

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Publication Date
2011-06-05

Peer reviewed

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doi: 10.1152/ajpendo.00634.2010

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Noninvasive measurement of plasma glucose from exhaled breath in healthy and type 1 diabetic subjects

Timothy D. C. Minh,[1] Stacy R. Oliver,[1] Jerry Ngo,[2] Rebecca Flores,[2] Jason Midyett,[3] Simone Meinardi,[3] Matthew K. Carlson,[3] F. Sherwood Rowland,[3] Donald R. Blake,[3] and Pietro R. Galassetti[1,2]

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Submitted 18 November 2010; accepted in final form 31 March 2011

Minh TD, Oliver SR, Ngo J, Flores R, Midyett J, Meinardi S, Carlson MK, Rowland FS, Blake DR, Galassetti PR. Noninvasive measurement of plasma glucose from exhaled breath in healthy and type 1 diabetic subjects. *Am J Physiol Endocrinol Metab* 300: E1166–E1175, 2011. First published April 5, 2011; doi:10.1152/ajpendo.00634.2010.—Effective management of diabetes mellitus, affecting tens of millions of patients, requires frequent assessment of plasma glucose. Patient compliance for sufficient testing is often reduced by the unpleasantness of current methodologies, which require blood samples and often cause pain and skin callusing. We propose that the analysis of volatile organic compounds (VOCs) in exhaled breath can be used as a novel, alternative, noninvasive means to monitor glycemia in these patients. Seventeen healthy (9 females and 8 males, 28.0 ± 1.0 yr) and eight type 1 diabetic (T1DM) volunteers (5 females and 3 males, 25.8 ± 1.7 yr) were enrolled in a 240-min triphasic intravenous dextrose infusion protocol (baseline, hyperglycemia, euglycemia-hyperinsulinemia). In T1DM patients, insulin was also administered (using differing protocols on 2 repeated visits to separate the effects of insulinemia on breath composition). Exhaled breath and room air samples were collected at 12 time points, and concentrations of ~100 VOCs were determined by gas chromatography and matched with direct plasma glucose measurements. Standard least squares regression was used on several subsets of exhaled gases to generate multilinear models to predict plasma glucose for each subject. Plasma glucose estimates based on two groups of four gases each (*cluster A*: acetone, methyl nitrate, ethanol, and ethyl benzene; *cluster B*: 2-pentyl nitrate, propane, methanol, and acetone) displayed very strong correlations with glucose concentrations (0.883 and 0.869 for *clusters A* and *B*, respectively) across nearly 300 measurements. Our study demonstrates the feasibility to accurately predict glycemia through exhaled breath analysis over a broad range of clinically relevant concentrations in both healthy and T1DM subjects.

volatile organic compounds; breath tests; gases; diagnostic techniques and procedures; diabetes mellitus

THE INCIDENCE OF BOTH TYPE 1 AND TYPE 2 DIABETES (T1DM and T2DM, respectively) has been rapidly increasing in recent years (7, 14, 28, 32). For T1DM, prevalence is estimated to double by 2020 in some populations (25); for T2DM, recent estimates indicate that in 2050 between 20 and 33% of all adults in the US may be diabetic (5). Because many of the complications of diabetes can be prevented by tight glycemic control, standard medical guidelines now call for patients to self-monitor their blood glucose multiple times a day (1). Current diabetes management typically relies on painful finger lancing for glucose testing, a daily practice that many patients have come to hate, often resulting in fewer measurements and worsened glycemic control.

Although alternative, noninvasive techniques such as near-infrared or ultrasound sensors, dielectric impedance, and ionophoresis (20, 39) are being actively pursued by several research laboratories, none have been developed sufficiently for clinical practice at the present time; furthermore, the most promising techniques appear to be rather costly. We are proposing the quantification of volatile organic compounds (VOCs) in the exhaled breath as a novel, noninvasive methodology for plasma glucose monitoring. Breath analysis offers many potential advantages; it is completely painless, it is easily acceptable even by children, it does not require patient interaction (important during sleep), and it can become much cheaper than current glucose meters because it does not require an interface between sample and machine (i.e., for most glucose meters, hundreds of test strips are needed every month, which can add up to thousands of dollars annually). Moreover, additional information can conceivably be captured simultaneously from the same breath sample to provide a snapshot of an individual's metabolic status, including insulin and lipid levels (12, 21).

Although breath analysis technology has been proposed for clinical diagnosis in multiple fields for decades (9, 23, 27, 35), surprisingly few practical applications have been developed, and very little has been reported previously in relation to diabetes. Several studies have found associations between higher acetone levels and the presence of diabetes in various patient populations without attempting to actually derive plasma glucose values (8, 29, 37, 38). To date, our laboratory has been the only one to have reported previously the ability to estimate glycemic levels through integrated analysis of the kinetic profiles of multiple exhaled gases; this was done in young healthy subjects during relatively brief and simple glycemic fluctuations, such as oral (13) and intravenous (iv) glucose tolerance tests (18). Furthermore, a strong correlation between exhaled methyl nitrate profiles in the low part-per-trillion range and glycemic levels in the 100 to 400 mg/dl range was observed in a cohort of T1DM children (22). These studies clearly indicated that any effective predictive model must incorporate the exhaled profiles of at least several VOCs. Each of these profiles is also likely influenced not only by glucose concentrations but possibly by a number of concomitant metabolic changes, including insulin, lipids, and oxidative status, which complicates data interpretation and potentially limits predictive ability across metabolic conditions and subject groups.

On the basis of these results and considerations, we hypothesized that stronger breath-derived predictive models for plasma glucose, applicable to both healthy and diabetic subjects, could be obtained through more prolonged and complex in vivo metabolic studies, encompassing multiple combina-

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tions of glycemic and insulinemic values. Therefore, we designed the current study in healthy and T1DM subjects with 4-h clamp experiments, during which hyperglycemia and hyperinsulinemia were induced both simultaneously and separately, and plasma glucose values were estimated accurately through analysis of the profiles of clusters of four exhaled gases collected at 12 time points during each study.

SUBJECTS AND METHODS

All procedures were approved by the University of California Irvine (UCI) Institutional Review Board and conducted by specialized personnel at the UCI Institute for Clinical and Translational Science (ICTS). VOC analysis was conducted in the Rowland and Blake Atmospheric Chemistry Laboratory at UCI.

Subjects

Seventeen healthy (9 females and 8 males, 28.0 ± 1.0 yr) and eight T1DM volunteers (5 females and 3 males, 25.8 ± 1.7 yr) were enrolled in our study. All signed informed consent forms prior to participation, did not smoke, and had no known allergies. Healthy subjects had no history of recent or chronic illnesses, nor were they taking any medications. T1DM subjects had been diagnosed with the condition ≥5 yr prior to study enrollment, did not have recent illness or other chronic conditions, and did not take any medications besides insulin. Given their well-documented, long-term clinical history of T1DM, C-peptide screening was not performed before enrollment.

