Cavity Enhanced Absorption Spectroscopy And Its Application To Molecular Detection Of Diabetes Mellitus

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Dedicated to My Grandfather and Supervisor…

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ABSTRACT

In recent years, the cavity enhanced absorption spectroscopy (CEAS) has been established as a powerful technique for high-sensitive gas absorption study in the infrared region. In CEAS, the optical cavity consists of two high-reflectivity mirrors which allow the laser light to move back and forth to enhance the effective sample path lengths of few kilometers. This arrangement allows the CEAS tool to achieve the ultra-high sensitivity of parts-per-billion (ppb) levels and even down to parts-per-trillion (ppt) levels. This thesis is focused on application of high resolution laser-based cavity enhanced absorption spectroscopy (CEAS) in molecular detection of biomedical disease. Here, we investigated the association of endogenously produced trace gases and their isotopic species in exhaled breath with diabetes mellitus. In this thesis work, we first checked the feasibility of breath carbon-13 isotope ($^{13}C^{16}O^{16}O$) analysis followed by non-radioactive ^{13}C-glucose administration for non-invasive understanding of metabolic defect in diabetes. We demonstrated how $^{13}CO_2$ measurements in breath exploiting a simple residual gas analyzer-mass spectrometry (RGA-MS) could precisely distinguish the non-diabetic control (NDC), pre-diabetes (PD) and type 2 diabetes (T2D). The diagnostic accuracy, precision and validity of the results by RGA-MS system were confirmed by the CEAS technique. In the next study, we proposed a new method for accurate and early detection of insulin resistance in T2D. Currently, insulin sensitivity index ($ISI_{0,120}$) is considered to be a viable invasive method of whole-body insulin resistance for use in clinical settings in comparison with other invasive sensitivity indexes like homeostasis model assessment (HOMA) and quantitative insulin sensitivity check index (QUICKI). Here, we showed that monitoring of isotopic breath CO_2 may be a novel method for accurate estimation of $ISI_{0,120}$ and thus may open new perspectives into the isotope-specific point-of-care evaluation of insulin resistance for large-scale diabetes screening. To promote the isotopic breath test for detection of T2D, it is important to replace the commercially prepared ^{13}C-labelled glucose with naturally available ^{13}C-enriched substrates. Therefore in the next study, we formulated a new test

meal comprising of naturally available ^{13}C-enriched foods and subsequently administered it to NDC and T2D. Our study showed that measurements of enrichments or depletions of oxygen-18 ($^{12}C^{18}O^{16}O$) and carbon-13 isotopes of breath CO_2 in T2D after administration of naturally ^{13}C-abundant nutrients may be a valid and potentially robust method devoid of any synthetically manufactured commercial ^{13}C-enriched glucose. Further, it is well known that the oxygen-16 (^{16}O) isotope in $^{12}C\ ^{16}O_2$ and the oxygen-18 (^{18}O) isotope of body water ($H_2\ ^{18}O$) are rapidly exchanged during the respiration process, catalyzed by carbonic anhydrase (CA), a common metalloenzyme present in human body. Here, we explored how CA activity links to ^{18}O of breath CO_2 to pre-diabetes and type 2 diabetes during metabolism. Our findings suggest that the alterations in erythrocytes CA activities may be the initial step of altered metabolism of type 1 diabetes (T1D) and T2D, and breath ^{18}O-isotope regulated by the CA activity is a potential diagnostic biomarker that can selectively and precisely distinguish the T1D from T2D. Now-a-days, the association of carbonic anhydrase with numerous diseases like edema, glaucoma, osteoporosis, neurological disorders, renal cancer, cervical cancer and lung cancer has been widely discussed. Although traditional method provides useful information regarding the enzymatic assay of CA, the practical application of this method is limited due to tedious and expensive processes including blood sample collection, long time for laboratory processing and standardization to cell counts. In this in-vitro study, we mimicked the isotopic fractionation reaction of human body within the experimental sample flasks. We found a quantitative relation between the oxygen-18 isotope of CO_2 and CA activity, suggesting a new and alternative method for estimation of CA activity from ^{18}O-isotope of CO_2 analysis. In conclusion, in this thesis, we have demonstrated and established the proof-of-concept of the new-generation non-invasive diagnostic methodologies exploiting few unique panels of molecular species and their stable isotopes in human breath that can precisely and selectively diagnose early-stage and type 2 diabetes.

LIST OF PUBLICATIONS

1. List of publications related to thesis work

1. **C. Ghosh**, S. Mandal, M. Pal, P. Mukhopadhyay, S. Ghosh and M. Pradhan "^{13}C-isotope abundance in natural nutrients: a new formulated test meal for type 2 diabetes", ***Journal of Breath Research***, 11, 026005 (2017).

2. **C. Ghosh**, S. Mandal, G. D. Banik, A. Maity, P. Mukhopadhyay, S. Ghosh and M. Pradhan "Targeting erythrocyte carbonic anhydrase and ^{18}O-isotope of breath CO_2 for sorting out type 1 and type 2 diabetes", ***Scientific Reports (Nature Publishing Group),*** 6: 35836 (2016).

3. **C. Ghosh**, P. Mukhopadhyay, S. Ghosh and M. Pradhan "Insulin sensitivity index ($ISI_{0,\ 120}$) potentially linked to carbon isotopes of breath CO_2 for pre-diabetes and type 2 diabetes", ***Scientific Reports (Nature Publishing Group)***, 5: 11959 (2015).

4. **C. Ghosh**, G. D. Banik, A. Maity, S. Som, A. Chakraborty, C. Selvan, S. Ghosh, S. Chowdhury and M. Pradhan "Oxygen-18 isotope of breath CO_2 linking to erythrocytes carbonic anhydrase activity: a biomarker for pre-diabetes and type 2 diabetes", ***Scientific Reports (Nature Publishing Group)***, 5: 8137 (2015) .

5. **C. Ghosh**, A. Maity, G. D. Banik, S. Som, A. Chakraborty, C. Selvan, S. Ghosh, B. Ghosh, S. Chowdhury and M. Pradhan, "Non-invasive ^{13}C-glucose breath test using residual gas analyzer-mass spectrometry: a novel tool for screening individuals with pre-diabetes and type 2 diabetes", ***Journal of Breath Research,*** 8 (3), 036001 (2014).

2. List of publications apart from thesis work

1. S. Das, **C. Ghosh** and S. Jana "Moisture induced isotopic carbon dioxide trapping from ambient air", ***Journal of Materials Chemistry A***, 4 (20), 7632-7640 (2016).

2. S. Jana, S. Das, **C. Ghosh**, A. Maity and M. Pradhan "Halloysite nanotubes capturing isotope selective atmospheric CO_2", ***Scientific Reports (Nature Publishing Group),*** 5:8711 (2015).

3. A. Maity, G. D. Banik, **C. Ghosh**, S. Som, S. Chaudhuri, S. B. Daschakraborty, S. Ghosh, B. Ghosh, A. K. Raychaudhuri and M. Pradhan "Residual gas analyzer mass spectrometry for human breath analysis: a new tool for the non-invasive diagnosis of Helicobacter pylori infection", ***Journal of Breath Research***, 8 (1), 016005 (2014).

4. A. Maity, S. Som, **C. Ghosh**, G. D. Banik, S. B. Daschakraborty, S. Ghosh, S. Chaudhuri and M. Pradhan "Oxygen-18 stable isotope of exhaled breath CO_2 as a non-invasive marker of *Helicobacter pylori* infection", ***Journal of Analytical Atomic Spectrometry***, 29 (12), 2251-2255 (2014).

5. S. Som, A. De, G. D. Banik, A. Maity, **C. Ghosh**, M. Pal, S. B. Daschakraborty, S. Chaudhuri, S. Jana and M. Pradhan "Mechanisms linking metabolism of Helicobacter pylori to ^{18}O and ^{13}C-isotopes of human breath CO_2", ***Scientific Reports (Nature Publishing Group)***, 5: 10936 (2015).

6. S. Som, A. Maity, G. D. Banik, **C. Ghosh,** S. Chaudhuri, S. B. Daschakraborty, S. Ghosh and M. Pradhan "Excretion kinetics of ^{13}C-urea breath test: influences of endogenous CO_2 production and dose recovery on the diagnostic accuracy of Helicobacter pylori infection", ***Analytical and Bioanalytical Chemistry***, 406 (22) 5405-5412 (2014).

7. G. D. Banik, A. Maity, S. Som, **C. Ghosh**, S. B. Daschakraborty, S. Chaudhuri, S. Ghosh and M. Pradhan "Diagnosis of small intestinal bacterial overgrowth in irritable bowel syndrome patients using high-precision stable $^{13}CO_2/^{12}CO_2$ isotope ratios in exhaled breath", ***Journal of Analytical Atomic Spectrometry***, 29 (10), 1918-1924 (2014).

List of Patents

1. **Patent Filed: 201631003758**; Title: "System and kit for monitoring blood glucose profile based on breath analysis", ***Indian Pat. Appl.*** (2016).
2. **Patent Filed: 201631038296**; Title: "Dioxo vanadium (v) complex as carbonic anhydrase inhibitor", ***Indian Pat. Appl.*** (2016).

Chapter 1

1.1. Introduction

Laser spectroscopy is a powerful technique which is widely used in chemistry. In recent years, high-sensitive laser absorption spectroscopy has been developed to study the precise concentration of trace molecular species in gas phase [1-7]. Cavity enhanced absorption spectroscopy (CEAS), an ultra sensitive absorption spectroscopy, exploiting the principle of high-finesse optical cavities, is a recent advance for specific detection of trace gas molecules in analytical and spectroscopic applications in chemistry [8-15]. CEAS, also known as integrated cavity output spectroscopy (ICOS), exploits the principle of enhancement of effective optical path length of the sample by using high-reflectivity cavity mirrors. The advantage of CEAS technique over the other commonly used laboratory techniques is that it can detect the gas phase molecular concentrations and their isotopes in parts per billion (ppbv) to parts per trillion (pptv) levels [16-18]. The laser-based CEAS offers a new method for the quantitative estimation of molecular isotopes in gas phase with high-precision [19-20]. Now-a-days, many sophisticated analytical methods are available for measurement of traces gases and their isotopes in gaseous samples like human breath. However, application of those methods is practically limited due to some methodological problems. The main reason behind the limitation is the fact that the presence of those isotopes in exhaled breath is very small to provide sufficient sensitivity for the analysis. Although isotope-ratio mass spectroscopy (IRMS) is capable to deliver sufficient sensitivity in gaseous phase, there are some intrinsic drawbacks including high cost of the instrument and requirement of specific expertise for operation [21]. Also, these techniques are not suitable for real time measurements of isotopic species in gaseous composition. CEAS technique may provide a potential

platform for quantitative estimation of trace gases and their isotopes in ultra low concentration in human breath.

Recently, breath analysis has proven to be a potential tool to track the physiological processes in human body. The use of breath as a diagnostic tool is becoming popular in modern medical science for evaluation of health and a variety of disease states [22-30]. The compositions in exhaled breath represent the blood-borne concentrations of the molecular species produced during metabolism in body. Breath test is preferred for direct measurement of volatile organic compounds in blood samples through gas exchange blood/breath interface in the lungs because it is much simpler to measure the target molecule in gas matrix than the biological tissue samples, more hygienic than blood analysis and overall painless to devoid of any risk for patients [26-37]. Therefore, detection of trace gases in exhaled breath can provide a reliable diagnostic method for a series of disease like lung cancer, *Helicobacter pylori* infection, small intestine bacterial overgrowth (SIBO) and chronic pulmonary obstructive disease (COPD) [32-43]. Other promising breath analysis like ^{13}C-Erythromycin for CYP3A4 activity, ^{13}C-Caffeine for CYP1A2 activity, ^{13}C-Aminopyrine for hepatic drug metabolism, ^{13}C-ketoisocaproate for mitochondrial function determination and ^{13}C-Trioctanoin for assessment of fat malabsorption have been developed during the last few years [44-55].

During the past few years, $^{13}CO_2/^{12}CO_2$ breath tests have been developed for the investigations of gastrointestinal disorder, colonization and liver function determination using an array of ^{13}C-enriched substrate [44]. The ^{13}C-breath test is a good tool to understand the metabolic defects within the body. The individual under investigation is allowed to take a certain amount of labeled carbon compound with its stable isotope of carbon-13. If the ^{13}C-tracer is converted into $^{13}CO_2$ in body and the production of this

$^{13}CO_2$ is rate determining step during metabolism, then we can characterize the physiological dysfunctions in human body from the excretion kinetics of the $^{13}CO_2$ in exhaled breath [21]. In this thesis, I have investigated the feasibility of laser-based cavity enhanced absorption spectroscopy (CEAS) technique for identifying the trace molecular compositions and their isotope ratios in exhaled breath for the non-invasive detection of biomedical disease like diabetes.

Diabetes mellitus is the most common deleterious metabolic disease in the 21st century and has become one of the most pressing human health concerns all over the world. Epidemiological study highlighted that about 415 million people are currently afflicted with diabetes worldwide and the risk factors associated with the disease can affect several millions of people in the near future [56-60]. The prevalence of diabetes is rapidly becoming a global pandemic and at present, India has the second highest number of diabetic patients. Unfortunately, half of the world population is totally unaware of the onset of the disease due to asymptomatic nature of diabetes at the early stage [61-65]. In general, diabetes is considered as a metabolic disorder in individuals with high blood glucose levels. There are two primary forms of diabetes: type 1 diabetes (T1D) and type 2 diabetes (T2D). Insulin dependent T1D occurs when insulin producing beta cells of pancreas are totally destroyed and the individuals live on only exogenous insulin. T1D has strong genetic components. The occurrence of T1D is the most common at very early age. However, T1D can also develop in adult patients having the auto immune antibody of T1D. T2D is the most common of the diabetes cases [66-68]. T2D can develop both in young and adult people. The prevalence of T2D is 85% of total diabetes cases and the numbers of affected individuals are increasing in alarming rate throughout the world. Several symptoms like polyuria, polydipsia, polyphagia are common in T2D. Early detection of type 2 diabetes is very important to avoid the diabetes complications like

cardiovascular disease, kidney failure and blurred vision. In T2D, either body does not produce enough insulin than body's need or body's cells become resistant to insulin action. Insulin resistance and pancreatic β-cell dysfunction play an important role in the pathogenesis of type 2 diabetes [69-70]. Pre-diabetes (PD) is a stage where insulin resistance is just switched on and is considered as the onset of T2D. The diagnosis of type 1 diabetes is based on autoantibody testing, C-peptide assay, insulin levels and blood glucose measurements etc. These all methods need repeated blood samples analysis and therefore these are very much cumbersome. Major drawbacks of those methods are the necessity for repeated invasive blood samplings, cumbersome laboratory processes, a prolonged testing time and overall the patient inconvenience. Breath analysis exploiting laser based technique has a great advantage over the traditional approaches.

During the last few years, ^{13}C-glucose breath test (^{13}C-GBT) has been proposed to track the impaired metabolism in human body [71-73]. The ^{13}C-GBT is based on the principle that when a dose of ^{13}C-labelled glucose substrate is orally ingested and metabolized, $^{13}CO_2$ is exhaled by the respiratory system. Individuals with type 2 diabetes would exhibit the less $^{13}CO_2$ in exhaled breath samples because of impaired glucose uptake by the cells after exogenous glucose load. The aim of the present study was to demonstrate the clinical utility of the ^{13}C-glucose breath test (^{13}C-GBT) using a simple residual gas analyzer-mass spectrometry (RGA-MS) method for non-invasive estimation of diabetes mellitus.

To delay or prevent the acute onset of type 2 diabetes, an accurate and early detection of insulin resistance is important. At present, the hyperinsulinemic-euglycemic clamp (HEC) and the surrogate techniques such as the quantitative insulin sensitivity check

index (QUICKI) and homeostasis model assessment (HOMA), derived primarily from the measurements of fasting blood glucose and fasting plasma insulin levels are performed to evaluate the insulin resistance in type 2 diabetes [72-75]. More recently, another invasive surrogate index called insulin sensitivity index, $ISI_{0,120}$, that exploits both the fasting (0 min) and post-dose (120 min) plasma insulin and blood glucose concentrations, has been proposed to be a viable method of whole-body insulin sensitivity for use in clinical settings. The $ISI_{0,120}$ has also been shown to be superior to the other indices such as HOMA-IR, which is now widely used in epidemiologic studies. In the next study, we wanted to explore the clinical feasibility of the non-invasive ^{13}C-GBT to evaluate the insulin resistance in individuals with different metabolic states.

However, the major limiting factor of ^{13}C-GBT is the requirement of consuming the artificially labelled glucose. The poor availability and the high cost of the ^{13}C-glucose have limited its clinical applicability for non-invasive diagnosis of type 2 diabetes [76]. Therefore, it is necessary to find out an alternative test meal containing easily available ^{13}C-enriched substrates for the ^{13}C-GBT. As reported by Duchesne and his co-workers, $^{13}C/^{12}C$-isotopic ratio in exhaled breath is largely dependent on the composition of the diet [77]. In general, photosynthesis goes through the two isotopic effects in such a way that carbon-12 is slightly higher enriched than the carbon-13 isotope in the plants. In nature, the plants having four carbon cycles (C-4 plants) fix more ^{13}C-atoms in comparison to C-3 plants. Therefore, the C-4 plants like the maize, sugarcane, corn etc have the higher enrichments of carbon-13 isotope than the normal vegetables and foods. After ^{13}C-enriched naturally available meal administration (C-4 plants based foods)), the subject will exhibit variations in $^{13}C/^{12}C$-isotopic ratio in their exhaled breaths depending on the insulin resistance in the body. Therefore in the next study, we investigated the

feasibility of the carbon-13 isotope analysis after naturally available isotopic enriched foods administration for distinguishing type 2 diabetes.

Carbonic anhydrase (CA), a well-characterized pH-regulatory metalloenzyme found in most tissues including human erythrocytes (red blood cells), rapidly catalyzes the hydration of carbon dioxide (CO_2) to form bicarbonate (HCO_3^-) and the reversible dehydration. Some early studies [78-79] demonstrated that the oxygen-16 (^{16}O) isotope in $^{12}C^{16}O_2$ and the oxygen-18 (^{18}O) isotope of body water ($H_2{}^{18}O$) are rapidly exchanged during the respiration process in humans, catalyzed by carbonic anhydrase;

$$C^{16}O^{16}O + H_2{}^{18}O \xrightarrow{\text{carbonic anhydrase}} C^{16}O^{18}O + H_2{}^{16}O$$

This efficient exchange suggests the possibility of exploiting the oxygen-isotope fractionations of CO_2 in exhaled breath for non-invasive assessment of early-stage pre-diabetes prior to the onset of T2D. The aim of the next study was to investigate whether the total enzymatic activity of CA in erythrocytes is altered when individuals are in pre-diabetic and type 2 diabetic states. We essentially aimed to derive the precise role of CA activity in erythrocytes in response to glucose-stimulated insulin secretion that might influence the change in oxygen-isotope fractionations of CO_2 in exhaled breath.

During the last few years, the inability to envisage the acute onset and progression of type 1 diabetes has been a major clinical stumbling block and an important area of biomedical research. Type 1 diabetes, a chronic autoimmune disorder resulting from destruction of insulin-producing β-cells in the pancreatic islets of Langerhans, is an important and serious health problem afflicting millions of people worldwide [80-82]. Over the last few decades much effort has been devoted towards identifying the T1D from several measureable markers of the autoimmune state as well as the progression of

islet destruction. The most commonly used indicators are glutamic acid decarboxylase autoantibodies (GADA), islet cell cytoplasmic autoantibodies (ICA), insulin autoantibodies (IAA) and insulinoma associated-2 autoantibodies (IA-2A). Although the occurrence of T1D is suggested by the presence of one or different types of antibodies, but it is still the subject of considerable debate within the healthcare community when and which antibody should be tested for precise identification of disease state. To our knowledge, as there is no clinical characteristic or diagnostic marker available till now to readily distinguish T1D from T2D, therefore there is a major challenge worldwide to develop a new and suitable diagnostic marker that can selectively and precisely track the progression of both T1D and T2D. Herein, we would like to check the feasibility of breath $^{12}C^{18}O^{16}O$ isotope regulated by erythrocytes CA activity to distinguish T1D and T2D.

Development of a simple methodology to estimate the enzymatic activity of carbonic anhydrase, is necessary for early detection of a series of diseases including edema, glaucoma, osteoporosis, renal cancer, cervical cancer, lung cancer and neurological disorders. Although traditional methods provide useful information regarding the CA assay, the practical application of these methods are limited due to tedious and expensive process for sample collection, long time for laboratory processing and analysis via traditional mass spectroscopy technique. Further, it is difficult to estimate the real time CA activity in human body by the traditional methods. In this present work of the thesis, we planned to mimic the isotopic exchange reaction in human body within the experimental sample flasks to estimate the CA activity quantitatively from oxygen-18 isotope of breath CO_2 analysis.

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Chapter 2

Materials and Methods

2.1. Cavity-enhanced absorption spectroscopy

The cavity-enhanced absorption spectroscopy is an important tool for the measurement of trace gases along with isotopic species with ultra low concentrations. We utilized a high-resolution carbon dioxide isotope analyzer based on off-axis integrated cavity output spectroscopy (ICOS) exploiting a cavity-enhanced laser absorption technique to measure the isotopic compositions of CO_2 of breath samples (figure 1).

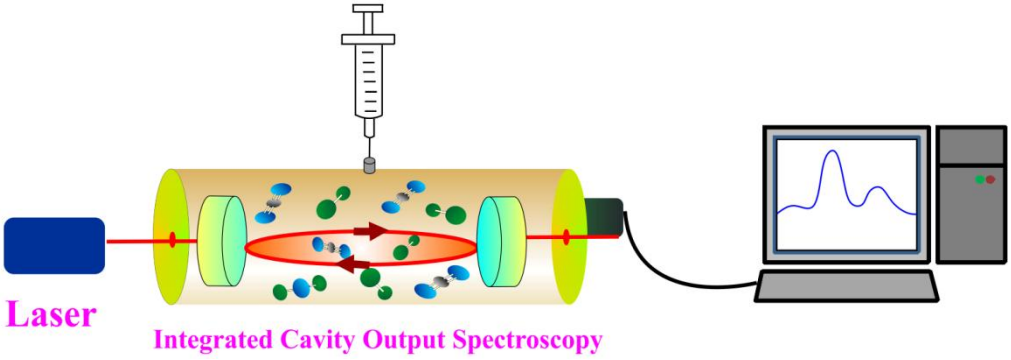

Figure 1: *Diagram of Integrated cavity output spectroscopy*

The ICOS system consists of two high reflectivity mirrors (R ~ 99.98%) placed at the two ends of a high-finesse optical cavity (59 cm long). The ICOS instrument is so designed that laser light can be directed into the off-axis of the optical cavity. The present ICOS spectrometer (CCIA 36-EP, Los Gatos research, USA) consists of a continuous wave distributed feedback diode laser operating at ~2.05 μm and a high-finesse optical cavity (~59 cm long) with two high-reflectivity mirrors (R~99.98%) at the ends of the measurement cell. This arrangement allows the laser light to move back and forth inside the cavity to reach an effective optical path-length of ~3 km and thus

enabling high sensitivities. The laser frequency was repeatedly tuned to scan over 20 GHz across the P(36), R(28) and P(16) ro-vibrational lines to record the absorption spectra of $^{12}C^{18}O^{16}O$, $^{12}C^{16}O^{16}O$ and $^{13}C^{16}O^{16}O$ at the wave numbers of 4874.178 cm^{-1}, 4874.448 cm^{-1} and 4874.086 cm^{-1} respectively, in the $(2,0^{\mathbf{0}},1)\leftarrow(0,0^{\mathbf{0}},0)$ vibrational combination band of the CO_2 molecule. The transmitted laser intensities were recorded by exploiting a photodetector after passing through a breath sample of interest. Absorption was determined from the measurement of voltage from photodetector. Beer-Lambert law was utilized to calculate the concentration after integrating the absorption spectrum. The data were acquired at a rate of 1 Hz. The temperature of the cavity was maintained at 46^0C by a resistive heater and feedback control system. The pressure of the cavity also regulated at 30 Torr by a diaphragm pump and solenoid valve in order to analyze the sample. There are few naturally occurring isotopes of carbon dioxide among which ^{12}C ^{16}O ^{16}O and ^{13}C ^{16}O ^{16}O are the most abundant isotopes of CO_2. The oxygen-18 isotope, i.e. $^{12}C^{16}O^{18}O$, is stable third major naturally occurring isotope of CO_2. The capability of the ICOS technique in comparison to conventional isotope ratio mass spectrometry (IRMS) to measure the stable isotope ratios of carbon dioxide ($^{12}C^{16}O^{16}O$, $^{13}C^{16}O^{16}O$ and $^{12}C^{16}O^{18}O$) has been well demonstrated elsewhere [1-3]. The $^{13}C^{16}O^{16}O$ and $^{12}C^{18}O^{16}O$ isotopic enrichments in the breath samples have been expressed by the conventional notation, $\delta^{13}C$ and $\delta^{18}O$ in per mil (‰), respectively relative to the standard Pee Dee Belemnite (PDB). It is described as below:

$$\delta^{13}C‰ = (R^{13}_{sample} / R^{13}_{standard} - 1) \times 1000$$

$\delta^{18}O‰ = (R^{18}_{sample} / R^{18}_{standard} - 1) \times 1000$, where R^{13}_{sample} and $R^{18}_{standard}$ are the $^{13}C/^{12}C$ and $^{18}O/^{16}O$ isotopes ratios of CO_2 in the sample, respectively. $R^{13}_{standard}$ and $R^{18}_{standard}$ are the international standard Vienna Pee Dee Belemnite values i.e. 0.0112372 and 0.0020672, respectively. We utilized three certified standard calibration gases of 5%

CO_2 in air with different $\delta^{13}C‰$ values (i.e. $\delta^{13}C‰ = -22.8‰$, $-13.22‰$ and $-7.3‰$, Cambridge Isotope Laboratory, CIL, USA) to verify the precision and accuracy of the measurements by the ICOS system for $\delta_{DOB}{}^{13}C‰$ $[(\delta^{13}C‰)_{2h\ post\text{-}dose} - (\delta^{13}C‰)_{pre\text{-}dose}]$ values in the exhaled breath samples. The typical precision of the ICOS system was 0.15‰ in the $\delta^{13}C‰$ measurements of calibration standards with accuracy in the range of 98–99%. The accuracy and precision for the $\delta^{18}O‰$ measurements of the breath samples were determined by using a standard NOAA air tank. Accuracy was determined from the measurements of seven flasks filled from the certified standard NOAA air tank, whereas precision was determined from six consecutive measurements of same breath sample. We have determined that the typical precision of $\delta_{DOB}{}^{18}O‰$ $[(\delta^{18}O‰)_{2h\ post\text{-}dose} - (\delta^{18}O‰)_{pre\text{-}dose}]$ measurements is ±0.18‰ .

