

## Breath Ethanol and Acetone as Indicators of Serum Glucose Levels: An Initial Report

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### ABSTRACT

**Background:** Many volatile organic compounds are present in exhaled breath and may represent by-products of endogenous biological processes. Ethanol is produced via alcoholic fermentation of glucose by gut bacteria and yeast, while acetone derives from oxidations of free fatty acids, influenced by glucose metabolism. We hypothesized that the integrated analysis of breath ethanol and acetone would provide a good approximation of the blood glucose profile during a glucose load.

**Methods:** We collected simultaneous exhaled breath gas, ambient air, and serum glucose and insulin samples from 10 healthy volunteers at baseline and during an oral glucose tolerance test (OGTT) (ingestion of 75 g of glucose followed by 120 min of sampling). Gas samples were analyzed by gas chromatography/mass spectrometry.

**Results:** Mean glucose values displayed a typical OGTT pattern (rapid increase, peak values at 30–60 min, and gradual return to near baseline by 120 min). Breath ethanol displayed a similar pattern early in the test, with peak values at 30 min; this was followed by a fast return to basal levels by 60 min. Breath acetone decreased progressively below basal levels, with lowest readings obtained at 120 min. A multiple regression analysis of glucose, ethanol, and acetone was used to estimate glucose profiles that correlated with measured glucose values with an average individual correlation coefficient of 0.70, and not lower than 0.41 in any subject.

**Conclusion:** The integrated analysis of multiple exhaled gases may serve as a marker of blood glucose levels. Further studies are needed to assess the usefulness of this method in different populations.

### INTRODUCTION

THE HUMAN BREATH contains numerous volatile organic compounds (VOCs), many of them present at concentrations in the part per trillion range.<sup>1</sup> While the origin and pathophysiological importance of most VOCs are yet poorly understood, elevation of breath levels of

some of these gases (ethane, *n*-pentane, butane, nitric oxide, ethanol, carbon monoxide, acetone) is beginning to be associated with a variety of metabolic and pathologic conditions, including lipid peroxidation,<sup>2–6</sup> heart failure,<sup>5</sup> asthma,<sup>7</sup> cystic fibrosis,<sup>8</sup> diabetic ketoacidosis,<sup>9</sup> alcohol intoxication,<sup>10</sup> and others. However, only marked elevations in these compounds,

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several orders of magnitude above basal levels, have been typically included in these reports.

Our group previously described quantification of atmospheric trace gases using an analytical system that is capable of precisely quantifying gas concentrations as low as 10 parts per quadrillion by volume.<sup>11,12</sup> This sensitive detection range and the wide variety of compounds measured (over 300 gases) may enable us to observe VOCs in exhaled breath that have not been previously reported and attempt to correlate them with known metabolic processes. Endogenous glucose metabolism may be one of the processes resulting in specific patterns of exhaled gases. Ethanol, for example, although not directly produced by any known mammalian cellular biochemical pathway, may increase in exhaled gas mixtures because of alcoholic fermentation of an excessive overload of carbohydrate-rich food, in conjunction with overgrowth of intestinal bacteria or yeast.<sup>13</sup> Acetone, a ketone body derived from oxidation of non-esterified fatty acids, is normally produced by humans in baseline conditions with very little circadian fluctuations.<sup>14</sup> Its production is known to increase with high-fat ketogenic diets,<sup>15</sup> in diabetic ketoacidosis,<sup>9</sup> and in other catabolic conditions, such as starvation, during which hypoinsulinemia favors lipolysis and oxidations of lipids as a preferential energy substrate.<sup>16</sup> We hypothesized that subtler changes in exhaled ethanol and acetone, significantly smaller than those observed in the conditions reported above, may occur during the comparatively small fluctuations in blood glucose that physiologically follow ingestion of a normal meal and that the integrated analysis of these exhaled gas profiles may yield an indirect assessment of circulating glucose levels.

In the present study, therefore, the pattern of exhaled VOCs was assessed, simultaneously with blood glucose and insulin measurements, in a group of healthy young volunteers during the 2-h period of a standard oral glucose tolerance test (OGTT).

