^{13}C-LABELLED BREATH TESTS IN MALABSORPTION

STUDIES OF PATIENTS WITH COELIAC DISEASE

Kari Tveito

Lovisenberg Diakonale Sykehus

Faculty of Medicine

University of Oslo

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Kari Tveito

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1 ABSTRACT

Breath tests have been widely used in gastroenterology for several decades. The most well known test nowadays is perhaps the ^{13}C-urea breath test for Helicobacter Pylori. Isotope-labelled breath test can, however, be applied in a wide range of conditions. The main aim of this thesis has been to determine the usefulness of ^{13}C-labelled substances to diagnose small intestinal malabsorption. To this end, we have focused on coeliac patients, since coeliac disease is one of the most common causes of small intestinal malabsorption in the industrialised part of the world today.

The main objective of the first study of this thesis was to determine the diagnostic accuracy of the 13C-D-xylose breath test in diagnosis and follow-up of coeliac disease. Two cohorts of coeliac patients were reviewed retrospectively. In one cohort patients had been tested with a 14C-D-xylose breath test before and after gluten-free treatment (GFD). In the other patients had been tested with a 13C-D-xylose breath test before and after GFD. Results were similar in the two cohorts, and confirmed that the non-radioactive isotope ^{13}C-D-xylose can replace the radioactive isotope ^{14}C-D-xylose without loss of diagnostic accuracy. Furthermore, we developed a diagnostic index for the D-xylose breath tests in which $^{13}CO_2$ or $^{14}CO_2$ levels in breath at 30 minutes were combined with levels in breath at 210 minutes in a gas fraction index: 30 minutes value/210 minutes value. Using this diagnostic index, we found sensitivities of 84-95% and specificities of 87-94% with the two breath tests. After treatment with a gluten-free diet, most patients improved their gas fraction index, but did not reach the levels of healthy control subjects.

In the second study we compared the ^{13}C-D-xylose breath test to measurements of D-xylose in plasma and urine in untreated coeliac patients and treated coeliac patients. Test sensitivity and specificity for coeliac disease versus healthy controls were 88% and 84% with the ^{13}C-D-xylose breath test, 65% and 71% with a 1-h plasma D-xylose test, and 55% and 74% with a 4-h urine D-xylose excretion test. Breath test results improved significantly in the treated coeliac group compared to untreated patients, but were not normalized compared to healthy control subjects.

The aim of the two last studies was to develop a novel ^{13}C-sorbitol breath test and determine the diagnostic accuracy of the test for coeliac disease. We performed a prospective study of coeliac patients who were tested at diagnosis before commencing on a gluten-free diet and after approximately one year on GFD. Coeliac patients were compared to a group of patients with similar gastrointestinal symptoms without coeliac disease and to healthy control subjects. All study subjects performed the H_2-sorbitol breath test concomitant with the ^{13}C-sorbitol breath test. We found that the ^{13}C-sorbitol breath test duration could be shortened to one-hour without loss of diagnostic precision versus a four-hour test. We proposed a diagnostic algorithm to divide patients into high, moderate and low-risk groups for coeliac disease. ^{13}C-sorbitol breath test results increased in treated coeliac patients, but did not reach levels of healthy control subjects. The H_2-sorbitol breath test had unsatisfactory specificity versus control groups.

We concluded that ^{13}C-labelled breath tests are sensitive tools for small intestinal malabsorption. Further studies are needed to determine how ^{13}C-labelled breath tests can be of value in follow-up of coeliac disease.

2 ABBREVIATIONS

^{13}C-SBT	^{13}C-sorbitol breath test
GC	gas chromatography
GFD	gluten-free diet
GF-index	gas fraction index
HLA	human leukocyte antigen
HPLC	high performance liquid chromatography
H_2-SBT	H_2-sorbitol breath test
IEL	intraepithelial lymphocyte
Ig	immunoglobulin
IRMS	isotope ratio mass spectrometry
tTGA	tissue-transglutaminase antibody
SIBO	small intestinal bacterial overgrowth

3 INTRODUCTION

3.1 Coeliac disease

Coeliac disease is a chronic inflammatory disease of the small intestine induced by ingestion of proline-rich and glutamine-rich gluten proteins in wheat, rye and barley in genetically susceptible people [1]. The first description of the disease dates from the 1st and 2nd centuries CE [2]. The disorder is characterized by a diverse clinical heterogeneity that ranges from asymptomatic to severely symptomatic. Patients with coeliac disease have increased morbidity due to the frequent association with autoimmune disorders [3-9], and increased mortality resulting from the emergence of T-cell clonal proliferation that predispose the patient to enteropathy-type T-cell lymphoma [10-12]. A strict gluten-free diet is the mainstay of safe and effective treatment.

3.1.1 Genes and environment

Coeliac disease is strongly linked with HLA-DQ genes. Most patients carry a variant of DQ2 and others carry a variant of DQ8 [13]. The association between HLA genes and coeliac disease is very strong compared with other HLA-linked diseases [14]. However, other non-HLA regions must be involved as well, since the concordance rate between HLA identical siblings is much lower than between monozygotic twins who show a concordance rate of 85% [15, 16].

Coeliac disease is induced in genetically susceptible people by gluten and possibly other environmental cofactors. Intestinal infections may be a trigger for coeliac disease, perhaps because an infection causes a transient rise in small-bowel permeability and

release of tissue transglutaminase that enhances gluten immunogenicity [17, 18]. Some drugs can enhance a person's susceptibility to gluten - such as a course of interferon alpha [19]. Furthermore, infant-feeding practices and whether infants are introduced to dietary gluten while still being breastfed might affect the risk of developing coeliac disease [20, 21].

3.1.2 Pathophysiology

After crossing the epithelium into the lamina propria, gluten peptides are deamidated by tissue transglutaminase and presented by DQ2+ or DQ8+ antigen-presenting cells to pathogenic CD4+ T cells. Activated CD4+ T cells drive a T-helper type 1 response that leads to the development of the coeliac lesion. The severity of the lesion - which can range from intraepithelial and lamina propria infiltration of inflammatory cells with normal architecture to crypt hyperplasia and varying degrees of villous atrophy to completely flat mucosa – can be graded by the Marsh classification, as modified by Oberhufer and colleagues [22].

3.1.3 Epidemiology

The accuracy of estimates of the true prevalence of coeliac disease has been substantially improved by the increased reliability of serological tests. The IgA antigliadin antibodies used initially had low sensitivity and specificity [23-25], and the antiendomysial antibody test significantly improved the diagnostic accuracy. An important break-through came with the discovery that antiendomysial antibodies detect tissue transglutaminase [26].

The IgA tissue transglutaminase antibody test used for screening of coeliac disease today has very high sensitivity and specificity for coeliac disease [27, 28]. By screening with serological tests, the prevalence of biopsy-proven coeliac disease in Finnish and Italian schoolchildren was reported to be 1:99 and 1:106, respectively [29, 30]. Similar rates of seroprevalence have been reported in adult populations in UK (1:87) and USA (1:105) [31, 32]. The highest rate of antiendomysial antibody positivity (5.6%) has been reported in Saharawi children [33].

3.1.4 Clinical aspects

Until the beginning of the 1980s, the use of intestinal biopsy was reserved for patients with overt malabsorption. Consequently, the prevalence of malabsorption among patients with coeliac disease was very high. As awareness of the disease improved and the threshold for investigation was lowered, more subtle forms of the disease and the variable clinical expression of the condition was acknowledged [1]. At the end of the 1980s- after the advent of serological tests - the number of patients with minor symptoms was twice the number of patients with overt malabsorption. There was a significant rise in the rate of diagnosis, and a reduction of the male to female ratio [34]. Coeliac disease is two to three times more common in women than in men, but this predominance falls after the age of 65 years.

The factors that determine the severity of symptoms at presentation are not known. Neither the degree of duodenal villous atrophy nor the extent of visible enteropathy assessed by capsule endoscopy correlates with clinical presentation [35, 36].

3.2 Breath analysis in gastroenterology

The first gastroenterological applications of breath analysis were carried out by the medical student Charles Dodds in 1920 [37, 38]. He reported that the pressure of carbon dioxide ($PC0_2$) in breath rose after meals, and that the rise in PCO_2 differed in patients with pernicious anaemia and pancreatic disease from healthy controls. Following these pioneering efforts, the area remained dormant until, in the 1960s, new techniques were developed simplifying the assessment of $^{14}CO_2$ after administration of ^{14}C-labeled compounds and of hydrogen (H_2) after administration of carbohydrate [39].

3.2.1 ^{14}C-breath tests and ^{13}C-breath tests

Recovery of $^{14}CO_2$ in breath after administration of ^{14}C-labeled compounds indicates that metabolism of the radioactively labelled compound has occurred. When the rate-limiting step in the metabolism of the compound is intestinal absorption, measurement of breath $^{14}CO_2$ output after oral administration of the compound may provide information about it's rate of absorption. If the compound is metabolized solely by intestinal bacteria measurement of breath $^{14}CO_2$ output after oral administration of the compound may provide information about the exposure of the compound to intestinal bacteria.

