

Serum Pepsinogens and *Helicobacter pylori* in Relation to the Risk of Esophageal Squamous Cell Carcinoma in the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study

Michael B. Cook¹, Sanford M. Dawsey¹, Lena Diaw², Martin J. Blaser³, Guillermo I. Perez-Perez³, Christian C. Abnet¹, Philip R. Taylor¹, Demetrius Albanes¹, Jarmo Virtamo⁴, and Farin Kamangar^{1,5}

Abstract

Background: *Helicobacter pylori* can induce gastric atrophy in humans, which in turn increases gastric cancer risk. Whether *H. pylori* and gastric atrophy also affect the risk of esophageal squamous cell carcinoma (ESCC), however, remains unresolved.

Methods: We performed a nested case-control study within the prospective Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study to assess these relationships. The Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study is composed of 29,133 Finnish male smokers, ages 50 to 69 years, who were recruited during 1985-1988. Using baseline sera, we assessed *H. pylori* status (via immunoglobulin G antibodies against whole-cell and CagA antigens) and gastric atrophy status [via the biomarkers pepsinogen I (PGI) and pepsinogen II (PGII)] in 79 ESCC cases and 94 controls. Logistic regression with adjustment for age, date of blood draw, education, cigarette smoking, alcohol, body mass index, and fruit and vegetable intake was used to estimate odds ratios (OR) and 95% confidence intervals (95% CI).

Results: Gastric atrophy (PGI/PGII <4) was associated with ESCC (OR, 4.58; 95% CI, 2.00-10.48). There was no evidence for an association between *H. pylori* and ESCC (OR, 0.94; 95% CI, 0.40-2.24).

Conclusions: These results could be explained by misclassification of *H. pylori* status due to serologic amnesia, ESCC risk being dependent on the functional consequences or interactions of *H. pylori* rather than the infection per se, gastric atrophy having a different histogenesis in ESCC without being primarily dependent on *H. pylori* acquisition, or a lack of statistical power to detect an effect.

Impact: Validation of these results may warrant mechanistic studies to determine the route of association between gastric atrophy and ESCC. *Cancer Epidemiol Biomarkers Prev*; 19(8); 1966-75. ©2010 AACR.

Introduction

During the last 40 years, incidence trends and global patterns of esophageal adenocarcinoma and esophageal squamous cell carcinoma (ESCC) have indicated that there are strong environmental risk factors that we are yet to eluci-

date. Esophageal adenocarcinoma has been rapidly increasing in many predominantly Caucasian, developed countries of the western world while ESCC has been declining (1, 2). Many countries in east and central Asia have not observed such trends and squamous cell carcinoma remains the principal esophageal malignancy reported (3-5).

Helicobacter pylori is a Gram-negative bacterium that has been colonizing the gastric epithelium of humans since at least before their migration from east Africa 58,000 years ago (6). This bacterium colonizes approximately half of the world population, but its prevalence in developed countries has been declining (7). In 1994, the IARC categorized *H. pylori* as a group I carcinogen (8) due to overwhelming evidence of its association with noncardia gastric adenocarcinoma (7). Its association with ESCC, however, is uncertain; a recent systematic review and meta-analysis endorsed the null hypothesis, albeit with a high degree of heterogeneity, and with study-specific statistically significant associations in both directions (9). A subgroup analysis of western studies showed a statistically significant association with ESCC, although this used a broader definition of *H. pylori* positivity and included only four studies.

Authors' Affiliations: ¹Division of Cancer Epidemiology and Genetics, National Cancer Institute and ²Pulmonary and Vascular Medicine Branch, National Heart, Lung, and Blood Institute, NIH, Department of Health and Human Services, Bethesda, Maryland; ³Departments of Medicine and Microbiology, School of Medicine, New York University, New York, New York; ⁴Department of Chronic Disease Prevention, National Institute for Health and Welfare, Helsinki, Finland; and ⁵Department of Public Health Analysis, School of Community Health and Policy, Morgan State University, Baltimore, Maryland

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Corresponding Author: Michael Blaise Cook, Division of Cancer Epidemiology and Genetics, National Cancer Institute, NIH, Department of Health and Human Services, 6120 Executive Boulevard, EPS/Suite 550/Room 5030, Bethesda, MD 20852-7234. Phone: 301-496-1613; Fax: 301-402-0916. E-mail: michael.cook@nih.gov

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Persistent *H. pylori* colonization promotes the induction of gastric atrophy, which is considered to be part of the causal pathway of intestinal-type gastric adenocarcinoma (10, 11). Pepsinogens are aspartic proteinases that are predominantly produced and secreted into the gastric lumen by gastric epithelial cells. They can be categorized into two groups: pepsinogen I (PGI), which is produced only by the gastric fundic mucosa, and pepsinogen II (PGII), which is produced by the fundic, cardiac, and antral mucosae of the stomach and also by the duodenal mucosa (12). The progression of gastric atrophy leads to reduced fundic chief cell mass, which decreases PGI (13). PGII, however, stays relatively stable or may undergo a slight increase (13). Therefore, the PGI/PGII ratio is a sensitive and specific biomarker of gastric atrophy (14, 15).

Prior studies that have assessed gastric atrophy in relation to ESCC risk came to conflicting conclusions (16-21). Evidence for a causal relationship has been limited because four of these six studies used a case-control design (16, 17, 20, 21). Therefore, we designed a nested case-control study within the prospective Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) Study to assess whether *H. pylori* and gastric atrophy were associated with risk of ESCC in this cohort.