## SUBJECTS AND METHODS

### *Subjects*

Enrollment in the study included 10 healthy adult volunteers (five men, five women), mean

age of  $27.4 \pm 3.1$  years, height  $172.2 \pm 1.9$  cm (female  $166.3 \pm 3.7$  cm, male  $175.1 \pm 1.9$  cm), and weight  $77.8 \pm 3.9$  kg (female  $66.4 \pm 5.1$ , male  $83.9 \pm 4.8$  kg). None of the subjects smoked, took long-term medications, or suffered from known chronic disease. The study was approved by the University of California, Irvine Institutional Review Board, and informed consent was obtained.

### *Protocol*

Subjects were admitted to the University of California, Irvine General Clinical Research Center in the morning following an overnight fast. An intravenous catheter was inserted in the basilic vein of the right forearm. Two baseline blood samples were drawn, at 10-min intervals, to assess basal fasting glucose levels. Simultaneously with each blood draw, VOCs were collected as described below. A standard OGTT was then performed. Subjects ingested 75 g of glucose diluted in 296 mL of orange-flavored drink solution (Trutol 75, NERL Diagnostics, East Providence, RI).

Following glucose ingestions, blood and VOC sampling occurred at 2.5, 5, 10, 15, 20, 30, 45, 60, 90, and 120 min.

### *Serum measurements*

*Glucose.* Serum glucose levels were determined by quantitative enzymatic measurements with the use of Sigma diagnostic kit # 510 (Sigma Diagnostics, St. Louis, MO).

*Insulin.* Insulin serum levels were determined by enzyme-linked immunosorbent assay with the use of the DSL-10-1600 Active kit (Diagnostic System Laboratories, Webster, TX). Intra-assay coefficient of variation was 1.3–2.6%, inter-assay coefficient of variation was 5.2–6.2%, and the sensitivity was 0.26  $\mu$ IU/mL.

*$\beta$ -Hydroxybutyrate.*  $\beta$ -Hydroxybutyrate levels in serum were measured by colorimetric determination using the  $\beta$ -hydroxybutyrate LiquiColor assay (StanBio Laboratory, Boerne, TX).

*Ethanol.* Ethanol levels in serum were determined by colorimetric determination by the use

of the Ethanol-L3K assay kit (Diagnostic Chemical Ltd., Equal Diagnostics, Exton, PA). Sensitivity of the assay is from 2.2 to 130.3 mmol/L (10 to 600 mg/mL).

#### VOC procedures

*Collection.* VOCs were collected by allowing subjects to exhale for 10–15 s into specially designed, electropolished 1.9-L stainless steel canisters. Canisters were sterilized before use by baking at 100°C for 12 h and evacuated to  $<10^{-5}$  atm.

The subject was asked to take a deep inspiration to total lung capacity, and then to slowly exhale until near residual volume through a mouthpiece connected to the canister via a three-way valve. Gas from the first 2–3 s of the exhalation maneuver was vented to the room to clear system and anatomic dead space. A single practice was normally sufficient to train subjects for this technique. With each exhaled gas sample, a room air sample was simultaneously collected in an identical canister. Canisters containing breath and room air samples were then stored at room temperature and analyzed within 2–3 weeks. Control samples have indicated that only minimal changes in canister gas content occur during this period of time.

*Gas analysis.* Briefly, VOCs were cryogenically trapped and injected into a multicolumn/multidetector gas chromatography system. The detectors included two flame ionization detectors (FIDs), two electron capture detectors (ECDs), and a quadrupole mass spectrometer (MS). The five different column/detector combinations were: PLOT/FID, DB-1/FID, DB-5/ECD, RTX1701/ECD, and DB-5ms/MS.<sup>12</sup> This analysis allows accurate quantification of a variety of different gas species. For a more detailed discussion of analysis and gas standardization see Colman et al.<sup>12</sup>

#### Statistical analysis

In each subject, a simple linear regression analysis was performed between blood glucose and each gas in exhaled breath. The two gases displaying the highest mean individual correlation coefficient ( $r$ ) with glucose, ethanol, and acetone were then used as independent variables in a multiple linear regression

analysis with glucose as the dependent variable. This resulted in 10 multiple linear correlations coefficients ranging between 0.41 and 0.95 (Table 1) showing a very good approximation of the dependent variable for all subjects.

## RESULTS

All subjects completed the study with no discomfort or reported adverse events.

