CHAPTER 5

Lactose (mal)digestion evaluated by the "C-lactose digestion test

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ABSTRACT

Background: The prevalence of genetically determined lactase non-persistence is based on the results of the lactose H₂ breath test. This test, however, is an indirect test, which might lead to misinterpretation.

Design: We determined lactase activity in healthy Chinese and Dutch students using a novel ¹³C-lactose digestion test. The cut-off value of this test was established in a Chinese population with a homogenous genetic background of lactase non-persistence and was compared with the results obtained in a Caucasian population. Twenty-five grams of a ¹³C-lactose solution was consumed by 12 known H₂-positive and 5 H₂-negative Chinese students and 48 Dutch students and, subsequently, ¹³C-glucose concentration in plasma and H₂ excretion in breath were measured.

Results: A similar ¹³C-glucose response curve was found in all Chinese students. The mean response curve in the Dutch students was more pronounced (P<0.01). The 1 h (peak) plasma ¹³C-glucose concentration was the best discriminator between lactose digesting and maldigesting subjects. The cut-off level of 2 mmol/L⁻¹ ¹³C-glucose in plasma was defined in the H₂-positive Chinese students group. Based on the ¹³C-glucose response, the prevalence of lactose maldigestion in the Dutch subjects was 25%; based on the lactose H₂ breath test 17%.

Conclusions: Using the ¹³C-lactose digestion test, the results demonstrate a higher prevalence of lactose maldigestion in a Caucasian population than indicated by the results of the H₂ breath test. A moderate increase in the plasma ¹³C-glucose concentration after consumption of ¹³C-lactose in the young adult Chinese subjects indicates a residual lactase activity in that age group, even when a positive H₂ breath test result is obtained. These results indicate that the ¹³C-glucose concentration in plasma more accurately reflects the small intestinal lactose digestion capacity than the lactose H₂ breath test.
INTRODUCTION
Prevalence of hypolactasia in different populations in the world has been well documented. Clear differences exist between Caucasians and individuals of non-Caucasian origin. In many of these studies, a lactose H$_2$ breath test with unphysiological high doses of substrate (50 g, equivalent to the consumption of 1 liter of cows milk), is used. The shortcomings of the H$_2$ breath test are well known. The prevalence of non-H$_2$ producers according to Arola is 2-20%. Moreover, antibiotics, low colonic pH and other extra-intestinal factors (smoking, hyperventilation) can interfere with the outcome of the test. False positive test results are nevertheless rare. In our recent clinical study in pediatric patients with various gastrointestinal diseases, the diagnosis of hypolactasia by measuring lactase activity in biopsy material and by performing the H$_2$ breath test were compared; false negative breath test results occurred in 46%.

In the present study, we used a more physiological dose of 25 g lactose and studied the H$_2$ breath response in 73 young healthy Chinese students. From this group with genetically determined lactase non persistence, a sample of individuals with a positive test result was used as a reference for lactose maldigestion. The results obtained from this group were compared with those of a group of Dutch students of comparable age with a known high prevalence of genetically determined lactase persistence and with those of the minority of Chinese subjects with a negative lactose H$_2$ breath test result.

SUBJECTS AND METHODS
Subjects
Seventy-three (35 male and 38 female, age range between 19 and 22 years) Chinese students at the West China University of Medical Sciences, Chengdu, China, and 48 (13 male and 35 female, aged 20 to 25 y) Dutch students at the University of Groningen, The Netherlands, were recruited for this study. All subjects were apparently healthy and had not been using antibiotics during the 10 days before the study. Verbal informed consent was obtained from all tested subjects. The study was approved by the ethical committee of both the West China University of Medical Sciences, Chengdu, China, and the University of Groningen, Groningen, The Netherlands.

