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RESEARCH PAPER

Natural ^{18}O and ^{13}C-urea in gastric juice: a new route for non-invasive detection of ulcers

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Abstract The ^{13}C-urea breath test (^{13}C-UBT), developed a few decades ago, is widely used as a non-invasive diagnostic method to detect only the presence of the gastric pathogen *Helicobacter pylori* infection; however, the actual disease state, i.e. whether the person harbouring *H. pylori* has peptic ulcer disease (PUD) or non-ulcerous dyspepsia (NUD), is still poorly understood. Nevertheless, the present ^{13}C-UBT has numerous limitations, drawbacks and pitfalls owing to the ingestion of ^{13}C-labelled external urea. Here, we show that *H. pylori* is able to utilize the natural ^{13}C and ^{18}O-urea inherently present in the gastric juice in humans for its urease activity which has never been explored before. In vitro measurements of isotopic fractionations of gastric juice urea provide new insights into the actual state of the infection of PUD or NUD. We also provide evidence of the unusual ^{13}C and ^{18}O-isotopic fractionations of breath CO_2 that are distinctively altered in individuals with PUD encompassing both gastric and duodenal ulcers as well as with NUD by the enzymatic activity of *H. pylori* in the gastric niche without oral administration of any ^{13}C-enriched external urea. This deepens our understanding of the UBT exploiting the natural ^{13}C and ^{18}O-gastric juice urea in the pathogenesis of *H. pylori* infection, reveals the actual disease state of PUD or NUD and thus offers novel opportunities for a simple, robust, cost-effective and non-toxic global strategy devoid of any ^{13}C-enriched urea for treating these common diseases by a single breath test.

Keywords Breath analysis · Ulcer · *Helicobacter pylori* · Urea breath test · Gastric juice · Non-invasive diagnosis

Introduction

Helicobacter pylori, one of the most common gastric pathogens in the human stomach, is strongly associated with chronic gastritis and peptic ulcer disease [1–6]. This microorganism has high urease activity which enables it to hydrolyse gastric juice urea with the production of CO_2 and NH_3, thus elevating the pH to neutral as required for the survival of the pathogen in a highly acidic environment [7, 8]. However, despite the vital role of urease enzyme in *H. pylori* pathogenesis, the relationship between non-ulcerous dyspepsia, peptic ulcer (encompassing both gastric and duodenal ulcers) and *H. pylori* infection still remains poorly understood.

Over the last few decades, the ^{13}C-urea breath test (^{13}C-UBT) based on the urease activity of the organism has widely been used as a non-invasive diagnostic method to detect exclusively the presence of *H. pylori* infection in the stomach [9, 10]. But thus far the current ^{13}C-UBT has not allowed one to accurately assess the actual disease state i.e. whether the person harbouring *H. pylori* infection has peptic ulcer or non-ulcerous dyspepsia. One salient disadvantage of the ^{13}C-UBT is the influence of urease-positive non-*H. pylori* bacteria in the oral cavity on the results of ^{13}C-UBT [11–13]. Numerous false positive results are thus still observed in the present ^{13}C-UBT because of the

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consumption of orally administered ^{13}C-enriched urea. Moreover, there are several other drawbacks and pitfalls in the existing ^{13}C-UBT. For example, the optimal diagnostic cut-off point discriminating positive and negative ^{13}C-UBT results is still a controversial issue [10]. The results of ^{13}C-UBT remain questionable and affect the diagnostic accuracy when the cut-off values are very close to the borderline at the onset of the infection or when the cut-off level lies in the so-called grey zone [10]. Therefore there is a pressing need to explore a new-generation strategy which can circumvent all these issues in the existing ^{13}C-UBT and consequently make the diagnosis more sensitive, precise and disease specific.

Studies in the past decade have also demonstrated that ^{16}O in $^{12}C^{16}O_2$ and ^{18}O of $H_2{}^{18}O$ are rapidly exchanged in the gastric juice catalysed by the metalloenzyme carbonic anhydrase (CA) of *H. pylori* to maintain the urease activity in its environment [14–16]. But the potential role of ^{18}O-isotopic fractionations in the gastric environment influenced by the enzymatic activity of *H. pylori* especially in the pathogenesis of peptic ulcer or non-ulcerous dyspepsia has not yet been fully explored.

Several lines of evidence [17, 18] also suggest that gastric juice contains urea in the range of 3–5 mM but its potential link to the infection remains controversial. Moreover, the natural isotopic abundances of ^{13}C-urea [$^{13}C^{16}O(NH_2)_2$] as well as ^{18}O-urea [$^{12}C^{18}O(NH_2)_2$] in the gastric juice of positive and negative *H. pylori*-infected persons are also largely unknown. It therefore suggests a tantalizing hypothesis that utilization of natural isotopic urea in the gastric juice by the *H. pylori* and subsequently monitoring of ^{18}O and ^{13}C of breath CO_2 levels in response to the enzymatic activity may distinctively track the pathogenesis of peptic ulcer, non-ulcerous dyspepsia and *H. pylori* infection. Hence this may afford a new-generation and cost-effective UBT strategy without any orally administered ^{13}C-labelled external urea for treating the risk of developing ulcer or ulcer-related complications associated with *H. pylori* infection. But until now there has been no study to support such a hypothesis. Moreover, without oral ingestion of any ^{13}C-labelled urea, how the accurate diagnosis of *H. pylori* infection along with the actual disease state (i.e. peptic ulcer or non-ulcerous dyspepsia) might be possible has never been explored before. The reason for the missing information is mainly due to the lack of knowledge of the actual isotopic abundances of the ^{18}O and ^{13}C-urea in human gastric juice. New insight into the role of natural ^{13}C and ^{18}O-isotopic urea in gastric juice is therefore essential to elucidate the pathophysiology of *H. pylori* infection and to distinctively track the pathogenesis of peptic ulcer or functional dyspepsia.

