Increased Risk of Atherosclerosis Is Confined to CagA-Positive *Helicobacter pylori* Strains

Prospective Results From the Bruneck Study

Manuel Mayr, MD; Stefan Kiechl, MD; Michael A. Mendall, MD; Johann Willeit, MD; Georg Wick, MD; Qingbo Xu, MD, PhD

Background and Purpose—Accumulating evidence indicates that a variety of infections contribute to the pathogenesis of atherosclerosis, but there is controversy concerning the impact of *Helicobacter pylori* infections in atherosclerosis.

Methods—We evaluated seropositivity to *H pylori* and to its cytotoxin-associated gene A (CagA) product in a large, prospective, population-based study (n=684). Intima-media thickness and atherosclerosis of carotid arteries were thoroughly assessed by high-resolution duplex scanning.

Results—In our study population, *H pylori* infections defined by seropositivity have no relationship with levels of classic cardiovascular risk factors or markers of systemic inflammation, except for elevated levels of immune reactions to mycobacterial heat shock protein 65. The latter showed a trend toward highest levels in those harboring virulent *H pylori* strains ($P=0.08$). Common carotid artery intima-media thickness—both absolute values and changes between 1995 and 2000—were significantly enhanced in subjects seropositive to CagA but not in those infected with CagA-negative *H pylori* strains. There was a clear dose-response relation between anti-CagA antibodies and both intima-media thickness and atherosclerosis risk. Notably, the risk of atherosclerosis associated with CagA seropositivity was amplified by elevated C-reactive protein levels.

Conclusions—Infections with virulent CagA-bearing *H pylori* strains may contribute to the pathogenesis of early atherosclerosis by aggravating immune-inflammatory reactions. **(*Stroke*. 2003;34:610-615.)**

Key Words: carotid arteries ■ *Helicobacter pylori* ■ infection ■ risk factors ■ seroepidemiologic studies

Seropositivity to *Helicobacter pylori* has been postulated to be a risk factor for cardiovascular and cerebrovascular disease.[1,2] However, from an epidemiological perspective, the role of *H pylori* in the pathogenesis of coronary artery disease remains controversial, and a recent meta-analysis revealed only limited evidence for a positive relationship.[3]

The virulence of pathogens may be a crucial determinant of its injurious and potential proatherogenic potencies. The most virulent *H pylori* strains bear a high-molecular-weight toxin, inducing vacuolation of gastric epithelial cells (VacA toxin). This toxin has the potential to cause severe damage to the gastric epithelium and is associated with an enhanced local inflammatory response. An immunodominant protein associated with VacA is the cytotoxin-associated gene A (CagA).[4] Seropositivity to CagA is widely used to detect infections with virulent *H pylori* strains. Significant associations of CagA-positive *H pylori* strains with coronary heart disease were reported previously in 3 small case-control studies[5–7] but could not be confirmed by larger-scale studies[8–11] in which a significant or nearly significant difference in the crude prevalence of CagA-positive strains between cases and controls was attenuated after adjustment for covariates. Studies focusing on cerebrovascular disease showed a preferential association of *H pylori* with atherothrombotic stroke,[2,12–14] but so far, only 1 study has discriminated between virulent and nonvirulent strains.[14]

All of these studies, however, had clinical end points. Because such advanced stages of vessel pathology differ substantially from early atherosclerosis with respect to underlying pathomechanisms and risk profiles,[15] these studies do not address whether CagA-positive *H pylori* strains contribute to the initiation and early progression of atherosclerosis.

In a prospective, population-based study, we previously demonstrated that all common types of chronic infections defined by clinical criteria are associated with early atherosclerosis.[16] In addition, seropositivity to certain bacteria correlated with lesion development in different vascular territories.[17] IgA antibodies to *Chlamydia pneumoniae* were most reliably associated with atherosclerosis. For anti–

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From the Institute for Biomedical Aging Research, Austrian Academy of Sciences (M.M., G. W., Q. X.), and Department of Neurology, University Clinic (S.K., J.W.), Innsbruck, Austria, and Mayday University Hospital, London, UK (M.A.M.).

Correspondence to Professor Qingbo Xu, Department of Cardiological Sciences, St George's Hospital Medical School, Cranmer Terrace, London SW17 0RE, UK. E-mail q.xu@sghms.ac.uk

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H pylori IgG, significant correlations were restricted to subjects of low social status, who are more likely to be infected with *H pylori* strains.[17]

Intima-media thickness (IMT) is a well-established surrogate and precursor of definite atherosclerosis.[18] The objective of the present study was to investigate whether virulent CagA-positive *H pylori* strains are those preferentially related to IMT and early stages of plaque development in carotid arteries.

