References

1. Selhub J, Miller JW. The pathogenesis of homocysteinemia: interruption of the coordinate regulation by *S*-adenosylmethionine of the remethylation and trans-sulfuration of homocysteine. Am J Clin Nutr 1992;55: 131–8.
2. McCully KS. Vascular pathology of homocysteinemia: implication for the pathogenesis of arteriosclerosis. Am J Pathol 1969;56:111–28.
3. Foley RN, Parfrey PS, Samak MJ. Clinical epidemiology of cardiovascular disease in chronic renal disease. Am J Kidney Dis 1998;32(5 Suppl 3):S112.
4. Welch GN, Loscalzo J. Homocysteine and atherothrombosis. N Engl J Med 1998;338:1042–50.
5. Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylentetrahydrofolate reductase. Nat Genet 1995;10:111–3.
6. Brattstrom L, Wilcken DEL, Öhrvik J, Brudin L. Common methylentetrahydrofolate reductase gene mutation leads to hyperhomocysteinemia but not to vascular disease: the result of a meta-analysis. Circulation 1998;98: 2520–6.
7. Födinger M, Mannhalter C, Wölfl G, Pabinger I, Muller E, Schmid R, et al. Mutation (677 C to T) in the methylentetrahydrofolate reductase gene aggravates hyperhomocysteinemia in hemodialysis patients. Kidney Int 1997;52:517–23.
8. Bostom AG, Shemin D, Bagley P, Massy ZA, Zanabli A, Christopher K, et al. Controlled comparison of L-5-methyltetrahydrofolate versus folic acid for the treatment of hyperhomocysteinemia in hemodialysis patients. Circulation 2000;101:2829–32.
9. Kaplan LN, Mamer OA, Hoffer LJ. Parenteral vitamin B_{12} reduces hyperhomocysteinemia in end-stage renal disease. Clin Invest Med 2001;24:5–11.
10. Dierkes J, Domrose U, Ambrosch A, Schneede J, Guttormsen AB, Neumann KH, et al. Supplementation with vitamin B_{12} decreases homocystein and methylmalonic acid but also serum folate in patients with end stage renal disease. Metabolism 1999;48:631–5.
11. Billion S, Tribout B, Cadet E, Queinnec C, Rochette J, Wheatley P, et al. Hyperhomocysteinaemia, folate, and vitamin B_{12} in unsupplemented haemodialysis patients: effect of oral therapy with folic acid and vitamin B_{12}. Nephrol Dial Transplant 2002;17:455–61.
12. Pastore A, Massoud R, Motti C, Lo Russo A, Fucci G, Cortese C, et al. Fully automated assay for total homocysteine, cysteine, cysteinylglycine, glutathione, cysteamine, and 2-mercaptopropionylglycine in plasma and urine. Clin Chem 1998;44:825–32.
13. Obeid R, Kuhlmann MK, Köhler H, Herrmann W. Response of homocysteine, cystathionine, and methylmalonic acid to vitamin treatment in dialysis patients. Clin Chem 2005;51:196–201.

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Genotyping of the Lactase-Phlorizin Hydrolase −13910 Polymorphism by LightCycler PCR and Implications for the Diagnosis of Lactose Intolerance,

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Background: **Hypolactasia and lactose intolerance are common conditions worldwide. Hypolactasia seems to be strongly correlated with genotype C/C of the genetic variant C→T_{-13910} upstream of the lactase phlorizin hydrolase (*LPH*) gene. We developed a rapid genotyping assay for *LPH* C→T_{-13910} and investigated the relationship of positive lactose breath hydrogen test (LBHT) results suggesting lactose intolerance with *LPH* C→T_{-13910} genotype.**

Methods: **Using automated DNA purification on the MagNA Pure LC and real-time PCR on the LightCycler, we examined samples from 220 individuals to estimate genotype frequencies; we then determined *LPH* C→T_{-13910} genotype in samples from 54 Caucasian patients with a positive LBHT result and symptoms of lactose intolerance.**

Results: **Genotyping of 220 individuals revealed frequencies of 21.4%, 41.8%, and 36.8% for genotypes C/C, C/T, and T/T. Of the patients with positive LBHT results, only 50% had the C/C genotype suggestive of primary adult hypolactasia in our study population. The other patients had various degrees of secondary hypolactasia or symptoms of lactose intolerance. Patients with C/C genotype had a mean (SD) peak H_2 increase in the LBHT [108 (58) ppm] that was significantly higher than in patients with the C/T [65 (54) ppm] and T/T [44 (34) ppm] genotypes.**

