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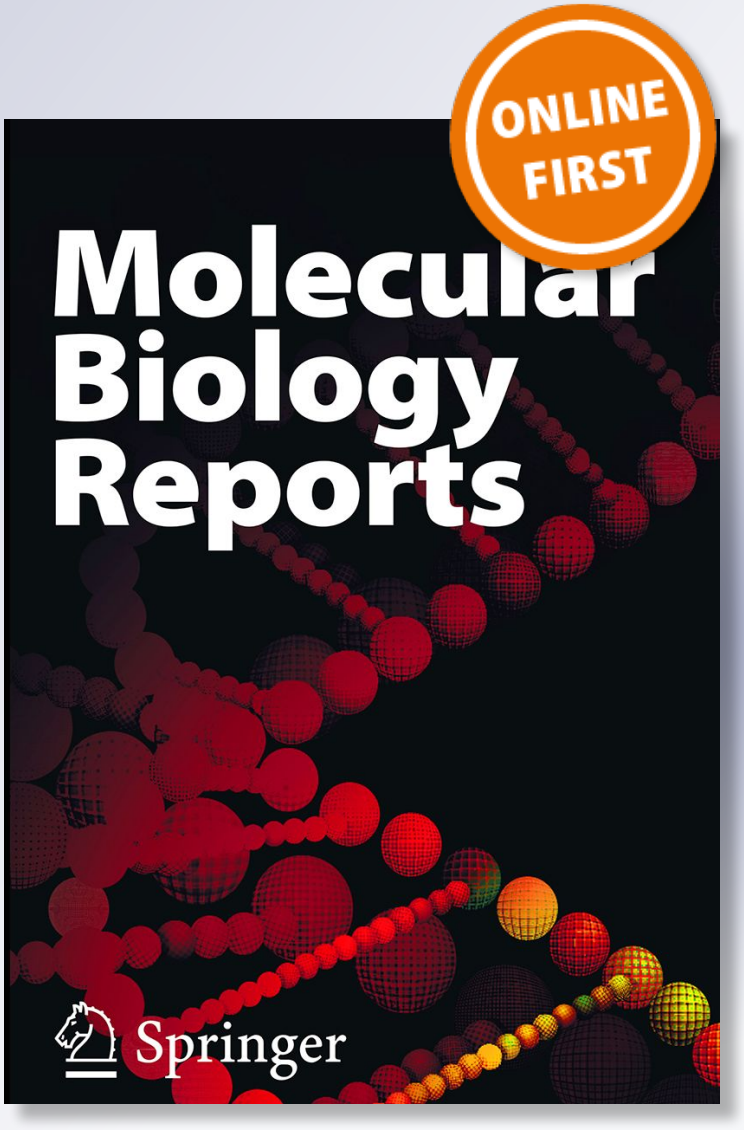

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ORIGINAL ARTICLE

Real-time PCR based detection of the lactase non-persistence associated genetic variant LCT-13910C>T directly from whole blood

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Abstract

Primary hypolactasia is the main cause of lactose intolerance in adults. It is strongly associated with the single genetic variant LCT-13910C>T, located upstream of the lactase encoding gene. Consequently, analysis of LCT-13910C>T has been recommended as a direct genetic test for the trait. The aim of our study was to develop a TaqMan probe based real-time PCR protocol for the detection of the LCT-13910C>T variant directly from whole blood, circumventing DNA isolation. The LCT-13910C>T variant was determined using the DirectBlood Genotyping PCR Kit (myPOLS Biotec, Konstanz, Germany) together with an in-house TaqMan primer-probe assay. Validity and specificity of the assay was evaluated using EDTA anti-coagulated whole blood samples and corresponding DNA samples. Results from real-time PCR were compared with results obtained by Sanger sequencing from 105 blinded whole blood samples. Validity and specificity of the assay using whole blood were comparable to those using purified genomic DNA as substrate in PCR. Genetic analysis of blood samples were in complete agreement with results obtained by Sanger sequencing. In conclusion, we present a reliable real-time PCR protocol for the detection of the LCT-13910C>T variant directly from whole blood further facilitating diagnosis of primary hypolactasia in symptomatic patients.