Study Procedures

Subjects reported to the ICTS at 7:30 AM after an overnight fast. T1DM subjects, if not on an infusion pump, were asked to not inject any long-acting insulin following their previous evening meal until after the study's end and inject only fast-acting insulin as per their normal regimen. For subjects on insulin pumps, the basal infusion rate was maintained until iv catheters were placed. At that point, the insulin pump was discontinued and replaced by iv insulin administration at the same rate.

Following the collection of anthropomorphic data, iv catheters were placed in the antecubital veins of both arms for subsequent blood drawing and iv glucose/insulin infusions. All experiments began at 8 AM and lasted 240 min, following this general format: a 60-min baseline period (t = 0–60 min), a 30-min transition to hyperglycemia (t = 60–90 min), a 60-min hyperglycemic period (t = 90–150 min), a 30-min transition back to euglycemia with sustained hyperinsulinemia (t = 150–180 min), and a 60-min euglycemic hyperinsulinemic period (t = 180–240 min). In all experiments, matched breath, room air, and 10-ml blood samples were collected at multiple time points: t = 40, 60, 90, 110, 130, 140, 150, 180, 200, 220, 230, and 240 min; additional 1-ml blood aliquots were collected every 5 min after the baseline period for the monitoring of plasma glucose (t = 60–240 min).

Glucose Infusion

In all participants, the glycemic levels at admission were maintained throughout the baseline period; T1DM subjects who presented with some degree of hyperglycemia were not corrected to euglycemia. This design was to allow baseline measurements with a stable metabolic milieu, minimizing any confounding effects of rapid metabolic changes on exhaled gases. Independent of initial glycemia, at t = 60 min, a variable-rate iv infusion of 20% dextrose was started to bring plasma glucose to a target level of 205–225 mg/dl over the following 30 min; glycemia was then kept at this level for 1 h (t = 90–150 min), with adjustments of the glucose infusion rate based on plasma glucose measurements obtained every 5 min. At the end of the hyperglycemic period, the glucose infusion rate was transiently reduced to maintain euglycemia by t = 180 min and constantly adjusted until study's end (t = 240 min).

Insulin Infusion

Healthy subjects. All of our 17 healthy subjects participated in *protocol H*, in which no exogenous insulin was infused until the end of the hyperglycemic period (t = 150 min); however, during hyperglycemia, a physiological endogenous insulin response increased plasma levels to ~10-fold basal concentrations. At t = 150 min, iv infusion of exogenous fast-acting insulin (Novolin R; Novo Nordisk, Princeton, NJ) was started at a constant rate of 1.5 $mU \cdot kg^{-1} \cdot min^{-1}$ and maintained until the end of the study. This design allowed us to sustain hyperinsulinemia despite the return to euglycemia during the last 60 min.

T1DM subjects. All eight T1DM participants underwent a first protocol (*protocol DM-1*) in which the plasma insulin profile was made to resemble that of healthy subjects (*protocol H*). Because T1DM subjects do not release endogenous insulin during hyperglycemia, a low-rate insulin infusion was started at t = 60 min and gradually increased through the hyperglycemic period. By t = 150 min, the infusion rate had reached 1.5 $mU \cdot kg^{-1} \cdot min^{-1}$ and was continued unchanged from this point until study end, similar to what was done in the healthy group.

Five of our T1DM subjects (2 males and 3 females) also participated in a second protocol (*protocol DM-2*) in which the exogenous insulin infusion profiles were identical to that used in healthy subjects; i.e., no exogenous insulin was infused during the hyperglycemic period. Therefore, because neither endogenous nor exogenous insulin was released in these studies from t = 60 to t = 150 min, a state of "unchallenged" hyperglycemia was obtained. This phase was developed specifically to separate the contributions of hyperglycemia and hyperinsulinemia to exhaled gases, simulating real life in T1DM patients, in whom prolonged hyperglycemia is often paralleled, and indeed caused by, absent or insufficient insulin concentrations. After t = 150 min, a constant infusion rate of 1.5 $mU \cdot kg^{-1} \cdot min^{-1}$ insulin was started, similar to what was done in the other two protocols.

Gas Sample Collection and Analysis

Exhaled breath was collected in 1.9-l custom-made stainless steel canisters that had been sterilized before use at 150°C, pumped to 10^{-5} atm, flushed with purified helium, and repumped to 10^{-5} atm. Study participants, after two tidal volume ventilations and a deep inspiration, slowly exhaled for ~10 s through a three-way valve mouthpiece into the canister. The first 3 s (~500 ml) of exhaled gas was vented to the room to clear anatomic dead space. A room air sample was collected simultaneously in an identical canister. These gas canisters were then taken to the Rowland and Blake Atmospheric Chemistry Laboratory, stored at room temperature, and analyzed within 1 wk.

On the day of analysis, a 275-ml sample aliquot (at standard temperature and pressure) was introduced in the system manifold and passed over glass beads chilled by liquid nitrogen (−196°C), with flow kept below 500 ml/min to ensure complete trapping of the relevant components. This procedure preconcentrated the relatively less volatile sample components (e.g., halocarbons, hydrocarbons) while allowing volatile components (e.g., N_2, O_2, and Ar) to be pumped away. The less volatile compounds were then revolatilized by immersing the loop containing the beads in hot water (80°C) and flushed into a helium carrier flow (head pressure at 48 psi). The sample flow was then split into five streams for chromatographic separation by a multicolumn/multidetector gas chromatography system utilizing two electron-capture detectors (sensitive to halocarbons and alkyl nitrates), two flame ionization detectors (sensitive to hydrocarbons), and a quadruple mass spectrometer detector (for unambiguous compound identification and selected ion monitoring). In total, concentrations of ~100 VOCs were quantified for analyses pertinent to this study. Each chromatographic peak was manually identified and integrated, peak shape was checked for possible coeluting com-

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pounds, and area was compared with VOC standards. More detailed methodological descriptions have been reported previously (6, 33).

Blood Analysis

Peripheral blood was collected into EDTA-treated Vacutainer tubes (BD Biosciences, Franklin Lakes, NJ). Samples were centrifuged immediately following each draw, and plasma glucose concentrations measured in triplicate using a Beckman Glucose Analyzer II (Beckman, Fullerton, CA). Remaining plasma was stored at −80°C until assays were performed. Insulin levels in plasma were determined by Human Insulin ELISA Kit EZHI-14K from Linco Research (St. Charles, MO) after extraction with the acid-ethanol method. Intra-assay coefficient of variation (CV) was 4.6–7.0%, interassay CV was 9.1–11.4%, and assay sensitivity was 2 μU/ml.

