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Association of Endotoxemia With Carotid Atherosclerosis and Cardiovascular Disease

Prospective Results from the Bruneck Study

Christian J. Wiedermann, MD,* Stefan Kiechl, MD,† Stefan Dunzendorfer, MD,*
Peter Schratzberger, MD,* Georg Egger, MD,‡ Friedrich Oberhollenzer, MD,‡ Johann Willeit, MD†
Innsbruck, Austria, and Bruneck, Italy

OBJECTIVES Focus of the current study was on the significance of bacterial endotoxin, which shows a variety of pro-atherogenic properties and may occur at high concentration in the circulation of infected subjects.

BACKGROUND The possibility of an infectious risk factor in atherogenesis and cardiovascular disease has stimulated research interest, but the nature of such process remains obscure.

METHODS We measured plasma endotoxin levels (LAL assay) in a random population of 516 men and women 50 to 79 years old at the 1990 baseline evaluation (Bruneck Study). End points of this prospective survey were incident (early) atherosclerosis in the carotid arteries as assessed with high-resolution Duplex ultrasound (five-year follow-up rate, 98%) and incident cardiovascular disease (follow-up rate, 100%).

RESULTS Median endotoxin concentration amounted to 14.3 pg/ml (range, 6.0 to 209.2 pg/ml). Subjects with levels beyond 50 pg/ml (90th percentile) faced a threefold risk of incident atherosclerosis (odds ratio [95% confidence interval] 2.9 [1.4–6.3]; $p < 0.01$). The risk associated with high endotoxin was most pronounced in subjects with chronic infections and in current and ex-smokers. Notably, smokers with low endotoxin levels and nonsmokers did not differ in their atherosclerosis risk, whereas smokers with high levels almost invariably developed new lesions. All findings emerged as independent of vascular risk factors. Similar results were obtained for incident cardiovascular disease.

CONCLUSIONS The current study yields first epidemiologic evidence that endotoxemia constitutes a strong risk factor of early atherogenesis in subjects with chronic or recurrent bacterial infections and a link in the association between cigarette smoking and atherosclerotic disease. (J Am Coll Cardiol 1999;34:1975–81) © 1999 by the American College of Cardiology

Circulating bacterial endotoxin (ETX) is well-established to provoke severe endothelial dysfunction and exhibits a variety of pro-atherogenic properties (1–6). Contra previous knowledge, prominent endotoxemia is not confined to sepsis but also occurs in apparently healthy subjects (7). Chronic or recurrent bacterial infections, smoker's bronchitis and gut barrier dysfunction all may be rich sources of circulating ETX (7–9).

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A growing body of evidence supports the concept that systemic inflammation plays a role in the initiation and progression of atherosclerosis and its complications. Plasma markers for underlying systemic inflammation such as C-reactive protein were shown to be risk predictors for future myocardial infarction or stroke (10). The inflammatory signals driving immunologic activity in atherosclerosis are unknown. They may be nonantigenic or antigenic but of noninfectious origin (10). We hypothesize that sustained exposure to high ETX levels in the clinical settings (mentioned previously) constitutes a prominent risk factor of early atherogenesis. The Bruneck Study, a prospective population survey on the course and etiology of atherosclerosis, offers a unique possibility to clarify this issue from an epidemiological perspective.

METHODS

Study subjects. The current evaluation was performed as part of the Bruneck Study (11,12). The survey area is located in the north of Italy (Bolzano Province). Agriculture, tourism, commerce, and light industry are the main

From the *Department of Internal Medicine and †Department of Neurology, Medical Faculty, University of Innsbruck, Innsbruck, Austria; and ‡Department of Internal Medicine, Federal Hospital, Bruneck, Italy. Part of the study was supported by the Austrian Science Foundation, grant number 09977, to C.J.W.

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Abbreviations and Acronyms

CV = coefficient of variation
ETX = endotoxin
HDL = high-density lipoprotein
LAL = Limulus amebocyte lysate
LDL = low-density lipoprotein
ln = log-normalized

sources of income. Geographic remoteness causes low population mobility (<0.2%/year). Plasma ETX levels were assessed in a random subsample of the Bruneck Study population. In brief, 86 men and 86 women each in their sixth to eighth decade were enrolled at the baseline examination (July to November 1990) after they had given their informed consent to join this study (n = 516). During follow-up, 41 subjects died. A total of six participants changed their residence within the Bruneck area and remained eligible. Among survivors (n = 475) follow-up was 98% complete for ultrasonographic reevaluation (July to October 1995; n = 466). Reliable data on fatal and nonfatal cardiovascular disease were available in virtually all subjects.

