Lactose Intolerance Quick Test CLINICAL TRIAL

Performance of the LACTOSE INTOLERANCE Quick Test in Diagnosis

of Adult-Type Hypolactasia Among Dyspeptic Patients.

Jointly Executed by:

Biohit Oyj (Helsinki, Finland); Hospital X (City Y, Country Z)

Research Team:

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Summary

Background: Hypolactasia, or **lactose intolerance (LI)** exists in three different forms: i) primary (adult-type), ii) secondary, and iii) congenital. **Adult-type hypolactasia**, also known as lactose malabsorption or lactase non-persistence, is an autosomal recessive condition that affects about **half of the world's population**. It is caused by the developmental down-regulation of the **lactase-phlorizin hydrolase (LPH) enzyme** in the brush border of epithelial cells in the small intestines. Affected persons have insufficient levels of lactase that catalyses hydrolysis of lactose into glucose and galactose in their digestive system. Consuming milk and other dairy products causes gastrointestinal symptoms which vary in severity and may include abdominal bloating and cramps, flatulence, diarrhoea, nausea, borborygmi (rumbling stomach), or vomiting. Similar **dyspeptic symptoms** can be caused by a wide variety of gastrointestinal disorders and diseases, and making a specific clinical diagnosis is essential to establish correct therapeutic measures. **Biohit Oyj** (Helsinki, Finland) has developed two diagnostic tests for patients with dyspeptic symptoms: 1) Biohit GastroPanel® test, and 2) Biohit **LACTOSE INTOLERANCE Quick Test (LIQT)**, for assessment of 1) gastric mucosal structure and function, as well as 2) for rapid (POC; point-of-care) diagnosis of hypolactasia (LI), respectively. Clinical validation studies in different settings are advocated for both tests.

Objective: To test the clinical performance of the Biohit Lactose Intolerance Quick Test (LIQT) in diagnosis of adult-type hypolactasia in patients with dyspeptic symptoms.

Study Design: This clinical trial evaluates the performance of LIQT in duodenal biopsies of patients referred for endoscopy (EGD) due to dyspeptic symptoms, using two gold standards: disaccharidase assay and genetic testing for C/C_{-13910} polymorphism. Hydrogen Breath Test (HBT) can be used as a comparison test, if appropriate.

Methods: Study subjects (both genders) for the cohort are enrolled among the consecutive adult patients with dyspeptic symptoms (with or without suspicion of LI), referred for endoscopic examination at Hospital X (City Y, Country Z). All patients are subjected to esophago-gastro-duodenoscopy (EGD). In addition to the usual sampling of gastric mucosa (interpreted according to Updated Sydney System; USS), two biopsies from the descending duodenum (below the level of the papilla Vateri) are needed for the lactase testing. LIQT is performed following the manufacturer's instructions using the biopsies from post-bulbar duodenum. The reference method used for confirming LI diagnosis is a direct biochemical assay for disaccharidase activity in duodenal biopsies, described by Dahlqvist (34). Another gold standard test in this study is PCR testing for C/T_{-13910} polymorphism, according to Kuokkanen et al. 2003 (48). Statistical analyses include calculation of the performance indicators (SE, SP, PPV, NPV) of the LIQT for two study endpoints (mild and severe adult-type hypolactasia), using the two gold standards as reference. If relevant, Biohit LIQT can be compared with HBT (hydrogen breath test), using the AUC (area under ROC curve) comparison (roccomb) test.

Specific Aims: The most important goal of this study is to assess the performance of the LACTOSE INTOLERANCE Quick Test in diagnosis of adult-type hypolactasia (LI) in patients with dyspeptic complaints, using biochemical and genetic testing as the reference (gold standards). The two primary study endpoints are i) mild, and ii) severe adult-type hypolactasia. If relevant to the study setting, the performance of LIQT can be compared with that of HBT (optional). In addition to these primary study endpoints, this study also provides information about the frequent co-existence of adult-type hypolactasia and **celiac disease (CD)**, diagnosed in duodenal biopsies using the Marsh classification.

Study execution and time table: The necessary preparations for the study execution at Hospital X will start immediately when the hospital has reached the agreement with Biohit Oyj. The study plan necessitates a review by the institutional review board (IRB, Ethical Committee) before permission to start. Given that the subjects in the study will be enrolled among consecutive patients with clinical symptoms of dyspepsia, attending the Outpatient Department of Endoscopy, Hospital X, it is estimated that at least 200 subjects need to be screened by LIQT to reach a cohort of 100 patents enriched with equal numbers (n=50) of both mild and severe hypolactasia. Subjects testing negative (normolactasia) in LIQT will be used as controls in calculations of the test indicators.

Impact of the study: Confirmatory diagnosis of adult-type hypolactasia (LI) is not possible on the basis of clinical (dyspeptic) symptoms but requires biochemical lactase testing in duodenal biopsies, or PCR assay for C/T_{-13910} polymorphism in a blood sample. Both tests require specialized laboratory and the test results are delayed. Designed to circumvent these disadvantages, Biohit LIQT is a biopsy-based method for rapid diagnosis of duodenal hypolactasia. If shown to favourably compete with the classical LI tests in this clinical trial, Biohit LACTOSE INTOLERANCE Quick Test should represent a major step forward towards a reliable and user-friendly **point-of-care diagnosis** of hypolactasia (LI).