Data Analysis and Statistics

Exhaled breath and room air samples as well as matching peripheral blood samples (drawn simultaneously and 5, 10, and 15 min before gas sampling to account for possible lag times) were collected at 10 time points for both healthy and T1DM subjects (2 additional preliminary time points were collected to document metabolic stability and were not included in the analysis). Of the potentially usable 300 matched data points across all subjects, gas measurements were missing or technically unsatisfactory in 10 instances, and the entire data point was dropped from the analysis. These 10 data points were dispersed randomly across participants and study phase, and therefore, no further adjustment to our data set was required. For all our calculations, Δ-values of exhaled gases (differences between room air and breath concentrations) were used.

We initially generated predictive models for each study participant, utilizing a set of four gases (*cluster A*: acetone, methyl nitrate, ethanol, and ethyl benzene) that we used to predict blood glucose during an earlier experiment only on health subjects (18) via multiple linear regression analysis and standard least squares fitting by JMP software, version 8 (SAS Institute, Cary, NC). We then explored alternative gas clusters from our total of ~100 measured exhaled VOC profiles. We first reduced the number of potential gas candidates for inclusion in our predictive algorithms to 20. Namely, we chose all gases from the subset that most accurately predicted corresponding plasma glucose concentrations according to best subset regression analysis (of subsets ≤20 variables) calculated by SAS software, version 9.2 (SAS Institute). We then proceeded to generate multiple predictive models for plasma glucose based on 4-VOC clusters within this restricted list of 20, using standard least squares regression as determined by JMP software. With this approach, we identified several gas clusters that were able to predict plasma glucose with reasonable accuracy, among which 2-pentyl nitrate, propane, methanol, and acetone (*cluster B*) displayed the highest correlation values. Therefore, data obtained with *clusters A* and *B* will comprise the main results of this study.

Prior observations from our group also indicated the possible presence of variable lag times between changes in a plasma metabolite concentration and corresponding changes in exhaled gas concentrations. If a time delay is not incorporated into the predictive models, breath-based estimates of blood glucose may be lower than the actual plasma concentration during rapid increases of glycemia and higher than actual plasma values during rapid decreases. Therefore, in the attempt to identify and account for possible lag times in our data set, we generated additional predictive models pairing VOC samples with glucose values determined 5, 10, and 15 min prior to breath collection.

Although glucose predictions generated with the simultaneous gas measurements were reasonably strong, the greatest accuracy in glucose prediction was indeed obtained with glucose readings taken 15 min prior to exhaled gas collection; therefore, study results will be presented utilizing predictions with this time frame. Matched plasma and gas samples at 290 and 295 data points (out of a total possible maximum of 300 time-offset points) were included in our final analysis for *clusters A* and *B*, respectively. The missing data points were distributed across the whole study population, with no individual study subjects having <9 out of 10 possible data points.

All equations followed the standard format of "[predicted glucose: $(t - c)$] = $X_0 + X_1$[gas 1(t)] + X_2[gas 2(t)] + X_3[gas 3(t)] + X_3[Gas 4(t)]", where t represents time, c is a time offset, and X_0, X_1, X_2, and X_3 are coefficients that represent the expected difference in glucose when the concentration of each corresponding gas is increased by one unit, whereas other gases are kept constant. To assess the statistical accuracy of our models from a clinically relevant point of view, measured and predicted glucose values were plotted against each other on a Parkes glucose consensus error grid (24) as well as analyzed via Bland-Altman plots (4). Pearson's product-moment correlation coefficients were also calculated.

RESULTS

Glycemic Targets

During the hyperglycemic period (t = 90–150 min) in all three experimental protocols, the target glycemic range of 205–225 mg/dl was achieved (207 ± 2.5 mg/dl in healthy subjects during *protocol H*, 208 ± 3.6 mg/dl in T1DM subjects during *protocol DM-1*, and 222 ± 4.5 mg/dl in T1DM subjects during *protocol DM-2*) (Fig. 1). In all test subjects, euglycemia was then restored by t = 180 min and maintained until study's end. During the final hyperinsulinemic period, plasma insulin increased similarly in all groups (86.9 ± 4.3 μU/ml in *protocol H*, 90.5 ± 4.5 μU/ml in *protocol DM-1*, and 93.5 ± 6.1 μU/ml in *protocol DM-2*); however, during the 60- to 150-min period, insulin concentrations were significantly lower in *protocol DM-2* vs. both *protocol DM-1* and *protocol H* (P = 0.0003–0.0065).

Plasma Glucose Estimations

Cluster A. Using predictive models based on *cluster A*, the mean correlation coefficient between predicted and measured glucose concentrations was 0.883; glucose predictions were found to deviate from our reference glucometer by an average of 15%.

When considering only healthy subjects, the mean correlation coefficient was 0.836, and all values ranged between r = 0.654 and r = 0.975. During all study visits in T1DM subjects, we reported a mean correlation coefficient of 0.950; the strongest correlation was 0.999, and the weakest correlation was 0.799. The strong correlation between measured and predicted values was also maintained when cumulative data from the healthy (r = 0.839, 167 data points) and T1DM groups (r = 0.945, 123 data points) were compared (Fig. 2, *top*). When all 290 data points from all study participants were included in a single plot, the overall correlation coefficient was 0.887 (n = 30). Representative overlays of measured and predicted glucose concentrations during the 4-h study visit are displayed in Fig. 2, *bottom*.

Cluster B. When predicted glucose concentrations that were generated using *cluster B* were plotted separately against measured glucose values, the mean correlation coefficient was 0.869 (n = 30); glucose predictions were found to deviate from our reference glucometer by an average of 17%.

