Letters to the Editor

analysis” of results of studies reporting both a difference in risk and a difference in homocysteine concentration between TT and CC individuals overcomes the problem.

Fig. 1 shows the IHD relative risk between TT and CC individuals for each of the 26 studies reporting homocysteine differences (TT minus CC) among control individuals (no history or angiographic evidence of IHD). Studies are ranked according to homocysteine differences into tertile groups specified before the analysis. IHD risk increases across tertile groups: an odds ratio of 0.86 (95% CI, 0.70–1.06) in the lowest group (no homocysteine difference between TT and CC individuals) and 1.52 (95% CI, 1.09–2.08) in the highest tertile (homocysteine, 3.8 μmol/L higher in TT individuals than in CC individuals). The trend is significant (odds ratio, 1.1 per 1-μmol/L increase in the homocysteine concentration; \( P = 0.017 \)) and is due mainly to the highest group (homocysteine differences ≥3 μmol/L, median 9 μmol/L in CC individuals). This result suggests a possible threshold effect. Therefore, folic acid would be expected to have a greater effect in preventing IHD in populations with low folate intake than in those with higher folate intake.

Publication bias is unlikely to explain the dose–response analysis, because to do so would require (a) a failure to publish studies with negative results involving large homocysteine differences and (b) positive results for studies with small homocysteine differences. Furthermore, both of these requirements would have to be satisfied in small studies but not in large studies.

A metaanalysis of randomized trials of homocysteine lowering with folic acid showed no reduction in IHD events (4), possibly because most patients in the trials took aspirin, which might negate the antiplatelet effect of lowering the homocysteine concentration (4). On this basis, folic acid would have a role in the primary prevention of IHD, when aspirin is not taken routinely, but not in secondary prevention, when it is.

Our analysis of all 107 MTHFR studies, together with the dose–response analysis of 26 studies also reporting homocysteine concentrations, suggests a causal effect of homocysteine on IHD. The result is not reasonably explained by publication bias, but chance cannot be confidently excluded. A role for homocysteine in causing IHD therefore remains open, as does a role for folic acid in preventing IHD. It would be a mistake, on the present evidence, to exclude homocysteine as a cause of IHD.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

Authors’ Disclosures or Potential Conflicts of Interest: Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:

Employment or Leadership: N.J. Wald, Polypill Limited; D.S. Wald, Polypill Limited.

Consultant or Advisory Role: None declared.

Stock Ownership: N.J. Wald, Polypill Limited; D.S. Wald, Polypill Limited.

Honoraria: None declared.

Research Funding: None declared.

Expert Testimony: None declared.

Patents: N.J. Wald, EU1272220.

References


David S. Wald*
Jonathan P. Bestwick
Nicholas J. Wald

Wolfson Institute of Preventive Medicine
Barts and the London School of Medicine and Dentistry
Queen Mary University London
London, UK

*Address correspondence to this author at: Wolfson Institute of Preventive Medicine
Barts and the London School of Medicine and Dentistry
Queen Mary University London
Charterhouse Square
London EC1M 6BQ, UK
E-mail d.s.wald@qmul.ac.uk

Previously published online at DOI: 10.1373/clinchem.2012.191791

Genotyping Efficiency of 2 Primer Sets and an Unlabeled Oligonucleotide Probe for the p.Phe508del in Exon 10 of the CFTR Gene as Determined with High-Resolution Melting Analysis

To the Editor:

Cystic fibrosis (OMIM 219700) is one of the most common autosomal recessive disorders in Caucasians. It is caused by mutations in the CFTR\(^1\) [cystic fibrosis transmembrane conductance regulator (ATP-binding cassette sub-family C, member 7)] gene, located on

\(^1\) Human genes: CFTR, cystic fibrosis transmembrane conductance regulator (ATP-binding cassette sub-family C, member 7).
chromosome 7q31.2. The frequency of the most common mutation, p.Phe508del (F508del, c.1521_1523delCTT), varies markedly among ethnic groups, but is always tested in both patient testing and carrier screening (1, 2).

At our institution, we screen for CFTR mutations with high-resolution melting analysis (LightScanner®; Idaho Technology). The method consists of PCR amplification of the fragment in question in the presence of a fluorescent dye (LCGreen Plus) that intercalates between bases in double-stranded DNA. The amplification products are melted after the PCR to reveal unique melting profiles according to their sequence compositions (3).

A successful experiment depends on primer design (gene scanning) and probe design (genotyping), as well as on DNA quantity and quality. Because gene scanning depends on melting curve differences created by mismatched bases, it is not always feasible to detect homozygous mutations when a gene-scanning protocol is used. An unlabeled probe is often used to achieve accurate genotyping. Because p.Phe508del is the most commonly occurring mutation, one would expect this mutation to be homozygous in a substantial number of patients and therefore be detected more accurately than with an unlabeled probe. Additionally, p.Phe508del is usually associated with different nonpathogenic polymorphisms in the same exon, which will alter the melting profile. For these reasons, use of the probe is essential to distinguish between pathogenic and benign genotypes.