Clinical history and examination. All participants underwent a clinical examination with cardiologic and neurologic priority (12). Regular alcohol consumption was quantified in terms of grams per day. Systolic and diastolic blood pressures were taken with a standard mercury sphygmomanometer after at least 10 min of rest while the subject was in a sitting position. The values used in the current analysis were means of three measurements taken by the same investigator at about 1-h intervals. Hypertension was defined by a blood pressure ≥160/95. A standardized oral glucose tolerance test (75 g glucose in 10% solution) was performed in all subjects except those with well-established diabetes mellitus. Diabetes mellitus was coded present for subjects with fasting glucose levels >7.8 mmol/liter (140 mg/dl) and/or a 2-h value >11.1 mmol/liter (200 mg/dl). Body mass index was calculated as weight divided by height squared. The number of cigarettes smoked and the pack-years were recorded for each smoker and ex-smoker. Lifetime nonsmokers and ex-smokers who quit more than 5 years before entering the study (1990) were referred to as current nonsmokers.

Myocardial infarction was deemed confirmed when World Health Organization criteria for definite disease status (13) were met, including compatible symptoms and either electrocardiographic changes or elevated cardiac enzymes. Stroke and transient ischemic attacks were classified according to the criteria of the National Survey of Stroke (14). The diagnosis of peripheral artery disease required a positive response to the Rose questionnaire, with the vascular nature of complaints confirmed by standard diagnostic procedures. Self-reported data were verified from hospital records, death certificates and information from general practitioners and supplemented by a thorough screening of the hospital database for arterial diseases to minimize recall bias and selective nonresponding.

In an effort to identify subjects with chronic bacterial infections or clinical conditions known to be associated with recurrent episodes of infectious exacerbation (asthma, emphysema), we commenced an extensive screening consisting of two consecutive phases. The first step involved a detailed self-reported medical and medication history, full clinical examination, spirometry, extensive laboratory evaluations including assessment of acute-phase reactants, markers of chronic infections and urinary analysis and a thorough review of Bruneck Hospital databases and medical records provided by general practitioners. In a second step, individuals suspected of having diseases of interest were referred for further optional examinations such as chest X-ray, endoscopy and serological examinations. A definite diagnosis was established applying standard diagnostic guidelines.

Laboratory methods. Blood samples were taken from the antecubital vein after subjects had fasted and abstained from smoking for at least 12 h (12). In the event of acute infections, blood samples were drawn up to six weeks later. Apolipoproteins were measured using a nephelometric fixed-time method (apolipoprotein AI and B, Behring; coefficients of variation [CV] 5.7% and 2.4%, respectively). High-density lipoprotein (HDL) cholesterol was determined enzymatically (CHOD-PAP method, Merck; CV 2.2% to 2.4%). Low-density lipoprotein (LDL) cholesterol was calculated with the Friedewald formula except in subjects with triglycerides >4.52 mmol/liter. Lipoprotein(a) (ELISA, Immuno; CV 3.5% to 6.3%), serum protein (biuret method; Merck), ferritin (fluorometric enzyme immunoassay, Baxter; CV 5.0% to 5.9%) and other variables were assessed according to standard procedures.

Endotoxin-levels were quantified using the chromogenic Limulus amebocyte lysate (LAL) test for ETX measurements in plasma samples (Chromogenix, Mölndal, Sweden) according to the manufacturer's instructions, as previously described (15). In brief, the LAL contains an enzyme system that is activated in the presence of ETX. The activated enzymes split off para-nitro aniline from the chromogenic substrate to produce a yellow color, giving the opportunity to measure photometrically (405 nm) the amount of ETX present in the system. Single measurements of aliquoted plasma samples were performed. A standard curve showing linear correlation between increasing absorbances and log concentrations of standard ETX was used to determine the ETX concentrations in unknown heat-treated samples. Data were calculated and transformed to pg/mL plasma ETX.

