Multiple Infections and Subsequent Cardiovascular Events in the Heart Outcomes Prevention Evaluation (HOPE) Study

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Background—Limited prospective epidemiological data are available on the relation between exposure to Chlamydia pneumoniae, Helicobacter pylori, cytomegalovirus (CMV), and hepatitis A virus (HAV), individually or as a total pathogen score, and human cardiovascular (CV) disease.

Methods and Results—We analyzed enrollment sera from 3168 Canadian patients in the Heart Outcomes Prevention Evaluation (HOPE) study for antibodies to C pneumoniae, H pylori, CMV, and HAV and measured the relation between serostatus and 494 adjudicated trial outcomes of myocardial infarction, stroke, or CV death over 4.5 years of follow-up. CV events were associated with CMV serostatus (covariate-adjusted hazard ratio [HR], 1.24; 95% CI, 1.01, 1.53). Neither C pneumoniae IgG (adjusted HR, 0.87; 95% CI, 0.68, 1.10), C pneumonia IgA (adjusted HR, 1.10; 95% CI, 0.90, 1.34), H pylori IgG (HR, 0.99; 95% CI, 0.82, 1.19), nor HAV IgG (HR, 1.01; 95% CI, 0.83, 1.24) predicted CV events. Total pathogen score was associated with CV events (adjusted HR for 4 versus 1 or 0 = 1.41; 95% CI, 1.02, 1.96).

Conclusions—Exposure to CMV but not to C pneumoniae, H pylori, or HAV was associated with a slight excess risk of subsequent myocardial infarction, stroke, or CV death in HOPE study patients, and total pathogen score based on these infections predicted a small increased hazard of CV events. (Circulation. 2003;107:251-257.)

Key Words: infection ■ cardiovascular diseases ■ prognosis

A possible role for infections in atherosclerosis has been intensely scrutinized since the demonstration of herpesvirus-induced atherosclerosis in chickens in 1978.1 Human atherosclerotic heart or cerebrovascular disease has been associated with previous exposure to the bacteria Chlamydia pneumoniae,2,3 Helicobacter pylori,4 or Porphyromonas gingivalis5,6 and with the viruses cytomegalovirus (CMV),7 herpes simplex virus types 1 and 2 (HSV-1, HSV-2),8,9 enteroviruses,10 or hepatitis A virus (HAV),9,11 but prospective studies remain limited.12–14 Large randomized trials of antibiotics for secondary prevention of cardiovascular (CV) disease are ongoing.15,16 Recently, Zhu and colleagues proposed and demonstrated that the aggregate sum of infectious exposures, expressed as a total pathogen burden, was a stronger prognostic marker than individual infections in cross-sectional17 and prospective studies.9 Rupprecht and colleagues18 also found a prospective relation between pathogen burden and CV outcome.

Our primary study objectives were to determine whether exposure to C pneumoniae and to 3 other infections, individually or as a total pathogen score, were a prospective risk marker for CV events among patients with preexisting CV disease or at high risk of disease. Our secondary objective was to explore the relation between infections and CV risk factors.

Methods

Description of the HOPE Study

The Heart Outcomes Prevention Evaluation (HOPE) study was a multicenter, randomized clinical trial of ramipril, vitamin E, both, or
Serological Methods: *Chlamydia pneumoniae*

All serological testing was performed by staff blinded to clinical outcomes. Serum was stored at -70°C until testing and batch-assayed for *C pneumoniae* after initial thawing. All remaining assays were performed at one additional freeze-thaw cycle. Serum was assayed for *C pneumoniae* IgG and IgA antibodies by microimmunofluorescence (MIF) end-point titration as described previously.\(^{21,22}\) Doubling dilutions of sera in PBS, pH 7.4, were incubated overnight at 4°C with *C pneumoniae* antigen (LabSystems Oy), conjugated for 30 minutes at 37°C with FITC-conjugated anti-human IgG or IgA (Dakopatts). All sera found to be positive for IgA in a screening test were treated with GullSorb (Gull Laboratories) to remove IgG antibodies, then retested. Slides were read with a Zeiss microscope with a UV light source at ×400 magnification by one experienced microbiologist. All tests were run under strict quality control, and test runs were accepted only if the high- and low-titer IgG and IgA controls were within one titer step of the predetermined values. Antibody levels are expressed as inverted titers. A priori, *C pneumoniae* seropositivity was defined as IgG ≥32 or IgA ≥16, and high titers defined as a composite of IgG ≥512 or IgA ≥64.

