Burden of Infection and Insulin Resistance in Healthy Middle-Aged Men

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OBJECTIVE — We hypothesized that burden of infection could be associated with chronic low-grade inflammation, resulting in insulin resistance. We aimed to study the effect of exposure to four infections on insulin sensitivity in apparently healthy middle-aged men ($n = 124$).

RESEARCH DESIGN AND METHODS — By inclusion criteria, all subjects were hepatitis C virus antibody seronegative. Each study subject's serum was tested for specific IgG class antibodies against herpes simplex virus (HSV)-1, HSV-2, enteroviruses, and *Chlamydia pneumoniae* through the use of quantitative in vitro enzyme-linked immunosorbent assays. Insulin sensitivity was evaluated using minimal model analysis.

RESULTS — The HSV-2 titer was negatively associated with insulin sensitivity even after controlling for BMI, age, and C-reactive protein (CRP). The associations were stronger when considering the infection burden. In particular, in those subjects who were seropositive for *C. pneumoniae*, the relationship between the quantitative seropositivity index (a measure of the exposure to various pathogens) and insulin sensitivity was strengthened ($r = -0.50$, $P < 0.0001$). We also observed decreasing mean insulin sensitivity index with increasing seropositivity score in subjects positive for enteroviruses. In the latter, the relationship between insulin sensitivity and seropositivity was especially significant ($r = -0.71$, $P < 0.0001$). In a multivariate regression analysis, both BMI and quantitative seropositivity index (7%) independently predicted insulin sensitivity variance in subjects with *C. pneumoniae* seropositivity. When controlling for CRP, this association was no longer significant.

CONCLUSIONS — Pathogen burden showed the strongest association with insulin resistance, especially with enteroviruses and *C. pneumoniae* seropositivity. We hypothesize that exposure to multiple pathogens could cause a chronic low-grade inflammation, resulting in insulin resistance.

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Inflammatory processes are increasingly being recognized as important players in the development of atherosclerosis. In this sense, pathogen burden has been identified as an important factor influencing both inflammation and atherosclerosis (1–4). Cross-sectional epidemiological studies indicate that patients with coronary artery disease (CAD) are more likely to have serological evidence of prior infection. In addition, prospective studies have shown increased risk for cardiovascular events (2) and endothelial dysfunction (3) in patients with serological evidence of prior infection. However, most tested pathogen candidates had a modest and variable predictive value. Epstein et al. (1) proposed the sum of relevant infectious exposures, expressed as a total pathogen burden. This score was demonstrated to constitute an improved prognostic seromarker of risk. Exposure to a panel of pathogens was tested and found to improve prediction of angiographic CAD in a cross-sectional study (5) and incident events among CAD patients in a succeeding prospective study (2). Subsequently, other investigators found an association between the number of exposures to a panel of pathogens and cardiovascular mortality in CAD patients in the AtheroGene study (6). Together with conventional risk factors, pathogen burden imposed an additional independent risk for the presence and severity of CAD (7–9). Several studies further found that risk was primarily attributable to seropositivity for the viral pathogens tested (2,6–8). Exposure to pathogens seems to trigger and amplify inflammatory signals (10–13).

Insulin resistance, the central pathophysiological mechanism of the metabolic syndrome, is a well-known risk factor for the development of CAD (14). Individual components of the metabolic syndrome, such as hypertension and type 2 diabetes, have been reported to be linked to herpes simplex virus (HSV)-1 IgG (15,16) and HSV-2 IgG seropositivity (17). A very recent study has also disclosed an association of type 2 diabetes and seroprevalence for cytomegalovirus (18). A serum lipid profile known to be a risk factor for atherosclerosis with increased levels of triglycerides and decreased HDL has also been found to be associated with HSV-2 seropositivity (19). Some other studies have described associations between *Chlamydia pneumoniae* seropositivity and the metabolic syndrome (20) and dyslipidemia (21,22, 23).

