

## ORIGINAL ARTICLE

# Lactase Non-Persistence Genotyping: Comparison of Two Real-Time PCR Assays and Assessment of Concomitant Fructose/Sorbitol Malabsorption Rates

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### SUMMARY

**Background:** Genetic testing is a standard technique for the diagnosis of primary adult-type hypolactasia, also referred to as lactase non-persistence. The aim of this study was to compare the lactase gene (*LCT*) C/T-13910 polymorphism genotyping results of two commercially available real-time (RT)-PCR assays in patients referred to our outpatient clinic for primary lactose malabsorption testing. Furthermore, concomitant conditions of fructose/sorbitol malabsorption were assessed.

**Methods:** Samples obtained from 100 patients were tested in parallel using the *LCT* T-13910C ToolSet™ for Light Cycler™ (Roche, Rotkreuz, Switzerland) and the *LCT*-13910C>T RealFast™ Assay (ViennaLab Diagnostics GmbH, Vienna, Austria). Additionally, patients were also screened for the presence of fructose/sorbitol malabsorption by functional hydrogen (H<sub>2</sub>)/methane (CH<sub>4</sub>) breath testing (HMBT). Cohen's Kappa ( $\kappa$ ) was used to calculate the agreement between the two genotyping methods. The exact Chi-Square test was performed to compare fructose/sorbitol HMBT with *LCT* genotyping results.

**Results:** Twenty-one (21.0%) patients had a *LCT* C/C-13910 genotype suggestive of lactase non-persistence, and 79 (79.0%) patients were identified with either a *LCT* T/C-13910 or T/T-13910 genotype (i.e., lactase persistence). In all genotype groups, concordance between the two RT-PCR assays was 100%. Cohen's  $\kappa$  demonstrated perfect observed agreement ( $p < 0.001$ ,  $\kappa = 1$ ). Fructose and sorbitol malabsorption was observed in 13/100 (13.0%) and 25/100 (25.0%) individuals, respectively.

**Conclusions:** Both RT-PCR assays are robust and reliable *LCT* genotyping tools in a routine clinical setting. Concomitant fructose and/or sorbitol malabsorption should be considered in individuals with suspected lactase non-persistence. However, standardization of clinical interpretation of laboratory HMBT results is required. (Clin. Lab. 2016;62:xx-xx. DOI: 10.7754/Clin.Lab.2015.150923)

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### KEY WORDS

lactase non-persistence, real-time PCR, carbohydrate malabsorption, breath tests

### INTRODUCTION

Primary (adult-type) hypolactasia, also known as lactase non-persistence, is a common autosomal recessive condition resulting in a reduced lactase enzyme activity in the small intestine brush border after weaning. A single nucleotide polymorphism (SNP) (C/T-<sub>13910</sub>) 14 kb up-

stream of the lactase gene (*LCT*) locus was found to be tightly linked to this inherited form in patients of European origin [1,2]. As a consequence the non-absorbable disaccharide lactose reaches the large intestine, where colonic bacteria generate degradation products such as short chain fatty acids, carbon dioxide (CO<sub>2</sub>), hydrogen (H<sub>2</sub>), and methane (CH<sub>4</sub>), leading to abdominal pain, bloating, and diarrhoea [3].

Diagnostic approaches for patients with lactase non-persistence include the functional lactose H<sub>2</sub>/CH<sub>4</sub> breath testing (HMBT) and molecular tests to determine the allelic *LCT* variants [2]. Since there is no genetic approach to fructose and sorbitol malabsorption testing, these conditions can only be detected with functional HMBT [4].

Conventional diagnostic HMBT is known to have false-negative rates due to hydrogen non-excretion [5] or poor patient preparation [6]. For the stated reasons a real-time (RT)-PCR assay (*LCT* T-13910C ToolSet™ for LightCycler™ [Roche, Rotkreuz, Switzerland]) for *LCT* genotyping based on fluorescence-resonance energy-transfer (FRET) was established at our institution in 2005, serving as the routine testing method. Because several different genotyping methodologies for the diagnosis of primary adult-type hypolactasia were developed in the last decade [7], the present study was conducted to evaluate the performance of a novel RT-PCR assay (*LCT*-13910C>T RealFast™ Assay [ViennaLab Diagnostics GmbH, Vienna, Austria]) in patient samples previously analyzed using the Roche's ToolSet™ chemistry. Since also fructose and sorbitol malabsorption are considered common types of non-immune-mediated food intolerance in European population [8], but are less well-studied conditions, *LCT* C/T<sub>-13910</sub> genotypes were matched against the fructose/sorbitol HMBT results that were obtained routinely for all patients included in this study.