Methods

ATBC Study

The rationale, design, and results of the ATBC Study have been described in detail (22). In brief, the ATBC Study was a randomized, double-blind, placebo-controlled, 2 × 2 factorial primary prevention trial that tested whether daily supplementation with α -tocopherol (50 mg) and/or β -carotene (20 mg) could reduce the incidence of lung and other cancers. A total of 29,133 male smokers, ages 50 to 69 years and living in southwestern Finland, were recruited from 1985 to 1988. The intervention concluded on April 30, 1993, but the participants continued to be followed as a cohort. The ATBC Study was approved by the institutional review boards of the National Cancer Institute, Bethesda, Maryland, and the National Public Health Institute, Helsinki, Finland. All subjects provided written informed consent. At the pre-randomization baseline visit, study participants completed questionnaires on demographic characteristics and provided information about their medical, dietary, and smoking history. Their weights and heights were measured by trained study staff. Blood samples were collected from participants at two time points: at baseline (1985-1988) and 3 years after randomization. The sera were stored at -70°C . All sera used for this analysis were collected at baseline.

Study subjects and serum pepsinogen and *H. pylori* measurement

Cases were individuals who were diagnosed with ESCC in the ATBC Study through April 30, 2005. ESCC was defined according to the International Classification

of Diseases, 9th Revision (23) code 150, and the International Classification of Diseases for Oncology, 2nd Edition (24) codes 8050-8078. Eligible cases were required to have at least 200 μL of serum available for analysis. Controls were matched to cases on age at randomization (± 5 years) and date of blood draw (± 30 days) and were required to be alive and cancer-free until the date of cancer diagnosis of their matched case.

Serum PGI and PGII were assayed blinded to case-control status in duplicate in the same batch using ELISA (Biohit ELISA kit). The averages of the duplicate values for PGI and for PGII were used for analysis. Duplicate results were strongly correlated: Pearson's correlation coefficient was 0.998 for PGI and 0.997 for PGII. In addition, 15 quality control samples, aliquoted from a single large pool of serum from ATBC Study participants, were distributed among the five assay plates (three samples per plate). On the basis of these samples, the coefficients of variation were 6.0% and 8.7% for PGI and PGII, respectively.

H. pylori seropositivity was assessed using immunoglobulin G antibodies against *H. pylori* whole-cell and CagA antigens by ELISA, as described (25). Seropositivity was defined as an absorbance ratio of ≥ 1.0 for the whole-cell antigen assay and ≥ 0.35 for the CagA antigen assay. Serum samples were assayed in duplicate in the same batch blinded to case-control status. Fifteen quality control serum samples, aliquoted from a single large pool, were equally distributed among the five batches. On the basis of these samples, the coefficients of variation for *H. pylori* whole-cell and CagA antigen assays were 7.8% and 11.8%, respectively.

Statistical analysis

Serum PGI, serum PGII, and the ratio of these biomarkers (PGI/PGII) were analyzed as continuous, dichotomous, and ordinal (quartiles) variables. For the continuous analyses, a single unit increase was equal to half the distance between the 25th and 75th percentile values (the average size of the two central quartiles) of

Table 1. Descriptive characteristics of controls and ESCC cases nested in the ATBC Study

| Variable | Controls | ESCC |
|--|---------------|---------------|
| Total, <i>n</i> | 94 | 79 |
| Mean age, y (SD) | 58.1 (4.8) | 57.7 (4.6) |
| Mean years of smoking (SD) | 36.7 (8.5) | 37.1 (8.2) |
| Mean cigarettes/day (SD) | 20.1 (10.3) | 21.3 (7.4) |
| Alcohol, g ethanol/d (SD) | 16.6 (17.3) | 30.4 (29.7) |
| Education—post-elementary school, <i>n</i> (%) | 16 (17.0) | 11 (13.9) |
| BMI (SD) | 26.4 (3.9) | 25.6 (4.0) |
| Fruit, g/d (SD) | 268.7 (247.5) | 189.7 (182.4) |
| Vegetables, g/d (SD) | 319.6 (114.0) | 278.0 (105.8) |

Table 2. Association of pre-diagnostic serum pepsinogens and *H. pylori* with subsequent ESCC in the ATBC Study