1.BACKGROUND

Lactose intolerance (LI), also called lactase deficiency (LD) and **hypolactasia**, is the inability to digest lactose, a sugar found in milk and to a lesser extent milk-derived dairy products. It is not a disorder as such, but a genetically-determined characteristic. Lactose intolerant individuals have insufficient levels of **lactase**, an enzyme that catalyzes hydrolysis of lactose into glucose and galactose, in their digestive system. In most cases this causes symptoms which may include abdominal bloating and cramps, flatulence, diarrhea, nausea, borborygmi (rumbling stomach), or vomiting (1), after consuming significant amounts of lactose. Some studies have produced evidence that milk consumption by lactose intolerant individuals may be a significant cause of inflammatory bowel disease (2,3). Most mammals normally cease to produce lactase, becoming lactose intolerant, after weaning, but some human populations have developed lactase persistence, in which lactase production continues into adulthood (2,4).

1.1.Epidemiology

It is estimated that 75% of adults worldwide show some decrease in lactase activity during adulthood (4). The frequency of decreased lactase activity ranges from 5% in Northern Europe through 71% for Sicily to more than 90% in some African and Asian countries (5). This distribution is now thought to have been caused by recent natural selection favoring lactase-persistent individuals in cultures in which dairy products are available as a food source (6,7). While it was first thought that this would mean that populations in Europe, India, Arabia and Africa had high frequencies of lactase persistence because of a particular mutation, it was later shown that lactase persistence is caused by several independently occurring mutations (8). Indeed, lactase persistence is a dominantly inherited state and is frequent in populations adapted to dairy products, especially in Northern Europe (9-11). A variant, C/T_{-13910}, located about 14 kb upstream from the initiation codon of lactase gene on chromosome 2q21, has been shown to be associated with lactase persistence/non-persistence (12-15).

1.2.Clinical presentation

Lactose intolerance primarily refers to a syndrome having one or more symptoms upon the consumption of food substances containing lactose. Individuals may be lactose intolerant to varying degrees, depending on the severity of these symptoms. Lactose malabsorption refers to the physiological concomitant of lactase deficiency (i.e., the body does not have sufficient lactase capacity to digest the amount of lactose ingested)(16).

1.2.1.Classification

Three distinct groups of patients with LI can be differentiated, based on their different pathogenesis:

1) Primary lactase deficiency (PLD) is a genetic disorder, only affecting adults and is caused by the absence of a lactase persistence allele (12,16). It is the most common cause of LI, because a majority of the world's population lacks these alleles (17).

2) Secondary, acquired, or transient lactase deficiency is caused by an injury to the small intestine, usually during infancy, from acute gastroenteritis, diarrhea, chemotherapy, intestinal parasites or other environmental causes (16,18,19).

3) Congenital lactase deficiency (CLD) is a very rare, autosomal recessive genetic disorder that prevents lactase expression from the birth (16). It is unusually common in Finland (4,20). People with CLD cannot digest lactose from the birth, and therefore cannot digest breast milk at all.

It is important to recognize that LI is **not an allergy** because it is not an immune response, but rather a problem with digestion caused by lactase deficiency. Milk allergy is a separate condition, with distinct symptoms that occur when the presence of milk proteins trigger an immune reaction.

1.2.2.Clinical symptoms

The principal symptom of LI is an adverse reaction to products containing lactose (primarily milk), including abdominal bloating and cramps, flatulence, diarrhea, nausea, borborygmi

(rumbling stomach) and vomiting (particularly in adolescents). These appear thirty minutes to two hours after consumption (1-3). The severity of symptoms typically increases with the amount of lactose consumed; most lactose-intolerant people can tolerate a certain level of lactose in their diet without ill-effect (21,22).

Lactose intolerance, biliary reflux, and *H. Pylori* infection are clinical conditions commonly found in general population and particularly in patients with gastrointestinal disorders. Frequently, **symptoms** reported in each condition are **similar** even if related to the presence of different pathogenetic factors. Therefore, it is essential to distinguish between different pathophysiological patterns of symptoms in order to establish correct therapy.

1.2.2.1.Bloating

Bloating is any abnormal general swelling, or increase in diameter of the abdominal area. As a symptom, the patient feels a full and tight abdomen, which may cause abdominal pain, and sometimes accompanied by increased stomach growling or more seriously the total lack of it. The most common symptom associated with bloating is a sensation that the abdomen is full or distended. Rarely, bloating may be painful or cause shortness of breath.

Pains that are due to bloating will feel sharp and cause the stomach to cramp. These pains may occur anywhere in the body and can change locations quickly (1,2,4). They are so painful that they are sometimes mistaken for heart pains when they develop on the upper left side of the chest. Pains on the right side are often confused with problems in the appendix or the gallbladder. One symptom of gas that is not normally associated with LI is the hiccup. Hiccups are harmless and will diminish on their own; they also help release gas that is in the digestive tract before it moves down to the intestines and causes bloating.