Subjects and Methods

Subjects and Clinical Examination

Human sera were derived from the Bruneck Study, a large, prospective, population-based survey on the epidemiology and cause of atherosclerosis.[19,20] The survey area is located in northern Italy (Bolzano Province). The study population was recruited between July and November 1990 as an age- and sex-stratified random sample of all inhabitants of Bruneck 40 to 79 years of age (125 men and 125 women in the fifth to eighth decade each, n=1000). A total of 93.6% participated, with data assessment completed in 919 subjects. During follow-up (1990 to reevaluation in 1995 and 2000), a subgroup of 63 and 97 individuals died or moved away before 1995 and 2000, respectively. In the remaining population, follow-up was 96.5% (1995) and 93.8% (2000) complete; ie, 684 subjects remained for the present analysis. The nearly complete participation and follow-up rates provide a considerable safeguard against a potential selection bias.

All participants gave informed consent before entering the study. Subjects underwent a clinical examination with cardiological and neurological priority and completed standardized questionnaires on current and past exposure to candidate vascular risk factors as described previously.[19,20] Chronic infections were assessed by an extensive screening procedure as detailed previously.[16] Socioeconomic status was defined on a 3-category scale based on information about the occupational status of the person with the highest income in the household and the educational level of subjects.[15,16]

TABLE 1. Means and Proportions of Demographic, Vascular Risk, and Inflammation Variables According to the Seroprevalence of CagA$^-$ and CagA$^+$ *H pylori* Strains

	H.p.$^-$ (n=136)	H.p.$^+$/CagA$^-$ (n=285)	H.p.$^+$/CagA$^+$ (n=263)	*P*
Demographic variables				
Age, y	55.7	56.6	55.6	0.77
Female sex, n (%)	67 (49)	134 (47)	152 (58)*	0.04
Low social status, n (%)	63 (46)	174 (61)**	189 (72)**	0.005
Established risk factors				
LDL cholesterol, mmol/L	3.75	3.80	3.95	0.15
HDL cholesterol, mmol/L	1.46	1.46	1.51	0.48
Smoking, n (%)	20 (15)	46 (16)	45 (17)	0.86
Glucose, mmol/L	5.66	5.59	5.69	0.55
Systolic blood pressure, mm Hg	140.0	138.3	140.4	0.37
Diastolic blood pressure, mm Hg	83.9	83.7	84.1	0.84
Inflammation/infection				
C-reactive protein, mg/L	1.7	1.8	2.0	0.29
α_1-Antitrypsin, g/L	1.92	1.93	1.98	0.26
Soluble ICAM-1, ng/mL	313.9	327.5	323.6	0.39
E-selectin, ng/mL	51.0	54.1	55.2	0.21
Soluble VCAM-1, ng/mL	646.9	668.8	671.4	0.71
P-selectin, ng/mL	196.6	198.5	201.5	0.70
Fibrinogen, g/L	2.88	2.90	2.92	0.74
Ferritin, ng/mL	126.2	130.5	105.9	0.34
Homocysteine, mmol/L	14.3	13.6	12.4*	0.06
Folic acid, ng/mL	5.9	5.6	6.0	0.21
mHSP65 antibody, titer	83.1	97.5*	107.8**	0.029
Others				
γ-glutamyl transferase, U/L	36.9	38.2	45.3	0.58
Acetyl salicylic acid, n (%)	14 (10)	37 (13)	39 (15)	0.68
HMG-CoA reductase inhibitors, n (%)	14 (10)	29 (10)	24 (9)	0.82

Data presented are means or absolute numbers (n) and percentage; for C-reactive protein levels the geometric mean is reported. *P* values in the far right column are derived from an ANOVA (adjusted for age, sex, and social status). Pairwise comparison between category *H pylori*$^-$ (H.p.$^-$) and the two other categories were done with Scheffé's test: *$P<0.05$, **$P<0.01$. *P* values for categorical variables were derived from a chi-square test.

ICAM indicates intercellular adhesion molecule; VCAM, vascular cell adhesion molecule.