## MATERIALS AND METHODS

### Subjects

A total of 100 adult patients referred to our outpatient clinic for primary lactose malabsorption testing from January 1, 2014 to December 31, 2014, were included in this cross-sectional study. Twenty-nine patients (29.0%) were male and 71 (71.0%) were female. The median age was 65 (range: 18 - 86) years. Children and adolescents below 18 years were excluded from the study. All patients gave their written informed consent. Approval was obtained from the Ethical Committee of Upper Austria, Linz, Austria (trial registration number: K-56-14). The study was carried out in accordance with the latest version of the Declaration of Helsinki given by the World Medical Association.

### Methods

*LCT* C/T<sub>-13910</sub> genotyping: VACUETTE® K2 EDTA tubes (2 mL) (Greiner Bio-one International GmbH,

Kremsmünster, Austria) were used for blood draw from a peripheral vein and stored frozen at -20°C until genetic analysis once a week. Genomic DNA was purified from 200 µL EDTA blood using the MagNA Pure Compact Nucleic Acid Isolation Kit I and the MagNA Pure Compact Instrument (Roche Diagnostics, Rotkreuz, Switzerland) according to the manufacturer's instructions. PCR with specific fluorescent labeled hybridization probes, followed by melting curve analysis for detecting the *LCT* C/T<sub>-13910</sub> polymorphism (*LCT* T-13910C ToolSet™), was performed on the LightCycler® 2.0 Instrument (both Roche Diagnostics, Rotkreuz, Switzerland). Consecutively, the same DNA samples were analyzed with a novel fluorogenic 5' nuclease assay (*LCT*-13910C>T RealFast™ Assay; ViennaLab Diagnostics GmbH) based on the TaqMan® technique. According to the manufacturer's instructions, this assay was performed on the LightCycler® 2.0 Instrument (Roche Diagnostics, Rotkreuz, Switzerland). Fructose and sorbitol HMBT: HMBT protocols were established to detect patients with fructose and/or sorbitol malabsorption. Gas chromatography was employed using the QuinTron Model DP Plus MicroLyzer™ (QuinTron, Milwaukee, WI, USA). After fasting overnight for 12 hours and determining baseline breath H<sub>2</sub>/CH<sub>4</sub> concentrations, on one day fructose was given in a dose of 25 g dissolved in 200 mL of water, on another following visitation sorbitol was given in a dose of 12.5 g dissolved in 200 mL of water [3]. The end-expiratory breath H<sub>2</sub> and CH<sub>4</sub> concentrations were measured at 15, 30, 45, 60, 75, 90, and 120 minutes after sugar ingestion. According to the literature [3,6], HMBT was defined positive if an H<sub>2</sub> and/or CH<sub>4</sub> increase ≥ 20 ppm above baseline concentrations was observed within 60 - 120 minutes after sugar ingestion (i.e., colonic passage). Patients were asked to avoid physical effort, smoking or eating until HMBT was completed.

### Statistical analysis

Descriptive statistics were performed to analyze *LCT* (C/T<sub>-13910</sub>) genotyping and HMBT results. The agreement between the RT-PCR methods was calculated using Cohen's Kappa (κ). The exact Chi-Square test for independence was used to compare the fructose and sorbitol HMBT results with the *LCT* (C/T<sub>-13910</sub>) genotyping results. Adjusted residuals were used for post-hoc analysis of the Chi-Square test. No adjustment for type I error was made. Therefore, the p-values are only descriptive. The software R 3.1.2 (R Foundation for Statistical Computing, Vienna, Austria) was used for statistical analysis.

## RESULTS

### *LCT* C/T<sub>-13910</sub> genotyping results

As shown in Table 1, 21/100 (21.0%) patients were C/C<sub>-13910</sub> homozygotes, the *LCT* genotype indicative of lactase non-persistence. Fifty-two out of 100 (52%) in-

**Table 1. *LCT* C/T-13910 genotyping results.**

n = 100	ToolSet™ (Roche)	RealFast™ (ViennaLab)
C/C-13910	21 (21.0%)	21 (21.0%)
T/C-13910	52 (52.0%)	52 (52.0%)
T/T-13910	27 (27.0%)	27 (27.0%)

Cohen's Kappa ( $\kappa$ ) for observed agreement between both assays was 1.0 ( $p < 0.001$ ).

**Table 2. Fructose/sorbitol H2/CH4 breath test (BT) results observed for three *LCT* C/T-13910 genotypes.**

	Fructose BT + *	Fructose BT -	Total
C/C-13910	4 (19.0%)	17 (81.0%)	21
T/C-13910	6 (11.5%)	46 (88.5%)	52
T/T-13910	3 (11.1%)	24 (88.9%)	27
<b>Total</b>	<b>13</b>	<b>87</b>	<b>100</b>
	Sorbitol BT + *	Sorbitol BT -	Total
C/C-13910	5 (23.8%)	16 (76.2%)	21
T/C-13910	14 (26.9%)	38 (73.1%)	52
T/T-13910	6 (22.2%)	21 (77.8%)	27
<b>Total</b>	<b>25</b>	<b>75</b>	<b>100</b>

\* BT result  $\geq 20$  ppm H<sub>2</sub> and/or CH<sub>4</sub> above baseline concentration within 60 - 120 minutes after sugar ingestion was positive (+).

dividuals could be identified as C/T<sub>-13910</sub> heterozygotes and 27/100 (27%) as T/T<sub>-13910</sub> homozygotes, with both genotypes indicating lactase persistence. In all genotype groups, concordance between the two RT-PCR methods was 100% with Cohen's  $\kappa$  demonstrating perfect agreement ( $p < 0.001$ ,  $\kappa = 1$ ).