We hypothesized that burden of infection could be associated with chronic low-grade inflammation, resulting in insulin resistance before established atherosclerosis develops (24). For that reason, we aimed to study the effect of exposure to four infections that had been previously associated with human atherosclerotic disease (2,6–8,25) on insulin sensitivity in apparently healthy middle-aged men.

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Abbreviations: CAD, coronary artery disease; CRP, C-reactive protein; ELISA, enzyme-linked immunosorbent assay; HSV, herpes simplex virus; TNFR, tumor necrosis factor receptor; WHR, waist-to-hip ratio.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

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RESEARCH DESIGN AND METHODS

— One hundred and twenty-four consecutive, unselected (except for inclusion criteria, see below) Caucasian subjects, participants in an ongoing epidemiological study of risk factors for cardiovascular disease in Northern Spain, were included in the study. Subjects were randomly localized from a census and invited to participate. The participation rate was 71%. Smokers were defined as any person consuming at least one cigarette a day in the previous 6 months. A food frequency questionnaire was obtained from all subjects. None of the subjects were taking any medication or had any evidence of metabolic disease other than obesity. All subjects reported that their body weight had been stable for at least 3 months before the study. Inclusion criteria were BMI <40 kg/m^2, absence of any systemic disease, absence of clinical symptoms and signs of infection in the previous month by structured questionnaire to the patient, and hepatitis C virus antibody seronegativity. Informed consent was obtained from all subjects. The local ethics committee approved the study.

BMI was calculated as weight (in kilograms) divided by the square of height (in meters). The subjects' waist was measured with a soft tape midway between the lowest rib and the iliac crest. The hip circumference was measured at the widest part of the gluteus region. The waist-to-hip ratio (WHR) was then calculated. Blood pressure was measured in the supine position on the right arm after a 10-min rest; a standard sphygmomanometer of appropriate cuff size was used, and the first and fifth phases were recorded. Values used in the analysis are the average of three readings taken at 5-min intervals. Patients were asked to abstain from consuming alcohol and caffeine for at least 12 h prior to testing.

Insulin sensitivity and secretion

All subjects had fasting plasma glucose <7.0 mmol/l and 2-h postload plasma glucose <11.1 mmol/l after a 75-g oral glucose tolerance test according to the American Diabetes Association criteria. Insulin sensitivity was measured using the frequently sampled intravenous glucose tolerance test on a different day. In brief, the experimental protocol started between 8:00 and 8:30 A.M. after an overnight fast. A butterfly needle was inserted into an antecubital vein, and patency was maintained with a slow saline drip. Basal blood samples were drawn at -30, -10, and -5 min, after which glucose (300 mg/kg body wt) was injected over 1 min starting at time 0 and insulin (Actrapid; Novo Nordisk, Bagsværd, Denmark; 0.03 units/kg) administered at time 20. Additional samples were obtained from a contralateral antecubital vein up to 180 min, as previously described (26). Insulin secretion was calculated as the insulin area during the first 10 min of the frequently sampled intravenous glucose tolerance test.

Analytical methods

Blood samples were drawn from each subject after an overnight fasting period. Serum was centrifuged at 4,000*g* for 10 min, immediately divided into aliquots, and frozen at -80°C until analysis. Each study subject's serum was tested for specific IgG class antibodies against HSV-1, HSV-2, enteroviruses, and *C. pneumoniae* through the use of quantitative in vitro enzyme-linked immunosorbent assays (ELISAs). In the IgG ELISA for HSV-1, HSV-2, and *C. pneumoniae*, a value of >18 relative units/ml was considered positive and 16–18 considered indeterminate according to the manufacturer's instructions. The anti–*C. pneumoniae* antibody test is based on broad-reactive chlamydial inclusions. In the IgG ELISA for enteroviruses, a value of >100 relative units/ml was considered positive and 80–100 considered indeterminate according to the manufacturer's instructions. Two pathogen scores were then constructed: in the semiquantitative seropositivity score, a score ranging from 0 to 8 was assigned according to the individual subject's seropositivity to each pathogen (0 for seronegative, 1 for indeterminate, and 2 for seropositive). In the quantitative seropositivity index, the arithmetical sum of the individual titers of each pathogen was calculated. The enterovirus titer was multiplied by 0.18 to reflect a compatible range of burden compared with the other seropositivities.