| Analysis | ESCC | | | | | | | |
|--|--------------------|-------|-------------------|--------|----------------|-------|-------------------|--------|
| | Minimally adjusted | | | | Fully adjusted | | | |
| | Controls | Cases | OR (95% CI) | P | Controls | Cases | OR (95% CI) | P |
| Pepsinogen I | | | | | | | | |
| PgI continuous per-unit decrease* | 93 | 79 | 1.43 (1.13-1.81) | 0.003 | 89 | 72 | 1.49 (1.13-1.96) | 0.004 |
| PgI >75th percentile (152) | 23 | 9 | Reference | | 21 | 8 | Reference | |
| PgI ≤75th percentile (152) | 70 | 70 | 2.52 (1.08-5.87) | 0.033 | 68 | 64 | 3.37 (1.19-9.54) | 0.022 |
| PgI >50th percentile (106) | 46 | 25 | Reference | | 44 | 23 | Reference | |
| PgI ≤50th percentile (106) | 47 | 54 | 2.13 (1.13-4.03) | 0.020 | 45 | 49 | 2.09 (1.01-4.33) | 0.047 |
| PgI >70 | 74 | 51 | Reference | | 71 | 45 | Reference | |
| PgI ≤70 | 19 | 28 | 2.22 (1.10-4.48) | 0.025 | 18 | 27 | 2.37 (1.07-5.23) | 0.033 |
| PgI >50 | 81 | 56 | Reference | | 78 | 50 | Reference | |
| PgI ≤50 | 12 | 23 | 2.86 (1.29-6.30) | 0.009 | 11 | 22 | 3.68 (1.51-8.97) | 0.004 |
| PgI >30 | 86 | 61 | Reference | | 83 | 54 | Reference | |
| PgI ≤30 | 7 | 18 | 3.63 (1.42-9.29) | 0.007 | 6 | 18 | 5.12 (1.79-14.67) | 0.002 |
| PgI ≥151.85 | 23 | 9 | Reference | | 21 | 8 | Reference | |
| PgI ≥105.30 and <151.85 | 24 | 17 | 1.77 (0.65-4.79) | 0.260 | 24 | 16 | 2.65 (0.80-8.80) | 0.111 |
| PgI ≥73.58 and <105.30 | 23 | 23 | 2.50 (0.94-6.64) | 0.066 | 22 | 20 | 3.00 (0.94-9.59) | 0.063 |
| PgI <73.58 | 23 | 30 | 3.40 (1.30-8.86) | 0.012 | 22 | 28 | 4.62 (1.44-14.81) | 0.010 |
| Pepsinogen II | | | | | | | | |
| PGII Continuous per unit* | 93 | 79 | 1.01 (0.87-1.18) | 0.891 | 89 | 72 | 0.99 (0.82-1.21) | 0.943 |
| PGII >50th percentile (16.45) | 47 | 35 | Reference | | 45 | 30 | Reference | |
| PGII ≤50th percentile (16.45) | 46 | 44 | 1.27 (0.69-2.36) | 0.440 | 44 | 42 | 1.58 (0.79-3.18) | 0.197 |
| PGII <10.57 | 23 | 12 | Reference | | 21 | 11 | Reference | |
| PGII ≥10.57 and <16.45 | 23 | 32 | 2.64 (1.08-6.41) | 0.032 | 23 | 31 | 2.86 (1.04-7.86) | 0.041 |
| PGII ≥16.45 and <22.43 | 24 | 13 | 1.04 (0.39-2.80) | 0.938 | 23 | 13 | 0.98 (0.33-2.98) | 0.978 |
| PGII ≥22.43 | 23 | 22 | 1.87 (0.74-4.73) | 0.186 | 22 | 17 | 1.57 (0.53-4.65) | 0.416 |
| Pepsinogen I/pepsinogen II ratio | | | | | | | | |
| PgI/PgII ratio Continuous per unit decrease* | 93 | 79 | 1.52 (1.23-1.87) | <0.001 | 89 | 72 | 1.48 (1.17-1.87) | 0.001 |
| PgR >75th percentile (8.85) | 23 | 9 | Reference | | 22 | 9 | Reference | |
| PgR ≤75th percentile (8.85) | 70 | 70 | 2.76 (1.17-6.51) | 0.020 | 67 | 63 | 1.86 (0.74-4.69) | 0.187 |
| PgR >50th percentile (6.75) | 46 | 24 | Reference | | 44 | 23 | Reference | |
| PgR ≤50th percentile (6.75) | 47 | 55 | 2.28 (1.21-4.29) | 0.011 | 45 | 49 | 1.89 (0.94-3.80) | 0.073 |
| PgI/PgII ratio >5 | 68 | 40 | Reference | | 65 | 38 | Reference | |
| PgI/PgII ratio ≤5 | 25 | 39 | 2.70 (1.42-5.13) | 0.002 | 24 | 34 | 2.50 (1.20-5.18) | 0.014 |
| PgI/PgII ratio >4 | 79 | 46 | Reference | | 76 | 43 | Reference | |
| PgI/PgII ratio ≤4 | 14 | 33 | 4.11 (1.98-8.54) | <0.001 | 13 | 29 | 4.58 (2.00-10.48) | <0.001 |
| PgI/PgII ratio >3 | 82 | 56 | Reference | | 79 | 49 | Reference | |
| PgI/PgII ratio ≤3 | 11 | 23 | 3.10 (1.39-6.91) | 0.006 | 10 | 23 | 4.32 (1.74-10.71) | 0.002 |
| PgI/PgII ratio >2 | 87 | 63 | Reference | | 84 | 56 | Reference | |
| PgI/PgII ratio ≤2 | 6 | 16 | 3.62 (1.34-9.81) | 0.011 | 5 | 16 | 5.16 (1.70-15.66) | 0.004 |
| PgI/PgII ratio >3 or PgI >70 | 84 | 61 | Reference | | 81 | 54 | Reference | |
| PgI/PgII ratio ≤3 and PgI ≤70 | 9 | 18 | 2.76 (1.15-6.61) | 0.023 | 8 | 18 | 3.70 (1.40-9.84) | 0.009 |
| PgI/PgII ratio >2 or PgI >30 | 88 | 63 | Reference | | 85 | 56 | Reference | |
| PgI/PgII ratio ≤2 and PgI ≤30 | 5 | 16 | 4.40 (1.53-12.70) | 0.006 | 4 | 16 | 6.28 (1.90-20.77) | 0.003 |
| PgI/PgII ratio ≥8.85 | 23 | 9 | Reference | | 22 | 9 | Reference | |
| PgI/PgII ratio ≥6.75 and <8.85 | 23 | 15 | 1.81 (0.65-5.06) | 0.260 | 22 | 14 | 1.24 (0.41-3.78) | 0.702 |
| PgI/PgII ratio ≥4.86 and <6.75 | 23 | 17 | 2.00 (0.73-5.48) | 0.177 | 22 | 16 | 1.35 (0.45-4.08) | 0.595 |
| PgI/PgII ratio <4.86 | 24 | 38 | 4.35 (1.70-11.16) | 0.002 | 23 | 33 | 2.98 (1.07-8.33) | 0.037 |

