A Serological Biopsy Using Five Stomach-Specific Circulating Biomarkers for Gastric Cancer Risk Assessment: A Multi-Phase Study

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OBJECTIVES: **We aimed to assess a serological biopsy using five stomach-specific circulating biomarkers—pepsinogen I (PGI), PGII, PGI/II ratio, anti-*Helicobacter pylori (H. pylori)* antibody, and gastrin-17 (G-17)—for identifying high-risk individuals and predicting risk of developing gastric cancer (GC).**

METHODS: **Among 12,112 participants with prospective follow-up from an ongoing population-based screening program using both serology and gastroscopy in China, we conducted a multi-phase study involving a cross-sectional analysis, a follow-up analysis, and an integrative risk prediction modeling analysis.**

RESULTS: **In the cross-sectional analysis, the five biomarkers (especially PGII, the PGI/II ratio, and *H. pylori* sero-positivity) were associated with the presence of precancerous gastric lesions or GC at enrollment. In the follow-up analysis, low PGI levels and PGI/II ratios were associated with higher risk of developing GC, and both low (<0.5 pmol/l) and high (>4.7 pmol/l) G-17 levels were associated with higher risk of developing GC, suggesting a J-shaped association. In the risk prediction modeling analysis, the five biomarkers combined yielded a C statistic of 0.803 (95% confidence interval (CI)=0.789–0.816) and improved prediction beyond traditional risk factors (C statistic from 0.580 to 0.811, *P*<0.001) for identifying precancerous lesions at enrollment, and higher serological biopsy scores based on the five biomarkers at enrollment were associated with higher risk of developing GC during follow-up (*P* for trend <0.001).**

CONCLUSIONS: **A serological biopsy composed of the five stomach-specific circulating biomarkers could be used to identify high-risk individuals for further diagnostic gastroscopy, and to stratify individuals' risk of developing GC and thus to guide targeted screening and precision prevention.**

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/ajg

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INTRODUCTION

Gastric cancer (GC) is the fifth most common cancer and the third leading cause of cancer deaths worldwide (1). It is well accepted that gastric adenocarcinoma, especially the intestinal type, is preceded by a prolonged *Helicobacter pylori* (*H. pylori*)-driven precancerous process (Correa's model (2)). Gastroscopy (with biopsies) is commonly accepted as the gold standard for detecting GC (3). However, gastroscopy use is limited by its invasiveness and an insufficient supply of skilled endoscopists and endoscopy facilities, even in highly developed countries such as Japan (3). Therefore, there is an urgent need for biomarker-based risk stratification so that limited gastroscopy resources can be allocated to high-risk individuals.

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Pepsinogen I (PGI), PGII, and gastrin-17 (G-17) are products of terminally differentiated gastric mucosa, and anti-*H. pylori* antibodies indicate the reaction of gastric mucosa to an exogenous pathogen. More than 30 years ago, Samloff *et al.* (4) first proposed that serum levels of PGI, PGII, and the PGI/II ratio could reflect the morphology and function of gastric mucosa, serving as a "serological biopsy". In the early 1990s, Miki *et al.* (5,6) promoted the use of a noninvasive serum PG test, and then combined it with the detection of anti-*H. pylori* IgG (so-called ABCD method) for GC risk assessment (7,8). Meanwhile, Sipponen *et al.* (9) pioneered serum G-17 as another marker of morphology and function of the gastric mucosa and advocated a joint test of serum PGs, anti-*H. pylori* IgG, and G-17 for a comprehensive evaluation of the morphologic and functional status of gastric mucosa (10,11).

Although a serological biopsy of gastric mucosa using stomach-specific circulating biomarkers, including PGI, PGII, the PGI/II ratio, anti-*H. pylori* antibodies, and G-17, has been applied for detecting GC and precancerous lesions for over 20 years (3,12,13), its practical utility in GC risk assessment has been controversial due to highly varied accuracy in different regions of the world. Most previous reports mainly focused on PGI, the PGI/II ratio, and anti-*H. pylori* antibodies while neglecting other biomarkers such as PGII and G-17. Also, most previous investigations were cross-sectional, but prospective studies on their performance for assessing GC risk are limited. In addition, most previous studies assessed individual biomarkers or combinations of a subset of these five biomarkers, but there is a lack of joint evaluations of the five.

In 1997, we started the Zhuanghe Gastric Diseases Screening Program, an ongoing population-based screening program using both serological tests and gastroscopy in a high GC risk area in northern China (14,15). To investigate a possible role of a serological biopsy using the five stomach-specific circulating biomarkers (PGI, PGII, the PGI/II ratio, anti-*H. pylori* IgG, and G-17) in GC risk assessment, we conducted a multi-phase study among a large cohort of participants in the Zhuanghe Gastric Diseases Screening Program. We first conducted a cross-sectional analysis to assess the associations of circulating biomarkers with the presence of precancerous gastric lesions and GC, then a follow-up analysis to assess their associations with risk of developing GC, and finally an integrative prediction modeling analysis for identifying individuals at high risk of developing GC.