1.2.2.2.Cramps

A cramp is an involuntary temporary strong muscle contraction or over-shortening, which may cause a severe pain. Usually the onset is sudden while the cramp resolves spontaneously

in a few seconds to minutes. Other common causes of skeletal muscle cramps include muscle fatigue, low sodium, low potassium, and/or low magnesium.

1.2.2.3.Flatulence

In people with LI, the intestinal bacteria feeding on lactose can give rise to excessive gas production when milk or lactose-containing substances have been consumed.

1.2.2.4.Diarrhea

In healthy individuals, too much magnesium or vitamin C or undigested lactose can produce osmotic diarrhea and distention of the bowel. A person who has LI can have difficulty absorbing lactose after an extraordinarily high intake of dairy products.

1.2.2.5.Borborygmi

A stomach rumble, also known as a bowel sound or peristaltic sound, is a rumbling, growling or gurgling noise produced by the small intestine that occurs due to peristalsis, a series of contractions that propel the contents of the gastro-intestinal tract forward, which is the ultimate cause of the rumble. Incomplete digestion of food can lead to excess gas in the intestine. In humans, this can be due to incomplete digestion of carbohydrate-containing foods, including **milk** and other **dairy products** (**lactose intolerance** or the use of α-glucosidase inhibitors by diabetics), gluten (protein in wheat, barley, and rye)(celiac disease), fruit, vegetables, beans, legumes, and high-fiber whole grains.

1.2.3.Nutritional implications

Congenital lactase deficiency (CLD), where the production of lactase is inhibited from the birth, can be dangerous in any society because of infants' initial dependence on human breast milk for nutrition until they are weaned onto other foods. Earlier, babies born with CLD often did not survive (16), but death rates decreased with soybean-derived infant formulas and manufactured lactose-free dairy products. Beyond infancy, individuals affected by CLD usually have the same nutritional concerns as any LI-adults (4,16,18).

1.3.Pathogenesis

LI is a consequence of **lactase deficiency**, which may be genetic or environmentally induced (see section 1.2.1). In either case, symptoms are caused by insufficient levels of the enzyme **lactase** in the lining of the duodenum. Lactose, a disaccharide molecule found in milk and dairy products, cannot be directly absorbed through the wall of the small intestine into the bloodstream: Thus, in the absence of lactase, lactose passes intact into the colon. Bacteria in the colon can metabolize lactose, and the resulting fermentation produces copious amounts of gas (a mixture of hydrogen, carbon dioxide and methane) that causes the various abdominal symptoms. The unabsorbed sugars and fermentation products also raise the osmotic pressure of the colon, causing an increased flow of water into the bowels (diarrhea)(2,4,16,18).

1.3.1.Genetics

The LCT (lactase) gene provides the instructions for making lactase. There is a specific DNA sequence in the MCM6 (mini-chromosome maintenance complex component 6) gene that helps control whether the LCT gene is turned on or off (23-29). The MCM6 gene provides instructions for making part of the MCM complex, a group of proteins that functions as a helicase. Helicases attach to particular regions of DNA and temporarily unwind the two spiral strands of these molecules. When a cell prepares to divide, helicases unwind the DNA so that it can be copied. The DNA that makes up the chromosomes is duplicated (replicated) so that each new cell will get a complete set of chromosomes.

A specific DNA sequence within the MCM6 gene called a regulatory element helps control the activity (expression) of the nearby LCT gene. The LCT gene which encodes the lactase protein and the MCM6 gene are both located on the long arm (q) of chromosome 2 in region 21, i.e., in the locus 2q21. (23-29). The LCT gene provides instructions for making an enzyme called lactase. This enzyme helps to digest lactose, a sugar found in milk and other dairy products. LI in adulthood is caused by gradually decreasing expression of the LCT gene after infancy, which occurs in most humans.

At least four variations have been identified in the regulatory element that modulates LCT gene expression. These variations change single DNA nucleotides in the regulatory element. Each of the variations results in sustained lactase production in the small intestine and the ability to digest lactose throughout life. People without these mutations have a reduced ability to digest lactose as they get older, resulting in the symptoms of LI.

2.DIAGNOSIS

To assess LI, intestinal function is challenged by ingesting more dairy products than can be readily digested. Clinical symptoms typically appear within 30 minutes, but may take up to two hours, depending on other foods and activities (2,4,16,18). Substantial variability in response (symptoms of nausea, cramping, bloating, diarrhea, and flatulence) is to be expected, as the extent and severity of LI varies among individuals.

LI is distinct from milk allergy, an abnormal immune response, (usually) to milk proteins. They may be distinguished in diagnosis by giving lactose-free milk, producing no symptoms in the case of lactose intolerance, but the same reaction as to normal milk in the presence of a milk allergy. An intermediate result might suggest that the person has both conditions. However, since LI is common (some degree of it being found in most adults worldwide), it is **not considered a disease** and a medical diagnosis is not normally required. If positive confirmation is necessary, optional tests are available (30).

2.1.Hydrogen breath test (HBT)

In a hydrogen breath test, after an overnight fast, 25 grams of lactose (in a solution with water) is swallowed (31). If the lactose cannot be digested, enteric bacteria metabolize it and produce hydrogen, which, along with methane, if produced, can be detected on the patient's breath by a clinical gas chromatograph or compact solid-state detector. The test takes about 2 to 3 hours to complete. For the breath hydrogen test, the specificity is reported to be 89–100% and the sensitivity 69–100%. (32).