(Continued on the following page)

Table 2. Association of pre-diagnostic serum pepsinogens and *H. pylori* with subsequent ESCC in the ATBC Study (Cont'd)

| Analysis | ESCC | | | | | | | |
|--|--------------------|-------|------------------|-------|----------------|-------|------------------|-------|
| | Minimally adjusted | | | | Fully adjusted | | | |
| | Controls | Cases | OR (95% CI) | P | Controls | Cases | OR (95% CI) | P |
| Pepsinogen I and pepsinogen II categorical | | | | | | | | |
| High PGI (>106) and high PGII (>16.45) | 32 | 19 | Reference | | 30 | 17 | Reference | |
| Low PGI (<106) and high PGII (>16.45) | 15 | 16 | 1.77 (0.71-4.40) | 0.218 | 15 | 13 | 1.62 (0.57-4.59) | 0.365 |
| High PGI (>106) and low PGII (≤16.45) | 14 | 6 | 1.03 (0.31-3.45) | 0.964 | | | | |
| Low PGI (≤106) and low PGII (≤16.45) | 32 | 38 | 2.03 (0.95-4.34) | 0.068 | 30 | 36 | 2.42 (1.00-5.86) | 0.051 |
| <i>Helicobacter pylori</i> | | | | | | | | |
| Negative | 20 | 14 | Reference | | 19 | 14 | Reference | |
| Positive | 71 | 64 | 1.34 (0.62-2.89) | 0.462 | 68 | 57 | 0.94 (0.40-2.24) | 0.892 |
| Negative | 20 | 14 | Reference | | 19 | 14 | Reference | |
| Whole cell positive and CagA negative | 29 | 29 | 1.50 (0.63-3.57) | 0.362 | 27 | 25 | 0.89 (0.33-2.40) | 0.816 |
| CagA positive | 42 | 35 | 1.23 (0.54-2.80) | 0.622 | 41 | 32 | 0.98 (0.39-2.44) | 0.959 |

NOTE: ORs and 95% CIs in the minimally adjusted models were estimated using unconditional logistic regression models with adjustment for the matching variables of age at randomization and date of blood draw. The fully adjusted models also included the covariates years of smoking, cigarettes per day, alcohol, education (dichotomous), BMI, fruit intake, and vegetable intake. Unless otherwise specified, these exposures were modeled as continuous variables.

*Each continuous unit was equal to half the distance between the 25th and 75th percentile values in the control subjects, which translated to units of 38.99 µg/L for PGI, 5.93 µg/L for PGII, and 2.00 for the PGI/PGII ratio. All PGI and PGII units are in micrograms per liter.

control subjects (19, 26). The units of such were 38.99 µg/L for PGI, 5.93 µg/L for PGII, and 2.00 for the PGI/PGII ratio. For the dichotomous analyses, the cutoff points were the 50th percentile of the control distribution for PGI and PGII and the value of 4 for PGI/PGII, the latter of which we have used in previous pepsinogen analyses (19, 26). We also conducted additional dichotomous analyses of PGI using the cutoff points of the 75th percentile of the control distribution, 70 µg/L, 50 µg/L, and 30 µg/L; analyses of PGI/PGII using the cutoff points of the 75th percentile of the control distribution, the 50th percentile of the control distribution, and the ratios 5, 3, and 2; and analyses of two combinations of PGI/PGII ratios and PGI values, including a PGI/PGII ratio of 3 and a PGI of 70 µg/L, and a PGI/PGII ratio of 2 and a PGI of 30 µg/L. These analyses were designed to assess the full range of the pepsinogen distributions, to provide results for comparison to previous publications, and for use in future meta-analyses. For the ordinal analyses, pepsinogen categories were based on quartiles of the control distributions.

H. pylori status was assessed as a dichotomous variable, whereby an individual was classed as exposed if the assay for antibodies to whole cell or CagA was positive (27). We also analyzed *H. pylori* as a categorical variable, comparing whole cell-positive/CagA-negative individuals and CagA-positive individuals to the reference group (whole cell negative/CagA negative).