METHODS

Study population

The study population selection and recruitment process of the Zhuanghe Gastric Diseases Screening Program is summarized in **Figure 1**. In brief, the program selected 50 geographically representative villages in Zhuanghe County, a rural county of northern China that has been noted for its high GC mortality since 1984 (16). The Zhuanghe Gastric Diseases Screening Program was initiated in 1997 to investigate the etiology of GC and to reduce the burden of gastric diseases in Zhuanghe County in northern China that has been ongoing since 1997. The program targets all residents in these 50 villages who are 35–70 years old or who have upper gastrointestinal symptoms (including abdominal bloating, heartburn, acid reflux, nausea, hiccups, belching, decreased appetite, and stomachache) or a positive family history of GC. The screening program is ongoing, but for the present analysis, we did not include participants recruited after 2012 to ensure adequate follow-up time. By the end of 2012, 17,844 adults had voluntarily participated in the screening program. After excluding those who migrated out of Zhuanghe County (n=1,763), those with invalid follow-up information (n=15), and those who did not have complete biomarker measurements (n=3,954), 12,112 participants remained for analysis. Among them, 9,002 participants underwent gastroscopy with biopsies for gastric histopathological evaluation and were included in the cross-sectional analysis. For the follow-up analysis, we further excluded those who had cancer diagnosed at enrollment (n=94), leaving 12,018 for the final analysis. Interviewer-administered questionnaires were used to collect information on age, sex, lifestyle (e.g., smoking and alcohol consumption), family history of GC among first degree relatives, and upper gastrointestinal symptoms.

Case ascertainment

Case ascertainment was conducted by three complementary methods: (1) Study participants were linked to the Zhuanghe Cancer Registry files and Zhuanghe Death Registry files between 1997 and 2013. Cancer cases were diagnosed based on histological discharge forms and oncology reports, and classified according to the International Classification of Diseases (ICD-10). Details of case ascertainment methods were reported previously (17). Zhuanghe Cancer Registry is part of the WHO Cancer Incidence in Five Continents project with well-established cancer registry quality. (2) In order to minimize potential under-reporting of cancer cases and deaths and to identify participants who had migrated out of Zhuanghe County, we conducted a separate active follow-up at the end of 2013 by directly contacting study participants. The active follow-up was led and supervised by Zhuanghe Gastric Diseases Screening Program investigators, and a questionnaire was specifically designed to ask information including past diagnosis of cancer and other diseases, diagnosis date, diagnosis hospital, vital status, and cause of death. Zhuanghe Center for Disease Control and Prevention (CDC) was responsible for implementing the active follow-up and training 117 local village doctors who conducted in-person interviews with the study participants or relatives of deceased participants. The completed questionnaires were double checked by 32 Zhuanghe CDC employees for completeness and logical errors. (3) Follow-up gastroscopies with biopsies for gastric histopathological evaluation were conducted on 2,845 high-risk participants to identify incident GC cases. Follow-up endoscopic examination was usually done within one or two years of the initial endoscopic examination. During a median of 11.6 years and 113,234 person-years of follow-up, 86 newly-developed GC cases (median time to diagnosis of 6.3 years) were recorded as of 12/31/2013.

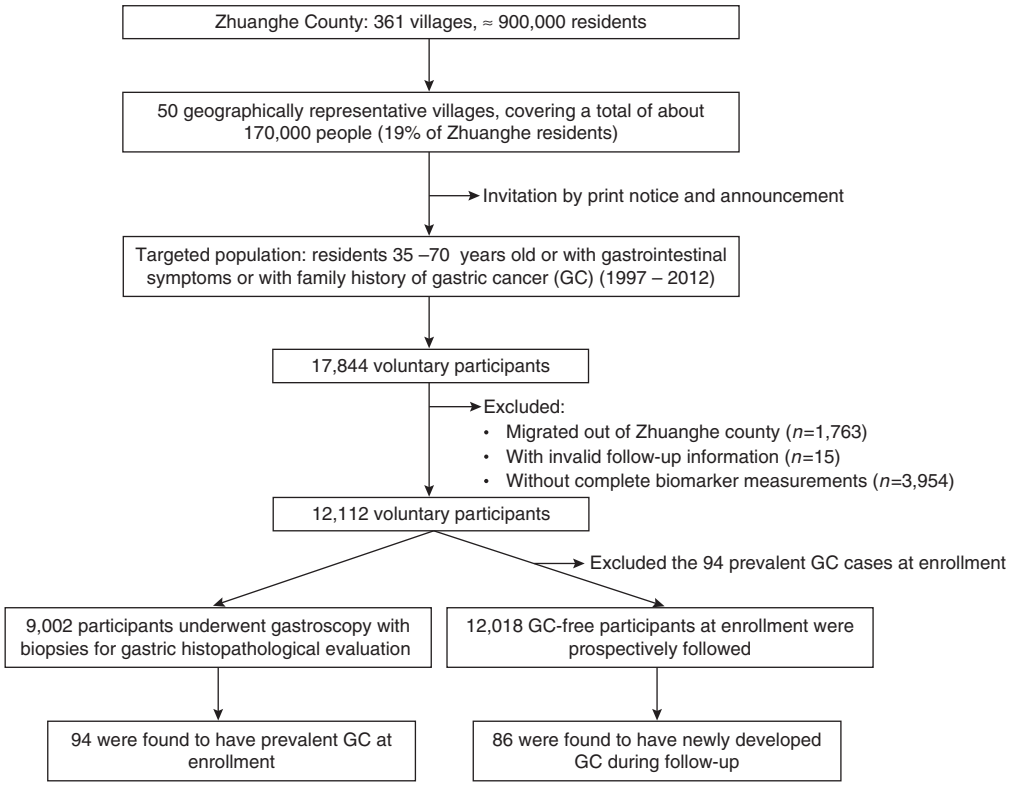

Figure 1. Flow diagram of included and excluded participants.

Serological measurements and endoscopic and histopathological examinations

Details on the serological measurements and endoscopic and histopathological examination procedures were previously described (18,19). Serum PGI, PGII, anti-*H. pylori* IgG, and G-17 concentrations in morning fasting blood samples were measured using enzyme-linked immunosorbent assays (ELISAs; Pepsinogen I ELISA; Pepsinogen II ELISA; *H. pylori* IgG ELISA; and Gastrin-17 ELISA kit, BIOHIT Plc, Helsinki, Finland).