The original radiospirometric studies applied to gastroenterological studies were performed by Schwabe et al., who administered ^{14}C-labeled trioctanoate and monitored breath $^{14}CO_2$ [40]. Cozetto el al., in 1964, applied the same technique to study intestinal absorption of fatty acids, glucose and lactose in children [41].

Tests in which breath $^{14}CO_2$ is measured require the administration of small amounts of ^{14}C-labeled compounds. The calculated radiation dose is extremely small. Nevertheless,

the tests should not be performed in children or pregnant women. When ^{13}C-compounds became widely available at a reasonable cost and methods to measure ^{13}C abundance in samples of CO_2 were developed, ^{14}C-labeled substrates were gradually replaced by non-radioactive ^{13}C-labeled substrates [42-45].

The ^{13}C-urea breath test for the diagnosis of Helicobacter Pylori infection is the most used stable isotope breath test in gastroenterology today [46, 47]. However, a range of substrates and functions can be monitored by means of ^{13}C-breath tests [48, 49]. The underlying concept of the ^{13}C-breath test is simple and similar to the ^{14}C-breath tests: ^{13}C is introduced into one or more functional groups in a substrate. The functional groups are linked to the rest of the molecule through bonds that are cleaved by specific enzymes. Once cleavage occurs, the functional group is further oxidized until CO_2 is produced and excreted in breath.

In 1978, Toskes et al. developed a new ^{14}C-D-xylose breath test [50]. In a series of studies they showed that the ^{14}C-D-xylose breath test was well suited to detect small intestinal bacterial overgrowth (SIBO) [51-53]. The test was later replaced by the ^{13}C-D-xylose breath test [54, 55]. However, the initial optimism regarding the utility of the ^{14}C-D-xylose and ^{13}C-D-xylose breath tests for SIBO has been challenged by several studies showing less convincing results [56-58].

3.2.2 Hydrogen breath tests

In 1975, Newcomer et al., demonstrated the superiority of H_2 measurements to $^{14}CO_2$-labelled lactose and blood sugar changes in detecting lactose malabsorption [59]. The H_2-lactose breath test has been shown to have high sensitivity for lactose malabsorption in

many repeated studies since then, and is accepted as the method of choice for lactose malabsorption by many gastroenterologists [60-63].

Soon after the introduction of the H_2-lactose breath test, the H_2-breath test was also applied to other complex sugars like fructose [64, 65], maltose [66], sucrose [67], sorbitol [68], and lactulose [69, 70]. In 1988, Corazza et al., presented a sorbitol H_2-breath test for coeliac disease [71]. The initial study as well as follow-up studies demonstrated high sensitivity of the H_2-sorbitol breath test for untreated coeliac disease [72, 73].

All cells produce CO_2 during metabolism, but only bacteria can produce H_2 and methane (CH_4) as metabolic by-products. An increase in H_2 concentration in breath after ingestion of a carbohydrate shows that bacterial fermentation of the carbohydrate has taken place in the intestine [74]. It has been shown that a relationship exists between H_2 and CH_4 production, in which methanogenic bacteria are able to convert H_2 to CH_4, and this exchange occurs in the colon [75, 76]. A significant proportion of humans have a methanogenic gut bacterial flora [77-80]. Therefore, when intestinal bacteria metabolize carbohydrates, sometimes only H_2 is produced, sometimes both H_2 and CH_4 appears in the exhaled air, and sometimes only CH_4 will be increased. Methane should also be measured in breath tests for carbohydrate malabsorption in order to allow correct interpretation of the test. The response to a challenge-dose of sugar is measured from the pre-dose baseline or the lowest level reached prior to an increase.

3.3 D-xylose testing in malabsorption

Absorption of D-xylose has been a gold standard test in the evaluation of small intestinal malabsorption since the 1960s [81, 82]. However, the diagnostic accuracy of the test has been subject to much controversy in later years [83-87]. D-xylose is a five carbon monosaccharide found naturally in plants [88]. The sugar is incompletely absorbed, which allows it to be used as an absorptive test [89]. The mechanism of D-xylose absorption in the small intestine is disputed. Data from intestinal perfusion studies in normal humans support predominantly passive absorption, transcellular or paracellular, with little or no carrier-mediated transport [90]. D-xylose is absorbed unchanged from the small intestine [91, 92]. Approximately 30% is metabolized by the liver to CO_2 and threitol [93, 94]. Five percent is excreted unchanged in the bile and undergoes enterohepatic cycling [95], and the remainder is excreted in the urine.

Normal subjects absorb approximately 70% of a 25g oral dose of D-xylose [96]. The absorption constant for D-xylose correlates closely with the 1-h serum concentration of D-xylose, and the 5-h urine content of D-xylose correlates closely with it's absolute bioavailability [97]. Essentially all of the absorption, metabolism, and excretion occur within the first five hours after administration.

From the 1960s and onwards a range of studies was performed which showed that a 5-h urine excretion test with 25g D-xylose distinguished clearly between normal individuals and patients with proximal intestinal malabsorption [98-110]. To improve the sensitivity, specificity and ease of D-xylose testing, various modifications of dosing and the inclusion of serum analyses have been suggested. Most studies have used the 25g or 5g D-xylose absorption test with either 5-h urine collection and/or 1-h serum levels. In

paediatrics, the 1-h serum level after 5g of D-xylose has been the preferred test due to the difficulty of collecting urine accurately in children [111-116].

Other factors than malabsorption may affect the result of the D-xylose test and must be considered in clinical practice. The excretion of D-xylose in urine is dependent on its renal clearance, and renal insufficiency complicates the interpretation of the test [117, 118]. Interpretation of D-xylose tests in geriatric patients must therefore take status of renal function into account [119-121]. Delayed gastric emptying may alter the absorption kinetics and give low 1-h serum levels [122].

The 25g D-xylose H_2-breath test was developed as an alternative to urine and serum analysis of D-xylose. This is an indirect test of D-xylose malabsorption, in which a rise in H_2 signals that unabsorbed D-xylose has been delivered to the colon and metabolized by enteric bacteria. The H_2-breath test circumvents some of the problems with the traditional D-xylose tests such as dependence on renal clearance and accurate urine collection. H_2 production after a 25g D-xylose dose was first shown to be well correlated with 5-h D-xylose excretion and 90-min D-xylose levels in serum in tropical sprue [123]. Similar results were found when H_2-breath testing was compared with 5-h urinary D-xylose for patients with various malabsorptive disorders [124]. One study showed that the 25g D-xylose H_2-breath test was more reliable in diagnosing coeliac disease than the 5-h urine D-xylose content [125]. Isotope-labelled breath tests with D-xylose have been evaluated for small intestinal bacterial overgrowth, but not for coeliac disease or other malabsorptive states.

3.4 Summary of introduction

Coeliac disease is a small intestinal enteropathy that can develop in genetically disposed individuals upon exposure to gluten. Before the development of serological tests for coeliac disease and easy access to endoscopic procedures, malabsorption testing played a pivotal role in the diagnosis of coeliac disease. D-xylose testing was the most commonly used malabsorptive test. Initially D-xylose measurements were made in serum and urine, but in later years the D-xylose H_2-breath test was developed as an alternative. Isotope-labelled breath tests have been much used in gastroenterology, but not as primary tests of coeliac disease or other malabsorptive states.

4 AIM OF THE STUDY

The aim of this thesis has been to evaluate isotope-labelled breath tests as diagnostic tools in small intestinal malabsorption. We chose to focus on coeliac disease; the most frequent cause of small intestinal malabsorption in the Western world today.

The specific aims of the present study were:

1. To determine the diagnostic accuracy of the ^{13}C-xylose breath test in diagnosis and follow-up of coeliac disease.
2. To compare the ^{13}C-xylose breath test with xylose absorption tests based on D-xylose measurements in blood and urine.
3. To develop a novel ^{13}C-sorbitol breath test and determine the diagnostic accuracy of this test in diagnosis and follow-up of coeliac disease.
4. To compare the ^{13}C-sorbitol breath test with the conventional H_2-sorbitol breath test.

5 LIST OF PAPERS

Paper I

Tveito K, Brunborg C, Sandvik L, Løberg EM, Skar V. 13C-xylose and 14C-xylose breath tests for the diagnosis of coeliac disease. Scand J Gastroenterol. 2008;43(2):166-173.

Paper II

Tveito K, Brunborg C, Bratlie J, Askedal M, Sandvik L, Lundin KE., Skar V. Intestinal malabsorption of D-xylose: comparison of test modalities in patients with coeliac disease. Scand J Gastroenterol. 2010 Nov;45(11):1289-94

Paper III

Tveito K, Hetta AK, Askedal M, Brunborg C, Sandvik L, Løberg EM, Skar V. A novel one-hour 13C-sorbitol breath test versus the H2-sorbitol breath test for assessment of coeliac disease. Scand J Gastroenterol. 2009;44(7):813-19.