In this study, we first report the natural isotopic abundances of ^{18}O and ^{13}C-urea in the gastric juice of *H. pylori* positive and negative patients together with the potential links between both ^{18}O and ^{13}C isotopes of breath CO_2 and the gastric pathogen *H. pylori.* We subsequently assessed the clinical validity and robustness of the new UBT without ingestion of any external isotopic urea in response to the standard eradication therapies of the infection and finally also determined several novel diagnostic parameters such as diagnostic cut-off values, sensitivity, specificity of ^{18}O and ^{13}C of breath CO_2 to gain a better insight into the diagnostic efficacy for the non-invasive assessment of peptic ulcer, non-ulcerous dyspepsia and *H. pylori.*

Materials and methods

Subjects

In this study, 145 individuals (83 male, 62 female; age 18–67 years), with different gastrointestinal disorders like gastritis, non-ulcer dyspepsia, and peptic ulcer, were enrolled after the initial screening. We categorized the enrolled individuals into the three distinct groups: non-ulcer dyspepsia (*H. pylori* positive, $n = 53$), peptic ulcer (*H. pylori* positive, $n = 57$) and *H. pylori* negative ($n = 35$) based on the reports of both invasive and non-invasive "gold-standard" tests i.e. endoscopy, biopsy-based rapid urease (RUT) and ^{13}C-urea breath test (^{13}C-UBT), respectively. For all the enrolled individuals, there was no contradiction between the test reports of *H. pylori* infection of both tests. In ^{13}C-UBT, individuals with the value of $\delta_{DOB}{}^{13}C$ (‰) ≥ 3 ‰ at 30 min were considered to be *H. pylori* positive [9, 10, 19]. Patients receiving antibiotics, proton pump inhibitors or H_2 receptor antagonists 4 weeks prior to the study were excluded at the initial screening. The Ethics Committee Review Board of AMRI Hospital, Salt Lake, Kolkata, India, approved the protocol of the current study (Study no.: AMRI/ETHICS/2013/1). The administration of S. N. Bose Centre, Kolkata, India, also approved the current study (Ref. no.: SNB/PER-2-6001/13-14/1769). All the patients gave their written consent prior to the study.

Collection of gastric juice

During the endoscopic examinations, ca. 10 mL of gastric juice from each enrolled subject was aspirated through the suction channel of the endoscope and collected in a mucus extractor inserted in the suction line. The gastric juice samples were then stored under −20 °C to maintain protein stability until analysis. Before analysis, the gastric juice samples were centrifuged at 10,000 rpm for 10 min to further remove the mucus and subsequently the filtrate parts were used for the Fourier transform infrared (FTIR) spectroscopy and integrated cavity output spectroscopy (ICOS) studies.

Preparations of chemical solutions

The protein, jack bean urease (Sigma Aldrich; EC 3.5.1.5), was purchased from Sigma Aldrich with the highest available purity. The ^{13}C-enriched urea was acquired from Cambridge Isotope Laboratory (CLM-311-GMP, Cambridge Isotopic Laboratories, Inc., USA) whereas all the other chemicals were procured from

Sigma Aldrich. All the chemicals were used without further purification. Milli-Q water was used to prepare the salt solutions. The aqueous solution (0.01 nM) of urease was prepared in citrate buffer (10 mM) at pH 7.0. The wide functionality range of citrate buffer was exploited to deal with the different pH (1–7.6) of the gastric juice samples. The standard solutions (5 mM) of both ^{12}C and ^{13}C-enriched urea were prepared in the same citrate buffer at pH 7.0. The pH measurements of different samples were made utilizing a standard pH meter (ecphtutor-ds).

FTIR study

FTIR spectroscopic measurements were carried out in a JASCO FTIR-6300 spectrophotometer using CaF_2 windows and a spacer thickness of 100 μm/200 μm in the mid-infrared region (1400–1500 cm^{-1}). For each measurement, 50 scans were acquired at 2 cm^{-1} resolution. All the spectra were collected taking the buffer solution as a background reference.