Mucosal biopsies were obtained from the gastric body (around 8 cm from the cardia in the greater curvature and around 4 cm from the angulus in the lesser curvature), angulus (in the lesser curvature), antrum (around 2–3 cm from the pylorus in the greater curvature and around 2–3 cm from the pylorus in the lesser curvature), and, if applicable, lesion site, and were evaluated by two gastrointestinal pathologists using standard criteria from the WHO classification for GC (20) and the visual analog scale of the updated Sydney System for gastritis (21). Each participant was assigned a global diagnosis based on the most severe lesion found among all the biopsy specimens. Accordingly, the 9,002 participants who underwent gastroscopy with biopsies for histopathological evaluation were grouped as: normal or nearly normal gastric mucosa (n=1,956, the reference group), non-atrophic gastritis (n=3,740), atrophic gastritis/intestinal metaplasia (n=2,812), intraepithelial neoplasia (formerly dysplasia, n=400), and GC (n=94).

Statistical analyses

H. pylori sero-positive was defined as anti-*H. pylori* IgG titer ≥34 enzyme immunounits (EIU) according to manufacturer's instructions. PGI and the PGI/II ratio were categorized according to commonly used cut-off points (i.e., 30 and 70 ng/ml for PGI; 3 and 7 for the PGI/II ratio). Biomarkers without well accepted cut-off points (PGII and G-17) were categorized according to quartiles of their distributions in the study cohort. For the cross-sectional analysis, odds ratios (ORs) with 95% confidence intervals (95% confidence intervals (CIs)) were calculated using logistic regression, and corresponding sensitivity and specificity were calculated. For the follow-up analysis, the time to event was calculated as from the date of enrollment to the date of GC diagnosis, death due to any causes other than GC, or the end of the study follow up (12/31/2013), whichever came first. We used Cox proportional hazards regression to estimate hazard ratios (HRs) and 95% CIs. We conducted a sensitivity analysis when we excluded incident cases within 1 year or 3 years of follow-up.

In the risk prediction modeling analysis, receiver operator characteristic curves with corresponding C statistics (area under the curve, AUC) based on logistic models were used to measure the discriminatory performance of combination of predictors where the pathology diagnosis was considered the "gold standard". The logistic regression coefficients of the five biomarkers were transformed into an integer risk point by rounding the quotient of

Table 1. Selected baseline characteristics of the study participants

Characteristics[a]	Cross-sectional analysis population[a]			Follow-up analysis population		
	Prevalent GC *N*=94	GC-free at enrollment *N*=8,908	*P* value	Incident GC *N*=86	GC-free during follow-up *N*=11,932	*P* value
Age, years, mean (s.d.)	61.2 (11.4)	50.7 (10.1)	<0.001	59.0 (10.6)	49.6 (10.7)	<0.001
Sex, no. (%)						
Male	68 (72.3)	4,179 (47.0)		71 (82.6)	5,375 (45.1)	
Female	26 (27.7)	4,716 (53.0)	<0.001	15 (17.4)	6,544 (54.9)	<0.001
Current smoker, no. (%)						
Yes	38 (52.1)	3,011 (40.2)		30 (39.0)	3,806 (36.4)	
No	35 (47.9)	4,481 (59.8)	0.04	47 (61.0)	6,649 (63.6)	0.64
Current drinker, no. (%)						
Yes	17 (23.3)	2,522 (33.7)		28 (36.4)	2,965 (28.4)	
No	56 (76.7)	4,970 (66.3)	0.06	49 (63.6)	7,490 (71.6)	0.12

GC, gastric cancer.
[a]Only those who underwent gastroscopies with mucosal biopsies were included.

dividing the regression coefficient by a single constant, which is the logistic regression coefficient for a 2-year increase in age in relation to GC risk in this population. We created a serological biopsy score which is the sum of the individual risk points corresponding to the five biomarkers. All statistical analyses were performed using STATA version 13 (StataCorp, College Station, TX, USA). A *P* value<0.05 (two-sided) was considered statistically significant.

RESULTS

Selected baseline characteristics of the study participants

Selected characteristics of the study participants in the cross-sectional analysis (only those who underwent gastroscopies with mucosal biopsies) and in the follow-up analysis are summarized in **Table 1**. In the cross-sectional analysis population, prevalent GC cases were on average, older, and more likely to be men and current smokers. In the follow-up analysis population, incident GC cases were more likely to be men and, on average, were older.

Cross-sectional associations of circulating biomarkers with baseline gastric histopathology

As shown in **Table 2**, all of the five biomarkers were associated with baseline gastric histopathology, and the associations were stronger for PGII, the PGI/II ratio, and anti-*H. pylori* IgG than for PGI and G-17. Specifically, extremely low PGI levels (<30 ng/ml vs. >70 ng/ml) were associated with increasing odds of having more advanced precancerous lesions or GC (following the Correa's model of gastric carcinogenesis). There was a dose-response relationship between higher PGII levels or lower PGI/II ratios and the presence of precancerous lesions or GC (*P* for trend <0.001). Those who were *H. pylori*-seropositive had statistically significant, 5-, 7-, 6-, and 5-fold higher odds of having non-atrophic gastritis, atrophic gastritis/intestinal metaplasia, intraepithelial neoplasia, and GC, respectively. There was a J-shaped association between G-17 and the presence of non-atrophic gastritis or atrophic gastritis/intestinal metaplasia. **Supplementary Tables S1** and **S2** online show the measures of accuracy corresponding to different cutoff points of circulating biomarkers for identifying GC and precancerous gastric lesions.