*Helicobacter pylori*, Cytomegalovirus, and Hepatitis A Virus

Serum IgG antibodies to *H pylori*, CMV, and HAV were determined in 3135, 3153, and 3128 patients, respectively, with the use of 96-well microtiter plate enzyme immunoassay and an automated washer and reader (Biotek Instruments Inc). Fewer than 3168 assays were performed because of inadequate blood volume and were considered missing completely at random. *H pylori* IgG antibody was measured by Hycor HP (Hycor Biomedical). Results of >40 arbitrary units (AU) were considered positive, and values of 27 to 40 AU were considered indeterminate. CMV IgG antibody was measured by using a quantitative CMV IgG assay (DiaSorin). The assay incorporates 4 control calibration sera set to proposed World Health Organization reference standards (1995), and results of >0.4 International Units (IU)/mL were considered positive. HAV IgG was determined by a qualitative assay (DiaSorin), and results of ≥20 IU/mL were considered positive. In all assays, we used cut-off values recommended by the manufacturers.

Statistical Analysis

The primary study outcome measure was the HOPE study primary event cluster of incident myocardial infarction (MI), incident stroke, or CV death. Secondary outcomes were MI alone, stroke alone, or the primary event cluster combined with revascularization procedures. Laboratory results were dichotomized as positive or negative for primary analysis for all assays except *H pylori* IgG. Indeterminate values for CMV and HAV were 0.3% and 2.3%, respectively, and classified as negative. For *H pylori*, results were analyzed as negative, indeterminate, or positive, as the indeterminate category included 5.1% of patients. Kaplan-Meier time-to-event curves were plotted for the cohort of 3168 patients, and serostatus was tested by the log-rank test. Cox proportional hazards modeling was performed in SAS 6.02, adjusting simultaneously for age, sex, smoking status (current, former or never), ramipril randomization, diabetes mellitus, hypertension, and history of hypercholesterolemia. For total pathogen score, 0 to 4 was assigned according to the individual patient’s seropositivity to *C pneumoniae* (IgG ≥512 or IgA ≥64), *H pylori* (>40 AU), CMV (>0.4 IU/mL), or HAV (>20 mIU/mL). SAS 6.02 (SAS Institute) was used for all analyses. An α level of 0.05 was set as the level of significance for the primary hypothesis of *C pneumoniae* serology and the primary CV outcome and 0.01 for all other infections and for subgroup analyses, to account for multiple testing.

Results

Patient Description

The patient characteristics among the 3168 patients were similar to those in the overall HOPE study, which has been described in detail previously.\(^{19}\) Patients had a mean age of 65.4 years; 77.6% were men; 13.9% were current smokers, 63.0% were former smokers, and 23.1% were never-smokers; 34.2% had diabetes, 41.4% had hypertension; 49.0% had a history of hypercholesterolemia; 57.9% had a previous MI; 9.5% had a previous stroke or transient ischemic attack, 16.9% had peripheral vascular disease; and 9.6% had no previous vascular event. In addition to random assignment to ramipril, vitamin E, both, or neither, patients were taking other CV medications including aspirin (79.0%), lipid-lowering drugs (40.1%), β-blockers (43.4%), and calcium channel blockers (18.3%).

Cardiovascular Events

For patients with sera available, the adjudicated primary event cluster of incident MI, stroke, or CV death occurred in 494 of 3168 patients (15.6%) during a mean follow-up of 4.5 years. MI alone occurred in 364 patients (11.5%), stroke alone in 107 (3.4%), and the composite measure of MI, stroke, CV death, or revascularization in 980 (30.9%).

*Chlamydia pneumoniae* and CV Events

*C pneumoniae* IgG antibodies, at a predefined reciprocal titer of ≥32, were present in 2627 of 3168 (82.9%) patients. By time-to-event analysis, *C pneumoniae* IgG serostatus was not associated with CV outcome (log-rank test=0.86). With the use of Cox proportional hazards models (Table 1), an unadjusted hazard ratio (HR) of 0.90 (95% CI, 0.71, 1.13) was obtained and an adjusted HR of 0.87 (95% CI, 0.68, 1.10) after accounting for covariates (age, sex, smoking, ramipril assignment, diabetes mellitus, hypertension, and hypercholesterolemia). *C pneumoniae* IgG ≥32 was not associated with the secondary end points of MI alone, stroke alone, or the primary end point combined with revascularization (Table 1). To examine the influence of various antibody levels, *C pneumoniae* IgG titers were divided approximately into quarters (Figure 1A, log rank test=0.16 for 4th versus 1st quarter). No association was found at any level of IgG antibody titer and CV outcomes (data not shown).

