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Individual pathogens, pathogen burden, and markers of subclinical atherosclerosis: the Multi-Ethnic Study of Atherosclerosis

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Abstract

We examined the cross-sectional relationships of subclinical atherosclerosis – expressed by carotid intimal–medial thickness and coronary calcification – with antibodies to *Chlamydia pneumoniae*, *Helicobacter pylori*, cytomegalovirus, herpes simplex virus, hepatitis A virus, and pathogen burden (number of positive pathogens). A random sample of 1056 individuals chosen from 5030 Multi-Ethnic Study of Atherosclerosis cohort participants were included. After multiple adjustment, no associations were found between atherosclerosis measures and either individual pathogens or pathogen burden. Interactions with inflammatory and endothelial function markers, demographic factors, BMI, high-density lipoprotein, diabetes, and smoking were also explored. The only interaction that was large, qualitative, statistically significant ($P < 0.05$) and in the expected direction was that between hepatitis A virus and soluble intercellular adhesion molecule-1 with regard to Agatston calcium score: the difference between hepatitis A virus-positive and hepatitis A virus-negative participants was −86 units in participants with soluble intercellular adhesion molecule-1 below the median, and +162 units in those with soluble intercellular adhesion molecule-1 equal or above the median. However, given the number of interactions that were explored, these results must be interpreted cautiously.

Findings from the present analyses do not provide support for an infectious etiology for subclinical atherosclerosis. However, the study's limitations, which include its cross-sectional design and insufficient statistical power, suggest that inferences from its findings should be made cautiously.

Keywords

atherosclerosis; infections; pathogens

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Introduction

Studies on both animals [1,2] and humans [3] have suggested a role for pathogens in the etiology of atherosclerosis, including cytomegalovirus [4,5], herpes simplex virus [6], hepatitis A virus [7], *Helicobacter pylori* [8], and particularly *Chlamydia pneumoniae* [9,10]. The observation that periodontitis is related to atherosclerosis [11] further supports an infectious etiology. In addition to individual pathogens, pathogen burden (number of pathogens) has been also found in association with atherosclerosis [12–15].

Baseline data from a multicenter cohort study, the Multi-Ethnic Study of Atherosclerosis (MESA), provided an opportunity to further examine the infectious hypothesis. In this report, we extend the evaluation of the relationship of infections with subclinical atherosclerosis – which heretofore has been mostly based on intimal–medial thickness (IMT) of the carotid or femoral arteries [13,16] – to also include coronary artery calcification. In addition, interactions between pathogen burden and markers of endothelial dysfunction and inflammation were studied, as these processes may link infections to atherosclerosis [17–21].

Methods

The MESA is a population-based cohort study of cardiovascular outcomes in 6814 white, African–American, Chinese, and Hispanic men and women aged 45–84 years who, at baseline (2000–2002), were free of prevalent cardiovascular disease. Cohort participants were recruited from six communities, with approval from all institutional review boards: Baltimore, Maryland; Chicago, Illinois; Forsyth County, North Carolina; Los Angeles, California; New York, New York; and St Paul, Minnesota, USA. A detailed description of the study design has been published [22]. The present report is based on cross-sectional data from a simple random sample of 1056 individuals in whom pathogens were measured, selected from the 5030 MESA participants enrolled prior to February 2002.

Pathogens

Antibodies to the following pathogens were evaluated in the random sample: *C. pneumoniae*, cytomegalovirus, hepatitis A virus, *H. pylori*, and herpes simplex virus. Serum samples for determination of pathogens were frozen at −70°C. Indirect enzyme immunoassays were used to determine IgG antibodies to herpes simplex virus types 1 and 2, cytomegalovirus, and *H. pylori* antigens (Diamedix Immunosimplicity Test Kits, Diamedix Corporation, Miami, Florida, USA). Total serum antibodies to hepatitis A virus were detected using the IM× HAVAB qualitative microparticle enzyme immunoassay (Abbott Laboratories, Abbott Park, Illinois, USA). Serum IgG antibodies to *C. pneumoniae* were detected using a microimmunofluorescent antibody assay employing a two-stage sandwich procedure (Focus Technologies, Cypress, California, USA). Positivity values were as follows: herpes simplex, at least 16 EU/ml; cytomegalovirus at least 8.0 EU/ml; *H. pylori*, at least 1.1 index value; hepatitis A virus, at least 20 mIU/ml; and *C. pneumoniae*, intensity of fluorescence $\neq 0$.