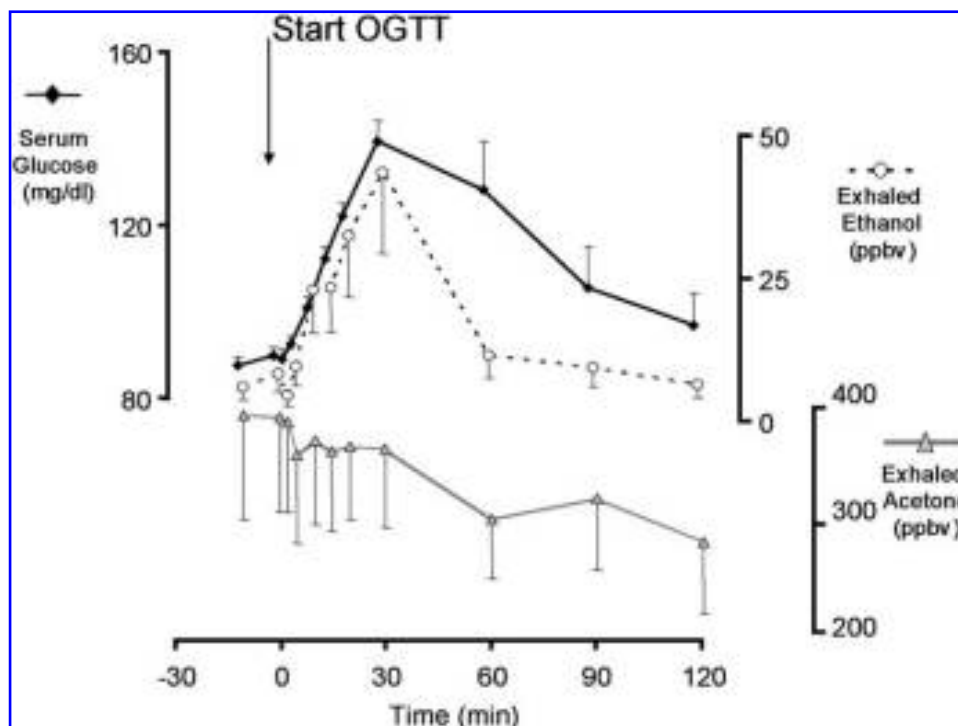
#### *Serum glucose, insulin, $\beta$ -hydroxybutyrate, and ethanol levels*

The mean baseline fasting serum glucose was  $88 \pm 2$  mg/dL (range 76–97 mg/dL) (Fig. 1). Peak serum glucose concentrations were recorded at 30 min ( $139 \pm 5$  mg/dL, range 116–162 mg/dL), although the highest reading in the whole data set was 185 mg/dL measured in one subject at 60 min. At the end of the OGTT (120 min), serum glucose returned to near-baseline ( $97 \pm 7$  mg/dL, range 69–142 mg/dL). Thus, according to current diagnostic standards (normal OGTT results if 120-min blood glucose  $<140$  mg/dL),<sup>17</sup> nine of 10 subjects displayed normal results. In one subject, a non-obese Hispanic female with family history of type 2 diabetes, a reading of 142 mg/dL at the 120-min time point resulted in a diagnosis of

TABLE 1. CORRELATION COEFFICIENTS ( $r$ ) BETWEEN MEASURED GLUCOSE PROFILES AND EXHALED ETHANOL OR ACETONE PROFILES, EITHER INDIVIDUALLY (VIA SLR) OR COMBINED (VIA MLR)

Subject number	Gender	SLR		
		(Ethanol; glucose)	(Acetone; glucose)	MLR (ethanol; acetone; glucose)
1	M	0.78	0.46	0.79
2	M	0.10	-0.41	0.42
3	M	0.54	0.21	0.56
4	M	0.22	-0.42	0.55
5	M	0.36	-0.70	0.85
6	F	0.41	0.10	0.41
7	F	0.68	-0.34	0.74
8	F	0.76	0.60	0.82
9	F	0.95	-0.06	0.95
10	F	0.66	-0.70	0.87

SLR, simple linear regression; MLR, multiple linear regression (using the gases as independent variables and glucose as the dependent variable).



**FIG. 1.** Serum glucose, exhaled ethanol and acetone profiles in 10 healthy volunteers during a standard OGTT after an overnight fast. Data are group mean  $\pm$  SE values.

glucose intolerance, and the subject was referred for appropriate clinical follow-up evaluation.