We investigated whether the five biomarkers were independently associated with the presence of precancerous lesions or GC. As shown in **Supplementary Table S3**, when all five biomarkers were included simultaneously, the strength of association was weaker than that in **Table 2**, and again the associations were stronger for PGII, the PGI/II ratio, and anti-*H. pylori* IgG than for PGI and G-17.

We further stratified GC by major histological types (intestinal and diffuse gastric adenocarcinoma), and the associations of the five biomarkers were stronger with intestinal adenocarcinoma than with diffuse adenocarcinoma (see **Supplementary Table S4**).

Prospective associations of circulating biomarkers with risk of developing gastric cancer

In univariate analyses, PGI, PGII, the PGI/II ratio, and G-17 were associated with risk of developing GC (**Table 3**). In multivariate analyses, extremely low PGI levels (<30 ng/ml vs. >70 ng/ml) were associated with a statistically significant 2.55-fold higher risk of developing GC. PGI/II ratios were inversely associated with risk of developing GC (*P* for trend <0.001), and risk for ratios <3 and ratios 3–7 (relative to ratios >7) was statistically significantly 3.13- and 2.15-fold higher, respectively. We found a J-shaped association between G-17 and risk of developing GC; relative to those in the second quartile of G-17, risk for those in the first and fourth quartiles was statistically significantly 2.42- and 2.95-fold higher, respectively. Further, using restricted spline regression (see

Table 2. Cross-sectional associations of circulating biomarkers levels with gastric histopathology

Biomarkers	Reference group	Non-atrophic gastritis		Atrophic gastritis/intestinal metaplasia		Intraepithelial neoplasia		Gastric cancer	
	n	*n*	OR[a] (95% CI)	*n*	OR[a] (95% CI)	*n*	OR[a] (95% CI)	*n*	OR[a] (95% CI)
PGI (ng/ml)									
>70	1,284	2,815	Reference	2,037	Reference	293	Reference	62	Reference
30–70	640	858	0.63 (0.55–0.71)	680	0.73 (0.64–0.83)	94	0.78 (0.60–1.01)	19	0.91 (0.52–1.58)
<30	32	67	0.99 (0.64–1.53)	95	1.72 (1.13–2.61)	13	1.84 (0.92–3.69)	13	5.75 (2.45–13.47)
PGII (ng/ml)									
Q_1 (≤6.00)	799	740	Reference	464	Reference	65	Reference	16	Reference
Q_2 (6.01–9.73)	659	845	1.39 (1.20–1.61)	559	1.33 (1.13–1.57)	83	1.28 (0.91–1.82)	26	1.37 (0.70–2.68)
Q_3 (9.74–16.78)	347	979	3.05 (2.60–3.59)	816	3.81 (3.20–4.54)	119	3.75 (2.66–5.28)	18	1.63 (0.78–3.40)
Q_4 (>16.78)	151	1,176	8.90 (7.27–10.90)	973	10.61 (8.59–13.11)	133	10.71 (7.42–15.47)	34	8.25 (4.18–16.28)
P for trend			< 0.001		< 0.001		< 0.001		< 0.001
PGI/II ratio									
>7	1,705	2,322	Reference	1,426	Reference	223	Reference	41	Reference
3–7	217	1,201	4.08 (3.48–4.78)	1,155	6.32 (5.37–7.46)	146	5.58 (4.26–7.29)	40	7.16 (4.32–11.85)
<3	34	217	4.85 (3.35–7.03)	231	7.60 (5.24–11.04)	31	6.93 (4.01–11.98)	13	9.85 (4.33–22.40)
P for trend			< 0.001		< 0.001		< 0.001		< 0.001
Anti-H. pylori IgG (EIU)									
Sero-negative (<34)	1,659	1,931	Reference	1,245	Reference	207	Reference	53	Reference
Sero-positive (≥34)	297	1,809	5.19 (4.51–5.97)	1,567	7.23 (6.23–8.38)	193	5.78 (4.52–7.40)	41	4.92 (3.06–7.92)
G-17 (pmol/l)									
Q_1 (≤0.50)	570	959	1.21 (1.04–1.40)	723	1.38 (1.18–1.63)	72	0.97 (0.69–1.35)	20	0.67 (0.36–1.24)
Q_2 (0.51–2.00)	667	898	Reference	619	Reference	99	Reference	32	Reference
Q_3 (2.01–4.80)	424	841	1.51 (1.29–1.76)	681	1.78 (1.50–2.10)	104	1.73 (1.27–2.36)	30	1.53 (0.88–2.67)
Q_4 (>4.80)	295	1,042	2.88 (2.43–3.41)	789	2.88 (2.41–3.44)	125	2.77 (2.04–3.77)	12	0.94 (0.46–1.93)

CI, confidence interval; EIU, enzyme immunounits; G-17, gastrin-17; *H. pylori*, *Helicobacter pylori*; IgG, immunoglobulin G; OR, odds ratio; PG, pepsinogen.
[a]Adjusted for age, sex, smoking status, and drinking status.