TABLE 2. Association of CagA Negative or Positive *H pylori* Strains With Intima-Media Thickness of Common Carotid Arteries (CCA-IMT 2000)

Infectious Status	CCA-IMT (mean±SD)		Difference in CCA-IMT	95% CI	*P*
H pylori neg. CagA neg. (n=136)	991 μm (±187)	Comparison group			
H pylori pos. CagA neg. (n=285)	986 μm (±184)	Differences in IMT (vs comparison group) adjusted for:			
		Age/sex	−4.7 μm	[−34.8 to 24.7]	0.725
		+ other variables*	−6.2 μm	[−34.8 to 22.4]	0.660
H pylori pos. CagA pos. (n=263)	1014 μm (±176)	Age/sex	+23.4 μm	[1.5 to 45.5]	0.040
		+ other variables*	+22.1 μm	[0.7 to 43.5]	0.045

*Multivariate adjustment was performed for age/sex/hypertension/smoking/LDL/HDL/ferritin/social status/chronic infection/fibrinogen/diabetes/alcohol consumption.

Laboratory Methods and Measurement of Anti–*H pylori*, Anti-CagA, and Anti–Mycobacterial Heat Shock Protein Antibodies by Enzyme-Linked Immunosorbent Assay

In each evaluation, blood samples were taken from the antecubital vein after subjects had fasted and abstained from smoking for ≥12 hours. In subjects with acute infections, blood drawing was delayed for at least 6 weeks. Laboratory parameters were examined by standard methods as extensively described previously.[16,19,20] C-reactive protein (CRP) concentrations were measured by the N High Sensitivity CRP assay (Dade Behring).

We used 2 commercial enzyme-linked immunosorbent assays (ELISAs; Dia.Pro) to detect IgG antibodies to *H pylori* and CagA in human sera. Antibody categories were defined in accordance with our previous publication (negative, 8, 16, 64, >64 AU/mL; see Reference 17). Total immunoglobulins against mycobacterial heat shock protein 65 (mHSP65) were determined according to an established protocol.[21] Measurements of anti-*H pylori*, anti-CagA, and anti-mHSP65 antibodies were part of the 1995 and 2000 evaluations. In all prospective analyses, means of antibody titers assessed at the beginning and end of the observation period were applied to determine the infectious load before and during the observation period simultaneously.

Scanning Protocol and Definition of Ultrasound End Points

The ultrasound protocol involved scanning of the internal (bulbous and distal segments) and common (proximal and distal segments) carotid arteries on either side with a 10-MHz imaging probe and 5-MHz Doppler.[19,20] Scanning was performed 3 times—in 1990, 1995, and 2000—by the same experienced sonographer who was unaware of the subjects' clinical and laboratory characteristics. Atherosclerotic lesions were defined by wall surface (protrusion into the lumen or roughness of the arterial boundary) and wall texture (echogenicity) as detailed previously.[19] Incident (early) atherosclerosis was defined by the occurrence of new plaques in previously normal segments (n=281, 1995 to 2000). IMT was measured in 1995 and 2000 at the far wall of common carotid arteries with the ultrasound beam directed through the axis of the vessel. It was defined as the distance between the lumen-intima interface and the leading edge of the media-adventitia interface.

Statistical Analyses

Means of vascular risk factors and other parameters in the 3 serological subgroups (HP^-, HP^+CagA^-, and HP^+CagA^+) were compared with 1-factorial analysis of variance (Table 1). Subsequent pairwise comparisons were done with Scheffé's test. The potential association of the 3 serological categories with magnitude and 5-year changes in common carotid artery IMT (IMT 2000 and ΔCCA-IMT 1995 to 2000, respectively) was assessed by means of linear regression analyses. A base model was adjusted for age and sex only. The multivariate model was additionally controlled for hypertension, smoking, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, ferritin, social status (3 categories), alcohol consumption, diabetes, fibrinogen levels, and clinical evidence of chronic infection (Tables 2 and 3). In separate equations, anti-CagA antibody concentration was treated as a continuous variable. These analyses were aimed at demonstrating a potential dose-response relation between immune reactivity to virulent *H pylori* strains and wall thickness (Table 4). Next, logistic regression models were built to analyze strength and type of association between antibody concentrations and prevalent/incident atherosclerosis (categorical outcome). The test procedure was based on maximum-likelihood estimators, and the goodness of fit of each model was assessed by the test of Hosmer and Lemeshow.[22] The logistic regression models adjusted for the same variables included in the linear regression model above except for inclusion of a baseline atherosclerosis score in the analysis of atherosclerosis incidence. Finally, we looked for a differential association between CagA