Serum glucose concentrations were measured in duplicate by the glucose oxidase method with the use of a Beckman Glucose Analyzer II (Beckman Instruments, Brea, CA). The coefficient of variation was 1.9%. The coefficients of variation for serum insulin were similar to those previously reported (26). HbA_{1c} (A1C) was measured by high-performance liquid chromatography by means of a fully automated glycated hemoglobin analyzer system (Hitachi L-9100). Normal range among 774 subjects with normal glucose tolerance was 4.71 ± 0.46%. Total serum cholesterol was measured through the reaction of cholesterol esterase/cholesterol oxidase/peroxidase. Total serum triglycerides were measured through the reaction of glycerol-phosphate-oxidase and peroxidase. Serum C-reactive protein (CRP) (ultrasensitive assay; Beckman, Fullerton, CA) was determined by routine laboratory test, with intra- and interassay coefficients of variation <4%. The lower limit of detection was 0.02 mg/l. Plasma soluble tumor necrosis factor receptor (TNFR)-1 and -2 (BioSource Europe, Fleunes, Belgium) were determined as previously described (26).

Statistical methods

Descriptive results of continuous variables are expressed as means $\pm$ SD if normally distributed or as median (interquartile range). Before statistical analysis, normal distribution and homogeneity of the variances were evaluated using Levene's test, and then variables were log transformed if necessary. These parameters (insulin sensitivity, insulin secretion, soluble TNFR-1 and -2, triglycerides, and seropositivities) were analyzed on a log scale and tested for significance on that scale. The anti–log-transformed values of the means are reported in the tables.

Differences between groups were tested by χ^2 test for categorical variables and ANOVA for continuous variables. Relation between variables was tested using Pearson's test and partial correlation analysis. Multivariate regression analysis was performed, including BMI, age, and CRP and the sum of titers for each pathogen as continuous variables for the dependent-variable insulin sensitivity. A P value ≤ 0.05 was considered statistically significant. Computations were carried out with SPSS version 11.0.

RESULTS

— The anthropometrical and biochemical characteristics of the subjects and serological status are shown in Table 1.

We observed significant relationships between the titer of some pathogens and selected variables in all subjects, as shown in Table 2 (all correlations shown in Table S1 [online appendix, available at http://care.diabetesjournals.org]). Of note was the association between diastolic blood pressure and the HSV-1 titer and the quantitative seropositivity index, which persisted after controlling for BMI, age, and CRP (Table 2).

Table 1—*Anthropometric, biochemical, and burden of infection variables of the study subjects*