Basal serum insulin levels were  $6 \pm 1$   $\mu$ U/mL; insulin levels then rapidly increased, reaching  $56 \pm 1$   $\mu$ U/mL by 30 min, peaking at  $59 \pm 2$   $\mu$ U/mL at 60 min, and remaining elevated for the rest of the study, with a value of  $45 \pm 1$   $\mu$ U/mL at 120 min (30, 60, and 120 min values,  $P < 0.01$ – $0.001$  vs. basal values).

Serum  $\beta$ -hydroxybutyrate was  $38 \pm 12$   $\mu$ M at baseline; levels then gradually decreased through the study, with values of  $27 \pm 6$  mM by 30 min,  $19 \pm 7$  mM at 60 min,  $9 \pm 1$  mM at 90 min, and  $6 \pm 1$  mM at 120 min (30, 60, 90, and 120 min values,  $P < 0.05$ – $0.01$  vs. basal values).

All serum ethanol measurements throughout the study were, as expected, below the lower detection point of the assay.

#### Breath ethanol and acetone

Baseline levels of ethanol in exhaled breath were  $9.6 \pm 3.1$  parts per billion in volume

(ppbv) (Fig. 1). Exhaled ethanol levels then increased during the first part of the OGTT, peaking by the 30-min time point at  $45.0 \pm 14.2$  ppbv. Exhaled ethanol levels then rapidly decreased, and by 60 min were back at near-baseline levels; by 120 min mean ethanol levels were  $7.9 \pm 2.5$  ppbv.

Baseline levels of exhaled acetone were  $392 \pm 85$  ppbv (Fig. 1). After the start of the OGTT, acetone levels displayed a continuous decreasing trend, with values of  $364 \pm 71$  ppbv at 30 min,  $300 \pm 64$  ppbv at 60 min, and  $280 \pm 64$  ppbv at 120 min ( $P < 0.05$  vs. baseline).

#### Correlations between serum glucose and exhaled gases

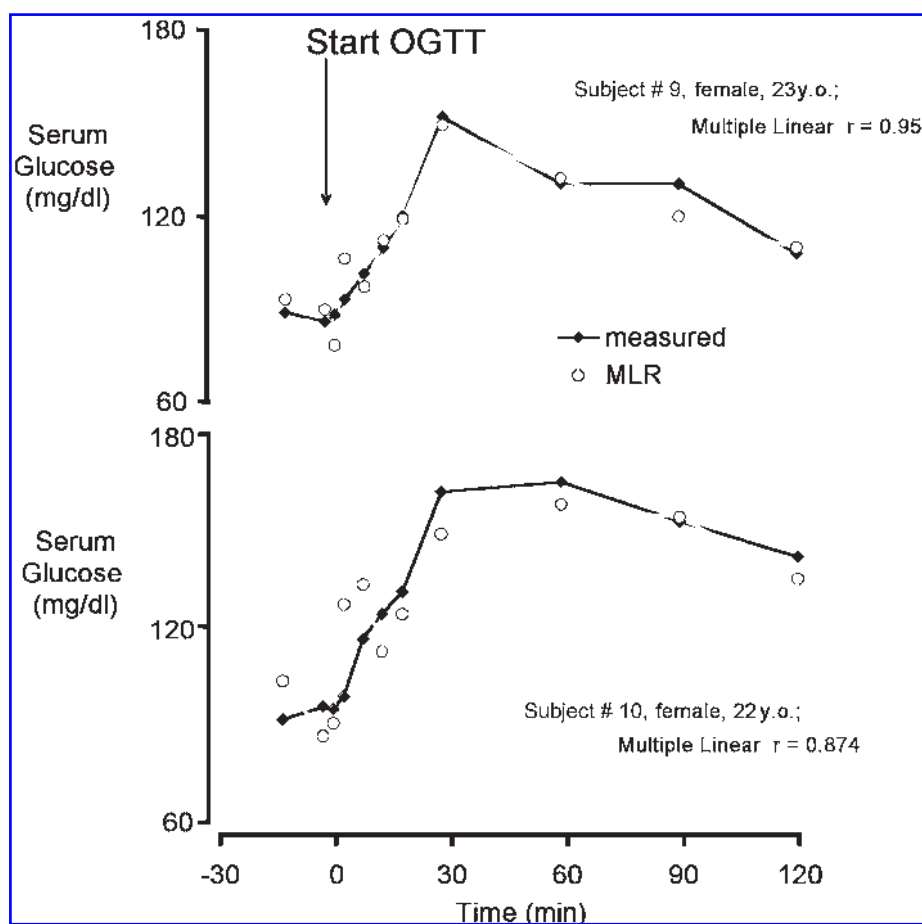
When a simple linear regression analysis was performed between either ethanol or acetone and glucose in each subject, both gases did correlate with glucose (ethanol vs. glucose, average individual  $r = 0.55$ ; acetone vs. glucose, average individual  $r = 0.40$ ) (Table 1). With either gas, however, in a few subjects the correlation was very low (lowest in ethanol–glucose 0.09,

