Modification of baseline status to improve breath tests performance

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Breath tests used to evaluate carbohydrates malabsorption require baseline H2 and CH4 levels as low as possible. Test cancellation is recommended when exceeding certain cut-offs (H2 ≥ 20 ppm and CH4 ≥ 10 ppm). Although following preparation protocols, many patients have baseline levels above those cut-offs. We investigated if light walking can reduce baseline H2 and CH4 levels. We retrospectively analyzed baseline H2 and CH4 levels from 1552 breath tests. Baseline levels (B1), especially in H2, were lower when obtained at later hours of the day. In those with baseline levels above cut-off, re-sampling (B2) after light walking for one hour, decreased H2 levels 8 ppm (Q1–Q3: 1–18 ppm), and 2 ppm (Q1–Q3: 0–3 ppm) for CH4. Consequently, 40% of tests with elevated B1 levels, presented B2 levels below mentioned cut-offs. Ten percent of tests considered negative when using B1 for calculations, turned positive when using B2 instead. All positive tests when using B1 values, remained elevated when using B2. Re-sampling after light walking for one hour could allow test performance in those with previous elevated baseline levels, avoiding diagnosis delays. Using the second sample for delta calculations identifies positive patients for malabsorption that would have been considered negative.

Malabsorption of carbohydrates can provoke several gastrointestinal symptoms including diarrhea, bloating, flatulence and abdominal pain due to intestinal fermentation of these non-absorbed sugars. Multiple pathologies can lead to this malabsorption, such as small intestine bacterial overgrowth (SIBO)1, lactase enzyme deficiency causing lactose malabsorption2, or the overload of fructose intestinal transporters (GLUT-2 and GLUT-5) causing fructose malabsorption3.

Currently, diagnosis of carbohydrates malabsorption is mainly based on breath tests. These null invasiveness tests consist on an oral load with the corresponding sugar and the monitoring of the altered absorption by measuring the fermentation gases (H2 and CH4) in exhaled air. An increase in H2 and/or CH4 levels reflects the intestinal fermentation of non-absorbed sugars. Rise in H2 or in CH4 levels depends on the type of anaerobic bacteria present. Although more prevalent intestinal bacteria produce H2, some patients present methanogenic bacteria that consume this H2 to produce CH4. As a consequence, both H2 and CH4 are recommended to be analyzed4.

To avoid false negative results, H2/CH4 baseline levels should be low5,6 and interpretation of elevated baseline levels is still uncertain7. We have previously shown that baseline high H2 levels are associated with more positive lactose breath test8. For this reason, patient preparation is required1,9, and it includes avoiding drugs such as antibiotic or laxatives, in the previous days, or procedures that could alter colonic microbiota such as colonoscopy. Besides, in the 24 h before the test, patient must follow a diet without fermentable fibers10, and the test must be performed in fasting state. In fasting conditions, baseline H2 levels are usually 7 ± 5 ppm11. However, elevated baseline levels are found in a significant number of patients due to different causes, such as SIBO, transgression in patient preparation, or constipation in the case of CH412,13. For example, Kumar et al.14 showed that patients with irritable bowel syndrome have a baseline H2 concentration double than healthy population, while constipation correlate with baseline CH4 higher than 10 ppm15. In fact, some guidelines recommend not to continue the test if baseline levels exceed certain cut-offs ranging 15–20 ppm for H25,7. This results in a delay of analytical results and diagnosis, and consequently in a detriment of patients. However, a recent survey stated that despite recommendations breath tests are still executed with a wide variety of conditions both in performance and in interpretation16. Although the requirement for baseline CH4 levels as low as possible also applies to CH4, guidelines do not clearly include a specific cut-off value5,7.

With the aim to decrease the number of high baseline H2 and/or CH4 levels detected during the performance of breath tests, we evaluated the effect of the time of procedure and a previous light walk.

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Results

Basal breath test and patients characteristics. Median baseline levels of \( H_2 \) were 6 ppm (Q1–Q3: 3–11 ppm) and 4 ppm (Q1–Q3: 2–10 ppm) for \( CH_4 \). Considering the baseline cut-offs for \( H_2 \) and \( CH_4 \) of 20 and 10 ppm respectively, 11% of tests presented an elevated baseline \( H_2 \) level and 25% an elevated baseline \( CH_4 \) level. It should be noted that in 2% of tests, both \( H_2 \) and \( CH_4 \) were above their corresponding cut-offs (Table 1).