#### Fructose and sorbitol HMBT results

Fructose and sorbitol malabsorption was observed in 13/100 (13.0%) and 25/100 (25.0%) individuals, respectively. Fructose and sorbitol HMBT results matched against *LCT*<sub>-13910</sub> genotypes are illustrated in Table 2. All in all, 4/100 (4.0%) and 5/100 (5.0%) *LCT* C/C<sub>-13910</sub> homozygotes were diagnosed with fructose and sorbitol malabsorption, respectively. One patient with lactase non-persistence and six patients with lactase persistence (three T/C<sub>-13910</sub> heterozygotes and three T/T<sub>-13910</sub> homozygotes) showed positive HMBT results with both sugars. When matched against lactase non-persistence, neither fructose nor sorbitol malabsorption ( $p = 0.726$  and  $p = 0.953$ ) was found to be associated in a statistically significant way.

## DISCUSSION

In this study, genomic DNA, isolated from 100 adult patients referred to our outpatient clinic for primary lactose malabsorption testing, was used to evaluate a novel RT-PCR assay using hydrolysis probe chemistry for the detection of the *LCT* C/T<sub>-13910</sub> polymorphism (*LCT*-13910C>T RealFast™ Assay, ViennaLab Diagnostics GmbH). With the Toolset™ molecular assay serving as the routine testing method in our laboratory, *LCT* genotyping results concurred with those of the RealFast™ Assay. Twenty-one percent of individuals were found lactase non-persistent (i.e., *LCT* C/C<sub>-13910</sub> genotype) thereby corroborating an earlier study of 220 Austrian patients that reported a prevalence rate of 21.4% [2]. At our institution, *LCT* genotyping was established to replace lactose HMBT because it is time consuming and prone to error [6,7,9]. We only recently showed moderate agreement ( $p < 0.001$ ,  $\kappa = 0.44$ ) between the two methods [6]. On the one hand, poor patient preparation and daily changes in the outpatient clinic staff can cause false negative results, on the other hand, hyperventilation or exercise may also influence the concentration of the exhaled gases [6]. However, since there is no genetic approach to fructose/sorbitol malabsorption testing and both carbohydrates are considered frequent condi-

tions of food intolerance [8], *LCT* genotyping results were matched against those obtained by HMBT. In total, 13.0% and 25.0% of individuals were found with a positive fructose and sorbitol HMBT, respectively. Interestingly, 4.0% and 5.0% of those patients with a *LCT* C/C<sub>-13910</sub> genotype (i.e., lactase non-persistence) showed positive fructose and sorbitol HMBT results, respectively, with one of them found to be positive for both sugars. However, when matched against lactase non-persistence, neither fructose nor sorbitol malabsorption was found to be associated in a statistically significant way. Fructose and sorbitol are considered potential sugars for carbohydrate malabsorption testing; however, clinical interpretation of functional HMBT results is limited. Sugar load represents a major influencing factor [10] and standardized criteria for HMBT interpretation are not available yet. The optimal HMBT dosage of fructose and sorbitol to detect malabsorption is unclear [4]. Furthermore, there is little experience with CH<sub>4</sub>, and a uniform cutoff value to define a H<sub>2</sub> and/or a CH<sub>4</sub> producer is still lacking [6].

Based on HMBT with a positive cutoff value of  $\geq 20$  ppm H<sub>2</sub> and/or CH<sub>4</sub> above baseline concentrations [3,5], this study identified 13/100 (13.0%) and 25/100 (25.0%) individuals with fructose and sorbitol malabsorption, respectively. These findings are in accordance with published data elsewhere, that, in general, malabsorption rates observed for sorbitol are higher than for fructose [8].

This work is limited by the small number of patients and the absence of lactose HMBT to detect secondary acquired and reversible forms of lactose malabsorption. Furthermore prospective studies are needed to define uniform criteria for clinical interpretation of HMBT results and to assess clinical relevance of concomitant fructose and sorbitol malabsorption.

## CONCLUSION

Both RT-PCR assays under investigation here are robust and reliable *LCT* genotyping tools in a routine clinical setting. Concomitant fructose and/or sorbitol malabsorption should be considered in individuals with suspected lactase non persistence. However, uniform criteria and standardization of interpretation of laboratory HMBT results are urgently required.

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### Declaration of Interest:

The authors declare that possible conflicts of interest are disclosed for each author of this study.

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