AC:180 results obtained for macroprolactinemic samples (range, −50% to 86%) indicates a highly variable, sample-dependent response of the ACS:180 assay to macroprolactin. The great disparity of values observed when comparing results from macroprolactinemic samples measured by the Elecsys or the Immulite assay with the results obtained by a low-reading method such as ACS:180 may reflect variation in the structure of macroprolactin. Macroprolactin is most probably not one unique molecule but rather a heterogeneous family of PRL-IgG complexes reacts differently depending on the type of immunoassay used for PRL determination.

In conclusion, our study reinforces the point that PRL assays from different manufacturers give highly variable prolactin results for samples containing macroprolactin (4–8). Our data additionally show that the reactivity of macroprolactin in a PRL immunoassay, be it a low-, medium-, or high-reading method, is not identical for all macroprolactinemic samples. This finding underscores the necessity of a systematic screening strategy for macroprolactin in all samples with increased PRL (4, 5, 10, 11). With the Elecsys PRL assay, PEG precipitation, with a cutoff of 50%, was an efficient and easy-to-use screening tool for the presence of macroprolactin. Because of the interference of PEG in some commercially available PRL assays, the confirmation of macroprolactinemia may require time-consuming methods, such as centrifugal ultrafiltration (9) and GFC.

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References

Multiplexed Mutagenically Separated PCR: Simultaneous Single-Tube Detection of the Factor V R506Q (G1691A), the Prothrombin G20210A, and the Methylene-tetrahydrofolate Reductase A223V (C677T) Variants, Georg Endler,1 Paul A. Kyrle,2 Sabine Eichinger,2 Markus Exner,2 and Christine Mannhalter1*(1) Department of Laboratory Medicine, Molecular Biology Division, and 2 University Clinic for Internal Medicine 1, Department of Hematology, AKH Wien, Währinger Gürtel 18-20, 1190 Wien, Austria; *author for correspondence: e-mail christine.mannhalter@univie.ac.at

Recently, mutations in several genes that encode for coagulation proteins, such as the factor V (FV):R506Q (G1691A) mutation or the prothrombin (FII):G20210A variant, have been identified as important risk factors for developing a venous thromboembolism (VTE) (1). These mutations contribute to the development of thrombosis in ~50% of all patients.

The FV:R506Q (G1691A) mutation currently is considered the most important genetic risk factor for venous thrombosis. Although 5% of the healthy population carry the mutant allele, the prevalence of this mutation in patients with venous thrombosis is ~40%. Compared with the wild type, heterozygous carriers of the mutation have an 8-fold higher risk and homozygotes have an 80- to 100-fold higher risk of developing a VTE (2). If and how this mutation contributes to arterial thrombosis is still under investigation, although recently Ardissovino et al. (3) showed correlations among the factor V mutation, smoking, and myocardial infarction. The FII:G20210A variant, which is associated with increased prothrombin activity and increased plasma prothrombin, has been identified as an independent risk factor for thrombosis (4). Compared with individuals with the wild-type genotype, heterozygous carriers of the mutation have a 2.8- to 5-fold higher risk of developing VTE. The influence of the FII:G20210A mutation on arterial thromboembolic disease is not clear, but there is evidence that it is associated with cerebrovascular ischemic disease (5) and myocardial infarction (6).

In addition to these two established thrombotic risk factors, the role of other genetic variations is still under investigation. The A223V (C677T) mutation in the thermolabile methylene-tetrahydrofolate reductase (MTHFR) gene is associated with mild hyperhomocysteinemia. Homozygosity for the mutation may be associated with an increased risk for cardiovascular events. The effect of the mutation on venous thrombosis is still controversial (7–10).

For diagnostic analyses and for scientific studies of large numbers of patients, fast and economic assays that can be performed with standard PCR instruments are highly desirable. We developed a mutagenically separated (MS) multiplex PCR for the simultaneous detection of mutations in the FV, FII, and MTHFR genes (11). Our MS-PCR is a single-tube PCR-based technique using allele-specific primers that differ in length by 8–10 bp. Base mismatches
in the allele-specific primers introduce deliberate differences into the allelic PCR products that minimize cross-reactions of the PCR products in subsequent cycles. The alleles are easily discernible by electrophoresis on high-resolution gels.

To evaluate the assay, we tested 70 known patients with recurrent venous thrombosis (40 males, 30 females; mean age, 55.5 ± 13.7 years; range, 28–84 years) and 83 selected healthy controls (30 males, 53 females; mean age, 54.4 ± 11.8 years; range, 25–83 years) for whom we had previously determined the genotypes for the FV:R506Q (G1691A), the FII:G20210A, and the MTHFR:A223V (C677T) variants by PCR followed by restriction enzyme digestion (12). Patients and controls were part of the Austrian Study of Recurrent Venous Thromboembolism (2, 3, 13). All participants in the study had given their informed consent, and the study was approved by the local ethics committee. Three patients and two controls were carriers of combined heterozygous FV:R506Q (G1691A) and FII:G20210A mutations.

DNA was extracted by established methods. Primer sequences and concentrations used in MS-PCR are given in Table 1. The primer sequences for FV:R506Q (G1691A) and MTHFR:A223V (C677T) have been published by Ulvik et al. (14). PCR products were generated in 50-μL volumes containing 1.5 U of AmpliTaq Gold (Perkin-Elmer Cetus), 1.5 mM MgCl₂, 200 μM each dNTP (Amer-sham Pharmacia Biotech), primers in the concentrations shown in Table 1 (MWG Biotech), and ~50 ng of DNA. Amplifications were performed in either a Perkin-Elmer 480 DNA Thermo Cycler (Perkin-Elmer Cetus) or a Biometra Thermocycler. The PCR conditions for the Perkin-Elmer 480 DNA Thermo Cycler were as follows: denaturation for 10 min at 95 °C; followed by 37 cycles at 95 °C for 1 min, 55 °C for 2 min, and 72 °C for 1 min; and a final extension step of 10 min at 72 °C. For the Biometra Thermocycler, the conditions were the same with a ramping temperature of 2 °C/s.