n	124		
Smokers	35 (28)	**HSV-1 serologic status**	129 (96.5–149)
BMI (kg/m^2)	27.4 ± 3.5	Negative	10 (8.4)
		Indeterminate	1 (0.8)
WHR (%)	0.93 ± 0.07	Positive	113 (90.8)
Systolic blood pressure (mmHg)	127.3 ± 16		
Diastolic blood pressure (mmHg)	80.8 ± 10	**HSV-2 serologic status**	9 (6–12)
		Negative	115 (93.1)
Fasting glucose (mg/dl)	97.5 ± 8	Indeterminate	3 (2.3)
		Positive	6 (4.6)
A1C (%)	4.7 ± 0.4		
Total cholesterol (mg/dl)	209 ± 39		
LDL cholesterol (mg/dl)	14 ± 70	**Enterovirus serologic status**	42 (26–82)
HDL cholesterol (mg/dl)	53.3 (12.6)		
		Negative	90 (72.6)
Triglycerides (mg/dl)	84 (58–117)	Indeterminate	11 (8.9)
		Positive	23 (18.5)
CRP (mg/l)	0.2 (0.11–0.43)		
Soluble TNFR-1 (ng/ml)	1.72 (1.33–2.16)	***C. pneumoniae* serologic status**	17 (7–40.7)
Soluble TNFR-2 (ng/ml)	4.9 (3.4–8)	Negative	58 (46.7)
		Indeterminate	17 (13.7)
Insulin sensitivity (10^{-4} * min^{-1} * mU/l)	2.42 (1.38–3.62)	Positive	49 (39.6)
Insulin secretion (mU/l)	332 (190–516)		
Quantitative seropositivity index	143 (176–212)	**Semi-quantitative Seropositivity Score**	
		0-2……45 (36.3)	
		3-4……56 (45.5)	
		5-7……23 (18.2)	

Data are means ± SD, *n* (%), and median (interquartile range).

The HSV-2 titer was significantly and negatively associated with insulin sensitivity and positively with soluble TNFR-1 (Table 2). These two associations persisted after controlling for BMI, age, and CRP. Interestingly, subjects with the HSV-2 titer over the median (9 units, n = 60) showed lower insulin sensitivity index (2.37 ± 0.18 vs. 3.14 ± 0.2, P = 0.04), similar BMI (27.3 ± 3.8 vs. 27.8 ± 3.5 kg/m^2, P = 0.4), WHR (0.93 ± 0.07 vs. 0.93 ± 0.07, P = 0.6), and age (50 ± 11 vs. 53 ± 10 years, P = 0.2) than subjects with HSV-2 titer below the median (n = 64).

The associations were stronger when considering the infection burden. The quantitative seropositivity index was inversely associated with insulin sensitivity (r = −0.21, P = 0.02). Furthermore, in those subjects that were seropositive for *C. pneumoniae*, the relationships between the quantitative seropositivity index and insulin sensitivity (Fig. 1*A*), soluble TNFR-1 (Fig. 1*B*), and systolic blood pressure (Table S1 [online appendix]) were strengthened. This observation was reflected in decreased mean insulin sensitivity index with increasing seropositivity score in subjects positive for *C. pneumoniae* (Fig. S1, *upper panel* [online appendix]) or those positive for enteroviruses (Fig. S1, *lower panel*). In the latter, the relationship between insulin sensitivity and seropositivity was especially significant (r = −0.71, P < 0.0001; r = −0.64, P =

Table 2—*Relationships of pathogen antibody titers and selected variables*

Variable	Relationship	After controlling for age and BMI	After controlling for age, BMI, and CRP
HSV-1 titer			
Systolic blood pressure	$r = 0.14$, $P = 0.1$	$r = 0.17$, $P = 0.08$	$r = 0.18$, $P = 0.05$
Diastolic blood pressure	$r = 0.20$, $P = 0.03$	$r = 0.20$, $P = 0.03$	$r = 0.19$, $P = 0.03$
HSV-2 titer			
Insulin sensitivity	$r = -0.29$, $P = 0.002$	$r = -0.23$, $P = 0.01$	$r = -0.21$, $P = 0.03$
Soluble TNFR-1	$r = 0.19$, $P = 0.03$	$r = 0.19$, $P = 0.03$	$r = 0.21$, $P = 0.02$
Enterovirus titer			
Systolic blood pressure	$r = 0.24$, $P = 0.009$	$r = 0.17$, $P = 0.05$	$r = 0.17$, $P = 0.07$
Diastolic blood pressure	$r = 0.18$, $P = 0.05$	$r = 0.14$, $P = 0.1$	$r = 0.17$, $P = 0.07$
Soluble TNFR-1	$r = 0.19$, $P = 0.03$	$r = 0.20$, $P = 0.02$	$r = 0.18$, $P = 0.06$
Soluble TNFR-2	$r = 0.19$, $P = 0.03$	$r = 0.21$, $P = 0.02$	$r = 0.17$, $P = 0.07$
Quantitative seropositivity index			
Systolic blood pressure	$r = 0.24$, $P = 0.01$	$r = 0.24$, $P = 0.01$	$r = 0.26$, $P = 0.01$
Diastolic blood pressure	$r = 0.22$, $P = 0.02$	$r = 0.22$, $P = 0.02$	$r = 0.22$, $P = 0.02$