2.2. Classification of diabetic stages

On the basis of the 2-h oral glucose tolerance test (OGTT), blood glucose levels and glycosylated haemoglobin (HbA1c) measurements, subjects were classified into four different groups as outlined by the American Diabetes Association [4]:

a) Non-diabetes controls (NDC) (2-h OGTT < 140 mg/dL and HbA1c <5.7%);

b) Pre-diabetes (PD) (140 mg/dL< 2-h OGTT < 199 mg/dL and 5.7% ≤ HbA1c <6.5%);

c) Type 2 diabetes (T2D) (2-h OGTT ≥ 200 mg/dL and HbA1c ≥ 6.5%);

d) Type 1 diabetes (T1D) (HbA1c ≥ 6.5% and GAD-65 antibody ≥ 10 IU/mL)

All participants gave their written informed consents before participating in the studies. The ethical permissions were received from the Institutional Ethics Committee of Post Graduate Medical Education & Research (IPGMER), Kolkata (Memo No. Inst/IEC/275)

and Institutional Ethics Committee of Vivekananda Institute of Medical Sciences (Registration No. ECR/62/Inst/WB/2013).

2.3. Breath sample analysis

Breath samples were collected in the breath sample collection bag (QUINTRON, USA, SL No.QT00892). Breath collection bags were designed in such a way that the oral-breath first passed into a dead space and then the endogenously produced deep-breath (end expiratory breath) entered into the 750 mL reservoir bags through a one-way valve. Breath samples were drawn from the reservoir bags by an air tight syringe (QUINTRON) fitted with a T-connector onto the bags.

2.4. Blood sample preparation

The collected venous blood samples were centrifuged at 4,000 rpm for 5 minutes to remove the plasma and the buffy coats. Then the erythrocytes were washed with 0.9% NaCl solution for cellular nutrition. The whole sample mixture was allowed to spin at 4,000 rpm for 20 minutes. Thereafter, the erythrocytes were lysed with ice cold distilled water to prepare hemolysate. Next, the hemolysate was again centrifuged at 10,000 rpm for 10 minutes to separate the ghost cells. The supernatant liquid was collected and used for the measurements of carbonic anhydrase activities.

2.5. Total erythrocyte carbonic anhydrase activity measurements

The total esterase activity of carbonic anhydrase was estimated by the method described by Armstrong *et al*. [5] with the modifications described by Parui *et al*. [6]. In this method, the carbonic anhydrase activity was measured spectrophotometrically from the hydrolysis rate of p-nitrophenyl acetate to p-nitrophenol. Many proteins, present in hemolysate have the ability to hydrolyse the p-nitrophenyl acetate to produce the p-

nitrophenol. Therefore, a specific inhibitor of carbonic anhydrase i.e. acetazolamide (AZM), was used to inhibit the carbonic anhydrase enzymes selectively in erythrocytes. From the difference of optical densities in presence and absence of AZM, the carbonic anhydrase activity was calculated. The assay system was comprised of 100 μL hemolysate placed in a 1 cm cuvette in presence of TRIS buffer (pH=7.4). The change of absorbance was measured by a UV-Vis spectrophotometer (Shimadzu UV-2600) over a time period of 3 min at 348 nm before and after addition of hemolysate. The net activity was calculated from the following formula:

$$\text{CA activity} = \frac{(A_1 - A_0)}{5000} \times \frac{1}{3} \times \frac{2000}{100} \times 1000 \mu\text{mol/min/mL} \quad (1)$$

where A_1 and A_0 are the absorbance at 3 minutes and 0 minute after addition of hemolysate respectively (equation 1). The molar absorptivity of p-nitrophenol is 5000 $M^{-1}cm^{-1}$. The carbonic anhydrase activity was calculated from the amount of p-nitrophenol released per minute per mL of hemolysate. The activity was normalized to 4.5×10^9 cells/mL.

2.6. Biochemical analysis:

The fasting and post-dose plasma blood glucose concentrations were estimated spectrophotometrically (2300 STAT Plus Glucose Analyzer). The insulin levels were estimated by using monoclonal antibody coated immunoassay DIAsource INS-EASIA Kits (DIAsource ImmunoAssays S. A. Rue du Bosquet, 2, B-1348 Louvain-la-Neuve, Belgium). The glycosylated hemoglobin (HbA1c %) was measured by HPLC method. The carbonic anhydrase activity was estimated by utilizing UV-Vis spectrophotometer (Shimadzu UV-2600 Spectrophotometer).

2.7. Statistical method

In these studies, Origin Pro 8.0 (Origin Lab Corporation, USA) and Analyse-it Method Evaluation software (Analyse-it Software Ltd, UK, version 2.30) were utilized for the statistical analyses. Normality test was performed to check whether the data were normally distributed or not. Based on the normality test results, one way analysis of variance (ANOVA) for normally distributed data and Kruskal-Wallis test and Mann-Whitney test for non-normal distributed data were performed to compare the data sets. Data were considered to be statistically significant when the two-sided p-value was less than 0.05. To check the diagnostic efficacy to differentiate the diseased and non-diseased states, receiver operating characteristic (ROC) curves were drawn to determine the optimal diagnostic cut-off values. The cut-off values were corresponded to the data points which showed the maximum sensitivity and specificity for the diagnosis of the disease. The p value was also calculated for demographic study.

2.8. References

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Chapter 3

^{13}C-glucose breath test exploiting a simple residual gas analyzer-mass spectrometry for non-invasive detection of pre-diabetes and type 2 diabetes

1. Introduction

Type 2 diabetes mellitus is the most common deleterious metabolic disease in the 21st century and has become one of the most pressing human health concerns all over the world with an estimated high prevalence of 101 million individuals by the year 2030 in India [1-3]. Several evidences suggest that insulin resistance and pancreatic β-cell dysfunction play an important role in the pathogenesis of type 2 diabetes [4, 5]. Insulin resistance is also associated with a cluster of risk factors (referred to as metabolic syndromes) for increased cardiovascular disease [6]. It is still the subject of debate when or how to recognize the individuals at high-risk for altered insulin action or during the preclinical phase of type 2 diabetes [7]. Hence an accurate and fast practical diagnosis of pre-diabetes prior to the onset of type 2 diabetes remains a challenge.

Recently, the ^{13}C-glucose breath test (^{13}C-GBT) has been proposed to be a non-invasive method for assessing impaired glucose tolerance in comparison to the gold standard direct invasive method called hyperinsulinemic-euglycemic clamp [5,8] and the surrogate methods, such as homeostasis model assessment (HOMA) and quantitative insulin sensitivity check index (QUICKI) [9], estimated from the measurement of blood

glucose and plasma insulin. The ^{13}C-GBT is based on the principle that when a dose of ^{13}C-labelled glucose substrate is orally ingested and metabolized, $^{13}CO_2$ is exhaled by the respiratory system. In diabetes mellitus, it is expected that less $^{13}CO_2$ will be exhaled because of impaired glucose uptake by the cells. However, the widespread clinical efficacy of the ^{13}C-GBT exhibiting the diagnostic sensitivity, specificity, test accuracy, positive and negative predictive values along with optimal diagnostic cut-off points for large-scale screening individuals with non-diabetes, pre-diabetes and type 2 diabetes mellitus have not yet been evidently explored. Furthermore, to the best of our knowledge, no studies till date have elucidated whether the endogenous CO_2 production related to the basal metabolic rates (BMR) in individuals would affect the diagnostic accuracy of the ^{13}C-GBT for pre-diabetes and type 2 diabetes. Therefore there is a pressing need to accurately evaluate the ^{13}C-GBT for routine clinical practice.

The ^{13}C-GBT to determine the $^{13}CO_2/^{12}CO_2$ isotope ratios in exhaled breath samples, usually expressed as the delta-over-baseline (DOB) values i.e. $\delta_{DOB}{}^{13}C‰$, has previously been demonstrated using the conventional high-precision gas-chromatography coupled with isotope ratio mass-spectrometer (GC-IRMS) [8]. Although traditional GC-IRMS methodologies are highly reliable, there remain some intrinsic drawbacks. In particular, the GC-IRMS system is fairly costly, not portable, require specialized expertise to operate and maintain, and are not suitable for real-time on-line measurements-facts that have hindered significantly the widespread applicability of GC-IRMS system for the breath analysis as a daily decision making tool for use at the point-of-care (POC) facilities. It is therefore of immense interest to develop a simple, robust and cost-effective alternative non-invasive diagnostic tool for the ^{13}C-GBT that can reliably and accurately analyze breath samples in real-time at the point-of-care (POC). We have recently developed and validated a simple and low-cost residual gas analyzer-mass

spectrometry (RGA-MS) method for exhaled breath analysis [10]. The aim of the present study was therefore to standardize and validate this novel analytical RGA-MS method in the ^{13}C-GBT for non-invasive assessment of pre-diabetes and type 2 diabetes mellitus in real-time and eventually to explore its true potential for routine clinical practices.

In the present study, we report for the first time, the clinical utility of the ^{13}C-GBT using a simple RGA-MS system as an alternative non-invasive method for screening individuals at risk for diabetes. The diagnostic accuracy and the feasibility of ^{13}C-GBT by RGA-MS were also validated by comparison with an established laser based cavity-enhanced absorption technique called integrated cavity output spectroscopy (ICOS). Finally, we determined statistically significant numerous diagnostic parameters of the ^{13}C-GBT including sensitivity, specificity, and the optimal cut-off point for the $\delta_{DOB}{}^{13}$C‰ values to delineate the applicability of the RGA-MS technique as a POC non-invasive diagnostic tool for large-scale diabetes screening tests.

2. Methods

2.1. Subjects

Sixty five individuals (n=18 non-diabetes controls, n=22 pre-diabetes, n=25 type 2 diabetes) within the age group of 25-79 yrs (mean age of 48.98 ±14.37 yrs) were selected for the present ^{13}C-GBT. Patients with history of any chronic respiratory disorders or on any inhalers were also excluded. Subjects were classified into three different groups: non-diabetes controls (NDC), pre-diabetes (PD) and type 2 diabetes (T2D). The detailed description of the subject characteristics is presented in table 1.

Table 1. *Characteristics of study subjects. Data are expressed as mean ± SD. The abbreviations M and F stand for male and female,* respectively. **Represents statistically significant difference among normal, pre-diabetes and type 2 diabetes*

	Non-diabetes control (NDC) (n=18)	**Pre-diabetes (PD) (n=22)**	**Diabetes (T2D) (n=25)**	**p values**
Sex(M/F)	11/7	12/10	15/10	
Age (Years)	41.72± 11.5	51.45± 15.3	50.08± 15.4	0.083
Weight (kg)	59.45± 9.5	63.51± 7.8	59.96± 11.4	0.342
BMI (kg/m^2)	23.37± 0.8	23.83± 0.7	23.36± 1.2	0.148
Fasting Blood Glucose (mg/dl)	98.61±11.8	110.64±10.8	221.52±80.3	<0.001*
Fasting Plasma Insulin (μIU/mL)	4.5±0.6	9.22±0.6	23.65±1.2	0.0015*
2-h postload plasma glucose	133.16±12.4	182.27±12.5	307.56±42.8	<0.001*
HbA1c (%)	5.28±0.2	6.11±0.2	9.63±1.6	<0.001*

2.2. Study protocol

Following an overnight fast (~10-12 hr), a baseline breath sample was collected in a breath sample collection from each subject. Then the subjects were instructed to ingest a test drink containing 75 mg U-$^{13}C_6$ labelled D-glucose (CIL-CLM-1396-CTM, Cambridge Isotope Laboratories, Inc., USA) along with 75 gm normal glucose dissolved in 150 mL water. Post-dose breath samples were collected in breath sample collection bags after 120 min of glucose load. All breath samples were repeated and analyzed immediately by both RGA-MS and ICOS systems for cross verification and validation of the experimental results as described below**.**

2.3. Residual gas analyzer-mass spectrometry (RGA-MS)

We have employed the residual gas analyzer-mass spectrometry (RGA-MS) that exploits a conventional quadrupole mass filter technology with a Faraday Cup detector to measure the masses of $^{13}C^{16}O^{16}O$ (45 amu) and $^{12}C^{16}O^{16}O$ (44 amu) isotopes in exhaled breath samples. A schematic diagram of the RGA-MS system for the analysis of exhaled breath samples is depicted in figure 1.

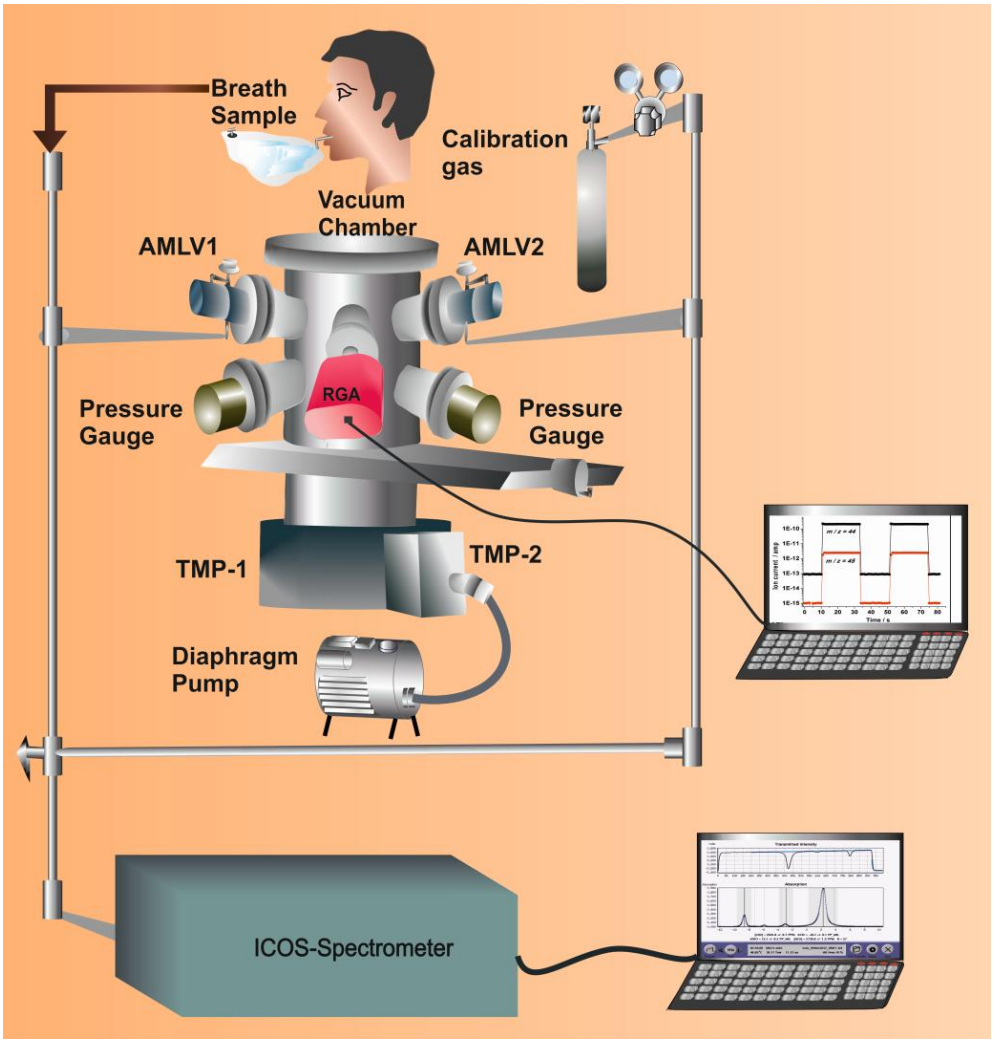

Figure 1. *The diagram shows residual gas analyser-mass spectrometry (RGA-MS) coupled with high vacuum (HV) chamber for breath analysis. Abbreviations AMLV & TMP stand for all metal leak valve and turbo molecular pump respectively.*

The RGA-MS system and its potential of measuring the carbon isotopes with a typical precision of ±0.25‰ in breath samples, have been described in detail in our previous study [10]. In brief, a RGA was coupled with a high vacuum chamber (~ 9.0×10^{-8} Torr) and the baseline vacuum was achieved by two turbo molecular pumps backed up with a diaphragm pump. The vacuum chamber was equipped with two all metal leak valves and a manually actuated gate valve to control flow of breath samples into the vacuum chamber as well as to sustain the working pressure ($2.1\text{-}2.4\times10^{-7}$ Torr) for the measurements. The ion currents for the masses 44 and 45 amu were measured with a scanning rate of 0.1s/amu by Quadera software (Prisma plus, version 4.50) in the selected multiple ion current detection (MID) mode. Typically a total number of 15-20 data points were recorded for each mass to calculate the $\delta^{13}C‰$ values.

2.4. Validation of $\delta_{DOB}{}^{13}C‰$ measurements by RGA-MS for ^{13}C-GBT

We utilized three certified standard calibration gases of 5% CO_2 in air with different $\delta^{13}C‰$ values (i.e. $\delta^{13}C‰$= -22.8‰, -13.22‰ and -7.3‰, Cambridge Isotope Laboratory, CIL, USA) to verify the precision and accuracy of the measurements by the RGA-MS system for $\delta_{DOB}{}^{13}C‰$ values in exhaled breath samples. The typical precision of the RGA-MS system was 0.25‰ in the $\delta^{13}C‰$ measurements.

Table 2. *ICOS and RGA-MS were calibrated by three certified calibration gases of 5% CO_2 in air with $\delta^{13}C$ values of -22.8‰, -13.22‰, and -7.3‰ .The uncertainties of difference of $\delta^{13}C$ values are expressed as the standard deviations of three consecutive measurements.*

Difference (‰) of $\delta^{13}C$ values	**Difference (‰) measured by ICOS method (±0.15‰)**	**Difference (‰) measured by RGA-MS (± 0.25‰)**
$\delta_I{}^{13}C$ (22.8-7.3)‰ = 15.5‰	15.48 ‰	15.56‰
$\delta_{II}{}^{13}C$ (22.8-13.22)‰ = 9.58‰	9.65 ‰	9.53‰
$\delta_{III}{}^{13}C$ (13.22-7.3)‰ = 5.92‰	5.92 ‰	5.96‰

3. Results and Discussion

In the present study, Box and Whisker plots were utilized to assess the distribution of $^{13}CO_2$ enrichments in exhaled breath samples for NDC, PD and T2D individuals. Figure 2 illustrates the Box and Whisker plots of $\delta_{DOB}{}^{13}C$‰ values measured by both RGA-MS and ICOS methods. We observed that the mean DOB value for the group with T2D (mean $\delta_{DOB}{}^{13}C$‰ = 13.46‰) measured by RGA-MS was significantly lower ($p < 0.001$) compared to the groups with NDC (mean $\delta_{DOB}{}^{13}C$‰ = 35.94‰) and PD (mean $\delta_{DOB}{}^{13}C$‰ = 24.80‰). In cases of T2D and pre-diabetes, glucose uptake would be impaired because of diminished pancreatic insulin secretion or impaired insulin action on the target tissue [11], resulting in blunted glucose oxidation and consequently the reduced rate of generation of $^{13}CO_2$ in exhaled breath samples.

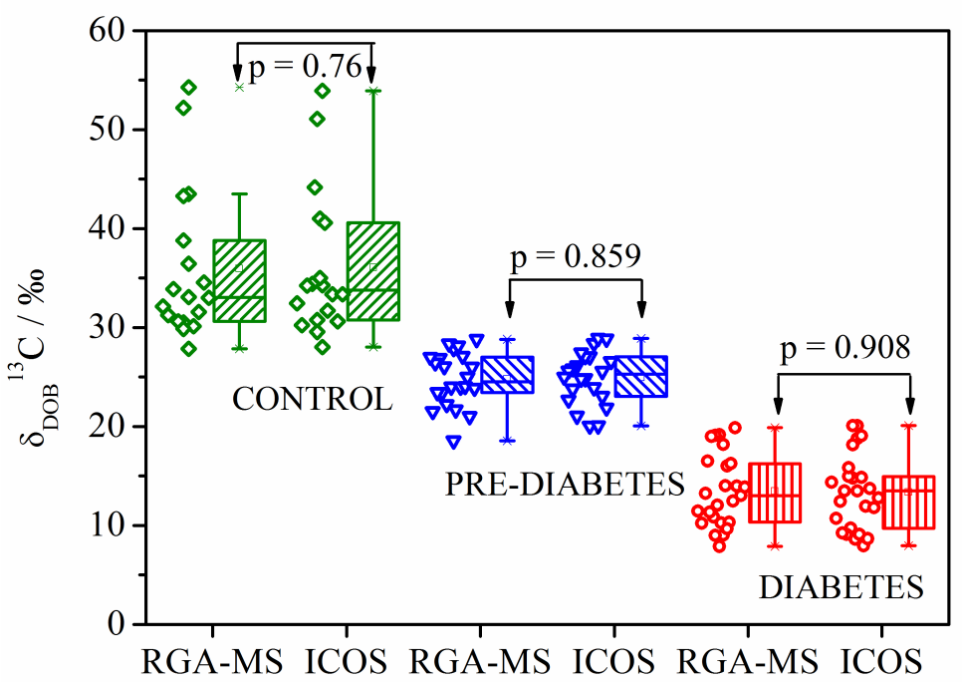

Figure 2. *Comparison of measured $\delta_{DOB}{}^{13}C$‰ values by both ICOS and RGA-MS at 120 min for non-diabetes control, pre-diabetes and diabetes using Box and Whisker plot. The scatter points represent the measured $\delta_{DOB}{}^{13}C$‰ values.*

Figure 2 also demonstrates that the present ^{13}C-GBT using the RGA-MS technique was capable of detecting marked differences in ^{13}C-enriched glucose-derived $\delta_{DOB}{}^{13}C$‰ values at 120 min in exhaled breath samples among the groups with NDC, PD and T2D, suggesting a robust approach for large-scale screening testing without the need for invasive blood sampling. We also observed that $\delta_{DOB}{}^{13}C$‰ values in breath samples analyzed by both mass spectrometry (RGA-MS) and laser based spectroscopy (ICOS) techniques were statistically almost similar (35.95‰ vs 36.04‰, 24.80‰ vs 24.94‰ and 13.46‰ vs 13.34‰ for NDC, PD and T2D, respectively) as depicted in figure 2, demonstrating the clinical potential of a simple RGA-MS technique as an alternative non-invasive analytical tool for real-time exhaled breath analysis to precisely diagnose individuals with NDC, PD and T2D. Moreover, to compare the $\delta_{DOB}{}^{13}C$‰ results of the

^{13}C-GBT analyzed by RGA-MS to those determined by ICOS system, the regression statistics was employed.

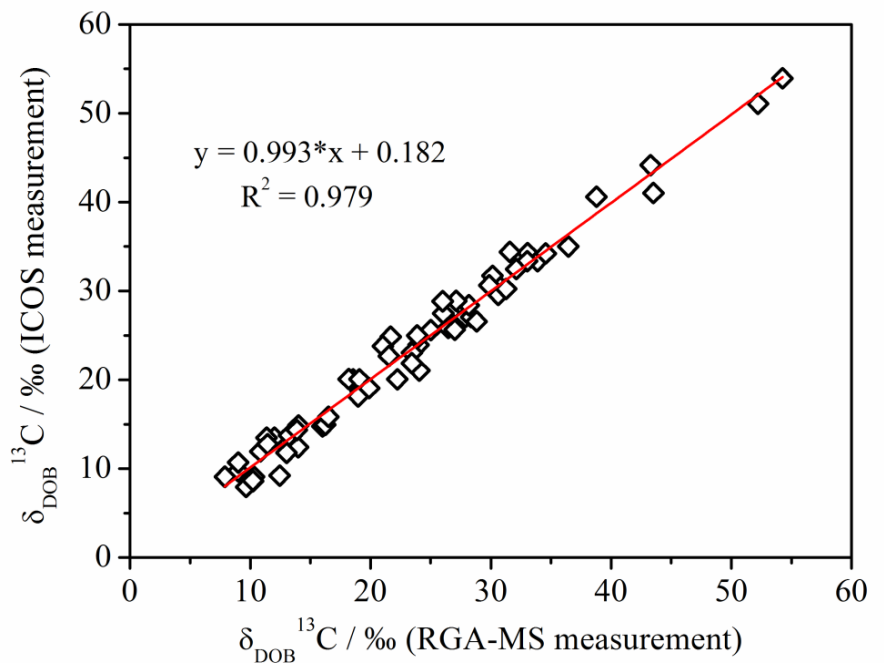

Figure 3. *Linear regression plot to compare RGA-MS measurements of $\delta_{DOB}{}^{13}C‰$ values with respect to ICOS measurements.*

Figure 3 shows a least-square regression plot of the correlation for $\delta_{DOB}{}^{13}C‰$ values of both methods. We found a close correlation as indicated by R^2=0.979, validating the RGA-MS technique to accurately detect $^{13}CO_2/^{12}CO_2$ isotope ratio measurements in exhaled breath samples for non-invasive evaluation of glucose metabolism in the diagnosis of diabetes.