2.2.Blood test

LI can also be diagnosed by measuring serial blood glucose levels after ingestion of lactose. Measuring blood glucose level every 10 to 15 minutes after lactose ingestion will show a "flat curve" in individuals with lactose malabsorption, while the lactase persistent subjects will have a significant glucose "peak", with a typical elevation of 50% to 100%, within one to two hours. However, due to the need for serial blood sampling, this approach has been largely replaced by breath testing. After an overnight fast, blood is drawn and then 50 grams of lactose (in aqueous solution) is swallowed. Blood is then drawn again at the 30 minute, 1-hour, 2-hour, and 3-hour mark. If the lactose cannot be digested, blood glucose levels will rise by less than 20 mg/dl (31). The diagnosis of **adult-type** hypolactasia is usually based on this lactose tolerance test, the specificity of which has been reported to be in the range of 77–96%, with a sensitivity of 76–94% (32).

2.3.Stool acidity test

This test can be used to diagnose LI **in infants**, for whom other forms of testing are impractical (33). The infant is given lactose to drink. If the individual is tolerant, the lactose is digested and absorbed in the small intestine; otherwise it is not digested and absorbed and it reaches the colon. The bacteria in the colon, mixed with the lactose, cause acidity in stools. Stools passed after the ingestion of the lactose are tested for level of acidity. If the stools are acidic, the infant is intolerant to lactose. Stool pH in LI is less than 5.5.

2.4.Disaccharidase activity measurement

A classical reference method of confirming LI diagnosis has been a direct biochemical assay of disaccharidase activity in duodenal or intestinal biopsies, described by Dahlqvist several decades ago (34). Biopsy specimens are stored at –70C and weighed in a cold room with a temperature of – 20C. Glass homogenizers are used, and homogenization is carried out in crushed ice to avoid warming of the sample. Each biopsy specimen is homogenized in 200 µl of 0.9% NaCl. The results are expressed as a U (mol substrate/min at 37C) disaccharidase/g protein. Values <10 U/g are interpreted as severe hypolactasia, those between 10-30 U/g

indicate mild hypolactasia, and those above 30 U/g implicate normolactasia (34).

2.5. Genetic testing

This method is used to confirm the primary LI. Lactase activity persistence in adults is associated with two polymorphisms: C/T_{13910} and G/A_{22018} located in MCM6 gene (23-29). These polymorphisms may be detected by molecular biology techniques at the DNA extracted from blood or saliva samples, and currently, genetic kits specific for this diagnosis are available. The procedure consists of extracting and amplifying DNA from the sample, following with a hybridization protocol in a strip. Colored bands are obtained as final result, and depending on the different combination, it would be possible to determine whether the patient is lactose intolerant. This test allows a non-invasive definitive diagnostic (23,36). Subjects with **C/C_{-13910} genotype** (indicating down-regulation of the lactase gene in adulthood) have severe hypolactasia, while those with **T/T_{-13910} genotype** (indicating persistence of lactase activity in adulthood) have normolactasia (i.e., no lactose intolerance). Interestingly, those with heterozygosity of the **C/T_{-13910} allele** demonstrate approximately half of the ability to down-regulate the lactase gene in adulthood, as compared with C/C_{-13910} homozygotes (23-29,36).

2.6. Biopsy of the small intestine

Although LI is not uncommonly associated with celiac disease (CD), causing distinct morphological alterations in the duodenal mucosa, isolated LI without CD does not cause histologically detectable morphological changes in the biopsies. First symptoms of LI are frequently non-specific dyspeptic complaints (see section 1.2.2.), and patients who undergo a diagnostic upper gastrointestinal endoscopy are frequently diagnosed as having hypolactasia in the small intestine and intolerance of dairy products. Prompted by this practice of frequent using of endoscopy as the routine diagnostic tool of LI in such patients, **Biohit Oyj** (Helsinki, Finland) recently developed a novel **biopsy-based** method for rapid endoscopic diagnosis of duodenal hypolactasia (4,37,38). This **LACTOSE INTOLERANCE Quick Test** (LIQT) is based on incubation of a post-bulbar endoscopic duodenal biopsy with

lactose in a plate in which a strong color reaction develops within 20 min if the lactase activity is "normal" and if glucose appears in the test plate from hydrolyzed lactose (4). Biohit LIQT has been tested in several studies and shown to favorably compete with the other diagnostic tests of LI (39-47).

3.THE LACTOSE INTOLERANCE QUICK TEST

Lactose Intolerance quick test (LIQT), developed by Biohit Oyj for detecting LI (hypolactasia of the small intestine) is based on the activity of the **lactase enzyme** in a biopsy specimen. Lactase enzyme in the biopsy specimen breaks down the lactose (milk sugar) in the test buffer. As a result **blue color** develops in the buffer indicating a NEGATIVE test result (=patient has normolactasia). However, if there is no or only a slight color reaction, the test is considered POSITIVE, indicating that the patient suffers from hypolactasia.

