To evaluate the influence of steroids, we performed a matched-pair analysis of 20 patients in groups A and B with respect to gender, age, weight, and GFR. Group A received ≥10 mg of prednisone per day [mean (SD), 14.8 (5.1) mg; n = 10], whereas group B received 5 mg of prednisone per day. Despite the small sample size, the matched-pair analysis showed significantly higher Cys C concentrations in group A [mean (95% CI), 2.86 (1.75–3.98) mg/L] than in group B [2.3 (1.48–3.14) mg/L; P = 0.049]. BTP concentrations were not affected by steroids [1.58 (0.93–2.22) mg/L vs 1.79 (1.21–2.38) mg/L; P = 0.1].

Our data show similar diagnostic performance for BTP, Cys C, and creatinine in RTx patients. In fact, when renal function decreased, BTP and Cys C detected a diminished GFR earlier and increased more prominently than creatinine.

We found only 5 published studies using ROC analyses for evaluation of the diagnostic performance of BTP. BTP was superior to creatinine in 2 investigations in children (8, 14). In adults, at cutoff points of 70 and 80 mL·min⁻¹·(1.73 m²)⁻², the diagnostic performance of BTP was similar to or slightly better than that of creatinine (9–11). However, none of these investigations showed an advantage of BTP over Cys C, which is in line with our results. In the cited studies, the range of GFR defined as normal was wide, giving decision points of 70–90 mL·min⁻¹·(1.73 m²)⁻². In fact, the mean GFR in our cohort was <40 mL·min⁻¹·(1.73 m²)⁻². Nonetheless, ROC analysis revealed similar AUCs for all tested analytes, irrespective of the cutoff value used.

In accordance with a recent study showing that BTP increased when GFR was <75 mL·min⁻¹·(1.73 m²)⁻² (11), we saw an earlier increase in BTP than creatinine. Because virtually all measured BTP (96.5%) and all Cys C (100%) concentrations were above the upper reference values, in contrast to 75% of creatinine results, we evaluated the impact of concomitant prednisone treatment. We found that steroid maintenance treatment (≥10 mg/day) was associated with higher Cys C concentrations, whereas we observed no association between steroid dose and BTP concentration.

On the basis of the above results, we believe that BTP may be a useful and reliable analyte to estimate GFR, particularly when RTx patients receive steroids that may falsly increase Cys C concentrations. Thus, despite similar diagnostic sensitivities and specificities, BTP may have a place alongside Cys C and creatinine as an alternative endogenous GFR marker in RTx patients.

Factor V Null Mutation Affecting the Roche LightCycler Factor V Leiden Assay, Mani S. Mahadevan* and Paul V. Benson†‡ († Department of Pathology, University of Virginia, Charlottesville, VA; ‡ Ohio State University, Columbus, OH; * address correspondence to this author at: Department of Pathology, University of Virginia, MR5 Building, Rm. 3330, 415 Lane Rd., PO Box 80904, Charlottesville, VA 22908-0904; fax 434-924-1545, e-mail mahadevan@virginia.edu)

Although the role of factor V as a coagulation factor is more familiar, it has an equally important alternative role as a cofactor for protein C. Activated protein C (APC) is important in a naturally occurring anticoagulant pathway in which it cleaves factor V, thereby controlling the concentrations of factor V. The factor V Leiden mutation (1), which has a frequency of ~1% in Caucasian populations and accounts for most cases of (APC) resistance, makes factor V resistant to cleavage by APC. Heterozygosity for the factor V Leiden mutation confers an increased lifelong relative risk for venous thrombosis, whereas homozygosity for the factor V Leiden mutation confers an even greater increased lifelong risk. Because of its high prevalence and association with thrombophilic disorders, a variety of assays have been developed to detect the G→A mutation at nucleotide 1691, codon 506, of the factor V gene, including assays based on use of the LightCycler™ (2, 3). In this report we present a case of an anomalous result obtained with the Roche LightCycler assay for factor V Leiden and discuss its implications.