For the 17 healthy subjects, the mean correlation coefficient was 0.829, with values ranging between 0.641 and 0.962. The mean correlation coefficient for all T1DM study visits was 0.920 (n = 13); the strongest correlation coefficient was 0.990, whereas the weakest was 0.776. The strong correlation between measured and predicted values was also maintained when cumulative data from the healthy (r = 0.829, 168 data

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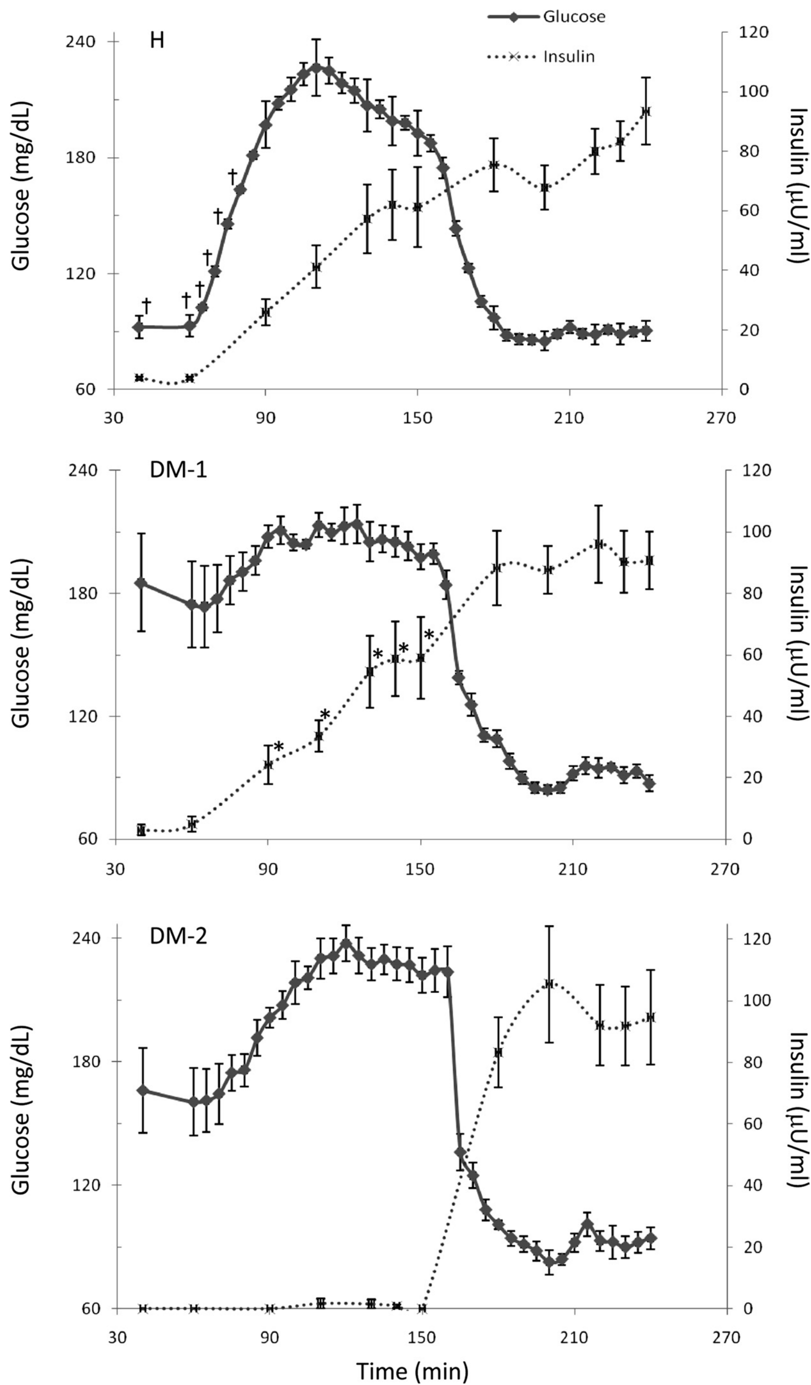

Fig. 1. Mean (± SE) glucose and insulin concentrations on healthy subjects (n = 17, 9 females and 8 males) and type 1 diabetic (T1DM) subjects (*protocol DM-1*: n = 8, 5 females and 3 males; *protocol DM-2*: n = 5, 2 males and 3 females) during 4 h of controlled induced metabolic fluctuations. Differences between insulin and glucose concentrations across protocols were compared using Student's t-test with Bonferroni correction (†*protocol H* vs. *protocol DM-1*, $P < 0.05$; **protocol DM-1* vs. *protocol DM-2*, $P < 0.05$).

points) and T1DM groups (r = 0.923, 127 data points) were compared (Fig. 3, *top*). When all 295 data points from all study participants were included in a single plot, the overall correlation coefficient was 0.934 (n = 30). Representative overlays of measured and predicted glucose concentrations during the 4-h study visit are displayed in Fig. 3, *bottom*.

Assessment of Clinical Relevance

The clinical relevance of the discrepancy between measurements from a novel glucose-measuring device and the gold standard can be assessed in several ways; among the most used are Parkes glucose consensus error grids and Bland-Altman plots.

In a Parkes consensus error grid, glucose estimates differing from gold standard measurements are categorized on the basis of their possible impact on clinical decision (24). By applying this methodology, 286 of 290 glucose concentrations predicted through our analysis on *cluster A* were shown to have "no effect on clinical action" or "little or no effect on clinical outcome" compared with those measured by the Beckman Glucose Analyzer II; only four values (1.4%) were considered "likely to affect clinical outcome" (Fig. 4). Similarly, using the

