made to both the dependent and independent variables to improve the model fit.

Changes in urinary M protein correlated strongly with changes in serum FLC concentrations when we used the random-effects model \( (P = 0.0001) \). In other words, although serum FLC does not quantitatively match urinary M-protein concentrations, changes in serum FLC over a period of time correlate with changes in the amounts of 24-h urinary M protein for an individual patient.

It appears, therefore, that the quantification of FLCs in serum by nephelometry correlates linearly on a log-log scale with changes in urinary FLC excretion. This correlation suggests that serum measurements may provide a logical and feasible alternative to 24-h urine collections in monitoring patients with LCMM.

This study was supported in part by Grant CA 62242 from the National Cancer Institute. We also wish to acknowledge Dr. A. R. Bradwell and The Binding Site, Ltd (Birmingham, United Kingdom) for providing the FLC reagents for analysis.

References


Genotyping of the Apolipoprotein B R3500Q Mutation Using Immobilized Locked Nucleic Acid Capture Probes, Nana Jacobsen,1* Mogens Fenger,2 Joan Bentzen,3 Søren Lind Rasmussen,4 Mogens Havsteen Jakobsen,1,2 Jørgen Fensholt,2 and Jan Skov2 (1 Department of Expression Microarrays, EURAY, and 2 Department of Chemistry, Exiqon A/S, Bygstubbjen 9, DK-2950 Vedbaek, Denmark; Departments of 3 Clinical Biochemistry and 4 Cardiology, University Hospital of Copenhagen, DK-2650 Hvidovre, Denmark; * author for correspondence: fax 45-45-661888, e-mail jacobsen@exiqon.com)

Hyperlipidemia and coronary heart disease (CHD) are associated with genetic variation in the apolipoprotein B (apoB) gene (1). Nonexchangeable apolipoprotein B-100 (apoB-100) is an important determinant of LDL-cholesterol in plasma; it plays a central role in cholesterol transport by its association to LDL particles as a ligand for the LDL receptor (2). One of the first mutations in the apoB gene to be discovered was the apoB-100 R3500Q mutation (apoBR3500Q) (3), a single nucleotide transition, CCG to CAG, in exon 26. This mutation reduces the affinity to the LDL receptor by at least 95% (4) and is the major cause of familial defective apoB-100 (FDB). The frequency of the mutation is 1:500 to 1:700 in Caucasians (5, 6). Because the cholesterol concentration is often within the reference interval in FDB patients, the only reliable way of detecting FDB is by genotyping. At present, genotyping of the apoB R3500Q mutation is based on PCR (7–9), but other methods, such as heteroduplex analysis and real-time PCR, have also been applied (10–12). In general, these methods are time-consuming and need to be optimized. Here we describe a simple, rapid, and sensitive assay for genotyping the apoBR3500Q mutation that is suitable for the 96-well microtiter plate format and relies on hybridization of PCR amplicons to allele-specific locked nucleic acid (LNA) capture probes (13, 14).

The microtiter plates were prepared by covalent photomobilization of 10 pmol/well of either wild-type (wt)-LNA8 [AQ-CONH-(CH2)3-TACATGTTATGCTGA-Gl-A1MeC1MeC1L1G1T1G1T1G] or mutant (m)-LNA8 [AQ-CONH-(CH2)3-TACATGTTATGCTGA-Gl-A1MeC1MeC1L1G1T1G1T1G1T1G1T1G] capture probes using an anthraquinone (AQ) moiety as described by Koch et al. (15). \( 1^\circ \) indicates 2′-O-4′-C-methylene-(2′-deoxy)-ribofuranosyl) (LNA) nucleotides, and MeC indicates 5-methylcytosine. After irradiation, the plates were treated as described by Ørum et al. (16).