Paper IV

Tveito K, Hetta AK, Askedal M, Løberg EM, Skar V. Follow-up of coeliac disease with the novel one-hour 13C-sorbitol breath test versus the H2-sorbitol breath test. (Submitted)

5.1 Paper I

The aim of the study was to evaluate the diagnostic properties of the ^{13}C-D-xylose breath test and the ^{14}C-D-xylose breath test in coeliac disease and develop a diagnostic breath test index.

Data from 41 patients with untreated coeliac disease who had performed the ^{14}C-D-xylose breath test, and 60 patients with untreated coeliac disease who had performed the ^{13}C-D-xylose breath test were reviewed retrospectively. All patients in the ^{14}C-D-xylose breath test cohort repeated the breath test on a gluten-free diet (GFD), and 37 patients in the ^{13}C-D-xylose breath test cohort repeated the breath test on GFD. Patients were compared to a healthy control group.

For both breath tests, combining breath test values at 30 minutes and 210 minutes (the gas fraction (GF) index: 30 min/210 min) resulted in the highest diagnostic accuracy. The breath tests had very similar diagnostic properties for untreated coeliac disease, with sensitivities of 84-95% and specificities of 87-94%.

After treatment with a gluten-free diet, most patients improved their GF-index, but did not reach the levels of healthy controls.

5.2 Paper II

The aim of this study was to compare the ^{13}C-D-xylose breath test with D-xylose measurements in plasma and urine in a retrospective cohort of patients with coeliac disease. Data from 91 untreated coeliac patients, and 98 treated coeliac patients, who had performed the ^{13}C-D-xylose breath test were evaluated. 1-h plasma D-xylose had been measured in 48 untreated and 41 treated coeliac patients. 4-h urine D-xylose excretion in urine had been measured in 47 untreated and 51 treated patients. Patients were compared to a healthy control group (n = 43).

Test sensitivity and specificity for coeliac disease versus healthy controls were 88% and 84% with the ^{13}C-D-xylose breath test, 65% and 71% with the 1-h plasma D-xylose test, and 55% and 74% with the 4-h urine D-xylose excretion test. Breath test results improved significantly in the treated coeliac group compared to untreated patients, but were not normalized compared to healthy control subjects. No difference was found between D-xylose levels in plasma and urine between treated coeliac patients and healthy control subjects.

5.3 Paper III

The aim of this study was to examine the diagnostic properties of a novel ^{13}C-sorbitol breath test in coeliac disease. We compared the ^{13}C-sorbitol breath test to the H_2-sorbitol breath test in 39 untreated coeliac patients, 40 patients with symptoms compatible with coeliac disease referred for evaluation who turned out not to have coeliac disease, and 26 healthy control subjects.

The ^{13}C- sorbitol breath test and the H_2-sorbitol breath test were performed simultaneously. Breath samples were gathered every 30 minutes for four hours. Single time point variables and combinations of single time point variables were analyzed statistically to chose the best test variable(s) for the ^{13}C-sorbitol breath test. Optimal combined sensitivity and specificity turned out to be obtained with single time point measurements at 60 minutes or 90 minutes. The 60 minutes variable was chosen to shorten test duration. A diagnostic algorithm dividing patients into high, moderate and low risk groups for coeliac disease was proposed. Following the algorithm, sixty-two per cent of coeliac patients were detected with 100% specificity. The H_2-sorbitol breath test had a sensitivity of 71%, a specificity of 46% versus healthy controls, and a specificity of 25% versus patient controls.

5.4 Paper IV

The aim of this study was to evaluate the novel ^{13}C-sorbitol breath test for follow-up of coeliac disease compared to the H_2-sorbitol breath test. Patients with coeliac disease from the first study of the ^{13}C-sorbitol breath study were invited to participate after approximately one year on a gluten-free diet. 27/39 patients repeated the breath tests, and 25 patients had a new duodenal biopsy. Patients were compared to 40 patients without coeliac disease and 26 healthy subjects from the previous study.

As in the previous study, the ^{13}C- sorbitol breath test and the H_2-sorbitol breath test were performed simultaneously. Breath samples were gathered every 30 minutes for four hours.

The rate of $^{13}CO_2$ excretion increased significantly at all time points before 210 minutes in treated versus untreated coeliac patients, but remained lower than in healthy control subjects and patient controls at all time points except 30 minutes. This also applied to treated patients who had completely normal histological duodenal mucosa after treatment with GFD. 20/26 patients had increased $^{13}CO_2$ breath levels after 60 minutes compared to their test before treatment. 17/27 patients had a negative H_2-sorbitol breath test after treatment with GFD. Peak H_2 concentrations did not correlate with ^{13}C-sorbitol breath test results. Mean time to reach peak H_2 concentration and mean peak H_2 concentration did not change after treatment.

5.5 Other publications during the PhD programme

Hope HB, Tveito K, Aase S, Messelt E, Utzon P, Skar V. Small intestinal malabsorption in chronic alcoholism determined by 13C-D-xylose breath test and microscopic examination of the duodenal mucosa. Scand J Gastroenterol. 2010;45(1):39-45. Erratum in: Scand J Gastroenterol. 2010 Dec;45(12):1519.

6 METHODS

6.1 Study subjects

6.1.1 Patients

The ^{13}C-D-xylose breath test studies had a retrospective design. Patient data were collected from the breath test database of ^{13}C-D-xylose breath tests and ^{14}C-D-xylose breath tests at Lovisenberg Diakonale Hospital and Ullevaal University Hospital. Clinical files and duodenal biopsies were re-assessed to confirm that the diagnosis of coeliac disease was correct.

In the ^{13}C-sorbitol breath test studies patients were included and followed up prospectively between 2006 and 2008 in the Department of Gastroenterology at Stavanger University Hospital and Lovisenberg Diakonale Hospital. Consecutive patients with suspected coeliac disease were included after giving written consent to participate in the study. Patients underwent gastroscopy with duodenal biopsies, routine laboratory testing and were questioned about gastrointestinal symptoms at inclusion and follow-up. Patients who did not have biopsy-confirmed coeliac disease served as patient controls. Patients with verified coeliac disease were followed-up after approximately one year.

6.1.2 Healthy control subjects

Healthy control subjects were recruited from the medical staff and medical students at Lovisenberg Diakonale Hospital. Some of the test subjects had performed the breath test as controls when the breath test was introduced at Lovisenberg Diakonale Hospital. They

had not been tested with serologic markers for coeliac disease. Healthy control subjects included after 2004 underwent serological testing for IgA and IgG tissue-transglutaminase antibodies, and levels within reference values were required to participate. All healthy control subjects had normal bowel habits, and had not been investigated for gastrointestinal disease in the past. In the ^{13}C-sorbitol breath test studies, serious illness in the past, having a 1st degree relative with coeliac disease and having used antibiotics in the previous month served as additional exclusion criteria.

6.2 Breath test protocols

All breath tests were performed after an overnight fast. Subjects were quietly seated and requested not to eat, drink or smoke during the test period. In the ^{13}C-sorbitol breath test where hydrogen and methane were also measured, subjects were instructed to ingest a meal consisting of fish or chicken and rice and avoid high fibre food the evening before the test. Breath was sampled in duplicates at every time point, and mean values of the duplicates were used.

6.2.1 ^{14}C-D-xylose breath test

1 g D-xylose and 10 μCi ^{14}C-D-xylose, 99% (Amersham, U.K.), were given orally in 500 ml water as described by Skar et al. [126]. The subjects exhaled through a disposable device directly into counting vials. Specific radioactivity of the breath samples was determined by liquid scintillation. The results were expressed as percentage of orally administered dose ^{14}C-D-xylose expired as $^{14}CO_2$ per mmol CO_2, corrected for the endogenous production of CO_2 (9 mmol CO_2/kg body weight/h).

6.2.2 ^{13}C-D-xylose breath test

100 mg ^{13}C-1, 2-D-xylose, 99% (Cambridge Isotope Laboratories, Massachusetts, USA), and 5 g D-xylose were dissolved in 250 ml tap water. Two end-expiratory breath samples were collected via a straw in 12 ml tubes (Labco Limited, High Wycombe, UK) at each time point. The $^{13}CO_2/^{12}CO_2$ ratio in the breath samples was determined by gas chromatography (GC) and continuous flow isotope ratio mass spectrometry (IRMS, Europe Scientific 20/20, Crewe, UK). The delta-value (δ) between the $^{13}CO_2/^{12}CO_2$ ratio of the samples and the PDB limestone standard was calculated [127], using the following formula:

$\delta = [(R_{sa}/R_{st})-1] \times 1000$, in which $R_{sa} = {}^{13}C/{}^{12}C$ in the CO_2 of the sample and $R_{st} = {}^{13}C/{}^{12}C$ in the CO_2 of the standard. The results were expressed as the percentage of ^{13}C-recovery per hour (% dose/h). For this calculation the formula of Schoeller et al. was used [128], and the CO_2 production was assumed to be 300 mmol/m^2 body surface area per hour. Body surface area was calculated by the weight-height formula of Haycock et al [129].