3.1.Test principle

The biopsy specimen taken from the mucosa of the upper part of the small intestine is examined immediately. The development of color in the test liquid **after 20 minutes** indicates whether there is functional lactase in the biopsy. Practical performing of the test is described in more detail in Methods (Section 4.3.) section, and illustrated step-by-step in **ANNEX 1**.

4. STUDY DESIGN

The present study is a 100% biopsy-controlled clinical trial testing the performance of Biohit LACTOSE INTOLERANCE Quick Test in detecting incident Lactose Intolerance (adult-type hypolactasia) among endoscopy-referral patients with dyspeptic symptoms. The primary study endpoints include detection of i) **mild,** and **ii) severe adult-type hypolactasia** confirmed by biochemical measurements: i) 10–30 U/g protein, and ii) <10 U/g protein, respectively; and by genetic testing for C/T_{-13910} polymorphism.

4.1. Aims of the study

The most important goal of this study is to assess the overall performance of the LACTOSE INTOLERANCE Quick Test in diagnosis of **adult-type hypolactasia** (LI) in patients with dyspeptic complaints, using biochemical and genetic testing as the reference (gold standards). This goal is reached through the following specific aims.

1. Sensitivity (SE), specificity (SP), negative predictive value (NPV), positive predictive value (PPV) and area under ROC curve (AUC) for LIQT in detecting **severe hypolactasia** (<10 U/g protein, C/C_{-13910} genotype) in duodenal biopsies.

2. SE, SP, NPV, PPV and AUC for LIQT in detecting **mild hypolactasia** (10-30 U/g protein; C/T_{-13910} genotype) in duodenal biopsies.

4.2. Patients

This clinical trial is conducted in collaboration between Biohit Oyj (Helsinki, Finland) and Hospital X (City Y, Country Z)(hereafter called "the Partners"). The study is performed exclusively in Hospital X, supervised by a steering committee consisting of members from both Partners.

Enrolment of the patients in the study will take place at Hospital X, including consecutive adult patients referred for esophagogastroduodenoscopy (EGD) at the Outpatient Department of Endoscopy, because of unexplained dyspeptic symptoms. The eligible patients are **adults** aging 18 years or older, in whom hypolactasia (LI) has not been previously diagnosed, i.e., who represent **incident cases of adult-type hypolactasia**. They have either 1) dyspeptic non-specific symptoms (Section 1.2.2.) that advocate endoscopic examination, and/or 2) might report personal experience that their symptoms are aggravated by ingestion of milk and other dairy products, implicating possible hypolactasia. All patients are referred for diagnostic endoscopy (EGD), accompanied by biopsies from the gastric and duodenal mucosa.

The total cohort to be screened by LIQT is estimated to be **at least 200 subjects** (adults, both genders), to reach a **cohort of 100 patients** enriched with **equal numbers (n=50)** of both **mild** and **severe** hypolactasia, needed to calculate the performance indicators for the two LIQT endpoints.

Patient enrollment is taking place **in a single step**. In brief, the potentially eligible patients are identified (by the clinical staff) among the endoscopy-referral adults, who have not been previously diagnosed as having LI. At this stage, every patient will be asked for their willingness to take part in the study and sign a written consent to participate.

Eligible patients are all adult subjects, referred for diagnostic endoscopy due to dyspeptic symptoms suggesting or not of adult-type hypolactasia. However, the following patients should be considered <u>**non-eligible**</u>: 1) those who refuse to sign written consent; 2) those who refuse duodenal biopsy (necessary to perform LIQT).

4.2.1.Patient preparation

Proper conduction of and reliable results from the LIQT does not necessitate any preparatory measures of the patient. The preparatory measures required for EGD for which the subjects are referred to the hospital are fully compatible with the sampling for LIQT.

4.3.Methods

4.3.1.Sample collection for LACTOSE INTOLERANCE Quick Test (LIQT)

Performance of LIQT in the laboratory, including the sampling procedure, is illustrated in **ANNEX 1.** The biopsy specimen taken from the mucosa of the **upper part** of the **small intestine** is examined <u>immediately.</u> Biopsy specimens are recommended to be taken with e.g. 5 mm forceps from the mucosa of the upper part of the small intestine at any site of the **post-bulbar duodenal** mucosa examined during gastroscopy. The bulbus should be avoided, because of the gastric acid-related changes in the lactase activity in the bulbar mucosa in some patients.

It is known that the total lactase content in the biopsy depends on the size of the biopsy used for testing. Recommended size of biopsies is between 1.5-2.0 mm, because the test was optimized for biopsies in this size range. If too small or too large biopsy is used, there is a risk of a false positive (hypolactasia) or a false negative (normolactasia) result, respectively.

4.3.2. Sample processing

Sample processing is illustrated in detail in ANNEX 1. Before preparing for testing, allow all the reagents to reach room temperature (20-25°C) for at least 15 minutes before use. Mix all the reagents before using by turning the bottles upside down for a few times. Then tap the bottles on the table to ensure the liquid returns to the bottle. When adding the drops into the wells, hold the bottles in vertical upside down position (Figures 1 and 2).