References


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Recently, a 52-year-old female was diagnosed with deep venous thrombosis at our institution. As part of her assessment, she underwent a routine work-up for hypercoagulation. Her partial thromboplastin time and prothrombin time were normal before she was treated with anticoagulants. Measurements of protein C, protein S, and anti-thrombin III were deferred until she was finished with coumadin. Meanwhile, results from molecular diagnostics testing included a negative result for the G→A mutation at nucleotide 20210 in the prothrombin gene. However, an assay for the factor V Leiden mutation performed on the LightCycler showed an abnormal melting peak, distinct from the usual factor V Leiden mutation (Fig. 1A).

We used the Roche analyte-specific reagent for factor V Leiden (cat. no. 3028526). This assay is performed by real-time PCR followed by melting curve analysis with fluorescence resonance energy transfer (FRET) probes targeted at the factor V Leiden mutation sequence. According to the Roche package insert, the acceptable intervals for the melting temperatures for the PCR products are 62.5–67.5 °C for the wild-type factor V allele and 54.5–59.5 °C for the factor V Leiden allele. As part of our quality-control (QC) procedures, for the past 2 years we have been running DNA samples from patients confirmed to be heterozygous for the factor V Leiden mutation in all of our factor V assays, tracking the melting temperatures of the wild-type and factor V Leiden PCR products. The mean (SD) melting temperatures (T<sub>ms</sub>) for our control materials were 64.59 (0.50) °C and 56.52 (0.50) °C for the wild-type and factor V Leiden alleles, respectively. Thus, in our hands, these results show that the assay has a much narrower range of variability (CV <1.0%) than that suggested by Roche. The mean (SD) difference in melting temperature (ΔT<sub>ms</sub>; i.e., the T<sub>ms</sub> of the wild-type allele minus the T<sub>ms</sub> of the mutant allele), an adjunct QC measure, was 8.07 (0.17) °C with a CV of 2.1%. For 27 random patients identified as heterozygous for the factor V Leiden mutation, the mean (SD) T<sub>ms</sub> were 64.50 (0.53) °C and 56.36 (0.54) °C for the 2 alleles, with a mean (SD) ΔT<sub>ms</sub> of 8.14 (0.15) °C, in keeping with the results from our QC material.

The patient in this report was heterozygous for the wild-type allele and a second allele (Fig. 1A). The wild-type allele had a melting temperature of 65.7 °C. This patient’s second allele showed melting temperatures of 58.7 and 59.7 °C for 2 separate DNA extracts, significantly different from that for our positive QC sample. However, if we used the package insert temperature range guidelines of 54.5–59.5 °C, the former value (58.7 °C) was within the range for the factor V allele and the latter value (59.7 °C) was just slightly out of that range. In addition, the ΔT<sub>ms</sub> (6–7 °C) were also significantly less than those for our QC materials or our usual results from heterozygous patients with the factor V Leiden mutation. Thus, because of our quantitative QC monitoring of this assay and the fact that the patient’s results were significantly outside the established variability for our heterozygous control material, we considered that the results from the Roche LightCycler assay were consistent with an anomalous melting peak and did not correspond to a factor V Leiden mutation. This prompted us to consider another mutation lying within the region detected by the FRET probes.

At least 19 different mutations in the factor V gene have been described (4, 5). Most of these mutations have been described in factor V-deficient patients (4, 5). Of significance to this patient, a mutation causing a “false positive factor V Leiden” result has been reported previously and was obtained by a strategy combining PCR and restriction fragment length polymorphism analysis with MnlI and Hpyhl restriction enzyme digestion (6). Sequencing of the PCR product from that patient revealed the mutation to be a C→T transition at position 1690 of the factor V gene (6). Given this information, we used the software associated with the LightCycler to show that this mutation would give a T<sub>ms</sub> that corresponded to that seen in our patient’s specimen (i.e., ~59 °C). Direct sequencing of the PCR product from our patient’s specimen revealed that she was indeed heterozygous for the C→T mutation at position 1690 (Fig. 1B). This mutation causes the arginine
at position 506 to change to a stop codon, producing a null allele and probably causing a 50% decrease in the measured factor V activity in a heterozygote. Unfortunately, quantitative assays for factor V activity or protein were not available.

Obviously, the premature stop codon that we identified would have effects on the coagulation pathway vastly different from those of a factor V Leiden mutation, as well as significantly different clinical and therapeutic implications. The C→T change at position 1690 would produce a factor V deficiency, whereas the factor V Leiden mutation (G→A at position 1691) causes a thrombophilic predisposition. Because of the proximity of the 2 melting peaks seen in the Roche LightCycler factor V assay, the results have the potential to be misinterpreted as positive for factor V Leiden mutation if the C→T 1690 mutation is present instead of the G→A 1691 mutation. Accordingly, based on our results and DNA sequence confirmation, the patient’s thrombotic condition was considered not related to a factor V Leiden mutation.