Table 3. Prospective associations of circulating biomarkers with risk of developing gastric cancer

Biomarkers	GC/GC-free	GC rate[a]	Crude HR (95% CI)	*P* value	Adjusted[b] HR (95% CI)	*P* value
PGI (ng/ml)						
>70	59/8,290	76.0	Reference	NA	Reference	NA
30–70	18/3,299	56.1	0.74 (0.44–1.26)	0.27	0.93 (0.55–1.59)	0.80
<30	9/343	256.8	3.39 (1.68–6.85)	0.001	2.55 (1.25–5.20)	0.01
PGII (ng/ml)						
Q_1 (≤6.00)	14/3,034	50.2	Reference	NA	Reference	NA
Q_2 (6.01–9.73)	13/2,948	48.2	0.96 (0.45–2.04)	0.92	0.75 (0.35–1.60)	0.46
Q_3 (9.74–16.78)	19/2,986	65.8	1.31 (0.66–2.62)	0.44	0.98 (0.49–1.96)	0.96
Q_4 (>16.78)	40/2,964	135.6	2.66 (1.44–4.91)	0.002	1.82 (0.98–3.38)	0.06
P for trend			<0.001		0.01	
PGI/II ratio						
>7	30/7,676	42.9	Reference	NA	Reference	NA
3–7	41/3,528	115.4	2.61 (1.62–4.19)	<0.001	2.15 (1.34–3.46)	0.002
<3	15/728	193.4	4.48 (2.41–8.34)	<0.001	3.13 (1.68–5.84)	<0.001
P for trend			<0.001		<0.001	
Anti-H. pylori IgG (EIU)						
Sero-negative (<34)	45/6,769	70.3	Reference	NA	Reference	NA
Sero-positive (≥34)	41/5,163	83.3	1.16 (0.76–1.77)	0.50	1.20 (0.78–1.83)	0.41
G-17 (pmol/l)						
Q_1 (≤0.50)	28/2,991	87.8	2.26 (1.15–4.45)	0.02	2.42 (1.23–4.78)	0.01
Q_2 (0.51–2.00)	13/3,080	42.3	Reference	NA	Reference	NA
Q_3 (2.01–4.80)	19/2,888	69.3	1.79 (0.87–3.69)	0.11	1.98 (0.96–4.09)	0.06
Q_4 (>4.80)	26/2,973	112.2	2.96 (1.49–5.89)	0.002	2.95 (1.48–5.87)	0.002

CI, confidence interval; EIU, enzyme immunoassay units; GC, gastric cancer; G-17, gastrin-17; *H. pylori*, *Helicobacter pylori*; HR, hazard ratio; NA, not applicable; PG, pepsinogen.
[a]Per 100,000 person-years.
[b]Adjusted for age, sex, smoking status, and drinking status.

Supplementary Figure S1), a decreasing PGI/II ratio was associated with increasing risk of developing GC, and the increasing risk seemed to accelerate below a PGI/II ratio of 7.

We investigated whether PGI, the PGI/II ratio, and G-17 were independently associated with risk of developing GC. As shown in **Supplementary Table S5**, when PGI, the PGI/II ratio, and G-17 were included simultaneously, only PGI/II ratio and G-17 remained statistically significantly associated with GC risk, and the strength of association was similar to that in **Table 3**.

As a sensitivity analysis, we excluded incident cases within 1 year or 3 years of follow-up (see **Supplementary Table S6**). PGI, the PGI/II ratio, and G-17 remained statistically significantly associated with risk of developing GC and the associations became stronger. In addition, when incident cases within 3 years of follow-up were excluded, the highest quartile of PGII became statistically significantly associated with higher risk of developing GC.

Integrative prediction model for identifying individuals at high risk of developing gastric cancer

Given the strong associations of circulating biomarkers with the presence of precancerous gastric lesions (i.e., non-atrophic gastritis, atrophic gastritis/intestinal metaplasia, and intraepithelial neoplasia) observed in the cross-sectional analysis, we built a prediction model based on the five biomarkers for identifying persons with precancerous gastric lesions at enrollment using the cross-sectional data. As shown in **Figure 2a**, the five biomarkers combined yielded a C statistic of 0.803 (95% CI=0.789–0.816). Furthermore, the five biomarkers in combination provided added predictive value to traditional risk factors (age, sex, smoking, family history of GC, and upper gastrointestinal symptoms), and the C statistic improved from 0.580 to 0.811 (P<0.001). The results were similar when non-atrophic gastritis (**Figure 2b**), atrophic gastritis/intestinal metaplasia (**Figure 2c**), and intraepithelial neoplasia (**Figure 2d**) were assessed separately.