Keywords Lactose intolerance · Primary hypolactasia · LCT-13910C>T · Real-time PCR · Direct blood PCR · TaqMan probe · Locked nucleic acids

Introduction

Lactose intolerance is one of the most common forms of food intolerances. It manifests itself in abdominal bloating, diarrhea, flatulence, nausea, and vomiting after ingestion of dairy products [1, 2]. Moreover, significant correlations between lactose intolerance and the incidence of osteoporosis, changes in mental status, and the presence of additional food intolerances have been reported [3–6]. In most people, lactose intolerance is a consequence of low lactase activity. Lactase hydrolyzes the disaccharide lactose into glucose and galactose monomers in order to enable mucosal absorption in the small intestine [7]. In the majority of human populations, lactase activity declines dramatically in early childhood, an autosomal recessive determined condition known as primary hypolactasia, adult hypolactasia or lactase non-persistence [8]. Notably, in various ethnic groups, particularly those from populations with a history of dairying, lactase activity persists in high levels throughout adulthood [9].

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Genetic association studies have shown that in people of European ancestry, the T-allele of the single nucleotide polymorphism (SNP) LCT-13910C>T (rs4988235), located 13,910 base pairs upstream of the lactase (*LCT*) gene on chromosome 2q21-22 and within intron 13 of the adjacent *MCM6* gene, shows complete correlation with lactase persistence [10, 11]. All included subjects showing lactase deficiency were homozygous with respect to the -13910*C allele of the LCT-13910C>T variant. Other studies demonstrated that the -13910*T allele enhances LCT transcription in vitro [12–14] as well as in vivo [15] in keeping with a functional role for this variant in mediating the human lactase persistence phenotype. Consequently, analysis of the LCT-13910C>T polymorphism is commonly used as a simple test for the diagnosis of primary hypolactasia in symptomatic patients [16–18].

Several different methods have been used for the detection of this genetic variant, including PCR-restriction fragment length polymorphism (RFLP)-based analysis [19], pyrosequencing [20], high resolution melting analysis (HRM) [21], competitive allele-specific PCR (KASP) [22], and real-time PCR based assays using either allele-specific fluorescence resonance energy transfer (FRET) probes [23] or TaqMan probes [24]. Real-time PCR based techniques are probably the most widely used due to the relatively fast time-to-result, the simple interpretation of analytical results, and the broad distribution of various real-time PCR instruments offered by different companies among laboratories. However, despite the given abundance of published genotyping techniques as well as the availability of several commercial PCR-based kits [25, 26] for the detection of the LCT-13910C>T polymorphism, the first step of genetic analysis generally is the isolation of genomic DNA from blood or other sources. DNA extraction is often laborious and cost-intensive and increases the risk of cross-contaminations or sample mistakes. PCR analysis directly using blood as substrate, without prior DNA isolation, is generally resrticted by PCR inhibition caused by blood substances, particularly hemoglobin and immunoglobulin G [27]. Furthermore, in real-time PCR quenching of fluorescence may lead to a reduced assay sensitivity and, consequently, false-negative results [27].

Notably, several suppliers have released powerful DNA polymerases tolerating inhibitors present in whole blood in the recent years [28, 29]. However, no real-time PCR based protocol for the detection of the LCT-13910C>T polymorphism directly from blood is available in the current literature, to date. In order to further simplify LCT-13910C>T analysis we evaluated the analytic validity of the DirectBlood Genotyping PCR Kit (myPOLS Biotec, Konstanz, Germany) in combination with an in-house TaqMan primer-probe assay designed for the detection of the LCT-13910C>T polymorphism. We (i) investigated the specificity of our in-house TaqMan primer-probe assay together with the DirectBlood Genotyping PCR Kit using both, DNA and blood as substrates, (ii) we evaluated the impact of different concentrations of whole blood on the efficiency of the DirectBlood Genotyping PCR Kit, and (iii) we investigated the reliability of our direct blood PCR protocol by genotyping over 100 blinded blood samples from patients who underwent LCT-13910C>T testing by Sanger sequencing.