Regarding the age of patients, we observed higher baseline \( H_2 \) levels in tests performed in underage patients (9 ppm; Q1–Q3: 4–17 ppm) than those in patients over 18 years (5 ppm; Q1–Q3: 3–10 ppm; \( p < 0.01 \); Table 1). On the contrary, in the case of \( CH_4 \) levels were higher in adults (5 ppm; Q1–Q3: 2–11 ppm) than in underage patients (4 ppm; Q1–Q3: 2–7 ppm; \( p < 0.05 \)). These differences were also reflected in the percentage of tests with baseline levels above the cut-off. In the case of \( H_2 \), elevated baseline levels were observed in 22% of tests performed by underage patients, while this percentage decreased to 8% (\( p < 0.01 \)) in those performed by older patients. Tests with elevated \( CH_4 \) baseline levels were 27% of tests in patients above 18 years and only 16% of tests in underage patients (\( p < 0.01 \)).

When considering the sex of patients, no differences were observed in baseline \( H_2 \) levels or in the percentage of tests with baseline levels above the cut-off (Table 1). Also, female patients had slightly higher \( CH_4 \) levels (5 ppm, Q1–Q3: 3–11 ppm) than male patients (4 ppm; Q1–Q3: 2–8 ppm; \( p < 0.01 \)). Accordingly, we observed a higher percentage of tests with elevated baseline \( CH_4 \) in female (28%) than in male patients (19%; \( p < 0.01 \)).

Basal breath test and time of the day. We also investigated the influence of the time of the day in baseline levels. We observed that baseline \( H_2 \) levels changed with the hour of testing (Fig. 1a), being significantly higher (\( p < 0.01 \)) in those performed before 10 am (6 ppm, Q1–Q3: 3–12 ppm) than those performed later (5 ppm, Q1–Q3: 2–9 ppm). Same occurred for \( CH_4 \) levels (Fig. 1b), with higher values in tests performed in the early hours (5 ppm, Q1–Q3: 3–10 ppm) than in those performed after 10 am (4 ppm, Q1–Q3: 2–9 ppm).

As mentioned before, 240 patients performed multiple breath tests in different days. The range for baseline \( H_2 \) levels in each patient among different days presented a median value of 4 ppm (Q1–Q3: 2–8 ppm), higher than 2 ppm observed for \( CH_4 \) (Q1–Q3: 1–4 ppm; \( p < 0.01 \) Fig. 2a). The difference observed in \( H_2 \) baseline levels did not depend on the hour of the day the sample was taken in or how distance were the different sampling hours (Fig. 2b). Median coefficient of variation of baseline levels among patients was 57% (Q1–Q3: 28–88%) for \( H_2 \) and 35% (Q1–Q3: 16–71%) in the case of \( CH_4 \).

Table 1. Baseline \( H_2 \) and \( CH_4 \) levels according to age and sex of patients, indicated as median and interquartile range (Q1–Q3) is shown in a bracket. Baseline samples classification according to \( H_2 \) and \( CH_4 \) levels compared to their corresponding cut-offs. \( H_2 \) high means ≥ 20 ppm, \( CH_4 \) high means ≥ 10 ppm, \( H_2 \) low means < 20 ppm and \( CH_4 \) low means < 10 ppm. Comparing with patients above 18 years, * represents \( p < 0.01 \) and ~ represents \( p < 0.05 \). # represents \( p < 0.01 \) compared with female patients.