Isotopic determination of gastric juice urea

A portion of the filtrate gastric juice was equally divided into two closed round-bottom flasks filled with an inert atmosphere (N_2). The jack bean urease solution (0.01 nM) was then added into the gastric juice of one of the flasks, and the other flask contained only the gastric juice sample. After 10 min, an adequate amount of H_3PO_4 acid was injected into both flasks to remove the dissolved CO_2 from the sample to its headspace. The headspace CO_2 was thereafter collected from the individual flasks and analysed separately for the measurements of isotopic mole fractions of CO_2 using an isotope-sensitive high-precision off-axis ICOS as described in the following section. The actual isotopic mole fractions of CO_2, generated from the in vitro chemical hydrolysis of gastric juice urea in response to the external jack bean urease, were calculated after subtracting the CO_2 mole fractions of the other flask that originated from the bicarbonate which was initially present in the gastric juice sample. This eliminated the contributions from bicarbonates, and the isotopic mole fractions of CO_2 were utilized for the measurements of isotopic abundances of gastric juice urea. It is noteworthy that an adequate amount of NaOH solution was additionally mixed with the gastric juice samples of pH 1–3 to increase the pH to 7 before the addition of external urease and as a result the functionality of the urease remained intact in the gastric juice samples.

Integrated cavity output spectroscopy (ICOS)

A CO_2 isotope analyser (CCIA 36-EP, LGR, USA) exploiting off-axis ICOS was utilized for extremely precise measurements of $^{12}CO_2$, $^{13}CO_2$ and $^{12}C^{16}O^{18}O$ isotopes of CO_2 in a gas sample. The details of the ICOS system and its capability for high precision isotope measurements were described elsewhere [19–21]. Here we briefly described its salient features. It consists of a high finesse optical cavity comprising two high reflectivity mirrors ($R \sim 99.98$ %) yielding an optical path length of ca. 3 km. A laser operating at ca. 2.05 μm scans over a range of 20 GHz and records the absorption spectra of $^{12}C^{16}O^{16}O$, $^{13}C^{16}O^{16}O$ and $^{12}C^{18}O^{16}O$ at the wavenumbers of 4874.448 cm^{-1}, 4874.086 cm^{-1} and 4874.178 cm^{-1}, respectively. The aforementioned wavenumbers correspond to R(28), P(16) and P(36) ro-vibrational lines in the $(2,0^0,1)\leftarrow(0,0^0,0)$ vibrational combination band of the CO_2 molecule. The isotopic ratios were expressed in the typical $\delta^{13}C$ ‰ and $\delta^{18}O$ ‰ notation relative to the standard Vienna Pee Dee Belemnite (PDB) values of $(^{13}C/^{12}C)_{Standard} = 0.0112372$ and $(^{18}O/^{16}O)_{Standard} = 0.0020672$ as described by Eqs. (1) and (2), respectively:

$$\delta^{13}C = \left(\frac{(^{13}C/^{12}C)_{sample}}{(^{13}C/^{12}C)_{standard}} - 1\right) \times 1000 \quad (1)$$

$$\delta^{18}O = \left(\frac{(^{18}O/^{16}O)_{sample}}{(^{18}O/^{16}O)_{standard}} - 1\right) \times 1000 \quad (2)$$

The accuracy and precision of the CO_2 spectrometer were determined by utilizing three calibration standards from CIL (Cambridge Isotope Laboratory, USA) with known $\delta^{13}C$ values (−22.71 ‰, −15.5 ‰ and −9.69 ‰) and a standard NOAA air tank with known $\delta^{18}O$ value (−1 ‰) (Electronic Supplementary Material (ESM) Tables S1 and S2).

Breath sample collection

The patients enrolled in the study underwent ^{13}C-UBT within 1–2 days of the endoscopic examination. Prior to the breath test, every patient was instructed to wash his mouth repeatedly to avoid any kind of contamination arising from the oral cavity bacteria. In ^{13}C-UBT, after an overnight fasting (10–12 h) a baseline breath sample was collected in a breath bag (QUINTRON, USA, SL No.QT00892) 10 min after administration of 4 g citric acid dissolved in 200 mL of water. The test meal consisting of 75 mg ^{13}C-labeled urea (CLM-311-GMP, Cambridge Isotopic Laboratories, Inc., USA) in 50 mL of water was then administered and subsequently breath samples were taken at 15-min intervals for a period of 1 h. The design of the breath collection bags was such that the oral breath was first passed through a dead space and then the end tidal breath was captured in the 750-mL reservoir bags utilizing a one-way valve. In this study, we collected an empty stomach breath sample additionally prior to the ^{13}C-UBT.

The next day, to exploit the natural isotopic urea present in the gastric juice, an empty stomach breath was taken after the

overnight fasting (10–12 h) and 4 g citric acid dissolved in 200 mL of water was then administered. Consequently, breath samples were collected at 15-min intervals for 1 h. The isotope ratios of $^{13}C/^{12}C$ and $^{18}O/^{16}O$ in the exhaled breath samples were analysed using the ICOS technique as mentioned before.

Statistical analysis

To analyse the results, one-way ANOVA test was used for the normally distributed data whereas Mann–Whitney test and Kruskal–Wallis test were utilized for non-normally distributed data. A two-sided p value less than 0.05 was considered to be statistically significant. All data are presented as mean ± standard deviation (SD) except in Figs. 2a, b and 4c, d where mean ± standard error (SEM) are plotted. The receiver operating characteristics curve (ROC), drawn by plotting the sensitivity against (1 − specificity), was utilized to demonstrate different diagnostic cut-off values. The optimal diagnostic cut-off value represents the maximum sensitivity and specificity. All the statistical analyses were performed using Origin Pro 8.0 and Analyse-it method evaluation software (Analyse-it software Ltd, UK, version 2.30).