Materials and methods

Patients' samples

The present study totally included 129 EDTA anti-coagulated whole blood samples together with their matching genomic DNA samples. Out of these, 24 patients' samples of known genotype were used to evaluate the specificity of an in-house TaqMan primer-probe assay for LCT-13910C>T analysis combined with the DirectBlood Genotyping PCR Kit; remaining 105 samples were used to elucidate the reliability of our direct blood PCR protocol.

DNA-extraction

Genomic DNA was extracted from 200 µL EDTA blood using the peqGOLD® Blood DNA Mini kit (VWR, Vienna, Austria) according to the manufacturer's instructions.

Genotyping by Sanger sequencing

PCR products of 384 bp were generated using PCR primers tagged with M13 and M13-40 universal primers to be used in the subsequent sequencing reactions. Primer sequences were as follows (M13 and M13–40 universal primers are given as lower case letters): Forward primer: tgtaaaacgacggccagTTGGTTGAAGCGAAGATGG; reverse primer: gttttcccagtcacgacACTGTTGAATGCTCATACGACC. PCR was performed under the following amplification conditions: 10 min at 95 °C, followed by 40 cycles at 95 °C for 20 s, 55 °C for 30 s, and 72 °C for 30 s, followed by a final extension step of 72 °C for 5 min. PCR-products were purified using the ExoProStar™ 1-STEP Kit (GE Healthcare, Chicago, IL, USA) and sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA) on a 3730 DNA Analyzer (Thermo Fisher Scientific).

Direct blood PCR

Primers and probes

We designed a TaqMan probe based genotyping assay to detect the LCT-13910C>T polymorphism. The genotyping

assay consists of a pair of PCR primers tightly spanning the genetic variant and two allele-specific TaqMan probes containing locked nucleic acids (LNAs). TaqMan probes labeled with the 5′ fluorescent reporter dye 6-carboxy-fluorescein (6FAM) are specific for the respective T-allele and TaqMan probes labeled with hexachloro-fluorescein (HEX) are specific for the C-allele of the LCT-13910C>T variant. A non-fluorescent Black Hole Quencher™ 1 (BHQ1) is attached to the 3′ end of each TaqMan probe. Primer and probe sequences are given in Table 1. All oligonucleotides were synthesized by Microsynth Inc. (Balgach, Switzerland). Synthesized primer and probes were used to produce a 40× genotyping assay with concentrations of 36 µM of each PCR primer and 8 µM of each TaqMan probe.

Blood sample preparation

Blood samples were homogenized by vortexing. To test the impact of different blood concentrations on the performance of the PCR, blood samples were diluted to 2–16% in nuclease-free water. Diluted blood samples were heated for 10 min at 95 °C. After the heating step, blood samples were placed on ice and immediately used as substrates in PCR.

Real-time PCR

PCR was performed in a 20 µL volume containing 10 µL of 2× DirectBlood Genotyping PCR master mix (myPols Biotec), 0.5 µL of the in-house manufactured 40× primer-probe assay, 4.5 µL nuclease-free water, and 5 µL of diluted blood samples (corresponding to a final blood concentration of 0.5–8% in PCR). Alternatively, 5 µL of undiluted genomic DNA samples of 24 patients were used to compare the power of the DirectBlood Genotyping PCR Kit using different substrates. All TaqMan PCRs were carried out on a LightCycler® 480 Real-Time PCR System (Roche Diagnostics, Vienna, Austria) under the following thermocycling conditions: 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Non-template controls were included in each run as duplicates.

Table 1 Primer and probe sequences for LCT-13910C>T detection included in the TaqMan assay

Primers and probes	
LCT forward	5′ TGC GCT GGC AAT ACA GAT AAG
LCT reverse	5′ AAT GCA ACC TAA GGA GGA GAG TTC
-13910*T-probe	6FAM—AAT gTa gtc CCT GG—BHQ1
-13910*C-probe	HEX—AAT GTa gcC CCT GG—BHQ1

Locked nucleotides (LNAs) are denoted in lower case; the nucleotide complementary to the target allele is underlined. 6FAM, 6-carboxy-fluorescein

HEX hexachloro-fluorescein, *BHQ1* Black Hole Quencher 1™

Endpoint genotyping analysis was performed by LightCycler® software 1.5. The software annotates a validity score between 0 and 1 to each call (0 = invalid; 1 = highly valid). Samples that failed to be automatically called were manually assigned to the most probably genotype by visual inspection whenever possible or determined as uncertain or negative.