To investigate the diagnostic performance of the RGA-MS technique and the accuracy of the ^{13}C-GBT to precisely distinguish between individuls with NDC and PD, and individuals with PD and T2D, we performed ROC curve analysis. A ROC curve was constructed by plotting the true positive rate (sensitivity) against the false postive rate (1-specficity) using the $\delta_{DOB}{}^{13}C‰$ values and subsequently the highest values of sensitivity and specificity were used to determine the optimal diagnostic "cut-off point"

for the present ^{13}C-GBT using the RGA-MS method. Figure 4 (a) and (b) depict ROC plots for the ^{13}C-GBT to distinguish NDC and PD cases, as well as PD and T2D cases, respectively. The optimal diagnostic cut-off point was determined to be $\delta_{DOB}{}^{13}C‰$ (cut-off) = 28.81‰ to correctly diagnose individuals with NDC and PD using the RGA-MS method and this corresponded to a sensitivity of 100% and specificity of 94.4%.

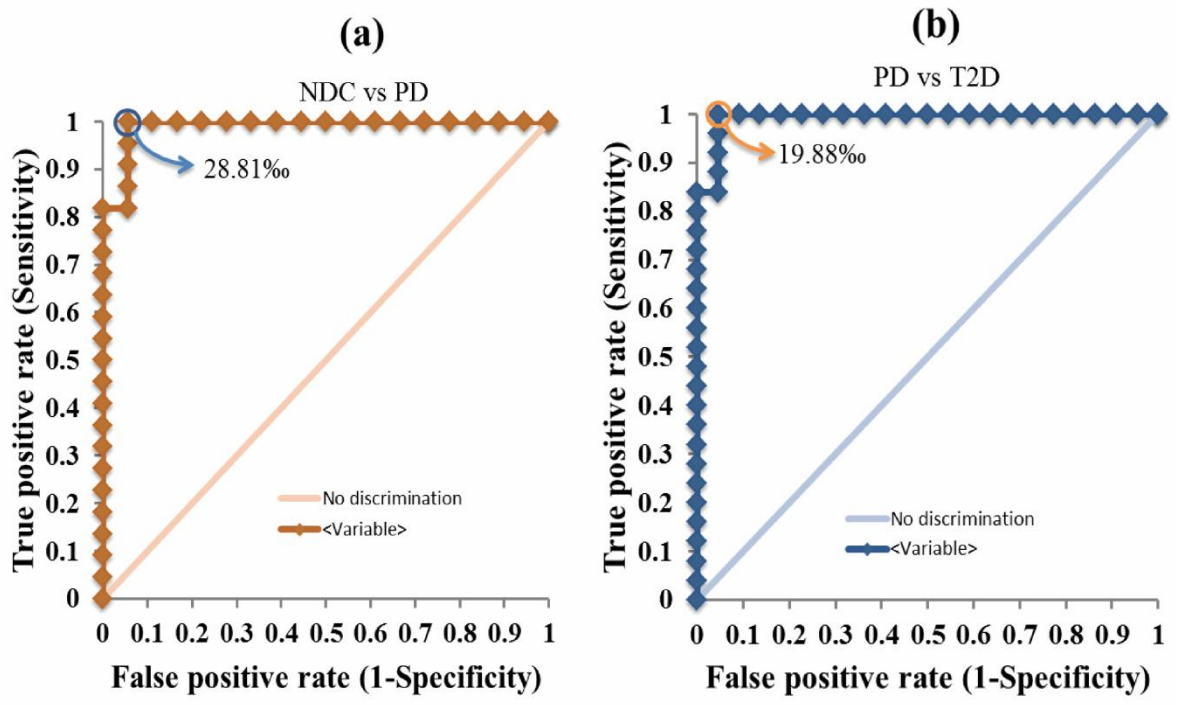

Figure 4. Receiver operating characteristic curves (ROC) to obtain the optimal cut-off points of $\delta_{DOB}{}^{13}C‰$ for screening (a) non diabetes control (NDC) and pre-diabetes (PD) & (b) pre-diabetes (PD) and type 2 diabetes (T2D).

Moreover, we also determined another diagnostic cut-off point of $\delta_{DOB}{}^{13}C‰$ (cut-off) = 19.88‰ between individuals with PD and T2D, which exhibited the sensitivity and specificity of 100% and 95.5%, respectively, establishing the clinical feasibility of the RGA-MS technique as a sufficiently robust detection method for an accurate diagnosis of PD and T2D. Consequently, in the present study individuals above $\delta_{DOB}{}^{13}C‰$ (cut-off) = 28.81‰ were considered as NDC and individuals with $\delta_{DOB}{}^{13}C‰$ (cut-off) ≤

19.88‰ were considered as T2D, whereas individuals with $28.81 \geq \delta_{DOB}{}^{13}C‰ > 19.88$ were considered to be pre-diabetes. In both cases, the area under the ROC curve which is a global measure of accuracy in a diagnostic test, was determined to be 0.99 ($p<0.001$), showing the validity of the ^{13}C-GBT. We subsequently explored the positive and negative predictive values i.e. PPV and NPV of the present ^{13}C-GBT by means of a RGA-MS method for diagnostic assessment. The PPV and NPV are usually defined by the patients' probability of getting diseased states when the actual test results are known [12]. We found a diagnostic PPV and NPV of 95.65% and 100%, respectively between subjects with NDC and PD, and 96.15% and 100%, respectively, between subjects with PD and T2D, indicating an excellent diagnostic predictions. Table 2 shows the important diagnostic parameters of the present ^{13}C-GBT that may be practical for large-scale screening purposes. Bargueno *et al*. [13] pointed out that an ideal diagnostic method should have sensitivity and specificity each close to 100; however, our RGA-MS methodology for the ^{13}C-GBT manifested a sufficiently robust method as indicated by the acquired values of sensitivity (i.e. 100%) and specificity (i.e. about 95%). Consequently, to the best of our knowledge, the results of the present ^{13}C-GBT using the RGA-MS method demonstrate for the first time, the clinical applicability of the use of a cost-effective diagnostic method as opposed to a GC-IRMS method as previously used.

Table 2. *Important diagnostic parameters of ^{13}C-GBT by RGA-MS for screening non-diabetes control (NDC), pre-diabetes (PD) and type 2 diabetes (T2D). AUC: area under the curve; PPV: positive predictive value; NPV: negative predictive value.*

Groups	Cut-off points $\delta_{DOB}{}^{13}C‰$	Sensitivity	Specificity	PPV	NPV	AUC	Accuracy
NDC vs PD	28.81‰	100%	94.4%	95.65%	100%	0.99	97.5%
PD vs T2D	19.88‰	100%	95.5%	96.15%	100%	0.99	97.87%

We finally investigated whether there was any effect of endogenous CO_2 production related to the basal metabolic rates (BMR) in individuals on the diagnostic accuracy of the present ^{13}C-GBT using the RGA-MS method. The accurate determination of the endogenous CO_2 production in the ^{13}C-breath test is still a controversial issue. The endogenous CO_2 production is usually influenced by age (adults > children), weight, height and sex (male > female) [14] and consequently the $\delta_{DOB}{}^{13}C‰$ values are also expected to vary in accordance with these factors. Therefore to assess the effect of endogenous CO_2 production in the present ^{13}C-GBT, we applied Mifflin-St Joer equations [15, 16] to calculate the BMR based on age, weight and height of either sex and the following equations have been used:

$$CO_2\ production rate(mmol/\min) = \frac{V_{CO_2} \times 1000}{24 \times 60} \quad (3.1)$$

$$V_{CO_2}(mol/day) = \frac{Energy\ Expenditure\ (EE)}{134.25} \quad (3.2)$$

$$Energy\ Expenditure\ (EE)\ (kcal/day) = Physical\ Activity\ Level\ (PAL) \times BMR \quad (3.3)$$

Figure 5 depicts Box-Whisker plots of the endogenous CO_2 production rate for three different groups of subjects. We observed that there were no statistically significant differences (p= 0.37 and 0.73) among the endogenous CO_2 production rates estimated for normal, pre-diabetes and type 2 diabetes, indicating the negligible effect of BMR-based endogenous CO_2 production on the diagnostic accuracy.

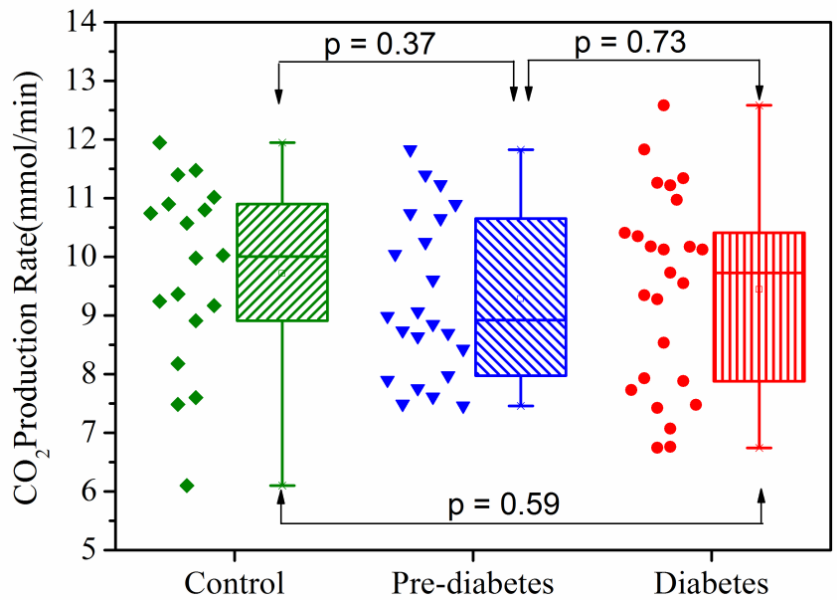

Figure 5. *Box and Whisker plot to compare the endogenous CO_2 production rates among the non-diabetes control, pre-diabetes and type 2 diabetes. The plot demonstrates statistically non-significant differences in CO_2 production rates.*

4. Conclusion

This study confirms the clinical feasibility of a novel RGA-MS method for accurate evaluation of the ^{13}C-GBT in the diagnosis of non-diabetes control, pre-diabetes and type 2 diabetes in real-time, thus making it a valid and potentially robust non-invasive diagnostic tool for routine clinical practices at the point-of-care. Our results also suggest that the ^{13}C-GBT using a simple RGA-MS method can reliably assesses the changes in glucose metabolism in real time. The present ^{13}C-breath analysis instrument is compact, easy-to-run, more portable and inexpensive compared to currently available optical spectroscopy and MS-based detection methods, suggesting a simple alternative non-invasive screening tool for the measurement of high-precision $\delta_{DOB}{}^{13}C‰$ values in exhaled breath samples, not only from diabetes or non-diabetes, but also from any other disease or metabolic disorder. However, the cost of the ^{13}C-glucose breath test may be considered to be of concern from the economic point of view, since ^{13}C-glucose is much more expensive than blood sugar analysis. This is the main limitation of the ^{13}C-glucose breath test (^{13}C-GBT) for screening diabetes. Nevertheless, utilizing the RGA-MS methodology rather than GC-IRMS, the overall cost of the ^{13}C-GBT will likely not be prohibitive in the near future in order to screen diabetes even in economically backward countries like India.

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Chapter 4

Estimation of insulin resistance from carbon-13 isotopes of exhaled breath CO_2 for non-invasive screening of pre-diabetes and type 2 diabetes

1. Introduction

Type 2 diabetes mellitus (T2D), the most common deleterious metabolic disease at present all over the world, is usually preceded by the combined effects of pancreatic β-cell dysfunction and insulin resistance [1, 2]. Several lines of evidence suggest that insulin resistance is the key risk factor in the pathogenesis of T2D. Hence an accurate and early detection of insulin resistance is important to delay or prevent the acute onset of T2D. However, there is still a real challenge on when or how to evaluate individuals who are at high-risk for developing insulin resistance or during the preclinical phase of T2D.

To quantify the insulin resistance (i.e. inverse of insulin sensitivity), the hyperinsulinemic-euglycemic clamp (HEC) study has been considered over the decades to be the "gold standard" method. But due to several complications like continuous infusion of insulin, frequent blood glucose infusion and overall withdrawals of blood samples at different time intervals, it is difficult to apply in large-scale screening purposes [3, 4]. Therefore, the different surrogate techniques such as the quantitative insulin sensitivity check index (QUICKI) and homeostasis model assessment (HOMA)

[5-7], derived primarily from the measurements of fasting blood glucose and fasting plasma insulin levels, are well accepted alternative methods for assessing insulin resistance [3]. More recently, another invasive surrogate index called insulin sensitivity index, $ISI_{0,120}$, that exploits both the fasting (0 min) and post-dose (120 min) plasma insulin and blood glucose concentrations, has been proposed to be a viable method of whole-body insulin sensitivity for use in clinical settings. The $ISI_{0,120}$ is calculated by the following formula: $1/ISI_{0,120}$ = log mean insulin/MCR, where mean of 0 and 120 min blood glucose values are utilized to calculate the metabolic clearance rate (MCR). Results obtained by utilizing $ISI_{0,120}$ were well correlated with the HEC study in comparison to the other surrogate sensitivity indexes. This is because of the fact that during oral glucose tolerance test (OGTT), the 0 and 120 min samples are more reproducible than other intermediate time points [10]. Moreover, HOMA-IR and QUICKI both estimate the hepatic glucose resistance, whereas the peripheral insulin resistance can be evaluated from the $ISI_{0,120.}$ Besides, the calculations for estimating HOMA-IR and other sensitivity indexes consider the insulin secretory capability, whereas $ISI_{0,120}$ evaluates the insulin sensitivity in body. Nevertheless, the samples required for $ISI_{0,120}$ measurement are less and it estimates better insulin sensitivity compared to the other sensitivity index formulas [8]. Therefore, the ability to non-invasively evaluate insulin sensitivity index ($ISI_{0,120}$) for diagnosis of pre-diabetes and type 2 diabetes has a substantial clinical significance. Recently, it has been proposed that ^{13}C-glucose breath test (^{13}C-GBT) may be a non-invasive approach for quantifying insulin resistance by contrast with the direct invasive HEC study [5]. The ^{13}C-GBT exploits the carbohydrate metabolism of orally administered ^{13}C-labelled glucose substrate. The substrate is metabolized and produces ^{13}C-labelled carbon dioxide ($^{13}CO_2$). This $^{13}CO_2$ is then transported to the lungs through the blood stream, and finally

it is excreted in exhaled breath. During OGTT, the glucose utilization for cellular fuel oxidation is strongly dependent on the insulin sensitivity. Postprandially, the alteration of insulin sensitivity in PD and T2D significantly decreases the cellular glucose uptake. In individuals with pre-diabetes and type 2 diabetes, when a dose containing isotopically labelled glucose [U-$^{13}C_6$] is ingested, the cellular response to this exogenous glucose is remarkably blunted. Individuals with insulin resistance or T2D will exhibit less carbon-13 isotopes of exhaled breath CO_2 because of impaired glucose uptake by the cells [6, 7]. However, the potential link between the exhaled breath $^{13}CO_2/^{12}CO_2$ isotope ratios and $ISI_{0,120}$ for the pre-diabetes and type 2 diabetes is not currently known.

The isotope tracer, $\delta_{DOB}{}^{13}C(t)‰$, quantified from the exhaled breath samples, can be followed to accurately evaluate the insulin resistance prior to the onset of T2D. Therefore, there is a pressing need to evaluate the clinical efficacy of the carbon isotopic fractionations of breath CO_2 during the glucose metabolism for large-scale screening of individuals with insulin resistance and type 2 diabetes. Moreover, unravelling the potential link between the stable isotopes of carbon in breath CO_2 and $ISI_{0,120}$ may specifically track the pathogenesis of the preclinical phase of T2D and hence may introduce a new strategy for non-invasive evaluation of insulin resistance.

To find the association between the $^{13}C/^{12}C$-isotope ratios of breath CO_2 and the $ISI_{0,120}$, we have analysed the exhaled breath carbon dioxide isotopes for the accurate and fast non-invasive assessment of insulin resistance in PD and T2D by means of a laser-based high-precision cavity-enhanced integrated cavity output spectroscopy (ICOS) system. In this study, we have demonstrated the associations between exhaled breath carbon-13 isotopes of CO_2 and invasive parameters like blood glucose, insulin and HbA1c levels. Furthermore, we also determined several diagnostic parameters of the breath isotope

analysis including sensitivity, specificity, optimal diagnostic cut-off points along with positive and negative predictive values to accurately evaluate the insulin resistance as well as the precise metabolic transition from normal to PD and then on to T2D.

2. Methods

2.1. Subjects.

A total of 116 human subjects (31 non-diabetic controls, 37 pre-diabetes and 48 type 2 diabetes) were recruited for the study. The clinical parameters are described in Table 2.

Table 2. Clinical characteristic of the study subjects. *Represents statistically significant differences among non-diabetic control (NDC), pre-diabetes (PD) and type 2 diabetes (T2D). M and F stand for male and female, respectively. *p<0.05 (statistically significant). Results are mean ± SD.

Parameters	Non-diabetic control (NDC) (n=31)	Pre-diabetes (PD) (n=38)	Type 2 Diabetes (T2D) (n=47)	p values
Sex (M/F)	18/13	26/12	31/16	
Age (Years)	35.48 ± 6.8	34.55 ± 6.6	38.63 ± 9.8	0.632
Weight (kg)	65.01 ± 6.5	65.93 ± 7.1	65.92 ± 5.6	0.789
BMI (kg/m^2)	24.43 ± 2.7	24.21 ± 2.5	23.93 ± 2	0.807
Fasting Plasma Glucose (mg/dL)	90.1 ± 12.5	107.2 ± 9.1	196 ± 17.6	< 0.001*
Fasting Plasma Insulin (μIU/mL)	7.57 ± 3.5	10.51 ± 2.6	22.94 ± 4.1	< 0.001*
2-hr Post-dose Plasma Glucose (mg/dL)	144.3 ± 11.2	182.1 ± 16.1	372.1 ± 26.5	<0.001*
HbA1c (%)	5.26 ± 0.2	6.07 ± 0.2	7.64 ± 0.5	<0.001*
HOMA-IR	1.68 ± 0.76	2.79 ± 0.8	7.92 ± 1.5	<0.001*
1/QUICKI	3.49 ± 0.08	3.04 ± 0.11	2.79 ± 0.19	<0.001*
$1/ISI_{0,120}$	0.014 ± 0.002	0.023 ± 0.002	0.046 ± 0.012	<0.001*

2.2. Study protocol

Subjects were asked for overnight fasting (~12 hours) before the study. A baseline breath sample was taken in a breath sample collection bag (QUINTRON, USA, SL No.QT00892), whereas blood sample was drawn from each participant in EDTA-vial. Thereafter, participants were instructed to ingest a test meal containing 75 mg U-$^{13}C_6$ labelled D-glucose (CIL-CLM-1396-CTM, Cambridge Isotope Laboratories, Inc., USA) along with the 75 g normal glucose dissolved in 150 mL water. From this starting point, the post-dose breath and blood samples were collected in every 30 minutes interval up to 5 h.

2.3. Insulin resistance index.

HOMA-IR, QUICKI and $ISI_{0,120}$ were calculated from the blood glucose and insulin levels in the fasting (0 min) and post-dose (120 min) conditions. Insulin resistance indexes obtained from insulin and glucose concentrations during 2h-OGTT study and these were measured by utilizing the following equations [9]

$$\text{HOMA - IR} = \frac{\text{Fasting glucose(mg/dL)} \times \text{Fasting insulin (μIU/L)}}{405} \quad (3)$$

$$1/\text{QUICKI} = \log(\text{Fasting glucose (mg/dL)} \times \log(\text{Fasting insulin (μIU/L)}) \quad (4)$$

$1/ISI_{0,120}$ = log (mean insulin(μIU/L))/MCR; where log (mean insulin (μIU/L)) =[(log (fasting insulin (μIU/L) + log (2h-post dose insulin (μIU/L))/2] and MCR (metabolic clearance rate) = $\frac{m}{\text{mean glucose}}$, m=[75000+((fasting glucose (mg/L) – 2h post dose

glucose (mg/L)) × 0.19 × weight (kg)] / 120 and mean glucose = [fasting glucose (mmol/L) + glucose at 2h (mmol/L)]/2 (5)

3. Results and Discussion:

We initially investigated the time-dependent excretion kinetics of stable carbon-13 isotopes, expressed as $\delta_{DOB}{}^{13}C(t)‰$ values, in exhaled breath samples by ICOS method to investigate the distribution of $^{13}CO_2$ isotopic abundance in breath samples associated with isotopically labelled glucose metabolism for NDC, PD and T2D and the results have been illustrated in figure 1a. We observed that the mean $\delta_{DOB}{}^{13}C(t)‰$ values for the group with T2D were significantly lower ($p< 0.01$) compared to the groups with PD and NDC between 90 min and 210 min after the glucose load. These findings suggest that carbon isotopic fractionations of breath CO_2 is capable of detecting marked differences in $\delta_{DOB}{}^{13}C(t)‰$ values in exhaled breath samples within 90 min of a 5h-OGTT among the groups with NDC, PD and T2D. However, there were no statistically significant differences ($p > 0.05$) in mean $\delta_{DOB}{}^{13}C(t)‰$ values among all the groups from 240 min in response to glucose ingestion. We also simultaneously studied how the concentration of blood glucose changes with time in response to oral glucose ingestion.

Figure 1b indicates that blood glucose levels were significantly ($p< 0.001$) higher in T2D and PD groups compared with the NDC group, as expected. For an individual with T2D or PD, the impaired glucose uptake plays an important role for blunted glucose oxidation in cells because of diminished pancreatic insulin secretion or impaired insulin action on the target tissues [11, 18].

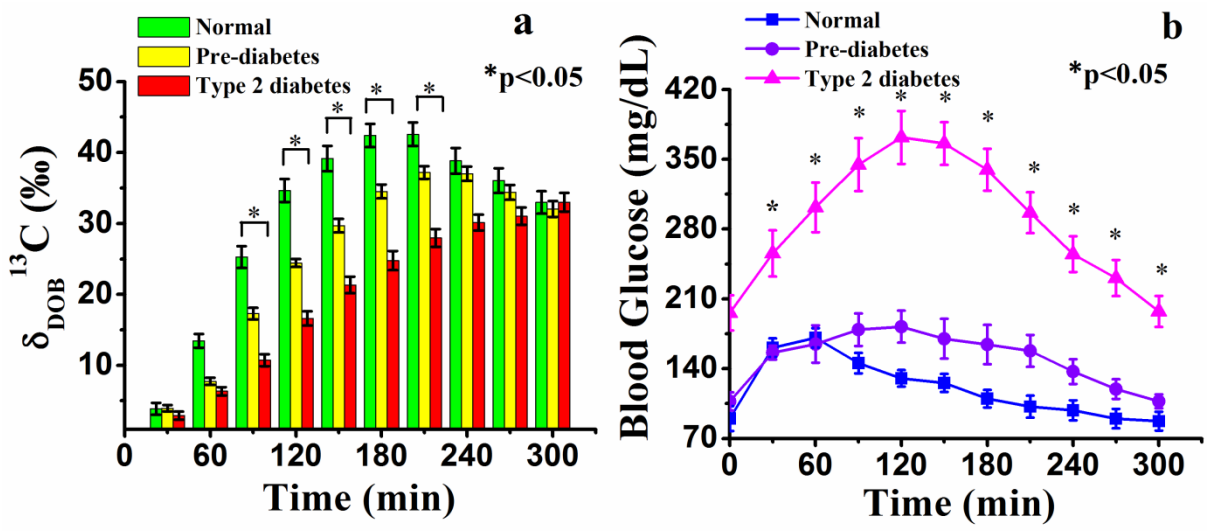

Figure 1. *Kinetics study of breath carbon-13 isotope excretions and blood glucose levels after administration of ^{13}C-glucose. a, Breath $\delta_{DOB}{}^{13}C$ (‰) enrichments.* ***b,*** *blood glucose concentrations for normal (NDC), pre-diabetes (PD) and Type 2 diabetes (T2D) individuals at every 30 min interval during 5h-OGTT. *indicates statistically significant difference ($p<0.05$). Data are expressed in terms of mean ± SEM.*

The individuals with PD and T2D accordingly produce less amount of $^{13}CO_2$ in exhaled breath samples compared with NDC. Our results support that the breath tests are more direct measure of intracellular glucose metabolism and impairments of exogenous (oral) glucose. Thus the monitoring of stable carbon isotopes may assist in non-invasive assessment of NDC, PD and T2D individuals by providing an alternative approach for large-scale screening purposes without the need for invasive repeated blood samplings.

We next explored whether there were any correlations of $\delta_{DOB}{}^{13}C‰$ values in exhaled breath at 120 min with the variables related to the insulin resistance, such as absolute changes in blood glucose levels, and plasma insulin levels at the particular time during the OGTT, in addition to glycated haemoglobin (HbA1c) measurements.