Results and discussion

To investigate the natural isotopic abundances of ^{12}C and ^{13}C-urea present in human gastric juice, we first studied the in vitro chemical reactions of human gastric juice in response to an external jack bean urease enzyme (Sigma Aldrich; EC 3.5.1.5) using FTIR spectroscopy. In the FTIR analyses, CN stretching vibrational frequencies at 1465 cm^{-1} and 1434 cm^{-1} [22, 23] were assigned to qualitatively examine the presence of ^{12}C and ^{13}C-urea, respectively, in the human gastric juice samples. The selected vibrational frequencies were also confirmed by utilizing the solutions of ^{12}C and ^{13}C-enriched pure urea substrates (ESM Fig. S1). In this in vitro investigation (Fig. 1a, b), we observed a significant decrease in peak intensity at different time intervals, demonstrating the possible chemical hydrolysis of urea present in gastric juice catalysed by external jack bean urease and thereby confirming the existence of the different isotopes of urea in the human gastric juice in a qualitative way.

To evaluate the precise abundances of individual isotopes, i.e. ^{12}C, ^{13}C and ^{18}O, of urea along with the total amount of urea present in the gastric juice, we analysed the gastric juice of positive and negative *H. pylori*-infected individuals by an isotope-selective high-resolution optical ICOS technique. In this unique approach, headspace CO_2, generated from the chemical breakdown of urea present in human gastric juice in response to the external jack bean urease, was exploited for the isotope specific measurements of gastric juice urea. In this study (Fig. 2a), we found statistically insignificant difference ($p > 0.05$) of total gastric juice urea between *H. pylori* positive (1.78 ± 0.3(SE) mM) and negative (2.68 ± 0.44(SE) mM) individuals. But, interestingly, a statistically significant difference ($p < 0.01$) of total gastric juice urea was observed between the subgroups of *H. pylori* positive individuals, i.e. between NUD (2.17 ± 0.37(SE) mM) and PUD (0.65 ± 0.20(SE) mM), whereas NUD was found to be statistically insignificant ($p > 0.05$) from *H. pylori* negative individuals (Fig. 2b). This result signifies that higher urease activity of PUD individuals eventually lowered the total gastric juice urea on average. Our study also sheds light on the contradictory results of previous reports [17, 18] in which specific disease states of the *H. pylori* infection had not been studied properly. However, the isotopic analyses (Fig. 2c, d) showed that there was statistically insignificant difference ($p > 0.05$) of isotopic abundances (%) between *H. pylori* positive and negative individuals irrespective of the isotopic nature of the gastric juice urea. Nevertheless, the main conclusive outcome of the isotopic analyses was that the abundances of the individual isotopes, i.e.

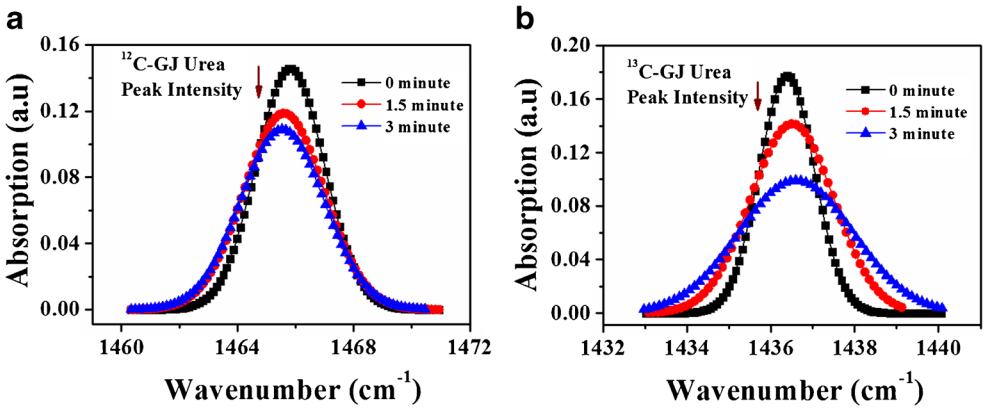

Fig. 1 FTIR study of gastric juice urea: **a**, **b** show reduction in peak intensity of urea with time in response to the external jack bean urease enzyme for ^{12}C and ^{13}C isotopes, respectively, exploiting C–N stretching vibrational frequencies. The decrease in peak intensity likely indicates the manifestation of the urea hydrolysis reaction. Larger volume of gastric juice has been exploited for ^{13}C isotope to achieve the reasonable peak intensity