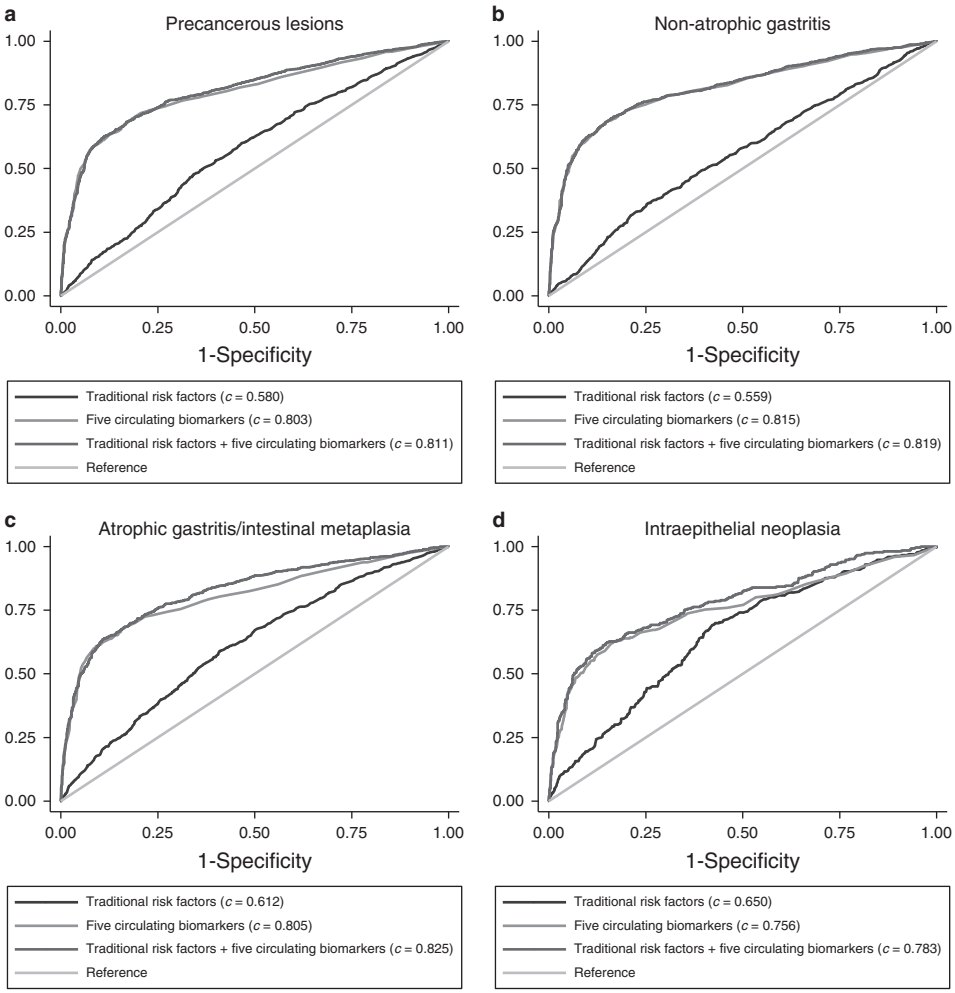

Figure 2. Receiver-operator characteristic curves of age, sex, smoking, family history of gastric cancer among first degree relatives, upper gastrointestinal symptoms, and circulating biomarkers (pepsinogen I, pepsinogen II, pepsinogen I/II ratio, anti-*H. pylori* antibody, and gastrin-17) for discriminating of precancerous gastric lesions combined (i.e., non-atrophic gastritis, atrophic gastritis, and intestinal metaplasia, **a**), non-atrophic gastritis (**b**), atrophic gastritis/intestinal metaplasia (**c**), or intraepithelial neoplasia (**d**) from the reference group. Note: because a history of upper gastrointestinal symptoms was collected only from 2008 onwards, this analysis was limited to 4,046 participants recruited from 2008 and on. A full color version of this figure is available at the *American Journal of Gastroenterology* journal online.

In order to make the serological biopsy more applicable in practice, we created the serological biopsy score. **Table 4** shows the individual risk points corresponding to different levels of the five biomarkers, and the serological biopsy score summarizes the five biomarkers into a single score. **Table 5** shows the measures of accuracy associated with different serological biopsy scores for identifying GC and precancerous gastric lesions combined. The maximal sum of sensitivity and specificity was achieved at a score of 5, 6, 7, or 8.

We further assessed the performance of the serological biopsy score using the follow-up data and investigated whether the serological biopsy score at enrollment was associated with risk of developing GC during follow-up. As shown in **Table 6**, higher serological biopsy scores were associated with higher risk of developing GC (P for trend <0.001), and those with a score ≥14 had a 3.9-fold (95% CI=1.70–8.90; P=0.001) higher risk of developing GC relative to those with a score ≤2.

DISCUSSION

Our results suggest that, at least in this Chinese population, (1) the five circulating biomarkers, (especially PGII, the PGI/II ratio, and anti-*H. pylori* IgG) are associated with the presence of precancerous gastric lesions or GC at baseline; (2) PGI, the PGI/II ratio, and G-17 are associated with risk of developing GC during follow-up; (3) a serological biopsy composed of the five stomach-specific circulating biomarkers yields adequate prediction accuracy for identifying precancerous lesions at enrollment and substantially improved prediction beyond traditional risk factors, and higher serological biopsy scores based on the five biomarkers at enrollment were associated with higher risk of developing GC during follow-up. To our knowledge, this study is the first joint evaluation of the five biomarkers for GC risk assessment using a multi-phase study design.

Table 4. Multivariate odds ratios and risk points of each circulating biomarker

Biomarkers	OR (95% CI)	Risk points[a]
PGI (ng/ml)		
>70	Reference	0
30–70	0.92 (0.80–1.05)	0
<30	1.21 (0.76–1.93)	1
PGII (ng/ml)		
Q1 (≤6.00)	Reference	0
Q2 (6.01–9.73)	1.17 (1.02–1.35)	1
Q3 (9.74–16.78)	1.82 (1.53–2.17)	3
Q4 (>16.78)	3.22 (2.50–4.15)	6
P for trend		
PGI/II ratio		
>7	Reference	0
3–7	2.10 (1.73–2.54)	4
<3	2.44 (1.58–3.77)	4
P for trend		
Anti-H. pylori IgG (EIU)		
Sero-negative (<34)	Reference	0
Sero-positive (≥34)	3.76 (3.27–4.32)	7
G-17 (pmol/l)		
Q1 (≤0.50)	1.27 (1.11–1.47)	1
Q2 (0.51–2.00)	Reference	0
Q3 (2.01–4.80)	1.33 (1.14–1.55)	1
Q4 (>4.80)	1.75 (1.49–2.06)	3

CI, confidence interval; EIU, enzyme immunoassay units; G-17, gastrin-1; *H. pylori*, *Helicobacter pylori*; OR, odds ratio; PG, pepsinogen.

[a]Calculated by rounding the quotient of dividing the regression coefficient by a single constant (0.20) to the nearest integer. The constant chosen was the logistic regression coefficient for a 2-year increase in age in relation to gastric cancer risk in this population.