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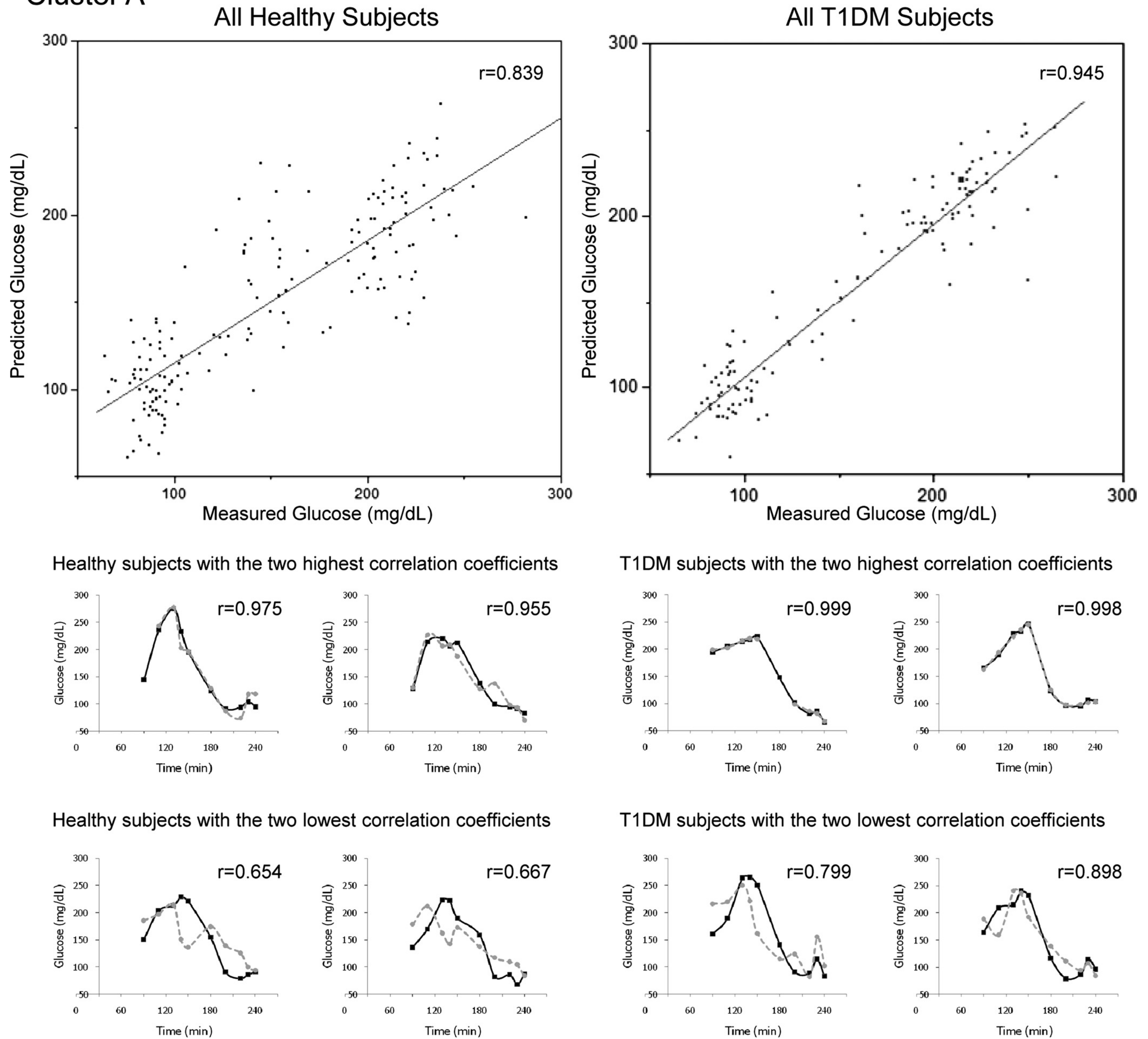

Fig. 2. *Top*: plasma glucose concentrations predicted from *cluster A* (acetone, methyl nitrate, ethanol, and ethyl benzene) are plotted against measured plasma glucose concentrations. Each dot represents a time point from our 17 study visits by healthy subjects (9 females and 8 males, 28.0 ± 1.0 yr) and 13 study visits by T1DM volunteers (*protocol DM-1*: $n = 8$, 5 females and 3 males, 25.8 ± 1.7 yr; *protocol DM-2*: $n = 5$, 2 males and 3 females). *Bottom*: time course overlays of predicted and measured glucose concentrations during a 4-h study visits displayed; the subjects from both healthy and T1DM cohorts with the 2 highest and 2 lowest correlations are presented. Dashed lines represent glucose concentrations predicted by breath gases (incorporating a 15-min time delay), and the solid lines represent plasma measurements from our Beckman Glucose Analyzer II.

alternative gases in *cluster B*, 293 of 295 predictions would have minimal effect, if any, on clinical action, whereas only two values (0.7%) were likely to affect clinical outcome.

The use of Bland-Altman plots adds an assessment of whether differences are biased or heteroskedastic (i.e., worse at high or low values of the variable of interest). Using this technique, we confirmed that the reported differences between measured and predicted glucose values have negligible bias (<0.000001 mg/dl), are normally distributed [17 of 295 points (6.01%) for *cluster A* and 13 of 295 points (4.41%) for *cluster B* are off >2 SD from the mean difference, the proposed cut point for agreement between measurements], and have overall limited heteroskedascity.

DISCUSSION

The main result of our study is the demonstration that noninvasive estimations of plasma glucose over a broad range of clinically relevant concentrations in both healthy and T1DM subjects are possible. This finding was achieved in controlled experimental conditions via the integrated analysis of four exhaled VOCs, indicating that, despite marked metabolic dif-

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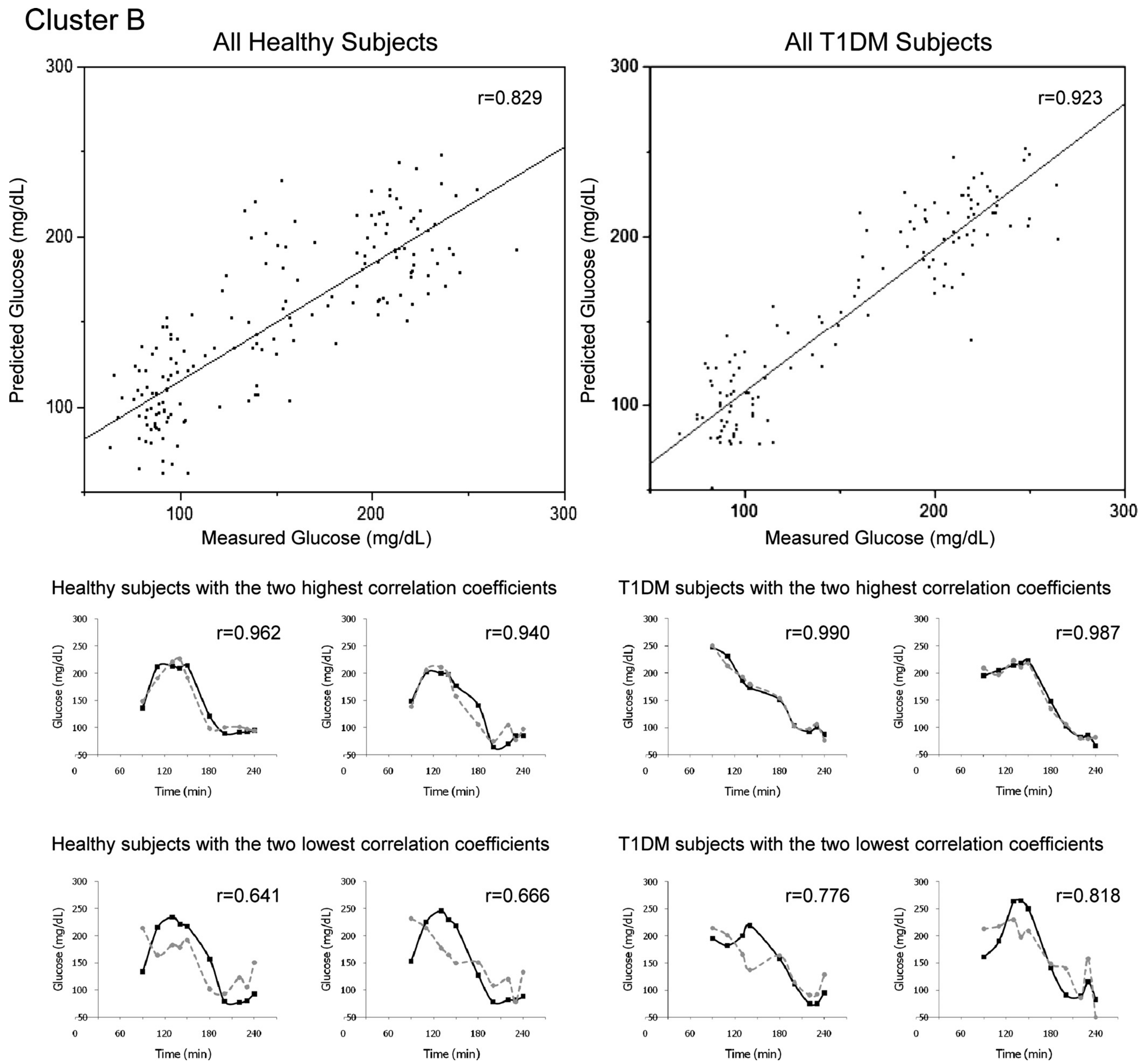