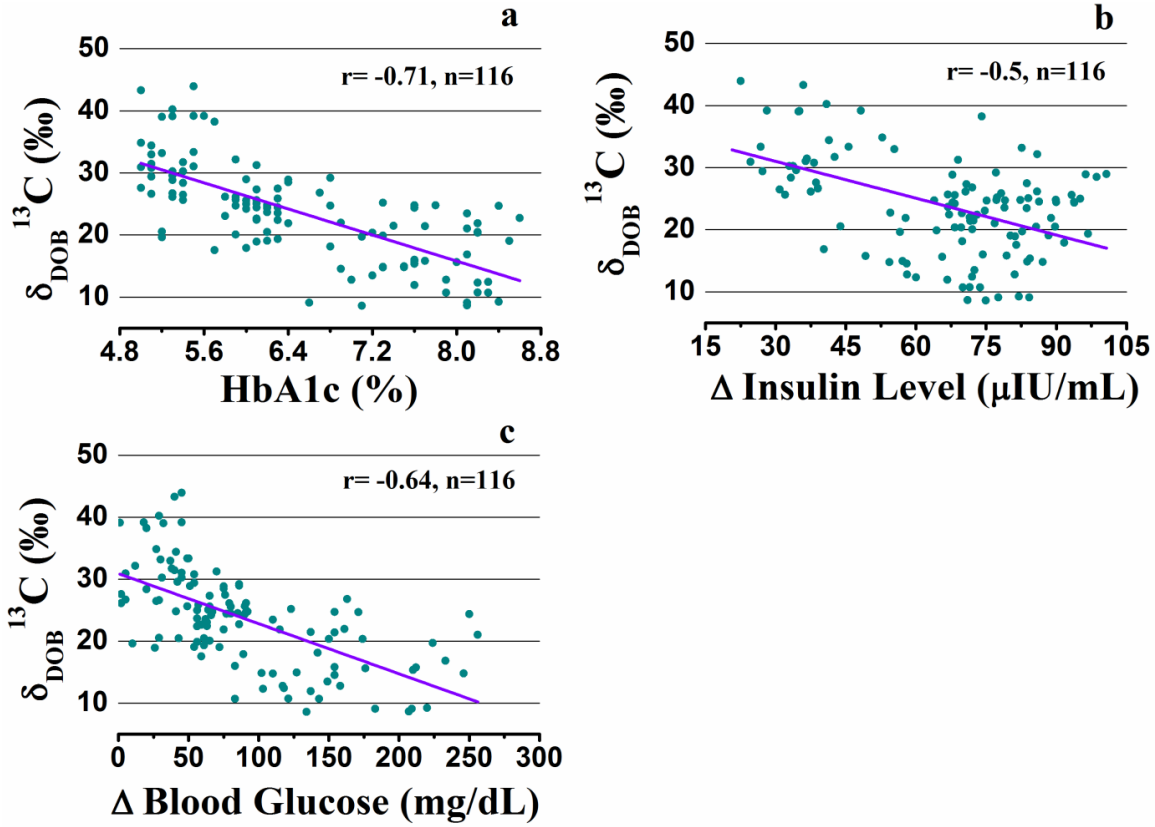

Figure 2. *Linear regression plots to show the correlations of $\delta_{DOB}{}^{13}C$ (‰) in breath with different invasive parameters. a, breath $\delta_{DOB}{}^{13}C$(‰) with glycosylated haemoglobin (HbA1c %). b, plasma insulin levels (Δ Insulin Level) from the baseline during 2h-OGTT. c, shows correlation of blood glucose concentrations (Δ Blood Glucose). The data are statistically significant different (p<0.001).*

Figure 2 depicts the inverse correlations of $\delta_{DOB}{}^{13}C$‰ values with all these measured parameters. The correlation of $\delta_{DOB}{}^{13}C$ (‰) with HbA1c (%) was strong with a correlation coefficient of r = -0.71 ($p < 0.001$) compared with the blood glucose (r = -0.64, $p < 0.001$) and plasma insulin levels (r = -0.5, $p < 0.001$). We further investigated the association of the $\delta_{DOB}{}^{13}C$‰ values at 120 min in breath samples with the three surrogate methods of HOMA-IR, QUICKI and $ISI_{0,120}$ to measure the insulin resistance in all individuals with different metabolic states. Significant inverse correlations ($p < 0.01$) between breath $\delta_{DOB}{}^{13}C$‰ values and all the measured indexes were observed, as shown in figure 3.

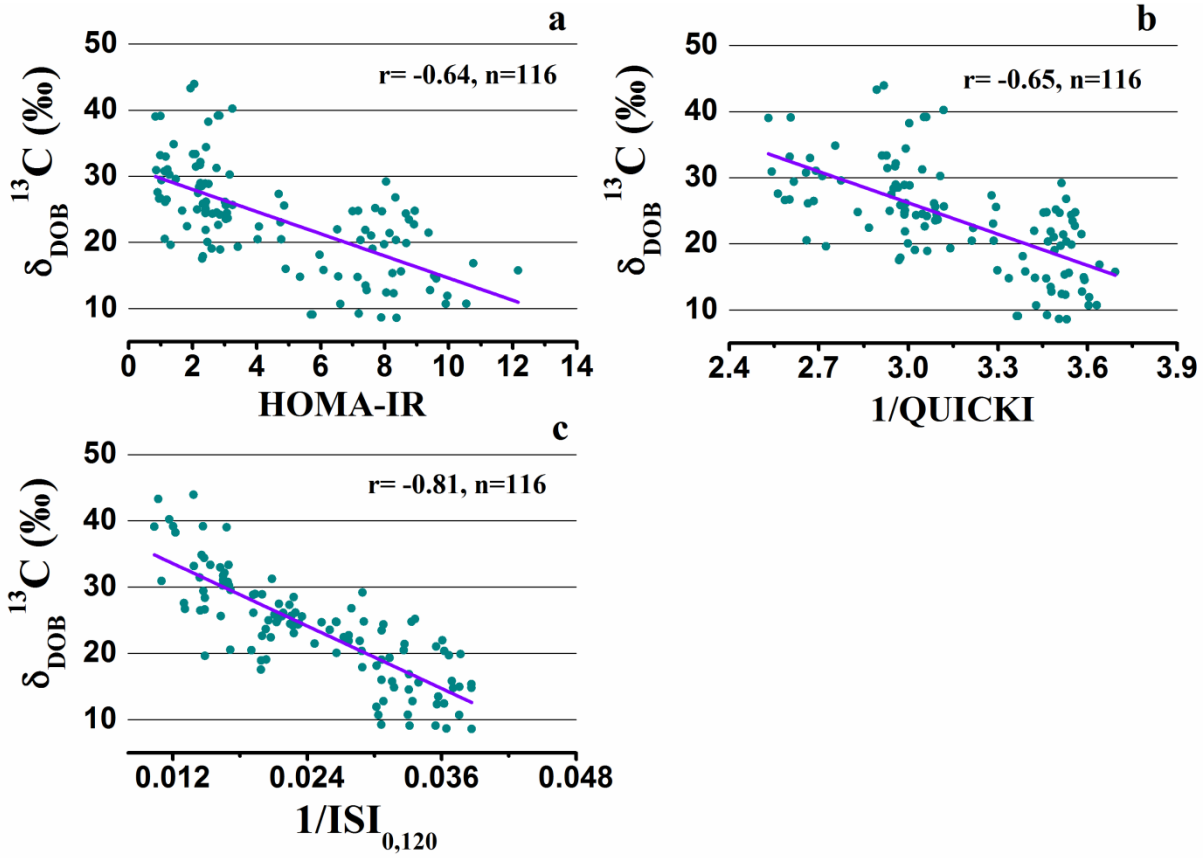

Figure 3. *Correlations of $\delta_{DOB}{}^{13}C(‰)$ in breath with currently available insulin resistance assessment methods. a, HOMA-IR. b, 1/QUICKI. c, $1/ISI_{0,120}$. The data are statistically significant different ($p<0.01$)*

However, the best correlation was observed between $\delta_{DOB}{}^{13}C‰$ values and $1/ISI_{0,120}$ ($r = -0.81$, $p < 0.001$), suggesting a significant association between the results of $\delta_{DOB}{}^{13}C‰$ and $ISI_{0,120}$ index.

Recent studies demonstrated that $ISI_{0,120}$ is superior to HOMA-IR for the assessment of insulin resistance because of essentially two intrinsic drawbacks of HOMA model [12, 13]. Firstly, HOMA index is based on the assumption that beta cell function is normal and therefore it can't be applied for patients with type 2 diabetes where beta cell is destroyed in many cases. Secondly, HOMA-IR considers that the relationship between insulin and glucose is linear, but practically it is parabolic [1]. The QUICKI has also the similar disadvantages as like HOMA-IR [12]. Furthermore, during the OGTT, the

majority of the insulin mediated glucose disposal takes place in peripheral tissues [14], suggesting that the estimation of insulin resistance in peripheral tissue is more important to correlate with $^{13}CO_2$ excretions from isotopically labelled glucose disposal for NDC, PD and T2D. It is noteworthy that HOMA-IR and QUICKI evaluate the hepatic insulin resistance essentially in fasting state, whereas $ISI_{0,120}$ estimates the peripheral insulin resistance which is primarily responsible for exogenous glucose uptake. Therefore, $ISI_{0,120}$ may be the best predictor of insulin resistance and hence the correlation of $\delta_{DOB}{}^{13}C$ ‰ with $ISI_{0,120}$ index is more practical to evaluate the efficacy of breath test to measure the insulin resistance.

Taken together, these findings suggest that monitoring stable $^{13}C/^{12}C$ isotopes of breath CO_2 in response to glucose ingestion may be an easy and non-invasive approach to evaluate the insulin resistance in individuals.

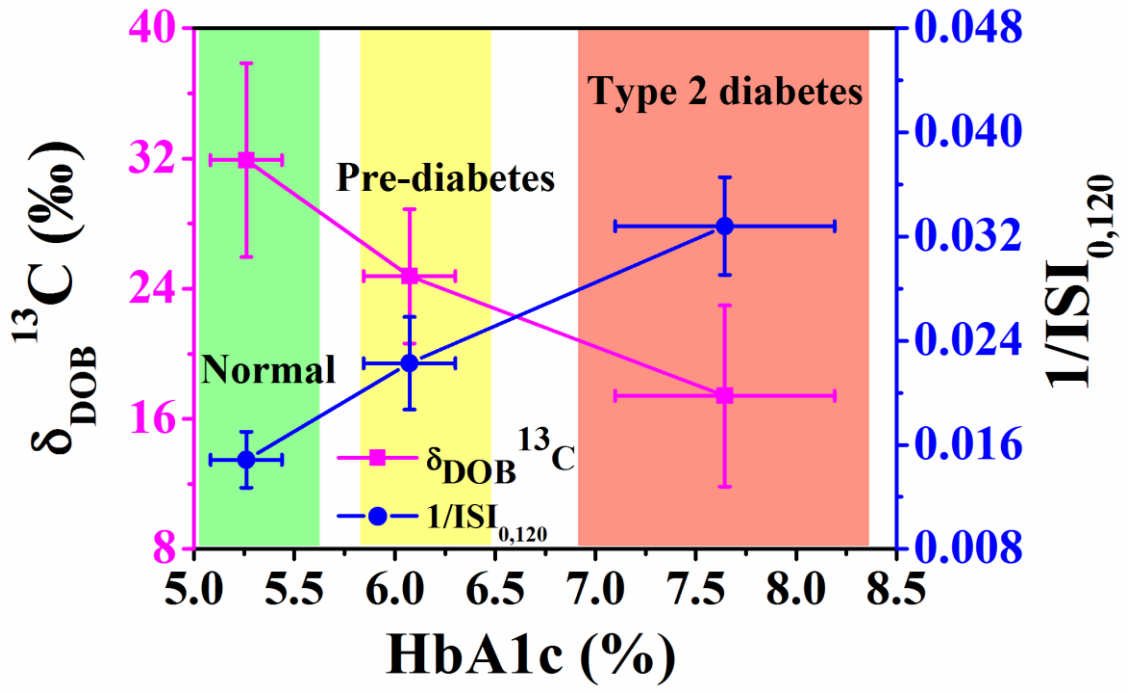

Figure 4. *Distribution of $\delta_{DOB}{}^{13}C$‰ and $1/ISI_{0,120}$ values against HbA1c (%) in normal (NDC), pre-diabetes (PD) and type 2 diabetes (T2D). Plot represents the clear transitions of $\delta_{DOB}{}^{13}C$ and $1/ISI_{0,120}$ from NDC (green) to PD (yellow) and T2D (red). Data are expressed as mean ± SD.*

In view of the present results, we posit that the measurements of $ISI_{0,120}$ index as a surrogate marker of insulin resistance and thus may distinctively track the evolution of pre-diabetes prior to the onset of T2D, as depicted in figure 4. The distributions of $\delta_{DOB}{}^{13}C‰$ and $1/ISI_{0,120}$ values illustrate that individuals can be classified into three distinct zones with NDC in green zone, PD (moderately insulin resistance) in yellow zone and T2D (sufficiently higher insulin resistance) in red zone.

Finally, to investigate the precise metabolic transition from NDC to PD and then on to T2D, we determined the optimal diagnostic cut-off points of $1/ISI_{0,120}$ and $\delta_{DOB}{}^{13}C‰$ values in exhaled breath using receiver operating characteristics curve (ROC) analysis (figures 5a and b). ROC curves were generated by plotting the true positive rate (sensitivity) against the false positive rate (1-specficity) using the values of $1/ISI_{0,120}$ and $\delta_{DOB}{}^{13}C‰$.

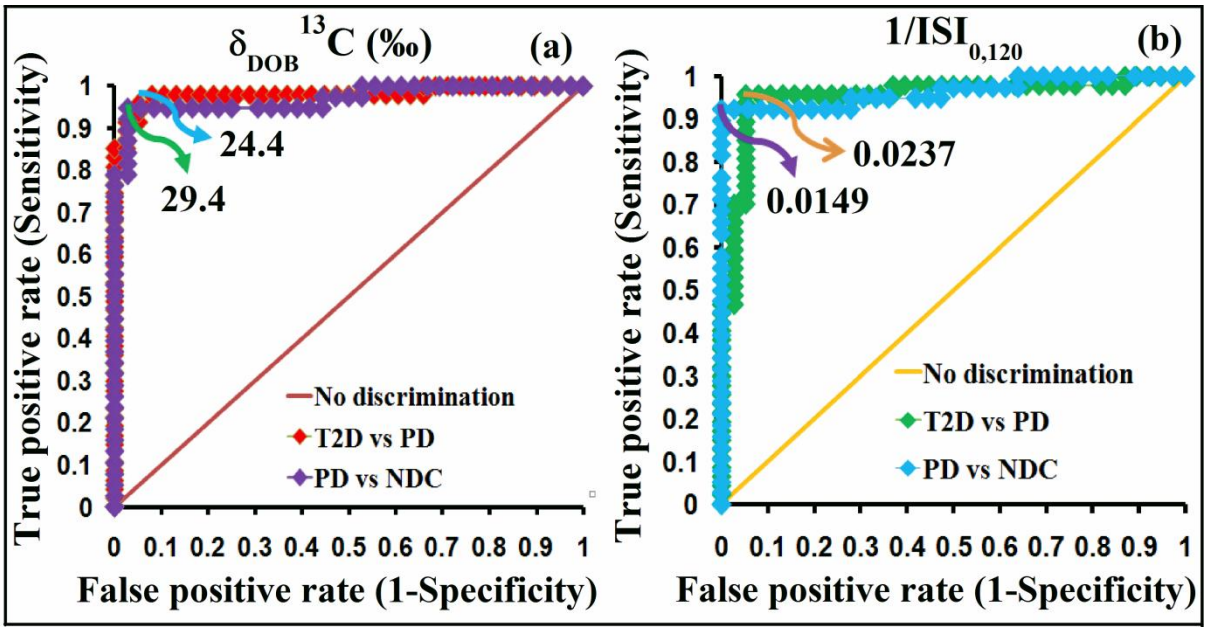

Figure 5. *Receiver operating characteristic (ROC) curves to determine optimal diagnostic cut-off points. a, $\delta_{DOB}{}^{13}C‰$ and b, $1/ISI_{0,120}$ for clinical diagnosis of normal (NDC), pre-diabetes (PD) and type 2 diabetes (T2D).*

The highest values of sensitivity and specificity were used to calculate the optimal diagnostic cut-off points. A diagnostic cut-off point of $1/ISI_{0,120}$ = 0.0237 between individuals with PD and T2D, exhibited the sensitivity and specificity of 96.1% and 95.1%, respectively, whereas $1/ISI_{0,120}$ = 0.0149 accurately diagnosed individuals with NDC and PD. We calculated the optical diagnostic cut-off values of carbon-13 isotopes of exhaled breath CO_2. We observed that individuals with $\delta_{DOB}{}^{13}C‰ < 24.4$ and $\delta_{DOB}{}^{13}C‰ > 29.4$ were considered as T2D and NDC respectively, whereas subjects with $29.4 > \delta_{DOB}{}^{13}C‰ > 24.4$ were suggested to be PD (table 1).

Table 1. *Determination of important diagnostic parameters related to cut-off values of $1/ISI_{0,120}$ and $\delta_{DOB}{}^{13}C$ (‰) by ICOS method for screening individuals with non-diabetic control (NDC), pre-diabetes (PD) and type 2 diabetes (T2D). AUC: area under the curve; PPV: positive predictive value; NPV: negative predictive value.*

Groups	**Cut-off points of $\delta_{DOB}{}^{13}C‰$**	**Sensitivity**	**Specificity**	**PPV**	**NPV**	**AUC**	**Accuracy**
NDC vs PD	29.4	94.7%	97.2%	97.3%	94.6%	0.97	95.9%
PD vs T2D	24.4	97.9%	92.1%	93.8%	97.2%	0.98	95.3%
Groups	**Cut-off points of $1/ISI_{0,120}$**	**Sensitivity**	**Specificity**	**PPV**	**NPV**	**AUC**	**Accuracy**
NDC vs PD	0.0149	92.1%	100%	100%	92.3%	0.963	95.9%
PD vs T2D	0.0237	95.7%	95.7%	95.7%	94.7%	0.954	95.3%

We have calculated these cut-off values with 95% confidence intervals. Thus the analyses of stable isotopes of the major metabolite of human breath CO_2 establish a broad clinical feasibility as a sufficiently robust non-invasive detection method for an accurate diagnosis of PD and T2D with different metabolic states of insulin resistance (figure 6).

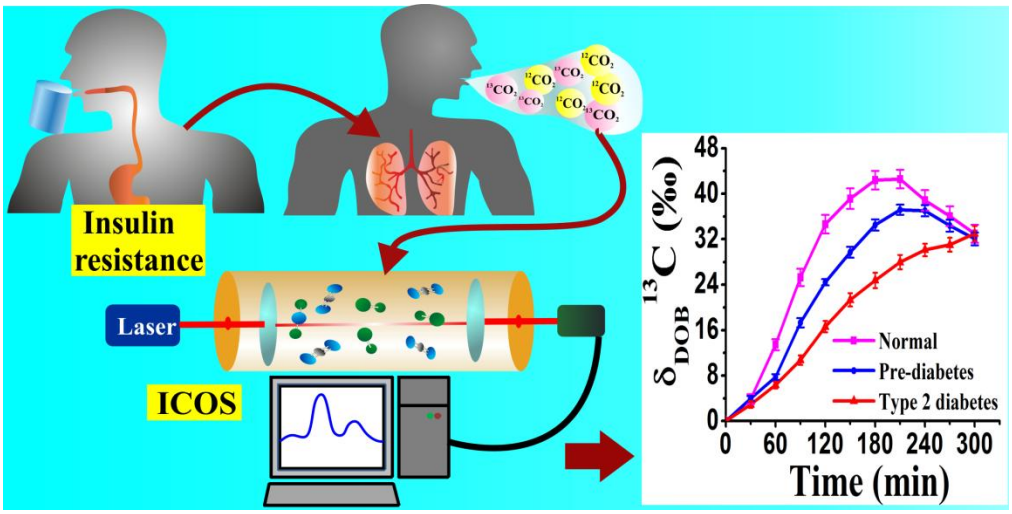

Figure 6. *Diagram shows how exhaled breath analysis by utilizing laser-based technique can be utilized to estimate the insulin resistance for diagnosis of pre-diabetes and type 2 diabetes*

We also finally explored the positive and negative predictive values (PPV and NPV) for the diagnostic assessment. These two parameters essentially indicate the probabilities of getting diseases once the actual test results of the patients are known [15]. The present method demonstrated diagnostic PPV of 98% between NDC vs PD, and 96% between PD vs T2D. It also exhibited diagnostic NPV of 94% between NDC vs PD, and 95% between PD vs T2D, indicating an excellent diagnostic accuracy for the accurate evaluation of insulin resistance in different metabolic states.

However, it is important to note that the cut-off values may depend on the food habits in the different populations of various countries and the isotopic compositions of labelled

glucose. We have determined the cut-off values based on the Indian populations utilizing the mentioned labelled glucose. Therefore, it would be interesting to estimate the cut-off values within the subject-variability and also to elucidate the probable dietary effects on breath isotope analysis in future studies.

4. Conclusion

In conclusion, our study confirms the clinical feasibility of the exhaled breath carbon dioxide isotopes analysis for estimating insulin resistance and thereafter the diagnosis of non-diabetic control, pre-diabetes and type 2 diabetes. When $ISI_{0,120}$ has been suggested as the most correlated alternative insulin resistance (1/insulin sensitivity) parameter with respect to euglycemic hyperinsulinemic clamp study, our observations demonstrated a strong correlation of $ISI_{0,120}$ index with breath carbon dioxide isotopes, suggesting a new perspective into the non-invasive evaluation of insulin resistance rather than the traditional invasive measurements. Additionally, we first estimated the cut-off values of $1/ISI_{0,120}$ for the diagnosis of pre-diabetes and type 2 diabetes, thus making it a potentially robust approach for accurate evaluation of insulin resistance. Overall, our calculated optimal cut-off values of both $1/ISI_{0,120}$ and carbon-13 isotopes of breath CO_2 also suggest that they may serve as useful methods for early detection and follow-up of individuals who are at high-risk for developing insulin resistance prior to the onset of type 2 diabetes that threaten modern society. Although, many important gaps may remain in understanding the potential link between $ISI_{0,120}$ index and breath CO_2 isotopes in the present study, our results, however, have significant implications in the isotope-specific molecular diagnosis of insulin resistant pre-diabetes and type 2 diabetes with broad clinical applications. Besides, this non-invasive approach for estimation of insulin resistance may assist in detecting the pre-diabetes stage of asymptomatic type 2 diabetic subjects in preclinical phase. This point-of-care diagnostic method may also help to

overcome the current compliance of invasive techniques for screening diabetes mellitus in future days. Therefore, our proposed breath isotopes analysis may be a new method to prevent or treat the deleterious effect of the most common metabolic syndrome in the world. Finally, as this breath analysis approach is safe, simple and non-invasive, it could be an attractive option for large-scale screening purposes in a wide variety of individuals including children, pregnant women and seniors.

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Chapter 5

^{13}C-isotopic abundances in natural nutrients: a newly formulated test meal for non-invasive diagnosis of type 2 diabetes

1. Introduction

It is well known that T2D involves a series of complications including cardiovascular disease, kidney damage (nephropathy), blurred vision (retinopathy) and nerve damage (neuropathy) during the course of the disease [1, 2]. As described in the previous chapters, T2D is diagnosed by oral glucose tolerance test (OGTT), which necessitates repeated blood samplings following oral administration of a certain amount of glucose. The glycosylated haemoglobin (HbA1c) has also been proposed to be a superior method than the blood glucose estimation [3]. But, the results of HbA1c (%) may vary from patient to patient because of different haemoglobin levels present in the subjects. Sometimes, insulin resistance (IR) is targeted to identify the diabetic condition of the patients and consequently hyperinsulinemic-euglycemic clamp study is performed to evaluate the IR of T2D patients. But, the clamp study is difficult for routine clinical applications because it not only requires several blood samples, but also the method is time-consuming and expensive [4].

Recently, ^{13}C-glucose breath test (^{13}C-GBT) has been proposed to be an alternative non-invasive method to monitor the insulin resistance in T2D [4-10]. The subject under the investigation is allowed to take an oral dose of carbon-13 (^{13}C) labelled glucose, which is

metabolized in the body to produce isotope-enriched carbon dioxide ($^{13}CO_2$) and subsequently detected in exhaled breath. Insulin assists to promote the glucose uptake into the cell of our body [11, 12]. At post-dose state, the utilization of exogenous glucose for the cellular energy production is decreased due to the insulin resistance and the decrease in exogenous glucose uptake in T2D is monitored non-invasively from the recovery rates of ^{13}C-enriched CO_2 levels in exhaled breath. However, the poor availability and the high cost of the synthetically labelled ^{13}C-enriched glucose have limited the widespread clinical applicability of the ^{13}C-GBT for non-invasive diagnosis of T2D. Therefore, there is a need to develop an alternative and effective test meal comprised of easily available naturally ^{13}C-enriched substrates for early detection of T2D by means of ^{13}C-breath tests and indeed, to our knowledge, so far no study has reported any particular test meal derived from ^{13}C-enriched natural nuitrients in the diagnosis of T2D mellitus.

However, as reported by Duchesne and his co-workers [13, 14], $^{13}C/^{12}C$-isotopic ratio in exhaled breath is largely dependent on the composition of the diet administered. In general, photosynthesis undergoes through the two isotopic effects in such a way that ^{12}C-isotope is slightly higher enriched than the ^{13}C-isotope in plants. In nature, the plants having four carbon cycles (C-4 plants) fix more ^{13}C-atoms in comparison to C-3 plants. Therefore, the C-4 plants such as maize, sugarcane and corn have the higher ^{13}C-enrichments than the normal vegetables and foods. Consequently, the subjects will exhibit quite different $^{13}C/^{12}C$-isotope ratios in their exhaled breath depending upon the consumption of ^{13}C-enriched foods [15].