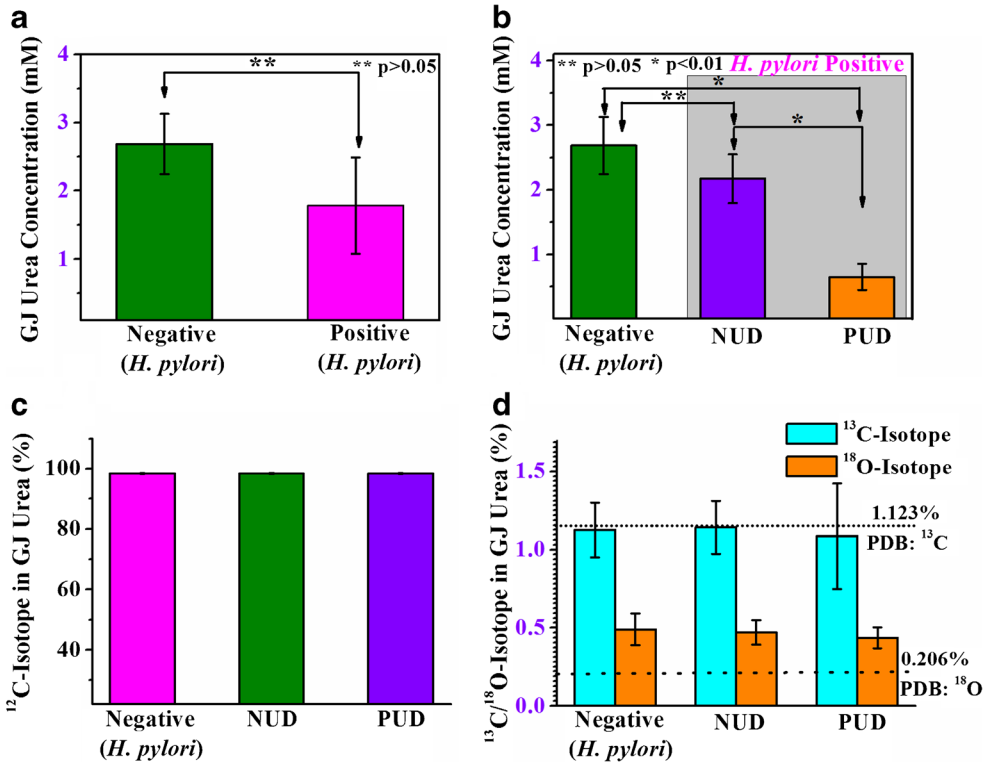

Fig. 2 Urea concentration and isotopic fractionations in gastric juice of NUD, PUD and *H. pylori* negative patients. **a** Total urea concentration in gastric juice of *H. pylori* positive and negative individuals. $p > 0.05$ indicates statistically insignificant difference between both the groups. **b** Total urea concentration in gastric juice of PUD can be statistically differentiated from NUD and *H. pylori* negative patients. **c**, **d** ^{12}C, ^{13}C and ^{18}O isotopic fractionations (%) of total urea content in gastric juice of PUD, NUD and *H. pylori* negative individuals. The abundances of ^{13}C and ^{18}O isotopes in gastric juice are comparable to those of the enriched international standard Pee Dee Belemnite (PDB)

^{12}C (~98.4 %), ^{13}C (~1.11 %) and ^{18}O (~0.45 %), of gastric juice urea were found to be sufficiently high enough to be utilized for non-invasive detection of *H. pylori* infection in conjunction with its urease activity.

To investigate how the ^{13}C and ^{18}O-isotopic fractionations of breath CO_2 changes without ingestion of any external ^{13}C-enriched urea but exploitation of natural isotopic urea present in the gastric juice by *H. pylori*, we studied the time-dependent excretion dynamics of both the isotopes in exhaled breath following ingestion of a citric acid-containing test meal for *H. pylori* negative ($n = 35$) and positive ($n = 110$) individuals with different gastrointestinal disorders such as peptic ulcers ($n = 57$) and non-ulcerous dyspepsia ($n = 53$). We monitored simultaneously $^{13}C^{16}O_2/^{12}C^{16}O_2$ and $^{12}C^{18}O^{16}O/^{12}C^{16}O^{16}O$ isotope ratios in exhaled breath samples associated with the enzymatic activities of urease in the citric acid-mediated bacterial environment using a laser-based high-resolution ICOS technique. The isotopic fractionations in breath were expressed as usual by the delta-over-baseline (DOB) with respect to the VPDB standard, i.e. $\delta_{DOB}{}^{13}C‰ = [(\delta^{13}C‰)_{t=t} - (\delta^{13}C‰)_{t=\text{basal}}]$ and $\delta_{DOB}{}^{18}O‰ = [(\delta^{18}O‰)_{t=t} - (\delta^{18}O‰)_{t=\text{basal}}]$. In this investigation (Fig. 3a, b), *H. pylori* positive subjects exhibited two notably distinct excretion kinetic profiles of both ^{18}O and ^{13}C in breath CO_2 depending on the state of the infection i.e. NUD and PUD during the 1-h breath excretion studies, while no significant enrichments of both the isotopic species were manifested for *H. pylori* negative individuals.