|               | Median baseline levels ppm (Q1–Q3) | Baseline samples                                                                 |
|---------------|-------------------------------------|----------------------------------------------------------------------------------|
|               | \( H_2 \) | \( CH_4 \) | \( H_2 \) high | \( CH_4 \) high | \( H_2 \) low/\( CH_4 \) low | \( H_2 \) high/\( CH_4 \) high | \( H_2 \) low/\( CH_4 \) high | \( H_2 \) high/\( CH_4 \) high |
| Age ≥ 18      | 5 (3–10) | 5 (2–11) | 101 (8%) | 340 (27%) | 837 (67%) | 77 (6%) | 316 (25%) | 24 (2%) |
| Age < 18      | 9 (4–17) * | 4 (2–7) ~ | 65 * (22%) | 48 * (16%) | 196 (66%) | 54 (18%) | 37 (12%) | 11 (4%) |
| Sex Female    | 6 (3–12) | 5 (3–11) | 106 (11%) | 252 (28%) | 628 (64%) | 80 (8%) | 252 (26%) | 26 (3%) |
| Sex Male      | 5 (3–10) | 4 (2–8) # | 60 (11%) | 110 # (19%) | 404 (72%) | 51 (9%) | 101 (18%) | 9 (2%) |
| Total         | 6 (3–11) | 4 (2–10) | 166 (11%) | 388 (25%) | 1033 (67%) | 131 (8%) | 353 (23%) | 35 (2%) |

Figure 1. Baseline levels of \( H_2 \) (a) and \( CH_4 \) (b) according to sampling hour. Baseline levels are represented in Box-Whisker plots.
This variation observed in baseline H₂ levels obtained in different days, resulted in that 16% of patients presented an H₂ baseline level below cut-off in one test and above that cut-off in another test performed in a different day (p<0.01).

**Light walking and basal breath test.** When baseline levels exceed established cut-offs, we obtained a new sample after 60 min of light walk. We performed this re-sampling in 158 tests, 90 (57%) due to high H₂ levels, 39 (25%) due to high CH₄ levels and 29 (18%) because of an elevation of both gases (Table 2). This re-sampling resulted in the decrease of baseline H₂ levels from an initial median of 25 ppm (Q1–Q3: 19–34 ppm) to 15 ppm (Q1–Q3: 6–20 ppm) (Fig. 3a; p<0.01). When considering CH₄ levels, we observed a decrease from 9 (Q1–Q3: 5–22 ppm) to 7 ppm (Q1–Q3: 4–20 ppm) (Fig. 3b; p<0.01). Median decrease in baseline H₂ levels was 8 ppm (Q1–Q3: 1–18 ppm), higher than the observed in the case of CH₄ (median: 2 ppm, Q1–Q3: 0–3 ppm, p<0.01; Fig. 3c). If patient age was considered, significant differences were observed in H₂ reduction (Fig. 3d), being much lower in the patients above 18 years (5 ppm; Q1–Q3: 0–14 ppm) than in underage patients (15 ppm; Q1–Q3: 7–23 ppm; p<0.01).
These decreases in baseline levels after re-sampling resulted in that 67% of samples with initial high H\textsubscript{2} levels, had a second baseline below 20 ppm cut-off (Table 2). In the case of CH\textsubscript{4}, only 12% decreased CH\textsubscript{4} levels below 10 ppm cut-off after 1 h. As a whole, after baseline sample re-drawing, baseline levels below both cut-offs were observed in 40% of samples, 21% maintained elevated H\textsubscript{2} levels, 33% elevated CH\textsubscript{4} levels and 6% kept both H\textsubscript{2} and CH\textsubscript{4} levels elevated. These frequencies differed significantly from those observed in the initial measurement (p < 0.01).

Breath test performance after baseline repetition. In 126 tests with initial baseline (B1) levels above cut-off, after light walking and re-sampling (B2), tests continued with stimulus intake. If first baseline B1 was considered for delta calculations, H\textsubscript{2} breath test was negative in 73% of the tests. However, 10% of these turned positive when delta was calculated with the repeated baseline B2 (Table 3). On the contrary, all the tests positive using B1, remained elevated when using B2 for calculations. This differences in classification reached statistical significance (p < 0.01).

Table 3. Performance of breath tests with baseline re-sampling. Tests were classified according to the presence of an increase in H\textsubscript{2} or CH\textsubscript{4} levels after stimulus intake, depending on whether first baseline of repeated baseline were considered for delta calculations. Increase in H\textsubscript{2} levels was considered when ≥20 ppm and in CH\textsubscript{4} levels when ≥10 ppm.

|       | Positive test using the first baseline | Positive test using the repeated baseline | P     |
|-------|----------------------------------------|------------------------------------------|-------|
|       | No                                     | Yes                                      |       |
| H\textsubscript{2} | No                                     | 83                                       | 9     | <0.01 |
|       | Yes                                    | 0                                        | 34    |       |
| CH\textsubscript{4} | No                                     | 96                                       | 8     | <0.01 |
|       | Yes                                    | 6                                        | 16    |       |