Real-time PCR products of the dilution series were further analyzed on a 2100 Bioanalyzer instrument (Agilent Technologies, Santa Clara, CA, USA) using the Agilent High Sensitivity DNA Kit.

Statistics

Differences in score values using either whole blood or genomic DNA as substrates were tested for statistical significance with the paired *t*-test. p-values < 0.05 were considered significant. Statistical analyses were performed with SPSS 25.0 for Windows (IBM, Armonk, New York, USA).

Results

First, we evaluated the specificity of our in-house TaqMan primer-probe assay combined with the DirectBlood Genotyping PCR Kit using 24 DNA samples of known genotype (eight samples for each genotype) as substrates. The obtained scatter plot is shown in Fig. 1a. All genotypes could be called automatically and correctly.

Next, we investigated the impact of different blood concentrations on the performance of the DirectBlood Genotyping PCR Kit together with our in-house TaqMan primer-probe assay using concentrations from 0.5 to 8% of whole blood as substrate in PCR. Samples homozygous of the LCT-13910*T allele were used. Amplification curves obtained by real-time PCR with final blood concentrations of 0.5%, 1%, 2%, 4%, and 8% in PCR are displayed in Fig. 2a. Threshold cycle (Ct) values decreased with increasing blood concentrations, reflecting the increase in DNA input. Also, analysis of PCR products on a 2100 Bioanalyzer instrument (Agilent Technologies) showed increased amounts of the final PCR products with increased blood concentrations indicating that efficiency of PCR was not affected by higher blood concentrations (Fig. 2b). However, maximum value of emitted fluorescence used in endpoint genotyping analysis decreased with increasing blood concentrations. The highest fluorescence value within the tested concentrations was reached with a final blood concentration of 0.5% in the reaction (Fig. 2a). Consequently, a blood concentration of 0.5% was used for the following experiments.

To evaluate the specificity of our assay using whole blood as substrate, we genotyped blood samples of the same 24 patients, whose DNA samples were used in the experimental approach described above. The scatter plot obtained by

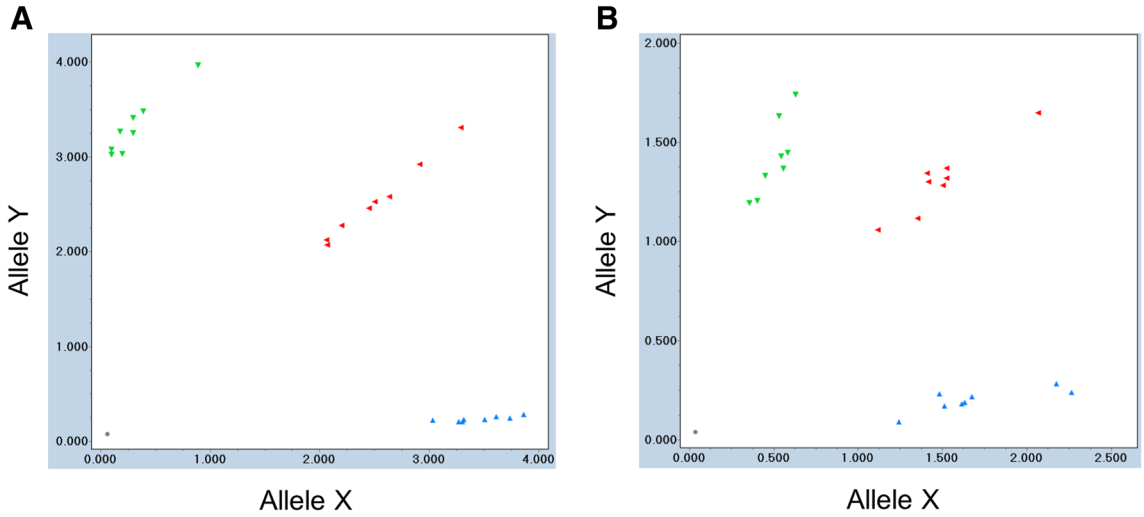

Fig. 1 Scatter plots from real-time PCR using either DNA (**a**) or blood (**b**) as substrates. Scatter plots obtained by real-time PCRs using the in-house LCT-13910C>T TaqMan primer-probe assay together with the DirectBlood Genotyping PCR Kit and **a** undiluted purified DNA samples or **b** whole blood with a final concentration of 0.5% in PCR as substrates

