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A NOVEL NON-INVASIVE BREATH TEST METHOD FOR SCREENING PERSONS AT RISK FOR DIABETES

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Objective- Diagnosis of pre-diabetes and early-stage diabetes occurs primarily by means of an oral glucose tolerance test (ogtt) which requires invasive blood sampling. The aim of this study was to determine whether differences exist in breath $^{13}co_2$ excretion during a ^{13}c-labeled ogtt between normal glucose tolerant (ngt) individuals and persons with pre-diabetes and early-stage diabetes (pded), and whether these differences correlated with blood glucose kinetics.

Methods- Blood and breath samples were collected at baseline and every 30 minutes for a 10 h period following ingestion of 75 g of glucose isotopically labeled with 150 mg of U-$^{13}C_6$-D-glucose.

Results- Age (56±5 vs. 47±3 y) and body mass index (BMI) (31±2 vs. 31±2 kg/m^2) were not different between NGT (n=10) and PDED (n=7), respectively. Blood glucose concentrations were significantly higher in PDED compared to NGT from baseline to 4.5 h post-glucose ingestion ($P \leq 0.05$). Glucose-derived breath $^{13}CO_2$ was significantly lower in PDED compared to NGT from 1 to 3.5 h post-glucose ($P \leq 0.05$). Peak breath $^{13}CO_2$ abundance occurred at 4.5 and 3.5 h in PDED and NGT, respectively (36.87±3.15 vs. 41.36±1.56 ‰ delta over baseline).

Conclusions- These results suggest that this novel breath test method may assist in recognition of pre-diabetes or early-stage diabetes in at-risk persons without the need for invasive blood sampling, thus making it an attractive option for large-scale testing of at-risk populations, such as children.

Type 2 diabetes and its cluster of disorders (i.e. metabolic syndrome) have become a major public health concern (1; 2), and forms a predictive risk profile for cardiovascular disease (3-5). Recently, the risk of developing metabolic syndrome has significantly increased in the pediatric population (6). Traditionally, pre-diabetes and early-stage diabetes are assessed in at-risk adult and pediatric populations using a standard oral glucose tolerance test (OGTT) requiring repeated invasive blood sampling over the 2-3 hours following a glucose load. Post-prandially, the mix of fuels used for cellular energy production is dramatically altered when insulin sensitivity is decreased (7-9). The ability to non-invasively detect the shift in fuel source utilization between individuals with normal glucose tolerance (NGT) and those at-risk for pre-diabetes or early-stage diabetics, (PDED) has considerable clinical utility.

It has been previously demonstrated that $^{13}CO_2$ in breath is as effective at assessing insulin resistance (IR) as the hyperinsulinemic-euglycemic clamp method (10). However, the clinical utility of detecting breath CO_2 kinetics using a standard diagnostic infrared $^{13}CO_2$ breath analyzer between normal (NGT) and PDED individuals has not been clearly established. Therefore, the aim of the present study was to evaluate the efficacy of a diagnostic breath analyzer available in many physician offices to characterize the kinetics of glucose-derived CO_2 in breath following administration of a standard oral glucose tolerance test (OGTT) in NGT and PDED individuals. We compared the breath CO_2 kinetics from NGT to PDED individuals for 10 hours following an OGTT (75 g glucose load) isotopically labeled with U-$^{13}C_6$-glucose**.** Additionally, breath CO_2 data were correlated to indices of IR (Whole body index of insulin sensitivity [WBISI], homeostasis model assessment of insulin resistance [HOMA-IR], and quantitative insulin sensitivity check index [QUICKI]) calculated from measures of blood glucose and plasma insulin. We hypothesized that the standard breath analyzer would be sufficiently sensitive to detect impaired glucose utilization for cellular respiration during a standard OGTT between NGT and PDED.