Lactose
The unlabeled lactose was pure lactose (>99%) obtained from Spruyt Hillen (Utrecht, The Netherlands). The $^{13}$C-lactose used in this study was obtained from the Dutch Institute of Dairy Research (NIZO), Ede, The Netherlands (Dr. R. van der Meer). This $^{13}$C-lactose consisted of naturally $^{13}$C-enriched lactose, derived from milk of cows fed with cattle fodder corn for 5 weeks. Milk of several cows was pooled. Lactose was obtained by standard procedures. The chemical purity of the $^{13}$C-lactose powder was 95%. The $^{13}$C abundance of $^{13}$C-
lactose was 1.098% ($\delta^{13}C_{POB} -12 \%$). Bacterial cultures of the $^{13}$C-labeled and unlabeled lactose powder for enterococci, salmonellae, and other enteropathogens were negative.

**Experimental protocol in Chengdu, China**

All Chinese students and two Dutch students, who conducted this investigation in Chengdu, were subjected to this protocol. The data obtained from the two Dutch students were used as an internal control and were subsequently added to the data of the Dutch group.

**Protocol breath hydrogen test**

Subjects were advised to consume a low-fiber diet during the day before the experiment to lower the fasting breath $H_2$ concentration. After an overnight fast (>12 h), 25g of unlabelled lactose dissolved in 250 mL water was consumed by the subjects. The subjects remained quietly seated during the test period. They were allowed to drink water, coffee or tea (without sugar and cream) from two h after the lactose ingestion. No smoking was allowed, since smoking has been shown to increase breath $H_2$ concentration$^6$. The end-expiratory air, which essentially represents alveolar air$^8$, was sampled by breathing through a straw into a 20-mL syringe. Breath air samples for $H_2$ determination were collected before and every 30 min after the lactose load for 6 hours.

**Protocol $13C$-lactose test**

The 5 Chinese subjects with an increase in breath $H_2$ concentration lower than 20 ppm in this 6-hour experiment (possibly non-producers) were further evaluated by using $^{13}$C-lactose as substrate. Additionally twelve of the 68 Chinese students with a breath $H_2$ concentration higher than 20 p.p.m. after consumption of 25 g of lactose were also recruited for the $^{13}$C-lactose test (randomly selected). The subjects were asked to refrain from consuming foods naturally enriched in $^{13}$C, such as cane sugar, corn, corn products and pineapple, for two days before the experiment. The substrate, 25 g $^{13}$C-lactose in 250 mL water, was administered orally. A venous catheter (Becton Dickinson GMBH, Heidelberg, Germany) was placed in an antecubital vein which allowed repetitive blood sampling. Samples of 2 mL of venous blood were taken 30 minutes before and 0, 60, 120, 180, 240, 300 and 360 min after substrate ingestion. The blood-sampling tubes (Vacutainer®, Becton Dickinson GMBH, Heidelberg, Germany) contained NaF and potassium oxalate. The other experimental conditions were similar to those as described above for the initially conducted breath hydrogen test.

**Experimental protocol in Groningen, The Netherlands**

All 48 Dutch students consumed 25 g $^{13}$C-lactose in 250 mL of water, while
simultaneously performing the H₂ breath test and the 13C-lactose digestion test. The experimental conditions were identical to those conducted in Chengdu.

Analytical methods
1. Breath H₂ determination: a H₂ monitor (type 60HP, Stimotron Medizinische Geraete, Wendelstein, Germany) was used to measure the breath hydrogen concentrations. The monitor was calibrated using a standard gas containing 96 ppm H₂. To evaluate the validity of the monitor, some breath air samples were both determined by the monitor and gas chromatography, which is routinely used in the Groningen laboratory⁹. The results showed that H₂-positive and -negative persons evaluated by H₂ monitor and by gas chromatography were identical.

