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. 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.

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). 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 but could not be confirmed by larger-scale studies 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, but so far, only 1 study has discriminated between virulent and nonviral strains.

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, 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. In addition, seropositivity to certain bacteria correlated with lesion development in different vascular territories. IgA antibodies to Chlamydia pneumoniae were most reliably associated with atherosclerosis. For anti-
Intragastric IgG, significant correlations were restricted to subjects of low social status, who are more likely to be infected with \textit{H pylori} strains.\textsuperscript{17}

Intima-media thickness (IMT) is a well-established surrogate and precursor of definite atherosclerosis.\textsuperscript{18} The objective of the present study was to investigate whether virulent \textit{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.\textsuperscript{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; i.e., 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. Chronic infections were assessed by an extensive screening procedure as detailed previously.\textsuperscript{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.\textsuperscript{15,16}

**TABLE 1. Means and Proportions of Demographic, Vascular Risk, and Inflammation Variables According to the Seroprevalence of CagA\textsuperscript{*} and CagA\textsuperscript{+} \textit{H pylori} Strains**

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

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 \textit{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.
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).

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.
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 CagA 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 3.71; 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).

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

<table>
<thead>
<tr>
<th>Anti-CagA Antibodies</th>
<th>Adjustment</th>
<th>Difference in CCA-IMT</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (AU/ml) Age/sex</td>
<td>+6.8 μm (per antibody category)</td>
<td>[0.2 to 13.5]</td>
<td>0.042</td>
<td></td>
</tr>
<tr>
<td>Seropositivity Age/sex</td>
<td>+5.5 μm (per antibody category)</td>
<td>[−1.2 to 12.2]</td>
<td>0.085</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Anti-CagA Antibodies</th>
<th>Adjustment</th>
<th>Difference in ΔCCA-IMT</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (AU/ml) Age/sex</td>
<td>+11.9 μm (per antibody category)</td>
<td>[3.9 to 19.9]</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td>Seropositivity Age/sex</td>
<td>+13.5 μm (per antibody category)</td>
<td>[5.3 to 21.7]</td>
<td>0.001</td>
<td></td>
</tr>
</tbody>
</table>

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.

CagA-positive compared with CagA-negative subjects (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 3.71; 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).
with vascular wall antigens, 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, indicating overall mortality, and are strongly correlated with seropositivity to bacterial infections incriminated in atherosclerosis. We now provide evidence that anti-mHSP65 antibodies 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. 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. Third, serum antibodies to mHSP65 show partial cross-reactivity to human HSP60 and mediate cytotoxicity on stressed endothelial cells. 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.

According to previous publications, CagA seropositivity is not linked to an increased systemic inflammatory response. 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. 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. 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.

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 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.

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. The presence of *H. pylori* in atherosclerotic lesions was associated with increased expression of intercellular adhesion molecule-1. Interestingly, anti-CagA antibodies show cross-reactivity
References


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