on the polyacrylamide gel were visualized by silver staining (Bio-Rad). PCR products were purified by polyacrylamide gel electrophoresis and used for sequence analyses. The sequencing reactions were carried out using the Taq Dye-Deoxy Termination Cycle Sequencing Kit and DNA Sequencer (Model 373) from Applied Biosystems.

The mutation screening showed heteroduplex formation only for exon 2. These heteroduplexes were detectable for every hypocatalasemic (n = 23) but not for the normocatalasemic (n = 26) members of families M, D, and G. No heteroduplex formation was detected in the hypocalasalemic and normocatalasemic members of the other three hypocatalasemic families.

The heteroduplex pattern showed the first band at 268 bp (wild-wild homoduplex), the second at ~270 bp (mutant-mutant homoduplex), and two heteroduplexes at 273 and 304 bp. It is rare to be able to distinguish these four bands so clearly. Separation of the patterns of the four bands in exon 2 were found in the same well when a smaller gel (150 x 150 x 1.5 mm) was prepared with molecular biology-grade polyacrylamide (Bio-Rad) and no sample treatment was used (Fig. 1, middle panel).

The nucleotide sequence analysis showed a GA insertion (Fig. 1, bottom panel) at position 138 of exon 2. This insertion increased the GA repeat number from four to five and caused a frameshift mutation. This frameshift insertion increased the GA repeat number from four to five and caused a frameshift mutation. This frameshift mutation yielded a truncated protein and the lack of histidine 74, which is required for the binding of a hydrogen peroxide substrate (12). These findings could explain the decreased blood catalase activities of the hypocatalasemic patients (49.2 ± 13.7 MU/L; n = 23) compared with the normocatalasemic family members (107.6 ± 19.5 MU/L; n = 26). The heteroduplex formed from a 268-bp wild-type and a 270-bp mutant PCR product required neither pretreatment nor a special gel for its detection. This simple method was checked when the screening of the 625 normocatalasemic subjects yielded no heteroduplex formation.

In conclusion, a simple heteroduplex analysis of PCR products could be used for screening of GA insertions in exon 2 of the catalase gene. This new syndrome-causing mutation was detected in three of the nine hypocatalasemic families in Hungary. These data confirm the heterogeneity of the acatalasemia/hypocatalasemia detected in Hungary.

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References


the morning were collected in sterile containers without preservatives and stored.

The bone metabolism marker analysis was carried out on samples stored at various temperatures at established intervals: storage time, 24 h, 48 h, 7 days, and 1, 3, 6, and 12 months. All of the testing was carried out by systematically following the instructions of the manufacturer of each diagnostic kit.

P1NP was measured by radioimmunoassay (Procollagen Intact P1NP; Orion Diagnostica) (5, 6), and NTx was measured with a competitive-inhibition ELISA (Osteomark; Ostex) (7–9).

The total pyridinium cross-links PYD and DPD, the end products of collagen breakdown (10, 11), were determined by HPLC (Chrom-links; Bio-Rad) (12). The results of the study are summarized in Table 1. Taking the result obtained at storage time as 100%, the deviations (mean \( \pm SD \)) of the groups of data obtained are reported.

Using one-way ANOVA, we carried out a statistical elaboration of the data with the aim of highlighting a significant difference: each group of data was compared with its respective baseline group, and the \( P \) values calculated are reported.

Only the PYD and DPD values were significantly different from the baseline data group \( (P \leq 0.05) \) after 48 h of storage at room temperature. We can conclude that:

- P1NP is stable in stored serum for at least 48 h at 23–25 °C, 7 days at 2–8 °C, and frozen for at least 6 months at −20 to −80 °C.
- NTx is stable in stored urine for at least 48 h at 23–25 °C, 7 days at 2–8 °C, 3 months when frozen at −20 °C, and for at least 1 year at −80 °C.
- PYD and DPD are not stable in urine samples stored at room temperature: even after 24 h, the respective mean concentrations compared with the baseline values were reduced by 30% and 39%, respectively. After 48 h, the PYD and DPD concentrations were reduced by more than 50%.
- PYD and DPD are, however, stable for at least 7 days in urine stored at 2–8 °C, for at least 3 months in urine frozen at −20 °C, and for at least 1 year in urine frozen at −80 °C.

The instability of PYD and DPP during storage at room temperature could be caused by the lack of acidification in the sample. Indeed, Bio-Rad, the manufacturer of the kit, suggests acidifying the sample with 10 mL of 6 mol/L HCl if it is a 24-h urine collection. Nevertheless, no advice is given regarding the second urine of the morning. Additional studies are under way in our laboratory to investigate this matter. In any case, it is important to note that acidification of the urine sample is incompatible with the ELISA for the NTx, which could not, therefore, be conducted together with the pyridinium cross-links determination in 24-h urine samples.