2. Plasma ¹³C-glucose determination: one aliquot of serum was analyzed for glucose concentration applying routine techniques by using an ECA-180 glucose analyzer (Medingen, Germany), and a second aliquot was prepared for ¹³C/¹²C analysis. This method was first described by Normand et al¹⁰; we used the same method with some modifications. 100 µL plasma was added to 1 mL ethanol for denaturation of plasma proteins. After vortex mixing and centrifugation the supernatant was transferred to 2 mL vials. After evaporation to dryness, sugars were derivatized to the penta-acetate derivative using 75 µL acetic acid anhydride / pyridine (10:5 v/v) reacting for 1.5 h at room temperature. After evaporation of the reagent, the derivatives were dissolved in 500 µL chloroform.

The ¹³C/¹²C ratio of glucose was determined applying Gas Chromatography/Combustion/Isotope Ratio Mass Spectrometry (GC/C/IRMS). A Delta S/GC instrument (Finnigan MAT, Bremen, Germany) was used. The GC conditions were as follows: 2 µL of the chloroform solution were injected in the splitless mode onto a 25m x 0.32 mm (0.2 µm film thickness) OV1701 column (CP Sil 19CB, Chrompack, Middelburg, The Netherlands) installed in a Varian 3300 GC. The oven temperature was programmed from 100°C (1 min) to 275°C (2 min) at a rate of 30°C min⁻¹. Helium was used as the carrier gas at a column head pressure of 20 psi. Eluting compounds were combusted on-line in a platin catalyzed CuO oxidation reactor operating at 800 °C. Solvent and compounds eluting before 4 min were kept out of the reactor applying a helium backflush gas flow. Water vapor was removed by a nafion tubing and the CO₂ pulses formed in the reactor are transferred to the IRMS through an open split interface. ¹³CO₂ /¹²CO₂ measured by IRMS is corrected for ¹⁷O abundance¹¹ and the final ¹³C /¹²C ratio is expressed as δ¹³C_PDB¹².

Calculations
The δ¹³C_PDB value is converted to the atom % value (AP). The AP values after
ingestion of substrate are corrected for the baseline abundance. The difference (atom % excess (APE)) is used for further calculations. For breath CO₂, the APE value is related to the standard CO₂ excretion (300 mmol/m² BSA h⁻¹) in order to calculate the substrate derived exhalation rate. This exhalation rate is calculated as % dose recovered (PDR h⁻¹). Total recovery after 6 h is calculated as the cumulative % dose (cPDR 6h). The ¹³C/¹²C of plasma glucose is converted to the AP ¹³C. The concentration of glucose derived from the ¹³C-lactose (exogenous glucose) in plasma is calculated as follows:

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[¹³C-glucose] = [total glucose] \times \frac{(AP^{¹³C}_t - AP^{¹³C}_{t=0})}{(AP_{substrate} - AP_{body}) \times 2.67}
\]

in which:

- \([total glucose]\) = glucose concentration in plasma (mmol L⁻¹)
- \(AP^{¹³C}_t\) = atom % ¹³C of plasma glucose at time point \(t\) after ingestion of ¹³C lactose
- \(AP^{¹³C}_{t=0}\) = atom % ¹³C of plasma glucose before ingestion of ¹³C lactose
- \(AP_{substrate}\) = atom % ¹³C of the administered lactose
- \(AP_{body}\) = atom % ¹³C of endogenous glucose, expressed by the basal ¹³C abundance in breath
- 2.67 = factor to correct for the dilution of glucose ¹³C abundance the ¹³C abundance of the derivatizing acetate C atoms

**Data analysis and Statistics**

The mean H₂ concentration (p.p.m.) of two breath samples before lactose ingestion was taken as the basal value. Subsequent ∆H₂ concentrations were expressed as the increment above this basal level. When the increment exceeded 20 p.p.m. the subject was considered to be a lactose maldigester (H₂-positive). This criterion was used in the non-labeled lactose pilot study as well in the ¹³C-lactose study.

The baseline value of plasma glucose ¹³C-abundance was calculated from two samples collected before ¹³C-lactose ingestion. Plasma glucose concentration enrichment was defined as the increment of ¹³C above the baseline ¹³C-abundance level.