0.007, after controlling for age and BMI). In subjects with *C. pneumoniae* seropositivity, pathogen burden correlated positively with WHR ($r = 0.33$, $P = 0.01$).

In a multivariate regression analysis, both BMI and quantitative seropositivity score, but not age or WHR, independently predicted 42 and 7% of insulin sensitivity variance, respectively. When controlling for CRP in both partial correlation and multivariate analysis, the relationship between quantitative seropositivity index and insulin sensitivity was no longer significant.

Systolic blood pressure also increased with the quantitative seropositivity score (Table 2) and the semiquantitative seropositivity score in all subjects (Fig. S2, *upper panel* [online appendix]) and subjects seropositive for *C. pneumoniae* (Fig. S2, *lower panels*). The former associations persisted after controlling for age, BMI, and CRP (Table 2). No significant associations were observed between pathogen burden and dyslipidemia after controlling for BMI, age, and CRP.

CONCLUSIONS — In this article, we describe a significant association between burden of infection and insulin sensitivity. We can exclude insulin secretion as a confounding factor because it showed no interaction with pathogen burden. The association between HSV-2 titer and insulin sensitivity was weak but significant in all subjects considered as a whole. However, it seems unlikely that one specific pathogen causes insulin resistance. This is supported by our findings that show a significant relation between the number of infectious pathogens to which an individual has been exposed (a combined antibody response, expressed as the semiquantitative and quantitative seropositivity indexes) and insulin sensitivity, which was especially significant in subjects who were seropositive for *C. pneumoniae* and for enteroviruses. This association persisted after controlling for age and BMI but not after controlling for CRP, suggesting that inflammation significantly influenced the relationship between insulin sensitivity and pathogen burden. On the other hand, CRP was <2 mg/l by inclusion criteria, excluding recent, acute infection as the common factor preceding inflammation and insulin resistance. The elevated exposure to HSV-1 in our sample of subjects only contributed to increase the seropositivity score. However, the small number of individuals negative for HSV-1 and the small number positive for HSV-2 limit the power of the study to find significant associations with these serologies. All subjects were hepatitis C virus antibody seronegative, so the confounding relationship between hepatitis C virus infection and insulin resistance (27) can be excluded.

There is little information available about the influence of any bacterial or viral infection on the development of chronic insulin resistance or the metabolic syndrome. Some studies have described associations between *C. pneumoniae* seropositivity and the metabolic syndrome and dyslipidemia (20–23). At least two reports disclosed increased prevalence of *C. pneumoniae* seropositivity in subjects with obesity (28,29) together with increased fasting insulin (29). A very small study found that type 2 diabetic patients were more frequently seropositive (IgA) for this pathogen (30). In another report, a significant positive correlation between this seropositivity and CRP was shown, but the association with homeostasis model assessment was not described (31). All of these findings suggest that inflammation is behind the link between pathogen burden and insulin resistance.

Dyslipidemia, hypertension, and type 2 diabetes have also been linked to viral seropositivity (15–19). In several studies, the risk associated with pathogen burden on the development of CAD was primarily attributable to seropositivity to the viral pathogens tested (2,6–8). In one report, the relative risk of myocardial infarction by high levels of enterovirus-specific antibodies depended on age: the risk was the highest in middle-aged men (25), precisely the subjects we investigated. In the latter study, significant interactions were seen between enterovirus antibody levels and systolic blood pressure (25). Our findings are in agreement with these previous observations and suggest that insulin resistance associated with seropositivity for enteroviruses could be the preceding factor for increased systolic blood pressure and myocardial infarction. On the other hand, infection with some enteroviruses led to insulin resistance and the presence of anti–insulin receptor autoantibodies in at least one case report (32).