Subclinical atherosclerosis

Coronary calcium was measured by multidetector computer tomographic scanning and electron beam computed tomography, as previously described [23]. Among participants with calcification, a total phantom-adjusted Agatston score [24], defined as the sum of calcium measures from the left anterior descending, circumflex, left and right coronary arteries, was calculated. High-resolution B-mode ultrasonography was used to capture images of the bilateral common carotid and internal carotid arteries using a Logiq 700 ultrasound machine (Logig-700 General Electric Medical Systems, Waukesha, Wisconsin, USA).

). Carotid IMT was computed as the mean of the maximum thickness of the near and far walls from the left and right sides [25].

Covariates

Interviews collected information on age, sex, education, ethnic background, and pack-years of smoking. Diabetes information was based on self-report of physician-diagnosed diabetes, use of hypoglycemic drugs, or fasting plasma glucose at least 126 mg/dl. Glucose was measured by rate reflectance spectrophotometry on the Vitros analyzer (Johnson & Johnson Clinical Diagnostics, Rochester, New York, USA). Fasting total cholesterol was measured using a cholesterol oxidase method (Roche Diagnostics, Indianapolis, Indiana, USA). High-density lipoprotein (HDL) cholesterol was measured similarly after precipitation of non-HDL cholesterol with magnesium–dextran. Resting blood pressure was the average of the last two of three measurements using a Dinamap automated oscillometric sphygmomanometer (model Pro 100, Critikon, Tampa, Florida, USA). BMI was calculated as weight in kilograms divided by height squared in meters. Height and weight were measured in light clothing and with no shoes.

Endothelial function and inflammatory markers were measured as follows: soluble intercellular adhesion molecule-1 (s-ICAM-1) by an ELISA (Parameter Human s-ICAM-1 Immunoassay; R&D Systems, Minneapolis, Minnesota, USA); von Willebrand factor (vWF) by an immunoturbidometric assay on the Star analyzer (Liatest vWF; Diagnostica Stago, Parsippany, New Jersey, USA); total homocysteine by a fluorescence polarization immunoassay (IMx Homocysteine Assay, Axis Biochemicals ASA, Oslo, Norway) using the IMx analyzer (Abbott Diagnostics, Abbott Park, Illinois, USA); interleukin-6 (IL-6) by ultrasensitive ELISA (Quantikine HS Human IL-6 Immunoassay; R&D Systems); and C-reactive protein (CRP) by immunonephelometry using the BNII nephelometer (N high-sensitivity CRP; Dade Behring Inc., Deerfield, Illinois, USA). Laboratory analytical coefficient of variability values for all analytes was less than 8%.

Statistical analysis

Distributions or means of demographic and cardiovascular risk factors, and measures of subclinical atherosclerosis were compared between the random sample and the total cohort. Linear regression was used to examine the associations of individual pathogens and total pathogen burden with carotid IMT, after adjustment for age (continuous), sex, education (less than high school, high school or some college degree, or college degree or higher), ethnic background, pack-years of smoking, diabetes, total and HDL cholesterol, systolic and diastolic blood pressure, and BMI. Because the calcium distribution is skewed, log-transformed Agatston score was used in the model. A generalized linear model with a log link was used to estimate prevalence ratios for coronary calcification, after adjustment for the same variables entered in the linear regression model. We examined the dependence of the pathogen–atherosclerosis associations on levels of the following endothelial function and inflammation markers, categorized as equal or above, or below the following median values: s-ICAM-1, 268 ng/ml; vWF, 130%; total homocysteine, 8.6 umol/l; IL-6, 1.12 pg/ml; and CRP, 2.03 mg/l. We also explored heterogeneity by age (<55 vs. ≥55), ethnic background (white vs. nonwhite), sex (men vs. women), BMI (>25 vs. ≤ 25), diabetes, HDL (<40 mg/dl vs. ≥40 in men and <50 vs. ≥50 in women), and smoking (former and current vs. never).

Results

Distributions and means of demographic and other variables, and median Agatston score were reasonably similar between the random sample on which this report is based and the total cohort (Table 1). However, mean age, proportions of male participants, and whites, and IMT and

calcium values were somewhat lower in the random sample. Infection prevalence proportions varied from a low of 45.4% for *H. pylori* to a high of almost 85% for herpes simplex virus (Table 2).