Scanning protocol and definition of ultrasound end points. The ultrasound protocol involves the scanning of the internal (bulbous and distal segments) and common carotid arteries (proximal and distal segments) of either side with a 10-MHz imaging probe and a 5-MHz Doppler. Atherosclerotic lesions were defined by two ultrasound

Table 1. Association of Potential Vascular Risk Factors With Incident Atherosclerosis

Characteristic	Incident Carotid Atherosclerosis (5-year)		p Value	p Value (Adjusted for Age/Gender)
	No (n = 227)	Yes (n = 239)		
Male gender, n (%)	87 (41.3)	129 (54.0)	0.006	—
Age, yrs	60.3 ± 8.3*	65.8 ± 8.1	< 0.001	—
Apolipoprotein B, mg/dl	118.9 ± 31.2	129.9 ± 46.8	0.003	0.012
Apolipoprotein AI, mg/dl	165.8 ± 27.3	162.6 ± 32.4	0.260	0.170
Lipoprotein(a), mg/dl	14.7 ± 14.9	16.9 ± 19.7	0.170	0.093
Ferritin, ng/ml	139.1 ± 138.4	181.1 ± 193.4	< 0.001	0.008
Blood pressure systolic, mm Hg	144.9 ± 19.5	153.1 ± 22.3	< 0.001	0.001
Hypertension, n (%)	62 (27.8)	108 (45.2)	< 0.001	0.010
Body mass index, kg/m^2	25.3 ± 3.9	25.1 ± 3.7	0.590	0.970
Diabetes mellitus, n (%)	19 (8.5)	30 (12.6)	0.150	0.710
Alcohol consumption, n (%)	—	—	0.004	0.030
≤50 g/d	64 (28.7)	59 (24.7)	—	—
51–99 g/d	17 (7.6)	44 (18.4)	—	—
≥100 g/d	20 (9.0)	27 (11.3)	—	—
Smoking, pack-years	17.9 ± 16.5	27.8 ± 15.3	< 0.001	0.017
White blood cells, 1000/μl	6.2 ± 1.7	6.5 ± 1.6	0.068	0.110
C-reactive protein, mg/dl	0.24 ± 0.73	0.45 ± 0.86	0.008	0.090
Endotoxin, pg/ml	20.4 ± 22.6	29.6 ± 43.2	0.005	0.002

n = number of subjects; *mean ± standard deviation.

criteria: i) wall surface (protrusion into the lumen or roughness of the arterial boundary) and ii) wall texture (echogenicity). Presence and maximum axial diameter of plaques were assessed in each of the eight vessel segments. An atherosclerosis summing score was calculated by adding all plaque diameters (12). Scanning was performed twice in 1990 and 1995 by the same experienced sonographer, who was unaware of the subjects' clinical and laboratory characteristics. The ultrasound method applied permitted us to monitor atherosclerosis in individuals (person-based approach) and to differentiate various stages of atherosclerosis progression (11). The current analysis focused on incident (early) atherosclerosis as the putative target of ETX attack. Reproducibility of this ultrasound end point was nearly perfect (16) as indicated by a kappa coefficient of agreement of 0.89 (double measurements, n = 100).

Statistics. Incidence rates of atherosclerosis were expressed as events per 1,000 person-years (incidence density) (17). Strength and type of association between plasma ETX level and incident atherosclerosis were assessed by logistic regression analysis. The test procedure was based on maximum likelihood estimators (18). To account for deviations from the normal distribution, ETX concentrations were log-normalized (ln). Multivariate regression models were built with either a forward stepwise selection procedure applying the default settings of the SPSS-X statistical software (19) or with forced entry of a fixed set of covariates including vascular risk factors and potential determinants of ETX level. Results were virtually identical. For ease of presentation, we provide data derived from the latter approach only (Tables 1 and 2). Separate equations were calculated for smokers and subjects with chronic infections. Effect modification was examined by the inclusion of interaction terms. In an attempt to obtain the most suitable parametric scale of association, 10 equally sized categories of plasma ETX concentrations were modeled with indicator variables in separate analyses. Trends were estimated by visual inspection of plots of the logit (log odds) against the midpoints of deciles. Finally, crude and adjusted hazard ratios of incident cardiovascular disease were calculated with Cox models (20). The proportional hazard assumptions were satisfied.

RESULTS

Five-year incidence of carotid atherosclerosis in our study population amounted to 103 per 1,000 person-years. Corresponding rates for incident cardiovascular disease and cardiovascular mortality were 10.8 and 3.9, respectively, per 1,000 person-years. For descriptive purposes Table 1 depicts main demographic characteristics, levels of selected vascular risk factors and ETX separately in subjects with and without incident atherosclerosis. Three out of five men and one out of five women reported current or previous cigarette smoking.