We observed a statistically significant differences of $\delta_{DOB}{}^{18}O‰$ and $\delta_{DOB}{}^{13}C‰$ values ($p < 0.001$) among the two subgroups i.e. NUD and PUD of *H. pylori*-infected positive individuals (Fig. 3c, d). In case of NUD patients, the higher amount of gastric juice urea as we experimentally observed (see Fig. 2b) in response to the urease enzyme secreted by *H. pylori* was possibly attributed to the isotopic enrichments of both $\delta_{DOB}{}^{13}C‰$ and $\delta_{DOB}{}^{18}O‰$ values, whereas for PUD patients a much lower amount of gastric juice urea was supposed to be responsible for the depletion of $\delta_{DOB}{}^{13}C$ ‰ and $\delta_{DOB}{}^{18}O‰$ values. In view of these results, cut-off ranges of -0.95 ‰ $\geq \delta_{DOB}{}^{13}C \geq 1.04$ ‰ and -0.96 ‰ $\geq \delta_{DOB}{}^{18}O \geq 1.01$ ‰ were calculated with 100 % sensitivity and specificity to diagnose the presence of *H. pylori* infection in this new UBT methodology. Taken together, these findings suggest that *H. pylori* has the ability to utilize the natural ^{13}C and ^{18}O-urea in the gastric juice and consequently the precise distinction between *H. pylori* infected and non-infected individuals is possible by monitoring ^{13}C and ^{18}O of breath CO_2; this reveals a missing link between *H. pylori* infection and ^{13}C

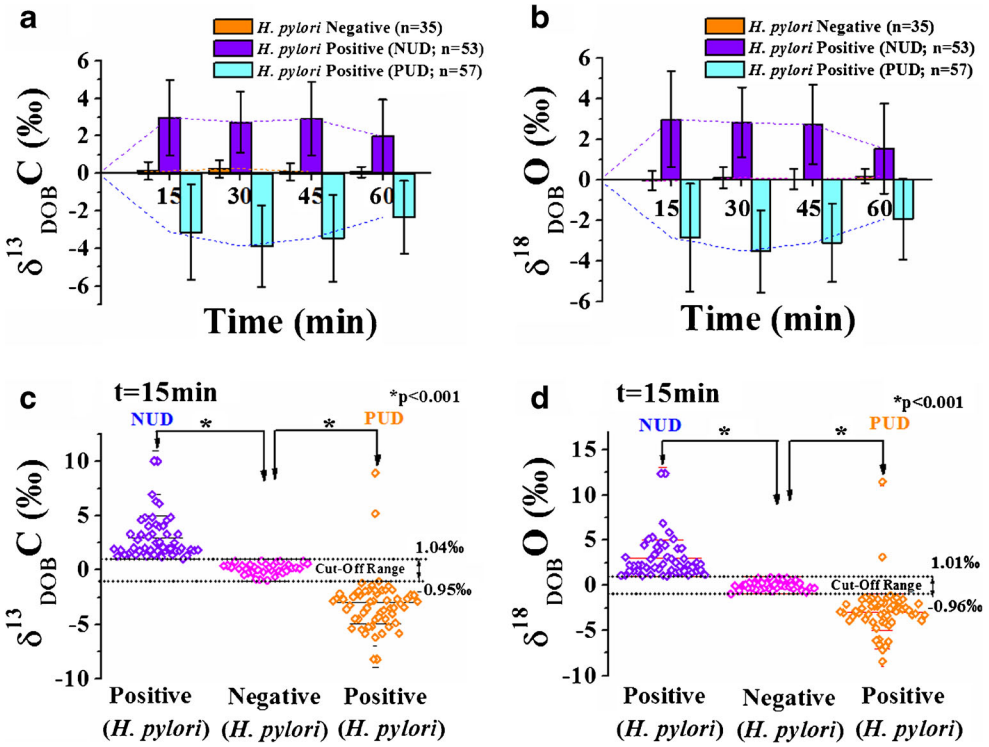

Fig. 3 Alterations in δ_{DOB} values for ^{13}C and ^{18}O in exhaled breath due to utilization of gastric juice urea by *H. pylori* in response to citric acid. **a** Change in $\delta_{DOB}{}^{13}C$ (‰) values in exhaled breath for NUD, PUD and *H. pylori* negative individuals. A significant enrichment and depletion is observed for NUD and PUD, respectively, while no significant change is observed for *H. pylori* negative patients. **b** Change in $\delta_{DOB}{}^{18}O$ (‰) values in exhaled breath for NUD, PUD and *H. pylori* negative individuals follows a similar trend as for $\delta_{DOB}{}^{13}C$(‰) values. **c**, **d** Statistical comparisons of $\delta_{DOB}{}^{13}C$(‰) and $\delta_{DOB}{}^{18}O$ (‰) values between the *H. pylori* positive and the *H. pylori* negative individuals. Cut-off ranges for the distinct diagnosis of *H. pylori* infection are demonstrated both for $\delta_{DOB}{}^{13}C$ (‰) and $\delta_{DOB}{}^{18}O$ (‰) values

and ^{18}O-istopic exchange in exhaled breath without oral administration of ^{13}C-labelled urea and hence may open a new UBT strategy to diagnose *H. pylori* infection. We have also confirmed the earlier hypothesis that the urease-catalysed hydrolysis of natural ^{13}C-urea and ^{18}O-urea in the gastric juice is strongly associated with the alteration of $^{13}C^{16}O_2$ and $C^{18}O^{16}O$ in breath samples.