No statistically significant relationships (at $P \leq 0.05$) were seen between individual pathogens or pathogen burden and either mean IMT or coronary calcium prevalence, and multiple adjustments did not change these patterns (Table 2). Similar null findings were observed for mean Agatston score in individuals with some coronary calcification (not shown in a table).

We also examined IMT and coronary calcium across quintiles of *Chlamydia* titer levels, but no significant trends were observed upon multiple adjustment (not shown). *Chlamydia* was the only pathogen tested in the whole cohort ($n = 6597$); however, results for all MESA participants were virtually the same as those seen in the random sample. Moreover, addition of antibiotic use in the previous year to the regression model did not change the results.

Interaction terms were tested for 156 combinations of individual pathogens/pathogen burden with endothelial/inflammatory markers, demographic variables, BMI, diabetes, HDL, and smoking status. Only eight interactions were found to be statistically significant ($P < 0.05$), comprising 5% of all interactions explored. However, for seven of these, heterogeneity was either very small or in a direction different from that expected (for example, calcium prevalence ratios slightly further away from 1.0 in the stratum below than in that above the median for the endothelial/inflammatory marker). Only one interaction was simultaneously statistically significant, large, qualitative, and in the expected direction: the difference in mean Agatston score between hepatitis A-positive and hepatitis A-negative participants with s-ICAM-1 below the median was −186 Agatston units, but in those with s-ICAM-1 equal or above the median, it was +162 Agatston units.

Discussion

Our results do not replicate those of previous studies showing associations between individual pathogens and atherosclerosis (e.g., [4,6,7,9,10,26,27]). On the other hand, our null findings are consistent with results of a very large cohort study [28] and those of a recent meta-analysis of randomized clinical trials [29], both suggesting that antichlamydial antibiotic therapy does not reduce clinical atherosclerosis risk.

Pathogen burden, rather than individual pathogens, has been postulated as a risk factor for atherosclerosis and, indeed, several studies support its relationship with clinical coronary artery disease or death [15], carotid atherosclerosis progression [13], poor prognosis of patients with coronary artery disease [30], ischemic stroke [31], peripheral arterial disease [32], and endothelial dysfunction [33]. Yet, even after adjustment for multiple potential confounders, and in agreement with some other studies [34–36], we could not observe an association of pathogen burden with proven markers of subclinical atherosclerosis.

Changes in the endothelium are related to infections [17,18,21], and expression of cytokines in endothelial cells has been proposed as a mechanism linking infections to atherosclerosis [19,20]. In addition, previous research suggests that pathogens may interact with inflammatory markers with regard to atherosclerosis [14,26]. However, regardless of statistical significance, we only observed a clear-cut statistical interaction when examining the joint association of hepatitis A virus and s-ICAM-1 with Agatston score. Given the large number of interactions explored in the present analyses, this result may have been due to chance; however, its qualitative ('cross-over') nature warrants confirmation in future studies.

Among the strengths of our study are its population-based design, careful exposure, covariate and subclinical atherosclerosis measurements, and adjustment for multiple potential

confounders. Among its limitations, inability to determine temporality given its cross-sectional nature is of particular concern, as antibodies may reflect prior, rather than current or chronic infections. Another possible reason for our null findings is past decreased survival for individuals with both infections and atherosclerosis, thus, resulting in survival bias at baseline. Because four of the five pathogens were measured in a random sample of about 20% of the cohort, insufficient statistical power may also explain our inability to detect associations; however, at least for *Chlamydia*, results for the whole cohort were also null. In addition, as prevalent cardiovascular disease was an exclusion criterion and as the distributions of some factors in the random sample and the whole cohort were not entirely comparable (Table 1), it is important to be cautious when generalizing the findings of the present study to the reference population.

In sum, we have investigated the link between individual pathogens, pathogen burden and subclinical atherosclerosis, and found no significant associations. It is possible that pathogen burden is related to a subset of the population prone to develop clinical manifestations of atherosclerosis, that is, those with both subclinical atherosclerosis and pathogen burden with its accompanying inflammatory process. Given the study's limitations, longitudinal data on IMT progression, incidence and progression of coronary calcification, and incident cardiovascular events are needed to either confirm or reject our cross-sectional findings.