Conclusions: **The new real-time PCR assay provides a rapid, labor-saving means for the genotyping of *LPH* C→T_{-13910}. Use of the assay may assist in differentiating patients with primary hypolactasia from those with secondary hypolactasia and lactose intolerance, who may need further clinical examinations to diagnose their underlying primary diseases.**

Deficiency of the brush border enzyme lactase-phlorizin hydrolase (LPH), which catalyzes the hydrolysis of lactose to yield glucose and galactose, leads to malabsorption of lactose and to lactose intolerance when unabsorbed lactose reaches the colon and is fermented by bacteria *(1, 2)*. Adult hypolactasia, which reflects declining *LPH* gene expression *(3–5)* during maturation, varies in frequency among populations *(6)*.

A single-nucleotide polymorphism (SNP) upstream of the *LPH* gene at position −13910 (C→T_{-13910}) was reported to perfectly match with phenotypic hypolactasia and lactose malabsorption for the C/C genotype *(7)*. Further studies demonstrated that the C/C genotype matched with low LPH-specific mRNA expression and low lactase activity in intestinal biopsies, suggesting primary adult hypolactasia, whereas the genotypes C/T and T/T matched with high LPH-specific mRNA expression and high lactase activity, strongly suggesting lactase persistence *(7–11)*. A second described SNP, *LPH* G→A_{-22018}, is thought to be only in linkage disequilibrium with the SNP C→T_{-13910} *(11)*.

The described SNPs may not necessarily explain all of the genetic variations of *LPH* gene expression in all ethnic groups *(12)*. In some sub-Saharan African populations, the C/C genotype is frequent and not associated with the epidemiologic data of lactose intolerance *(13)*. In many ethnically diverse populations of Europe and of European descent, however, data on the frequency of the C/C_{-13910}

Table 1. Characteristics of C/T and T/T genotyped patients with positive LBHT and suspected lactose intolerance.

Patient age, years	Sex	Genotype	ΔH_2, ppm	Symptoms at lactose challenge during LBHT	Lactose-free diet	Primary diagnosis	Symptoms at genotyping
43	M	CT	21	None	Response	NE[a]	No complaints under diet
54	M	CT	22	None	No response	Pancreas insufficiency	Diarrhea
74	M	CT	22	None	No response	NE	None
83	F	CT	29	Diarrhea	No response	Pancreas insufficiency	None
27	F	CT	30	None	No response	Crohn disease	Diarrhea
60	F	CT	39	Bloating	No response	NE	Bloating
48	F	CT	44	Abdominal pain	No response	Gastric bypass	Abdominal pain; diarrhea
56	F	CT	44	Abdominal pain; bloating; diarrhea	No response	NE	Diarrhea
71	M	CT	45	Abdominal pain; bloating; diarrhea	Response	NE	No complaints under diet
71	M	CT	47	None	No response	Eosinophilic duodenitis	Abdominal pain; diarrhea
26	F	CT	62	Abdominal pain; diarrhea	ND	Celiac sprue	No complaints with gluten-free diet
82	M	CT	66	None	No response	NE	Abdominal pain; diarrhea
66	F	CT	72	Abdominal pain; nausea	No response	NE	None
81	F	CT	133	Abdominal pain; diarrhea	No response	NE	Abdominal pain; diarrhea
61	F	CT	162	Abdominal pain; diarrhea	No response	NE	None
60	M	CT	200	Abdominal pain; diarrhea	No response	Pancreas insufficiency	Diarrhea
24	F	TT	22	None	No response	NE	None
40	M	TT	22	None	No response	Pancreas insufficiency	Diarrhea
85	M	TT	22	None	No response	NE	None
74	F	TT	23	None	No response	NE	None
65	F	TT	28	None	ND	Celiac sprue	No complaints with gluten-free diet
76	F	TT	30	Abdominal pain; bloating; diarrhea	No response	Soybean allergy	Diarrhea
30	F	TT	41	None	No response	NE	None
18	M	TT	43	Abdominal pain; bloating	No response	NE	Abdominal pain; bloating
83	F	TT	49	None	No response	NE	Diarrhea
81	F	TT	64	Bloating; diarrhea	No response	NE	Bloating; diarrhea
82	F	TT	137	Bloating; diarrhea	No response	NE	Diarrhea

[a] NE, primary diagnosis not established at time of LBHT; ND, no lactose-free diet.

genotype concur with the prevalence of primary adult hypolactasia *(2)*.

Tests for the diagnosis of hypolactasia consist of biochemical tests such as the lactose breath hydrogen test (LBHT), lactase enzyme activity tests performed with biopsy samples of the small intestine, and most recently, molecular tests to determine the allelic variants of *LPH* $C \rightarrow T_{-13910}$ *(14–17)*.