lowest in acetone–glucose 0.06). After data analysis with multiple linear regression including glucose as the dependent variable, and both gases as independent variables, however, an approximation of the glucose profile could be derived that correlated with measured glucose values with an average individual  $r$  of 0.70. No subject displayed an  $r < 0.41$ , and six of 10 subjects displayed an  $r > 0.74$ . Data from two representative subjects are shown in Figure 2, including the only subject in our experimental group with an elevated 120-min serum glucose, corresponding to a clinical diagnosis of glucose intolerance. This latter subject displayed one of the strongest correlations ( $r = 0.874$ ) between measured glucose values and results of multilinear analysis of gases.

## DISCUSSION

In this study, we made novel observations regarding the ability of breath ethanol and acetone analysis to estimate serum glucose levels in human subjects. We hypothesized that using a highly sensitive VOC breath analysis system would enable us to identify patterns of exhaled gases that correspond with serum glucose levels. Indeed, we found that the integrated analysis of breath ethanol and acetone, both associated with glucose metabolism, correlated with changes in serum glucose levels.

As noted, ethanol is not produced by mammalian cells. Its measurement in the breath of our subjects is likely due to alcoholic fermentation of glucose by gut bacteria and yeast<sup>18</sup> and subsequent movement from the gut into



**FIG. 2.** Measured serum glucose profiles and glucose profiles approximations obtained via multiple linear regression (MLR) using ethanol and acetone as independent variables and glucose as the dependent variable. Data were obtained during a standard OGTT after an overnight fast in one subject with normal OGTT (**top panel**) and one subject with high 120-min glucose levels, indicating glucose intolerance (**bottom panel**).

the portal circulation. The probable bacterial origin of ethanol in mammals was recently confirmed by a 50% decrease in breath ethanol in mice after administration of oral neomycin, which markedly reduced gut flora.<sup>19</sup> Quantitatively, however, ethanol production by the intestinal flora is so small that results in mean blood concentrations of only  $\sim 0.3$  mg/dL,<sup>20</sup> not detected by standard laboratory techniques (typical commercial assays sensitivity is  $\sim 10$  mg of ethanol/dL of blood). Only in pathological circumstances does bacterial production of ethanol markedly increase, as first described by Ladkin and Davis<sup>13</sup> in 1948 and later by other authors.<sup>21–23</sup> In this exceptional situation, referred to as the “auto-brewery syndrome,” an excessive overload of carbohydrate or glucose is introduced in a digestive system with abnormal yeast or bacterial proliferation—particularly *Candida* species<sup>24</sup>—and massive alcoholic fermentation and ethanol production ensues.<sup>22,23</sup> The biochemical pathway follows the steps of anaerobic glycolysis to pyruvate, as occurs in mammals, but bacteria and yeast also contain the enzyme pyruvate decarboxylase, not present in humans, that converts pyruvate to acetaldehyde, which is then converted to ethanol by alcohol dehydrogenase (working in the opposite direction from its role in humans).