LIQT is very simple, performed in two separate steps (ANNEX 1). First, open the label covering the well on the plate, and place the biopsy specimen into the well. Then, add 2 drops (80 µl) of the substrate solution (Bottle 1) into the well, and close the label. Mix the plate by shaking 5-6 times vigorously sideways on the table. The, incubate for 15 minutes at room temperature (20-25°C).

In Step 2, open the label again, and add 1 drop (10 µl) of the chromogen solution (Bottle 2) into the well. Immediately after that, add 2 drops (80 µl) of the signal reaction solution (Bottle 3). Close the label, and mix the plate by shaking for 5-6 times vigorously sideways on the table. Finally, incubate for 5 minutes at room temperature (20-25°C) and interpret the lactase content with the color chart included in the kit package.

4.3.3. Interpretation of the LIQT results

The development of color in the test liquid after 20 minutes indicates whether there is functional lactase in the specimen. Lactase enzyme in the biopsy specimen breaks down the lactose in the test buffer. As a result blue color develops in the buffer indicating a negative

test result. However, if there is no or only a slight color reaction it can be concluded that the patient suffers from lactose intolerance (ANNEX 1; Figure 3). In the case of a negative LIQT result, hypolactasia can be excluded.

4.3.4.Hydrogen breath test (HBT)

In addition to hypolactasia, a hydrogen breath test (HBT) is used for clinical diagnosis of irritable bowel syndrome, and some other common food intolerances. The test is simple, non-invasive, and is performed after a short period of fasting (typically 8–12 hours). Even though the test is normally known as a "Hydrogen Breath Test" some physicians may also test for methane in addition to hydrogen. Many studies have shown that some patients (approximately 35% or more) do not produce hydrogen but actually produce methane. Some patients produce a combination of the two gases. Other patients, who are known as "Non-Responders", don't produce any gas. It has not been determined yet, whether they may actually produce another gas. In addition to hydrogen and methane, some facilities also utilize carbon dioxide (CO_2) in the patients' breath to determine if the breath samples that are being analyzed are not contaminated (either with room air or bronchial dead space air).

In testing LI, the patient takes a base reading of hydrogen levels in his/her breath. The patient is then given a small amount of pure lactose (typically 20 to 25 g), and then required to take readings every 15, 30 or 60 minutes for two to three hours. If the level of hydrogen rises above 20 ppm (parts per million) over the lowest preceding value within the test period, the patient is typically diagnosed as having LI. If the patient produces methane, then the readings for the methane typically rise 12 ppm over the lowest preceding value to be considered positive. If the patient produces both hydrogen and methane, then the values are typically added together and the mean of the numbers is used to determine positive results, usually 15 ppm over the lowest preceding value. However, recent study suggest that testing may not correlate with any actual diagnosis (31,32). For the BHT, the specificity is reported to be 89–100% and the sensitivity 69–100%. (32). Several commercial test kits are available to perform HBT, with no major difference in their performance.

4.3.5.Disaccharidase activity measurement

The classical reference method of confirming LI diagnosis is a direct biochemical assay for disaccharidase activity in duodenal or intestinal biopsies, described by Dahlqvist several decades ago (34). Biopsy specimens are stored at –70C and weighed in a cold room with a temperature of –20C. Glass homogenizers are used, and homogenization is carried out in crushed ice to avoid warming of the sample. Each biopsy specimen is homogenized in 200 µl of 0.9% NaCl.

In brief, disaccharidase measurement is carried out in a water bath at 37C in thin-walled polymerase chain reaction (PCR) tubes. The disaccharidase reaction is started by adding a substrate buffer solution (lactose, sucrose, or maltose in a 0.1-mol/l sodium maleate buffer, pH 6.0) to a final concentration of 0.26 mol/l. In addition, a blank sample is prepared by adding water instead of the substrate solution. The total reaction time at 37C is 60 min. Thereafter, the reaction is stopped by placing the tubes in ice and adding perchloric acid at a final concentration of 0.43 mol/l and shaking. The samples are then neutralized with 0.34 mol/l KOH – 0.075 mol/l imidazole base –0.075 mol/l KCl. The precipitate that is formed in neutralization is centrifuged down, and liberated glucose in supernatant is measured fluorometrically, using a hexokinase/glucose-6-phosphate dehydrogenase reaction with NADP as a cofactor (35). The samples are diluted to 40 times the original volume in a reaction buffer solution containing 0.14 U/ml glucose-6-phosphate dehydrogenase, 0.1 mmol/l NADP+, 0.5mmol/l dithiothreitol (DTT), 0.3 mmol/l ATP,1 mmol/l EDTA, 5 mmol/l $MgCl_2$ and 50 mmol/l Tris-HCl buffer pH 8.1. The buffer blank is measured and the reaction started by adding hexokinase (in 20 mmol/l Tris-HCl buffer pH 8.1) to a final concentration of 0.35 U/ml. Protein concentrations in the homogenate samples are determined using the BioRad Dc Protein A Assay with BSA as a standard (BioRad Laboratories, Hercules, California, USA) in accordance with the manufacturer's instructions. The results are expressed as a U (mol substrate/min at 37C) disaccharidase/g protein. The results are expressed as a U disaccharidase/g protein. Values <10 U/g indicate severe hypolactasia, those between 10-