Pepsinogens are products of terminally differentiated gastric mucosa. There are two isoforms of PG: PGI and PGII (22). PGI is produced only in the glandular mucosa of the stomach fundus and body, whereas the distribution of PGII-producing cells includes the entire stomach and duodenum (23). Low PGI levels and/or low PGI/II ratios (e.g., PGI≤30–70 ng/ml plus PGI/II ratio≤3.0) in the serum are commonly used to indicate chronic atrophic gastritis in the fundus/body (24), a precancerous lesion for GC (25), and the results of a microsimulation study suggest that the PG test could be a cost-effective strategy to reduce intestinal-type of GC mortality among high-risk individuals in United States (26). Consistent with previous studies, we found that low PGI levels and low PGI/II ratios were both associated with the presence of atrophic gastritis/intestinal metaplasia. As expected, the associations of low PGI levels and low PGI/II ratios with GC were stronger for the intestinal type of gastric adenocarcinoma than for the diffuse type because the intestinal type is more closely related to atrophic gastritis/intestinal metaplasia. Further, we found that low PGI levels and low PGI/II ratios were associated with higher risk of developing GC, which is consistent with the results from previous studies (7,27–36). Unlike most previous studies which used one specific cutoff point (e.g., PGI/II ratio≤3.0), we used restricted spline regression in which the PGI/II ratio was analyzed as a continuous variable, and our results indicated that a lower PGI/II ratio was associated with a higher risk of developing GC without a threshold effect. The spline analysis results also suggested that the increasing risk accelerated below a PGI/II ratio of 7, a finding that was consistent with the results that PGI/II ratios ≤7.0 were associated with statistically significantly higher risk of developing GC when the PGI/II ratio was analyzed using two pre-specified cutoff points (i.e., 3 and 7).

Interestingly, we found that extremely low (i.e., <30 ng/ml) but not intermediate (30–70 ng/ml) PGI levels were associated with statistically significantly higher risk of developing GC. However, this significant association disappeared when PGI and the PGI/II ratio were simultaneously included in one model. This observation suggests that PGI is not a significant predictor independent of the PGI/II ratio, which already takes into account atrophy in the stomach fundus and body (via the PGI component). In addition, elevated serum PGII levels were strongly associated with the presence of precancerous gastric lesions or GC and was associated with higher risk of developing GC when incident cases within three years of follow-up were excluded.

H. pylori infection has been consistently associated with risk of developing GC (37). We found that *H. pylori* sero-positivity (EIU≥34 vs.<34) was strongly associated with the presence of precancerous gastric lesions or GC but not with risk of developing GC. However, when anti-*H. pylori* IgG titers were categorized into three groups according to tertiles, intermediate levels compared with low levels (i.e., second tertile vs. first tertile) were associated with significantly higher risk of developing GC (data not shown). This is probably because that at the final stage of the gastric carcinogenesis sequence, the gastric mucosa may be a more inhospitable environment for *H. pylori*, leading to a reduction or eradication of the infection (12,38–43). Another explanation is the high prevalence of *H. pylori*-related precancerous gastric lesions among our study populations.

G-17 is released by the G cells in the antrum of the stomach in response to various stimulating factors, including low acidity in the stomach (44), and serum G-17 levels are, therefore, mainly dependent on the number of G cells and intra-gastric acidity (3). Our study is the first to report a J-shaped association between circulating G-17 levels and risk of developing GC, with higher risks for those with either low (<0.5 pmol/l) or high G-17 (2.0–4.7 or >4.7 pmol/l) levels. A limited number of studies have investigated this association. One recent study with a small sample size found that low (<1 vs. ≥1 pmol/l) G-17 levels were associated with higher risk of developing GC (36), but the finding was

Table 5. Measures of accuracy associated with different serological biopsy scores for identifying gastric cancer and precancerous gastric lesions combined

Serological biopsy score	Sensitivity (%)	Specificity (%)	Sum of sensitivity and specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Likelihood ratio+	Likelihood ratio–
0	100	0	100	78	Undefined	1.0	Undefined
1	96	12	108	80	45	1.1	0.3
2	86	41	126	84	44	1.4	0.4
3	80	54	134	86	43	1.7	0.4
4	76	63	140	88	43	2.1	0.4
5	71	72	143	90	41	2.5	0.4
6	70	73	143	90	40	2.6	0.4
7	67	76	143	91	39	2.8	0.4
8	64	79	143	92	38	3.1	0.5
9	57	85	142	93	35	3.9	0.5
10	52	88	141	94	34	4.5	0.5
11	47	92	139	95	33	5.6	0.6
12	37	95	131	96	29	6.8	0.7
13	36	95	131	96	29	6.9	0.7
14	30	97	126	97	28	8.5	0.7
15	26	97	123	97	27	8.9	0.8
16	22	98	120	97	26	9.5	0.8
17	20	98	118	97	26	10.0	0.8
18	15	99	114	97	24	10.5	0.9
19	8	99	107	98	23	13.4	0.9
20	7	99	107	98	23	13.2	0.9
21	0	100	100	100	22	Undefined	1.0

Table 6. Prospective associations of the serological biopsy score at enrollment based on five circulating biomarkers with risk of developing GC during follow-up

Serological biopsy score	GC/GC-free	GC rate[a]	Crude HR (95% CI)	*P* value	Adjusted[b] HR (95% CI)	*P* value
≤2	7/3,302	24.1	Reference	NA	Reference	NA
3–8	24/3,120	77.7	3.19 (1.37–7.40)	0.007	2.83 (1.22–6.57)	0.02
9–13	25/2,879	86.8	3.58 (1.55–8.27)	0.003	3.15 (1.36–7.29)	0.007
≥14	30/2,631	122.7	4.92 (2.16–11.24)	<0.001	3.89 (1.70–8.90)	0.001
P for trend			<0.001		0.002	

CI, confidence interval; GC, gastric cancer; HR, hazard ratio; NA, not applicable.
[a]Per 100,000 person-years.
[b]Adjusted for age, sex, smoking status, and drinking status.

not statistically significant. Another study found that individuals with higher combined serum gastrin −34 and −17 levels had higher risks of GC (30). Our finding of a J-shaped association between serum G-17 levels and GC risk is biologically plausible. The results of cross-sectional studies suggest that low serum G-17 may be a biomarker for atrophic gastritis in the stomach antrum (i.e., fewer G cells in the antrum) (10,45–47), while high serum G-17 may be an indication of atrophic gastritis limited to

the stomach fundus/body where acid-secreting glands are located (47,48), which is also supported by our previous results (18). In addition to being a marker of atrophic gastritis in the gastric corpus, high G-17 levels directly promoted gastric carcinogenesis in a mouse model (49), possibly due to the proliferative effect of gastrin on gastric mucosa (44).