Despite major differences in the biological and clinical consequences that result from human infection with several pathogens, there may be shared pathways

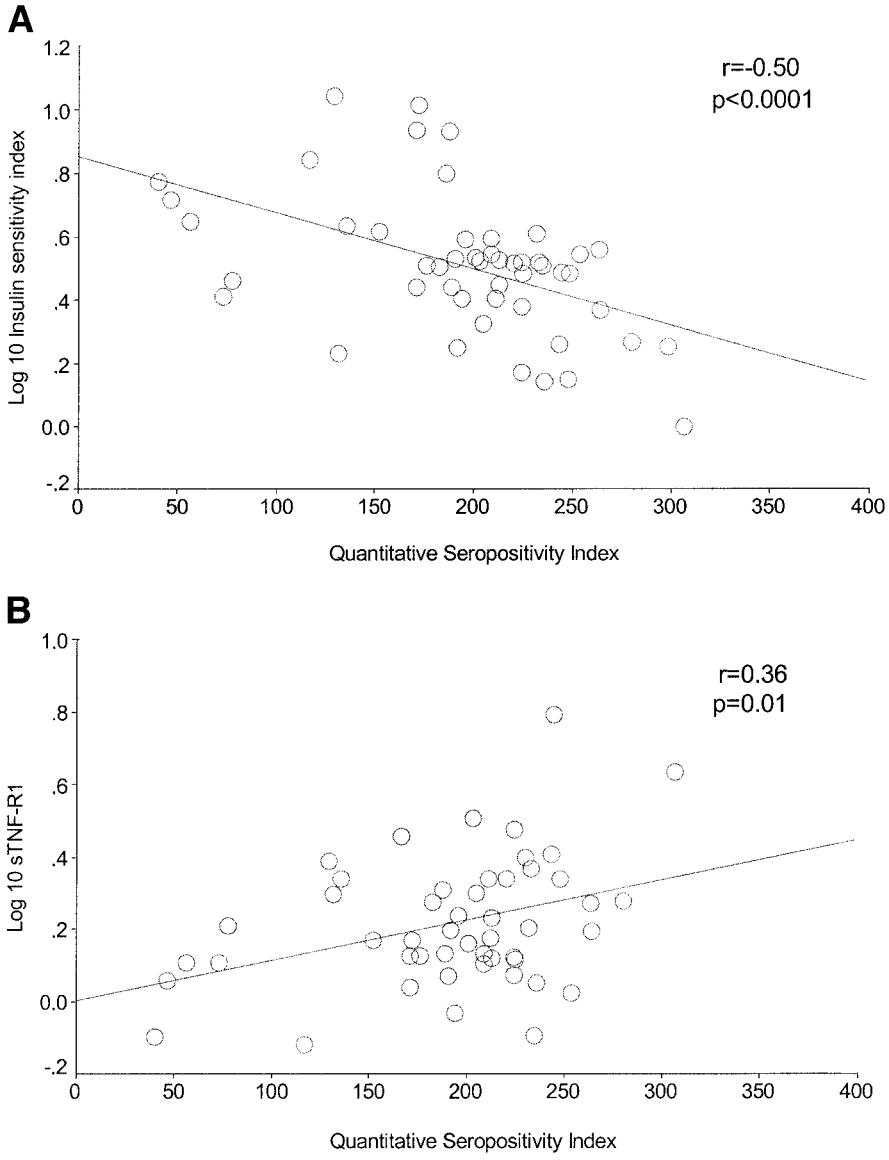

Figure 1—*Linear association between quantitative seropositivity index and insulin sensitivity* (A) *and log-transformed plasma soluble TNFR-1 concentration* (B) *in subjects seropositive for C.* pneumoniae (n = *49*).

by which diverse organisms produce insulin resistance (24). The stimulation of monocytes/macrophages by several bacterial and viral products induces overexpression of various cytokines and inflammatory mediators (33,34) that amplify and diversify these signals, resulting in insulin resistance (24).