6.2.3 ^{13}C-sorbitol breath test

5 g D-sorbitol and 100 mg ^{13}C-D-sorbitol, 99% (Aldrich, Milwaukee, USA) dissolved in 250 ml tap water was given orally. Two parallel end-expiratory breath samples were collected via a straw in 12 ml tubes (Exetainers, Labco Limited, High Wycombe, UK) at every time point for analysis of $^{13}CO_2$. The $^{13}CO_2/^{12}CO_2$ -ratio in the breath samples was determined by gas chromatography (GC) and continuous flow isotope ratio mass

spectrometry (IRMS, Europe Scientific 20/20, Crewe, UK), and expressed as the percentage of ^{13}C-recovery (% of dose ^{13}C/h) for every time point with a similar formula as in the in the ^{13}C-D-xylose breath test studies.[128] The CO_2 production was assumed to be 300 mmol/m^2 body surface area per hour. Body surface area was calculated by the weight-height formula of Haycock et al.[129].

6.2.4 H_2-sorbitol breath test

Duplicate end-expiratory breath samples were collected in 30 ml plastic syringes fitted with a T-piece and immediately analyzed for H_2 and methane (CH_4) by a QuinTron Model SC Microlyzer (QuinTron Instrument Company, Milwaukee, USA).
Concentrations used for calculation were means of duplicate samples. Hydrogen increase ≥ 20 ppm from the lowest previous level reached prior to an increase counted as a positive test.

6.3 D-xylose in plasma and urine

Blood samples were drawn after one hour, and urine was collected for four hours after starting the test with an empty bladder. Duplicate samples of blood and urine were frozen at -20 degrees C° before analysis.
D-xylose was measured in urine by high-performance liquid chromatography (HPLC) modified after Larew and Johnson,[130] using an electrochemical detector equipped with a gold electrode (ESA coulochem, model 5040, Chelmsford, Massachusetts, USA), a Rheodyn injector (Rheodyn inc., California, USA), 20 mikroliter loop and a Supelco C-611 column with Supelcogel C-611 guarding column (Supelco, Bellefonte, Pennsylvania,

USA). Mobil phase was freshly prepared 1mmol/l NaOH (Sigma , St.Louis, Missouri, USA) delivered at 1.0 ml/minute isocratic run at 65 degrees C°. Lactose (Sigma) was used as internal standard and D-xylose (Sigma) as external standard. Peak heights were measured and corrected according to the internal standard. A standard curve was used to determine D-xylose concentration. The coefficient of variation was 6%.

1-h plasma D-xylose levels were measured in duplicate samples by the method of Roe and Rice [131]. The coefficient of variation was 10%.

6.4 Statistics

Statistical analyses were performed using SPSS 14.0, 15.0 or 18.0. Comparison between independent groups was made using either independent sample Student's *t*-test or one-way ANOVA for normally distributed variables, and Mann Whitney U test or Kruskal-Wallis test for continuous variables with skewed distribution. Linear regression was used when comparing two continuous variables. Pearson's chi-square test was used for categorical outcome variables. Pearson's correlation coefficient or Spearman's correlation coefficient was used to estimate correlation between variables. Single time point test variables were analyzed by logistic regression. Receiver operating characteristic (ROC) curves were used to determine diagnostic accuracy. Findings with p values < 0.05 were considered statistically significant.

6.5 Ethics

The regional ethical committee approved all studies. Informed consent was given by all control subjects and by patients included after 2004.

7 Discussion

In this first series of studies of ^{13}C-labelled breath tests in coeliac disease we found high sensitivity for untreated coeliac disease compared to healthy control subjects, and in the ^{13}C-sorbitol breath test study, also compared to a patient group with comparable clinical symptoms. The latter finding is especially important as it demonstrates the specificity of ^{13}C-breath testing in clinical practice.

In follow-up studies of coeliac patients on a gluten-free diet, we found improved breath test results, but treated coeliac patients did not reach healthy control levels. Furthermore, we found that the ^{13}C-D-xylose breath test has comparable diagnostic properties to the ^{14}C-D-xylose breath test, and is superior to D-xylose measurements in plasma and urine. Finally, when comparing the ^{13}C-sorbitol breath test to the H_2-sorbitol breath test, we showed superiority of the ^{13}C-labelled breath test versus H_2-testing.

7.1 Strengths and limitations of the studies

The validity of our findings is strengthened by the fact that we carried out studies with three different isotope-labelled breath test protocols with very similar test results. Furthermore, many patients were tested before and after treatment with a gluten-free diet, which gave us the opportunity to investigate individual changes in breath test results. Comparison with other test modalities was performed simultaneously. Thus we could exclude intraindividual variance over time as a confounding factor. The prospective design of the ^{13}C-sorbitol breath test study is considered a major strength of this study, especially since we were able to collect both clinical, biochemical and histological data.

Other factors than malabsorption such as dysmotility and small intestinal bacterial overgrowth can interfere with breath test results. A limitation of our study is that we did not carry out additional studies of this. A limitation of the study of D-xylose modalities is that we carried out a 4-h xylose excretion test in urine instead of the traditional 5-h D-xylose excretion test and measured 1-h D-xylose levels in plasma instead of serum. Further studies are needed to assess the relationship between mucosal changes in patients on a gluten-free diet and breath test results as this was only done in a relatively small number of patients in the ^{13}C-sorbitol breath test study.

7.2 The ^{13}C-D-xylose breath test and the ^{13}C-sorbitol breath test

One of the aims in the studies with both isotopes was to decide what is the optimal test duration and determine a diagnostic algorithm. We reached different conclusions for the two breath tests. In the study of the ^{13}C-D-xylose breath test (and the ^{14}C-D-xylose breath test), we concluded that the highest diagnostic accuracy was achieved combining excretion of breath $^{13}CO_2$ in the early and late test phase. The 30min/210 min index was proposed. With the ^{13}C- sorbitol breath test, we found that a single measurement at 60 minutes was equally good as a test index combining several variables. How can we explain this discrepancy?

When looking at the breath test curves from the ^{13}C-D-xylose breath test and the ^{13}C-sorbitol breath test, a striking difference is apparent in the late test phase. The $^{13}CO_2$ overshoot in the late test phase in coeliac patients with the ^{13}C-D-xylose breath test has no equivalent in the ^{13}C-sorbitol breath test. This probably explains why adding a late test

variable did not increase diagnostic accuracy in the ^{13}C-sorbitol breath test study. We do not know the reason for the different patterns of $^{13}CO_2$ excretion with the two different ^{13}C-substrates. We predict that it has to do with the amount of substrate reaching colon and colonic fermentation of the substrates.

As for the question of which breath test should be preferred in clinical practice, several aspects should be considered. The best combined sensitivity and specificity for coeliac disease versus healthy controls was found with the ^{13}C-D-xylose breath test. The classic sprue syndrome of steatorrhoea and malnutrion has become rare, and there has been a trend over time for a greater proportion of coeliac patients presenting with subclinical/silent coeliac disease and having partial villous atrophy [35, 132]. In line with the reports on the changing clinical spectre of coeliac disease, we found that most of the patients in our studies had mild if any signs of malabsorption. This change in clinical presentation might well explain the reduced sensitivity and specificity we found for D-xylose testing in plasma and urine compared to early studies of D-xylose tests. Our results indicate that the ^{13}C-D-xylose breath test is a sensitive test for subtle small intestinal malabsorption. This conclusion is supported by the fact that D-xylose levels in plasma and urine did not distinguish between treated coeliac disease and healthy control subjects, whereas the ^{13}C-D-xylose and the ^{14}C-D-xylose breath test did.

The ^{13}C-sorbitol breath test had lower optimal combined sensitivity and specificity than the ^{13}C-D-xylose breath test, but offers a breath test alternative that is short (60 minutes), and can be used to stratify patients into high, medium and low risk for coeliac disease. Furthermore, the ^{13}C-sorbitol substrate is less expensive than ^{13}C-D-

xylose. Finally, the changes in ^{13}C-sorbitol breath test results were similar to the changes in the ^{13}C-D-xylose breath test in coeliac patients after treatment with a gluten-free diet.