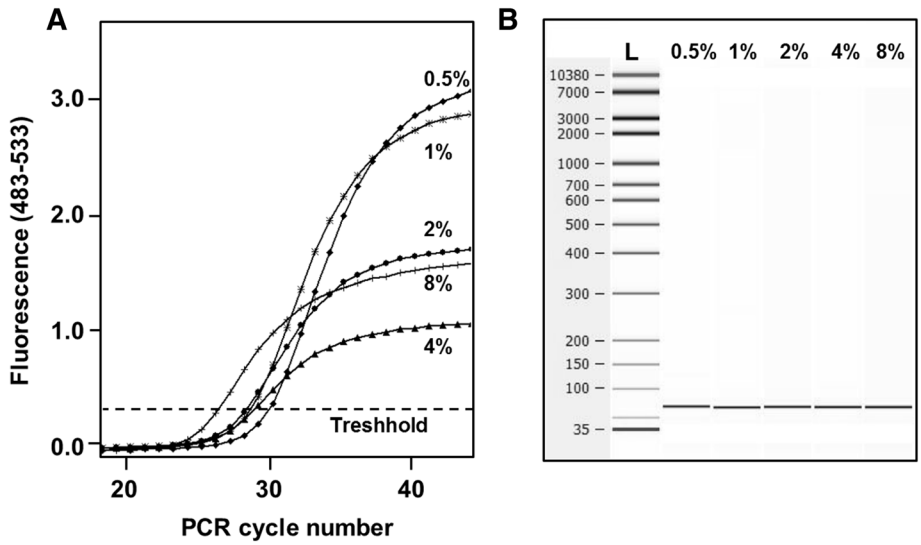

Fig. 2 Impact of different blood concentrations on PCR. **a** Amplification curves obtained by real-time PCRs with concentrations of 0.5%, 1%, 2%, 4%, and 8% of whole blood. Threshold cycle (Ct) values decreased from 30.3 over 29.0, 27.7, and 27.1 to 25.2 using blood concentrations of 0.5%, 1%, 2%, 4%, and 8% respectively. Maximum values of emitted fluorescence were 3.17, 2.97, 1.80, 1.16, and 1.68 for PCRs with concentrations of 0.5%, 1%, 2%, 4%, and 8% blood, respectively. **b** Results of electrophoresis of PCR products on a 2100 Bioanalyzer instrument (Agilent Technologies) using the Agilent High Sensitivity DNA Kit. Quantification of PCR products showed DNA amounts of 21.0 ng/µL, 24.6 ng/µL, 25.5 ng/µL, 31.9 ng/µL, and 31.4 ng/µL for PCRs with concentrations of 0.5%, 1%, 2%, 4%, and 8% blood, respectively. *L* ladder

direct blood PCR is shown in Fig. 1b. Direct blood PCR showed lower fluorescence signals compared to PCRs using genomic DNA as substrate (Fig. 1a) pointing to reduced input DNA amounts. However, all genotypes from PCRs using whole blood samples were called automatically and correctly by the genotyping analysis software. Moreover, assigned validity scores from direct blood PCR were in high concordance and did not differ significantly compared to those using genomic DNA. [Mean score value = 0.95 ± 0.03 and 0.96 ± 0.06 for whole blood and for genomic DNA, respectively ($p = 0.460$)].

To elucidate the reliability of our direct blood PCR protocol, we compared results obtained by Sanger sequencing with results obtained from direct blood PCR totally including

105 further subjects. Sanger sequencing revealed 37 carriers homozygous for the T-allele, 45 carriers of the heterozygous genotype, and 23 carriers homozygous for the C-allele of the LCT-13910C>T polymorphism. Furthermore, Sanger sequencing identified heterozygous carriership of an additional genetic variant, LCT-13913T>C (rs41456145), in one subject of putative Middle Eastern descent (homozygous for the LCT-13910*C allele). No other genetic variants within the LCT-13910C>T region were detected.