MATERIALS AND METHODS

Subjects: The protocol was approved by the University of Texas Medical Branch (UTMB) Institutional Review Board and conducted in the UTMB General Clinical Research Center (GCRC). All subjects provided written informed consent before participating in the study. Screening of all subjects included fasting blood glucose, blood cell count, thyroid function test, lipid panel, urinalysis, and a urine pregnancy test for pre-menopausal women. Individuals previously diagnosed with type 2 diabetes or taking any medications known to affect glucose or lipid metabolism were excluded from participation. Based on their 2-h OGTT blood glucose levels on the study day, subjects were grouped as either normal glucose tolerant (NGT, 2-h OGTT blood glucose < 7.8 mmol/L, n=10) as outlined by the American Diabetes Association (www.diabetes.org), or pre-diabetic and early-stage diabetic (PDED, 2-h OGTT blood glucose 7.8 - 16 mmol/L, n=7). All NGT subjects required fasting glood glucose ≤ 5.6 mmol/L for inclusion. Subject characteristics are shown in Table 1.

Methods: The study protocol consisted of 4 days of study activities (Figure 1). On days 1 – 3 subjects were placed on a standardized, low carbohydrate diet containing 15% carbohydrate, 25% protein, and 60% fat. Individual dietary requirements were determined by a registered dietitian and calculated using the Harris Benedict Equation (11). During this diet stabilization period all subjects were provided breakfast, lunch, and dinner from the UTMB General Clinical Research Center (GCRC) metabolic kitchen. All meals were picked up on a daily basis for consumption off site. Subjects were required

to completely consume these meals and nothing else except water. Height and weight were recorded daily during the visits to the GCRC for pick up of the meals. On the morning of the fourth day, a 10 h OGTT was performed in the GCRC.

After an overnight fast (water allowed), subjects were placed in a bed at the GCRC and an antecubital venous IV was placed for the collection of blood samples. Baseline blood samples were collected twice over a fifteen minute period. During the second baseline blood collection, simultaneous breath samples were collected by having the subjects breathe into breath collection bags fitted with one-way valves. Following the baseline sample collection, a drink containing 75 g glucose and 150 mg U-$^{13}C_6$-glucose was administered and consumed within 1 minute. From this point (t=0 minutes) blood and single-point breath samples were collected every 30 minutes for 10 hours. For the collection of breath samples, the subjects were instructed to breathe normally, hold their breath for 3 seconds, and exhale completely into a provided collection bag. Subjects remained at rest throughout the 10 h OGTT and were only allowed to move around the room to use the restroom. Water was provided *ad libitum* throughout the 10 h OGTT. After collection of the final samples at t=10 hours and removal of the IV, the subjects were fed and discharged.

Blood glucose: Glucose concentrations were determined in whole blood immediately after collection of the samples using a 2300 STAT Plus Glucose analyzer (intra-assay coefficient of variance (CV) = 2.1 %).

Plasma insulin: Insulin concentrations were determined in plasma using an Immulite 2000 chemiluminescence immunoassay system (Diagnostic Products Corporation, Los Angeles, CA, CV = 3.3 %).

Breath CO_2: The ratios of $^{13}CO_2$ to $^{12}CO_2$ in single breath samples were measured using a UBiT-IR300 infrared spectrophotometer (Otsuka Electronics Co., Ltd, Hirakata, Osaka, CV ≤ 1.0 %). All results are calculated as per mille (‰) change of $^{13}CO_2$ abundance from the baseline breath sample and expressed as ‰ delta over baseline (‰DOB).

Indices of insulin resistance: Whole body index of insulin sensitivity (WBISI) (12), homeostasis model assessment of insulin resistance (HOMA-IR) (13), and quantitative insulin sensitivity check index (QUICKI) (14) were calculated from blood glucose (mg/dL) and plasma insulin (μIU/mL) measurements using the following equations:

$$\text{WBISI} = \frac{10{,}000}{\sqrt{(\text{Fasting Glucose} \bullet \text{Fasting Insulin}) \bullet (\text{Mean OGTT Glucose} \bullet \text{Mean OGTT Insulin})}}$$

$$\text{HOMA} = \frac{\text{Fasting Glucose} \bullet \text{Fasting Insulin}}{405}$$

$$\text{QUICKI} = \frac{1}{\log(\text{Fasting Glucose}) + \log(\text{Fasting Insulin})}$$