TABLE 3. Association of CagA Negative or Positive *H pylori* Strains With Changes in Intima-Media Thickness of Common Carotid Arteries (Δ CCA-IMT 1995–2000)

Infectious Status	Δ CCA-IMT (mean±SD)		Difference in ΔCCA-IMT	95% CI	*P*
H pylori neg. CagA neg. (n=189)	+70.2 μm (±164)	Comparison group			
H pylori pos. CagA neg. (n=244)	+61.9 μm (±164)	Differences in IMT (vs comparison group) adjusted for:			
		Age/sex	−6.3 μm	[−44.9 to 32.3]	0.751
		+ other variables*	−6.3 μm	[−45.4 to 32.8]	0.751
H pylori pos. CagA pos. (n=251)	+107.5 μm (±156)	Age/sex	+38.9 μm	[2.1 to 75.7]	0.034
		+ other variables*	+39.8 μm	[2.6 to 77.0]	0.037

*For adjustment see Table 2 footnote.

TABLE 4. Association of Anti-CagA Antibody Concentration and Seropositivity to CagA With Intima-Media Thickness of Common Carotid Arteries

Anti-CagA Antibodies	Adjustment	Difference in CCA-IMT	95% CI	*P*
Concentration (AU/ml)	Age/sex	+6.8 μm (per antibody category)	[0.2 to 13.5]	0.042
	+ other variables*	+5.5 μm (per antibody category)	[−1.2 to 12.2]	0.085
Seropositivity	Age/sex	+26.6 μm (<8 vs ≥8 AU/ml)	[9.3 to 48.9]	0.020
	+ other variables*	+21.5 μm (<8 vs ≥8 AU/ml)	[1.7 to 41.3]	0.038
Anti-CagA Antibodies	**Adjustment**	**Difference in ΔCCA-IMT**	**95% CI**	***P***
Concentration (AU/ml)	Age/sex	+11.9 μm (per antibody category)	[3.9 to 19.9]	0.004
	+ other variables*	+13.5 μm (per antibody category)	[5.3 to 21.7]	0.001
Seropositivity	Age/sex	+39.5 μm (<8 vs ≥8 AU/ml)	[15.6 to 64.6]	0.001
	+ other variables*	+44.4 μm (<8 vs ≥8 AU/ml)	[20.1 to 68.7]	<0.001

Differences in CCA-IMT and ΔCCA-IMT were calculated per change of 1 antibody category in anti-CagA concentration and in separate equations for seropositive vs seronegative subjects.

*For adjustment see Table 2 footnote.

CCA-IMT indicates intima-media thickness of common carotid arteries; ΔCCA-IMT, changes in intima-media thickness of common carotid arteries during the 5-year follow-up.

seropositivity and atherosclerosis risk in population subgroups defined by CRP, sex, and age (by inclusion of interaction terms).

Results

Seropositivity to *H pylori* and the virulence-associated *H pylori* antigen CagA was common in the Bruneck population (80% and 38.5% of subjects, respectively). Immunity to CagA strongly was correlated to anti–*H pylori* antibodies (only 13 subjects who were seronegative for *H pylori* were positive for CagA). Changes in anti–*H pylori* and anti-CagA antibody concentrations during the 5-year follow-up period were low: ≈80% of subjects remained in the same or adjacent antibody category.

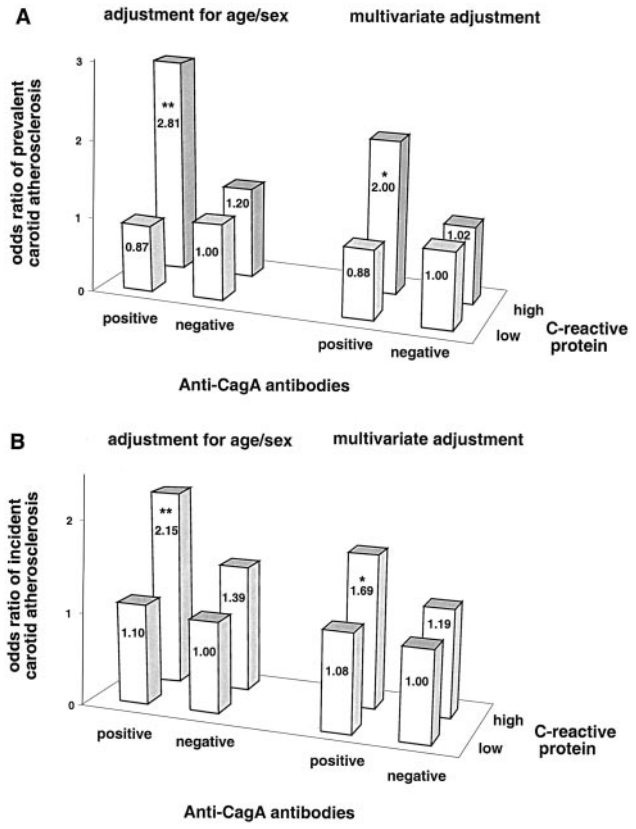
Table 1 depicts means and proportions of selected demographic characteristics and risk factors according to infectious status. Subjects did not differ in the levels of established vascular risk factors except for an overrepresentation of women in CagA-seropositive individuals. Notably, no difference in the levels of various inflammatory parameters was observed. However, anti-mHSP65 antibodies were significantly elevated in subjects seropositive to *H pylori* ($P=0.029$). Statistical analysis revealed a nearly significant trend toward even higher anti-mHSP65 antibody levels in CagA-positive compared with CagA-negative subjects ($P=0.08$).