In conclusion, this study shows that the storage of serum and urine samples at 2–8 °C for the bone metabolism markers in question can be delayed for at least 1 week; for long-term storage, freezing of the sample provides molecular stability for several months. PYD and DPD are very sensitive to the storage temperature, and therefore, immediate refrigeration of a sample determines the accuracy of their measurement.

We thank Dr. Romolo Dorizzi from the Laboratorio Analisi Ospedale Civile Maggiore (Verona, Italy) for invaluable assistance.

References


LDL Particle Size by Gradient-Gel Electrophoresis Cannot Be Estimated by LDL-Cholesterol/Apolipoprotein B Ratios, Daisuke Furuya, Atsuhito Yagihashi, Syunichi Nasu, Teruo Endoh, Tohru Nakamura, Reiko Kaneko, Chinatsu Kagamata, Daisuke Kobayashi, and Naoki Watanabe* (Department of Clinical Laboratory Medicine, Sapporo Medical University School of Medicine, South-1, West-16, Chuoku, Sapporo 060-8543, Japan; * author for correspondence: fax 81-11-622-7502, e-mail watanabn@sapmed.ac.jp)

LDL particles have been shown to be heterogeneous in size, density, and composition. Heterogeneity of LDL particles with respect to size has been demonstrated by analytic ultracentrifugation (1), density-gradient ultracentrifugation, gradient-gel electrophoresis (GGE), high-performance gel-filtration chromatography, dynamic light scattering, and electron microscopy. Among these methods, GGE is the most reliable and widely used, but it has the drawback of being labor-intensive. Recently, the ratio of the LDL-cholesterol (LDL-chol) concentration to the LDL-apolipoprotein B (LDL-apo B) concentration has been used as an alternative index of hyperapobetalipoproteinemia and small dense LDL (2–5). We therefore studied the relationship between LDL particle size as measured by GGE and as estimated by the LDL-chol/LDL-apo B ratio.

LDL particle size was quantified by the above two methods in samples from healthy controls (n = 49) and from hyperlipemic subjects (n = 81). The hyperlipemic samples in this study were defined according to the criteria of the Japan Atherosclerosis Society [total cholesterol ≥2200 mg/L; triglycerides (TGs) ≥1500 mg/L] (6). GGE was carried out using a 2.4–15.2% Multilipo nonde-naturing gradient gel (Daichi Pure Chemical). Serum samples (5 μL) were electrophoresed on the gel at 25 mA for 2 h. Colloidal gold with standard particle sizes (19.9 and 25.9 nm as confirmed by electron microscopy) was used as a marker for LDL particle size. The colloidal gold particle sizes were also estimated by GGE from a linear calibration curve of the diameter vs the migration distance, with l-lactate dehydrogenase (8.4 nm), catalase (10.4 nm), ferritin (12.2 nm), and thyroglobulin (17.0 nm) as calibrators of known size (7). Bands were analyzed by

### Table 1. Comparison of lipid markers between control and hyperlipemic subjects.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Control</th>
<th>Hyperlipemic</th>
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<tbody>
<tr>
<td>TGs, mg/L</td>
<td>650 ± 230</td>
<td>2616 ± 1520</td>
</tr>
<tr>
<td>HDL-chol, mg/L</td>
<td>649 ± 137</td>
<td>579 ± 170</td>
</tr>
<tr>
<td>LDL-chol, mg/L</td>
<td>908 ± 195</td>
<td>1607 ± 323</td>
</tr>
<tr>
<td>apo B, mg/L</td>
<td>713 ± 140</td>
<td>1404 ± 218</td>
</tr>
<tr>
<td>LDL particle size&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.7 ± 0.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22.9 ± 1.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>By LDL-chol/LDL-apo B</td>
<td>1.27 ± 0.06&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.15 ± 0.14&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are means ± SD.
<sup>b</sup> P < 0.0002, hyperlipemia vs control.
<sup>c</sup> P < 0.0001, hyperlipemia vs control.

Fig. 1. Comparison of LDL particle size between GGE and LDL-chol/LDL-apo B in control subjects (A) and subjects with hyperlipemia (B). △, misleadingly high LDL-chol/LDL-apo B ratios; □, spuriously low LDL-chol/LDL-apo B ratios (reflecting hypertriglyceridemia).