Raw data including fluorescence signal strength and score values of each individual analysis are given in Supplemental Table S1. Genotypes of 104 samples were automatically called correctly (mean score = 0.93 ± 0.06; range 0.58–1) and one sample was automatically called as unknown. The analysis of the sample with the unknown genotype was repeated showing the correct automatically called genotype (score = 0.99) in the second analysis pointing to handling errors in the initial analysis.

Discussion

Primary hypolactasia is the main cause of lactose intolerance and affects about 28% of the European population, with significant regional differences [30, 31]. The LCT-13910C>T polymorphism shows complete correlation with the lactase persistence/non-persistence phenotype [10, 11, 32] and analysis of this variant has been recommended as a direct genetic test for the trait [16–18, 33]. In our study, we successfully developed a valid real-time PCR protocol for the detection of the LCT-13910C>T polymorphism directly from blood further facilitating diagnosis of primary hypolactasia in symptomatic patients.

The protocol combines the commercially available DirectBlood Genotyping PCR Kit (myPOLS Biotec) together with an in-house developed TaqMan-assay for real-time detection of the LCT-13910C>T variant. The DirectBlood Genotyping PCR Kit contains an inhibition-resistant mutant of *Thermus aquaticus* (Taq) DNA polymerase and potentially non-disclosed substances to increase PCR inhibitor resistance. A variety of PCR enhancers have been described in the literature enabling Taq DNA polymerase to amplify DNA in the presence of blood including bovine serum albumin, 1,2-propanediol, and D-(+)-trehalose [34–36]. Notably, these PCR additives may significantly lower DNA melting temperature [34] and, therefore, may reduce assay specificity. That said, our in-house developed TaqMan-assay combined with the DirectBlood Genotyping PCR Kit shows clear genotype clustering either using DNA or blood as substrate allowing automated genotype calling without the need of manual editing.

Dilution series of whole blood showed that the efficiency of the DirectBlood Genotyping PCR Kit was not affected by increasing blood concentrations. However, in real-time PCR maximum value of emitted fluorescence used in endpoint genotyping analysis strongly decreased with increasing blood concentrations. This points to fluorescence quenching effects of blood components in real-time PCR as also previously described [27]. Therefore, quenching of fluorescence rather than amplification inhibition limits the amount of blood advisable for use with the DirectBlood Genotyping PCR Kit. That said, 5 µL of 2% diluted whole blood (corresponding to a final blood concentration of 0.5%) used in real-time PCR appears sufficient for variant detection: All 129 blood samples of the present study could be successfully genotyped following the given protocol.

It should be noted that accuracy of our in-house primer-probe assay may be influenced by other genetic variants occurring nearby the LCT-13910C>T variant. Particularly, in individuals of African or Middle Eastern descent several rare variants, including LCT-13907C>G (rs41525747), LCT-13913T>C (rs41456145), and LCT-13915T>G (rs41380347), have been identified [37, 38]. In fact, Sanger sequencing identified a heterozygous carriership of the LCT-13913T>C variant in one subject of putative Middle Eastern origin, who was also homozygous for the -13910*C allele as determined by our TaqMan assay and confirmed by sequencing analysis. Notably, however, in this case binding of the -13910*C probe exclusively on the -13910*C/-13913*T allele would have resulted in the correct genotype. Alternatively, in case of double heterozygous genotypes, binding of one of the two -13910C/T probes may fail, potentially leading to a false-positive homozygous LCT-13910C>T genotype. However, according to the NCBI dbSNP database [39] variants adjacent to LCT-13910C>T could not be found in subjects of European ancestry. Therefore, in non-European individuals, at least in those showing a homozygous LCT-13910*C genotype in TaqMan analysis, alternative detection methods such as Sanger sequencing or pyrosequencing should be considered, so as not to miss further genetic variants or to misclassify results from TaqMan genotyping.

Conclusions

In conclusion, we present a reliable real-time PCR protocol for the detection of the LCT-13910C>T variant directly from whole blood. The described method is valid, cost-saving, and accelerates time-to-result and, therefore, supports fast clarification of a putative underlying genetic background of lactose intolerance.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest related to the contents of this article.

Informed consent All patients gave written confirmed consent for clinical LCT-13910C>T testing.

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