7.3 The H_2-sorbitol breath test

Both studies of the H_2-breath test showed unsatisfactory diagnostic accuracy with the H_2-sorbitol breath test. The main weakness of the H2-sorbitol breath test was the high prevalence of positive breath tests in control groups and in treated coeliac patients.
The lack of specificity may be specific to the H_2-sorbitol breath test, but could also reflect a more general problem with the H_2-breath tests. Several factors point to the latter conclusion. The general use of H_2-breath tests might be thwarted by the variability of individual gut flora to produce H_2 [133-136]. As many as 10% of some populations may be non-H_2 producers [137-139]. False positive results may also result due to the oral bacterial flora and if the subject has failed to adhere to a low fibre diet the day before the test [140]. In line with previous studies, we showed that H_2-excretion is dependent on the presence of methanogenic colonic flora [75, 76, 80]. Methane should also be measured and considered when interpreting the H_2-breath test. Previous studies of the H_2-sorbitol breath test have not measured CH_4, and we find this a major weakness of these studies. The specificity of the H_2-sorbitol breath test depends critically on the sorbitol absorption capacity in healthy subjects. Further studies may elucidate if a changing the amount and concentration of sorbitol in the test solution could improve test specificity.

7.4 Pathophysiological considerations

We have concluded that the ^{13}C-breath tests are sensitive markers of small intestinal malabsorption. However, other factors than small intestinal damage such as small intestinal bacterial overgrowth and dysmotility can also give rise to pathological breath test results. Fordtran et al. first demonstrated a relationship between small intestinal transit time and extent of D-xylose absorption [92, 97]. Subsequently, Goldstein et al. showed that bacteria in the small intestinal lumen can metabolize substantial amounts of D-xylose [141].

Studies of small bacterial overgrowth in coeliac disease are scarce, but the few studies that have been done conclude that small intestinal bacterial overgrowth occurs in a small percentage of untreated coeliac patients, and should be suspected in coeliac patients with persistent clinical symptoms on a gluten-free diet [142, 143]. We did not carry out any experiments to solve this issue. However, the maximum rise in H_2 occurred at a late time point in the ^{13}C-sorbitol breath test and did not differ from the time to reach peak H_2 concentration in controls. If small intestinal bacterial overgrowth had been present in coeliac patients, we would expect bacterial metabolism of sorbitol and H_2-rise in the early test phase before colonic fermentation occurred [123]. The time to reach peak H_2 concentration did not change in coeliac patients after gluten-free diet. If small bacterial overgrowth had been present in untreated patients and resolved after treatment, we would expect the time to reach H_2 peak concentration to change. Furthermore, the shape of the isotope-labelled breath test curves did not suggest small intestinal bacterial overgrowth. In SIBO, we would expect high rates of $^{13}CO_2$ excretion in the early test phase (first two hours) due to bacterial metabolism of the substrate in the small intestine.

Several studies have found evidence for gastrointestinal dysmotility in coeliac disease [144-146]. We would expect changes in gastric emptying and small intestinal transit time to reduce and/or delay small intestinal absorption of the substrate. We cannot exclude the possibility that our breath tests reflect dysmotility rather than small intestinal malabsorption per se. However, time to reach peak H_2 concentration was similar in all groups, indicating that there were no significant differences in intestinal transit time between groups. Furthermore, Bjarnarson et al. carried out a series of permeability studies in coeliac disease with ^{51}Cr EDTA absorption test and found very similar results to our ^{13}C-breath test studies [147]. Since the ^{51}Cr-EDTA absorption test consists of a 24 hours urine excretion test, we can exclude the influence of dysmotility on their results.

Interestingly, we found similar changes in treated coeliac disease as Bjarnarson et al. They reported that although permeability was significantly reduced in treated coeliac patients, permeability remained increased compared to healthy control subjects. Furthermore, they found that 11/14 patients on gluten-free diet with a completely normal mucosa, still had a pathological ^{51}Cr EDTA absorption test. Based on their findings they raised the intriguing question if patients with coeliac disease have an inherent mucosal defect that gives rise to increased permeability even when histological findings have resolved. In the ^{13}C-sorbitol breath test study we reached the same conclusion. Breath test results remained abnormal compared to healthy control subjects even in patients with normal duodenal biopsies according to the Marsh classification.

Studies of patients with HIV and studies of patients with alcoholism have showed abnormal ^{13}C-D-xylose breath tests in the absence of histological small mucosal changes [148, 149]. However, when electron microscopy was performed, subtle mucosal changes

were detected. We propose that similar findings might be affecting small intestinal absorption capacity in coeliac patients on a gluten-free diet. Further studies are needed to investigate this issue, and to determine the clinical importance of the findings.

Bjarnarson et al. found no association between clinical variables in coeliac patients and permeability test results [150]. Likewise, there was no correlation between clinical symptoms and biochemical signs of malabsorption and breath test results in untreated coeliac patients in the ^{13}C-sorbitol breath test studies. However, we did find a significant association between IgA TGA levels and breath test results in the untreated coeliac group. Our findings are in line with a previous study showing lack of correlation between clinical presentation and histological changes in coeliac disease [35, 36].

7.5 Clinical considerations

Malabsorption testing in coeliac disease was developed and studied for many years before the development of excellent serological tests for coeliac disease and easy access to endoscopic procedures. Although breath testing is probably not useful as a routine test in coeliac disease, it might be a valuable additional test in complicated cases, and in patients where duodenal biopsies cannot be obtained. The non-invasiveness of the test and lack of serious side effects, makes it useful in children and pregnant women. We suggest that the ^{13}C-breath test protocols should be evaluated also in children with coeliac disease. Further studies are needed to decide how breath testing can be of value in follow-up of coeliac disease. Several researchers have emphasized the importance of early diagnosis of coeliac disease. Further studies are needed to determine if isotope labelled

breath tests may be of value in the investigation of patients with subtle duodenal histological pathology and so called borderline coeliac disease.

Recent studies show that despite a good clinical response, abnormal endoscopic and histopathological appearances persist in the majority of adult patients with coeliac disease on a gluten-free diet [151-153]. The clinical significance of these findings is uncertain. One study from Finland showed that patients without histological recovery on a gluten-free diet were at greater risk of developing complications of coeliac disease such as malignancy [154]. The normalization of tissue transglutaminase antibodies does not in itself guarantee that the small intestinal villous structure has improved [155]. We do not know if the ^{13}C-labelled breath tests can be used to determine which patients should be followed up with repeated duodenal biopsies, but this question could be addressed in further studies.

7.6 Conclusion

Patients with coeliac disease are excellent experimental models for small intestinal malabsorption since they can be examined before and after treatment with a gluten-free diet. Based on our studies of patients with coeliac disease, we conclude that ^{13}C-labelled breath tests are sensitive tests for small intestinal malabsorption.

8 References

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9 Errata

Paper II:

Page 1292, Figure 2A

The correct number of healthy controls should be $n = 43$.

10 Original papers

I

II

III

IV

Follow-up of coeliac disease with the novel one-hour ^{13}C-sorbitol breath test versus the H_2-sorbitol breath test

KARI TVEITO[1], ANNE KRISTINE HETTA[2], MIA ASKEDAL[1], CATHRINE BRUNBORG[3], LEIV SANDVIK[3], ELSE MARIT LØBERG[4], VIGGO SKAR[1]

[1]Department of Medicine, Lovisenberg Diakonale Hospital, Oslo, Norway,[2]Department of Medicine, Stavanger University Hospital, Stavanger, Norway, [3]Unit for epidemiology and biostatistics, Oslo University Hospital - Ulleval, Oslo, Norway, [4]Department of Pathology, Oslo University Hospital-Ullevaal, Oslo, Norway

Corresponding author:

Kari Tveito, M.D., Department of Medicine, Oslo University Hospital, Aker,

Trondheimsveien 235, N-0514 Oslo, Norway

Email: kari.tveito@oslo-universitetssykehus.no

Running head: ^{13}C-sorbitol breath test in follow-up of coeliac disease

Abbreviations: H_2-SBT, hydrogen-sorbitol breath test; ^{13}C-SBT, ^{13}C-sorbitol breath test; GFD, gluten-free diet; tTGA, tissue transglutaminase antibody

Key words: breath test, carbon isotopes, coeliac disease, malabsorption, sorbitol

Abstract

Background: We recently developed a ^{13}C-sorbitol breath test (^{13}C-SBT) as an alternative to the H_2-sorbitol breath test (H_2-SBT) for coeliac disease. In this study we compared the diagnostic properties of the H_2-SBT and the ^{13}C-SBT in follow-up of coeliac disease.

Material and methods: Twenty-seven coeliac patients on a gluten-free diet performed the breath tests. All had been tested before treatment in the intial study of the ^{13}C-SBT, in which 39 untreated coeliac patients, 40 patient controls, and 26 healthy volunteers participated.

5 g sorbitol and 100 mg ^{13}C-sorbitol were dissolved in 250 ml tap water and given orally. H_2, CH_4 and $^{13}CO_2$ were measured in end-expiratory breath samples every 30 minutes for four hours. Increased H_2 concentration ≥ 20 ppm from basal values was used as cut-off for the H_2-SBT. 60 minutes values were used as diagnostic index in the ^{13}C-SBT.