On the basis of the two distinct excretion dynamics profiles of both $\delta_{DOB}{}^{13}C‰$ and $\delta_{DOB}{}^{18}O‰$ in individuals with *H. pylori* infection (Fig. 3a, b), we next explored whether the new UBT without the utilization of any external ^{13}C-labelled urea has sufficient efficacy to selectively track the actual disease state i.e. whether the subject has PUD or NUD associated with the *H. pylori* infection. We used ROC analysis to determine the optimal diagnostic cut-off values of $\delta_{DOB}{}^{13}C$ ‰ and $\delta_{DOB}{}^{18}O‰$ of the new UBT to selectively track the PUD and NUD. In the new UBT not including any ^{13}C-labelled urea, individuals with $\delta_{DOB}{}^{13}C‰ \geq 1.04$ and $\delta_{DOB}{}^{18}O$ $‰ \geq 1.01$ were considered to be NUD and these afforded a diagnostic sensitivity and specificity of 100 % (Fig. 4a, b). In contrast, $\delta_{DOB}{}^{13}C‰ \leq -0.95$ and $\delta_{DOB}{}^{18}O‰ \leq -0.96$ indicated PUD with ca. 96 % sensitivity and 100 % specificity and two false negative outcomes (Fig. 4a, b). We then critically assessed the false negative results and found significant enhancements (>1.5 ‰) of both $\delta_{DOB}{}^{13}C‰$ and $\delta_{DOB}{}^{18}O‰$ values in the exhaled breath as a direct consequence of high urea concentrations in the gastric juices of these PUD individuals (ESM Fig. S2). Furthermore, the detailed characteristic study of the specific state of the infection revealed that these individuals were suffering from peptic ulcer bleeding. We therefore speculate that blood urea may play a vital role in the enhancement of urea concentration in the gastric juice of these two PUD individuals. We further assessed clinical validity and widespread efficacy of the new UBT for eradication purposes. Figure 4c, d demonstrate the clinical feasibility of the new UBT to monitor the response to the standard antibiotic therapies and thus provide a new-generation methodology for the early detection and follow-up of patients after eradication of *H. pylori* infection. In view of these results, our findings point to new perspectives into the pathogenesis of the disease and the potential mechanisms linking breath ^{13}C and ^{18}O-isotopes to ulcer and non-ulcerous dyspepsia.

Finally, we addressed the missing link of the grey-zone problem in standard ^{13}C-UBT and the efficacy of the new UBT not including any ^{13}C-labelled urea to overcome this problem. After incorporating the empty stomach breath sample