30 U/g represent mild hypolactasia, and those above 30 U/g are consonant with normolactasia (34).

4.3.6. Genotyping for C/T$_{-13910}$ polymorphism

Genetic testing for C/T$_{-13910}$ polymorphism is completed using the method described by Kuokkanen et al. 2003 (48). In brief, DNA is isolated from peripheral blood samples by submerging a blank strip (Merck, Darmstadt, Germany) into EDTA blood. Strips are dried and heated in 1×PCR reaction buffer (Dynazyme; Finnzymes, Espoo, Finland) containing 10 mmol/l Tris HCl (pH 8.8 at 25°C), 50 mmol/l KCl, 1.5 mmol/l MgCl2, and 0.1% Triton X-100, and degraded proteins are centrifuged. The supernatant (15–20 μl) containing the DNA will be amplified in a total volume of 50 μl containing: 1× PCR buffer, 10 nmol deoxynucleotide triphosphates (dNTPs) (Amersham Pharmacia Biotech, Uppsala, Sweden), and 30 pmol forward and reverse primers amplifying the SNPs or exons 1, 2, 6, 10, 13, or 17 (36,48). All PCR reactions are amplified under the following conditions: after heating at +94°C for four minutes, PCR was initiated by a hot start (1 U of Taq polymerase), samples are cycled 34 times with a 30 second annealing step at +55°C, a 30 second extension step at +72°C, and a 30 second denaturing step at +94°C, followed by the last 30 second annealing step at +55°C and 10 minutes extension at +72°C. PCR products are run on a 1.5% agarose gel electrophoresis with ethidium bromide to verify the size of the PCR product. PCR products are sequenced on both strands (BigDye Terminator RR mix), run on an ABI 377 Sequencher, and analysed using ABI Sequencing Analysis 3.3 (Applied Biosystems, Perkin Elmer, Foster City, California, USA) Sequencher 4.1 (Gene Godes, Ann Arbor, Michigan, USA) to determine the genotypes of the C/T$_{-13910}$ polymorphisms of each patient (48). Subjects with **C/C$_{-13910}$ genotype** (indicating down-regulation of the lactase gene in adulthood) have severe hypolactasia, while those with **T/T$_{-13910}$ genotype** (indicating persistence of lactase activity in adulthood) have normolactasia (i.e., no lactose intolerance). Those with heterozygosity of the **C/T$_{-13910}$ allele** usually demonstrate approximately half of the ability to down-regulate the lactase gene in adulthood, as compared with C/C$_{-13910}$ homozygotes (23-29,36).

4.4.Gastroduodenoscopy (EGD) and biopsy procedures

All patients in this study are examined for dyspeptic symptoms and will be subjected to gastroduodenoscopy (EGD), providing the histological samples from duodenal mucosa to be used for the lactase assays (LIQT, dishaccaridase assay). In EGD, all observed abnormal mucosal lesions are noted and photographed, and if necessary subjected to additional biopsy. **Two biopsies** from the descending duodenum below the level of the papilla Vateri from the same or an adjacent duodenal fold are needed for lactase testing. Two additional biopsy specimens from the duodenum will be taken for histological examination as part of the diagnostic routine. As emphasized, duodenal bulbus should be avoided, because of the gastric acid-related changes in the lactase activity in the bulbar mucosa in some patients.

4.4.1.Preparation of the microscopy slides

The biopsies from formalin tubes are embedded in paraffin using the routine procedures at the Pathology Laboratory of Hospital X. The blocks are cut into 4µ-sections, and stained with hematoxylin eosin (HE) for routine diagnosis and with Alcian Blue PAS staining for different intestinal mucins.

4.4.2. Interpretation of the biopsies

LI in itself does not influence on morphology of the duodenal mucosa. LI is not infrequently associated with **celiac disease (CD),** however, with characteristic morphological changes in the villus structure. The diagnosis CD concomitant with LI is reported using the **Marsh classification** of duodenal histology, classified into one of the 5 categories (49). In Marsh scoring, normal intestinal histology is scored a Marsh 0. The presence of intraepithelial lymphocytes alone is a Marsh 1. A biopsy specimen with crypt hyperplasia and increased numbers of intraepithelial lymphocytes is a Marsh 2. A specimen with any degree of villous atrophy is a Marsh 3. Score 4 represents the end stage of a full-blown (undiagnosed) CD. A Marsh score of 2 or 3 is consistent with CD (49).

4.5.Statistical analyses

All statistical analyses will be performed using the SPSS 25.0.0.1. for Windows (IBM, NY, USA) and STATA/SE 15.1 software (STATA Corp., Texas, USA). The descriptive statistics will be calculated according to routine procedures. Performance indicators (sensitivity, specificity, positive predictive value, PPV, negative predictive value, NPV and their 95%CI) of LIQT test will be calculated separately for both study endpoints (severe and mild hypolactasia), with the two gold standards (disaccharidase assay and C/T_{-13910} genotyping) as reference, using the STATA/SE software and the *diagti* algorithm introduced by Seed et al. (2001). This algorithm also calculates the area under ROC (Receiver Operating Characteristics) called AUC, for each study endpoint. If LIQT is compared with HBT, the significance between the AUC values can be estimated using STATA's roccomb test with 95%CI.

