Novel Risk Factors for Systemic Atherosclerosis
A Comparison of C-Reactive Protein, Fibrinogen, Homocysteine, Lipoprotein(a), and Standard Cholesterol Screening as Predictors of Peripheral Arterial Disease

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Several novel risk factors for atherosclerosis have recently been proposed, but few comparative data exist to guide clinical use of these emerging biomarkers.

Objective To compare the predictive value of 11 lipid and nonlipid biomarkers as risk factors for development of symptomatic peripheral arterial disease (PAD).

Design, Setting, and Participants Nested case-control study using plasma samples collected at baseline from a prospective cohort of 14,916 initially healthy US male physicians aged 40 to 84 years, of whom 140 subsequently developed symptomatic PAD (cases); 140 age- and smoking status–matched men who remained free of vascular disease during an average 9-year follow-up period were randomly selected as controls.

Main Outcome Measure Incident PAD, as determined by baseline total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), total cholesterol–HDL-C ratio, triglycerides, homocysteine, C-reactive protein (CRP), lipoprotein(a), fibrinogen, and apolipoproteins (apo) A-I and B-100.

Results In univariate analyses, plasma levels of total cholesterol (P < .001), triglycerides (P < .01), apo B-100 (P < .001), fibrinogen (P = .02), CRP (P = .006), and the total cholesterol–HDL-C ratio (P < .001) were all significantly higher at baseline among men who subsequently developed PAD compared with those who did not, while levels of HDL-C (P = .009) and apo A-I (P = .05) were lower. Nonsignificant baseline elevations of lipoprotein(a) (P = .40) and homocysteine (P = .90) were observed. In multivariable analyses, the total cholesterol–HDL-C ratio was the strongest lipid predictor of risk (relative risk [RR] for those in the highest vs lowest quartile, 3.9; 95% confidence interval [CI], 1.7–8.6), while CRP was the strongest nonlipid predictor (RR for the highest vs lowest quartile, 2.8; 95% CI, 1.3–5.9). In assessing joint effects, addition of CRP to standard lipid screening significantly improved risk prediction models based on lipid screening alone (P < .001).

Conclusions Of 11 atherothrombotic biomarkers assessed at baseline, the total cholesterol–HDL-C ratio and CRP were the strongest independent predictors of development of peripheral arterial disease. C-reactive protein provided additive prognostic information over standard lipid measures.

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directly compare the magnitude of predictive value associated with a large series of lipid and nonlipid risk factors in a prospective cohort of apparently healthy men. We elected to use as our clinical end point the future development of symptomatic peripheral arterial disease (PAD), a disorder for which few data exist regarding the potential usefulness of novel risk factors for atherosclerosis.

**METHODS**

**Study Population**

The study population consisted of apparently healthy middle-aged men participating in the Physicians' Health Study, a prospective, randomized trial of aspirin and beta-carotene in the primary prevention of cardiovascular disease and cancer. In brief, 14,916 men aged 40 to 84 years who had no prior history of cardiovascular disease or cancer provided baseline plasma samples that were collected in EDTA and then frozen at –80°C until the time of analysis. Participants were monitored over an average follow-up period of 9 years for the occurrence of incident health events, including the development of intermittent claudication and hospitalizations for peripheral arterial revascularization procedures. For the purposes of this analysis, case subjects were defined as those apparently healthy men who subsequently developed either of these PAD end points during the study period; there were 140 such subjects. By contrast, 140 control subjects were selected at random from the remaining study subjects who remained free of reported cardiovascular disease. Controls were matched to cases on the basis of age, smoking status, and length of follow-up. No study participant had a baseline history of intermittent claudication or prior lower extremity revascularization procedures. Because the study design focused on symptomatic lower extremity PAD, participants who underwent revascularization of either the renal or carotid arteries were not considered as case subjects.

**Measurement of Risk Factors**

Baseline plasma samples from case and control subjects were thawed and assayed for each plasma parameter using commercially available analytic systems. Total cholesterol (TC), high-density lipoprotein (HDL) cholesterol, direct low-density lipoprotein (LDL) cholesterol, and triglycerides were assayed on a Hitachi 911 analyzer (Roche Diagnostics, Indianapolis, Ind) using reagents from Roche Diagnostics and Genzyme Corporation (Cambridge, Mass). Total plasma homocysteine was measured with an IMx homocysteine assay on an IMx analyzer (Abbott Laboratories, Abbott Park, Ill). High-sensitivity CRP and lipoprotein(a) were assayed using a latex-enhanced immunonephelometric assay on a BN II analyzer (Dade Behring, Newark, Del), while fibrinogen, apo A-I, and apo B-100 were simultaneously measured on this device by immunoassay.