We tested the set of primers for the CFTR gene described by Montgomery et al. (4), who reported 2 different primer sets for scanning exon 10. One set yielded a 277-bp product, and the other, exon 10 – ΔF508 locus, produced a 201-bp product. The authors suggested the use of a primer pair in an asymmetric PCR reaction to detect the p.Phe508del mutation [exon 10 – ΔF508 locus (201-bp ampli-
con) forward primer, 5’-ACCTTTCTGCTTGAGTTATATGGG-3’; exon 10 – ΔF508 locus reverse primer, 5’-ACATAGTTTCTTACCTCTTCG-3’ (underlined bases indicate sites of known polymorphisms)] in combination with the unlabeled probe (5’-TAAAGAAAATCATCTTTGAGTTATATGGG-3’).

As we evaluated this primer–probe set, we identified 2 major drawbacks that made it unsuitable for genotyping the p.Phe508del mutation. The forward primer is designed to include the p.Met470Val (c.1504A>G) polymorphism and is complementary to the Val allele. The A allele (coding for Met) is in cis with p.Phe508del in the majority of genotypes analyzed, whereas
tributions to the conception and design, actual content of this paper and have met the confirmed they have contributed to the intellectual content and sensitivity, regardless of the polymorphisms in cis with the wild-type sequence. This condition leads to preferential amplification of the wild-type allele. In addition, the forward primer includes an extra polymorphism, p.Leu470Val and p.Leu467Phe with this primer–probe set, we observed a failure to identify the p.Phe508del mutation, because the forward primer has a design that leads to amplification of the wild-type allele only.

We also tested the primer set for exon 10 in combination with the same probe, as described by Montgomery et al., which gives a 277-bp PCR product (exon 10 – 277 bp, forward primer, 5'-TTGA TAATGACCTAATAATGATGGG TT-3'; reverse primer, 5'-GTGAAGGGTTCATATGCATAATCACA- 3'). The results showed that all samples were correctly genotyped, regardless of the polymorphisms in cis with the mutation.

To follow up on these findings, we used the 2 different primer sets to test samples with various exon 10 genotypes. The true exon 10 genotypes (as assigned by sequencing) and the genotyping results (as assigned by each genotyping assay) are presented in Table 1. We conclude that the primer pair that produces a 201-bp amplicon should not be used in genotyping assays and should be replaced by the primer pair that yields the 277-bp product, which shows high specificity and sensitivity, regardless of the other sequence variants present in cis to the p.Phe508del allele.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

Authors' Disclosures or Potential Conflicts of Interest: No authors declared any potential conflicts of interest.

References

Myrto Poulou2*, Aspasia Destouni2,3 Irini Fylaktou2 Emmanuel Kanavakis2,3 Maria Tzetis2

2 Department of Medical Genetics and 3 Research Institute for the Study of Genetic and Malignant Disorders in Childhood
Athens University
St. Sophia’s Children’s Hospital
Athens, Greece

*Address correspondence to this author at:
Department of Medical Genetics
Athens University
St. Sophia’s Children’s Hospital
Choremio Research Laboratory
Thivon and Levadias Str.
Athens 11527, Greece
Fax +30210-779-5553
E-mail mrpoulou@med.uoa.gr

Previously published online at DOI: 10.1373/clinchem.2012.189696

Cardiac Biomarker Responses to Dobutamine Stress Echocardiography in Healthy Volunteers and Patients with Coronary Artery Disease

To the Editor:

Cardiac stress testing provides important diagnostic and prognostic information in ischemic heart disease (1). The sensitivity and specificity of stress testing are limited, however (2). If a circulating biomarker could reliably reflect the short-lived ischemia that occurs with stress testing and if differences in marker-release patterns could be associated with grades of risk for negative outcomes, one would expect the sensitivity, specificity, and overall utility of stress testing to improve substantially. High-sensitivity cardiac troponin assays have potential to be earlier markers of myocardial ischemia (3, 4). B-type natriuretic peptide signal peptide (BNPsp) is a recently discovered circulating biomarker (5). In the setting of ST-segment elevation myocardial infarction, BNPsp concentrations increase early, preceding myoglobin peaks. BNPsp has not been reported to date in less extreme forms of cardiac ischemia.

We document the release of BNPsp, N-terminal pro–B-type natriuretic peptide (NT-proBNP), and troponin T as measured with a high-sensitivity assay (hsTnT) during dobutamine stress echocardiography (DSE) in patients with coronary artery disease (CAD) and in healthy volunteers. Blood samples were collected from 16 CAD patients and 10 healthy volunteers.