Serum pepsinogens and *H. pylori* were assessed in relation to risk of ESCC using conditional logistic regres-

sion to estimate odds ratios (OR) and 95% confidence interval (95% CI). We also conducted unconditional logistic analyses adjusted for the matching factors of age of randomization and date of blood draw. Fully adjusted estimates were also ascertained from models that included the additional covariates of education (dichotomous), duration and intensity of cigarette smoking, alcohol, body mass index (BMI), fruit consumption, and vegetable consumption. Unless otherwise specified, these exposures were modeled as continuous variables. Estimates derived from conditional and unconditional logistic regression models were similar; thus, only the results from the unconditional models are presented herein as this method allowed inclusion of a greater number of individuals. We also examined the shape of the association between pepsinogens and ESCC using nonlinear models adjusted for all covariates via PROC GAM in SAS. After fitting the generalized additive model, we plotted the ORs of ESCC on the logarithmic scale versus PGI, PGII, and PGI/PGII ratio on the linear scale. Lastly, we conducted some exploratory analyses of the effect of the primary exposures on ESCC risk stratified by cancer site [upper (ICD9 150.3), middle (150.4), and lower (150.5) esophagus]; tertiles of time between date of blood draw and cancer diagnosis; alcohol consumption dichotomized by the median alcohol consumption per day of controls; and cigarette smoking dichotomized by median pack-years of controls. The first two of these exploratory analyses were adjusted only for the matching

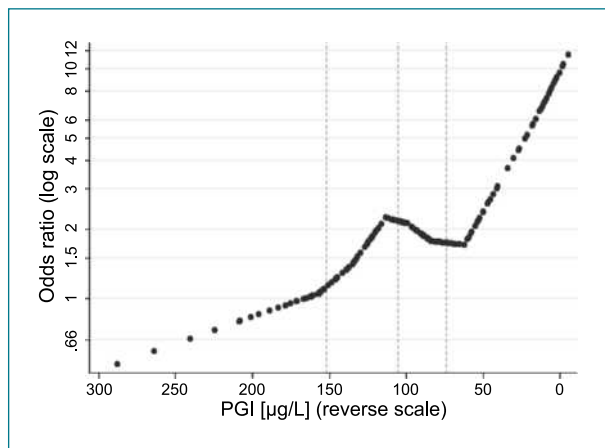


Figure 1. Association between PGI and the risk of ESCC. PGI ($\mu\text{g/L}$) was plotted on a reverse linear scale and the odds of ESCC was plotted on a logarithmic scale. The vertical lines demarcate the 25th, 50th, and 75th percentiles of PGI in control subjects.

variables due to small numbers, and the latter two analyses were fully adjusted. In addition, product-term models, with continuous metrics centered (28), were conducted to assess potential multiplicativity (29) between *H. pylori* and alcohol consumption and between *H. pylori* and cigarette smoking in relation to ESCC. Two-sided *P* values of <0.05 were considered to be statistically significant. All analyses were conducted using STATA version 10.1 (Stata-Corp LP) and SAS version 9.2 (SAS Institute).

Results

The descriptive characteristics of ESCC cases and controls are shown in Table 1. There were no differences in age, years of smoking, cigarettes per day, or BMI. On average, ESCC cases consumed more alcohol, received

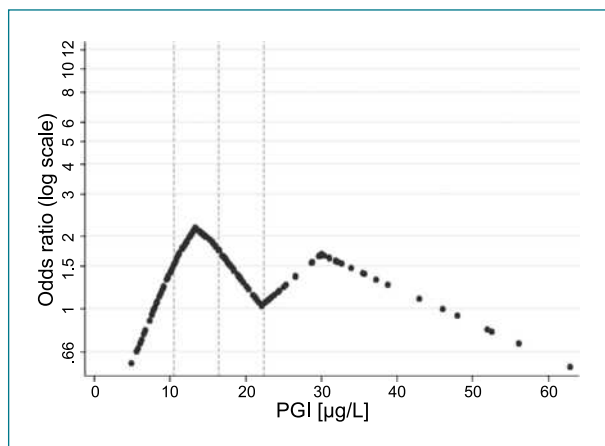


Figure 2. Association between PGII and the risk of ESCC. PGII ($\mu\text{g/L}$) was plotted on a linear scale and the odds of ESCC was plotted on a logarithmic scale. The vertical lines demarcate the 25th, 50th, and 75th percentiles of PGII in control subjects.

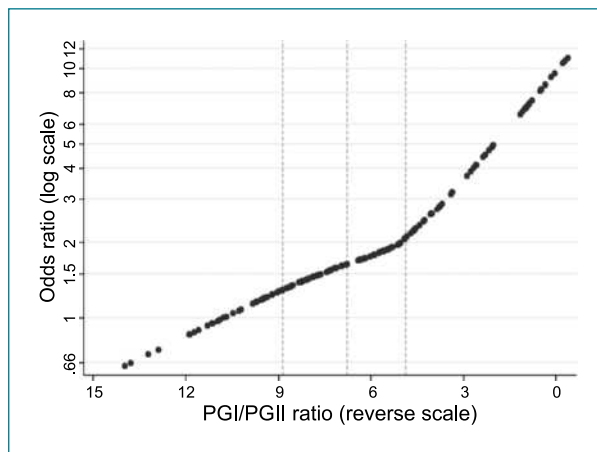


Figure 3. Association between PGI/PGII ratio and the risk of ESCC. The PGI/PGII ratio was plotted on a reverse linear scale and the odds of ESCC was plotted on a logarithmic scale. The vertical lines demarcate the 25th, 50th, and 75th percentiles of the PGI/PGII ratio in control subjects.

less formal education, and consumed less fruit and vegetables, compared with controls. The median time between date of randomization and date of diagnosis for the ESCC cases was 9.94 years.