(12), (13), (14)

Statistics: A repeated measures analysis utilizing restricted maximum likelihood estimation (REML) was used to obtain parameter estimates using the MIXED procedure in SAS® (SAS Institute INC. 2004. SAS/STAT® 9.1 User's Guide. Cary, NC: SAS Institute Inc.). Each set of measurements from the same subject were considered a correlated cluster of observations. The AR (1) covariance structures was used. These models allow for inclusion of subjects with small amounts of missing data. The mixed model tested the effects for group, time and the (group x time) interaction. When the interaction was significant ($P<0.05$) we computed 2-sample t-tests at each time point for descriptive purposes used only in the figures.

RESULTS

Subject characteristics: There were no differences in age, height, weight, BMI, gender, or fasting plasma insulin between the NGT and PDED groups (Table 1). HOMA-

IR was significantly higher in PDED, and QUICKI was significantly lower in PDED compared to NGT ($P \leq 0.05$). WBISI was not significantly different between the groups.

Blood glucose: By design, 2-h OGTT blood glucose concentrations were higher in the PDED (11.4 ± 0.95 mmol/L) compared to NGT (6.2 ± 0.19 mmol/L) ($P<0.00001$). The repeated mixed model analysis indicated that blood glucose had significant group ($P<0.0001$) time ($P<0.0001$) and (group x time) interaction ($P<0.0001$). The interaction indicated that blood glucose concentrations were significantly lower in PDED compared to NGT between 0 and 3 hours, as shown in Figure 2A. Fasting blood glucose concentrations were higher in PDED (6.8 ± 0.56 mmol/L) compared to NGT (5.1 ± 0.13 mmol/L) ($P<0.01$). Blood glucose levels were significantly elevated post-glucose load in PDED to NGT from t=0 to 3.5 h ($P<0.05$). There were no differences in blood glucose concentrations from 4 through 10 hours post-glucose load between the groups.

Plasma insulin: The repeated mixed model analysis indicated that insulin gave effects for group (P=0.53) time ($P<0.0001$) and (group x time) interaction (P=0.03). The interaction indicated that the differences between PDED and NGT were stronger at later time points than early ones, as shown in Figure 2B.

Breath CO_2: Breath $^{13}CO_2$ abundances had significant group (P=0.02) time ($P<0.0001$) and (group x time) interaction (P=0.0002). The interaction indicated that breath $^{13}CO_2$ abundances were significantly lower in PDED compared to NGT between 1 and 3 hours post-glucose load as shown in Figure 3A. Peak breath $^{13}CO_2$ abundance occurred at 3.5 hours in NGT (41.36 ± 1.56 ‰DOB) and 4.5 hours in PDED (36.87 ± 3.15 ‰DOB). Inter-subject CV at peak $^{13}CO_2$ appearance was 12% and 22% for NGT and PDED respectively. Areas under the curve (AUC) for breath $^{13}CO_2$ abundance were calculated from 0 to 4 hours and from 0 to 10 hours in Figure 3B as mean ± SEM. Mean breath $^{13}CO_2$ abundance AUC was lower in PDED compared to NGT from 0 to 4 hours post glucose load ($P<0.05$). The difference between PDED and NGT disappeared when AUC was calculated from 0 to 10 hours.

Correlation of Breath CO_2 to indices of insulin sensitivity: The 2-hour breath $^{13}CO_2$ abundance measurements correlated directly with WBISI (Pearson correlation coefficient r = 0.502, P=0.04), QUICKI (r = 0.629, P=0.007), and fasting glucose (r = -0.631, P=0.007), and inversely with 2-hour glucose (r = -0.689, P=0.002), HOMA-IR (r = -0.555, P=0.02), weight (r = -0.546, P=0.02). Significant correlations were found between all three measured indices of IR ($P \leq 0.002$). No correlations were found between age and any of the measured parameters.