Results: $^{13}CO_2$ levels at 60 minutes increased in 20/26 treated coeliac patients (77%) after GFD, but were significantly lower than in control groups. Out of 20 patients who had a positive H_2-SBT before GFD, 12 had a negative H_2-SBT after GFD. Peak H_2 concentrations were not correlated with ^{13}C-SBT results.

Conclusions: The study confirms the sensitivity of a one-hour ^{13}C-SBT for small intestinal malabsorption. The ^{13}C-SBT has superior diagnostic properties compared to the H_2-SBT in follow-up of coeliac disease.

Introduction

Sorbitol is only partially absorbed in the small intestine, and its malabsorption is dose- and concentration-dependent.[1-3] Corazza et al. were the first to show that administration of sorbitol at a low dose and concentration to patients with untreated coeliac disease resulted in increased excretion of hydrogen (H_2) in breath compared to healthy controls.[4] Since then a series of studies have suggested high sensitivity of the H_2-sorbitol breath test (H_2-SBT) when screening for coeliac disease in adults.[5-8]

We recently developed a novel ^{13}C-sorbitol breath test, and compared it's diagnostic properties to that of the H_2-SBT. We showed that a one-hour ^{13}C-sorbitol breath test had better sensitivity and specificity for coeliac disease versus healthy controls and patient controls than the H_2-SBT. A diagnostic algorithm was proposed for the ^{13}C-sorbitol in which patients were stratified as being at high, moderate or low risk for coeliac disease. In this study we aimed to study the diagnostic properties of the ^{13}C-sorbitol breath test in follow up of treated coeliac patients compared to the H_2-SBT. Coeliac patients who participated in the previous ^{13}C-sorbitol study were invited to participate.

Methods

Study subjects

Coeliac patients who had participated in our previous study of the ^{13}C-sorbitol breath test were invited to perform another breath test after approximately one year on a gluten-free diet. Twenty-seven out of 39 patients agreed to participate. Patients underwent gastroscopy with duodenal biopsies and routine laboratory testing. The pathologist reported the biopsies according to the 1992 Marsh criteria [9], as modified by Oberhuber et al. [10]. Serological testing for coeliac disease included measurement of IgA and IgG tissue-transglutaminase antibodies (tTGAs, U/ml), IgA gliadin antibodies (Units) and IgA levels (g/l). Patients were asked to range their symptoms of diarrhoea, constipation, bloating, abdominal pain, nausea, and fatigue on a 4-point Likert scale. A mean item score was used to provide a score from 1-4 (no discomfort to severe discomfort) for each dimension.

Patient controls (n = 40) and healthy controls (n = 26) were recruited in the previous study of the ^{13}C-SBT [11]. Coeliac disease had been excluded by duodenal biopsies in the patient control group. The majority of patients in this group suffered from irritable bowel syndrome (IBS).

The study was conducted in accordance with the Declaration of Helsinki-Tokyo and approved by the regional ethical committee. All subjects gave written consent to participate in the study.

Breath test protocol

Test subjects were examined after an overnight fast of at least 10 hours. They were instructed to avoid high fibre food, and to consume a meal of rice with fish or meat the evening before the test. During the test period, all subjects continued fasting, were quietly seated and refrained from smoking., An oral load of 5 g sorbitol and 100 mg ^{13}C-D-sorbitol, 99% (Aldrich, Milwaukee, USA), dissolved in 250 ml tap water, was given directly after obtaining four basal breath samples. Breath samples were collected at 30 minutes intervals for four hours. Duplicate end-expiratory breath samples were collected in 30 ml plastic syringes fitted with a T-piece and immediately analyzed for hydrogen (H_2) and methane (CH_4) by a QuinTron Model SC Microlyzer (QuinTron Instrument Company, Milwaukee, USA). Concentrations used for calculation were means of duplicate samples. Hydrogen increase ≥ 20 ppm from the lowest previous values (basal values) counted as a positive test.

Two parallel end-expiratory breath samples were collected via a straw in 12 ml tubes (Exetainers, Labco Limited, High Wycombe, UK) at every time point for analysis of $^{13}CO_2$. The $^{13}CO_2/^{12}CO_2$ -ratio in the breath samples was determined by gas chromatography (GC) and continuous flow isotope ratio mass spectrometry (IRMS, Europe Scientific 20/20, Crewe, UK), and expressed as the percentage of ^{13}C-recovery (% of dose ^{13}C/h) for every time point. The formula of Schoeller et al. was used for this calculation.[12] The CO_2 production was assumed to be 300 mmol/m^2 body surface area per hour. Body surface area was calculated by the weight-height formula of Haycock et al. [13].

Statistics

Data from patient characteristics are outlined in numbers and percentages. Mean with standard deviation (SD) or median with range are presented, as appropriate. Comparison between independent groups was made using either independent sample Student's *t*-test or one-way ANOVA for normally distributed variables, and Mann Whitney U test or Kruskal-Wallis test for continuous variables with skewed distribution. The paired Student's *t*-test was used for comparison of variables before and after gluten-free treatment. Linear regression was used when comparing two continous variables. Spearman´s correlation coefficient was used to test for correlation between variables. When comparing dichotomised variables, a chi-square test was used. Findings with *p* values < 0.5 were considered significant. All statistical analyses were performed using SPSS software package version 18.0 (SPSS Inc., Chicago, Illinois, USA).

Results

Subject characteristics

The 39 coeliac patients enrolled in the study had Marsh IIIa-c villous atrophy in small intestinal biopsies when they were diagnosed with coeliac disease. Twenty-seven patients agreed to participate in the follow-up study after treatment with gluten-free diet (median duration 12 months, range 6-24 months). Twenty patients had normal duodenal biopsies by this time. Four biopsies were classified as Marsh I, one was classified as Marsh IIIa, and two patients refused to have a new biopsy. Patient controls were described in detail in a previous article [11]. Patients and healthy control characteristics are summarised in table I.

H_2-sorbitol breath test results

Positive H_2-sorbitol breath tests were obtained in 27/38 untreated coeliac patients (71%). After gluten-free diet, 17/27 coeliac patients (63%) had a negative H_2-sorbitol breath test, while 10/27 coeliac patients (37%) had a positive H_2-sorbitol breath test (Figure 1). One of the intital H_2-sorbitol breath tests from the 27 coeliac patients tested twice was discarded due to technical problems. Twenty of the remaining 26 breath tests were positive before GFD. After GFD, twelve of these patients had a positive H_2-sorbitol breath test (60%), and eight were negative (40%). Out of the six coeliac patients who had negative H_2-sorbitol breath tests before treatment, five also had a negative test after GFD, but one patient had a positive breath test after GFD. As shown in the previous study,

30/40 patient controls (75%), and 14/26 healthy subjects (54%) had a positive H_2-sorbitol breath test.

Mean peak H_2 concentration was 37 ± 37 ppm in coeliac patients, 25 ± 28 ppm in treated coeliac patients, 42 ± 32 ppm in patient controls, and 28 ± 24 ppm in healthy controls. Mean time to reach peak H_2 concentration was 188 ± 49 min in coeliac patients, 190 ± 48 min in treated coeliac patients, 178 ± 58 min in patient controls, and 190 ± 49 min in healthy controls. Peak H_2 concentration differed significantly between treated coeliac patients and patient controls ($p = 0.01$), but not between other groups. Time to reach peak H_2 concentration did not differ significantly between groups.

^{13}C-sorbitol breath test results

Mean breath $^{13}CO_2$ levels were significantly higher in the 26 treated coeliac patients (one test was discarded due to technical problems) than in untreated coeliac patients at all time points before 240 minutes, and significantly lower in treated coeliac patients than in control subjects without coeliac disease at all time points exept 30 minutes (Figure 2). On an individual level, $^{13}CO_2$ levels at 60 minutes increased in 20/26 treated patients (77%).

In our previous study of the ^{13}C-sorbitol breath test, 60 minutes $^{13}CO_2$ levels in breath were chosen as the diagnostic test variable, and the following diagnostic algorith proposed: Excreted dose of ^{13}C/h at 60 minutes: < 2% = high probability for coeliac disease, 2-4% = moderate probability for coeliac disease, and > 4% = low probability for coeliac disease. Sixteen of the treated coeliac patients (59%) had a 60 minutes $^{13}CO_2$ level < 2% before treatment. $^{13}CO_2$ levels increased above the 2% cut-off in 12/16 patients (75%, Figure 3). The patient with the lowest level of exhaled $^{13}CO_2$ at 60 minutes

had Marsh 3a villous atrophy in the control biopsy performed after GFD. The other three patients with excreted dose of ^{13}C/h at 60 minutes <2% had normal control duodenal biopsies. Seventeen treated patients (63%) excreted between 2-4% of ingested dose of ^{13}C after 60 minutes, and five patients (19%) had $^{13}CO_2$ levels > 4% of ingested dose.

