The 204 specimens were first ranked separately by (a) triglyceride concentration (TRIG), (b) total cholesterol/total triglyceride ratio (CHOL/TRIG), (c) cholesterol: triglyceride ratio in VLDL (VLDL ratio), (d) IDL-cholesterol concentration (IDL), and (e) cholesterol:triglyceride ratio in IDL (IDL ratio). The centile rank for the 36 LDL-cholesterol results in error (i.e., those for which the Friedewald formula gave a result that differed from that of ultracentrifugation by >10%) was then found as shown. To provide a consistent comparison, an inverse ranking for total cholesterol/totai triglyceride ratio was used.

The ranking by total triglyceride concentration and total cholesterol:triglyceride ratio demonstrates that these values are far better suited as criteria for deciding whether it is valid to calculate LDL-cholesterol. Moreover, the results confirm that total triglyceride concentration alone seems to be the most simple and appropriate criterion to use, with total cholesterol:triglyceride ratio offering no improvement in discrimination.

In our study, 24 of 41 specimens (59%) with triglyceride concentration >3.2 mmol/L had an unreliable estimate of LDL-cholesterol from the Friedewald formula, with all but two being an overestimate. While such overestimation is typical of abnormal VLDL composition as seen in Type III hyperlipoproteinemia [5], there appeared to be no clear association between cholesterol:triglyceride ratio in VLDL and the incidence of error (Fig. 1). It seems improbable that the disparity between our results and those of others [8] could be explained by effects of drug treatment. Indeed, the conclusion was unchanged when the 50 specimens from patients on an hydroxymethylglutaryl-CoA reductase inhibitor were removed from analysis.

In summary, use of the Friedewald formula in a group of patients expected to give aberrant results provided reliable data in the majority of cases. Moreover, triglyceride concentration was a suitable index of reliability. However, restricting use of the formula to those specimens having a triglyceride concentration of <4.5 mmol (4 g)/L [13] included a group above the 80th centile in which the error rate exceeded 50%. Limiting use of the formula to those below the 80th centile and having a triglyceride concentration of <3.2 mmol (2.8 g)/L excluded the majority of errors and left 151 of 163 (93%) with a valid estimate. By contrast with the results of others [8, 14], we found little influence of VLDL or IDL on the reliability of the Friedewald formula and no advantage with total cholesterol:triglyceride ratio as a discriminant.

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References

Rapid Detection of the Fibrinogen Aa16Arg→His Mutation, Hayley J. Ridgyway, Stephen O. Brennan, Andrew P. Fellows, and Peter M. George (Molec. Pathol. Lab., Christchurch Hosp., Canterbury Health Ltd., Christchurch, New Zealand; author for correspondence: fax 64-3-364-0545, e-mail hridgway@chmeds.ac.nz)

We have now identified mutations in 17 families with dysfibrinogenemias. Over half of these families have the Aa16Arg→His mutation. This mutation is the most com-
commonly reported cause of dysfibrinogenemia and, like other dysfibrinogenemias, is readily detected because of the associated prolonged thrombin and reptilase times [1-3]. The mutation alters the thrombin cleavage site such that release of fibrinopeptide A is delayed. However, fibrinopeptide release assays are difficult and do not directly confirm the molecular basis of the impaired fibrinopeptide release. We have therefore designed a rapid and technically simple PCR-based method for detection of the Aα16Arg→His mutation. This allows reliable identification of a common dysfibrinogenemia, in its heterozygous form, is usually asymptomatic and does not pose any substantial threat to the health of the patient. Application of this method will allow clinical laboratories to determine the molecular defect in many of the cases that they detect during coagulation studies.

We examined nine families with the Aα16Arg→His mutation. These had been referred for further investigation when routine coagulation studies were consistent with dysfibrinogenemia. All procedures were carried out in accordance with the guidelines of our local ethics committee. Blood samples were collected into Na+ citrate Vacutainer Tubes (Becton Dickinson), and coagulation studies were performed by routine clinical tests for thrombin and reptilase times. There was considerable variation both within and between families in thrombin times [range 36-70 s (reference range 20 ± 2)] and reptilase times [range 45-72 s (reference range 20 ± 2)]. In each case fibrinopeptide release assays [4] demonstrated reduced fibrinopeptide A concentrations with an additional eluting peak. Either amino acid analysis of the abnormal fibrinopeptide or DNA sequence analysis then confirmed the mutation.

Genomic DNA was isolated from whole blood [5]. The oligonucleotides Fn1111α (ATT GCT GCT GCT TTC TTT TG) and Fn1309α (AAT CTC CTT CCC CCG CT) were used to amplify a 199-bp region spanning exon 2 of the Aα gene by PCR [6]. Each 100-μL amplification reaction contained 50 mmol/L KCl, 10 mmol/L Tris-HCl, pH 8.3, 1.5 mmol/L MgCl2, 200 μmol/L of each dNTP, 1 μmol/L of each primer, 1 μg of DNA template, and 2 units of Taq DNA polymerase (Boehringer Mannheim). Amplification was for 30 cycles with denaturation for 30 s at 94 °C, annealing for 30 s at 60 °C, and extension for 1 min at 72 °C with a final extension at 72 °C for 7 min. The PCR products were digested for 4 h at 37 °C with 5 units of NlaIII according to the manufacturer’s instructions (New England Biolabs). Typically 7 μL of PCR product was diluted to 10 μL by the addition of 0.5 μL of 10 units/μL NlaIII, 1 μL of NEBuffer 4 (New England Biolabs), 1 μL of 1 mg/mL bovine serum albumin, and 0.5 μL of sterile distilled water. Digestion was assayed by gel electrophoresis in 2% agarose, 50 mmol/L Tris base, 45 mmol/L boric acid, 0.5 mmol/L EDTA for 30-40 min at 100 V. Products were visualized by staining in 20 μg/mL ethidium bromide for 5 min followed by transillumination at 302 nm.