In this study, we have used two measures of pathogen burden. Most published studies on pathogen burden use the number of seropositivities as a measure of exposure (6,7). The quantitative index might add information concerning persistence for the different pathogens. In this sense, we report serologies against pathogens that are characterized by persistent infection. After HSV-1 infection, this virus replicates in epithelial cells at peripheral sites of infection, and later it is transported by retrograde axonal transport to the neuronal nuclei within the sensory ganglia, where it establishes a latent infection that persists for the life of the individual. HSV-1 sporadically reactivates from latency and is transported by anterograde axonal transport, being shed at peripheral sites and leading to recurrent disease (35). Long-term persistence and frequently recurring disease also occur after HSV-2 infection (36). Chlamydia infections are notorious for causing chronic infections, and treatment failure is common. These failures may be due to the establishment of a nonreplicating but viable state of chlamydia in the host cells (37,38). Enteroviruses are members of the Picornaviridae, which are small, nonenveloped, single-stranded, positive-sense RNA viruses. Although the enteroviruses are still considered very cytolytic, it is now known that they can establish persistent infection in vivo. It is becoming evident that enteroviruses are able to persist in their host after the primary infection (39). The study of persistence in vivo is complicated by the fact that the immune system might be the factor that permits the persistence (39).

Genetic factors that increase the risk of developing insulin resistance might also enhance the extent of antibody response to several pathogens. As we previously hypothesized, high cytokine responders may be at an advantage in an environment where infectious risk is prevalent but at a disadvantage where obesity, insulin resistance, and atherosclerosis dominate (40).

The importance of the findings of this study is highlighted by the prevalence of these infections and for the potentially high attributable risk for the different pathogens. An analysis, which used nationally representative data, showed that ~73% of the population aged ≥12 years in the U.S. had antibodies to one or both types of HSV (41). *C. pneumoniae* is one of the most widespread pathogens in humans, although up to 90% of infected people have few or no symptoms. Several studies have estimated the population prevalence of antibodies to *C. pneumoniae* at 40–55% in the northern hemisphere and >60% in underdeveloped countries (42). The range of enteroviruses circulating in a population is highly diverse and changes rapidly over time as new strains emerge and spread in previously nonexposed individuals. In a recent investigation in a coastal area of the U.S., the high prevalence of enteroviruses throughout the study area suggested a chronic pollution problem and potential risk to recreational swimmers in certain coastal areas (43).

The strengths of this research are that we studied a homogenous sample of healthy men and that a robust tool was used to measure insulin sensitivity.

Study limitations

This is a cross-sectional investigation that establishes an association but not causality. That insulin resistance might cause increased susceptibility to infection can-

not be excluded. These findings need to be confirmed in further studies with a larger number of patients. Further investigations to determine whether antibody scores represent reinfection, reactivation, persistence, or nonspecific immune stimulation are required.

We conclude that among apparently healthy men, HSV-2 seropositivity was modestly linked to insulin resistance, whereas a total pathogen burden based on HSV-1, HSV-2, enteroviruses, and *C. pneumoniae* IgG serostatus showed the strongest association with insulin resistance, especially when these two last pathogens caused seropositivity.

Exposure to multiple pathogens could cause a chronic low-grade inflammation, resulting in insulin resistance and finally leading to atherosclerosis. In fact, reduction in lifetime exposure to infectious diseases and other sources of inflammation has made an important contribution to the historical decline in old-age mortality (44).

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