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Table 1

Distributions and means (with SD) of selected demographic and cardiovascular risk factors and of markers of subclinical atherosclerosis in a random sample and in the total Multi-Ethnic Study of Atherosclerosis cohort, 2000–2002

	[0,2–3]Mean (SD) or percentage	
Variable	**Random sample (n = 1056)[a]**	**MESA cohort (n = 6814)**
Age in years (mean)	59 (10)	62 (10)
Male (%)	42	47
Whites (%)	45	39
Chinese	10	12
African–American	22	28
Hispanic	23	22
Pack-years of smoking (mean)	12 (23)	11 (22)
Total cholesterol (mean, mg/dl)	195 (35)	194 (36)
HDL cholesterol (mean, mg/dl)	51 (15)	51 (15)
LDL cholesterol (mean, mg/dl)	117 (31)	117 (31)
Systolic blood pressure (mean, mmHg)	124 (21)	127 (21)
Diastolic blood pressure (mean, mmHg)	71 (10)	72 (10)
Normal (%)	81	77
Impaired fasting glucose	6	8
Diabetes	13	15
BMI (mean, kg/m^2)	28 (6)	28 (5)
IMT[b] (mean, mm)	0.94 (0.3)	1.00 (0.4)
Coronary calcification prevalence (%)	46	50
Agatston score, median (interquartile range)[c]	62 (228)	89 (288)

HDL, high-density lipoprotein; IMT, intimal–medial thickness; LDL, low-density lipoprotein; MESA, Multi-Ethnic Study of Atherosclerosis.

[a] The random sample was drawn prior to completion of cohort recruitment.

[b] Average of internal and common carotid artery's intimal–medial thickness.

[c] Agatston scores are shown only for those with Agatston score greater than zero.

Table 2

Unadjusted mean carotid intimal–medial thickness values, adjusted absolute differences in intimal–medial thickness,[a] unadjusted coronary calcification prevalence proportions, and adjusted coronary calcification prevalence ratios[a] according to individual pathogens and pathogen burden in a random sample (n = 1056) of the Multi-Ethnic Study of Atherosclerosis cohort, 2000–2002

			[0,4–5]Mean IMT (mm)		[0,6–7]Coronary calcium	
Pathogen	***N***	**%**	**Unadjusted IMT means**	**Adjusted differences[b]**	**Unadjusted prevalence proportions**	**Adjusted prevalence ratios (95% confidence intervals)[b]**
[0,1–7]Cytomegalovirus						
Negative	232	23.3	0.94	Reference	0.45	1
Positive	765	76.7	0.93	−0.02	0.45	0.97 (0.90, 1.19)
[0,1–7]*Chlamydia pneumoniae*						
Negative	306	29.1	0.92	Reference	0.50	1
Positive	746	70.9	0.94	+0.01	0.44	0.89 (0.80, 1.00)
Hepatitis A virus						
Negative	425	42.5	0.95	Reference	0.49	1
Positive	574	57.5	0.93	−0.02	0.44	0.97 (0.86, 1.11)
[0,1–7]*Helicobacter pylori*						
Negative	538	54.6	0.95	Reference	0.44	1
Positive	448	45.4	0.93	−0.02	0.46	1.03 (0.92, 1.16)
[0,1–7]Herpes simplex virus						
Negative	151	15.1	0.93	Reference	0.41	1
Positive	847	84.9	0.94	+0.01	0.46	1.08 (0.92, 1.28)
[0,1–7]Pathogen burden						
0 and 1	110	11.2	0.89	Reference	0.49	1
2	161	16.4	0.96	+0.02	0.40	0.83 (0.69, 1.01)
3	215	21.9	0.97	+0.03	0.49	0.95 (0.81, 1.12)

Pathogen	*N*	%	[0,4–5]Mean IMT (mm)		[0,6–7]Coronary calcium	
			Unadjusted IMT means	Adjusted differences[b]	Unadjusted prevalence proportions	Adjusted prevalence ratios (95% confidence intervals)[b]
4	251	25.5	0.94	−0.01	0.43	0.86 (0.73, 1.02)
5	246	25.0	0.91	−0.03	0.45	0.92 (0.77, 1.10)

HDL, high-density lipoprotein; IMT, intimal–medial thickness.

[a]Adjusted for age, sex, ethnic background, education, pack-years of smoking, diabetes, total and HDL cholesterol, systolic and diastolic blood pressure, and BMI.

[b]Linear regression for differences between intimal–medial thickness means and Poisson regression for prevalence ratios. None of the differences shown in the table was statistically significant at $P < 0.05$.