The main aim of this study was therefore to explore a newly formulated test meal comprised of naturally available ^{13}C-enriched foods which can be used in the ^{13}C-breath test for precise classification of non-diabetic control (NDC) and T2D.

Moreover, some early studies showed that the oxygen-18 (^{18}O) isotope of breath CO_2 is associated with the altered metabolism in T2D, regulated by the enzymatic activity of carbonic anhydrase (CA), a ubiquitous metalloenzyme present in the human body [16-18]. Therefore, the another aim of the present study was to investigate the clinical feasibility of ^{18}O-isotopes of breath CO_2 analysis for non-invasive diagnosis of T2D after ingestion of naturally available ^{13}C-enriched test meal.

2. Methods

2.1. Subjects

Fifty one subjects including non-diabetic control (NDC, n=28) and type 2 diabetes (T2D, n=23) were recruited in the current study. Table 1 demonstrates the clinical parameters of the study subjects.

Table 1. *Clinical parameters of the subjects. *Represents statistically significant differences (p<0.05) among non-diabetic controls (NDC) and type 2 diabetes (T2D). The abbreviations M and F stand for male and female, respectively. Data are expressed as mean ± SD.*

Parameters	**Non-diabetic controls (NDC) (n=28)**	**Type 2 Diabetes (T2D) (n=23)**	**p values**
Sex (M/F)	17/11	14/9	
Age (Years)	33.2 ± 10.1	36.1 ± 8.5	>0.01
Weight (kg)	67.3 ± 9.5	63.74 ± 8.3	>0.01
BMI (kg/m^2)	23.7 ± 1.9	24.8 ± 1.7	>0.01
Fasting Plasma Glucose (mg/dL)	93.2 ± 10.3	138.6 ±12.8	< 0.001*
2-hr Post-dose Plasma Glucose (mg/dL)	119.6 ± 13.4	237.2 ± 49.2	<0.001*
HbA1c (%)	5.3 ± 0.3	8.6 ± 1.1	<0.001*

2.2. Study protocol

We first selected few control subjects (n=10) for standardization of test meal in our study. After overnight fasting (~ 10-12 hours), we administered 150 mL sugarcane juice (test meal-1) and collected the post-dose breath samples every 30 minutes interval. The breath samples were analyzed by a laser-based spectroscopy technique to measure the $^{13}C/^{12}C$ and $^{18}O/^{16}O$ isotopic ratios of breath CO_2. We enrolled the initial 10 control subjects to ingest the following three test meals: test meal-2 (150 mL sugarcane juice and 50 g normal glucose), test meal-3 (150 mL sugarcane juice, 50 g normal glucose and 40 g cornflakes) and test meal-4 (150 mL sugarcane juice, 50 g normal glucose, 40 g cornflakes, 100 g yogurt and 20 g baby-corn). The test meal-4 was considered as the final composite meal for our study. This final composite meal was administered to few more NDC (n=28) and T2D (n=23). Post-dose breath samples were collected in every 30 minutes interval up to 2.5 hours, whereas blood samples were collected after 2 hours of meal ingestion. We also performed the same breath test in presence of 75 mg U-$^{13}C_6$ labelled D-glucose (CLM- 1396-CTM, Cambridge Isotope Laboratories, Inc. USA) along with 75 g normal glucose dissolved in 150 mL water. During the duration of the administration of the breath test, the subjects were not allowed to consume any kind of food or drinks. Blood samples were utilized for the measurements of different clinical parameters such as plasma glucose concentrations and HbA1c (%).

3. Results

No significant difference was found in weight, height and BMI between the NDC and T2D. The plasma glucose (fasting and postprandial) and HbA1c (%) were significantly higher in T2D than NDC.

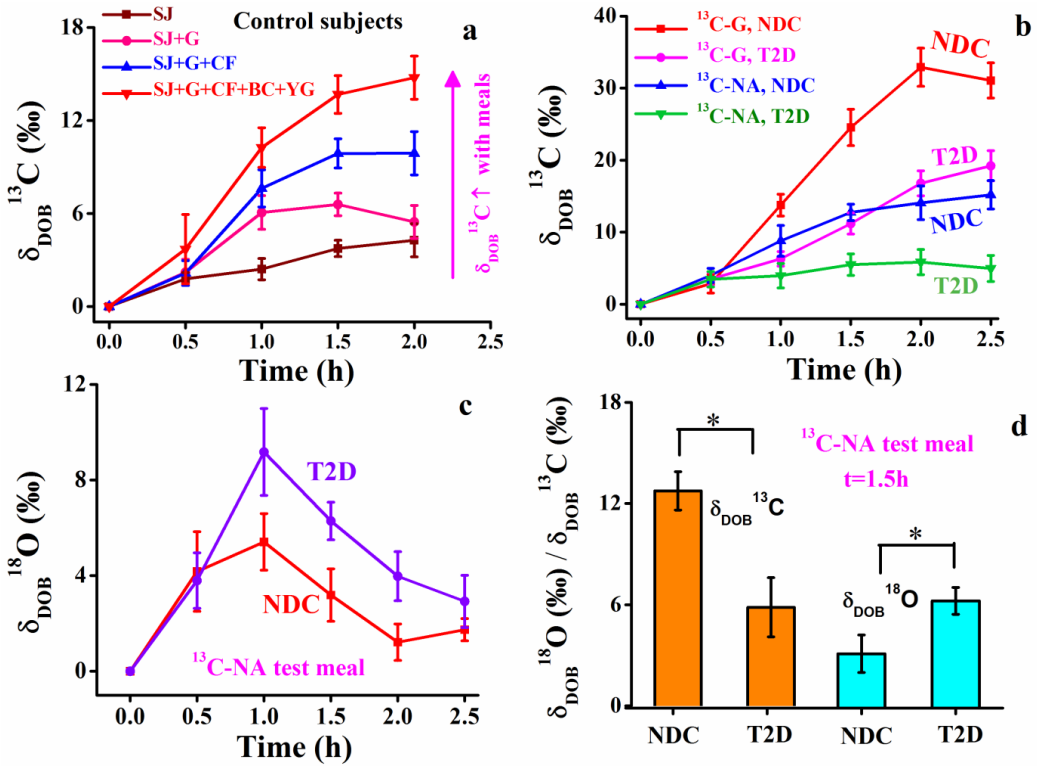

Figure 1. *Figure 1a, 1b show the excretion kinetic pattern of $\delta_{DOB}{}^{13}C$ (‰), whereas figure 1c demonstrates the $\delta_{DOB}{}^{18}O$ (‰) in NDC and T2D after the natural abundance (NA) test meal administration. Figure 1d is a comparative representation of $\delta_{DOB}{}^{13}C$ (‰) and $\delta_{DOB}{}^{18}O$ (‰) at 1.5h after test meal load. SJ: Sugarcane juice, G: Glucose, CF: Cornflakes, BC: Baby corn, YG: yogurt. Here, * represents that $p<0.05$.*

To standardize the composition of the test meal, initially a test dose containing sugarcane juice (1.0971% excess of ^{13}C-isotope) [25] was administered to few control subjects (n=10) at the fasting state. We found very little increment of mean $^{13}CO_2/^{12}CO_2$-isotope ratio (mean delta-over baseline (DOB) i.e. $\delta_{DOB}{}^{13}C=4.2\pm1.1$‰ at 2h of study) from the basal value in exhaled breath of the subjects (figure 1a). Moreover, natural glucose has been reported to be enriched with 1.0958% excess of carbon-13 isotope [19]. Therefore, we next administered a test meal containing 150 mL sugarcane juice and 50 g normal glucose to the same control subjects at the fasting state. This time, we observed a slight increment of mean $^{13}CO_2/^{12}CO_2$ isotopic ratio (mean $\delta_{DOB}{}^{13}C=6.6\pm0.7$‰ at 1.5h of

study) in breath CO_2 of those subjects (figure 1a). Furthermore, maize glucose has been reported to be associated with more carbon-13 isotopic enrichment (1.0995% excess of ^{13}C-isotope) than the normal C-3 foods [19]. To check whether the incorporation of the maize in our test meal would also increase the $^{13}CO_2$ excretion in exhaled breath, we further added cornflakes (maize) in the composite test meal. Here, we found that after addition of 40 g cornflakes in the test meal, the $\delta_{DOB}{}^{13}C‰$ value was further enhanced to reach the mean value of 9.8±0.9‰ at 1.5h after administration of the test meal (figure 1a). Moreover, few studies [19, 20] reported that yogurt and baby corn are highly enriched with carbon-13 isotopes than the normal foods. Therefore, we added these two foods in the test meal and made a composite meal consisting of 150 mL sugarcane juice, 50 g normal glucose, 40 g cornflakes, 20 g baby corn and 100 mL yogurt. After administration of the modified meal, we found a marked enhancement in the production of $^{13}CO_2/^{12}CO_2$-isotopic ratio (mean $\delta_{DOB}{}^{13}C$=14.7±1.3‰) after 2h of the study (figure 1a). We considered it as the final naturally ^{13}C-enriched composite test meal for our study in the diagnosis of T2D.

To address the feasibility of our new standardized test meal for detecting diabetes mellitus, we administered the final composite meal to several non-diabetic controls (NDC, n=28) and type 2 diabetes (T2D, n=23) patients after overnight fasting. We observed that the enrichment of $\delta_{DOB}{}^{13}C$ (‰) from the basal value was significantly higher in NDC than T2D after 1h of meal ingestion. To check the efficiency of our proposed method, we performed the whole study separately in presence of the artificially prepared ^{13}C-enriched glucose and naturally produced ^{13}C-enriched composite meal (figure 1b). Our study reveals that although the separation of $\delta_{DOB}{}^{13}C$ (‰) values between the two groups (NDC and T2D) for the artificially ^{13}C-glucose load was larger than the ulitization of naturally ^{13}C-enrihced foods, we can still selectively distinguish

the T2D from NDC after 1h of the excretion kinetics when the naturally available ^{13}C-enriched composite meal is administered.

We next investigated the isotopic fractionations of ^{18}O-isotopes of breath CO_2 in response to the ingestion of our newly formulated naturally ^{13}C-enriched test meal. In human body, the ^{16}O-isotopes of $^{12}C^{16}O_2$ and ^{18}O-isotopes of body water ($H_2{}^{18}O$) are rapidly exchanged during the metabolism to produce $^{12}C^{18}O^{16}O$ in exhaled breath. We therefore measured the $^{18}O/^{16}O$ isotope ratios of breath CO_2 (i.e.$\delta_{DOB}{}^{18}O‰$) in T2D and NDC (figure 1c). Our results showed that the enrichment of ^{18}O-isotopes of breath CO_2 was significantly higher in case of T2D compared to the NDC and this difference can clearly distinguish the two types of subjects in 1h of the study period.

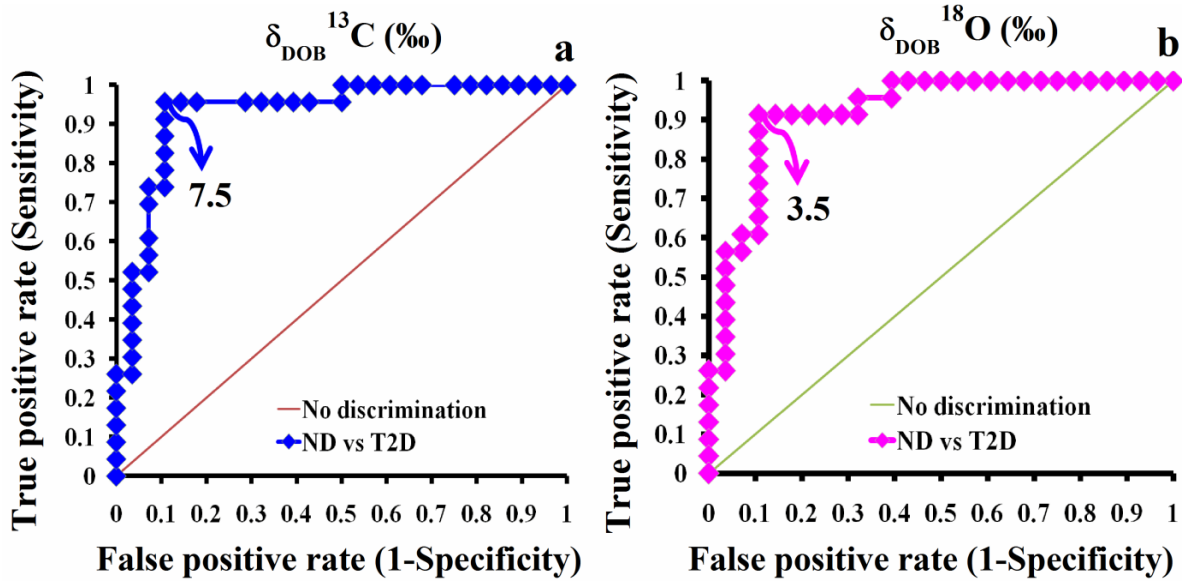

Figure 2. *The optimal diagnostic cut-off values of $\delta_{DOB}{}^{13}C‰$ (figure 2a) and $\delta_{DOB}{}^{18}O‰$ (figure 2b) were estimated by utilizing the receiver operating characteristic curves (ROC) analysis for screening non-diabetic control (NDC) and type 2 diabetes (T2D) after the natural abundance test meal load.*

We finally determined the optimal diagnostic cut-off values of both ^{13}C and ^{18}O-isotopes of breath CO_2 for precise classification of T2D from the NDC. We utilized the receiver

operating characteristic (ROC) curves to determine the cut-off levels. We found that the individuals with $\delta_{DOB}{}^{13}C‰ < 7.5$ and $\delta_{DOB}{}^{18}O‰ > 3.5$ were considered as T2D (figure 2) with diagnostic sensitivity about 91% and specificity about 89% (Table 2), suggesting the broad clinical efficacy of our methodology exploiting a newly formulated test meal for non-invasive diagnosis of T2D.

Table 2. *Optimal diagnostic parameters corresponding to cut-off values of $\delta_{DOB}{}^{13}C$ (‰) and $\delta_{DOB}{}^{18}O$ (‰) by ICOS method for screening non-diabetic control (NDC) and type 2 diabetes (T2D) individuals after naturally available ^{13}C-enriched test meal administration. AUC: area under the curve; PPV: positive predictive value; NPV: negative predictive value.*

Groups	**Cut-off points of $\delta_{DOB}{}^{13}C(‰)$**	**Sensitivity**	**Specificity**	**PPV**	**NPV**	**AUC**	**Accuracy**
NDC vs T2D	7.5	95.7%	95.8%	88.0%	96.0%	0.93	92.1%
Groups	**Cut-off points of $\delta_{DOB}{}^{18}O(‰)$**	**Sensitivity**	**Specificity**	**PPV**	**NPV**	**AUC**	**Accuracy**
NDC vs T2D	3.5	91.3%	89.3%	88.0%	93.0%	0.92	90.1%

4. Discussion

During the last few years, ^{13}C-breath test has been considered for non-invasive assessment of insulin resistance. To enable the isotopic breath test for routine clinical applications, considerable efforts have been devoted to formulate an alternative test meal rather than synthetically manufactured ^{13}C-labelled glucose. Here, our study demonstrates that a newly formulated test meal containing naturally available ^{13}C-enriched foods can detect the differences of $^{13}CO_2$ abundances in exhaled breath of NDC and T2D which has never been explored before. When an oral dose of naturally ^{13}C-enriched food is administered, the isotope enriched glucose entry is restricted into the cell due to insulin resistance in T2D and consequently the production of $^{13}CO_2$ is impaired in exhaled breath. In our study, the data shows the slower rate of excretion of $^{13}CO_2$ in individuals with T2D than NDC, supporting our assumption that the insulin resistant T2D patients utilize the isotopic substituent at a slower rate as compared to the NDC individuals after the administration of naturally available ^{13}C-enriched composite meal.

However, the study needs to be carried out in a large cohort of diabetic patients worldwide to confirm the accuracy of the ^{13}C-enriched composite meal as a cost viable substitute for ^{13}C-labeled glucose to identify patients with insulin resistance (T2D). In our study, we prepared the test meal from the foodstuffs obtained from the south-east continent of Asia (India). Therefore, it would be interesting to perform further study with the variations of foodstuffs produced from the different parts of the world in future days. The meal will also need to be standardized and packaged to make it commercially available in all parts of the world. The incorporation of ^{13}C-enriched C-4 crops like corn or maize (Zea mays), sugarcane (Saccharum officinarum), sorghum (Sorghum bicolor),

and millets, in the meal would enhance the accuracy of the breath test to identify patients with insulin resistance. However, the need of an expensive mass or laser spectrometer to analyze breath samples not available at hospitals and clinics limits the clinical applicability of the breath test and negates the benefits of substituting expensive $^{13}C_6$-glucose with the naturally ^{13}C-enriched composite meal. Therefore further research is necessary to develop a simple cost-effective system for breath analysis to validate this method as a potential diagnostic tool for practical clinical application.

5. Conclusion

In conclusion, we have formulated a new test meal comprised of naturally available ^{13}C-enriched foods which can be used for accurate evaluation of T2D without ingestion of commercially available ^{13}C-labelled glucose. By utilizing the newly formulated test meal, we were able to clearly distinguish T2D patients from NDC through the excretion kinetics of breath $^{13}CO_2$, thus opening a new perspective into the non-invasive diagnosis of T2D. Moreover, the determination of new cut-off values of ^{13}C and ^{18}O-isotopes of breath CO_2 may assist to track the NDC and T2D for practical clinical utilization. Finally, our new protocol for the ^{13}C-breath test exploiting the naturally ^{13}C-enriched foods is simple, thus suggesting the widespread clinical applicability for non-invasive diagnosis of the type 2 diabetes in a more robust and better way.

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Chapter 6

Erythrocyte carbonic anhydrase activity linking to ^{18}O-isotopes of breath CO_2 to pre-diabetes and type 2 diabetes

1. Introduction

Carbonic anhydrase (CA), a well-characterized pH-regulatory metalloenzyme found in most tissues including human erythrocytes (red blood cells), rapidly catalyzes the hydration of carbon dioxide (CO_2) to form bicarbonate (HCO_3^-) and the reversible dehydration [1, 2]. It also plays an important role in the transport of CO_2 and ions (such as H^+, Na^+ and Cl^-) along with pH-regulation in a variety of physiological processes ranging from respiration to intermediary metabolism at the cellular level [3-5]. Some early evidences suggest that the changes in CA activities in erythrocytes may be an initial step of altered metabolism in diabetes mellitus [2, 6]. However, the precise role of CA activity, especially in the pathogenesis of type 2 diabetes mellitus (T2D), the most common deleterious metabolic disease at present worldwide [7], is not currently known. Furthermore, the potential links between CA activity and T2D have not yet been fully elucidated.

Some early studies [8-10], demonstrated that the oxygen-16 (^{16}O) isotope in $^{12}C^{16}O_2$ and the oxygen-18 (^{18}O) isotope of body water ($H_2{}^{18}O$) are rapidly exchanged during the respiration process in humans, catalyzed by carbonic anhydrase. This efficient exchange suggests the possibility of exploiting the oxygen-isotope fractionations of CO_2 in exhaled breath for non-invasive assessment of early-stage pre-diabetes prior to the onset of T2D.

It also suggests a tantalizing hypothesis that monitoring stable $^{18}O/^{16}O$ isotope ratios of breath CO_2 in response to CA activity may track the pathogenesis of the preclinical phase of T2D and hence may introduce a new strategy for treating T2D. Moreover, unravelling the exact metabolic pathways involved in causing the isotopic changes of $^{12}C^{16}O^{18}O/^{12}C^{16}O^{16}O$ in breath influenced by the enzymatic activity of CA in erythrocytes remains a challenge, whenever an individual is at high-risk for altered insulin action or for the acute onset of T2D.

In this study, we investigated whether the total enzymatic activity of CA in erythrocytes is altered when individuals are in pre-diabetic and T2D states, and subsequently we assessed the precise role of CA activity in erythrocytes in response to glucose-stimulated insulin secretion that might influence the change in oxygen-isotope fractionations of CO_2 in exhaled breath. We further explored the potential metabolic pathways underlying CA alteration in the pathogenesis of T2D and the mechanisms linking breath oxygen-isotopes to pre-diabetes (PD) and T2D.

2. Methods

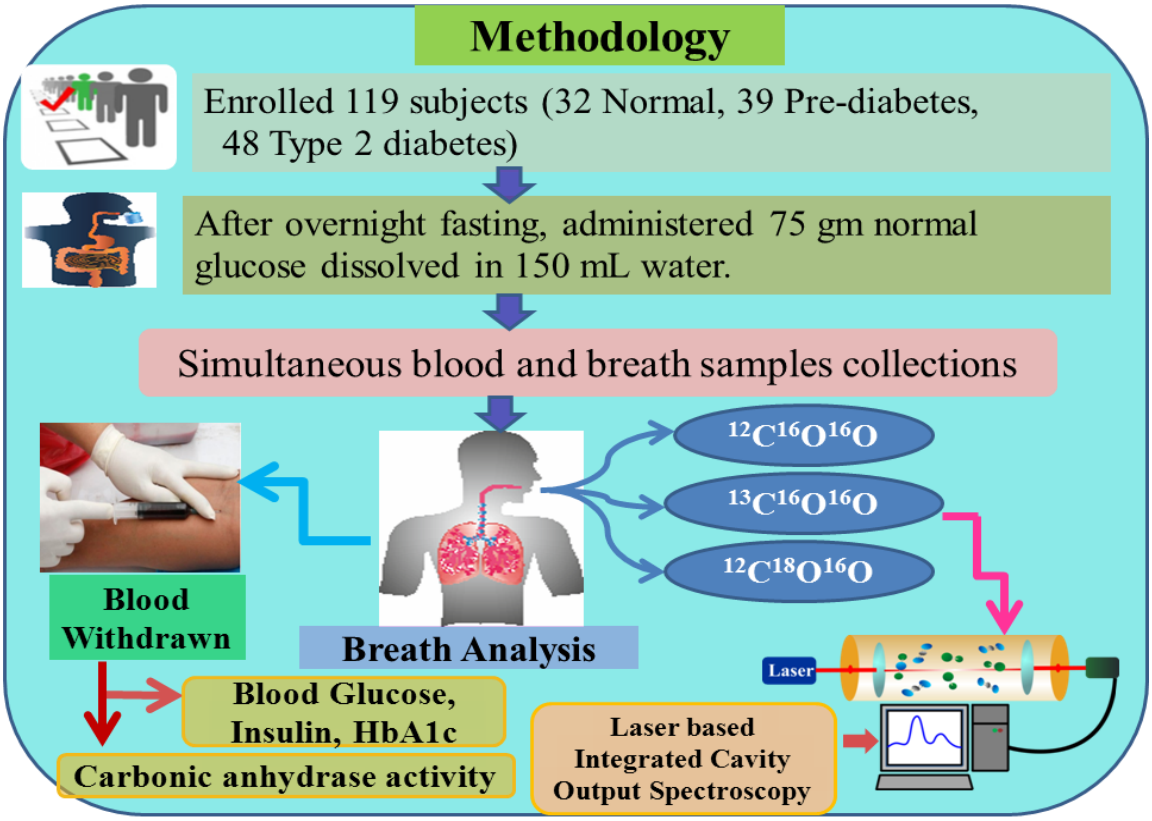
2.1. Subjects

119 participants (n=32 non-diabetic control, n=39 pre-diabetes, n=48 type 2 diabetes) were selected for the study. Subjects were classified into three groups of type 2 diabetes (T2D), pre-diabetes (PD) and non-diabetic controls (NDC).

2.2. Study protocol

After overnight fasting (~12 hours), subjects were placed on chairs, and a baseline breath sample from each subject was taken in a breath sample collection bag (QUINTRON, USA, SL No.QT00892). A baseline blood sample (10 mL) was also

collected simultaneously. A test meal containing 75-gm normal glucose dissolved in 150-mL water was orally administered and consumed within 1 minute.



From this starting point, breath samples were collected in every 30-min interval, whereas blood samples were drawn after 2-hr of glucose load. The blood samples were utilized for measurement of glycated hemoglobin i.e. HbA1c (%), carbonic anhydrase activities and insulin levels. Breath samples were immediately analyzed to measure $\delta^{18}O$ of breath CO_2.

3. Results and Discussion

To investigate the potential role of CA in the pathogenesis of T2D, we first tested whether the total basal CA activity altered in the pre-diabetic state and T2D after overnight fasting. We measured the total erythrocytes CA activity, which includes primarily three isozymes of CA (i.e. CAI, CAII and CAIII), spectro-photometrically in patients with non-diabetic controls (NDC) (n=32), pre-diabetes (PD) (n=39) and T2D

(n=48). In this investigation (figure 1a), individuals with T2D exhibited significantly lower basal CA activity as compared with PD and NDC, whereas no significant difference in basal CA activities was evident between subjects with PD and NDC. Several lines of evidence suggest that glycosylation of CA decreases its enzymatic and immunological activities and the glycosylation of CA is also enhanced during the peripheral circulation of erythrocytes [2, 6]. Therefore, the decrease in basal CA activity for T2D individuals is possibly attributed to the increased level of glycosylation of the enzyme, caused by the exposure of erythrocytes to a higher concentration of plasma glucose compared to the PD and NDC. Taken together, these findings indicate that the glycosylation plays a vital role in reduction of CA activity in individuals with T2D, whereas the glycosylation of CA does not play a significant role in either causing or facilitating the considerable differences in basal CA activities when an individual is in a normal or pre-diabetic state owing to their slight difference in blood glucose levels [11].