DISCUSSION

Results of the present study demonstrate that a standard infrared breath analyzer can detect differences in the excretion pattern of glucose-derived breath CO_2 between individuals with NGT and PDED. Moreover, these differences were detectable in the timeframe of a standard OGTT and correlated with the differences in glucose kinetics between the two groups. Thus, this non-invasive breath method may assist in recognition of PDED by providing an alternative approach for large-scale population testing without the need for blood sampling. This is particularly important to improve acceptance of pre-diabetes screening for certain patient populations, such as pediatric patients, who may be reluctant to undergo screening due to the prospect of repeated blood sampling.

Remarkably, the breath analyzer was capable of detecting marked differences in glucose-derived breath CO_2 kinetics between NGT and PDED individuals within 60 minutes of a 10 h OGTT. The initial rate of glucose-derived CO_2 appearance in breath was significantly lower in PDED compared to NGT. Consistent with the blood glucose measurements, the breath test was able to

detect the impairments in clearance of exogenous (oral) glucose from circulation in PDED individuals. While the traditional blood glucose measurements rely on the accumulation of substrate in circulation, the breath test is a more direct measure of intracellular glucose metabolism. Impaired glucose uptake due to diminished pancreatic insulin secretion or impaired insulin action on the target tissue (i.e. skeletal muscle) results in blunted glucose oxidation in PDED individuals.

A strength in our present study is that breath CO_2 kinetics were followed over a longer period (10 hours) and included multiple breath CO_2 measurements in conjunction with simultaneous measurements of both blood glucose and plasma insulin concentrations. The data expressed as AUC support the assumption that, while slower in PDED, the eventual fate of the oral glucose was the same as for NGT individuals. While the mean AUC was higher in NGT than PDED from 1.5 through 4.5 hours post-glucose ingestion, this difference disappeared within the 10-h period. Thus, our results support the concept that individuals with PDED switch at a slower rate between lipid and glucose as a source of metabolic fuel when compared to NGT individuals. Impaired glucose tolerance has been described as "metabolic inflexibility" of the target tissue to respond to a switch from lipid oxidation to glucose oxidation (7). Under normal physiologic conditions, cellular fuel consists primarily of free fatty acids (FFAs) and glucose, with protein making a minor contribution (7; 9; 15). In the basal state, > 60% of cellular fuel for insulin-dependent peripheral tissues (muscle) is derived from FFAs (15-19). In normal glucose tolerant (NGT) individuals, when an oral dose of glucose [75 – 100 g] is administered, contribution of free fatty acids (FFAs) to whole-body fuel oxidation is reduced dramatically, with glucose becoming a prominent cellular fuel (15). In individuals with IR or pre-diabetes, this response is significantly blunted, resulting in a disproportionate contribution of FFAs to post-prandial whole-body energy consumption (20; 21). Glucose uptake through GLUT-4 is mediated by insulin whereas non-insulin dependent glucose uptake occurs through other glucose transporters or mechanisms. In disorders such as type 2 diabetes, the non-insulin dependent glucose uptake may be upregulated to compensate for the lack of insulin dependent GLUT-4 recruitment, contributing to a higher than normal glucose oxidation in the fasting state but lower insulin mediated glucose oxidation in response to a meal (7; 20; 22).

While resting energy expenditure (REE) measurements are not available from these experiments, future studies that include indirect calorimetry measures are needed to confirm how REE differences between subjects, as well as metabolic changes in response to glucose ingestion, affect CO_2 kinetics in breath. However, it is important to underscore that the development of this breath test technique is intended as a non-invasive alternative to blood draws for the determination of glucose tolerance during a standard OGTT, not as an alternative to indirect calorimetry for measurements of whole body energy expenditure. The application of this technique is simple and relatively economical as it requires few single-breath collections and no special training, which may allow for the development of at-home kits.

Our results are in general consistent with the correlations between glucose derived CO_2 in breath and indices of insulin resistance reported by Lewanckzuk et al. (10). This group has recently compared a single measurement of ^{13}C-glucose derived CO_2 in breath taken 90 minutes after ingestion a lower dose of glucose to results obtained from a hyperinsulinemic-euglycemic clamp performed on a separate occasion. Interestingly, although the methods utilized in that study were profoundly different from those employed in ours, their correlation

coefficient was remarkably similar to our 2 h correlation coefficient between breath CO_2 and HOMA-IR (ref. 10: -0.531; ours: -0.555) (10). However, to the best of our knowledge no studies have explored whether glucose derived CO_2 in breath correlated with blood glucose and plasma insulin measurements collected simultaneously during labeled OGTT. Our study is also the first demonstrating feasibility of the use of a cost-effective diagnostic breath analyzer instead of an isotope ratio mass spectrometer as previously used.