**Statistical Analysis**

To ascertain the potential clinical usefulness of each putative risk marker, we followed an a priori analysis plan in which the assignment for each parameter was first compared between case and control groups using the nonparametric Wilcoxon rank sum test. After dividing subjects into quartiles based on the distribution of control values, we then used conditional logistic regression analyses to compute relative risks (RRs) of future PAD associated with increasing levels of each study parameter. By so doing, the magnitude of risk associated with levels in the highest vs the lowest quartile could be directly compared for each of the 11 parameters. Multivariable estimates of risk were computed in similar models which, in addition to the matching variables of age and smoking, adjusted further for hypertension, body mass index, family history of premature atherosclerosis, diabetes, and exercise frequency. Finally, we addressed the potential additive value of each novel parameter to standard lipid screening in a 2-step process. Initially, for any marker found to be positively associated with risk of PAD in univariate analyses, we used likelihood ratio testing to determine whether the addition of that marker to standard cholesterol screening significantly improved risk prediction models. Similar logistic regression analyses were then performed after dividing study subjects into 4 groups based on median levels for standard lipid markers and on median levels for each novel risk parameter. All P values are 2-tailed and all confidence intervals (CIs) computed at the 95% level (SAS Version 8.2, SAS, Cary, NC).

**RESULTS**

Due to matching, mean age and smoking patterns were virtually identical in the case and control groups. As expected, traditional atherosclerotic risk factors such as diabetes, hypertension, and a family history of cardiovascular disease were more prevalent at baseline among those who subsequently developed symptomatic PAD (cases) compared with those who did not (controls) (TABLE 1).

The mean time to diagnosis of incident PAD for the study population was 60 months (range, 3-155 months). As shown in TABLE 2, median levels of TC, LDL cholesterol, triglycerides, apo B-100, and the TC/HDL-C ratio were all significantly higher at baseline among case subjects. In contrast, HDL-C levels were significantly lower among those who subsequently developed PAD while levels of apo A-I were marginally reduced and no significant difference was observed for lipoprotein(a). Median levels of the 2 inflammatory markers, CRP and fibrinogen, were significantly higher at baseline among case subjects compared with controls. No significant difference was observed for total plasma homocysteine.

Age- and smoking-matched RRs of developing PAD for increasing quartiles of each of the measured parameters are presented in TABLE 3. Among the lipid parameters, TC, HDL-C, LDL-C, triglycerides, and apo B-100 were all significant predictors of risk, although the TC/HDL-C ratio was the single strongest predictor (highest quartile vs lowest quartile: RR, 3.4; 95% CI, 1.7-7.0). A modest but nonsignificant trend was observed for apo A-I, while there was no discernable risk gradient in these data for lipoprotein(a).
Among the nonlipid parameters, CRP was the single strongest predictor (highest quartile vs lowest quartile: RR, 2.5; 95% CI, 1.3-5.0) (Table 3). Also, increasing quartiles of fibrinogen were a significant predictor of risk of developing PAD. There was no discernable risk gradient for homocysteine.

None of these associations were materially altered in analyses which, in addition to the matching variables of age and smoking status, additionally controlled for hypertension, body mass index, family history of premature atherosclerosis, exercise frequency, and diabetes (Table 4). In these adjusted analyses, the strongest lipid predictor remained the TC/HDL-C ratio (highest vs lowest quartile: RR, 3.9; 95% CI, 1.7-8.6) while the strongest nonlipid predictor remained CRP (highest vs lowest quartile: RR, 2.8; 95% CI, 1.3-5.9).

Similar findings, although with wider CIs, were observed in analyses limited to participants who underwent peripheral arterial revascularization procedures in addition to developing intermittent claudication. For example, in this subgroup of 30 participants, the RR of incident PAD for those with baseline TC/HDL-C ratios in the highest vs the lowest quartile was 3.2 (95% CI, 1.0-10.9) while that for CRP was 2.1 (95% CI, 0.8-5.6). In these subgroup analyses, as in the study as a whole, statistically significant trends in risk were observed across quartiles of TC (P = .01), CRP (P = .02), apo B-100 (P = .02), LDL-C (P = .02), and triglycerides (P = .03).