A plasmid was constructed by cloning the wild-type PCR amplicon to test the specificity of the apoBR3500Q genotyping assay. This was generated from genomic DNA (extracted from 5 mL of EDTA-anticoagulated blood using a Roche DNA Isolation reagent set) with the forward primer 5′-CACCTTATTTTTCATGAGT-3′ and the reverse primer 5′-TTGATCATTTAGTT-
melting temperature (single mismatch. In addition, measurements of duplex probes were hybridized with DNA targets containing a No detectable signals were obtained from either the Biosystems); 2.5 mM MgCl2; 200 HCl (pH 8.0); 50 mM KCl (GeneAmp Gold buffer; PE Biosystems); and 100 ng of genomic DNA as template into the pCR 2.1-TOPO plasmid using the TOPO™ TA Cloning™ reagent set (Invitrogen). The primers amplify a region within the apoB gene encompassing nucleotide position 9775 (GenBank Accession. No. M19828). A mutant plasmid was then constructed by use of a primer covering the position of interest. The PCR product was generated using the splicing by overlap extension-PCR method (17). Purified plasmids were confirmed by DNA sequencing and used for validating the apoB<sub>R3500Q</sub> genotyping assay. The specificity of the capture probes was subsequently tested using plasmids where mismatched nucleotides were introduced 5’ and 3’ of the second base in apoB codon 3500, using site-directed mutagenesis (17). No detectable signals were obtained from either the wild-type or the mutant capture probes when the capture probes were hybridized with DNA targets containing a single mismatch. In addition, measurements of duplex melting temperature (T<sub>m</sub>) confirmed the affinity and specificity of the LNA capture probes. Melting curves were constructed without the AQ and the DNA linker moieties. The thermostability of the duplexes was determined as described by Wahlestedt et al. (18). As seen in Table 1, the T<sub>m</sub> values of the LNA capture probes were substantially higher than the T<sub>m</sub> values of identical DNA probes. A selected panel of archival patient samples was analyzed twice by the apoB<sub>R3500Q</sub> assay without prior knowledge of the genotype. The primers for the apoB<sub>R3500Q</sub> PCR were as follows: forward primer, 5’-biotin-CTAGTGAG-GCCAACACTTACTTGAATTCCAAGAGC-3’ (position 9736–9770); reverse primer, 5’-GTTTTCTGCTGTCTCCAGAG-3’ (position 9879–9902); these primers produce a 167-bp amplicon from genomic DNA. PCR reactions (50 μL) were prepared by mixing 15 mM Tris-HCl (pH 8.0); 50 mM KCl (GeneAmp Gold buffer; PE Biosystems); 2.5 mM MgCl2; 200 μM each of dATP, dCTP, dGTP, and dTTP (Amersham Pharmacia Biotech); 1 μM forward primer; 1 μM reverse primer; 1.25 U (0.25 μL of a 5 U/μL solution) of AmpliTaq Gold polymerase (PE Biosystems); and 100 ng of genomic DNA as template (purified as described above). After an initial 15 min denaturation step at 95 °C, 30 cycles of PCR were carried out (40 s at 95 °C, 40 s at 65 °C, and 40 s at 72 °C), followed by extension at 72 °C for 10 min.

The microtiter plate assay was performed by mixing 20 μL of the PCR amplicon with 20 μL of denaturation buffer (125 mmol/L NaOH, 8 mmol/L EDTA, 0.2 g/L phenol red) and incubating for 5 min at room temperature. We added 200 μL of hybridization buffer (50 mmol/L sodium phosphate buffer, pH 7.0, 0.1 mL/100 mL Tween 20); 100 μL of this reaction was transferred to the coated microtiter wells containing either the wt-LNA8 or m-LNA8 capture probe and hybridized for 30 min at 37 °C. The wells were washed three times in washing buffer [300 μL/well; 0.5× standard saline citrate buffer (75 mmol/L NaCl, 7.5 mmol/L sodium citrate, pH 7.0, 1 mL/L Tween 20)]. We then added 100 μL of conjugate solution (1 mg/L horse-radish peroxidase–streptavidin; Pierce) diluted in the washing buffer to each well and incubated the plate for 15 min at 37 °C. Finally, the wells were washed six times with washing buffer (300 μL/well), and 100 μL of 3,3’,5,5’-tetramethylbenzidine substrate (TMB one; KemEnTec) was added. The plate was then incubated in the dark for 10–15 min at room temperature, and the reaction was stopped by the addition of 100 μL/well of 0.5 mol/L H<sub>2</sub>SO<sub>4</sub>. The absorbance was measured at 450 nm on a Wallac Victor<sup>2</sup> (Perkin-Elmer). Fig. 1 shows that the apoB<sub>R3500Q</sub> assay yielded an unambiguous response for all archival patient samples, concurring with the results obtained by DNA sequencing.