While low-carbohydrate diets are not typically prescribed before administration of a standard OGTT, the 3-day low-carbohydrate diet stabilization period was intended to remove dietary intake as a variable in the present study. Additionally, we intended to decrease hepatic and muscle glycogen stores, thus minimizing these tissues as immediate sources of endogenous glucose. Future research is warranted to characterize the impact of diet and altered gastrointestinal absorption of glucose on the kinetics of breath CO_2 appearance.

In conclusion, the results of the present study demonstrate the clinical applicability of a standard breath analyzer to detect differences in glucose-derived breath CO_2 kinetics between individuals with NGT and PDED, which correlate with differences in blood glucose kinetics. Following a baseline sample, the optimal time for breath collection was between 1 and 3 hours after the oral glucose load, similar to the timeframe of a standard OGTT. Both the area under the curve, when multiple measurements are made over time, and the slope when only a pre- and post measurement are available, were shown to be effective. This noninvasive method may assist in recognition of undiagnosed PDED in at-risk individuals during the pre-diabetes stage of type 2 diabetes during a standard OGTT, and in the future may also help increase compliance with diabetes screening in specific populations, such as obese children. Additionally, the use of a point-of-care diagnostic breath $^{13}CO_2$ analyzer and storable breath collection bags lends itself for large-scale population testing in research as well as the clinical setting. However, further research is necessary to validate this method as a viable diagnostic tool to be used in addition to, or in place of, existing methods. For example it will be important to establish cut-off values between true NGT and PDED individuals, determine within-subject variability and repeatability, elucidate the effects of dietary intake on breath test results, and explore the applicability of this method in pediatric care.

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Table 1: Subject Characteristics.

	NGT	PDED	P-value
2-h OGTT Blood Glucose (mmol/L)	6.2 ± 0.17	11.4 ± 0.94 *	<0.0001
Gender	5Female/5Male	4Female/3Male	0.79
Age (yr)	47 ± 3	56 ± 5	0.15
Weight (kg)	89 ± 6	92 ± 6	0.74
Height (cm)	166 ± 2	172 ± 3	0.09
BMI (kg/m^2)	31 ± 2	31 ± 2	0.96
Fasting Blood Glucose (mmol/L)	5.1 ± 0.11	6.8 ± 0.56 *	0.003
Fasting Plasma Insulin (µIU/mL)	7.0 ± 1.7	10.39 ± 2.77	0.29
WBISI	8.00 ± 1.22	5.48 ± 1.47	0.10
HOMA-IR	1.63 ± 0.43	3.23 ± 0.94 *	0.05
QUICKI	0.37 ± 0.01	0.34 ± 0.01 *	0.04

Subject characteristics of the NGT and PDED subjects. * Significantly different from NGT ($P \leq 0.05$).

Figure 1: Study Timeline.

Subjects followed a three day standardized low carbohydrate diet composed of 15% carbohydrate, 25% protein, and 60% fat. Individual dietary requirements were determined by a registered dietitian and calculated using the Harris Benedict Equation. On the fourth day, after an overnight fast, a 10 h stable-isotopically labeled OGTT was performed and blood and breath samples were collected every 30 minutes.