Table 2 shows the results of the logistic regression analyses. PGI was found to be inversely associated with ESCC risk (OR, 1.49 per unit decrease; 95% CI, 1.13-1.96). When dichotomized using the 50th percentile of the control distribution (152 $\mu\text{g/L}$), the risk of ESCC was increased 2-fold in the lower PGI category compared with the higher PGI category. Moreover, risk estimates increased in magnitude in a monotonic manner as the PGI cutoff point decreased, culminating in an OR of 5.12 (95% CI, 1.79-14.67) for individuals with a PGI value of ≤ 30 $\mu\text{g/L}$. The ordinal analysis of PGI also supported an association with ESCC risk [OR_{Q2}, 1.77 (95% CI, 0.65-4.79); OR_{Q3}, 2.50 (95% CI, 0.94-6.64); OR_{Q4}, 3.40 (95% CI, 1.30-8.86)]. The results for PGII were mostly null. These observations are strengthened by plots of the relationship of PGI and PGII with ESCC risk (Figs. 1 and 2).

The PGI/PGII ratio was, like PGI, found to be inversely associated with ESCC risk. When analyzed as a continuous metric, the OR per unit decrease was 1.48 (95% CI, 1.17-1.87). A PGI/PGII ratio ≤ 4 was associated with a 4.5-fold increased risk of ESCC (OR, 4.58; 95% CI, 2.00-10.48) and this risk increased with more stringent (lower) cutoff points. The highest risk was observed when using the combined cutoff point of PGI/PGII ratio ≤ 2 and PGI ≤ 30 ; these individuals had an increased ESCC risk of 6.28 (95% CI, 1.90-20.77). When analyzed by quartiles, only the lowest quartile (PGI/PGII ratio <4.86) was associated with an increased risk of ESCC. However, the various dichotomous analyses and the generalized additive model of the relationship (Fig. 3) indicate that the relationship between PGI/PGII and ESCC is monotonic.

Analysis of a combined, categorical variable of PGI and PGII, defined using the 50th percentiles of the control distributions, found that only the category of low PGI and low PGII was associated with risk of ESCC (OR, 2.42; 95% CI, 1.00-5.86), as may be expected given the previously discussed results.

H. pylori was not associated with ESCC (OR, 0.94; 95% CI, 0.40-2.24). Similarly, null estimates were obtained for whole cell-positive/CagA-negative and CagA-positive groups, compared with individuals negative for *H. pylori* (Table 2).

Lastly, exploratory analyses of pepsinogens stratified by ESCC site (upper, middle, and lower thirds) provided similar estimates of association across sites (Supplementary Table S1). Exploratory analyses of ESCC risk stratified by time between blood draw and cancer diagnosis suggested a stronger association between *H. pylori* seropositivity and ESCC in the longest time category (>13.24 years; OR, 3.45; 95% CI, 0.74-16.01) relative to estimates of the middle (>7.89 and ≤13.24 years; OR, 1.07; 95% CI, 0.37-3.07) or shortest (≤7.89 years; OR, 0.90; 95% CI, 0.31-2.59) time categories (Supplementary Table S2). However, this highest risk did not reach statistical significance, the numbers for analysis were fairly modest, and the derived estimates were only minimally adjusted. Exploratory analyses stratified by median alcohol consumption (Supplementary Table S3), median pack-years of cigarette smoking (Supplementary Table S4), and product-term models of such ($P = 0.128$ and $P = 0.844$, respectively) indicated no evidence for multiplicativity of risk.

Discussion

This case-control study, nested within the prospective ATBC Study, provides evidence that serologic biomarkers of gastric atrophy are associated with risk of ESCC. PGI levels and the PGI/PGII ratio were both statistically significantly related to ESCC when analyzed as continuous, dichotomous, and ordinal metrics. The full range of analyses and nonlinear modeling of relationships indicated that PGI and PGI/PGII ratio have fairly monotonic relationships with ESCC risk. Thus, gastric atrophy may play a role in the pathogenesis of ESCC, although no association was found with *H. pylori*, the primary inducer of gastric atrophy.

The first indication of a potential relationship between gastric atrophy and ESCC was provided in 1979 when a case-series of 97 esophageal cancer patients found 63 cases to harbor moderate to marked atrophic gastritis (30). In 1993, a second study confirmed this observation (31), and a Swedish cohort study noted a 3-fold increased risk of esophageal cancer (88% of which was ESCC) in individuals with pernicious anemia, an ailment associated with severe gastric atrophy (32).

Subsequent to these initial observations, six studies (16-21) have formally tested the hypothesis that gastric atrophy is associated with ESCC (Table 3). As can be seen, all studies that assessed this relationship, whether using serology, histology, or endoscopic appearance for ascertainment of gastric atrophy, found evidence for

an association with ESCC, which, in totality, provides convincing evidence that the association is real. It remains less clear, however, whether this relationship is causal or results from confounding. If increased severity of gastric atrophy were associated with increased risk of ESCC, this would strengthen the case for the association being causal. Indeed, this is what we found in the current study from the statistical models and graphical representations of the association between PGI, PGI/PGII ratio, and ESCC. Yokoyama et al. (20) also found evidence for a monotonic pattern of association between serum pepsinogens and ESCC in a Japanese population. Further evidence for this pattern has also been provided by a previous study that we conducted in China (26) in which we showed a monotonic association between PGI/PGII ratio and esophageal squamous dysplasia, which is a known precursor lesion of ESCC (33, 34). Conversely, there is evidence from serum pepsinogen analyses that do not support a monotonic pattern with ESCC risk (refs. 17, 19; Table 3); although it should be noted that the results from Iijima et al.'s (17) ordinal analysis of PGI/PGII⁶ are not too dissimilar to the equivalent analysis contained herein, and more extensive scrutiny of the data could prove informative.