In our study, the association of immune reactions to *H pylori* with IMT of the common carotid arteries (CCA-IMT 2000) was restricted to subjects seropositive to CagA (≥8 AU/mL; Table 2). Similarly, IMT significantly increased over a 5-year period among *H pylori*–positive individuals with but not in those without immune reactions to CagA (ΔCCA-IMT 1995 to 2000; Table 3). Results remained significant after adjustment for numerous risk factors, including age, sex, hypertension, smoking, LDL, HDL, ferritin and fibrinogen levels, social status, alcohol consumption, diabetes, and clinical evidence of chronic infections.

In separate analyses, anti-CagA antibodies were included as a continuous variable (antibody concentration in AU/mL). These equations yielded a significant dose-response relation between antibody categories and both CCA-IMT 2000 and changes of CCA-IMT over time (ΔCCA-IMT 1995 to 2000; Table 4).

The above findings extended to definite carotid atherosclerosis. High CagA antibody concentration (≥8 AU/mL) emerged as a (nearly) significant risk predictor of prevalent atherosclerosis. Prevalence rates adjusted for age and sex were as follows: $CagA^-$, 52.5%; $CagA^+$, 59.9%; age- and sex-adjusted odds ratio (OR), 1.48 (95% CI, 1.02 to 2.14; $P=0.040$); multivariate OR, 1.44 (95% CI, 1.00 to 2.07; $P=0.050$). Age- and sex-adjusted frequencies of incident carotid atherosclerosis (1995 to 2000) were as follows: $CagA^-$, 37.5%; $CagA^+$, 44.2%; OR adjusted for age and sex, 1.41 (95% CI, 1.01 to 2.03; $P=0.049$); multivariate OR, 1.36 (95% CI, 0.94 to 1.97; $P=0.107$).

The association between CagA antibody levels and atherosclerosis applied to both men and women and tended to be more pronounced in younger age groups. Notably, high CRP levels (≥66th percentile) and seropositivity to CagA appeared to synergistically affect atherosclerosis risk (Figure, A; $P=0.015$ and $P=0.046$ for effect modification in the age- and sex-adjusted and multivariate models). Prevalence of atherosclerosis in the subgroups given in Figure, A was as follows: $CagA^+CRP^+$, 71%; $CagA^-CRP^+$, 56%; $CagA^+CRP^-$, 51%; and $CagA^-CRP^-$, 53%. ORs of prevalent atherosclerosis for subjects with both risk conditions were 2.81 (95% CI, 1.58 to 5.00; $P=0.0004$, model adjusted for age and sex) and 2.00 (95% CI, 1.11 to 5.71; $P=0.021$, multivariate adjustment) (Figure, A). Corresponding data for the incidence of atherosclerosis in given subgroups were as follows: $CagA^+CRP^+$, 55%; $CagA^-CRP^+$, 45%; $CagA^+CRP^-$, 38%; $CagA^-CRP^-$, 39%. ORs of incident atherosclerosis for a coexistence of high CRP levels and CagA seropositivity were 2.15 (95% CI, 1.21 to 3.69; $P=0.008$, model adjusted for age and sex) and 1.69 (95% CI, 1.08 to 3.37; $P=0.035$, multivariate adjustment; Figure, B; $P=0.15$, effect modification for CRP and CagA after adjustment for age and sex).