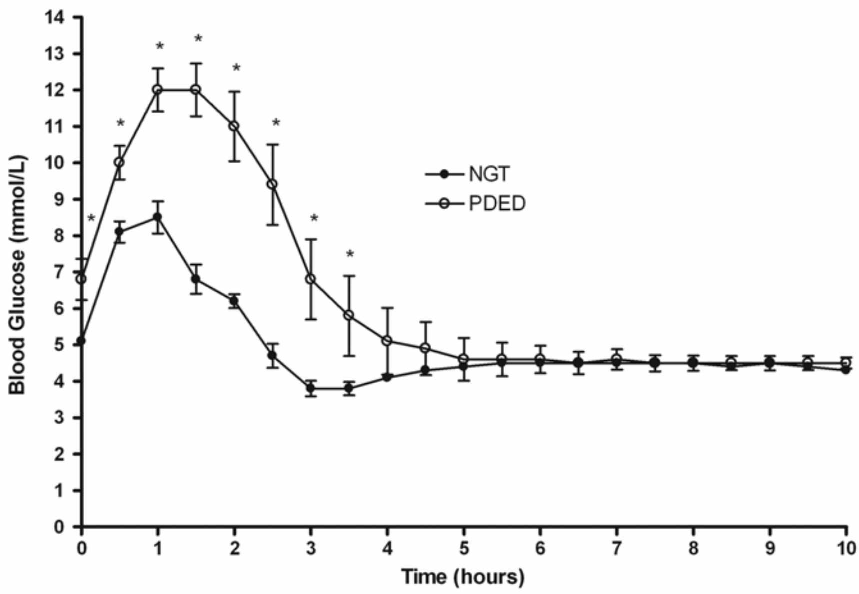
Figure 2: Blood glucose **(A)** and plasma insulin **(B)** concentrations in NGT and PDED during the 10-h OGTT.

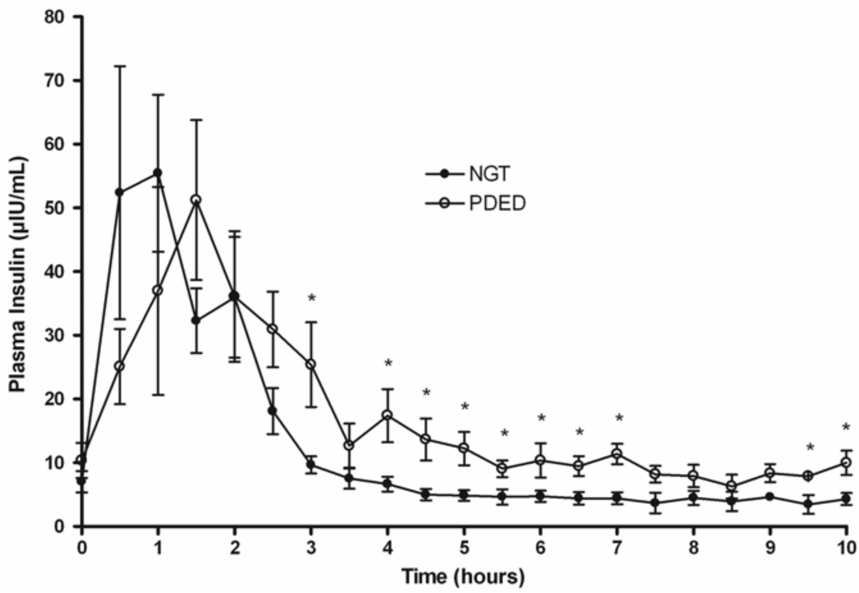
(A) Blood glucose was different between NGT and PDED over time as determined by Mixed Model Repeated Measures ($P<0.05$). *Different between NGT and PDED (T-test, $P<0.05$). **(B)** Plasma insulin was different between NGT and PDED over time as determined by Mixed Model Repeated Measures ($P<0.05$). *Different between NGT and PDED (T-test, $P<0.05$).