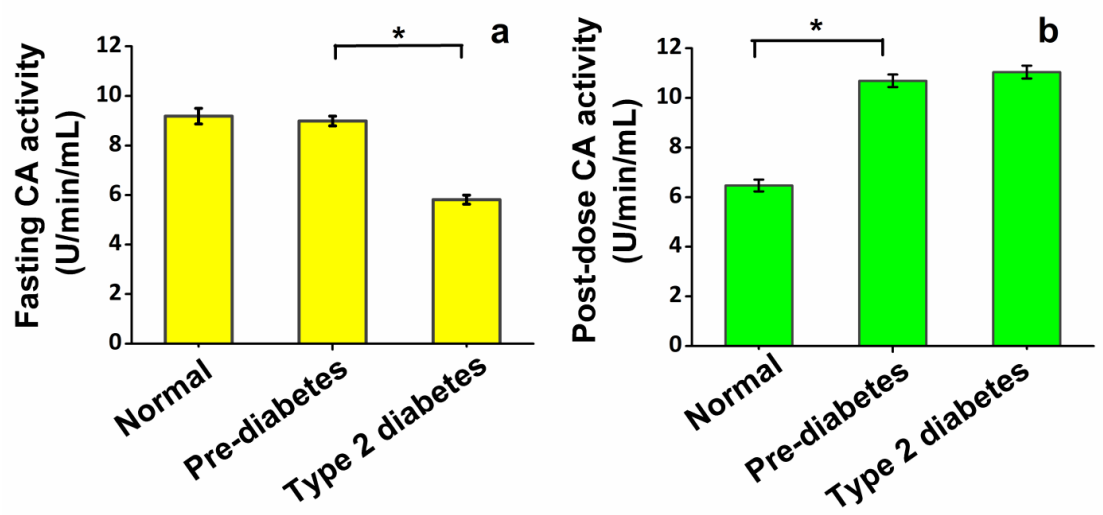


Figure 1. *Erythrocyte CA activities associated with plasma glucose, insulin levels during the oral glucose tolerance test in non-diabetic control (NDC), pre-diabetes (PD) and type 2 diabetes (T2D). a, Fasting CA activities [p<0.01 for T2D (5.81 ± 0.18) versus PD (8.99 ± 0.19) and NDC (9.18 ± 0.31)]. b, Post-dose CA activities [p=0.34 for T2D (11.03 ± 0.26) as compared with PD (10.68 ± 0.25), whereas p<0.01 among T2D, PD and NDC (6.47 ± 0.24)]. *p<0.01. Data are means ± SEM.*

We next explored the effect of CA activity in erythrocytes after administration of glucose, as the potential role of CA in response to glucose ingestion for NDC, PD and T2D remains unknown. To investigate this, we performed the 2h-oral glucose tolerance test (2h-OGTT). The post-dose CA activity (figure 1b), in both T2D and PD individuals, was markedly enhanced as compared with that of basal CA activity, although no significant difference of CA activities between these two groups was evident. In contrast, the NDC individuals exhibited a considerable reduction in post-dose CA activity when compared with basal CA activity. Erythrocyte receptor bound insulin has been proposed to alter the carbonic anhydrase activity [12], suggesting that post-dose glucose stimulated plasma insulin levels may play an important role in changing the post-dose CA activities in erythrocytes in NDC, PD and T2D.

It is noteworthy that only pre-dose or post-dose erythrocyte CA activities do not allow one to distinguish the exact pathological state of the diabetes mellitus. We therefore, examined the absolute changes in CA activities (i.e. ΔCA) between the pre-dose (basal) and post-dose of glucose ingestion among all individuals (figure 2c). We found marked differences in ΔCA activities between subjects with NDC and PD, and subjects with PD and T2D, suggesting a potential link between changes in CA activities and altered metabolism responsible in individuals with PD and T2D. We have also established the previous hypothesis that the changes in CA activities in erythrocytes might signify the onset of altered metabolism in diabetes mellitus [2]. In view of this result, our findings suggest ΔCA activity in erythrocytes may contribute to the pathogenesis of T2D and might be considered as a potential biomarker for the detection of T2D.

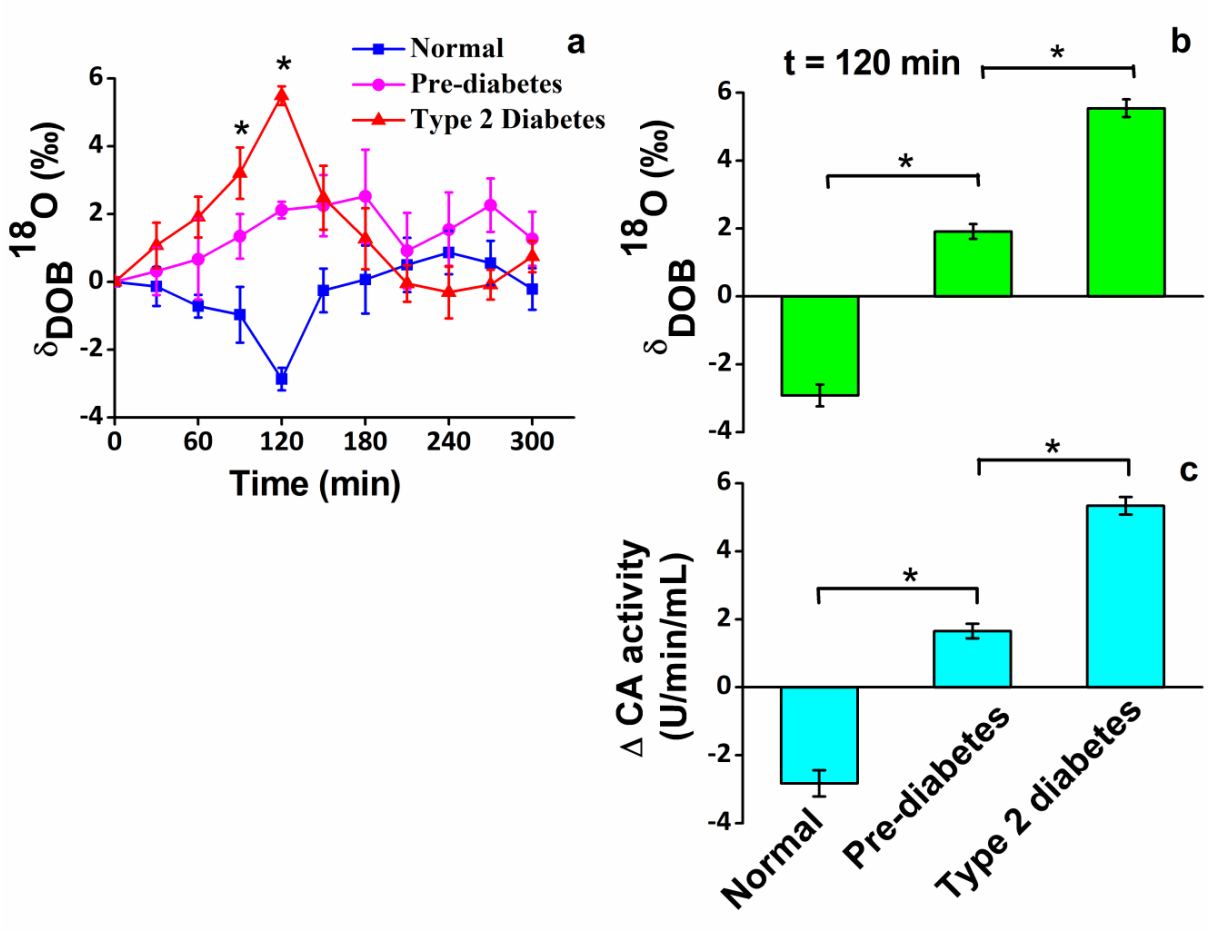


Figure 2. *Erythrocyte ΔCA activity links with the $\delta_{DOB}{}^{18}O$ of breath CO_2 for non-diabetic control (NDC), pre-diabetes (PD) and type 2 diabetes (T2D). a, The maximum difference ($p<0.0001$) of $\delta_{DOB}{}^{18}O$ (‰) was found at 120 min after glucose load. b, c, The ΔCA activities for T2D (5.34 ± 0.26), PD (1.65 ± 0.21) and NDC (-2.82 ± 0.38)] closely associated with $\delta_{DOB}{}^{18}O$ (5.54±0.26, 1.91±0.21 and -2.92±0.32 for T2D, PD and NDC, respectively) in exhaled breath. *$p<0.01$. Values are means ± SEM.*

We next investigated the oxygen-isotope fractionations of CO_2 by monitoring the $^{18}O/^{16}O$ stable isotope ratios in exhaled breath, expressed as delta-over-baseline (DOB) relative to the Vienna Pee Dee Belemnite standard, i.e., $\delta_{DOB}{}^{18}O‰ = [(\delta^{18}O‰)_{t=t} - (\delta^{18}O‰)_{t=basal}]$, associated with CA activities. To explore whether the changes in CA

activities are associated with changes in $^{18}O/^{16}O$ isotope ratios of breath CO_2, we investigated the excretion kinetics of $\delta_{DOB}{}^{18}O‰$ values in breath samples from NDC, PD and T2D (figure 2a), using an optical cavity-enhanced integrated cavity output spectrometer. In this investigation, individuals with T2D exhibited significantly higher isotopic enrichments of ^{18}O in CO_2 compared with PD during the 2h-OGTT, while a marked depletion of ^{18}O in CO_2 was manifested in individuals with NDC (figure 2b). These outcomes suggest oxygen-isotope fractionations of CO_2 in breath play an important role for non-invasive assessment of diabetes mellitus and hence these findings may open a new route to treat diabetes mellitus. However, the observations of isotopic depletion of ^{18}O in CO_2 for NDC and enrichments of ^{18}O in CO_2 for both individuals with T2D and PD are likely to be the effects of inhibition and promotion of catalytic activities of CA respectively, thus unveiling a potential link between CA activity in erythrocytes and the ^{18}O-isotopic exchange in exhaled breath. As a result, we therefore posit that the monitoring of ^{18}O-exchange between carbon dioxide ($C^{16}O_2$) and body water ($H_2{}^{18}O$) influenced by the enzymatic activity of CA may distinctively track the evolution of pre-diabetes prior to the onset of T2D in a non-invasive way (figure 3a).

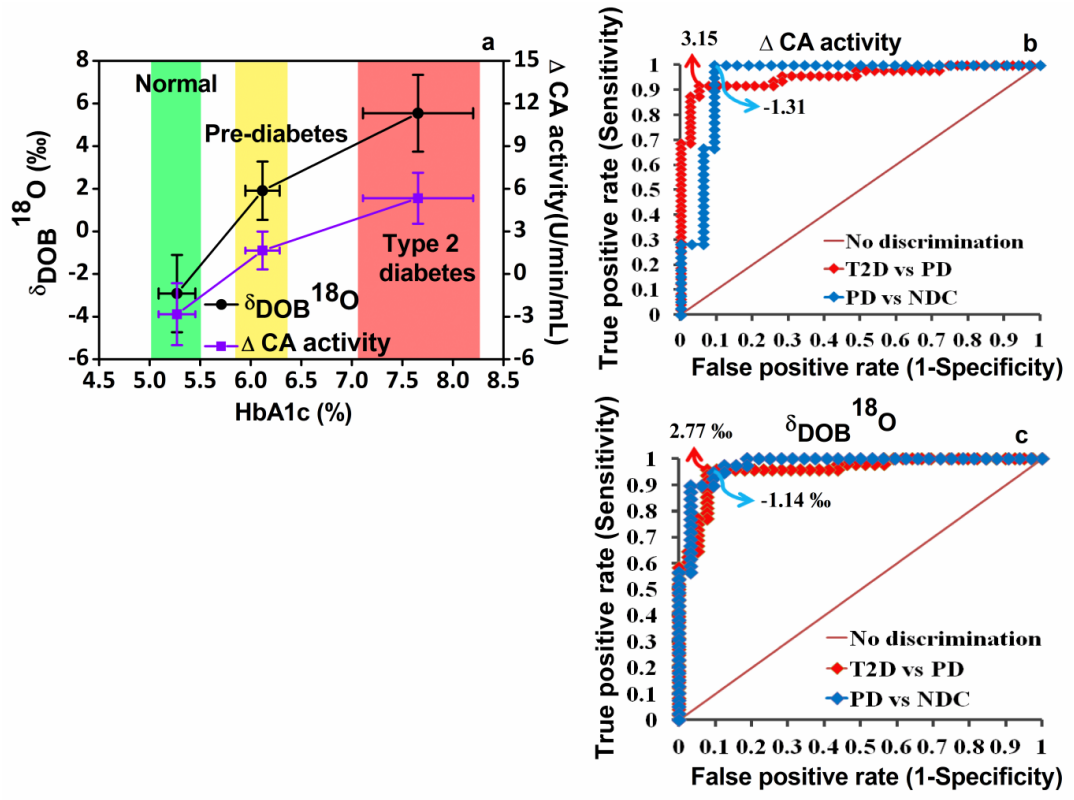


Figure 3. *Distribution of ΔCA activities and $\delta_{DOB}{}^{18}O$ values against HbA1c (%) for non-diabetic control (NDC), pre-diabetes (PD) and type 2 diabetes (T2D). a, Plot represents the clear transitions of ΔCA activities and $\delta_{DOB}{}^{18}O$ from NDC (green) to PD (yellow) and T2D (red). b, c, From receiver operating characteristic (ROC) curves analysis, the optimal diagnostic cut-off points were determined. The ΔCA activity < -1.31 for NDC; ΔCA activity > 3.15 for T2D and -1.31 ≤ ΔCA activity ≤ 3.15 for PD, whereas $\delta_{DOB}{}^{18}O$ ‰ < -1.14 for NDC; $\delta_{DOB}{}^{18}O$ ‰ >2.77 for T2D and -1.14 ≤ $\delta_{DOB}{}^{18}O$ ‰ ≤ 2.77 for PD. Data are means ± SD.*

To investigate the precise transition from normal to pre-diabetes and then on to type 2 diabetes, we determined optimal diagnostic cut-off points of $\delta_{DOB}{}^{18}O‰$ values in breath and ΔCA activity in erythrocytes using receiver operating characteristics curve (ROC) analysis (figure 3b and 3c). Individuals with $\delta_{DOB}{}^{18}O‰$ > 2.77‰ and $\delta_{DOB}{}^{18}O‰$ < -1.14‰ were considered T2D and NDC respectively, whereas subjects with 2.77 ≥ $\delta_{DOB}{}^{18}O‰$ ≥ -1.14 were suggested to be PD and these corresponded to the diagnostic

sensitivity and specificity of ~ 95% and ~ 91%, respectively. Conversely, a diagnostic cut-off point of ΔCA = 3.15 U/min/mL between individuals with PD and T2D, exhibited the sensitivity and specificity of 91.7% and 94.9%, respectively, whereas ΔCA = -1.31 U/min/mL correctly diagnosed individuals with NDC and PD corresponding to similar levels of diagnostic sensitivity and specificity. Taken together, these findings may have a broad clinical efficacy for accurate evaluation of diabetes mellitus either invasively or non-invasively and therefore provide a unique approach to treat the world's most common metabolic disease.

Finally, we elucidated the potential metabolic pathways (figure 4) both in basal and postprandial (2h-OGTT) states underlying the mechanisms responsible for the alteration of CA activity and ^{18}O-isotopic exchange in breath CO_2. Under basal conditions, the majority of endogenous glucose disposal takes place in insulin-independent tissues like the brain, liver and gastrointestinal tract [13, 14]. In T2D state, an elevated rate of hepatic glucose production is the major abnormality for the increased basal plasma glucose concentration [14-16] and erythrocytes are then exposed to excessive glucose concentration which in turn makes a higher glycosylated form of CA than that in healthy individuals, resulting in reduction of total enzymatic activity of CA. However, in the basal state, more than 60% of cellular fuel for insulin-dependent muscle tissues is derived from free fatty acids for whole-body fuel oxidation [17]. The major metabolite CO_2 produced by cellular oxidation reaches erythrocytes through the bloodstream and reacts with body water to form carbonic acid (H_2CO_3). The isotopes ^{16}O of $^{12}C^{16}O_2$ and ^{18}O of $H_2{}^{18}O$ are rapidly exchanged, catalyzed by CA activity, leading to the generation of $H_2C^{18}O^{16}O_2$ together with $H_2C^{16}O^{16}O_2$. All these carbonic acids rapidly degas to produce both $C^{18}O^{16}O$ and $C^{16}O^{16}O$ which subsequently bind to haemoglobin molecules to form carbaminohemoglobin compounds ($Hb.CO_2$), which are then transported to the lungs where $^{12}C^{16}O^{18}O$ and $^{12}C^{16}O^{16}O$ are excreted in exhaled breath.

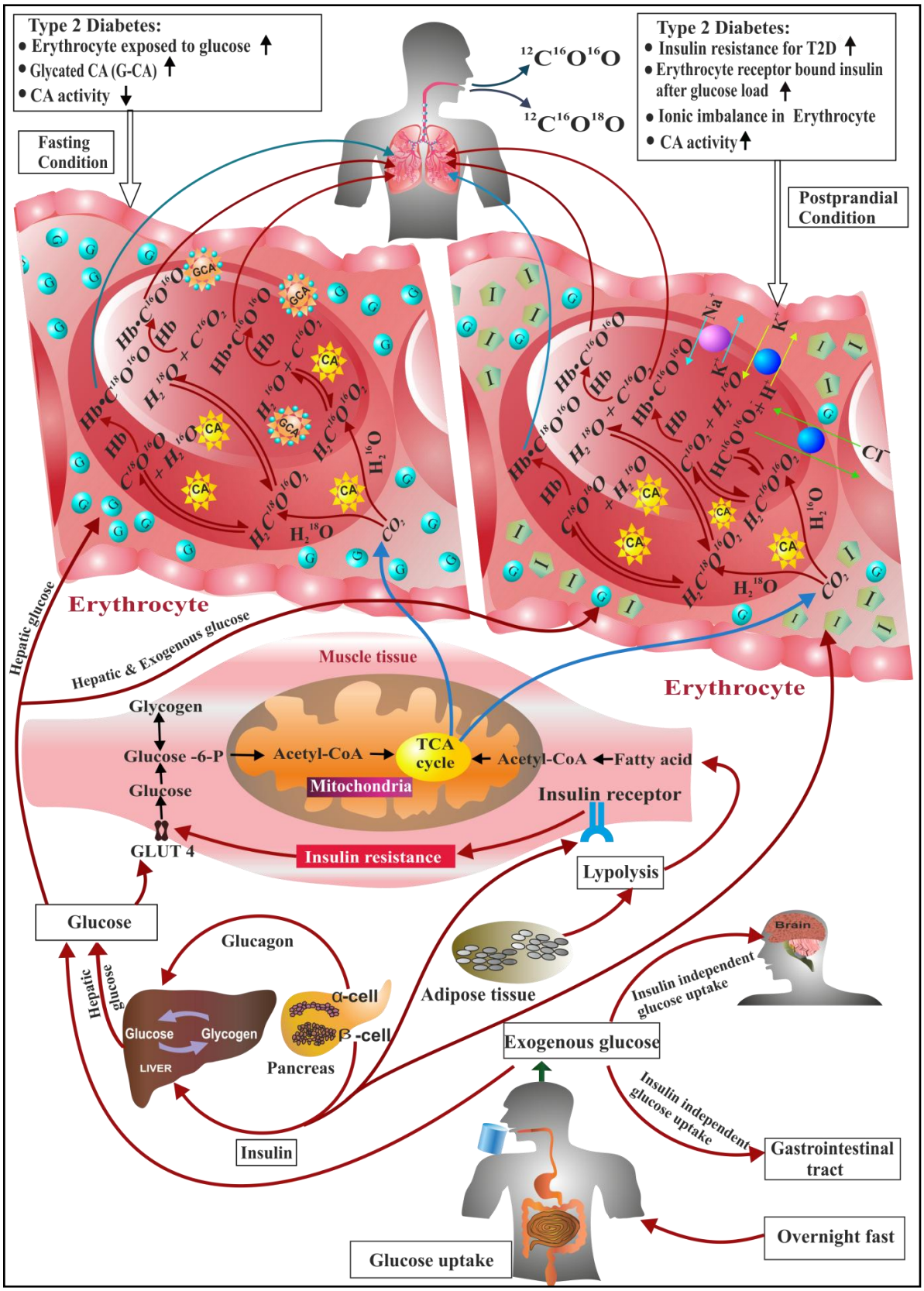


Figure 4. *Potential metabolic pathways for ^{18}O-isotopic exchange in response to CA activity.*

However, under postprandial condition, the exogenous glucose stimulates the release of insulin which facilities the majority of ingested glucose to uptake predominantly in peripheral tissues [18] through the GLUT 4 transporter and a small fraction to adipose tissue. For T2D, the inhibition of insulin-receptor signalling due to insulin resistance and accordingly the lack of GLUT 4 recruitment plays a central role for the blunted rate of glucose transportation especially in insulin-dependent muscle tissues [15]. After glucose enters into muscle cell, it is phosphorylated to produce glucose-6-phosphate which later equilibrates between glycogenesis and glycolytic metabolic pathways. About one-third of the total glucose-6-phosphate, which enters into the glycolytic pathway, is predominantly oxidised into CO_2 which is then transported to the erythrocyte and eventually excreted as $^{12}C^{16}O^{18}O$ and $^{12}C^{16}O^{16}O$ in breath. In case of pre-diabetes and newly diagnosed type 2 diabetes, the pancreas produces more insulin to compensate the insulin resistance. Therefore, erythrocyte is exposed to the environment of elevated receptor bound insulin in pre-diabetes and newly diagnosed type 2 diabetes, creating an ionic imbalance within the erythrocytes owing to increased proton production, Na^+ ion uptake and possibly exchange of other ions such as K^+ and Cl^- between plasma and erythrocytes [2, 12], leading to the enhancement of CA activity and accordingly alter the ^{18}O-isotopic fractionations of CO_2 in breath.

In conclusion, our findings point to a fundamental mechanism underlying the cause of altered CA activity in erythrocytes in individuals with dysglycemia. We have also taken a step towards unraveling the potential link between erythrocyte CA activity and ^{18}O-isotopic fractionations of breath CO_2, thus suggesting that ^{18}O in CO_2, the major metabolite of human breath, could be used as a potential molecular biomarker for the identification of accurate metabolic transition from normal to pre-diabetes and then on to type 2 diabetes in a non-invasive approach. Although many important gaps remain in our

understanding of the exact metabolic pathways involved in causing the ^{18}O-isotopic exchange and in the pathophysiology of T2D, our findings may open new perspectives in the molecular diagnosis of diabetes mellitus with broad clinical applications. Moreover, new insights into the mechanisms linking changes in CA activities to ^{18}O-isotopic exchange are fostering exploration of the molecular basis of the pathogenesis of type 2 diabetes and new methods along with new pharmacological targets to prevent or treat the deleterious effects of this metabolic disease that threatens modern society.

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Chapter 7

Role of oxygen-18 isotope of breath CO_2 and erythrocytes carbonic anhydrase activity for distinguishing type 1 and type 2 diabetes

1. Introduction

Type 1 diabetes (T1D), a chronic autoimmune disorder resulting from destruction of insulin-producing β-cells in the pancreatic islets of Langerhans, is an important and serious health problem afflicting millions of people worldwide [1]. Over the last few decades much effort has been devoted towards identifying the T1D from several measureable markers of the autoimmune state as well as the progression of islet destruction. The most commonly used indicators are glutamic acid decarboxylase autoantibodies (GADA), islet cell cytoplasmic autoantibodies (ICA), insulin autoantibodies (IAA) and insulinoma associated-2 autoantibodies (IA-2A) [2]. Although the occurrence of T1D is suggested by the presence of one or different types of antibodies, but it is still the subject of considerable debate within the healthcare community when and which antibody should be tested for precise identification of disease state. Moreover, the potential role of autoantibodies in the pathogenesis of disease is highly controversial [3, 4]. The underlying mechanisms are also poorly understood. Sometimes, maturity onset-diabetes of young (MODY) are misdiagnosed as T1D and incorrectly treated with insulin [5, 6]. Moreover, the autoantibody-based diagnosis of T1D is a major challenge as there remains a subset of patients who does not express the features reflective of autoantibody characteristics at the onset of disease. Therefore, the inability to visualize the acute onset and progression of T1D has been a

major area of research and clinical stumbling block over the last decade. The uncertainty of autoantibodies testings suggests that there is a pressing need to develop a new and effective strategy for early detection of T1D. Indeed, to our knowledge, so far no study has reported any specific diagnostic biomarker for T1D that can accurately track the initiation and progression of the disease as well as can also be used for routine clinical practices.

Type 2 diabetes (T2D) is another form of diabetes mellitus, where body either does not produce enough insulin or body is resistant to insulin action. The occurrence of T2D is very common and it accounts for 80% of total diabetes cases. The insulin resistant individuals with genetically higher rate of β-cell apoptosis are highly susceptible for developing type 1 diabetes [7] and consequently features of T1D and T2D may be present in the same patient, making the precise identification and classification of T1D and T2D extremely difficult. Although diabetic ketoacidosis (DKA) is a common incident for the onset of T1D, but the history of DKA is not a reliable way to distinguish between T1D and T2D. Now-a-days, C-peptide, an amino-acid polypeptide, has also been reported to be a potential candidate to distinguish the T1D from T2D [8]. Furthermore, the measurement of adiponectin-to-leptin ratio has been suggested to exhibit considerable advantages to detect T1D than the other proposed methods [9]. But, neither C-peptide nor adiponectin-to-leptin ratio has yet showed potential routine clinical applications for precise diagnosis of T1D and T2D. To our knowledge, as there is no clinical characteristic or diagnostic marker available till now to readily distinguish T1D from T2D, therefore there is a major challenge worldwide to develop a new and suitable diagnostic marker that can selectively and precisely track the progression of both T1D and T2D.