Comparison of breath tests and correlation with clinical variables

No significant association was found between peak H_2 concentrations and $^{13}CO_2$ values at 60 minutes in any of the groups. In the coeliac group there was an inverse association between $^{13}CO_2$ values at 60 minutes and age ($p = 0.037$), which was not found in the other groups. Age was inversely associated with peak H_2 concentration in the treated coeliac group ($p = 0.007$), but not in the other groups. Duration of gluten-free diet was not significantly associated with $^{13}CO_2$ values at 60 minutes or to peak H_2 concentrations.

IgA tTGA levels were inversely associated to 60 minutes $^{13}CO_2$ values ($p = 0.025$) in the untreated coeliac group, but there was no association in the treated coeliac group. Peak H_2 concentrations were not associated to IgA tTGA levels in any of the groups. In the coeliac group, peak H_2 concentrations increased significantly with increasing severity of duodenal lesions, classified from Marsh IIIa to Marsh IIIc ($p < 0.001$). IgA tTGA levels increased and $^{13}CO_2$ excretion rates at 60 minutes decreased from Marsh IIIa to Marsh IIIc, but these associations did not reach significance.

Symptom scores decreased significantly for all symptom variables except bloating after GFD (Table 2). The 60 minutes variable after GFD was inversely associated to fatigue after GFD ($p = 0.039$), but not to other symptom scores.

S-folate and s-vitamin B_{12} increased significantly after GFD (s-folate: mean difference 4,44 nmol/L, p = 0.001, and vitamin B_{12}: mean difference 55,3 pmol/L, p < 0.001). S-ferritin, but not b-haemoglobin (b-Hb), increased significantly in both gender after GFD (women: mean difference 20,1 mikrogram/L, p=0.038, men: mean difference 71,2 mikrogram/L, p= 0.001). In the treated coeliac group, $^{13}CO_2$ excretion rates at 60 minutes were not associated with levels of s-folat, s-vitamin B_{12}, b-Hb and s-ferritin. Peak H_2 concentration in the treated group was positively associated to b-Hb in men (n = 11, p = 0.018), but not in women. There was no association between peak H_2 concentration and s-folat, s-vitamin B_{12} or s-ferritin in the treated group.

Discussion

This first study of the ^{13}C-sorbitol breath test in patients with treated coeliac disease, confirms the sensitivity of the ^{13}C-sorbitol breath test for small intestinal malabsorption. Seventy-seven percent of coeliac patients tested both before and after gluten-free diet increased their ^{13}C-sorbitol breath test score after GFD. The H_2 breath test with a cut-off level at 20 ppm had a sensitivity of 63% and a specificity of 71% for treated versus untreated coeliac patients.

The improvement in breath test results was accompagnied by normalization of duodenal biopsies in all but four patients, significant decline in symptom scores and increased levels of s-ferritin, s-folate and s-vitamin B_{12}. Generally, laboratory results were not associated with breath test results, as in our previous study of the ^{13}C-sorbitol breath test and the H_2-sorbitol breath test in untreated coeliac disease. That increasing peak H_2 concentration was associated with increasing b-Hb in treated male coeliac patients was unexpected, and may be due to a type I statistical error.

There was a significant association between peak H_2 concentration and increasing histopathology in the untreated coeliac group. A parallell, but non-significant trend was seen for $^{13}CO_2$ excretion rates at 60 minutes. Furthermore, a non-significant increase in IgA tTGA levels from Marsh IIIa-IIIc was seen. Our results are in line with studies by Tursi et al., who found higher serum values of IgA tTGA with increasing histopathology in untreated coeliac disease, and a strict correlation between H_2-sorbitol breath test cut-off values (in ppm and minutes) and histological lesions [14, 15]. However, $^{13}CO_2$ excretion rates remained lower in treated coeliac patients with normal duodenal biopsies than healthy controls and patients without coeliac disease. Furthermore, 40% of the

patients who had a positive H_2-sorbitol breath test before GFD, also had a positive H_2-sorbitol breath test after GFD. These results are in line with a report from Bjarnason et al. who, using the ^{51}Cr EDTA absorption test, found persistent small intestine permeability in treated coeliac patients despite small intestinal mucosal healing [16]. Likewise, studies of patients with HIV and diarrhoea [17], and a study of patients with high alcohol consumption and diarrhoea [18], have shown ^{13}C-xylose breath test results compatible with small intestinal malabsorption despite light microscopically normal duodenal mucosa. Electron microscopic studies of the two latter patient groups revealed subtle architectural damage of the small intestinal mucosa. Similar minor small intestinal histopathology may persist also in treated coeliac patients. We also cannot exclude, as Bjarnason et al. proposed, the possibility of an inherent increase in small intestinal mucosal permeability in coeliac patients. Further studies are needed to investigate this issue and to determine the clinical importance of the findings.

In a kinetic study of D-xylose absorption, Breiter et al. found that total breath H_2 excretion was not correlated with the extent of D-xylose absorption [19]. Likewise, we could not show any correlation between the results of the ^{13}C-sorbitol breath test and peak H_2 excretion. H_2 production in the large intestine due to small intestinal malabsorption of carbohydrate has been showed to vary considerably between individuals, and also interindividually over time [20-22]. This may explain the lower sensitivity of the H_2-sorbitol breath test compared to the ^{13}C-sorbitol breath test, and also the lack of correlation between the two breath tests. Our previous study of the H_2-sorbitol breath test showed very low specificity of the H_2-sorbitol breath test for untreated coeliac disease versus patients with gastrointestinal symptoms without coeliac disease

and healthy control subjects [11]. The present study shows that the H_2-sorbitol breath test is also not ideally suited for follow-up of coeliac disease.

Some studies have showed changes in intestinal motility after treatment of coeliac disease with GFD [23-25]. Time to reach peak H_2 concentration was unchanged after GFD in our study. If there had been a significant change in motility with a gluten-free diet, we would expect a difference in rate of delivery of sorbitol to the large intestine and a change in time to reach peak H_2 concentration. Our study did not provide such evidence, and we believe that the changes in $^{13}CO_2$ excretion rate after GFD is mainly due to increased small intestinal sorbitol absorption capacity.

As expected, coeliac patients reported reduced gastrointestinal symptoms after GFD. Interestingly, the fatigue score after GFD was inversely associated to ^{13}C-sorbitol breath test results. Studies of patients with untreated coeliac disease have not showed any correlation between symptom presentation and severity of mucosal damage [26]. Further studies are needed to examine the possibility of a correlation of fatigue in patients with treated coeliac disease and persistently reduced small intestinal absorption capacity.

In conclusion, we recommend that the H_2-sorbitol breath test be abandoned due to low specificity versus patients with other gastrointestinal disorders such as IBS, and versus healthy controls. The novel ^{13}C-sorbitol breath test shows superior diagnostic properties to the H_2-sorbitol breath test for diagnosis and follow-up of coeliac disease.

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Table I. Clinical characteristics of study patients.

	Untreated coeliac patients, n = 39	Treated coeliac patients, n = 27	Patient controls, n = 40	Healthy control subjects, n = 26
Age, median (range)	36 (19-82)	44 (20-83)	29 (17-65)	30 (22-60)
Female/Male	24/15	15/12	26/14	18/8
BMI, mean (SD)	22.9 (3.6)	23.7 (3.4)	22.3 (2.8)	22.3 (2.5)
Small bowel histology:				
Marsh 0	0	20	36	
Marsh I	0	4	4	
Marsh IIIa	15	1	0	
Marsh IIIb	12	0	0	
Marsh IIIc	12	0	0	
IgA tTGA > 5U/ml	36	2	5	0
IgA tTGA 2-5U/ml	0	6	1	0
IgA tTGA < 2U/ml	3	19	0	26

BMI, body mass index, kg/m^2; IgA tTGA, IgA tissue transglutaminase antibody

Table II. Symptoms graded 1-4 (none-mild-moderate-severe) in coeliac patients tested twice.