Virtually all plasma specimens contained bacterial ETX (median, 14.3 pg/ml; interquartile range, 10.6–19.8) though at highly variable concentrations (range, 6.0 to 209.2 pg/ml; for distribution, see Fig. 1). Levels were on average 22% higher in women than in men (p = 0.001) and positively correlated with total protein concentrations (36% increase for each 1 g/dl-increment of serum protein; p = 0.011) and cigarette smoking (10% increase per 10 cigarettes; p = 0.006).

Table 2. Risk of Incident Atherosclerosis by Plasma Endotoxin Levels

	ETX*	p Value	ETX ≤50	ETX >50	p Value
All subjects, n = 466					
Cases, n (%)	—	—	208 (49)	31 (74)	—
Odds ratio (95% CI)					
Adjusted age/gender/baseline AS	1.4 (1.1–1.7)	0.005	1.0	2.9 (1.4–6.3)	0.005
Multivariate adjustment	1.3 (1.1–1.7)	0.016	1.0	3.3 (1.5–7.5)	0.003
Smokers/Ex-Smokers, n = 196					
Cases, n (%)	—	—	95 (53)	16 (84)	—
Odds ratio (95% CI)					
Adjusted age/sex/baseline AS	1.7 (1.2–2.3)	0.007	1.0	9.4 (2.3–39.8)	0.002
Multivariate adjustment	1.7 (1.2–2.5)	0.008	1.0	13.2 (2.9–61.3)	0.001

Odds ratios and 95% confidence intervals (CI) were derived from logistic regression analyses of incident atherosclerosis on endotoxin (ETX) levels or ETX categories and a variety of covariates (age, gender, baseline atherosclerosis, or in the multivariate analysis: age, gender, baseline atherosclerosis, hypertension, apolipoprotein AI, apolipoprotein B, pack-years of smoking, fibrinogen, ferritin, body mass index, alcohol consumption, and serum protein).
*Odds ratio calculated for a 1-SD unit change in plasma ETX; cases = subjects with incident atherosclerosis; AS = atherosclerosis.

In the risk analysis ln-transformed ETX concentrations were applied rather than native data to account for deviations from the normal distribution. The ln-ETX emerged as a significant and independent risk predictor of five-year incidence of carotid atherosclerosis. In an effort to characterize type and scale of association, analysis was repeated after categorizing ETX levels into 10 equally spaced groups (deciles). Figure 2 demonstrates a clear-cut binary-type association, with the excess risk confined to the highest decile (threshold, 50 pg/ml). All results remained virtually unchanged when subjects with prevalent cardiovascular disease (n = 47) and/or regular medication (n = 281) and/or gastrointestinal ulcers (n = 26) and/or malign neoplasm (n = 30) were excluded. Sample size does not allow potential modifying effects of aspirin treatment to be established.

As an outstanding finding, the association between endotoxemia and incident atherosclerosis was especially relevant to current and ex-smokers (Table 2, Fig. 3) ($p < 0.001$ for effect modification). In this subgroup of particular interest (n = 196) the scale-fitting procedure described above replicated the results obtained in the entire population sample (Fig. 2, lower panel), that is, revealed a clear-cut binary association with the threshold for the switch in atherosclerosis risk identified as 50 pg/ml (90th percentile). In current and ex-smokers, measurement of ETX permitted us to accurately identify subjects at high risk of incident (active) atherosclerosis. As visualized in Figure 4, age- and sex-adjusted incidence rates of atherosclerosis in smokers with ETX levels ≤50 pg/ml were no higher than those of non-smokers, whereas almost all smokers with ETX levels >50 pg/ml developed new lesions within the five-year follow-up period. Except for smoking, the association be-

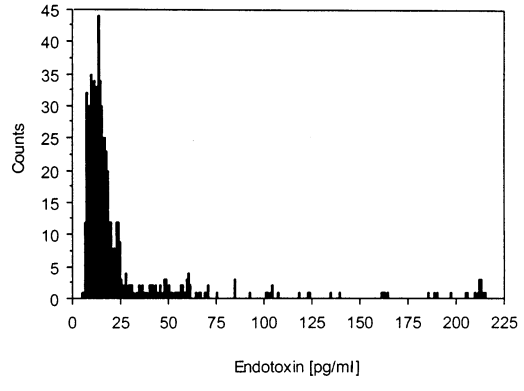

Figure 1. Frequency distribution of endotoxin levels (pg/ml) (n = 516).