Fig. 3. *Top*: plasma glucose concentrations predicted from *cluster B* (2-pentyl nitrate, propane, methanol, and acetone) are plotted against measured plasma glucose concentrations. Each dot represents a time point from our 17 study visits by healthy subjects (9 females and 8 males, 28.0 ± 1.0 yr) and 13 study visits by T1DM volunteers (*prootcol DM-1*: n = 8, 5 females and 3 males, 25.8 ± 1.7 yr; *protocol DM-2*: n = 5, 2 males and 3 females). *Bottom*: time course overlays of predicted and measured glucose concentrations during 4-h study visits displayed; the subjects from both healthy and T1DM cohorts with the 2 highest and 2 lowest correlations are presented. Dashed lines represent glucose concentrations predicted by breath gases (incorporating a 15 min time delay), and the solid lines represent plasma measurements from our Beckman Glucose Analyzer II.

ferences across participant groups, some clusters of gases can provide similar predictive accuracy.

Our results expand prior findings based on shorter and less complex glycemic and insulinemic fluctuations (i.e., glucose tolerance tests) in healthy subjects only (13, 18). From these early studies, it became apparent that predicting plasma glucose would require simultaneous analysis of several exhaled gas profiles, some of which may reflect plasma glucose indirectly through parallel changes in other energy substrate metabolites. Therefore, a number of accuracy problems could have potentially arisen when the technique was applied to conditions with altered metabolic milieu, such as diabetes. A certain exhaled VOC combination, for instance, may be associated with hyperglycemia in healthy subjects; because these individuals are expected to display a relatively constant relationship between glucose and insulin, some of the gas changes may have been caused by spontaneous reactions to hyperinsulinemia rather than by hyperglycemia per se. The same exhaled VOC combination might not accurately predict glycemia in a T1DM subject, in whom the same levels of hyperglycemia may be associated with a wide range of insulin values. Therefore, breath-based glycemic predictions applicable to both healthy

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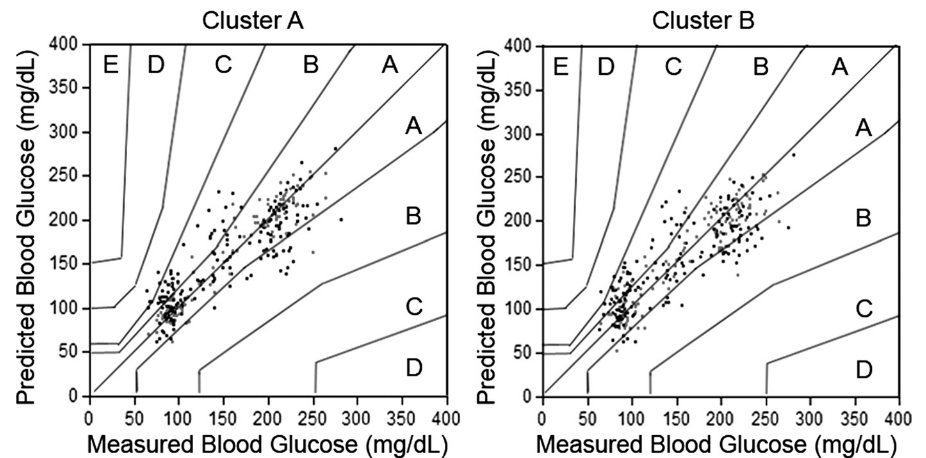

Fig. 4. Parkes consensus error grid plot for T1DM (24). Glucose concentrations predicted from *clusters A* and *B* were plotted against actual plasma measurements by the Beckman Glucose Analyzer II for all 30 study visits (both healthy and T1DM participants). *Zone A* represents no effect on clinical action; *zone B* represents altered clinical action, little or no effect on clinical outcome; *zone C* represents altered clinical action, likely to affect clinical outcome; *zone D* represents altered clinical action, could have significant medical risk; and *zone E* represents altered clinical action, could have dangerous consequences. Using *cluster A*, 286 of 290 glucose predictions fell into in *zone A* or *B* ($r = 0.887$; *left*). Using *cluster B*, 293 of 295 points in *zone A* or *B* ($r = 0.872$; *right*).

and T1DM subjects must rely on VOCs that are relatively unaffected by insulinemia, identifiable through experimental conditions in which plasma glucose and insulin fluctuations occur independent of each other. The multiple experimental protocols included in this study addressed this concern specifically and allowed us to identify multiple VOC clusters that maintained similar predictive accuracy for blood glucose concentrations in both healthy and diabetic subjects. Conversely, analysis of broader exhaled gas databases on larger populations may lead to the discovery of exhaled gases uniquely present in specific metabolic conditions (hyper- or hypoglycemia) or in population subsets (obesity, type 2 diabetes, children), potentially enhancing the diagnostic vs. monitoring applications of this methodology.

Despite the recognized potential of breath analysis, practical clinical applications seem to remain elusive, likely for two fundamental reasons. First, many breath VOCs, present in the part-per-trillion range or less, were below the detection range of many analytical systems in past decades. Only recent technological advances in VOC analysis have allowed our group to reliably measure the concentrations of these gases as low as 10 parts/quadrillion (6, 15, 43). Second, one must consider that most existing studies have measured exhaled breath at single time points. Although this experimental design may detect diagnostic markers to rule a condition in or out [e.g., heart allograft rejection (26) or liver disease (31)], it is likely to greatly underutilize the potential of VOC analysis. Although this approach is based on the assumption that an exhaled gas profile is constant in a given disease, it is likely that VOCs are instead generated from dynamic endogenous biochemical processes. Kinetic measurements of multiple gases could detect these changes for the monitoring of evolving, complex metabolic conditions, including hyperglycemia in diabetic patients. Therefore, we have developed and are utilizing a repeated-measure approach to VOC analysis for our experiments.