To explore whether any of the novel risk parameters added to the predictive value of standard lipid-based screening, a series of additional analyses were undertaken. First, likelihood ratio tests were used to compare the fit of risk prediction models based on the measurement of each novel marker in combination with standard lipid screening. Second, we computed the RR of developing PAD in analyses that stratified study participants into 4 groups based on median lipid levels and on median levels for each of the other measured parameters (Figure). To address the robustness of these findings, analyses were performed separately using either TC or the TC/HDL-C ratio as the method for standard lipid screening.

As might be expected due to intercorrelation among many of the lipid parameters, there was little evidence of benefit of adding LDL-C, apo A-I, or apo B-100 to analyses that already incorporated the TC/HDL-C ratio. Similarly, there was minimal evidence of benefit of adding either lipoprotein(a) or homocysteine to standard lipid screening with either TC or the TC/HDL-C ratio.

In contrast, both of the inflammatory markers (CRP and fibrinogen) significantly improved the predictive value of models based on either TC (P < .001 for CRP; P = .01 for fibrinogen) or the TC/HDL-C ratio (P < .001 for CRP; P = .04 for fibrinogen). These effects were stronger for CRP than for fibrino-
Table 3. Relative Risk of Developing Future Peripheral Arterial Disease According to Baseline Levels of Lipid and Nonlipid Risk Factors

<table>
<thead>
<tr>
<th>Variable</th>
<th>Relative Risk (95% CI) by Quartile</th>
<th>P Value†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>1.0</td>
<td>1.3 (0.6-2.8)</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>1.0</td>
<td>0.9 (0.4-2.0)</td>
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<tr>
<td>HDL cholesterol</td>
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<td>0.6 (0.3-1.1)</td>
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<tr>
<td>Triglycerides</td>
<td>1.0</td>
<td>1.3 (0.6-2.7)</td>
</tr>
<tr>
<td>TC/HDL-C ratio</td>
<td>1.0</td>
<td>1.4 (0.7-3.1)</td>
</tr>
<tr>
<td>Apolipoprotein A-I</td>
<td>1.0</td>
<td>0.8 (0.4-1.4)</td>
</tr>
<tr>
<td>Apolipoprotein B-100</td>
<td>1.0</td>
<td>1.0 (0.4-2.1)</td>
</tr>
<tr>
<td>Lipoprotein(a)</td>
<td>1.0</td>
<td>0.7 (0.4-1.4)</td>
</tr>
<tr>
<td>Homocysteine</td>
<td>1.0</td>
<td>1.0 (0.5-1.9)</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>1.0</td>
<td>1.2 (0.6-2.6)</td>
</tr>
<tr>
<td>C-reactive protein</td>
<td>1.0</td>
<td>1.6 (0.6-2.7)</td>
</tr>
</tbody>
</table>

*All models conditioned on age and smoking status. CI indicates confidence interval; LDL, low-density lipoprotein; HDL, high-density lipoprotein; and TC, total cholesterol. (All P values are tests for trend across quartiles.)

Table 4. Adjusted Relative Risk of Developing Future Arterial Disease According to Baseline Levels of Lipid and Nonlipid Risk Factors

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<tr>
<td>Triglycerides</td>
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<tr>
<td>TC/HDL-C ratio</td>
<td>1.0</td>
<td>1.6 (0.7-3.5)</td>
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<tr>
<td>Apolipoprotein A-I</td>
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<td>0.7 (0.4-1.4)</td>
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<tr>
<td>Apolipoprotein B-100</td>
<td>1.0</td>
<td>0.9 (0.4-2.1)</td>
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<tr>
<td>Lipoprotein(a)</td>
<td>1.0</td>
<td>0.7 (0.4-1.5)</td>
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*All models conditioned on age and smoking, and additionally adjusted for diabetes, hypertension, family history of premature atherosclerosis, exercise frequency, and body mass index. See Table 3 footnote for expansion of terms. (All P values are tests for trend across quartiles.)

In a post hoc analysis evaluating the addition of both CRP and fibrinogen to standard lipid screening, there was little evidence of any further improvement in risk prediction models, most likely because levels of CRP and fibrinogen were also intercorrelated (r=0.6; P=.001).