Table 2. Baseline sample classification depending on initial and repeated baseline levels compared to the corresponding cut-offs (H\textsubscript{2} ≥ 20 ppm; CH\textsubscript{4} ≥ 10 ppm). H\textsubscript{2} high means ≥ 20 ppm, CH\textsubscript{4} high means ≥ 10 ppm, H\textsubscript{2} low means < 20 ppm and CH\textsubscript{4} low means < 10 ppm.
irritable bowel syndrome patients and not in suspected for carbohydrates malabsorption. Contrary to $H_2$, fasting $CH_4$ levels were higher in adult patients than in underage patient. This can be a consequence of the higher prevalence of constipation among adults\cite{20}, which has been related to $CH_4$ production, and other intestinal alterations such as diverticulosis\cite{21}. We can reject the confounding effect of smoking in the results as patients were instructed not to smoke in the day of test performance and previous smoking effect in $H_2$ and $CH_4$ levels disappears after 10–15 min\cite{22,23}.

Concerning time of day, we observed that $H_2$ levels fluctuated more than $CH_4$ levels, with higher levels earlier in the morning. To check if this was just a consequence of interindividual variability we focused in those patients with multiple tests in different days. We found that $CH_4$ was much more stable than $H_2$, contrary to that described by Jonderko et al\cite{24}. This could be due to sample size differences since our study comprised 240 patients compared to only 12 of Jonderko et al. In any case, $H_2$ range across different days was reduced (although not significantly) when looking in those tests performed at same time of day (less than 60 min apart), suggesting that at least some of the variability observed can be attributed to the time of the day the test is performed. Shibata et al\cite{25} found in healthy controls slightly higher levels (an increase of 2 ppm) of fasting $H_2$ in the morning than before sleep the previous night. Related to this, we should consider that during the night there is a nocturnal hypoventilation that can cause these slightly elevated fasting levels of breath $H_2$ produced by persisting fermentable substrates in the colon\cite{26}.

Vigorous exercise is not recommended prior breath tests because it can alter ventilation rate and subsequently exhaled $H_2$ and $CH_4$ and its relation to $CO_2$\cite{27}. However, our indication is to go for a walk, light enough to not alter ventilation rate before and during the test. Even more, there was a minimum 5 min delay between patient returns and repeated sampling, and a visual inspection of potential hyperventilation. This light walk resulted in a reduction of baseline levels (mainly in $H_2$ levels) that allowed to perform 40% of these tests. Interestingly, all tests in which only $CH_4$ baseline levels were elevated, $CH_4$ continued also elevated in the repeated baseline sample. Consequently, it seems unnecessary to perform the light walk and resampling when only $CH_4$ is elevated in baseline samples.

The decrease in $H_2$ levels after the light walk and the influence of time of day may be indeed related, as light activity could induce intestinal motility and feces stirring causing the release of preformed $H_2$ trapped in the feces\cite{28,29}. After one hour, this $H_2$ would have been already released and the baseline levels would reflect more precisely the baseline state.

To date, it is not yet clear if elevated $H_2$ baseline levels are related with diet transgressions, reflect SIBO presence\cite{30,31}, or even pancreatic alterations such as exocrine insufficiency\cite{32} or pancreatic duct stenosis\cite{33}. Although our protocol includes oral washing to avoid oral microbiota interference and patients are also questioned about potential diet transgressions that could affect breath performance, we have found that elevated baseline $H_2$ levels were quite frequent (11%). However, they are critical since (i), when occurred, test cancelation is recommended and diagnosis is delayed and (ii), in the case of continuing, they can conceal a higher increase in $H_2$ levels than those reflected in delta calculations, and thus lead to false negative results. In fact, we have showed that in those tests with high baseline levels and negative result, 10% of them would turn positive if the delta value were calculated with the repeated baseline level after light walking instead. However, all tests with high baseline levels and positive result would remained positive when repeated baseline was used to calculate the delta value.

In summary, breath status can be modified in patients by light walking, which reduces $H_2$ and $CH_4$ baseline levels. This allows tests continuation and avoids tests re-scheduling and diagnosis delays in a significant percentage of patients.