Association of CagA seropositivity (≥8 AU/mL) and high CRP levels (>66th percentile) with risk of prevalent (A) and incident (B) carotid atherosclerosis. Values were obtained after adjustment for age and sex (left) or multivariate adjustment (right) as described in Subjects and Methods. Prevalence of atherosclerosis in the subgroups given in A: $CagA^+CRP^+$, 71%; $CagA^-CRP^+$, 56%; $CagA^+CRP^-$, 51%; $CagA^-CRP^-$, 53%. Corresponding data for incidence of atherosclerosis in B: $CagA^+CRP^+$, 55%; $CagA^-CRP^+$, 45%; $CagA^+CRP^-$, 38%; $CagA^-CRP^-$, 39%. *$P<0.05$, **$P<0.01$.

Discussion

To the best of our knowledge, the present study is the first prospective survey of anti-CagA antibodies and atherosclerosis. We provide evidence that infections with CagA-positive but not CagA-negative *H pylori* strains significantly increase the risk of early atherosclerosis in carotid arteries, suggesting that the association of *H pylori* infections with atherosclerosis is restricted to the more virulent genotype. There was a significant dose-response relation between anti-CagA antibodies and both IMT and atherosclerosis risk. Furthermore, anti-CagA antibodies were associated with changes in IMT and the occurrence of new lesions during the 5-year follow-up. These findings are consistent with a recent study demonstrating higher seroprevalences of anti-CagA antibodies in patients with atherosclerotic stroke.[14]

Infections with *H pylori* are thought to be restricted to the gastric mucosa, but recently, *H pylori* has been found in human atherosclerotic plaques by use of polymerase chain reaction and immunohistochemistry.[23–25] The presence of *H pylori* in atherosclerotic lesions was associated with increased expression of intercellular adhesion molecule-1.[25] Interestingly, anti-CagA antibodies show cross-reactivity with vascular wall antigens,[26] supporting a possible role of *H pylori* in inflammatory processes within the vessel wall.

We demonstrated previously that anti-mHSP65 antibodies are elevated in subjects with atherosclerosis,[21] are predictive of overall mortality,[27] and are strongly correlated with seropositivity to bacterial infections incriminated in atherosclerosis.[17] We now provide evidence that anti-mHSP65 antibody levels are on average higher in patients infected with virulent than nonvirulent *H pylori* strains. The enhanced immune reactions to mHSP65 may well be of pathogenetic relevance in early atherosclerosis. There are several clues for experimental evidence supporting such an interpretation. First, immunization with mHSP65 induces arteriosclerosis in normocholesterolemic rabbits.[28] Lesions were reversible after the immunization protocol was discontinued but become irreversible when the rabbits were fed a high-cholesterol diet. Second, lesion formation can be suppressed by simultaneous immunosuppressive treatment.[29] Third, serum antibodies to mHSP65 show partial cross-reactivity to human HSP60 and mediate cytotoxicity on stressed endothelial cells.[30,31] These findings tempt us to speculate that CagA-positive *H pylori* strains might enhance the atherosclerotic process by inducing a persistent, low-grade inflammatory response in the intima of the arterial wall with increased immunity to mHSP65.[32]

According to previous publications, CagA seropositivity is not linked to an increased systemic inflammatory response.[8] However, among subjects seropositive to CagA, there was a clear tendency for atherosclerosis risk to increase when CRP levels were elevated. Subjects with high CRP levels tend to have a higher risk of atherosclerosis if exposed to infectious agents.[16,17,33] CRP serves as a pattern-recognition molecule in innate immunity. It may directly contribute to a proinflammatory state in atheroma by inducing adhesion molecule expression on endothelial cells, stimulating cytokine release of monocytes, and activating the complement cascade.[34] Alternatively, high CRP levels may identify subjects capable of producing a prominent inflammatory response to pathogens and other stress factors. This capacity has a complex genetic control and was recently shown to enhance the risk of atherosclerosis.[35]

Possible limitations of our study are as follows. First, although seropositivity to CagA is widely used as a surrogate of infections with toxic *H pylori* strains, individuals seronegative to CagA may still be infected with virulent *H pylori* expressing VacA in culture. Second, the results of the Bruneck study may not necessarily apply to other populations with a different prevalence of *H pylori* infection.

In summary, we report here the first prospective, population-based study demonstrating that infections with CagA-positive but not CagA-negative *H pylori* strains significantly increase the risk of carotid atherosclerosis, and we provide evidence that this association is more pronounced in subjects with an enhanced immune inflammatory response.

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