*C pneumoniae* IgA antibodies ≥16 were present in 1995 of 3168 patients (63.0%). There was no clear relation between *C pneumoniae* IgA serostatus and CV outcomes (log rank test=0.13) and no association after covariate adjustment for primary or secondary outcomes (Table 1). An association between higher IgA antibody titers and CV events was sought (Figure 1B and Table 1), with no clear relation demonstrated (log rank test=0.09). *C pneumoniae* IgA titers of ≥512, which represented 9.5% of the patients, had an unadjusted HR of 1.39 (1.04, 1.88) for the primary outcome and 1.34 (1.06, 1.61) for incident MI alone, but neither of these associations was statistically significant after covariate adjustment.
The composite measure of *C. pneumoniae* IgG titer ≥512 or IgA titer of ≥64 was also examined (Table 1). A weak relation was found for the primary event cluster: unadjusted HR of 1.19, (95% CI, 1.00, 1.42), adjusted HR = 1.11 (95% CI, 0.92, 1.34).

The association of CV risk factors with serological status was sought by multiple logistic regression, with serostatus as the dependent variable (see Table 2). The composite measure of *C. pneumoniae* IgG or IgA antibody was strongly associated with male sex and with smoking status but not with age, diabetes mellitus, hypercholesterolemia, or hypertension.

**Interaction Between *C. pneumoniae* and Smoking**

We sought an interaction between *C. pneumoniae* IgA titers and current smoking status by examining smoking-hyperseroLOGY interactions in Cox models. Interaction terms were statistically significant (data not shown). Among 2727 former smokers or never-smokers, *C. pneumoniae* IgA ≥512 was associated with an adjusted HR of 1.59 (95% CI, 1.14, 2.22) for the primary study outcome, compared with 1.21 (95% CI, 0.98, 1.49) for IgA 32 to 256 and 1.00 for IgA <32. Among 441 current smokers, *C. pneumoniae* IgA was not associated with CV outcomes: adjusted HR (IgA ≥512)=0.69 (95% CI, 0.35, 1.34); HR (IgA 32 to 256)=0.70 (95% CI, 0.46, 1.08).

**Helicobacter pylori**

*H. pylori* IgG antibody (≥40 AU) was present in 1934 of 3135 patients (61.7%), and indeterminate (27 to 40 AU) in 5.1%. *H. pylori* serostatus was not associated with CV outcome (Figure 2A and Table 1), with an HR of 0.99 (0.82, 1.19). *H. pylori* serostatus was related to age and with smoking status but not with sex or with other CV risk factors (Table 2).

**Cytomegalovirus**

CMV IgG antibody >0.4 IU/mL was present in 2220 of 3153 patients (70.4%). CMV serostatus was associated with an excess of CV events (log rank test=0.03, Figure 2B). CMV serostatus was associated with an adjusted HR of 1.26 (95% CI, 1.03, 1.54, *P*<0.02) and an adjusted HR of 1.24 (1.01, 1.53, *P*<0.04). CMV was associated with the outcomes of MI alone and with the primary events combined with revascularization (Table 1). CMV was associated with the primary outcome with adjusted HR of 1.05 (95% CI, 0.59, 1.88, *P*<0.05) for women and 1.28 (95% CI, 1.03, 1.59, *P*<0.03) for men, although the interaction term was not significant (*P*=0.62). CMV interactions with CRP and fibrinogen were assessed by tertile of inflammatory marker. Adjusted HRs for CMV were 1.68, 1.32, and 1.11, respectively, for lowest to highest concentrations of CRP (interaction term *P*<0.04) and 1.35, 1.25, and 1.21, respectively, for lowest to highest concentrations of fibrinogen (*P*<0.01). CMV IgG seropositivity was associated with male sex and with age but not with smoking or with other CV risk factors (Table 2).