We developed an automated rapid genotyping assay for *LPH* $C \rightarrow T_{-13910}$ based on real-time PCR on the LightCycler instrument (Roche Applied Science) and used this assay to perform *LPH* $C \rightarrow T_{-13910}$ genotyping on DNA samples from 220 persons and to investigate the relationship between positive LBHT results and genotyping of *LPH* $C \rightarrow T_{-13910}$ in patients with symptoms of lactose intolerance.

DNA was purified from EDTA blood (200 μL) on the MagNa Pure LC instrument (Roche Applied Science) with use of the MagNa Pure LC Total Nucleic Acid Isolation Kit (Roche Applied Science) according to the manufacturer's instructions. DNA was eluted in 50 μL of elution buffer.

We designed primers and probes targeting a region around nucleotide −13910 upstream of the *LPH* gene (GenBank accession number AY220757; nucleotides 26481–26672) with the LightCycler probe design software 1.0 (Roche; see Fig. 1 in the Data Supplement that accompanies the online version of this Technical Brief at www.clinchem.org/content/vol52/issue1). Real-time PCR, followed by melting curve analysis for genotyping of *LPH* $C \rightarrow T_{-13910}$, was performed on the LightCycler instrument 1.0 (Roche; software version 3.5.3). The master-mix reagent Fast Start DNA Master Hybridization Probes (Roche) was used and supplemented to 4 mmol $MgCl_2$, 0.2 μM each of the *LPH*-specific primers (LPH-for, 5′-TGAGTGTAGTTGGACGG-3′; LPH-rev, 5′-CAACCTAAGGAGGAG-AGT-3′), and 0.2 μM each of the *LPH*-specific fluorescence resonance energy transfer hybridization probes (5′-GCCTCTGCGCTGGCAATACA-GATA-fluorescein-3′ and 5′-LC-Red705-ATAATGTAGC-CCCTGGCCT-phosphate-3′). PCR was carried out in 20-μL capillaries (2 μL of sample DNA and 18 μL of master-mix) at 95 °C for 8 min, followed by 40 cycles of 0 s at 95 °C, 10 s at 54 °C, and 15 s at 72 °C. Genotyping was performed by melting curve analysis: 95 °C for 0 s and 40 °C for 30 s, followed by a temperature increase from

40 °C to 80 °C (0.2 °C/s), with continuous fluorescence recording on channel F3. Each assay run included a reference sample for each of the 3 genotypes and a reagent control containing water instead of sample DNA.

We obtained 3 DNA reference samples, genotyped for the *LPH* C→T$_{-13910}$ SNP by PCR followed by restriction fragment length polymorphism analysis, from the Clinical Institute of Medical and Laboratory Analysis, Medical University Graz, Austria *(15)*. *LPH* C→T$_{-13910}$ genotyping of the reference DNA samples and all additional DNA samples led to distinct melting temperatures at [mean (SD)] 64.5 (0.3) °C (n = 20) and 56.0 (0.2) °C (n = 20) for the C and T alleles, respectively (see Fig. 2 in the online Data Supplement).

We tested the robustness of the assay in the routine clinical laboratory by assaying samples from 220 persons in a screening setup. All samples returned an unequivocal genotyping result with genotypes C/C, C/T, and T/T in 47, 92, and 81 samples, representing frequencies of 21.4%, 41.8%, and 36.8%, respectively. These frequencies are similar to those found in comparable European populations *(15, 16)*. In addition, our measured C/C genotype frequency matched the frequency in persons with phenotypic lactose malabsorption identified by use of the LBHT in an earlier study of apparently healthy adult Austrians *(18)*.

In a retrospective study, we randomly selected 54 European Caucasian outpatients (18 men and 36 women; General Hospital Linz, Austria) with a mean (SD) age of 55.8 (20.5) years (range 18–87 years) for *LPH* C→T$_{-13910}$ genotyping based on a positive LBHT result obtained after the appearance of abdominal symptoms suggesting lactose intolerance, such as diarrhea *(19)*. Routine LBHT was performed with 50 g of lactose, and breath hydrogen (H_2) excretion was recorded on the EC60 Gastrolyzer 2 (Bedfont Scientific Ltd) *(20)*. We began recording within the first hour after the participants ingested lactose, then recorded every 15 min, and thereafter every 30 min, for 4 h. The mean H_2 concentration of 2 breath samples measured before lactose ingestion was used as the baseline value, and an increase in excess of 20 parts per million (ppm) was considered indicative of LBHT positivity *(20)*.