As our subjects did not ingest alcohol for at least 12 h prior to the study and had no evidence of abnormal intestinal flora, blood ethanol remained below the minimal detection ability of the standard assay throughout the study. Blood ethanol levels are in equilibrium with breath levels, with concentrations 2,000–4,000 times lower in the breath than coexisting concentrations in an equal volume of blood.<sup>25,26</sup> Assuming an exhaled gas mixture saturated with water vapor, with a pressure of 760 mm Hg and a temperature of 310°K, we can estimate that our peak values of exhaled ethanol corresponded to blood ethanol levels of  $<0.02$ – $0.04$  mg/dL, similar to previously reported data.<sup>20</sup> These levels are 2,000–4,000 times lower than the legal limit of 80 mg/100 mL, an important point given the fact that in forensic medicine the attempt to justify increased blood ethanol levels with increased endogenous production rather than

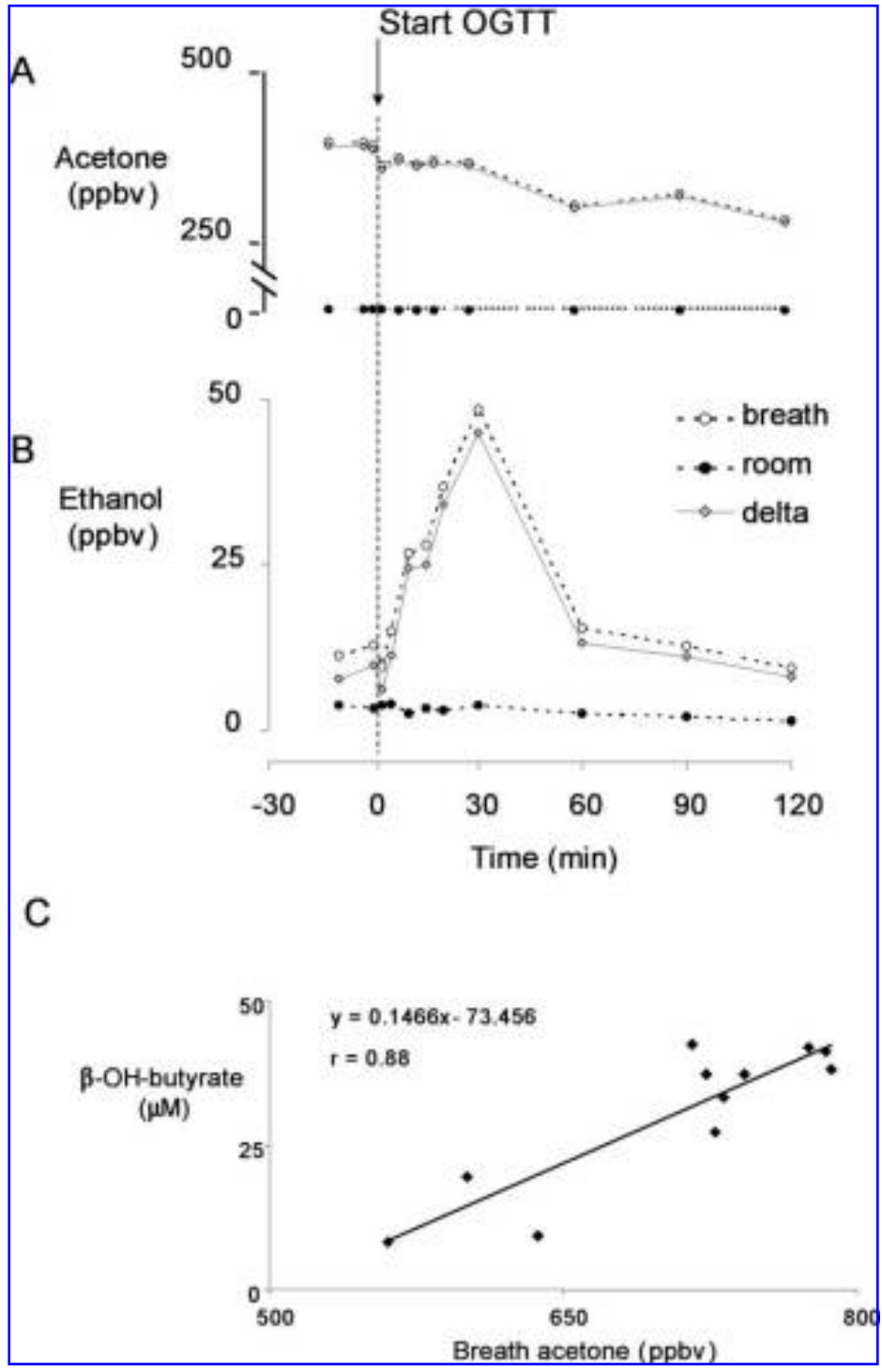
alcohol ingestion is a frequently controversial issue.<sup>27</sup>