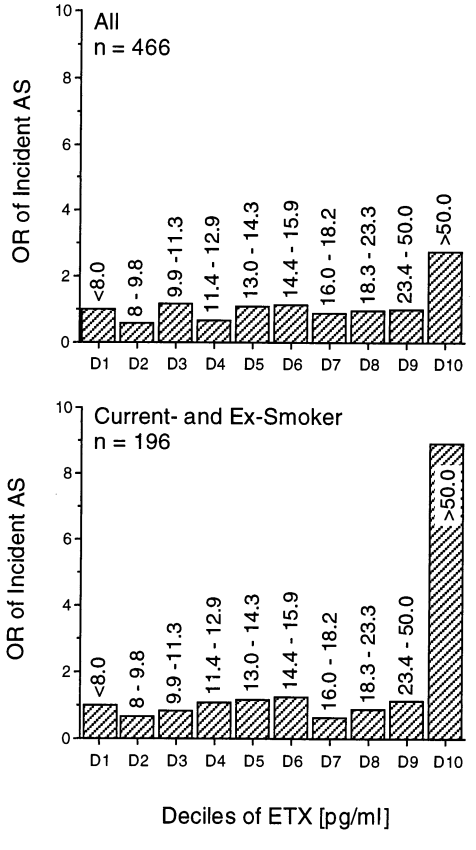

Figure 2. Binary-type association between endotoxin plasma level and atherosclerosis risk. OR = odds ratio; AS = atherosclerosis.

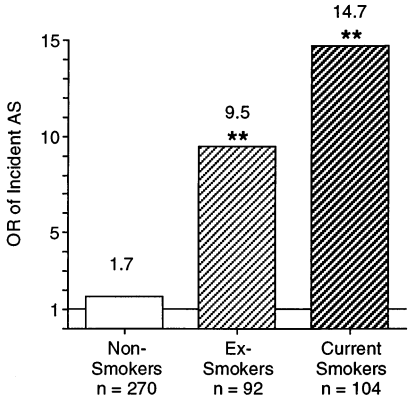

Figure 3. Risk of incident carotid atherosclerosis associated with endotoxin levels >50 pg/ml in nonsmokers, ex-smokers, and current smokers. **p < 0.01 for effect modification; OR = odds ratio; AS = atherosclerosis.

tween ETX and atherosclerosis was not appreciably different at distinct levels of vascular risk factors.

A total of 220 men and women showed laboratory and/or clinical evidence of one or more chronic bacterial infections and/or clinical conditions associated with frequent episodes of infections: recurrent urinary tract infections (n = 25), chronic bronchitis (n = 77), asthma (n = 15), emphysema (n = 141), recurrent upper respiratory tract infections (n = 9), chronic gastrointestinal infections (including *Helicobacter pylori*) (n = 7) and periodontal infections (n = 39). Mean serum c-reactive protein level was 0.32 mg/dl in subjects with ETX levels ≤50 pg/ml, and 0.64 mg/dl in subjects with ETX >50 pg/ml (p = 0.03). As expected, ETX level emerged as a prominent risk predictor of incident atherosclerosis in this subgroup. Predictive significance of high ETX according to smoking status and presence of infectious

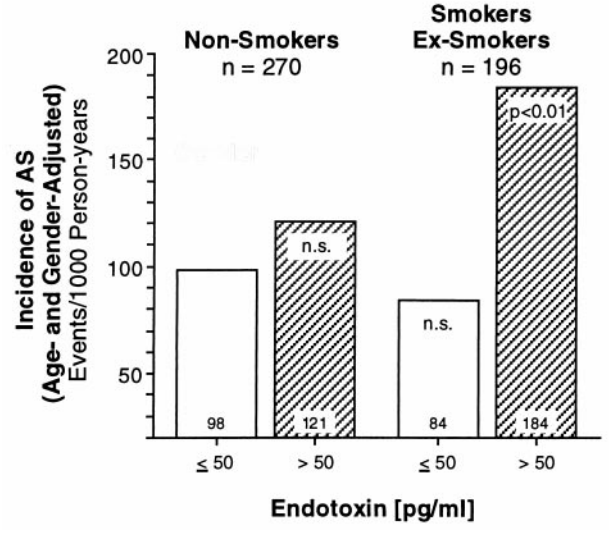

Figure 4. Age- and gender-adjusted incidence of carotid atherosclerosis according to endotoxin level and smoking status. AS = atherosclerosis. **p < 0.01; n.s. = not significant (vs. nonsmokers with endotoxin ≤50 pg/ml).