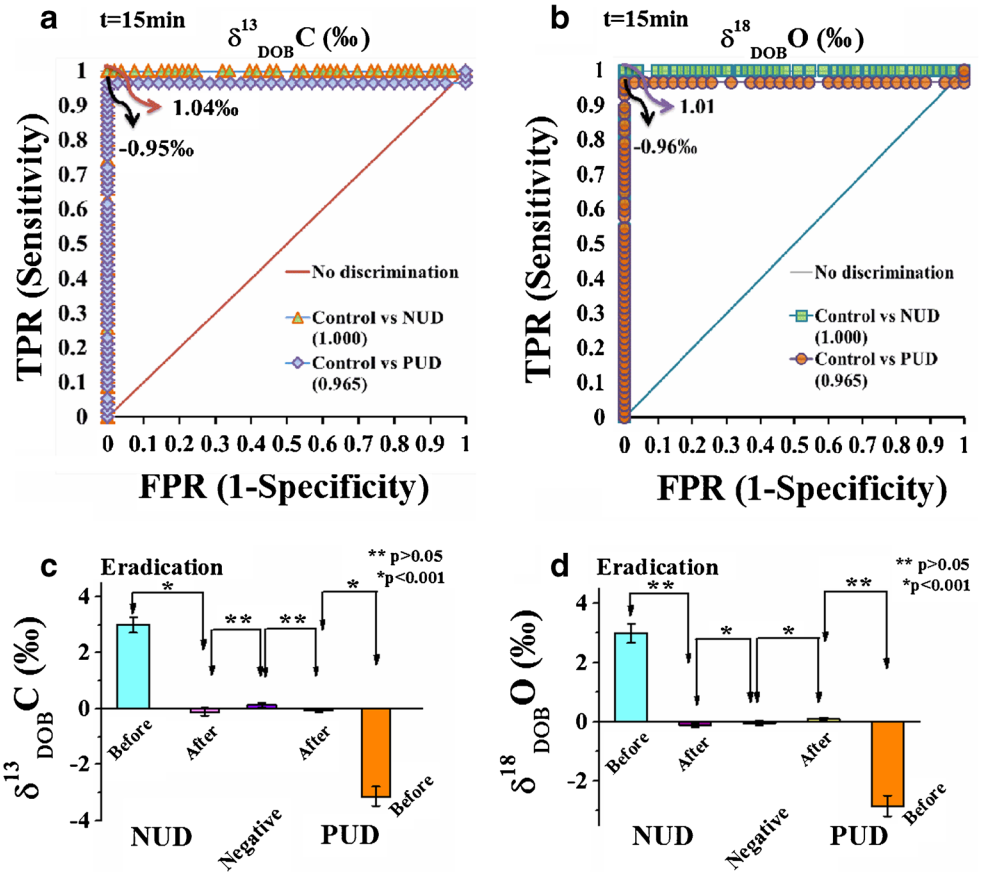

Fig. 4 Receiver operating characteristics (ROC) curve for determining diagnostic cut-off values. Viability of the new UBT before and after the eradication of the disease. **a**, **b** ROC analysis for the determination of cut-off values for the specific discrimination of PUD, NUD and *H. pylori* negative patients for $\delta_{DOB}{}^{13}C(‰)$ and $\delta_{DOB}{}^{18}O(‰)$, respectively. NUD patients could be well distinguished with 100 % sensitivity and specificity while PUD showed ca. 96 % sensitivity and 100 % specificity. **c**, **d** Comparison of the values of $\delta_{DOB}{}^{13}C(‰)$ and $\delta_{DOB}{}^{18}O(‰)$ before and after the eradication therapy depicting the absence of the infection after the therapy with no significant difference to the *H. pylori* negative group

into the standard ^{13}C-UBT protocol where ^{13}C-enriched urea was administered 10 min after ingestion of citric acid, we observed (Fig. 5) a significant enrichment of $\delta_{DOB}{}^{13}C‰$ value for NUD within the 10 min of acidification of the bacterial environment through the administration of citric acid and thereby indicating that *H. pylori* already started to utilize the gastric juice urea to alkalize the medium. Now, administration of ^{13}C-labelled urea thereafter may eventually show less enrichment of $\delta_{DOB}{}^{13}C‰$ value for the on-set of the infection and thus give an inconclusive result or grey-zone problem. Therefore, the new UBT, exploiting only gastric juice urea, provides great promise for a better, more robust and non-toxic global methodology compared with the existing ^{13}C-UBT for precise detection of the *H. pylori* infection. Moreover, several earlier pieces of evidence [11–13] demonstrated that numerous urease-containing microorganisms other than *H. pylori* are present in the human oral cavity possessing strong urease activity. Therefore the observations of the false positive results of the existing ^{13}C-UBT are likely to be the effects of the

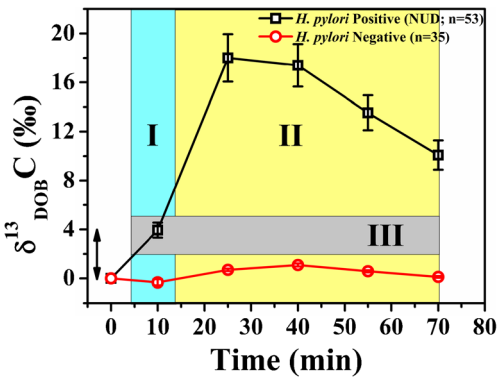

Fig. 5 Effect of gastric juice urea in the standard ^{13}C-UBT protocol. The figure shows the increment in the $\delta_{DOB}{}^{13}C(‰)$ value for NUD within 10 min of ingestion of citric acid where the endogenous urea in gastric juice is utilized by the *H. pylori* and therefore causes the grey-zone problem for the onset of the infection. 0 min denotes the empty stomach breath sample i.e. before the ingestion of citric acid. The entire excretion dynamics of ^{13}C-UBT is divided into two regions with region *I* demonstrating the effect of citric acid and region *II* illustrating the effect of ^{13}C-labelled urea, whereas the *grey region* (*III*) indicates the grey zone of ^{13}C-UBT

urease activity by the urease-positive non-*H. pylori* bacteria in response to the orally administered ^{13}C-enriched external urea.

Conclusion

Our findings suggest that devoid of any external ^{13}C-enchried urea, the new UBT strategy exploiting the natural ^{18}O and ^{13}C-urea in human gastric juice selectively reveals the specific disease state (i.e. whether it is a peptic ulcer or non-ulcer) and allows the correct diagnosis of the *H. pylori* infection with unprecedented diagnostic sensitivity and specificity. Consequently, our new UBT overcomes all the existing issues that the existing ^{13}C-UBT often fails to do. Nonetheless, the direct exploitation of gastric juice urea in the new UBT methodology should enhance our ability to devise new and better approaches to treat the deleterious effects of all these common diseases. The new knowledge is also fostering exploration in our understanding of the relationship between peptic ulcer disease, non-ulcerous dyspepsia and *H. pylori* infection.

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Author contributions Manik Pradhan arranged the funding; Manik Pradhan and Abhijit Maity conceived the study; Manik Pradhan, Sujit Chaudhuri supervised the whole study; Manik Pradhan and Abhijit Maity designed the study; Abhijit Maity, Mithun Pal, Suman Som and Sanchi Maithani collected and analysed the samples; all authors drafted the manuscript and critically reviewed it.

Compliance with ethical standards The present study has been approved by the Ethics Committee Review Board of AMRI Hospital, Salt Lake, Kolkata, India (Study no.: AMRI/ETHICS/2013/1). All the patients provided their written consent prior to the study.

Conflict of interest The authors declare no conflict of interests.

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