Unpaired Student’s \(t\)-test (two tailed) was used to test the differences of mean values when two (sub)groups were assessed. \(P<0.05\) was considered to be significant.

**RESULTS**

To evaluate the role of the ¹³C-lactose digestion test for the diagnosis of hypolactasia, this test was first applied in healthy Chinese students presumed to be lactose maldigesters. First a screening was made in 73 Chinese students with 25 g unlabeled lactose: 93% of these subjects were H₂-positive and 7%
H₂-negative. From the H₂-positive Chinese students (considered to be true maldigesters), 12 were randomly included in consecutive studies with ¹³C-lactose together with all 5 Chinese H₂-negative students. In all subjects, the serum ¹³C-glucose concentration rise and H₂ response were measured after substrate ingestion. In the 12 initially H₂-positive individuals, the H₂ response result was identical in the repeated test, in one of 5 screened negative individuals the H₂ response became positive in the repeated test. The results of this individual are not included because of the fact that our suspicion of false negative H₂ result in the first test could not be proven retrospectively. Fig.1 shows the mean total glucose response and the mean fractional ¹³C-glucose response in plasma in the 12 H₂-positive Chinese students. The mean total glucose concentration did not show an increase after lactose digestion. However, a clear increase in ¹³C-glucose concentration in plasma was found, representing ¹³C-substrate derived serum glucose, which was not expected in these adult individuals with genetically determined low lactase activity. A maximal ¹³C-glucose concentration increase

Figure 1. The (total) glucose concentration (lactose digestion test, LDT) (○○) and the ¹³C-glucose concentration (¹³C-LDT) (●●) in plasma of 12 H₂-positive Chinese students after consumption of 25 g ¹³C-lactose. Mean values ± SD. *: P<0.05; **: P<0.01.

Figure 2. ¹³C-glucose concentration in plasma of H₂-positive Chinese students (n=12) (malabsorbers; ●●) and of H₂-negative Dutch students (n=40) (absorbers; ○○) after consumption of 25 g ¹³C-lactose. Mean values ± SD. **: P<0.01.
was found 1 h after substrate ingestion. In Fig. 2 the $^{13}$C-glucose response in these $H_2$-positive Chinese students is compared with the response in the 40/48 $H_2$-negative Dutch students, assumed to be lactose digesters because of their genetical background. The difference was maximal at 1 h after lactose consumption ($P<0.01$). Calculating the area under the curve of $^{13}$C-glucose showed no improvement in discriminating digesting and maldigesting subjects (data not shown). Therefore, the 1 h-value of plasma $^{13}$C-glucose concentration was used as criterion for evaluation of hypolactasia. The students of Chengdu, a population which can be considered as relatively genetically homogenous, were considered as a reference group for lactase non-persistence. From this group, 12 $H_2$-positive individuals were randomly selected. The 1 h $^{13}$C-glucose cut-off value (mean value of the group plus 2 SD) turned out to be 2.0 mmol L$^{-1}$. With this cut-off value the $H_2$-negative Chinese students (considered as possibly false negative maldigesters, due to their genetical background) and a population of 48 Dutch students were analyzed. From the Dutch group, 17% were $H_2$-positive and 83% $H_2$-negative after consumption of 25 g lactose. In Fig. 3 the results of the $^{13}$C-lactose digestion test are shown. All $H_2$-positive Chinese students had $^{13}$C-glucose concentrations below 2 (by definition); all but

**Figure 3.** 1 h $^{13}$C-glucose values in plasma after consumption of 25 g $^{13}$C-lactose. Four groups were compared: 1: $H_2$-positive Chinese students (n=12), 2: $H_2$-negative Chinese students (n=4), 3: $H_2$-positive Dutch students (n=8) and 4: $H_2$-negative Dutch students (n=40).
one of the H₂-negative Chinese students had values below the cut-off value. From the 8 Dutch H₂-positive students, 5 exhibited ¹³C-glucose values below the cut-off value for ¹³C-glucose. From the 40 Dutch H₂-negative group, 7 had low ¹³C-glucose values.