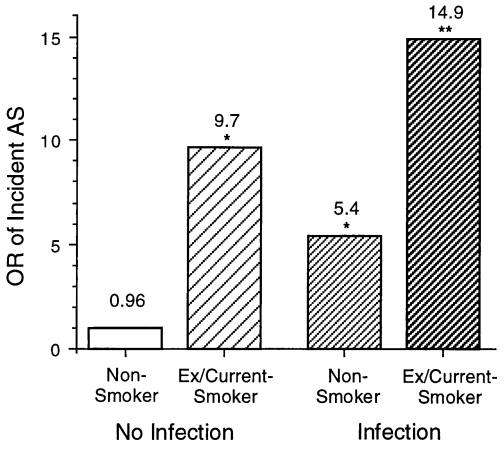

Figure 5. Risk of incident carotid atherosclerosis associated with endotoxin levels >50 pg/ml according to smoking status and presence of infectious diseases. The p values were derived from multivariate logistic regression analysis (*p < 0.05; **p < 0.01). OR = odds ratio; AS = atherosclerosis.

diseases is summarized in Figure 5. Highest priority was obtained for smokers with manifest infections, mainly smoker's bronchitis followed in descending order by smokers without evidence of infectious disease and nonsmoking subjects with bacterial infections. In subjects with ETX levels ≤50 pg/ml, chronic infection significantly increased risk of incident atherosclerosis (OR [odds ratio] = 1.6; p = 0.034).

Finally, once incident cardiovascular disease (n = 38 of 516) was substituted for incident atherosclerosis as the outcome measure, results were similar. Endotoxin level >50 pg/ml emerged as a significant risk predictor, with the association being more pronounced in smokers and subjects with infectious diseases (age/gender-adjusted hazard ratio at 95% CI [confidence interval], 2.3 [1.1 to 5.1]). Exclusion of men and women with cardiovascular disease prevalent at the 1990 baseline (n = 47) yielded similar results (age/gender-adjusted hazard ratio at 95% CI, 3.9 [1.2 to 12.7]). These results should be viewed in light of the comparatively low number of subjects with incident cardiovascular disease, which precludes multivariate adjustment and further subgroup analysis.

DISCUSSION

Infections and atherosclerosis. The possibility that infectious agents crucially contribute to early atherogenesis was first proposed in the late 1970s based on animal-experimental research (Mark's disease model) and recently experienced a resurgence of interest (10,21–24). Elevated level of C-reactive protein, an indicator of ongoing inflammation, was reported to predict a high risk of atherosclerotic disease in apparently healthy men (9,10,21,25). The bulk of seroepidemiologic studies supported an involvement of common chronic infections in atherogenesis (10,24). Special focus was on herpes viridae, gram-negative bacteria (*Helicobacter pylori*, *Chlamydia pneumoniae*) and chronic dental

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infections (2,10,26–30). Preliminary results from two interventional trials suggested beneficial effects of macrolid antibiotics on the course of coronary heart disease (31,32). All these studies, however, did not furnish proof that infections are causally related to atherosclerotic disease and not simply an epiphenomenon or a commensal without pathological relevance (24,33).

Lack of a consensus in this important field originates mainly from the fact that the nature of infectious attack against human vasculature remains obscure. Potential pathophysiological mechanisms include direct infection of the arterial wall (33,34), inference of acute-phase response with blood clotting (procoagulant state) and homocystein metabolism, and induction of autoimmunity (35–38). In addition, endotoxemia may cause endothelial dysfunction in the course of severe systemic infections, thus driving vasculature into a pro-atherogenic mode (response-to-injury hypothesis) (33,39). Lipopolysaccharides from *Escherichia coli* augment the expression of endothelial adhesion molecules and subsequent leukocyte adherence (40–42) and rank among the classic activators of cytokine production in atheroma (6,43,44). Further atherogenic properties include induction of intravascular coagulation and lowering of HDL, which is a natural scavenger of ETX (45). Exposure of human volunteers to ETX provokes prolonged stunning of the endothelium in vivo (5). In all, atherogenic capacity of bacterial ETX is experimentally well-founded but has so far attracted only limited attention for its putatively rare occurrence in human circulation (sepsis). A recent survey unexpectedly suggested a commonplace occurrence of, in part, high ETX levels in apparently healthy elderly subjects (7) and thus shed new light on the possibility of ETX involvement in atherogenesis.