**Hepatitis A Virus**

HAV IgG antibody ≥20 mIU/mL was present in 2377 of 3128 patients (76.0%). HAV serostatus was not associated with CV events (log rank test=0.33, Figure 2C). HAV was not associated with the primary outcome (HR=1.01, 95% CI, 0.83, 1.24), or with any of the secondary outcomes (Table 1). HAV IgG antibody was more frequent with older age and among men but was not associated with other CV risk factors (Table 2).
Total Pathogen Score
A total pathogen score consisting of 1 point each for *C pneumoniae* status (IgG ≥512 or IgA ≥16), *H pylori* status (>40 AU), CMV (>0.4 IU/mL), and HAV (>20 mIU/mL), for a total of 0 to 4 points, was examined for an association with CV events (Figure 3 and Table 3). The unadjusted event rates were associated with increasing total pathogen score ($\chi^2$ for trend = 6.23, df = 1, $P$ = 0.01). Because only 8 events occurred in the group with a pathogen score of 0, groups 0 and 1 were combined for regression models. In Cox models, pathogen scores of 2, 3, or 4 were associated with an excess hazard for CV events. The highest pathogen score was associated with an adjusted HR of 1.41 (95% CI, 1.02, 1.96) for CV events, compared with a score of 0 or 1. This relation was stronger for incident MI than for incident stroke or for the primary end point combined with revascularization procedures.

Discussion
In a large cohort of clinical trial patients with previous CV events or at high risk of events, we determined the prognosis associated with exposure to 4 infections that had been previously associated with human atherosclerotic disease. We found a modest association between trial-adjudicated CV events and CMV serostatus or with total pathogen score but no consistent association with individual exposure to *C pneumoniae*, *H pylori*, or HAV.

In a recent meta-analysis, Danesh and colleagues found a total of 15 prospective studies, including 3169 cases consisting primarily of case-control studies nested within primary prevention cohorts. They estimated a pooled, covariate-adjusted odds ratio of 1.15 (95% CI, 0.97, 1.36) for the association between *C pneumoniae* IgG and cardiac events. Our study results are compatible with this meta-analysis, with an estimated CV risk of 0.87 for IgG and 1.10 for IgG or IgA, respectively, and complement existing data in three ways. First, our data firmly establish that *C pneumoniae* antibodies have little prognostic value in patients with established CV disease. Second, we carried out a cohort study rather than a nested case-control, with time-to-event data. We were thus able to estimate the influence of serological and CV variables with considerably more precision than many previous studies. Third, we also measured *C pneumoniae* IgA, which may be a better marker for recent exposure to infection. Although we found a modest relation with *C pneumoniae* IgA at high titer
and among former smokers or never-smokers, these associations were not robust to covariate adjustment. Thus, neither chlamydial IgG nor IgA, or a combination of the two, was independently associated with subsequent CV events.

Our findings demonstrate that chlamydial antibodies had no prognostic value in high-risk patients but do not prove that \textit{C pneumoniae} plays no role in the genesis, progression, or clinical complications of atherosclerosis. Most HOPE study patients had preexisting CV disease, hence our study cannot examine the role of these infections in the genesis of atherosclerosis and first CV events. Although we found no independent association between \textit{C pneumoniae} antibodies and CV events, infections may be cofactors together with established CV risk factors. We found that chlamydial antibodies were significantly more common among men and smokers, validating associations noted by others.\textsuperscript{23,24} We specifically sought an interaction between \textit{C pneumoniae}, smoking, and CV events, hypothesizing synergy between smoking and infection. Although the interaction term was statistically significant, the relation between \textit{C pneumoniae} IgA and CV events persisted only in former smokers or never-smokers. Further analysis showed this risk only among former smokers (data not shown). This may simply represent a spurious subgroup analysis, but two other explanations are possible. Higher chlamydial titers may relate to a more recent cessation of smoking and hence a higher residual risk of smoking-associated CV disease. Thus, smoking may be a confounding factor in the \textit{C pneumoniae}–heart disease association, as originally suggested by Hahn and Golubjatnikov.\textsuperscript{23} Alternatively, smoking and chlamydial infection may be part of the same causal pathway, such that no excess risk is associated with chlamydial infection after controlling for smoking.