However, studies in the past decade have also demonstrated that oxygen isotopes (i.e. ^{16}O and ^{18}O) are rapidly interchanged between carbon dioxide ($^{12}C^{16}O^{16}O$) and water

($H_2{}^{18}O$) to produce stable $^{12}C^{18}O^{16}O$ isotope in exhaled breath during the human respiration process mediated by the carbonic anhydrase (CA), a widespread metalloenzyme in human body [10, 11]. Moreover, some early evidences [11, 12] suggest that changes in CA activity in human erythrocytes (red blood cells) may be the initial step of altered metabolism in diabetes mellitus. But the precise role of CA activity in the pathogenesis of T1D or T2D is completely unknown. Moreover, the potential role [13, 14] of ^{18}O-isotopic exchange regulated by CA activity suggests a tantalizing but unproven hypothesis about the possibility of exploiting breath ^{18}O-isotopic fractionation may specifically track the progression of T1D and T2D, and hence may introduce a novel noninvasive strategy for an accurate and early detection of diabetes as well as to distinguish T1D specifically from T2D. A new insight into the role of CA activity linking to ^{18}O-isotopic fractionations of breath CO_2 is important to delay or even prevent the acute onset of T1D or T2D and its complications. In addition, unraveling the precise metabolic pathways underlying breath ^{18}O isotopic alternation remains a challenge, whenever an individual is at high-risk of developing diabetes.

In this chapter, we therefore studied how the enzymatic activity of CA in human erythrocytes is altered in individuals with T1D and T2D, and subsequently we investigated how the ^{18}O-isotopic fractionations of breath CO_2 are linked to the alteration of CA activity in both T1D and T2D subjects. We also provide evidences that the breath $^{12}C^{18}O^{16}O$ isotope can be utilized as a potential noninvasive biomarker for selectively distinguishing T1D cases from those with T2D. We furthermore explored several optimal diagnostic parameters of CA activity in human erythrocytes and ^{18}O-isotopes in exhaled breath CO_2 keeping an eye on the widespread clinical efficacy of our new methodology for the diagnosis and precise classification of T1D and T2D. We finally elucidated the potential metabolic pathways underlying the mechanisms linking breath

^{18}O-isotopes to T1D and T2D to gain a better insight into the pathogenesis of diabetes mellitus.

2. Methods

2.1 Subjects

Sixty five subjects (n=27 non-diabetic control, n=24 type 2 diabetes and n=14 type 1 diabetes) were enrolled for the study. Based on the HbA1c (%), 2h-OGTT and autoantibody testing, the study subjects were allocated into three different groups: non-diabetic control (NDC), newly diagnosed type 2 diabetes (T2D) and type 1 diabetes (T1D). The clinical characteristics parameters are described in table 1.

Table 1. *Clinical parameters of the study subjects. Data are expressed as mean ± SD. *Represents statistically significant differences (p<0.05) among non-diabetic control (NDC), type 2 diabetes (T2D) and type 1 diabetes (T1D). The abbreviations M and F stand for male and female, respectively*

Parameters	Non-diabetic control (NDC) (n=27)	Type 2 Diabetes (T2D) (n=24)	Type 1 Diabetes (T1D) (n=14)	p values
Sex (M/F)	16/11	15/9	8/6	
Age (Years)	31.7 ± 8.9	36.2 ± 11.7	21.7 ± 13.8	<0.001*
Weight (kg)	61.2 ± 7.2	60.1 ± 5.8	47.1 ± 11.8	<0.001*
BMI (kg/m^2)	23.9 ± 2.4	24.6 ± 2.3	19.4 ± 2.9	<0.001*
Fasting Plasma Glucose (mg/dL)	87.7 ± 9.8	132.08 ± 12.2	272.6 ±107.6	< 0.001*
Fasting Plasma Insulin (μIU/mL)	9.68±3.1	5.2±1.3	1.1±0.7	< 0.001*
2-hr Post-dose Plasma Glucose (mg/dL)	126.6 ± 10.3	269.8 ± 37.9	452 ± 99.9	<0.001*
HbA1c (%)	5.2 ± 0.2	7.5 ± 0.5	11.3 ± 2.9	<0.001*
Fasting CA Activity (U/min/mL)	8.9 ± 1.3	5.2 ± 1.3	3.1 ± 1.4	<0.001*
Post-dose CA Activity (U/min/mL)	7.5 ± 1.36	11.1 ± 2.8	4.5 ± 1.9	<0.001*

2.3 Study protocol

After overnight fasting, breath and blood samples were taken from each participant. The subjects were then instructed to ingest a test meal containing 75 gm normal glucose dissolved in 150 mL water. Post-dose end-tidal breath samples were collected in breath samples collection bags at 30, 60, 90 and 120 minutes and analyzed immediately by laser-based ICOS spectrometer to measure the isotopes of carbon dioxides in exhaled breath. After 2h of glucose load, the blood samples were collected again to determine the erythrocytes carbonic anhydrase activities and post-dose blood parameters.

3. Results and Discussion

To investigate how carbonic anhydrase activity is associated with altered metabolism in T1D and T2D, we first estimated the total erythrocytes CA activity involving three primary isoenzymes of CA (i.e. CAI, CAII and CAIII), spectrophotometrically in the fasting state (figure 1a). The basal CA activity was found to be significantly lower in diabetic individuals as compared to non-diabetic controls (NDC, n=27). However, in between T1D (n=14) and T2D (n=24), the individuals with T1D exhibited considerably lesser CA activity than T2D individuals.

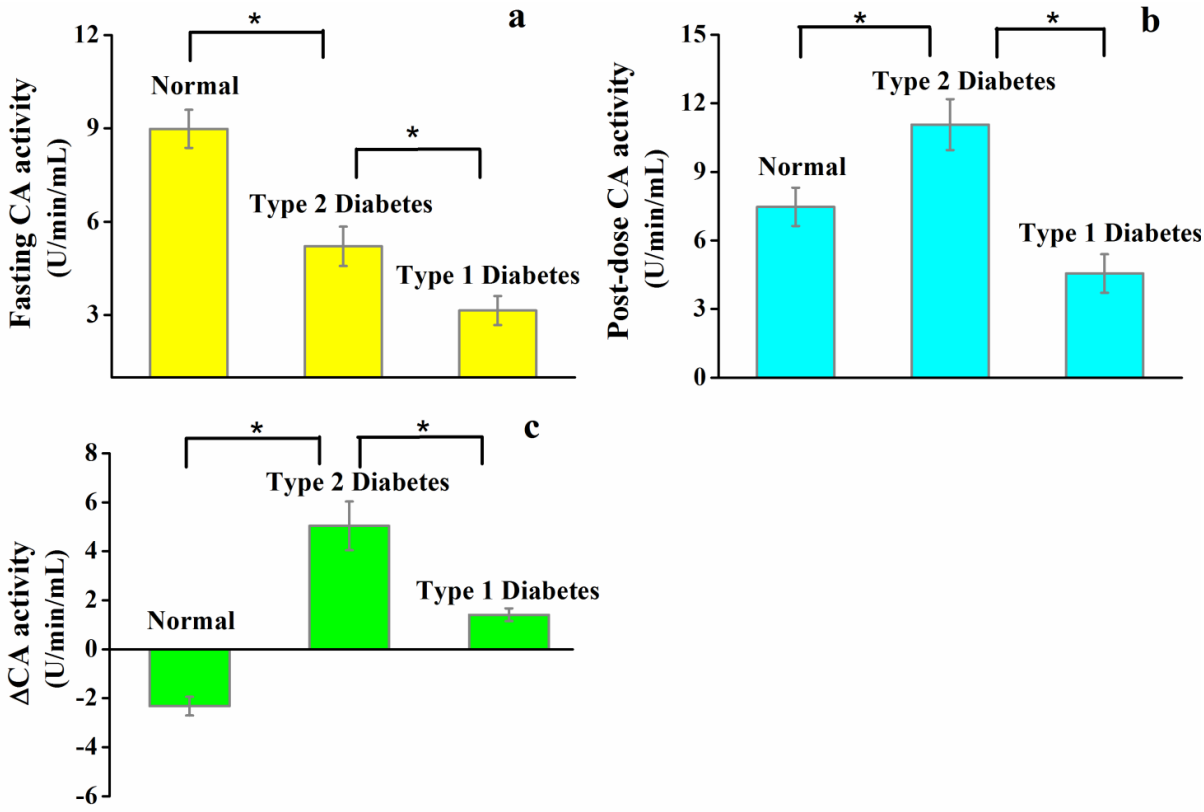

Figure 1. *Pre-and post-dose erythrocytes CA activities during oral glucose tolerance test in non-diabetic control (NDC), type 2 diabetes (T2D) and type 1 diabetes (T1D). a, Fasting CA activities [p<0.01 for T1D (3.1 ± 1.4) versus T2D (5.2 ± 1.3) and NDC (8.9 ± 1.3)]. b, Post-dose CA activities [p<0.001 for T1D (4.5 ± 1.9) as compared with T2D (11.1 ± 2.8) and NDC (7.5 ± 1.36)]. *p<0.01. Data are means ± SEM. c, ΔCA activities for T1D (1.4 ± 0.26) as compared with T2D (5.03 ± 0.9) and NDC (-2.3 ± 0.4). *p<0.01. Values are means ± SEM.*

Several earlier studies [11, 12] suggest that glycosylation is a negative regulator of erythrocytes CA activity in human body and the specific enzymatic activity of glycosylated CA (G-CA) was reported to be 40% of that of the unglycosylated CA. Therefore, glycosylation of CA may play an important role for the reduction of basal CA activities in T1D and T2D. In T1D, because of insulin deficiency, the erythrocytes are exposed to the highest levels of glucose-mediated environment, suggesting higher chance

of glycosylation of CA to reduce its enzymatic activity more than T2D. These results suggest that the measurement of basal CA activity in erythrocytes may be an important parameter for the identification and classification of T1D and T2D.

To address the potential role of CA activity in response to glucose ingestion, we next determined the post-dose CA activities in both T1D and T2D. Our results (figure 1b) showed that post-dose CA activities were enhanced in diabetic subjects with respect to the basal CA activities during the 2h-oral glucose tolerance test (2h-OGTT). However, the enhancement of post-dose CA activity was significantly higher in T2D patients when compared to patients with T1D. Several lines of evidence [15] suggest that the lack of erythrocyte receptor bound insulin creates an ionic imbalance within the erythrocytes cell membrane and subsequently alter the erythrocytes CA activity, indicating that in our observations the alterations of post-dose CA activities in individuals with T1D and T2D are likely to be the effect of ionic imbalance within the erythrocytes. However, our study demonstrates that the post-dose CA activity may also be a good candidate to distinguish T1D from T2D.

We next explored whether the absolute changes in erythrocytes CA activities between pre-and post-dose states (i.e. ΔCA) allow us to distinguish the exact state of the metabolic disorder. We observed marked differences in ΔCA activities in all individuals (figure 1c), suggesting a potential link between changes in erythrocytes CA activities and altered metabolism in diabetes mellitus. Our results also signify that changes in the activities of CA in human erythrocytes may contribute to the development of T1D and T2D. In view of these results, we also propose that measurements of ΔCA activities have an enormous potential to clearly distinguish between diabetic and non-diabetic subjects.

However, to investigate whether the alteration in CA activity in erythrocytes during the 2h-OGTT could influence the rate of isotopic fractionation reaction during metabolism, we monitored the $^{18}O/^{16}O$-stable isotope ratios of the major metabolite, CO_2 in exhaled breath by using a laser-based high-resolution cavity-enhanced integrated cavity output spectroscopy (ICOS) technique. Here, we expressed the $^{18}O/^{16}O$-isotope ratios of CO_2 by the delta-over-baseline (DOB) with respect to the standard Vienna Pee Dee Belemnite, i.e. $\delta_{DOB}{}^{18}O‰ = [(\delta^{18}O‰)_{t=t} - (\delta^{18}O‰)_{t=basal}]$.

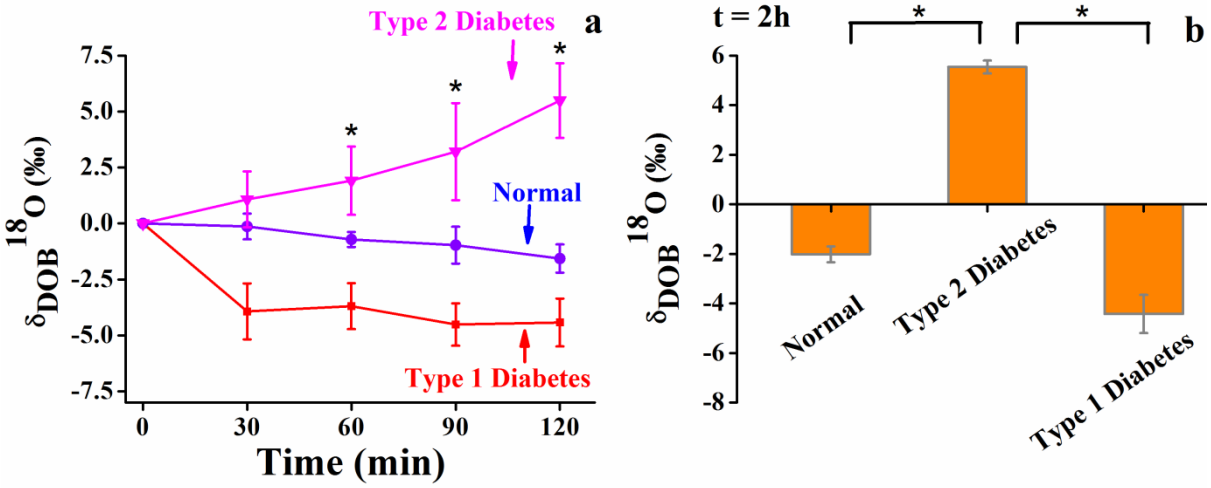

Figure 2. *Excretion kinetics study of oxygen-18 isotopes in exhaled breath CO_2 (expressed as $\delta_{DOB}{}^{18}O$) for type 2 diabetes (T2D) and type 1 diabetes (T1D). a, Breath $\delta_{DOB}{}^{18}O(‰)$ values are clearly distinguishable from 60 min to 120 min after glucose load in NDC, T2D and T1D. b, $\delta_{DOB}{}^{18}O‰$ for T1D (-4.4 ± 0.7) as compared with T2D (5.54 ± 0.2) and NDC (-2.9 ± 0.3). *p<0.01. Values are means ± SEM.*

Figure 2a shows the excretion kinetics patterns of ^{18}O-isotopes of breath CO_2 in NDC, T2D and T1D. In this investigation, T1D patients exhibited marked depletions of ^{18}O-isotopes from the basal value as compared to NDC, whereas T2D patients manifested the highest isotopic enrichments of ^{18}O-isotopes in their breath samples during the 2h-OGTT, thus unveiling a potential link between the breath ^{18}O-isotopic fractionations and

altered metabolisms in T1D and T2D subjects, which has never been explored before. It is noteworthy that in non-insulin dependent T2D patients, the isotopic enrichments of ^{18}O is possibly attributed to the effect of the enhancement of post-dose CA activity where it takes an important role to promote the isotopic exchange reaction during the metabolism to produce more ^{18}O-isotopes in exhaled breath CO_2.

However, in T1D subjects, although the post-dose CA activity was slightly higher than the basal value, but those subjects exhibited a significant depletion of ^{18}O values in breath CO_2 after glucose load. This is possibly due to the fact that in absence of insulin in T1D, the body switches to non-insulin dependent free fatty acids (FFAs) oxidation to supply energy for cellular work [16]. The FFAs are broken down in liver to produce ketone bodies which are then accumulated in bloodstream to lower its pH, thus making it acidic. As a result, the decrease of pH may facilitate the reverse hydrolysis of the isotopic fractionation reaction which may eventually reduce the production of ^{18}O-isotope in breath CO_2.

It is quite evident (figure 2b) that $\delta_{DOB}{}^{18}O‰$ in exhaled breath was depleted in NDC and T1D subjects, whereas T2D exhibited significant enhancement of $\delta_{DOB}{}^{18}O‰$ values after the 2h-OGTT. Therefore, our evidences suggest that monitoring $^{18}O/^{16}O$-isotopic exchange between $^{12}C^{16}O^{16}O$ and $H_2{}^{18}O$ influenced by the enzymatic activity of CA may be a new and robust route to selectively distinguish T1D from T2D and thus breath $^{12}C^{18}O^{16}O$ might be considered as a potential biomarker for the precise evaluation of T1D and T2D in a non-invasive way.

However, to establish the widespread clinical applicability of ^{18}O-isotopes of breath CO_2, we attempted to estimate the optimal diagnostic cut-off values of $\delta_{DOB}{}^{18}O‰$ by utilizing the receiver operating characteristic (ROC) curve analysis (figure 3).

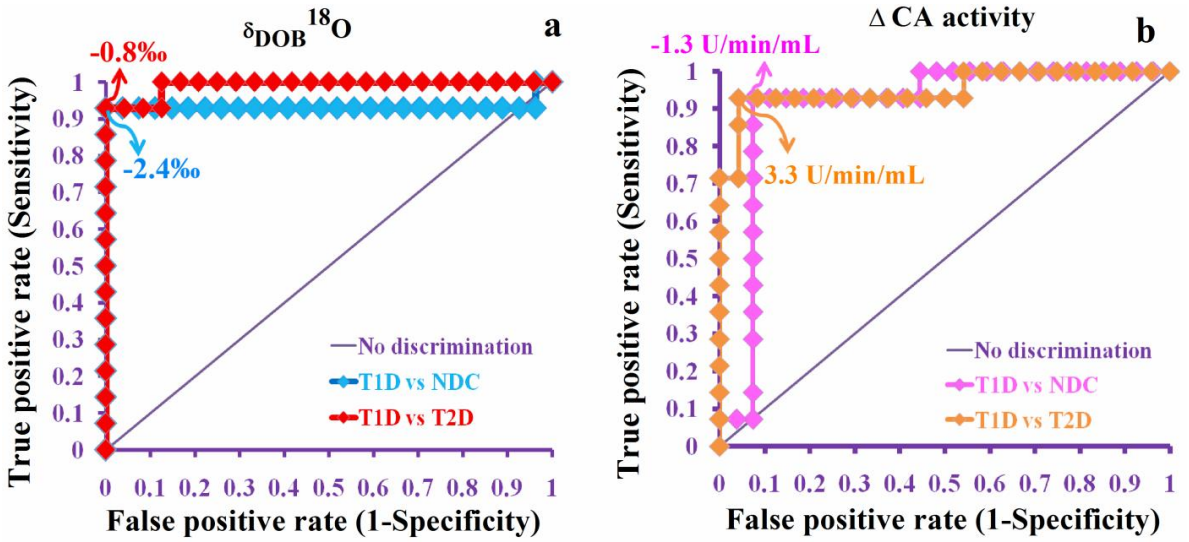

Figure 3. *Clinical feasibility of $\delta_{DOB}{}^{18}O$ (‰) and ΔCA activity (U/min/mL) measurements. The optical diagnostic cut-off points were estimated by utilizing the receiver operating characteristic (ROC) curves analysis. a, $\delta_{DOB}{}^{18}O$‰< -2.4‰ for T1D; $\delta_{DOB}{}^{18}O$ ‰ >-0.8 for T2D, whereas b, The -1.3 <ΔCA activity < 3.3 U/min/mL for distinguishing T1D from T2D. Data are means ± SD.*

From the ROC curve analysis, the individuals with $\delta_{DOB}{}^{18}O$‰< -2.4‰ were considered as T1D, whereas subjects with $\delta_{DOB}{}^{18}O$‰>-0.8‰ were suggested to be T2D and these corresponded to the diagnostic sensitivity and specificity of ~92% and ~100%, respectively. Similarly, a diagnostic cut-off point of -1.31<ΔCA<3.3 U/min/mL was considered as T1D to specifically distinguish T1D from T2D corresponding to the similar levels of diagnostic sensitivity and specificity. Taken together, our findings therefore suggest a potential unifying strategy for precise classification and diagnosis of T1D and T2D because carbonic anhydrase (CA) is a ubiquitous enzyme in nature present in the human body and occurs in most cells and tissues.

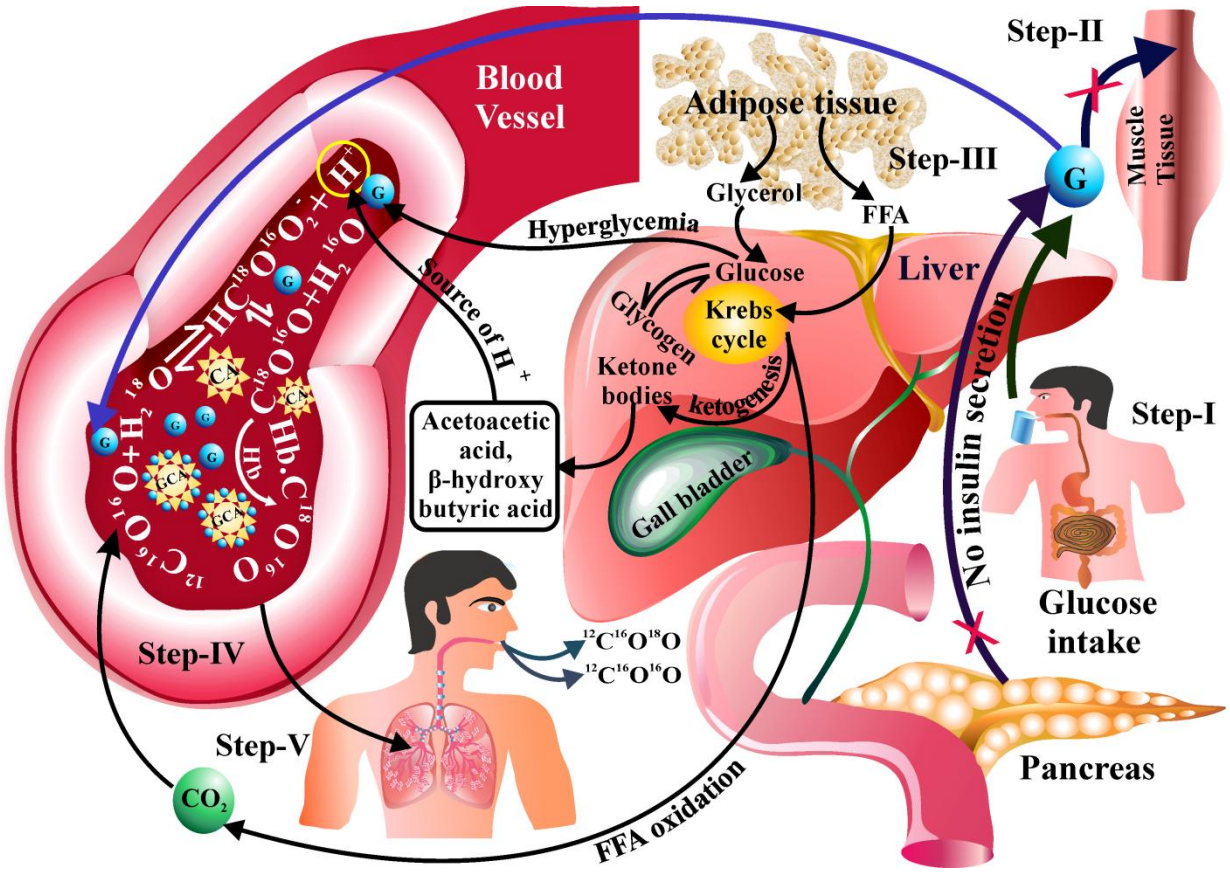

Figure 4. *Proposed physiological pathways of oxygen-18 isotopic fractionation in diabetes.*

At fasting state, carbonic anhydrase (CA) loses its enzymatic activity in diabetes subjects due to formation of excess glycosylated carbonic anhydrase molecules (G-CAs) in erythrocytes. The decrease in enzymatic activity of CA is the maximum in T1D as erythrocytes are exposed to the highest level of glucose-mediated environment in T1D than T2D. When a dose containing of 75 g of normal glucose is administered (step-I), due to lack of insulin, the exogenous glucose cannot enter into muscle tissue (step-II) in T1D. Therefore, the non-insulin dependent free fatty acids (FFAs) oxidation is facilitated to produce ketone bodies during metabolism in liver (step-III).This may alter the reverse isotopic fractionation reaction to deplete the ^{18}O-isotope in exhaled breath CO_2 at post-dose state (step-IV to step-V).

However, in order to understand the mechanisms underlying the alteration of breath ^{18}O-isotopic fractionations regulated by the CA activity, we finally elucidated the possible metabolic pathways to explicate our findings (figure 4). It is known that insulin promotes the cellular glucose uptake and removes the excess glucose from the blood. In T1D individuals, due to lack of insulin, glucose molecules build up in the bloodstream to create hyperglycemia, suggesting the possibility of erythrocytes CA to be exposed in higher level of glucose-mediated environment, which in turn makes a higher glycosylated CA (G-CA) enzymes than that in T2D individuals. Therefore, the maximum suppression of basal CA activity in T1D is possibly attributed to the formation of large number of G-CAs in erythrocytes at fasting state. After overnight fasting, when a test meal containing 75 g of normal glucose is administered, the majority of glucose disposal takes place in insulin-dependent peripheral tissues (primarily muscle tissue), whereas adipose tissue accounts for only small amount of exogenous glucose uptake. In case of T1D, the lack of insulin may be responsible to facilitate the non-insulin dependent free fatty acids (FFAs) oxidations to supply the metabolic fuel. This may accumulate the excess ketone bodies in the blood. This may be responsible to alter the rate of the reverse isotopic fractionation reaction to deplete the ^{18}O-isotope in exhaled breath CO_2 at the post-dose state in T1D.