Gel electrophoretic analysis of the PCR products was carried out on Spreadex EL 400 S-26 N-Polyacrylamide minigels (Elchrom Scientific) at 160 V for 2 h. After staining with Sybr Green (1:10,000 dilution; Molecular Probes) for 20 min and destaining with doubly distilled water for 40 min, the bands were visualized on an ultraviolet transilluminator at 306 nm and photographed with a Polaroid camera. DNA of an individual heterozygous for all three mutations was included in each experiment as a positive control. If available, such a triply heterozygous DNA represents an ideal control sample because successful amplification from all investigated alleles can be assessed. However, such a specimen may not be on hand in every laboratory; it can be replaced by three individual samples that are heterozygous for the FV:R506Q (G1691A), the FII:G20210A, and the MTHFR:A223V (C677T) genotypes (15). A reduction of the individual time segments to 20 s produced very weak amplification signals. This impairs the interpretation of the results and cannot be recommended.

Thirty-one of 70 patients (44%) were heterozygous, and 3 were homozygous for the FV:R506Q (G1691A) mutation, and 8 of 70 (11%) patients carried the heterozygous factor II:G20210A mutation. One patient was homozygous for the FII:G20210A allele. This patient exhibited a wild-type genotype for the other two mutations. Among the 83 selected controls, 9 (11%) were heterozygous for FV:R506Q (G1691A), whereas the FII:G20210A mutation was found in 4 of 83 (5%) controls. The combined FV:R506Q (G1691A) and FII:G20210A variants could easily be identified by MS-PCR in the five carriers. The evaluation of combined gene defects is currently a matter of great interest, and economical methods for simple detection of combined gene defects are useful for this purpose.

Several methods for simultaneous analyses of the FV:R506Q (G1691A), the FII:G20210A, and the MTHFR:A223V (C677T) variants have already been described, using multiplex allele-specific PCR (16, 17), multiplexed heteroduplex analysis (18), or LightCycler amplification (19).

Multiplexed allele-specific PCR as described by Hazard

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### Table 1. Primer sequences and amounts used in the multiplex MS-PCR.

<table>
<thead>
<tr>
<th>Primer</th>
<th>pmol/50 μL</th>
<th>Sequence*</th>
<th>Amplicon length, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>FV:506Q (1691G) (antisense)</td>
<td>2</td>
<td>5'AA CA AAT ACC TGT ATT CĄT C-3'</td>
<td>233</td>
</tr>
<tr>
<td>FV:506Q (1691A) (antisense)</td>
<td>2</td>
<td>5'GTC TGT CTG TCT CTT CAA GGA CAA ATT ACC TGT ATT CIT T-3'</td>
<td>246</td>
</tr>
<tr>
<td>FV common primer (sense)</td>
<td>2.5</td>
<td>5'GGC AGG AAC ACC ATG AT-3'</td>
<td>207</td>
</tr>
<tr>
<td>MTHFR:223A (677C) (sense)</td>
<td>1.5</td>
<td>5'GTC TCT CTC TGA AGG AGA AGG TGT CTG CGG TAG C-3'</td>
<td>197</td>
</tr>
<tr>
<td>MTHFR:223V (677T) (sense)</td>
<td>1.5</td>
<td>5'GA AGG AGA AGG TGT CTG CCG AGA T-3'</td>
<td>180</td>
</tr>
<tr>
<td>FII:G20210G (antisense)</td>
<td>10</td>
<td>5'G C ACT GCG AGC ATT GCG C-3'</td>
<td>188</td>
</tr>
<tr>
<td>FII:G20210A (antisense)</td>
<td>40</td>
<td>5'ATG AAT AGT AAT GGA GCG ATT GAG GAT T-3'</td>
<td>197</td>
</tr>
<tr>
<td>FII common primer (sense)</td>
<td>40</td>
<td>5'ATG TGT TCC GCC TGA AGA GTG-3'</td>
<td>233</td>
</tr>
</tbody>
</table>

* Mismatches with wild-type sequences are underlined.
et al. (17) has been a reliable method for mutation detection. However, it requires almost twice the amount of reagents and considerably more working time than our multiplexed MS-PCR. With the multiplexed heteroduplex analysis described by Bowen et al. (18), interpretation of the results can sometimes be difficult because the heteroduplexes have no defined size. This is not the case in our MS-PCR, in which PCR products of known length are generated that can easily be identified and allocated to a genotype. The recently reported simultaneous amplification of all three genetic variants by real-time fluorescence PCR on the LightCycler (19) is fast and elegant, but it requires expensive equipment. Our MS-PCR is a stable and reproducible single-tube reaction. No special equipment and only a small amount of standard PCR reagents are needed. During the last 9 months, we have tested >1,500 samples with analyses performed by eight different individuals, with various master mixes, and with three different lots of primers. The method has also been tested successfully in three external quality-control trials (Hematology QAP; Royal College of Pathologists, Australasia; and Deutsche Gesellschaft für Klinische Chemie, personal communication). Nevertheless, as with any multiplex PCR, we recommend that users optimize the conditions, especially the primer concentrations for the MS-PCR, in each laboratory.

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References

18. Bowen D, Bowley S, John M, Colling PW. Factor V (R506Q), the prothrombin 3′-untranslated region variant (G20210A) and thermolabile methylenetetrahydrofolate reductase (C677T): a single test genotypes all three loci—determination of frequencies of the S. Wales population of the UK. Thromb Haemost 1998;79:949–54.