In the present study, we also measured CMV, \textit{H pylori}, and HAV antibodies, with the a priori expectation that no association would be demonstrated. Thus, the modest association between CMV and CV disease may be spurious, and indeed the probability value of 0.03 did not cross our threshold of \(P<0.01\) for a secondary analysis. However, the result was robust to adjustment for CV risk factors and in keeping with other reports of an association between CMV and native vessel or posttransplantation atherosclerosis.\textsuperscript{25} In a companion manuscript, we measured 4 inflammatory markers in this same cohort of patients (Smieja et al, unpublished data, 2002) and found that fibrinogen and soluble intercellular adhesion molecule-1 (sICAM-1) were associated with CV events. CMV status remained statistically significantly associated with CV events when added to the fully adjusted model.
TABLE 3. HRs for 4.5-Year CV Outcomes by Total Pathogen Score Among 3089 HOPE Study Patients

<table>
<thead>
<tr>
<th>Pathogen Score*</th>
<th>Total (%)† Primary Outcome: MI, Stroke, or CV Death</th>
<th>HR‡ (95% CI) for Outcome Primary Outcome</th>
<th>MI</th>
<th>Stroke</th>
<th>Primary Outcome or Revascularization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Score=0</td>
<td>8/85 (9.4)</td>
<td>1.00§</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Score=1</td>
<td>49/406 (12.1)</td>
<td>1.00§</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Score=2</td>
<td>151/929 (16.3)</td>
<td>1.45 (1.07, 1.96)¶</td>
<td>1.41 (0.99, 2.02)</td>
<td>1.00 (0.55, 1.84)</td>
<td>1.26 (1.02, 1.56)¶</td>
</tr>
<tr>
<td>Adjusted</td>
<td></td>
<td>1.42 (1.05, 1.92)¶</td>
<td>1.41 (0.99, 2.01)</td>
<td>0.94 (0.59, 1.85)</td>
<td>1.26 (1.02, 1.56)¶</td>
</tr>
<tr>
<td>Score=3</td>
<td>175/1133 (15.5)</td>
<td>1.37 (1.01, 1.84)¶</td>
<td>1.33 (0.94, 1.88)</td>
<td>1.17 (0.66, 2.08)</td>
<td>1.21 (0.98, 1.49)</td>
</tr>
<tr>
<td>Adjusted</td>
<td></td>
<td>1.29 (0.95, 1.74)†</td>
<td>1.28 (0.90, 1.82)</td>
<td>1.04 (0.59, 1.85)</td>
<td>1.18 (0.96, 1.46)</td>
</tr>
<tr>
<td>Score=4</td>
<td>97/536 (18.1)</td>
<td>1.62 (1.16, 2.24)¶</td>
<td>1.74 (1.19, 2.53)¶</td>
<td>0.93 (0.46, 1.85)</td>
<td>1.27 (1.00, 1.61)</td>
</tr>
<tr>
<td>Adjusted</td>
<td></td>
<td>1.41 (1.02, 1.96)§</td>
<td>1.57 (1.08, 2.30)§</td>
<td>0.75 (0.37, 1.50)§</td>
<td>1.19 (0.94, 1.51)§</td>
</tr>
</tbody>
</table>

*Pathogen burden score assigned 1 point each for C pneumoniae IgG ≥512 or IgA ≥64, H pylori >40 AU, CMV >0.4 IU/mL, or HAV >20 IU/mL.
†Unadjusted χ² for linear trend: 6.23 (df=1), P=0.012.
‡Numbers denote HRs with 95% CI for association between total pathogen score and CV events (Cox proportional hazards models, SAS).
§Categories 0 and 1 combined due to low No. of events for score=0.
‖Adjusted for age, sex, smoking, diabetes mellitus, hypercholesterolemia, hypertension, and ramipril allocation.
¶P<0.05. All other P>0.05.

C pneumoniae, Mycoplasma pneumoniae and Hemophilus influenzae, compared with a score of 3 or fewer, predicted 12.6% CV mortality versus 3.7% in a cohort of 1018 German patients undergoing coronary angiography. In our study, a total pathogen score based only on the first 4 of these infections was a weak predictor of CV outcome after adjustment for clinical covariates, with a hazard of 1.41 for those with exposure to all 4 infections compared with 0 or 1. In the future, we will examine whether these other serological tests improve risk prediction for a total pathogen score in HOPE patients. Further studies of the total pathogen score concept are required in other populations, alongside investigations to determine whether antibody scores represent reinfection, reactivation, persistence, or nonspecific immune stimulation.

We conclude that among patients with preexisting CV disease at high risk for CV events, C pneumoniae, H pylori, and HAV antibodies were not individual risk markers, whereas CMV IgG serostatus or a total pathogen burden based on these 4 infections had a modest association with subsequent clinical CV events.

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