To analyze the discrepancy in more detail, ¹³C-glucose values from all 48 Dutch students and 16 Chinese students were plotted against their increase in H₂ values (fig. 4). In the disconcordant area (indicated as blank area) a considerable number of H₂-negative Dutch students were found with low ¹³C-glucose concentrations. Two of the three H₂-positive students with high ¹³C-glucose concentrations had a late H₂-response (increase in H₂ values after 3 h instead of 2 h) possibly indicating non lactose related colonic fermentation processes.

Using the combined H₂ and ¹³C-glucose values (at least one of both tests positive), we extrapolated that 99% and 25% of the Chinese and Dutch population, respectively, had lactase non-persistence.

**DISCUSSION**

Adequate diagnosis of hypolactasia or lactase non-persistence is relevant for clinical and epidemiological studies. The existing techniques of the lactose tolerance test (LTT), H₂ breath test and small intestinal lactase enzyme activity assay have well-known shortcomings. Therefore, we developed a ¹³C-lactose digestion test. This test allows for the discrimination between the origin of exogenous (substrate derived) plasma glucose and endogenous glucose. To prove the diagnostic power and establish the cut-off values, we applied this technique first in a genetically homogenous population in Chengdu, China, with a very high (presumably 100%) prevalence of genetically determined low...
intestinal lactase activity. The individuals with a positive lactose H₂ breath test result in this population were considered as the reference group of lactose maldigestion, as it is known that this test has an almost negligible proportion of false positive results. To our surprise, a considerable rise in ¹³C-glucose concentration in plasma was observed after ¹³C-lactose consumption. Obviously, this adult population still has some lactase activity available, which allows lactose digestion and absorption of the hydrolysis products (glucose and galactose) to a certain extent. Despite this observation, we still consider this group as the best available reference group of lactose maldigestion. A mutation, which caused the phenotype of lactase persistence into adulthood, was genetically selected especially in cattle breeding communities in Northern European regions. Therefore our homogenous Chinese reference group, without known selection of the lactase persistence mutation, is preferable over any heterogeneous patient group with symptoms and biopsy proven low lactase activity. In such a patient group the overall lactose digesting capacity is difficult to establish reliably and cannot simply be derived from measuring lactase activity in one single biopsy sample.

The largest difference in ¹³C-glucose response between lactose digesting and maldigesting individuals was found 1 h after consumption of labeled lactose. Also, theoretically, one can expect that with a time period < 1 h variations in gastric emptying will influence the outcome, and with a time period > 2 h variations in hepatic glucose metabolism will play a more prominent role. The 1 h ¹³C-glucose rise was therefore used as parameter to discriminate between both groups, with a cut-off level of 2 mmol L⁻¹. The H₂-negative Chinese students had a similar ¹³C-glucose concentration compared to their H₂-positive group members, which indicates that all but one were false negative in the H₂-response. When we combine the results of the initially positive Chinese subjects in the unlabeled lactose H₂-screening test (confirmed by the ¹³C-labeled lactose test) and the serum ¹³C-glucose concentration rises in the subsequent ¹³C-lactose digestion test, a prevalence of hypolactasia in the Chinese population of 99% is extrapolated. This finding confirms the expected fact that in lactose maldigesting subjects, false negative H₂ breath test results occur.

Using the same criteria for the Dutch students, we also found a considerable number of false negative H₂ responders. With the H₂ test a prevalence of 17% lactose maldigestion was found, which is higher than found in previous studies in Dutch population samples (2% (children) – 8% (adults with gastrointestinal complaints))³. Using the new ¹³C-lactose digestion test, a prevalence of 25% was found. It is clear that additional studies with larger numbers of subjects have to be performed to obtain an accurate value for the prevalence in the whole Northern European population. Our novel test could possibly be performed by a two sample venous micro puncture technique at time zero and one hour after substrate ingestion.
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