The Aα16Arg→His mutation changes the sequence CGTG to CATG creating an NlaIII cleavage site near the middle of the PCR product (Fig. 1, lower panel). Cleavage at this site generated 104-bp and 95-bp products that were not resolved on the agarose gel, but were clearly separated from the uncut product. DNA from apparently healthy individuals remained uncut. The upper panel of Fig. 1 shows the restriction pattern produced from apparently healthy individuals (lanes 2 and 5) and the pattern produced by individuals heterozygous for the Aα16Arg→His (CGT→CAT) mutation (lanes 3, 4, and 6). Additionally, the assay should be able to detect homozygotes because no uncut product should remain; however, appropriate controls were not available.

The mutation Aα16Arg→His affects the thrombin cleavage site at the N-terminal of the Aα chain. Normal cleavage at this site exposes the Gly-Pro-Arg (A) site, which interacts with a preformed complementary site located in the C-terminal of the γ chain, thereby initiating polymerization [7]. The net effect of replacing the arginine at position 16 of the Aα chain is only to delay the thrombin-catalyzed exposure of the A polymerization site. Therefore, it is not surprising that this mutation is usually asymptomatic. Despite this, two reported cases have been associated with mild bleeding tendencies [8, 9]. In these cases, the bleeding tendency generally can be attributed to additional abnormalities in other coagulation proteins. In fibrinogen Milano VI, the patient showed...
defective platelet aggregation [8], whereas in fibrinogen Birmingham, abnormalities in von Willebrand factor were seen [9]. The only reported case of this mutation in its homozygous form, fibrinogen Giessen I, is associated with more severe symptoms and displays a severe bleeding tendency and miscarriage [10].

Dysfibrinogenemias with the Aa16Arg → His mutation are usually detected by prolonged thrombin-clotting times. Once detected, the mutation can be characterized by reversed-phase monitoring of fibrinopeptide release [4]. In patients with the Aa16Arg → His mutation, the A peptide peak is reduced by half, and there is an additional, earlier-eluting peak that represents the histidine-containing A peptide. Subsequent protein sequencing of the abnormal peptide is required to confirm this mutation. Although the method does provide a definitive result, the apparatus and technical expertise required are well beyond the scope of most clinical laboratories. With the method described here, the detection of this mutation, which in our experience accounts for 50% of all cases of dysfibrinogenemia, is straightforward, requiring only a simple PCR and restriction digest. The absolute identification of this mutation will enable the clinician to reassure the patient that their dysfibrinogenemia is unlikely to cause any bleeding disorder.

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References

Urine Pyridinium Cross-Links Determination by Beckman Cross Links Kit, Isabella Fermo, Cinzia Arcelloni, Erminia Casari, and Rita Paroni (Lab. of Chromatogr. Techniques, Dept. of Lab. Med., IRCCS H. San Raffaele, Milano, Italy; 1 Ist. Clin. Humanitas, Rozzano (MI), Italy; *address for correspondence: Lab. HPLC, H. San Raffaele, Via Olgettina 60, 20132 Milano, Italy; fax 39-2-26432640)

Increased understanding of bone turnover has led to the development of several biochemical tests of bone metabolism. Among the biochemical indexes of bone resorption is measurement of urinary excretion of pyridinoline (Pyr) and deoxypyridinoline (Dpyr), molecules that cross-link the collagen chains and are released into the systemic circulation after the breakdown of mature bone collagen [1–3]. Because they are not metabolized in vivo, they are excreted directly into urine in free (~40%) and peptide-bound forms (~60%) [4]. Methods to measure cross-links in urine involve mainly two technical approaches: HPLC analysis, which, after hydrolytic and purification steps, allows the determination of the total forms of cross-links [5–7], and monoclonal antibody immunoassay methods able to quantify the sum of free Pyr and Dpyr or only the free Dpyr form [8, 9]. The quantification of total or free cross-links forms provides, in any case, similar clinical information [9, 10].

Here we report the evaluation of the Cross Links™ HPLC kit recently introduced by Beckman Labs. to quantify the total forms of Pyr and Dpyr. We compared the performance of this procedure with the Chrom-Links™ HPLC method from Bio-Rad Labs. In addition, we investigated the determination of free Dpyr by the Cross Links method by comparing it with the Pyrilinks™-D immunoassay (Metra Biosystem) [11].

After the Beckman procedure 0.25 mL of urine was hydrolyzed with 0.25 mL of HCl (12 mol/L) (R1 reagent) at 115 °C overnight. After the addition of 0.5 mL of the internal calibrator solution and 2 mL of 1-butanol (R2 reagent), samples were loaded onto solid-phase columns and washed with 9 mL of R3 reagent (a mixture of butanol:water:acetic acid). After the addition of 0.5 mL of an organic solvent reagent (R4) to clean up the columns from the previous R3 reagent, cross-links were eluted with 0.5 mL of the extraction reagent (R5) containing heptfluorobutyric acid in water, and 50 μL was injected into the HPLC. Unlike the Bio-Rad assay, which employs gravimetric columns, purification of urine is carried out under vacuum by solid-phase extraction, with a notable time saving and minimal solvent exposure for the technical staff.

The HPLC equipment consisted of a Beckman System Gold Model 116 on line with an autosampler Model 507 and a Shimadzu Model RF 551 fluorometric detector with excitation at 295 nm and emission at 395 nm. The chromatographic separation lasted 10 min (Fig. 1) and was performed on a reversed-phase column (100 × 4.6 mm) in isocratic mode, at a flow rate of 1.0 mL/min. The eluting