This report highlights the importance of recognizing the difference between these 2 peaks so that a patient with a potentially hemorrhagic condition is not mistakenly diagnosed with a thrombophilic condition. Although recognition of the C→T mutation at position 1690 by other methods has been described previously, to our knowledge this is the first report of this mutation detected with the LightCycler methodology. Given the increasing popularity of such assays, providers need to be cognizant of this potentially false-positive result. Furthermore, it underscores the value of quantitative QC measures for allele-specific assays that use melting-temperature analyses. We emphasize the importance of establishing and maintaining QC material and carefully examining the resulting data.

We wish to thank Dr. D. Haverstick and the staff of the molecular diagnostics laboratory.

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We wish to thank Dr. D. Haverstick and the staff of the molecular diagnostics laboratory.

Gas Chromatographic–Mass Spectrometric Analysis for Measurement of p-Cresol and Its Conjugated Metabolites in Uremic and Normal Serum, Henriette de Loor,1,2 Bert Bammens,3 Pieter Eeneveld,1 Vicky De Preter,1 and Kristin Verbeke1,2 (1 Laboratory of Digestion and Absorption, 2 Laboratory of Nephrology, and 3 Department of Medicine, Division of Nephrology, University Hospital Gasthuisberg K.U. Leuven, Leuven, Belgium; * address correspondence to this author at: Laboratory of Digestion and Absorption, University Hospital Gasthuisberg, Herestraat 49, B-3000 Leuven, Belgium; fax 32-16-344399, e-mail Kristin.Verbeke@uz.kuleuven.ac.be)

p-Cresol (4-methylphenol; 108 Da) is a protein-bound solute retained within the body in renal failure (1). p-Cresol is of interest because it has several toxic effects in vitro (2–6) and clinical correlates have been demonstrated (7,8). In the absence of external exposure (9), p-cresol originates uniquely from bacterial tyrosine fermentation in the large intestine (10). During passage through the colonic mucosa and liver, it is detoxified by conjugation processes (sulfation and glucuronidation) (11–13). Thus, one might expect to find p-cresylsulfate and p-cresylglucuronide in the serum, but reports on conjugated p-cresol in renal failure patients are scarce (14–16). Most techniques to deproteinize serum samples (e.g., heat and acidification) may also partially hydrolyze sulfate esters and glucuronide bonds. Hence, the “total” (i.e., protein-bound and unbound) and “unbound” p-cresol reported in most studies probably reflect both unconjugated and (part of the) conjugated forms of the solute (17–21). We determined the extent of desulfation and deglucuronidation by deproteinization with heat and acid followed by gas chromatographic–mass spectrometric (GC-MS) analysis (19) with p-nitrophenylglucuronide and p-nitrophenylsulfate as model substrates. We also calculated exact amounts of unconjugated p-cresol, p-cresylsulfate, and p-cresylglucuronide in serum of hemodialysis patients and healthy controls.

Percentage desulfation and deglucuronidation by different methods (see below) was determined for random serum samples. Further analyses were performed on 9 serum pools from hemodialysis patients [n = 86; 49 male; mean (SD) age, 69.8 (1.5) years] and 5 serum pools from healthy controls [n = 29; 10 male; 31.0 (1.4) years; creatinine clearance, 87.1 (1.4) mL min−1 (1.73 m²)−1]. Serum pools were stored at −80°C until analysis. The ethics committee of the University Hospital Leuven approved the study, and informed consent was obtained from all participants. p-Nitrophenylglucuronide, p-nitrophenylsulfate, and 2,6-dimethylphenol (all >98% purity) were from Sigma-Aldrich; p-cresol and p-nitrophenol were from Supelco; and β-glucuronidase (Escherichia coli K12) was from Roche. Other materials were of analytical grade.

Several methods were used for sample preparation. Method A [acid and heat deproteinization (19)] consisted of dilution of 500 µL of serum with 350 µL of water; addition of 100 µL of p-nitrophenylglucuronide (1.2 g/L)