Table 3 also shows that some studies have used other proxy measures to estimate atrophic severity: endoscopy for ascertainment of maximal atrophic extent and histopathology to ascertain whether an individual harbors intestinal metaplasia or dysplasia. Both of these measures are likely to be inferior relative to serum pepsinogens as proxies of atrophic severity. Endoscopic assessment of atrophic extent is a subjective measurement, and it can be difficult to delineate between atrophic and normal tissues using standard endoscopic visualization (35). Histologic diagnosis is subject to sampling bias and inconsistent interpretation (36, 37), both of which may cause misclassification and bias results toward the null. Conversely, serum pepsinogens are an objective measure that provides a global representation of the extent of gastric atrophy.

The Dutch study (18) reasoned that because ESCC risk did not increase with gastric carcinogenic progression (gastric atrophy to intestinal metaplasia to dysplasia), this was evidence for noncausality. Although the degree of intestinal metaplasia, using the Sydney Classification scheme, and the number of gastric biopsies positive for intestinal metaplasia have been correlated with severity of atrophic gastritis (38, 39), it has not, to our knowledge, been shown that gastric carcinogenic progression (gastric atrophy to intestinal metaplasia to dysplasia) is similarly correlated. In addition, atrophic gastritis is likely to be sufficient for an increased risk of ESCC regardless of intestinal metaplasia and dysplasia status, especially given that the sites of exposure and outcome are distinct.

⁶ Personal communication: Dr. K. Iijima, Division of Gastroenterology, Tohoku University Graduate School of Medicine, Japan.

Table 3. Studies that have formally tested the relationship between gastric atrophy, severity of gastric atrophy, and ESCC

| First author | Year of publication | Population | Study design | Controls/ controls | Cases (n) | Ascertainment of gastric atrophy | Effect of gastric atrophy on ESCC risk |
|--------------|---------------------|-----------------|---------------------|-----------------------|--------------|---------------------------------------|--|
| Ye, W | 2004 | Sweden | Case-control | 499 | 85 | PGI | ↑ |
| Iijima, K | 2007 | Japan | Case-control | 73 | 73 | PGI/histology (1 biopsy) | ↑/↑ |
| de Vries, AC | 2009 | The Netherlands | Cohort | 97,728 | 126 | Histology (variable biopsy number) | ↑ |
| Ren, JS | 2009 | China | Case-cohort | 1,050 | 300 | PGI/PGII | ↑ |
| Yokoyama, A | 2009 | Japan | Case-control | 99 | 180 | PGI/PGII | ↑ |
| Akiyama, T | 2009 | Japan | Case-control | 253 | 253 | — | — |
| Cook, MB | 2010 | Finland | Nested case-control | 93 | 79 | PGI/PGII | ↑ |

(Continued on the following page)

The Dutch study (18), however, presents the most persuasive evidence that the relationship between gastric atrophy and ESCC could be the result of confounding. In addition to their analyses of ESCC, they reported an association between gastric atrophy and small-cell lung carcinoma, a relationship which may be difficult to reconcile biologically. In our analyses, adjustment for the major risk factors of ESCC did not change the results, although we cannot rule out the possibility of residual confounding, a problem inherent to all observational epidemiologic studies. Further studies are needed to clarify the issue of causality in the relationship between gastric atrophy and ESCC risk.

We found no association between *H. pylori* and ESCC, in agreement with the results of a recent meta-analysis (9). This lack of association is not necessarily contradictory to our pepsinogen results; there are at least three pos-

sible explanations. First, by the age of entry into this study, *H. pylori* colonization may have burned itself out, by inducing gastric atrophy and thus destroying the environment in which it thrives, which could result in serologic amnesia (40). However, we found no difference in the estimates for the association of *H. pylori* and ESCC risk when we stratified the analysis by time between blood draw and cancer diagnosis (Supplementary Table S2); even in those with serum drawn with the shortest time period between blood draw and cancer diagnosis, 20 of 26 individuals were still *H. pylori* seropositive. A second alternative is that ESCC risk could be dependent on the functional consequences or interactions of *H. pylori*, rather than its presence per se; for example, whether it is able to induce hypochlorhydria (41) or whether an additional exposure, such as alcohol (42), increases the likelihood of atrophy, although an analysis stratified by alcohol