Independent of the metabolic processes resulting in VOC production, diabetic subjects may also present with microstructural changes in pulmonary tissue that could then lead to impaired lung function and reduced alveolar blood gas exchange (41). Chronic nonenzymatic cellular glycosylation resulting from chronic hyperglycemia may lead to vascular basement membrane thickening and remodeling as well as altered gap junction intercellular communication (30, 42), resulting in microangiopathy. As a result, gas diffusion patterns may be altered and affect breath gas composition. We cannot exclude that the overall presence of diabetes (or specific metabolic conditions such as hypoinsulinemia) may have affected some exhaled gas patterns, but it did not appear to significantly impact our selected gases or the accuracy of our predictive models. Although we lack the elements to correctly conduct such analysis, not having powered the study for this outcome, we feel it may be informative to report how the three protocols influenced the scatter of correlation coefficients between measured and predicted glucose values through a variability summary plot (Fig. 5). Interestingly, this distribution appears to be tighter in T1DM subjects vs. healthy subjects and similar between *protocols DM-1* and *DM-2*.

In the current study, which included T1DM subjects, we confirmed our prior findings observed in healthy subjects that methyl nitrate, acetone, ethyl benzene, and ethanol (*cluster A*) can provide a useful basis for glucose prediction models (18). Although the complete elucidation of the complex biochemical pathways connecting breath levels of these gases to systemic glycemia is well beyond the scope of this article, some established characteristics of each gas allow us to generate plausible hypotheses. Methyl nitrate derives from oxidative processes in the atmosphere and is the putative by-product of oxidative stress in biological systems, where it was observed to increase acutely after ingestion of high-fat meals (3). In a previous report, exhaled methyl nitrate also closely paralleled hyperglycemia in a group of T1DM children (22). Our current findings also show a similar relationship between methyl nitrate and glucose in T1DM adults (Fig. 6); this gas had a weaker but still positive correlation with plasma glucose in healthy subjects. Ethyl benzene is an aromatic VOC that is present at very low concentrations in room air and is typically inhaled, metabolized by the liver, and exhaled at lower concentrations. Its rate of metabolism is modulated by conditions affecting overall hepatic enzymatic activity, such as hyperglycemia or increased hepatic blood flow, transiently increasing exhaled profiles (36). Acetone, generated via oxidation of free fatty acids, is suppressed with the concomitant increase in insulin during hyperglycemia (16). Ethanol is produced during fermentation of glucose by gut flora and exhaled in the breath at very low concentrations. Interestingly, this appears to occur not only after glucose ingestion but also to a smaller extent with iv-induced hyperglycemia.

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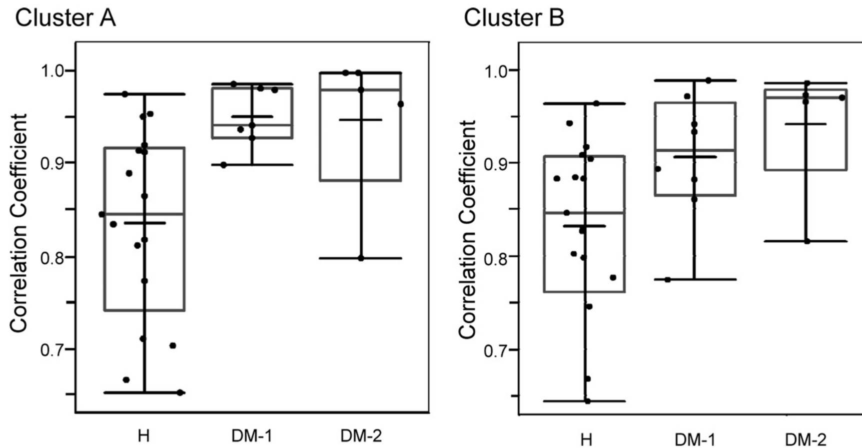

Fig. 5. A variability summary plot of correlation coefficients for glucose predictions from *clusters A* and *B* against direct measurements by protocol. The *top* and *bottom* lines of each column indicate the maximum and minimum correlation coefficients, and the box indicates the interquartile range and median value.

Although inclusion of *cluster A* into our predictive models allowed accurate glycemic predictions, the inclusion of ethanol as one of the gases raised a series of concerns, because measurement of this gas was technically problematic at a number of time points. Therefore, to remove this constraint, we proceeded to identify additional gas clusters that would allow glycemic predictions with similar accuracy. This process is a reflection of the remarkable flexibility of our technique, in which at least a dozen exhaled gases appear to be influenced by glycemic fluctuations, and will be a key feature in the development of future portable breath-testing devices. After analysis of several alternative gas clusters, 2-pentyl nitrate, propane, methanol, and acetone (*cluster B*) displayed the highest predictive accuracy. Again, without claiming to clarify in-depth biochemical links between plasma and exhaled variables, we identified several possible connections. Like the aforementioned methyl nitrate, 2-pentyl nitrate belongs to the akyl nitrate family and can be generated through pathways involving an organic peroxy ($RO_2\cdot$) radical with either NO and NO_2 (2). Similarly to methyl nitrate, generation of 2-pentyl nitrate could, therefore, conceivably be modulated by acute changes in systemic oxidative status, which is affected by hyperglycemia. Propane can be generated by three pathways, all of which are in equilibrium with or affected by glucose metabolism: n-4 fatty acid (18:3) peroxidation (17); protein oxidation, especially oxidation of branched-chain amino acids (17a); or production by colonic bacteria (which is affected by substrate availability) (17a). Methanol appears to reflect gut flora activity (40), similarly to ethanol and propane, and therefore, it is responsive to glycemic fluctuations. Although this likely accounts for the majority of its exhaled concentrations, methanol is also a commonly used industrial chemical and may be partially inhaled (40). It is also a natural component of ripe fruits and alcohol, which may contribute to systemic methanol levels when consumed (19), and is a by-product of pectin degradation by human colonic bacteria (34) as well as endogenous hydrolysis of the artificial sweetener aspartame (10). The definitive biochemical links of these exhaled VOCs with plasma variables relevant to diabetes, their reliability as glucose predictors in all metabolic conditions and in all dysmetabolic patient groups, and their stability over time will have to be documented in future studies stemming from our current observations.