Association of endotoxin with atherosclerosis. Our study is among the first to assess in a systematic fashion plasma levels of bacterial ETX in the general population. Blood specimens from virtually all subjects contained ETX though at highly variable concentrations (Fig. 1). A considerable proportion even exhibited levels higher than 50 pg/mL without evidence of underlying severe systemic illness. Endotoxemia originates from episodes of bacterial translocation (chronic infections or colonization), inhalation of ETX in cigarette smoke or gut barrier dysfunction (7,8). The assay system applied in the current study identifies lipopolysaccharides from chlamydia, *E. coli*, *Helicobacter* and *Haemophilus*, to name only a few (15), and it thus mirrors a broad spectrum of gram-negative bacteria. As an outstanding finding, baseline endotoxemia emerged as one of the strongest risk predictors of five-year incidence of carotid atherosclerosis and cardiovascular disease in our survey. The risk burden afforded by high ETX levels was mainly confined to levels beyond 50 pg/ml (90th percentile; Fig. 2) and applied to subjects with chronic infection and cigarette smoking (Table 2, Fig. 5). Notably, prediction significance of ETX declined in strength after smoking cessation but did not normalize within a five-year period (Fig. 3), which is in close agreement with a recent comparable cohort study (46).

This finding fits well into the concept that it is not smoking itself (exposure to nicotine and other smoke ingredients) but chronic bronchial infections and/or bacterial colonization that represent the actual atherogenic culprit. Elevation of C-reactive protein and clinical evidence of infection usually persist for several years after quitting (25). In this context, high coincidence of smoking and chronic chlamydial infections merits attention. The significance of chronic minor infections in the mediation of smoking effects on atherosclerosis is substantiated by our finding that the incidence of carotid atherosclerosis was no higher in current and ex-smokers with ETX concentrations lower than 50 pg/ml than in non-smokers (Fig. 4). All these subjects faced only a moderate base risk of atherosclerosis, whereas smokers and ex-smokers with high ETX almost obligatorily developed new atherosclerotic lesions (Fig. 4). In other words, ETX measurements permitted us to identify smokers at high risk of incident atherosclerosis in our population. The predictive significance of high ETX was more pronounced in smokers with manifest bronchitis but also extended to those without clinical (and laboratory) evidence of infectious disease (Fig. 5).

As anticipated, subjects with various chronic and recurrent bacterial infections constitute a second large population in which high ETX predicts a markedly elevated risk of incident carotid atherosclerosis. Assessment of infectious diseases, even when performed with maximum effort and best possible precision, remains subject to diagnostic inaccuracies. Results should be interpreted in the light of these potential limitations.

Finally, in nonsmokers without detectable infections, ETX levels higher than 50 pg/ml were rare and did not show a clear association with incident atherosclerosis. This may well be a matter of low statistical power but may also arise from poor estimation of long-term exposure to ETX by a single measurement in this subgroup.

Study limitations. Several limitations of our study deserve further consideration: first, there is no consensus on how to measure plasma ETX concentrations. The assay system applied (LAL) detects a broad spectrum of bacterial ETX and showed adequate performance in previous studies (15). It bears the disadvantage of undetermined interlaboratory variability and is not approved for use in clinical practice. Therefore, analyses have been performed on deciles of levels, thus enabeling reproducibility for other laboratories that may define their own risk ranges (i.e., upper decile of risk). Second, the level of ETX in human circulation is subject to substantial changes and consequently to high measurement variability. However, inaccuracies in assessing risk (independent) variables tend to weaken true relations rather than create spurious ones.

Conclusions. Endotoxemia is a promising candidate risk factor of atherosclerosis and cardiovascular disease and

probably represents a pathophysiological link in the association among cigarette smoking, chronic infections and atherosclerosis. Biological plausibility, consistency of results, strength of association and the prospective study design all advocate such an interpretation of our results. Nevertheless, the possibility that endotoxemia is only a surrogate for the actual (unknown) atherogenic agent in infectious disease cannot ultimately be ruled out.

Reprint requests and correspondence: Dr. Christian J. Wiedermann, Department of Internal Medicine, University of Innsbruck, Anichstrasse 35, A-6020 Innsbruck, Austria. E-mail: christian.wiedermann@uibk.ac.at.

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