Symptoms	*n*	Untreated coeliac patients Mean (SD)	Treated coeliac patients Mean (SD)	*p*-value
Diarrhoea	27	2.7 (1.0)	1.6 (1.0)	0.001
Bloating	22	2.3 (0.9)	2.1 (1.1)	0.55
Abdominal pain	26	2.9 (1.1)	1.4 (0.7)	0.001
Nausea	26	3.3 (1.1)	1.2 (0.5)	< 0.001
Constipation	27	2.9 (1.3)	1.2 (0.5)	< 0.001
Fatigue	20	2.7 (1.1)	1.8 (1.1)	0.03

***n*;** number of individuals in whom the paired *t*-test was performed.

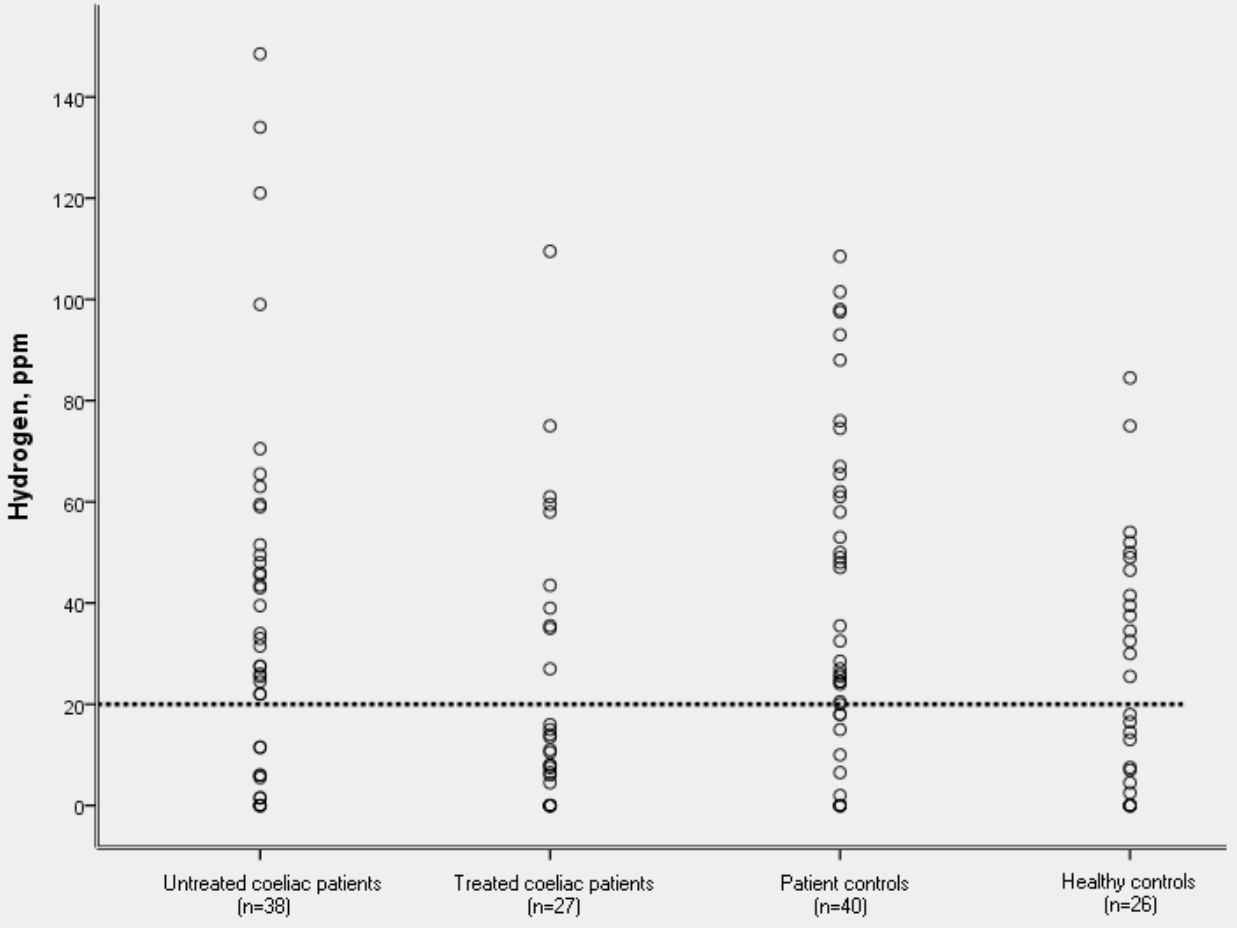

Figure 1. Scatterplot of peak H_2 concentrations in the four study groups. Dottet line represents diagnostic cut-off value.

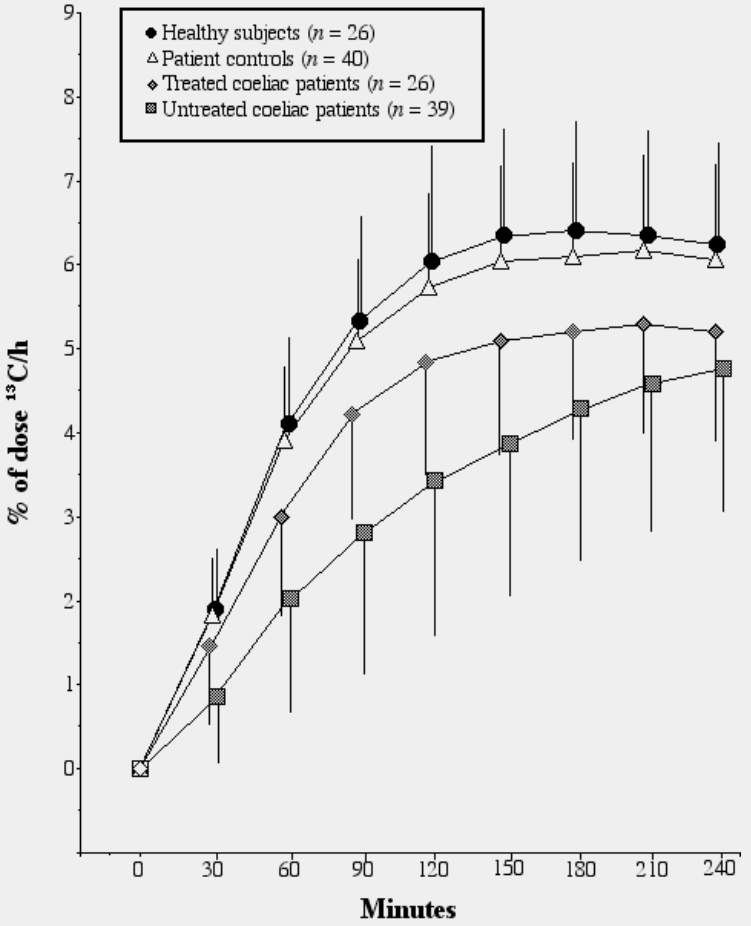

Figure 2. ^{13}C-sorbitol breath test: Mean (SD) $^{13}CO_2$ excretion in study subjects measured every 30 minutes for 240 minutes.

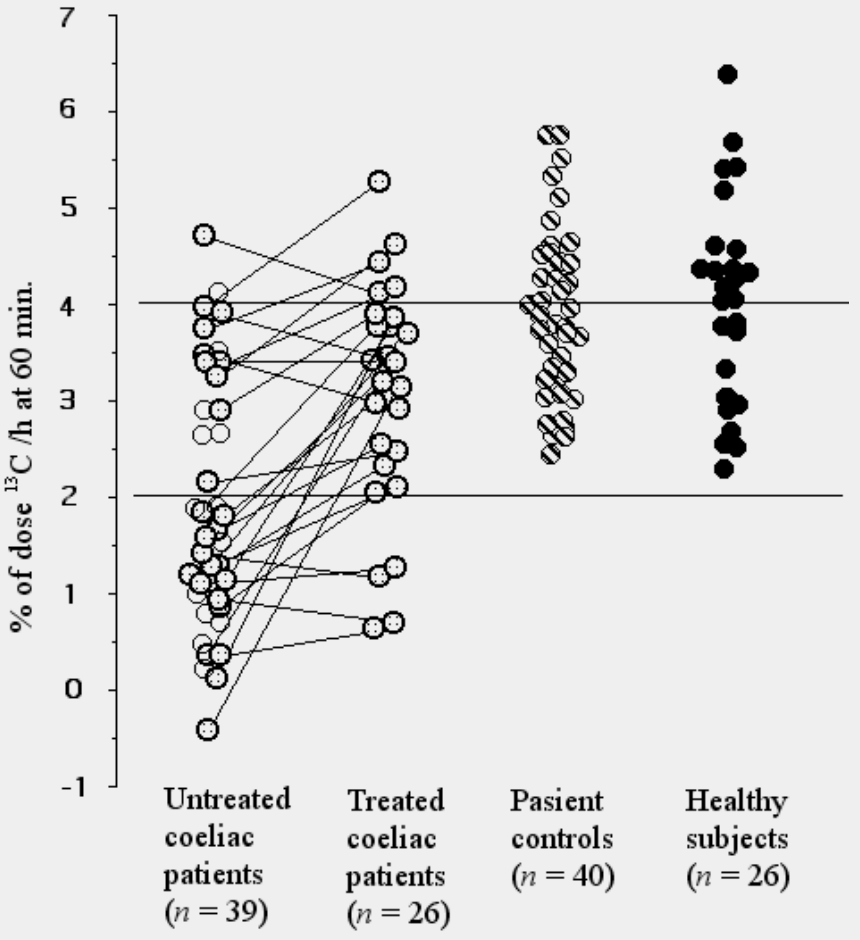

Figure 3. ^{13}C-sorbitol breath test: Individual $^{13}CO_2$ levels in breath after 60 minutes in study subjects. Horizontal lines represent cut-off values in the diagnostic algorithm.