It is also worth noting how the seven gases included in our two clusters displayed considerable variability in their kinetic patterns. For instance, acetone, methyl nitrate, and 2-pentyl nitrate displayed consistent downward trends through the study (2-pentyl nitrate, being actively taken up by the body, shifted

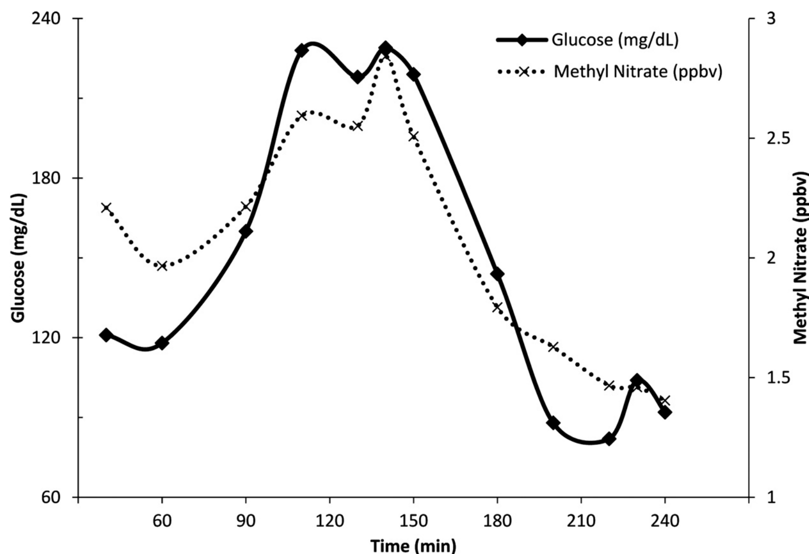

Fig. 6. A representative time course overlay of glucose and methyl nitrate concentrations for one T1DM subject undergoing *protocol DM-2* (r = 0.974) is displayed. In a previous study, we observed methyl nitrate to parallel hyperglycemia in a cohort of T1DM children (22). A weaker correlation was observed in healthy subjects.

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toward a more negative breath room air delta). For these three gases, the covariate coefficients in our model were positive, a phenomenon we had already observed and attributed, at least in healthy subjects, to the antiketogenic effect of insulin when it increases in response to glucose. It was intriguing to observe that this persists in T1DM, even when insulin replacement was delayed after hyperglycemia. The remaining four gases (ethylbenzene, ethanol, propane, and methanol) displayed inverse relationships to blood glucose in the majority of the subjects. Although methanol profiles were relatively homogeneous across subjects, a progressive drop in net uptake of ethylbenzene was noted only in healthy subjects, whereas T1DM subjects displayed very little uptake even at baseline. For ethanol and pentane, transient biphasic bell-shaped profiles were observed in all three groups, each with different time courses.

The main downstream goal of this project is the development of portable, lightweight, and inexpensive breath-testing devices that may replace current blood-based glucose meters. It is important to highlight here that although the profiles of the same four gases were used in all subjects in our study to predict each variable, the actual predictive algorithms were unique to each subject. This means that in its current state of development, even if the technique was translated into a portable testing device, it would require an initial individual calibration study for each subject. Although this would imply an inability to use the methodology for screening purposes, it should be noted the that the overwhelming majority of current blood glucose tests are not performed for diagnostic purposes but rather for repeated daily monitoring by already diagnosed diabetic patients. For these subjects, undergoing a calibration procedure (that probably will be similar to a glucose tolerance test) may represent a very appealing trade-off for them not having to lance their skin five to seven times a day, even if the calibration had to be repeated annually. This being said, the possibility is still very real that accurate, common predictive equations that will allow direct testing in all subjects may be derived, even those not previously calibrated. Our technology for VOC analysis has in fact been improving steadily, with detection and incorporation of more known but previously unused VOCs into our models as well as identification of completely novel gas species.

An additional technical issue that needs to be considered is that our glycemic predictions, although reasonable when based on simultaneous breath and plasma samples (roughly the same accuracy as current continuous glucose-monitoring systems), were most accurate when matching plasma glucose values obtained 15 min before breath collection. First, the multiple expected improvements in our core technology may render the impact of these lag times negligible. Furthermore, as stated above, considerable flexibility in model development is afforded by the relatively broad range of usable VOC combinations, and negative selection of VOCs that are susceptible to lag time effects may prove effective. Models without these gases may yield slightly less accurate (but still clinically relevant) predictions and may end up being selected for prototype development. Finally, although some gases with lag times might be indispensable for model accuracy, a lag effect could potentially be incorporated in the model, given that its timing is stable, by estimating the magnitude of glycemic change between time of sampling and time of most accurate estimate (i.e., −15 min). This approach would also require estimating the direction of the glycemic change (increase, no change, decrease) between these two time points, which is difficult with a single measurement point. However, we have identified some gases (e.g., xylenes) whose breath/room air ratio reflects the presence of an acute change in glycemia rather than absolute glucose concentrations; integration of this information may help solve the issue.

It should also be noted that accuracy of glycemic prediction in the hypoglycemic range was not tested in our study. Obviously, any technique claiming to effectively replace current glycemic monitoring in T1DM must be able to accurately capture hypoglycemic values. In our case, it would be naïve to expect our reported predictive models to automatically work during hypoglycemia because the unique admixture of metabolic and hormonal changes induced by hypoglycemia may significantly affect exhaled VOC concentrations. Because addressing this issue requires additional comprehensive, carefully designed protocols, hypoglycemic testing was beyond the current scope of our study but will certainly be the focus of future work from our group.

In summary, this study marks the first time that we were able to reconstruct glucose concentrations from exhaled gas profiles in both healthy and T1DM subjects. These predictions were achievable via integrated analysis of several clusters of four gases (of which 2 are reported), a number that we believe sufficiently small to develop into a portable breath-testing device that could have considerable impact on the approach to diabetes monitoring in broad populations. Further evolution of the methodology, possibly including breath tests for plasma insulin and lipids, may extend to large-scale screening projects for populations where blood draws would be impractical or impossible.

ACKNOWLEDGMENTS

We acknowledge Tu-Anh Nguyen, Alexandra Ly, and Nicole Tse for their contributions to this project. Our work would not have been possible without the excellent technical support of the UCI ICTS research nurses (Barbara Bodenhoefer, A. Diane Capobianco, and Connie Parido), biostatistician Sharon Leu, and other laboratory staff.

GRANTS

This work was supported by grants from the Juvenile Diabetes Research Foundation (no. 1-2006-76), the Division of Research Resources (M01-RR-00827-28), and the American Diabetes Association (7-08-CR-22). T. D. C. Minh is supported by a National Institutes of Health/National Institute of Diabetes and Digestive and Kidney Diseases (NIH/NIDDK) Kirschstein Predoctoral Fellowship (F30-DK-088401). P. R. Galassetti is supported by an NIH/NIDDK Mid-Career Development Grant (K24-DK-085223).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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