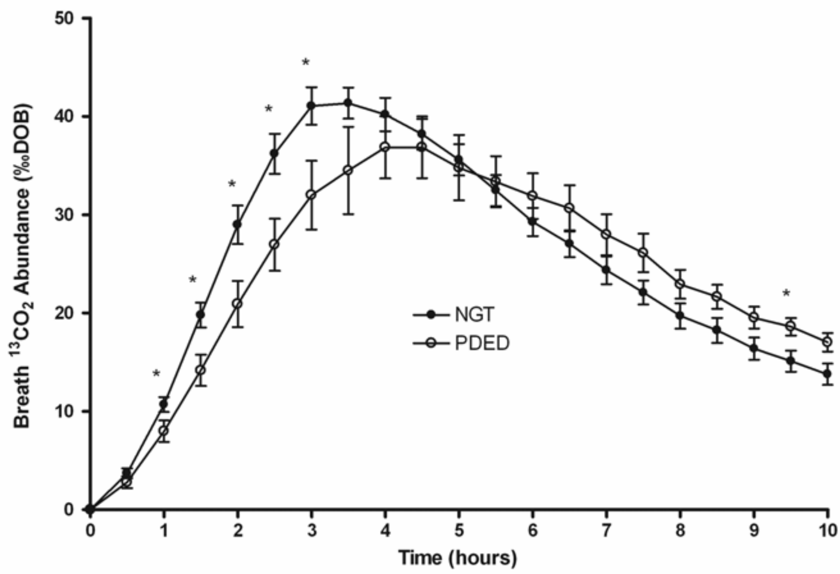
Figure 3: Breath $^{13}CO_2$ abundance **(A)** and areas under the curve **(B)** for NGT and PDED during the 10-h OGTT.

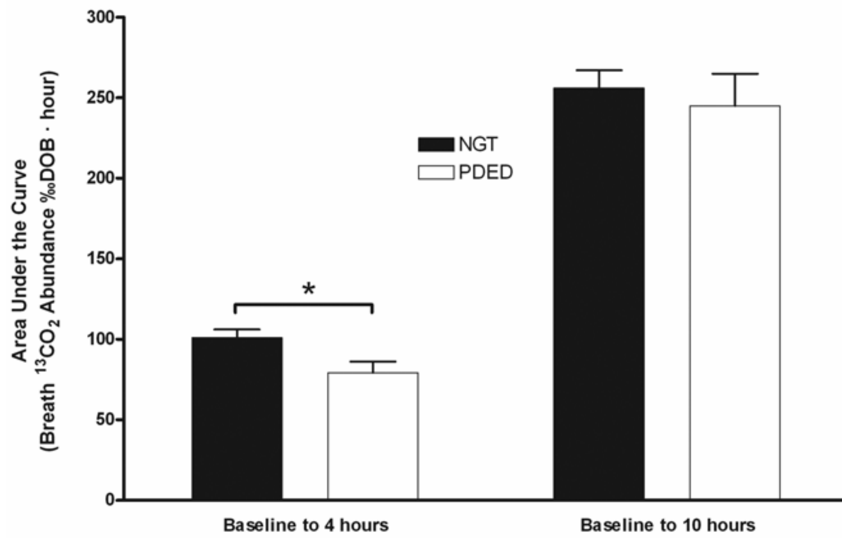
(A) Breath $^{13}CO_2$ abundance was different between NGT and PDED over time as determined by Mixed Model Repeated Measures ($P<0.05$). *Significant difference between NGT and PDED (T-test, $P<0.05$). **(B)** Areas under the curve (AUC) were different between NGT and PDED when measured from 0 to 4 hours but not when measured from 0 to 10 hours. *Significant difference between NGT and PDED (T-test, $P<0.05$).

Day 1 Day 2 Day 3
Low Carbohydrate Diet
(15% carbohydrate, 25% protein, 60% fat)
Day 4
10-h OGTT
Glucose Drink
75 g glucose +
150 mg U-$^{13}C_6$-glucose
Time (min) 0 30 60 90 120 150 180 210 240 270 300 330 360 390 420 450 480 510 540 570 600
Blood Sample
Breath Sample


A
Blood Glucose (mmol/L)
Time (hours)
NGT
PDED
*


B
Plasma Insulin (μIU/mL)
Time (hours)
NGT
PDED
*


A
Breath $^{13}CO_2$ Abundance (‰DOB)
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10
Time (hours)
NGT
PDED
*


B
Area Under the Curve
(Breath $^{13}CO_2$ Abundance ‰DOB · hour)
300
250
200
150
100
50
0
NGT
PDED
*
Baseline to 4 hours
Baseline to 10 hours