Here, we have elucidated a possible mechanism underlying the cause of change in erythrocytes CA activity during the metabolism in T1D and T2D. However, many important gaps remain in our understanding of the physiological pathways regarding the oxygen-18 isotopic exchange and metabolic pathways involved during metabolism. But, our method shows a potential perspective of exhaled breath analysis as the innovative diagnostic tool in future days. Although organ specific metabolic study is not available from these experiments, but future studies that include the experimental animal models

are needed to be ensured how different organs are associated with the oxygen-18 isotopic fractionations during the metabolism in T1D.

4. Conclusion

Our findings reveal a fundamental mechanism underlying the potential role of erythrocytes CA activity in the pathogenesis of T1D and T2D. We have also taken a major step towards unravelling a missing link between CA activity in human erythrocytes and ^{18}O- isotopic fractionations in human breath CO_2 which has never been explored before. Our studies suggest that breath $^{12}C^{18}O^{16}O$ and erythrocytes CA activity could be used as potential biomarkers for precise classification and diagnosis of T1D from T2D in a more robust and better way compared to the traditional methods which usually utilize one or more antibody testing. Another salient advantage of our methodology is that it may assist for non-invasive early evaluation of those individuals who are now T2D with higher rate of genetically driven beta cell apoptosis, but are highly susceptible to exhibit metabolic transition from T2D to T1D in future days. Moreover, new insights into the linkages between ^{18}O of breath CO_2 and CA activity in erythrocytes are fostering to devise new and better approaches to understanding the pathophysiology of T1D and T2D along with new pharmacological targets for the treatment and prevention of the world's most common long-lasting metabolic diseases.

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Chapter 8

In-vitro monitoring of oxygen-18 isotope of breath CO_2 for quantitative estimation of carbonic anhydrase activity in human body

1. Introduction

New insights into the development of a simple methodology to estimate the enzymatic activity of carbonic anhydrase is necessary for early detection of a series of diseases including edema, glaucoma, osteoporosis and neurological disorders [1-3]. Considerable data is now available to confirm the role of carbonic anhydrase (CA) during cell growth in renal cancer, cervical cancer and lung cancer. Diagnostic, prognostic and therapeutic implications of carbonic anhydrase in cancer have widely been discussed [4-8]. There is interesting evidence that prognostic value of carbonic anhydrase expression may be an important predictor of survival for renal cell carcinoma [9-11]. With growing interest, it is really necessary to find out a simple assay method for easily accessing the CA activity. Although traditional methods provide useful information regarding the enzymatic assay of CA, the practical application of this method is limited due to tedious and expensive process for sample collection, long-time for lab processing and analysis via traditional mass spectroscopy technique. In general, the most common assay is based on the spectrophotometric measurement of hydrolysis rate of para-nitro phenyl acetate to produce para-nitro phenol in presence and absence of a specific inhibitor of CA. However, the barriers for effective utilization of this method are the necessity of standardization from the knowledge of cell counts of the subject, maintaining the

medium temperature throughout the process and overall processing the blood sample for the long time, suggesting an alternate approach is desperately needed.

Early studies suggest [11-13] that oxygen-16 isotope and oxygen-18 isotope are rapidly exchanged between CO_2 and body ^{18}O-water to produce $^{12}C^{16}O^{18}O$ isotope in human exhaled breath:

$$C^{16}O^{16}O + H_2{}^{18}O \xrightarrow{\text{carbonic anhydrase}} C^{18}O^{16}O + H_2{}^{16}O$$

This isotopic exchange during physiological process has a large impact on isotopic composition of carbon dioxide during exhalation in human .The oxygen-18 isotopic composition of exhaled breath CO_2 ($^{12}C^{18}O^{16}O$) reflects the isotopic fractionation between CO_2 and $H_2{}^{18}O$ in body, providing an important tracer of CO_2. Therefore, this fractionation suggests that there is a possibility to non-invasively estimate the erythrocytes CA activity from monitoring of oxygen-18 isotope in exhaled breath. Although major advances in our knowledge regarding the mechanism of oxygen-18 isotopic exchange have occurred, no study still date has reported to exploit the isotopic exchange phenomenon to determine the CA activity in human body.

In this chapter, we explored a new method which can quantitatively estimate the CA activity from monitoring of oxygen-18 isotope in exhaled breath. During the equilibrium of the reaction, the oxygen isotope of CO_2 is relatively enriched with oxygen-18 isotope than body water. However, the subject-specific body temperature, pH, ^{18}O of H_2O and cellular produced CO_2 may alter the exchange kinetics resulting in variation of $^{12}C^{18}O^{16}O$ isotopic compositions in exhaled breath. Therefore, the aim of the present study was to explore the feasibility of isotopic fractionation reaction occurring in human body for quantitative estimation of CA activity. The main aim of our in-vitro study was to mimic the isotopic exchange reaction in human body.

2. Results and Discussion

To explore the feasibility of isotopic exchange phenomenon and subsequently to estimate the erythrocytes CA activity, pure carbon dioxide gas (5% CO_2) was injected into the flasks and kept for desired time (1h) to allow it to come into equilibrium within the closed flasks. We artificially prepared a wide variety of CA activities of hemolysate solutions (prepared from blood samples) within the flasks by addition of CA inhibitor (acetazolamide) at desired concentrations. The kinetics of isotopic fractionation reaction was measured by utilizing the high-precision laser-based ICOS technique as depicted in chapter 2. The productions of oxygen-18 isotope of CO_2 in sample flasks were compared with the blank flasks. We observed the enrichment of oxygen-18 isotope with increase of CA activity within the flasks, suggesting the significant role of CA enzyme to promote the rate of isotopic fractionation reactions. However, it is noteworthy that the kinetics of a chemical reaction primarily depends on concentrations of reactants ([CO_2] and [$H_2{}^{18}O$]), temperature and pH.

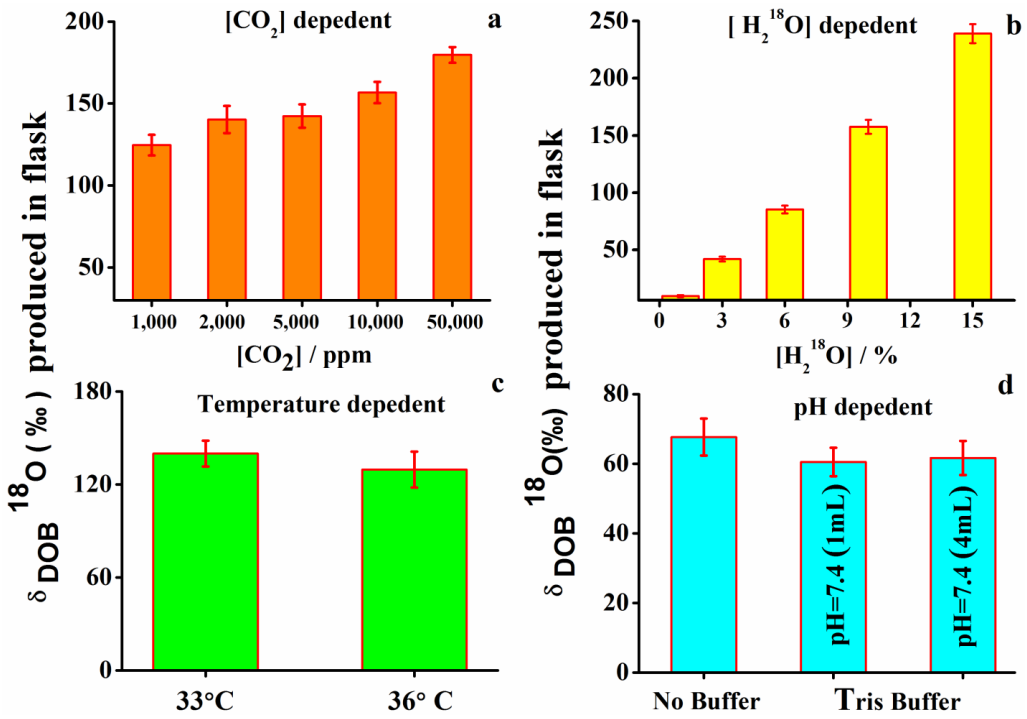

Figure 1. *Effects of [CO_2], [$H_2{}^{18}O$], temperature and pH on isotopic exchange reaction between oxygen-16 isotope of CO_2 and ^{18}O isotope of $H_2{}^{18}O$ within closed flasks*

To investigate the effect of CO_2 on this isotopic exchange reaction, we performed the reaction at wide variety of CO_2 concentrations (1000 to 50000 ppm). Our study demonstrated that the increase in [CO_2] facilitates the rate of the isotopic fractionation reaction to produce more and more ^{18}O of CO_2 (figure 1a) within the flasks, suggesting the alteration in cellular produced carbon dioxide during the metabolism in human body due to variation of subject-specific basal metabolic rate would have strong influence on exchange kinetics. Next, to examine the influence of ^{18}O-isotope of H_2O, we further investigated the exchange kinetics in presence of [$H_2{}^{18}O$] at different concentrations. The gradual increase of oxygen-18 isotope was found to be increased with [$H_2{}^{18}O$], suggesting the alteration of individual's [$H_2{}^{18}O$] in body can largely alter the rate of the isotopic exchange reaction (figure 1b). To gain insight into the effect of temperature on this in-vitro study, we again examined the reaction kinetics at different temperatures. Here, we found that the rate of oxygen-18 isotopic exchange reaction was affected insignificantly with slight increment of temperature (figure 1c). This observation suggests that the subject-specific variation of body temperature may not alter the isotopic fractionation reaction during physiological process in human body. pH is an important factor to regulate the rate of the reaction. To check the pH dependency on the isotopic fractionation reaction, we next performed our study at different pH (tris buffer) mediums. Here, we found that the rate of exchange reaction depends on the pH, indicating the variation in pH may alter the rate of exchange kinetics during our in-vitro study (figure 1d).

It is noteworthy that we need to consider all the factors including of [CO_2], [$H_2{}^{18}O$], pH and temperature during our in-vitro study to mimic the isotopic exchange reaction in human body. Therefore, we next performed the whole study with [CO_2] = 50,000 ppm, [$H_2{}^{18}O$] = 5‰, pH= 7.4 and temperature = 38^0C to create the equivalent environment as

of human body and monitored the exchange kinetics at different CA activities within the sample flasks. When acetazolamide (CA inhibitor) inhibits the total CA activity in the medium, the ^{18}O-enriched CO_2 isotope was found to be almost disappeared in the sample flask. Gradual increase of CA activity enhances the exchange kinetics to produce more and more ^{18}O of CO_2. The kinetics of isotopic exchange reaction was obtained by plotting the oxygen-18 isotope of CO_2 as a function of CA activity. Here, we fitted the curve with the acquired experimental data. We observed that the isotopic exchange reaction followed the first order reaction kinetics. The rate equation can be expressed as follows:

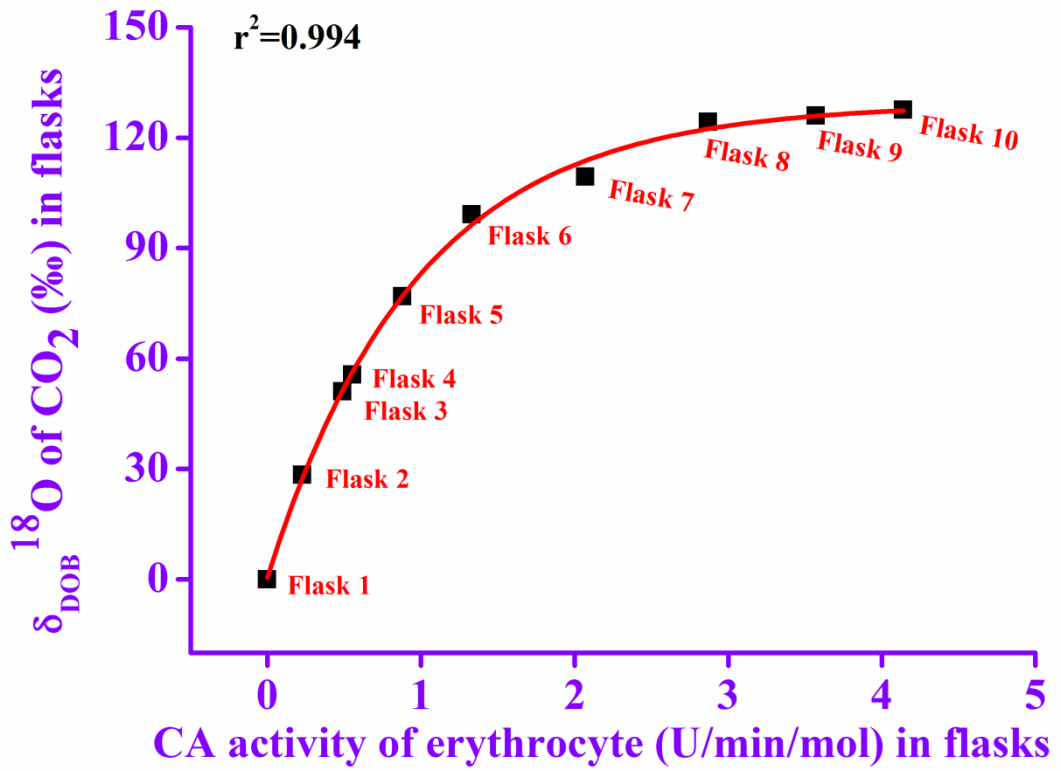

Figure 1: *Fittings of the kinetics curve of isotopic exchange reaction within the sample flasks*

$y=A_1 \exp(-x/t)+y_0$, where A_1, y_0 and t are the constants. Here, A_1= 128.6, y_0 = -129.1 and t=0.97, whereas the 'y' represents the oxygen-18 isotope of CO_2 ($\delta_{DOB}{}^{18}O‰$) and 'x'

represents the CA activity. From the knowledge of ^{18}O isotope of CO_2, one can estimate the CA activity quantitatively by utilizing the above equation.

3. Conclusion

We have developed a new method for quantitative estimation of CA activity from the oxygen-18 isotope of CO_2 analysis. This study shows the feasibility of carbon dioxide isotope analysis for non-invasive estimation of carbonic anhydrase activity. We created the similar environment as of human body to monitor the real time isotopic fractionation reaction. When the conventional method consists of several limitations associated with tedious and expensive process of sample preparation to estimate the CA activity, current method shows a new approach to track the real time CA activity from the ^{18}O of CO_2 analysis. Future research is necessary to validate this method. Finally, this non-invasive method is a new approach than the conventional technique.

4. Experimental Analysis

4.1. Blood sample preparation

10 mL of venous blood samples were collected from each participant in EDTA vacutainer tubes. The collected blood samples were centrifuged at 2000 r.p.m for 15 minutes. Plasma was isolated and buffy coat was removed. The red blood cells (RBC) were washed with 0.9% NaCl solution and allowed to spin against 4000 r.p.m for 20 minutes. The erythrocytes were lysed with ice-cold distilled water. The hemolysate was centrifuged at 10,000 r.p.m for 30 minutes to remove the ghost cells and the supernatant liquid was collected. The fresh supernatant liquid was used for analysis of carbonic anhydrase activity.

4.2 Gas samples analysis

Gas samples were drawn from the sample flasks by an air-tight syringe (QUINTRON) through a T-connector fitted onto the reservoir bag. All gas samples were analyzed by the high-resolution isotopic CO_2 integrated cavity output spectrometer (ICOS). $^{12}C^{18}O^{16}O$ isotopic data were expressed as delta-over-baseline (DOB) values and represented as follows:

$$\delta_{DOB}{}^{18}O‰ = (\delta^{18}O‰)_{sample} - (\delta^{18}O‰)_{blank}$$

$$\delta^{18}O‰ = \left[\frac{(\frac{^{18}O}{^{16}O})_{sample}}{(\frac{^{18}O}{^{16}O})_{standard}} - 1 \right] \times 1000$$

Where $\left(\frac{^{18}O}{^{16}O}\right)_{standard}$ corresponds to the international standard Pee Dee Belemnite (PDB) values i.e. 0.0020672. RBC is normalized to 4.5×10^9 cells/mL.

4.3. Carbon dioxide isotopes measurements

To monitor the production of oxygen-18 isotope of CO_2 during isotopic exchange reaction, we performed the whole experiments in round bottom flasks, which were fitted with rubber septum and adaptors. To ensure the complete removal of residual gases (if any), we pre-treated all the flasks with ultra pure nitrogen gas. We compared the results with respect to the blank flasks. Pure carbon dioxide gas was injected within the flasks. Carbon dioxide gas and $H_2{}^{18}O$ were kept into the closed flasks for 1h to reach the equilibrium of the fractionation reaction. After the equilibrium, the amount of oxygen-18 isotope of CO_2 was measured by laser-based cavity-enhanced absorption spectroscopy.

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Chapter 9

Summary and Future Perspective

In this thesis, we have investigated the feasibility of high resolution laser-based cavity enhance absorption spectroscopy for the measurement of the major metabolite of human breath i.e. CO_2 and its isotopic compositions with ultra low concentration for non-invasive identification and selective identification of type 1 diabetes (T1D), type 2 diabetes (T2D) and pre-diabetes (PD). We have extremely demonstrated how a simple RGA-MS system can be applied during the ^{13}C-glucose breath test in the diagnosis of T2D. Our study confirms the clinical feasibility of a novel residual gas analyzer-mass spectrometry (RGA-MS) method for accurate evaluation of the ^{13}C-GBT in real-time, thus making it a valid and potentially robust non-invasive diagnostic tool for routine clinical practices at the point-of-care (POC). Our results also suggest that the ^{13}C-GBT using a simple RGA-MS method can reliably assesses the changes in glucose metabolism in real time. The present ^{13}C-breath analysis instrument is compact, easy-to-run, more portable and inexpensive compared to currently available optical spectroscopy and MS-based detection methods, suggesting a simple alternative non-invasive screening tool for the measurement of high-precision $\delta_{DOB}{}^{13}C‰$ values in exhaled breath samples, not only from diabetes or non-diabetes, but also from any other disease or metabolic disorder. However, the cost of the ^{13}C-glucose breath test may be considered to be of concern from the economic point of view, since ^{13}C-glucose is much more expensive than blood sugar analysis. This is the main limitation of the ^{13}C-glucose breath test (^{13}C-GBT) for screening diabetes. Nevertheless, utilizing the RGA-MS methodology rather than gas

chromatography-isotope ratio mass spectrometry (GC-IRMS), the overall cost of the ^{13}C-GBT will likely not be prohibitive in the near future in order to screen diabetes even in economically backward countries like India.

However, new strategies for an accurate and early detection of insulin resistance are important to delay or prevent the acute onset of type 2 diabetes (T2D). Currently, insulin sensitivity index ($ISI_{0,120}$) is considered to be a viable invasive method of whole-body insulin resistance for use in clinical settings in comparison with other invasive sensitivity indexes like homeostasis model assessment (HOMA), and quantitative insulin sensitivity check index (QUICKI). In this study, we showed that $^{13}C/^{12}C$-isotope ratios of breath CO_2 were well correlated with blood glucose, insulin, glycosylated-hemoglobin as well as with HOMA-IR and 1/QUICKI. Conversely, the strongest correlation was observed between $1/ISI_{0,120}$ and breath CO_2 isotopes. Consequently, we determined the several optimal diagnostic cut-off points of $1/ISI_{0,120}$ and $^{13}CO_2/^{12}CO_2$-isotope ratios to distinctively track the evolution of PD prior to the onset of T2D. Our study confirms the clinical feasibility of the exhaled breath carbon dioxide isotopes analysis for estimating insulin resistance and thereafter the diagnosis of non-diabetic control, pre-diabetes and type 2 diabetes. When $ISI_{0,120}$ has been suggested as the most correlated alternative insulin resistance (1/insulin sensitivity) parameter with respect to euglycemic hyperinsulinemic clamp study, our observations demonstrated a strong correlation of $ISI_{0,120}$ index with breath carbon dioxide isotopes, suggesting a new perspective into the non-invasive evaluation of insulin resistance rather than the traditional invasive measurements. Additionally, we first estimated the cut-off values of $1/ISI_{0,120}$ for the diagnosis of pre-diabetes and type 2 diabetes, thus making it a potentially robust approach for accurate evaluation of insulin resistance. Overall, our calculated optimal

cut-off values of both $1/ISI_{0,120}$ and carbon-13 isotopes of breath CO_2 also suggest that they may serve as useful methods for early detection and follow-up of individuals who are at high-risk for developing insulin resistance prior to the onset of type 2 diabetes that threaten modern society. Although, many important gaps may remain in understanding the potential link between $ISI_{0,120}$ index and breath CO_2 isotopes in the present study, our results, however, have significant implications in the isotope-specific molecular diagnosis of insulin resistant pre-diabetes and type 2 diabetes with broad clinical applications. Besides, this non-invasive approach for estimation of insulin resistance may assist in detecting the pre-diabetes stage of asymptomatic type 2 diabetic subjects in preclinical phase. This point-of-care diagnostic method may also help to overcome the current compliance of invasive techniques for screening diabetes mellitus in future days. Therefore, our proposed breath isotopes analysis may be a new method to prevent or treat the deleterious effect of the most common metabolic syndrome over the world. Finally, as this breath analysis approach is safe, simple and non-invasive, it could be an attractive option for large-scale screening purposes in a wide variety of individuals including children, pregnant women and seniors.

Furthermore, new methodology to replace the commercially prepared ^{13}C-labelled glucose by naturally available ^{13}C-enriched substrates is necessary to promote the clinical applicability of the isotopic breath test for early detection of type 2 diabetes (T2D). Here, we have formulated a new test meal comprised of naturally available ^{13}C-enriched foods which can be used for accurate evaluation of T2D without ingestion of commercially available ^{13}C-labelled glucose. By utilizing the newly formulated test meal, we were able to clearly distinguish T2D patients from NDC through the excretion kinetics of breath $^{13}CO_2$, thus opening a new perspective into the non-invasive diagnosis

of T2D. Moreover, the determination of new cut-off values of ^{13}C and ^{18}O isotopes of breath CO_2 may assist to track the precise classification of NDC prior to the onset of T2D. Finally, our new protocol for the ^{13}C-breath test exploiting naturally ^{13}C-enriched foods is simple, safe, non-toxic and relatively economic, thus suggesting the widespread clinical applicability for non-invasive diagnosis of the type 2 diabetes in a more robust and better way.

We have also shown that carbonic anhydrase (CA) is associated with oxygen-18 (^{18}O)-isotopic fractionations of CO_2. To investigate how CA activity links the ^{18}O of breath CO_2 to pre-diabetes (PD) and type 2 diabetes (T2D) during metabolism, we studied pre- and post-dose CA activities in erythrocytes with simultaneous monitoring of $^{18}O/^{16}O$-isotope ratios of breath CO_2 and thereafter elucidated potential metabolic pathways underlying CA alteration in the pathogenesis of T2D. Here we showed that the post-dose CA activity in both T2D and PD was markedly enhanced, whereas the non-diabetic controls (NDC) exhibited a considerable reduction in post-dose CA activity when compared with their basal CA activities. However, T2D and PD exhibited isotopic enrichments of ^{18}O in breath CO_2, while a marked depletion of ^{18}O in CO_2 was manifested in NDC. Thus, the isotopic enrichments and depletions of ^{18}O in breath CO_2 were well correlated with the changes in CA activities for controls, PD and T2D. Our findings point to a fundamental mechanism underlying the altered CA activity in erythrocytes that can change the intracellular ion imbalance and may contribute to the onset of insulin resistance, which in turn can lead to the pathogenesis of T2D. We have also taken a step towards unravelling the potential link between erythrocyte CA activity and ^{18}O-isotopic fractionations of breath CO_2, thus suggesting that ^{18}O in CO_2, the major metabolite of human breath, could be used as a potential molecular biomarker for the

identification of accurate metabolic transition from normal to pre-diabetes and then on to type 2 diabetes in a non-invasive approach. Although many important gaps remain in our understanding of the exact metabolic pathways involved in causing the ^{18}O-isotopic exchange and in the pathophysiology of T2D, our findings may open new perspectives in the molecular diagnosis of diabetes mellitus with broad clinical applications. Moreover, new insights into the mechanisms linking changes in CA activities to ^{18}O-isotopic exchange are fostering exploration of the molecular basis of the pathogenesis of type 2 diabetes and new methods along with new pharmacological targets to prevent or treat the deleterious effects of this metabolic disease that threatens modern society.

The inability to envisage the acute onset and progression of type 1 diabetes (T1D) has been a major clinical stumbling block and an important area of biomedical research over the last few decades. Therefore there is a pressing need to develop a new and an effective strategy for early detection of T1D and to precisely distinguish T1D from type 2 diabetes (T2D). Here we describe the precise role of the enzymatic activity of carbonic anhydrase (CA) in erythrocytes in the pathogenesis of T1D and T2D. We show that CA activities are markedly altered during metabolism of T1D and T2D and this facilitates to the oxygen-18 (^{18}O) isotopic fractionations of breath CO_2. In our observations, T1D exhibited considerable depletions of ^{18}O-isotopes of CO_2, whereas T2D manifested isotopic enrichments of ^{18}O in breath CO_2, thus unveiling a missing link of breath ^{18}O-isotopic fractionations in T1D and T2D. Our studies suggest that breath $^{12}C^{18}O^{16}O$ and erythrocytes CA activity could be used as potential biomarkers for precise classification and diagnosis of T1D from T2D in a more robust and better way compared to the traditional methods which usually utilize one or more antibody testing. Another salient advantage of our methodology is that it may assist for non-invasive early evaluation of

those individuals who are now T2D with higher rate of genetically driven beta cell apoptosis, but are highly susceptible to exhibit metabolic transition from T2D to T1D in future days. Moreover, new insights into the linkages between ^{18}O of breath CO_2 and CA activity in erythrocytes are fostering to devise new and better approaches to understanding the pathophysiology of T1D and T2D.