COMMENT

In this prospective evaluation of 11 plasma biomarkers associated with the development of PAD, the TC/HDL-C ratio proved to be the single strongest lipid predictor of risk. Indeed, once this ratio was taken into account we found little evidence that additional screening for other lipid parameters including LDL-C, apo A-I, apo B-100, or lipoprotein(a) had significant clinical usefulness. However, the addition of either CRP or fibrinogen to standard lipid screening significantly improved the predictive value of computed risk prediction models. Of these 2 intercorrelated inflammatory variables, CRP was the stronger univariate predictor of risk and had the greater additive value when combined with either TC or the TC/HDL-C ratio, although the magnitude of these latter differences was small. Finally, we found little efficacy for homocysteine evaluation in these data, either alone or in combination with standard lipid screening. The current data for CRP were generated with a commercially available high-sensitivity assay and thus corroborate prior results from these study participants in which CRP was measured using an in-house research-based assay. Our observation that CRP testing combined with standard lipid screening appears to provide an improved method of detecting subclinical atherosclerosis also confirms prior work for myocardial infarction in men and for coronary heart disease and stroke in women.

To our knowledge, these data are the first to directly address the magnitude of predictive value of novel risk factors associated with the development of symptomatic PAD. We believe the strongly positive findings in this study—particularly those for the TC/HDL-C ratio, CRP, and fibrinogen—merit careful consideration among clinicians interested in programs of general population-based screening to improve the detection of subclinical atherosclerosis. However, we also believe that our null data for homocysteine and lipoprotein(a) should not be construed as suggesting no role for these latter markers in the detection of vascular risk. Recent overview analyses for both homocysteine and lipoprotein(a) have reported statistically significant relationships, albeit with a magnitude of effect smaller than that observed for the inflammatory markers CRP and fibrinogen. Thus, despite the null data presented here, clinical evaluation for homocysteine and lipoprotein(a) might nonetheless be considered in the setting of markedly premature atherosclerosis or when there is a strong family history of atherothrombosis in the absence of other major risk factors. Homocysteine evaluation is also likely to have greater yield among patients with renal failure. On the other hand, our null data for homocysteine and lipoprotein(a) argue against population-based screening for these parameters, and as such are in accordance with recent recommendations from the American College of Cardiology and the American Heart Association.

The use of self-reported symptomatic PAD as our primary a priori endpoint represents a potential limitation of these data. However, we believe this...
Figure. Relative Risk of Developing Peripheral Arterial Disease Among Apparently Healthy Middle-Aged Men

A. Stratification by baseline levels of total cholesterol and C-reactive protein. Referent, below study median levels for both risk parameters. B. Stratification by baseline levels of total cholesterol–high-density lipoprotein cholesterol ratio and C-reactive protein. Referent, below study median levels for both risk parameters. Relative risks and 95% confidence intervals (CIs) were derived from logistic regression models.

clinically relevant end point is valid for several reasons. First, our study participants were physicians, a group in whom validation rates for several other self-reported vascular and nonvascular end points have consistently been excellent. In particular, among Physicians’ Health Study participants, self-reported incident angina pectoris has been validated by either exercise stress testing or positive angiography in more than 98% of cases. Second, any potential misclassification on this basis would tend, if anything, to bias these data toward the null, not a false-positive finding. Finally, in the subgroup of study participants who not only self-reported incident intermittent claudication but who also underwent lower extremity revascularization, the point estimates of effect were minimally altered. Thus, we believe the use of self-reported symptomatic disease in our study is not only valid, but represents a true clinical end point rather than a surrogate marker for disease.

The current study was specifically designed to assess the magnitude of predictive value of several inexpensive plasma biomarkers for systemic atherosclerosis as well as their potential usefulness as adjuncts to standard lipid screening. It is important to note, however, that bedside techniques that assess peripheral blood flow also have clinical usefulness in the detection of PAD. In particular, simple physical examination of the distal pulses as well as assessment of the ankle-brachial index are useful for the detection of clinically relevant peripheral arterial obstruction and for the prediction of subsequent vascular events. Other modalities such as magnetic resonance imaging or electron beam computed tomography have also been advocated as tools for the detection of subclinical atherosclerosis. However, in marked contrast to physical examination or the measurement of a small panel of plasma-based biomarkers, these direct imaging techniques are quite expensive, a major concern for primary prevention screening programs. Thus, carefully designed studies comparing plasma biomarkers with direct imaging techniques will be needed to assess the clinical efficacy and cost-effectiveness of these different approaches.

Author Contributions: Study concept and design, acquisition of data, and analysis and interpretation of data: Ridker, Stampfer, Rifai. Drafting of the manuscript, obtained funding: Ridker. Critical revision of the manuscript for important intellectual content: Stampfer, Rifai. Statistical expertise: Ridker, Stampfer.

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REFERENCES

Administrative, technical, or material support: Ridker, Rifai.