We next genotyped DNA samples from 309 patients admitted to the emergency room at the University Hospital of Copenhagen with clinical and biochemical signs of acute myocardial infarction according to the guidelines from the American College of Cardiology (19). Only samples from patients who survived initial treatment were analyzed by the apoB<sub>R3500Q</sub> assay. The results of 53 randomly selected samples were confirmed by DNA sequencing. All 309 patient samples were genotyped as wild type. Thus, no apoB<sub>R3500Q</sub> heterozygotes were detected in this population of acutely admitted coronary patients. Considering the impact of the apoB<sub>R3500Q</sub> mutation on CHD (20, 21), we initially expected the prevalence of the apoB<sub>R3500Q</sub> mutation to be increased in patients with acute myocardial infarction. In the Danish popula-

---

**Table 1. Thermostability of the LNA and DNA capture probe sequences against complementary and single-base-mismatched DNA targets.**

<table>
<thead>
<tr>
<th>Entry</th>
<th>Capture probe</th>
<th>Sequence&lt;sup&gt;a&lt;/sup&gt;</th>
<th>wt-DNA target&lt;sup&gt;b&lt;/sup&gt;</th>
<th>m-DNA target&lt;sup&gt;c&lt;/sup&gt;</th>
<th>ΔT&lt;sub&gt;m&lt;/sub&gt;&lt;sup&gt;d&lt;/sup&gt; °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Wildtype LNA</td>
<td>5′-G&lt;sup&gt;a&lt;/sup&gt;A&lt;sub&gt;mic&lt;/sub&gt;C&lt;sub&gt;mic&lt;/sub&gt;C&lt;sub&gt;mic&lt;/sub&gt;G&lt;sup&gt;5&lt;/sup&gt;T&lt;sup&gt;5&lt;/sup&gt;G&lt;sup&gt;5&lt;/sup&gt;T&lt;sup&gt;5&lt;/sup&gt;G&lt;sup&gt;5&lt;/sup&gt;3′</td>
<td>68.8</td>
<td>33.8</td>
<td>34.8</td>
</tr>
<tr>
<td>2</td>
<td>Wildtype DNA</td>
<td>5′-GAC&lt;sup&gt;a&lt;/sup&gt;C&lt;sub&gt;mic&lt;/sub&gt;G&lt;sup&gt;5&lt;/sup&gt;TG&lt;sup&gt;5&lt;/sup&gt;3′</td>
<td>34.7</td>
<td>&lt;5</td>
<td>&gt;29.7</td>
</tr>
<tr>
<td>3</td>
<td>Mutant LNA</td>
<td>5′-G&lt;sup&gt;a&lt;/sup&gt;A&lt;sub&gt;mic&lt;/sub&gt;C&lt;sub&gt;mic&lt;/sub&gt;T&lt;sup&gt;5&lt;/sup&gt;G&lt;sup&gt;5&lt;/sup&gt;T&lt;sup&gt;5&lt;/sup&gt;G&lt;sup&gt;5&lt;/sup&gt;3′</td>
<td>28.9</td>
<td>55.3</td>
<td>26.4</td>
</tr>
<tr>
<td>4</td>
<td>Mutant DNA</td>
<td>5′-G&lt;sup&gt;a&lt;/sup&gt;C&lt;sub&gt;mic&lt;/sub&gt;T&lt;sup&gt;5&lt;/sup&gt;G&lt;sup&gt;5&lt;/sup&gt;TG&lt;sup&gt;5&lt;/sup&gt;3′</td>
<td>13.9</td>
<td>25.1</td>
<td>11.2</td>
</tr>
</tbody>
</table>