5.ETHICAL ISSUES

The study plan necessitates a review by the institutional review board (IRB, Ethical Committee) before permission to start. The study design and its execution do not involve any significant ethical issues except those in other clinical studies of similar type. The study is conducted in accordance with the Declaration of Helsinki.

Patients are enrolled among consecutive patients with dyspeptic symptoms referred for EGD at the outpatient Department of Endoscopy, Hospital X, to complete their diagnostic procedure. Thus, they represent regular outpatients who have clinical symptoms compatible with adult-type hypolactasia, and who are scheduled for confirmatory clinical examinations. The only additional tests carried out to these patients are a blood sampling (for C/T genotyping) and two additional biopsies from the duodenum (for the two lactase assays). All patients must sign the informed consent for their participation. When the results of the LIQT, disaccharidase test, C/T genotyping and biopsies are available, the patients will be informed about the results, following the usual hospital practices. This includes an explanation of the meaning of these test results, and the appropriate measures for further conduct, i.e., adherence to milk/dairy product-free diet to avoid symptoms.

6.TIME TABLE

Given that the subjects in the study will be enrolled among consecutive patients with clinical symptoms of dyspepsia, attending the Outpatient Department of Endoscopy, Hospital X, it is estimated that at least 200 subjects (adults, both genders) need to be screened by LIQT to reach a cohort of 100 patents enriched with equal numbers (n=50) of both mild and severe hypolactasia, needed to calculate the performance indicators for the LIQT. Subjects testing negative (normolactasia) in LIQT will be used as controls in these calculations.

Because of the test characteristics (quick test), the laboratory arm of this study is completed in parallel with the patient enrollment and performed endoscopies. Usually, there will be some delay (of days) due to the biopsy examination at the Department of Pathology, as well as the disaccharidase tests and C/T genotyping, until the results of each subjects are available. Accordingly, the full database of the patients will be ready for statistical analysis almost on real-time after completion of the enrollment and their LIQT testing.

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ANNEX 1.

SAMPLING AND PERFORMANCE OF LACTOSE INTOLERANCE QUICK TEST

SPECIMEN COLLECTION AND HANDLING
Biopsy specimens are recommended to be taken with e.g. 5 mm forceps from the mucosa of the upper part of the small intestine at any site of the post-bulbar duodenal wall during gastroscopy. The bulbus should be avoided, because of the gastric acid-related changes in the lactase activity in the bulbar mucosa in some patients.

Total lactase content in the biopsy depends on the size of the biopsy. Recommended size of biopsies is between 1.5 and 2.0 mm, because the LIQT was evaluated using biopsies in this size. If the biopsy is smaller than recommended, there is a risk of a false positive (hypolactasia) result. If the biopsy specimen is too large, there is a risk of a false negative (normolactasia) result. Before performing the test, blood can be removed from the biopsy specimen by placing it briefly on the sterile gauze pad. It is recommended to perform the test immediately.

TEST PROCEDURE
Allow all the reagents to reach room temperature (20-25 °C) for at least 15 minutes before use. Mix all the reagents before using by turning the bottles upside down for a few times. Then tap the bottles on the table to ensure the liquid returns to the bottle. When adding the drops into the wells, hold the bottles in vertical upside down position (Figures 1-2).
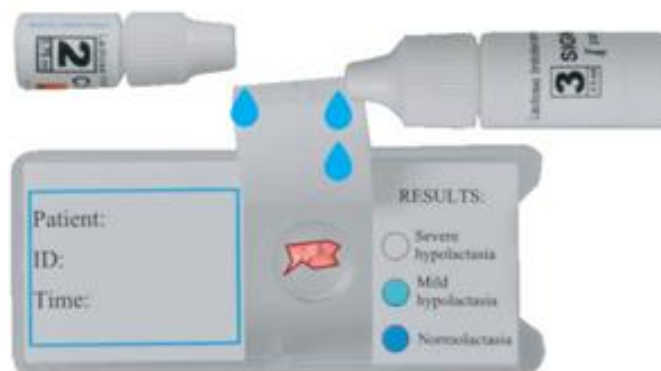
STEP 1: Lactase Reaction

1. Open the label covering the well on the plate. Place the biopsy specimen into the well (**Figure 1**).
2. Add 2 drops (80 µl) of the substrate solution (Bottle 1) into the well. Close the label.
3. Mix the plate by shaking 5-6 times vigorously sideways on the table.
4. Incubate for **15 minutes** at room temperature (20-25 °C).

Figure 1.

STEP 2: Signal Reaction

1. Open the label again. Add 1 drop (10 µl) of the chromogen solution (Bottle 2) into the well (**Figure 2**).
2. Immediately add 2 drops (80 µl) of the signal reaction solution (Bottle 3). Close the label.
3. Mix the plate by shaking for 5-6 times vigorously sideways on the table.
4. Incubate for **5 minutes** at room temperature (20-25 °C) and interpret the lactase content with the color chart included in the kit package.

Figure 2.

STEP 3: Reading of the results

Presence of functional lactase in the specimen can be interpreted with the color chart given on the label. See the example below.

Figure 3.

In case of a negative result hypolactasia can be excluded. For the more detailed interpretation of the results please refer to the interpretation chart included in the kit.