Of the 54 patients with a positive LBHT result, 27 (50%) were found to have genotype C/C, 16 (29.6%) genotype C/T, and 11 (20.4%) genotype T/T. Patients with the C/C genotype had a mean (SD) H_2 peak increase in LBHT of 108 (58) ppm, which was significantly higher than that in patients with the C/T [65 (54) ppm] and T/T [44 (34) ppm] genotypes when compared by the independent Student *t*-test (significance at $P < 0.05$). The difference in the mean peak H_2 increases between patients with genotypes C/T and T/T was not significant. Our results contrast with those of 2 recent reports showing a 97% and a 91.4% correlation of the C/C genotype with positive LBHT results in patients with suspected lactose malabsorption *(21, 22)*. In those 2 studies, patients were selected for LBHT and genotyping on the basis of history and clinical symptoms, whereas we selected our patients exclusively on the basis of their positive LBHT results. Thus, patients with secondary hypolactasia or with other causes for symptoms of lactose intolerance leading to a positive LBHT result were more likely to be present in our study population. In fact, previous studies have reported that up to 57% of individuals who experience symptoms of lactose intolerance do not exhibit hypolactasia *(23)*.

Of our 27 patients with either the C/T or T/T genotype, 10 patients had a diagnosis concomitant with secondary hypolactasia (Table 1). Five other patients exhibited a small increase, just above the threshold, of H_2 in LBHT and presented with no complaints at the time of genotyping. We considered those 5 patients and 3 other patients who no longer had complaints at the time of genotyping to have had false-positive results or suffered lactose intolerance symptoms for causes other than hypolactasia. The remaining 9 patients still complained of chronic diarrhea and other abdominal symptoms at the time of genotyping, and we advised their referring physicians to perform further diagnostic testing. Thus, the genotyping of *LPH* C→T$_{-13910}$, in our study population, identified those patients who needed further follow-up because the diagnosis of lactose intolerance appeared to be not sufficiently substantiated.

Lactose intolerance usually leads to avoidance of milk and dairy products, often leading to lower calcium intake. Reduced calcium intake has correlated with reduced bone mineral density and increased risk for bone fractures *(24)*. In postmenopausal women, the C/C genotype was correlated with higher bone fracture incidence and lower bone mineral density than in women with C/T and T/T genotypes *(18)*. In addition, the C/C genotype has been correlated with an increased frequency of bone fractures in older individuals in European populations *(18, 25)*. Because milk and dairy product restrictions can probably be revoked in patients with secondary hypolactasia and other causes of lactose intolerance symptoms after successful diagnosis and therapy of their primary diseases, it is important to distinguish these patients from those with primary adult hypolactasia.

Larger studies are warranted to further clarify the diagnostic value of *LPH* C→T$_{-13910}$ genotyping in lactose intolerance in patients of diverse ethnic populations, particularly those of non-European descent. Our almost fully automated assay may assist in those studies because it is rapid, robust, suitable for the screening of large numbers of samples, and considerably less labor-intensive than previously reported assays based on PCR and restriction fragment length polymorphism analysis. Costs for consumables and reagents per sample are higher to some extent but are balanced by less labor time; hands-on time in our assay was only 1 h for the analysis of 32 samples, including DNA purification.

In conclusion, this real-time PCR assay provides a rapid, labor-saving means for the genotyping of *LPH* C→T$_{-13910}$. The genotyping *LPH* C→T$_{-13910}$ may assist in differentiating patients with primary hypolactasia from those with secondary hypolactasia and lactose intolerance.