<sup>a</sup> L, 2′-O-(2-hydroxyethyl)-N′-methylthymidine (LNA); a<sub>mic</sub>, 5-methylcytosine.<br><sup>b</sup> Only 0.25 μL of the two complementary strands was used. The sodium ion concentration of the T<sub>m</sub> buffer corresponds to that of the hybridization buffer in the apoB<sub>R3500Q</sub> microtiter plate assay. The complementary DNA sequences were either fully matched or contained one mismatch opposite the underlined nucleotide, corresponding to wild-type (wt) or mutant (m) DNA targets.<br><sup>c</sup> The difference in melting temperature between perfectly matched and one mismatch in the complementary sequences.
tion, 70% of the mutation carriers suffer from CHD at age 60 compared with ~10% in the general population. It has been shown that 16% of all patients in the 30–74 age group suffering from myocardial infarcts die before admission to the hospital (M. Davidsen et al., submitted for publication), just as the finding of dyslipidemia in adolescents carrying the apoB_{R3500Q} mutation (22) supports the hypothesis that the carriers of the apoB_{R3500Q} mutation are at increased risk of a sudden death. The mutation is codominant and probably leads to development of atherosclerosis and CHD at an early age (5, 23, 24). In Denmark, ~5000–10 000 individuals go undetected as carriers of the mutation, and genotyping appears to be the only reliable alternative because determination of fasting cholesterol is an unreliable marker in diagnosing FDB in the young and in middle-aged adults (22, 25–28). Three persons were found to harbor the apoB_{R3500Q} mutation (unpublished results) when the apoB_{R3500Q} analysis was extended to the Danish MONICA 10 cohort (29), which consists of 2656 persons. This is in accordance with the prevalence of the mutation in the general population (5, 6).

In summary, we have established a genotyping method based on LNA technology for the detection of the apoB_{R3500Q} mutation in a microtiter plate format. The assay consists of a single hybridization step in which the DNA target is captured, followed by detection of the hybrids. It demonstrates that LNA oligonucleotides are uniquely suited as probes in mutation and single-nucleotide polymorphism detection assays. Conformational fixation of the sugar moiety in the LNA nucleotide enables more specific hybridization with DNA targets compared with the corresponding DNA-based capture probes (Table 1). The apoB_{R3500Q} assay includes capture probes for both the wild-type and the apoB_{R3500Q} mutation and detects both homozygous and heterozygous patient samples. The design ensures that no false-negative results occur in the genotyping of the apoB_{R3500Q} mutation. The assay is also suited for automation and high-throughput screening and can be adapted to genotyping microarrays.

This study was supported by Grant 1998-503/0002-1 from The Danish Agency for Trade and Industry.

References

29. In this study, we compared the 1H NMR spectra of urine samples from children and adolescents with type 1 diabetes, but without microalbuminuria or any other renal complication, with spectra of samples from healthy individuals matched for sex and age. The aim of this comparison was to obtain basic knowledge of possible differences in the urinary excretion or concentrations of a series of metabolites between patients with type 1 diabetes and nondiabetic individuals, with the prospect of evaluating the utility of diabetes-associated differences in the assessment of the patients’ metabolic control.

Proton Nuclear Magnetic Resonance Spectral Profiles of Urine from Children and Adolescents with Type 1 Diabetes, Cecilia Zuppi,1,2 Irene Messana,2,3 Pativi Tapranainen,4 Mikael Kulp,5 Federica Vincenzoni,1 Bruno Gardina,1,2 and Matti Nuutinen4 (1 Institute of Biochemistry and Clinical Biochemistry, Catholic University Rome, 00168 Rome, Italy; 2 Center for the Chemistry of Biologically Active Molecules, National Council of Research, 00168 Rome, Italy; 3 Department of Sciences Applied to Biosystems, University of Cagliari, 09100 Cagliari, Italy; 4 Department of Pediatrics, University of Oulu, FIN-90014 Oulu, Finland; 5 Hospital for Children and Adolescents, University of Helsinki, FIN-00029 Helsinki, Finland; address correspondence to this author at: Department of Pediatrics, University of Oulu, PO Box 5000, FIN-90014 Oulu, Finland; fax 358-8-315-5559, e-mail matti.nuutinen@oulu.fi)

The assessment of renal function is crucial in type 1 diabetes because nephropathy is one of the major complications. The measurement of urinary albumin excretion is the most widely used method for monitoring kidney involvement (1), although the use of other markers, such as enzymes or proteins derived from tubular cells (2), has also been proposed. 1H nuclear magnetic resonance (NMR) spectroscopic analysis of urine may provide useful information because it allows the analysis, in a single image, of several metabolites that reflect diverse renal functions, including intermediary metabolism as well as tubular and medullary cell function.

In this study, we compared the 1H NMR spectra of