For the first time, we built an integrative prediction model for identifying persons with precancerous gastric lesions at enrollment, and our results suggest that the five biomarkers combined yielded adequate prediction accuracy and substantially improved prediction beyond traditional risk factors. Then, we further assessed the performance of the prediction model using our follow-up data, and our results suggest that serological biopsy scores based on the five biomarkers at enrollment were positively associated with risk of developing GC during follow-up, which supported that the serological biopsy comprised of the five biomarkers could predict risk of developing GC. The results from the follow-up analysis indicate that the PGI/II ratio and G-17 components of the serological biopsy might contribute more than other components. As mentioned above, the PGI/II ratio and G-17 provide complementary information.

Our study has several limitations. First, our study population was from a high-risk area in northern China, so caution should be taken when generalizing our results to populations in other regions. Second, further external validation of the usefulness of the serological biopsy for GC risk assessment is needed. Third, our study was conducted mainly in population-based settings, a so called "natural population"; therefore, the value of the serologic biopsy in clinical settings should be prospectively tested in a separate set of patients. In fact, recently the serological biopsy has been gradually applied in clinical practices such as monitoring curative effect of *H. pylori* eradication, monitoring the evolution of precancerous lesions, indicating recurrence after curative treatments of GC, and assisting diagnosis of gastroesophageal reflux disease. However, there is no consensus on its role in routine clinical practice due to some unsolved questions. For example, proton pump inhibitors (PPI), which can increase serum PG value (especially PGI) and G-17 value, has been widespread in clinical settings, and we will need to answer whether the subjects on long-term PPI can be evaluated by the serological biopsy. In our opinion, long-term PPI users not only can receive delayed serological biopsy after stopping PPI to determine their baseline levels but also can receive repeated real-time serological biopsy to monitor the dynamic change of serological biomarkers and determine their responses to PPI stimulation, which is similar to the oral glucose tolerance test for evaluating the function of the insulin island to respond to glucose intake. This is a very interesting research topic and has important clinical implications. It did not get enough attention in the past, and more studies are needed to demonstrate the usefulness of the serological biopsy among PPI users. Our study utilized a population-based sample from a rural area in China and the rate of PPI use in our study population is expected to be low, so the impact of PPI treatment on our results is minimal.

In summary, we evaluated the potential role of a serological biopsy (comprised of five circulating stomach-specific biomarkers: PGI, PGII, the PGI/II ratio, anti-*H. pylori* IgG, and G-17) in GC risk assessment using a multi-phase study design. Each of the five biomarkers represents an independent aspect of the morphology and function of gastric mucosa, and a serological gastric biopsy comprised of the five biomarkers could suggest the presence of precancerous gastric lesions and GC, serving as a tool to identify high-risk individuals for further diagnostic gastroscopy. Furthermore, a serological gastric biopsy using the five biomarkers, especially the PGI/II ratio and G-17 components, was independently associated with risk of developing GC, supporting the serological gastric biopsy as a non-invasive tool to stratify risk of developing GC and thus to guide targeted screening/precision prevention. Applying a serological gastric biopsy in GC risk assessment may help to develop cost-effective GC prevention strategies.

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CONFLICT OF INTEREST

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Specific author contributions: Study concept and design: Yuan Yuan, Huakang Tu, and Liping Sun; acquisition of data: Liping Sun, Yuehua Gong, Qian Xu, Jingjing Jing, and Yuan Yuan; case ascertainment: Liping Sun and Yuan Yuan; statistical analysis and interpretation of data: Huakang Tu, Yuan Yuan, Xiao Dong, Roberd M. Bostick, and Xifeng Wu; drafting of the manuscript: Huakang Tu; critical revision of the manuscript for important intellectual content: all authors; obtained funding: Yuan Yuan; administrative, technical, or material support: Yuan Yuan; study supervision: Yuan Yuan; all authors read and approved the final version of the report.

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ETHICS APPROVAL

This study was approved by the Human Ethics Review Committee of the First Affiliated Hospital of China Medical University (Shenyang, China), and written informed consent was obtained from each participant.

Study Highlights

WHAT IS CURRENT KNOWLEDGE

- ✓ Pepsinogen I (PGI), PGII, PGI/II ratio, anti-*H. pylori* antibody, and gastrin-17 (G-17) are potential biomarkers for gastric cancer (GC) risk assessment.
- ✓ Few studies have evaluated PGII and G-17.
- ✓ There is a lack of joint evaluations of the five biomarkers.

WHAT IS NEW HERE

- ✓ The five circulating biomarkers (especially PGII, the PGI/II ratio, and *H. pylori* sero-positivity) were associated with the presence of precancerous gastric lesions or GC.
- ✓ We found a J-shaped association between G-17 levels and risk of developing GC during follow-up in addition to inverse associations with PGI levels and PGI/II ratios.
- ✓ The five biomarkers combined substantially improved prediction beyond traditional risk factors for identifying precancerous lesions at enrollment, and higher serological biopsy scores based on the five biomarkers were associated with higher risk of developing GC during follow-up.

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