Methods

Patients. We retrospectively analyzed 1552 breath tests, from 1305 patients (Table 4), 756 (49%) fructose breath test and 796 (51%) lactose breath test. Of these, 232 (18%) performed two breath tests in different days and 8 (0.5%) performed three breath tests. Those tests were performed with a median of 9 days apart (Q1–Q3:
Female patients performed 64% of the tests studied. Median age was 36 years (Q1–Q3: 20–48). Patients younger than 18 years were considered underage, and performed 298 tests (19%). All methods were performed in accordance with the relevant guidelines and regulations. The need of informed consent was waived and the study approved by Clínica Universidad de Navarra Ethic’s committee (project number 2020.222).

**Breath tests performance.** Patients were required to perform the test in fasting and without having smoked that day. They had to accomplish a diet the previous day without fermentable fibers, lactose or fructose. Adherence to prescribed diet was checked before the beginning of the test. Fructose and lactose breath tests were not performed if patients had recently received antibiotics or laxatives or procedures such as colonoscopy. Prior to breath sampling, adults patients rinsed their mouths with a 10 mL of commercial oral antisepsic solution containing chlorhexidine. In underage patients rinses were performed with water. The stimulus were 25 g of fructose or lactose, respectively. In the case of pediatric patients, dose was adjusted to 1 g/kg with 25 g as the maximum dose.

Breath baseline samples and each 30 min for three hours after stimulus intake were obtained and 20 mL of them were analyzed for H₂ and CH₄ levels (ppm) in a Breath Tracker SC (Quintron, Milwaukee, USA). CO₂ levels (in %) were also assayed simultaneously in the same analyzer to evaluate potential contamination of samples with ambient air. If any sample presented CO₂ < 2%, sample results were rejected and a new sample was obtained. The breath analyzer was daily calibrated and verified before processing any sample, using the gas calibrator provided by manufacturer. Our data indicate that imprecision and bias were respectively 3.1% and 4.7% for H₂ measurement, and 3.4% and 4.1% for CH₄.

According to guidelines, breath test lacks of reliability if baseline H₂ exceed 20 ppm. If baseline samples (B1) rendered H₂ above 20 ppm, a new baseline sample should be obtained. According to our own protocol and in order to achieve levels below that threshold, patients were indicated to go for a light walk outdoors for one hour with no goal in terms of distance. Although no specific cut-off is established for CH₄ in guidelines, our protocol order to achieve levels below that threshold, patients were indicated to go for a light walk outdoors for one hour.

Prior to breath sampling, adults patients rinsed their mouths with a 10 mL of commercial oral antiseptic solution. Breath tests performance. Patients were required to perform the test in fasting and without having smoked that day. They had to accomplish a diet the previous day without fermentable fibers, lactose or fructose. Adherence to prescribed diet was checked before the beginning of the test. Fructose and lactose breath tests were not performed if patients had recently received antibiotics or laxatives or procedures such as colonoscopy. Prior to breath sampling, adults patients rinsed their mouths with a 10 mL of commercial oral antiseptic solution containing chlorhexidine. In underage patients rinses were performed with water. The stimulus were 25 g of fructose or lactose, respectively. In the case of pediatric patients, dose was adjusted to 1 g/kg with 25 g as the maximum dose.

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**Data analysis.** Data were statistical analyzed with SPSS v20 software (IBM). Data are indicated as median and interquartile range (Q1–Q3). According to the normality tests performed (Shapiro–Wilk and Kolmogorov–Smirnov), both initial and repeated H₂ and CH₄ baseline levels followed non-normal distributions. Therefore, comparison between different patients were performed with Mann–Whitney U test whereas comparison between samples of the same patient was performed with Wilcoxon signed rank test. Frequencies were compared with χ² test. *P* < 0.05 was considered as statistically significant.

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**Author contributions**

E.A. and A.G. have made substantial contributions to conception and design, acquisition of data, analysis and interpretation of data. A.S, S.C. and S.D. were involved in acquisition of data, analysis and interpretation of data. E.A. and A.G. have made substantial contributions to conception and design, acquisition of data, analysis and interpretation of data. A.S, S.C. and S.D. were involved in acquisition of data, analysis and interpretation of data. E.A. and A.G. have been involved in drafting the manuscript or revising it critically for important intellectual content. All authors have given final approval of the version to be published. All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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