In our study breath ethanol rapidly increased following ingestion of 75 g of glucose, initially paralleling the increase in blood glucose, and then returned to near-baseline much more rapidly than blood glucose. Although increased breath ethanol after a mixed meal has been previously reported,<sup>28</sup> to our knowledge the specific kinetic correlation between breath ethanol and ingested glucose, as induced by a standard OGTT, has not been previously reported. We speculate that the conversion of glucose to ethanol in the intestine is possible only at a threshold level of glucose availability; as soon as intestinal glucose decreases below this threshold, bacterial ethanol production is almost completely abolished. Whether bacterial ethanol production requires the presence of glucose within the gut lumen, or can be independently supported by systemic hyperglycemia, cannot be ascertained at this time. In favor of the latter hypothesis, however, is the observation that in the one subject with abnormal OGTT results, breath ethanol remained elevated until the end of the study; as there is no reason to believe that her gut glucose absorption was delayed, it would appear that systemic hyperglycemia was driving breath ethanol production in this subject.

Acetone is a three-carbon ketone body derived from acetoacetate through spontaneous decarboxylation or enzymatic conversion (via acetoacetate decarboxylase).<sup>29</sup> Because of its volatile nature, acetone can be detected in breath where, at high concentrations, a typical fruity aroma results. As breath acetone increases during diabetic ketoacidosis,<sup>9</sup> fasting, and high-fat diets,<sup>14,15,30</sup> it is a reliable indicator of circulating ketone bodies.<sup>9,16</sup> To date, however, only substantial increases in exhaled acetone have been useful as biomarkers. To our knowledge, our study is the first to report the potential use of a decreasing profile of exhaled acetone below basal levels, paralleling physiological postprandial hyperglycemia (at 120 min, mean acetone levels were  $>30\%$  lower than baseline,  $P < 0.01$ ). Breath acetone during our OGTT displayed a pattern similar to that of blood  $\beta$ -hydroxybutyrate, another ketone body in equilibrium with acetone but markedly

more stable. Measurement of blood acetone, especially on samples frozen for several weeks, as in this study, is notoriously unreliable and was not performed; however, serum  $\beta$ -hydroxybutyrate and breath acetone were very

strongly correlated ( $r = 0.88$ , Fig. 3C). It is likely that the relative hyperinsulinemia caused by increased glucose decreased the availability of non-esterified fatty acids for oxidation, and less acetoacetate was formed and



**FIG. 3.** Room air, total exhaled, and net exhaled concentrations of ethanol (A) and acetone (B) and correlation between serum  $\beta$ -hydroxybutyrate and breath acetone (C) in 10 healthy volunteers during a standard OGTT after an overnight fast. Data are group means.

converted to acetone. In this respect, acetone is probably only indirectly reflecting glucose levels via the integrated glucose/insulin profile.

A common technical problem in the analysis of exhaled gases is potential interference of ambient gases. In our study, simultaneous with each breath sample, room air was also collected so that ambient gas concentrations could be subtracted from breath samples, then presented as net values. As shown in Figure 3, ethanol and acetone room concentrations were very stable and significantly lower than breath concentrations (average room/breath ratio of 1:11 and 1:70, respectively), suggesting that room air concentrations had little impact, if any, on the biological significance of our observations.

The diagnosis and follow-up of diabetes, one of the leading causes of morbidity and mortality in the western world, are based upon blood glucose measurements. Glucose loading is also used for the diagnosis of gestational diabetes. Millions of these measurements are performed daily, sometimes with significant discomfort or pain for the patient, and at considerable expense. The necessity of devising alternative, non-invasive, low-cost, and yet reliable diagnostic methodologies is further underscored by the impending epidemic of pediatric obesity, insulin resistance, and type 2 diabetes among children and adolescents in western societies.<sup>31-33</sup> Over the next decades, managing the increasing prevalence of these conditions may translate to broad-based screening for fasting and stimulated glucose levels at the school or community level. Our preliminary report raises the intriguing possibility that the integrated analysis of exhaled gases may serve as a non-invasive tool for measurement of glucose levels.

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