plasma sulfite, after the consumption of a glass of wine, to a concentration that may account for increased plasma antioxidant activity. We believe that these data justify further study of the role of sulfite as an antioxidant and antiatherogenic agent. Several important questions should be addressed, including whether there is any correlation between plasma sulfite concentrations and wine consumption or development of cardiovascular events in a large cohort.

References

Hideki Mitsuhashi
Hidekazu Ikeuchi
Yoshihisa Nojima*

Third Department of Internal Medicine
Gunma University
School of Medicine
Maebashi
Gunma, 371-8511 Japan

*Author for correspondence. E-mail ynojima@med.gunma-u.ac.jp.

Real-Time PCR Assay with Fluorescent Hybridization Probes for Rapid Interleukin-6 Promoter (−174G→C) Genotyping

To the Editor:
Interleukin-6 (IL-6) is a central protein in the regulation of the inflammatory and immunologic response (1). A G→C polymorphism at position −174 has been associated with osteoporosis (2), juvenile rheumatoid arthritis (3), and atherosclerosis (4) with increased risks in the absence of the CC genotype.

Restriction fragment length polymorphism analysis has been used for IL-6 genotyping, but it is time-consuming and requires multiple manual steps. To improve throughput, we developed a rapid-cycle PCR method on the LightCycler™ instrument (Roche Diagnostics) with fluorescent probe melting analysis. This assay is completed in 60 min.

DNA from 115 German Caucasians was extracted from whole blood according to standard procedures. Our study population consisted of 40 healthy blood donors and 75 patients from the intensive care unit of our hospital. The reliability of the proposed assay was confirmed by restriction enzyme digestion with SfaNI. PCR was performed in disposable capillaries (Roche Diagnostics) with a reaction volume of 20 µL containing 2 µL of DNA (20–80 ng), 0.5 µM each of the primers (sense, 5'-TTA CTC TTT GTT AAG ACA TGC CA-3'; anti-sense, 5'-ATG AGC CTC AGA CAT CTC CAG-3'), 2 µL of reaction buffer [LightCycler fast start DNA master hybridization probes 10× buffer (1× = 1.75 mM); Roche Diagnostics], 1 µL of MgCl2 (final concentration, 2.25 mM), and 0.2 µM each of the labeled probes. The anchor probe (5'-CTA AGC TGC ACT TTT CTC CCT CAG-3') was labeled at the 3' end with fluorescein. The sensor probe, which is specific for the G allele (5'-GTG TCT TGC GAT GCT AAA GGA-3'), was labeled with LightCycler Red 640 at the 5' end and modified at the 3' end by phosphorylation to block extension. The PCR conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 45 cycles of denaturation (95 °C for 10 s, 20 °C/s), annealing (53 °C for 10 s), and extension (72 °C for 10 s). The melting curve consisted of one cycle at 95 °C for 0 s and 45 °C for 90 s, and then increasing the temperature to 85 °C at a rate of 0.1 °C/s.

The fluorescence signal (F2) was monitored continuously during the temperature ramp and then plotted against the temperature (T). These curves were transformed to derivative melting curves (−d(F2)/dT vs T).

Representative results for the three different genotypes (GG, GC, and CC) are shown in Fig. 1. Of the 115 samples tested, 33% were GG, 46% were GC, and 21% were CC. In a larger study (383 Caucasians), the most frequent genotype was GC, followed by GG and CC (3). In a direct method comparison, our proposed new technique and the restriction enzyme technique with SfaNI gave identical genotyping results (data not shown). We conclude that the assay is rapid and accurate and seems especially suited for laboratories that process large numbers of samples.

We thank Andreas Nitsche and Olbert Landt (TIB MOLBIOL, Berlin, Germany) for designing the hybridization probes and reading the manuscript.

References
2. Ferrari SL, Garniero P, Emond S, Montgomery H, Humphries SE, Greenspan SL. A functional poly-
Recentl, a novel form of human prion disease (nvCJD) that is thought to be linked with bovine spongiform encephalopathy has been described. Almost all cases of nvCJD with available genetic analysis were methionine homoygotes at codon 129 of the PRNP gene and died at an early age (mean, 29 years). This finding suggests that Met/Met homozygosity at codon 129 of the PRNP gene is a risk factor for nvCJD.

The majority of available methods for the study of single-nucleotide polymorphisms and point mutations are time-consuming and require multiple manual steps. To overcome these problems, new and single-tube genotyping methods are being proposed. We describe a rapid and accurate method for the genotyping of the PRNP codon 129 polymorphism based on real-time PCR mutation detection by melting-point analysis with fluorescent hybridization probes with fluorescence resonance energy transfer (3, 4).

DNA was extracted from peripheral blood by a phenol-chloroform procedure. The primers and hybridization probes were designed and synthesized by TIB MOLBIOL (Berlin, Germany). The primers used for the amplification were as follows: PRNP Forward (5′-CCAAAAAC-CAACATGAAGCAC-3′) and PRNP Reverse (5′-TGTTGTTGTTGTACCGTGT-3′). The sequence of the sensor 3′-fluorescein-labeled probe was 5′-TTCCCAGCACGTAGCCGCC-3′, and that of the anchor 5′-LC-Red 640-labeled probe was 5′-AGGCCCACACCTGCCCCA-3′.

LightCycler (Roche, Mannheim, Germany) amplification was performed in a final volume of 15 µL containing 1.5 µL of DNA solution of a concentration ~100 ng/µL, 0.75 µL of each primer (10 µmol/L), 0.3 µL of each probe (4 µmol/L), 1.8 µL of MgCl₂ (25 mmol/L), 8.1 µL of distilled water, and 1.5 µL of the DNA Master hybridization probes (Roche). A negative control without DNA was included in all assays. Reaction mixtures were loaded into glass capillaries (Roche), centrifuged, and placed in the LightCycler carousel. The reaction mixture was denatured at 95 °C for 20 s, followed by 35 cycles of denaturation at 95 °C for 0 s, annealing at 55 °C for 5 s, and extension at 72 °C for 10 s. After amplification, the melting was performed by denaturation at 95 °C for 5 s, annealing at 55 °C for 10 s, and increasing the temperature to 90 °C with a ramp rate of 0.2 °C/s. The fluorescence emitted was measured during this process, and the melting curves were generated by plotting fluorescence (F) vs temperature (T).