Table 3. Studies that have formally tested the relationship between gastric atrophy, severity of gastric atrophy, and ESCC (Cont'd)

| Risk estimates | Ascertainment of atrophic severity | Effect of increasing gastric atrophy severity on ESCC risk | Risk estimates |
|---|--|--|--|
| PGI <28 vs PGI 28-158: OR, 4.3 (95% CI, 1.9-9.6) | — | — | — |
| PGI <25 vs PGI ≥25: OR, 8.2 (95% CI, 2.2-30.1) | PGI*/histology (1 biopsy; GA/IM) | Null/↑ | PGI quartiles*: OR _{Q1} , reference; OR _{Q2} , 0.5 (95% CI, 0.1-2.0); OR _{Q3} , 0.7 (95% CI, 0.2-2.8); OR _{Q4} , 7.6 (95% CI, 1.6-35.7) |
| Histologic atrophy: OR, 4.2 (95% CI, 1.5-11.7) | — | — | Gastric atrophy: OR, 4.2 (1.5-11.7) Intestinal metaplasia: OR, 10.7 (95% CI, 2.3-50.4) |
| Histologic atrophy: OR, 2.2 (95% CI, 1.8-2.6) | Histology (variable biopsy number; GA/IM/DYS) | Null | Gastric atrophy: OR, 2.3 (95% CI, 1.6-3.3) Intestinal metaplasia: OR, 2.1 (95% CI, 1.7-2.6) Dysplasia: OR, 2.3 (95% CI, 1.4-4.0) |
| PGI/PGII ≤4 vs PGI/PGII >4: OR, 1.6 (95% CI, 1.0-2.0) | PGI/PGII | Null | PGI/PGII ≤3 vs PGI/PGII >3: OR, 1.1 (95% CI, 0.6-2.0) PGI/PGII ≤4 vs PGI/PGII >4: OR, 1.6 (95% CI, 1.0-2.0) PGI/PGII ≤5 vs PGI/PGII >5: OR, 1.4 (95% CI, 1.0-2.0) PGI/PGII ≤6 vs PGI/PGII >6: OR, 1.1 (95% CI, 0.8-1.5) |
| PGI <30 and PGI/PGII <2 μg/L vs PGI ≥30 or PGI/PGII ≥2: OR, 3.2 [†] (95% CI, 1.6-6.4) | PGI/PGII | ↑ | PGI <70 and PGI/PGII <3 μg/L vs PGI ≥70 or PGI/PGII ≥3: OR, 1.5 [†] (95% CI, 0.9-2.6) |
| — | Endoscopic extent of GA | ↑ | GA open-type 2 or 3 vs all other GA types or no GA: OR, 1.6 (95% CI, 1.0-2.4) |
| See Table 2 herein | PGI/PGII | ↑ | See Table 2 and figures herein |

Abbreviations: GA, gastric atrophy; IM, intestinal metaplasia; DYS, dysplasia.

*Personal communication: Dr. K. Iijima, Tohoku University Graduate School of Medicine, Japan, December 2009.

[†]Crude ORs were estimated by the current authors from data presented in Yokoyama et al. 2009, Table 1.

consumption (Supplementary Table S3) and a product-term model of our data ($P = 0.128$) indicated no evidence for multiplicativity. A third alternative is that gastric atrophy/hypochlorhydria could have a different histogenesis in ESCC and may not be primarily dependent on *H. pylori* acquisition. A subset of patients with atrophic gastritis are *H. pylori* negative (43, 44), and *Acinetobacter lwoffii* is known to cause gastric atrophy in mice (45). However, in the present study, only a single case and no controls were *H. pylori* negative and had a PGI/PGII ratio of <4.

Regardless of the contribution of *H. pylori* to ESCC carcinogenesis, the evidence indicates that gastric atrophy and hypochlorhydria (41) are associated with an increased risk of ESCC, but the mechanistic link remains to be elucidated. Bacterial overgrowth and duodenal

reflux are possible consequences of atrophy that may play a role. Quantitative and qualitative changes in the microbiota of the gut resulting from hypochlorhydria (46, 47) could cause increased N-nitrosation reactions, exerting carcinogenic effects on the esophageal mucosa (10, 11). Duodenal reflux is also known to be carcinogenic (48), but requires both gastroesophageal and pyloric sphincters to be defective. Distally located ESCC has been associated with severe hypochlorhydria (41) and gastrectomy (49, 50), the latter of which vastly increases the propensity for duodenal reflux (51). Countering this are our exploratory analyses that provided similar estimates of association between gastric atrophy and ESCC across esophageal sites (upper, middle, and lower thirds; Supplementary Table S1). However, there is also

evidence from animal models (52-55) and human epidemiologic studies (56-59) that other squamous cell carcinomas of the upper gastrointestinal tract may also be associated with duodenal reflux.

Strengths of our study include the prospective design with long-term follow-up; use of prediagnostic serum for analysis of PGI, PGII, and *H. pylori* antibodies; use of continuous, dichotomous, and ordinal models to interrogate associations; and the availability of covariate information for adjustment of potential confounders. Limitations include modest sample size, a population that includes only male smokers, the possibility of residual confounding, and imperfect correlation between pepsinogen metrics and the true severity of gastric atrophy. Residual confounding could be manifested in the microbiome, which is known to adjust to changes in gastric pH and may also be associated with ESCC risk.

In summary, we have shown that biomarkers of gastric atrophy (PGI and PGI/PGII ratio) are associated with risk of ESCC in the ATBC Study. We also find no evidence to suggest that *H. pylori*, per se, is causal in this relationship. Studies investigating the mechanism relat-

ing gastric atrophy and resultant hypochlorhydria to ESCC risk are warranted.

Disclosure of Potential Conflicts of Interest

M. Blaser has a potential royalty interest in CagA diagnostics (licensed by Vanderbilt University) based on his discovery of CagA.

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