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References

1. Sibley E. Genetic variation and lactose intolerance. Am J Pharmacogenomics 2004;4:239–45.
2. Järvelä IE. Molecular genetics of adult-type hypolactasia. Ann Med 2005; 37:179–85.
3. Lloyd M, Mevissen G, Fischer M, Olsen W, Goodspeed D, Genini M, et al. Regulation of intestinal lactase in adult hypolactasia. J Clin Invest 1992; 89:524–9.
4. Escher JC, de Koning ND, van Engen CG, Arora S, Buller HA, Montgomery, et al. Molecular basis of lactase levels in adult humans. J Clin Invest 1992;89:480–3.
5. Wang Y, Harvey CB, Hollox EJ, Phillips AD, Poulter M, Clay P, et al. The genetically programmed down-regulation of lactase in children. Gastroenterology 1998;114:1230–6.
6. Sahi T, Genetics and epidemiology of adult-type hypolactasia. Scand J Gastroenterol 1994;29(Suppl 202):7–20.
7. Enattah NS, Sahi T, Savilahti E, Terwilliger JD, Peltonen L, Jarvela I. Identification of a variant associated with adult-type hypolactasia. Nat Genet 2002;30:233–7.
8. Kuokkanen M, Enattah NS, Oksanen A, Savilahti E, Orpana A, Jarvela I. Transcriptional regulation of the lactase-phlorizin hydrolase gene by polymorphisms associated with adult-type hypolactasia. Gut 2003;52:647–52.
9. Troelsen JT, Olsen J, Moller J, Sjostrom H. An upstream polymorphism associated with lactase persistence has increased enhancer activity. Gastroenterology 2003;125:1686–94.
10. Olds LC, Sibley E. Lactase persistence DNA variant enhances lactase promoter activity in vitro: functional role as a cis regulatory element. Hum Mol Genet 2003;12:2333–40.
11. Rasinperä H, Savilahti E, Enattah NS, Kuokkanen M, Töttermann N, Lindahl H, et al. A genetic test which can be used to diagnose adult-type hypolactasia in children. Gut 2004;53:1571–6.
12. Poulter M, Hollox E, Harvey CB, Mulcare C, Peuhkuri K, Kajander K, et al. The causal element for the lactase persistence/non-persistence polymorphism is located in a 1 Mb region of linkage disequilibrium in Europeans. Ann Hum Genet 2003;67:298–311.
13. Mulcare CA, Weale ME, Jones AL, Connell B, Zeitlyn D, Tarekegn A, et al. The allele of a single-nucleotide polymorphism 13.9 kb upstream of the lactase gene (LCT) (C-13.9kbT) does not predict or cause the lactase-persistence phenotype in Africans. Am J Hum Genet 2004;74:1102–10.
14. Chao CK, Sibley E. PCR-RFLP genotyping assay for a lactase persistence polymorphism upstream of the lactase-phlorizin hydrolase gene. Genetic Testing 2004;8:190–3.
15. Obermayer-Pietsch BM, Bonelli CM, Walter DE, Kuhn RJ, Fahrleitner-Pammer A, Berghold A, et al. Genetic predisposition for adult lactose intolerance and relation to diet, bone density, and bone fractures. J Bone Miner Res 2004;19:42–7.
16. Büning C, Ockenga J, Krüger S, Jurga J, Baier P, Dignass A, et al. The C/C_{-13910} and G/G_{-22018} genotypes for adult-type hypolactasia are not associated with inflammatory bowel disease. Scand J Gastroenterol 2003; 38:538–42.
17. Nilsson TK, Johansson CA. A novel method for diagnosis of adult hypolactasia by genotyping of the −13910 C/T polymorphism with pyrosequencing technology. Scand J Gastroenterol 2004;39:287–90.
18. Rosenkranz W, Hadorn B, Müller W, Heinz-Erian P, Hensen C, Flatz G. Distribution of human adult lactase phenotypes in the population of Austria. Hum Genet 1982;62:158–61.
19. Fine KD, Schiller LR. AGA technical review on the evaluation and management of chronic diarrhea. Gastroenterology 1999;116:1464–86.
20. Peuhkuri K, Poussa T, Korpela R. Comparison of a portable breath hydrogen analyser (Micro H2) with a Quintron MicroLyzer in measuring lactose maldigestion, and the evaluation of a Micro H2 for diagnosing hypolactasia. Scand J Clin Lab Invest 1998;58:217–24.
21. Högenauer C, Hammer HF, Mellitzer K, Renner W, Krejs GJ, Toplak H. Evaluation of a new DNA test compared with the lactose hydrogen breath test for the diagnosis of lactase non-persistence. Eur J Gastroenterol Hepatol 2005;17:371–6.
22. Büning C, Genschel J, Jurga J, Fiedler T, Voderholzer W, Fiedler EM, et al. Introducing genetic testing for adult-type hypolactasia. Digestion 2005;71: 245–50.
23. Carroccio A, Montalto G, Cavera G, Notarbatolo A. Lactose intolerance and self-reported milk intolerance: relationship with lactose maldigestion and nutrient intake. Lactase Deficiency Study Group. J Am Coll Nutr 1998;17: 631–6.
24. Di Stefano M, Veneto G, Malservisi S, Cecchetti L, Minguzzi L, Strocchi A, et al. Lactose malabsorption and intolerance and peak bone mass. Gastroenterology 2002;122:1793–9.
25. Enattah NS, Sulkava R, Halonen P, Kontula K, Jarvela I. Genetic variant of lactase-persistent C/T−13910 is associated with bone fractures in very old age. J Am Geriatr Soc 2005;53:79–82.

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