Stomach-specific Biomarkers (GastroPanel) Can Predict the Development of Gastric Cancer in a Caucasian Population: A Longitudinal Nested Case-Control Study in Siberia

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Abstract. *Background/Aim: Atrophic gastritis (AG) is the most important risk condition for gastric cancer (GC). A panel of stomach-specific serum biomarkers: pepsinogen (PG) I, pepsinogen (PG) II, gastrin-17 (G-17), and IgG antibodies to H. pylori (HP-Ab) detects the extent and grade of AG. The aim of the present study was to assess the predictive value of this 4-biomarker panel (GastroPanel, Biohit Oyj, Helsinki, Finland) in a case-control setting nested within a cohort of Caucasian population in Western Siberia. Patients and Methods: Both the cases and controls for the study derived from a population-based cohort of 45-69-year-old subjects (n=9,360) in the HAPIEE (Health, Alcohol and Psychosocial Factors In Eastern Europe) study, enrolled in Novosibirsk , Siberia during 2003-2005. Cases represent all GCs reported to the Cancer Registry until 2012, being matched (1:2) with healthy controls (COs). Altogether 156 (52 GCs and 104 COs) serum samples collected at study entry were available for GastroPanel analysis. Conditional logistic regression models (uni- and multivariate) were used to analyze this matched case-control setting. Results: The biomarker levels below cut-off at baseline predicted the development of GC as follows: PGI (OR=2.9; 95%CI=1.3-6.4), PGII (OR=9.0; 95%CI=1.8-44.3), PGI/PGII (OR=3.3; 95%CI=1.5-7.3); G-17 (OR=1.8; 95%CI=0.7-4.8), and HP-Ab (OR=0.4; 95%CI=0.1-1.3). In the multivariate model adjusted for sex, age, and all GastroPanel markers, PGI/PGII ratio was the most powerful independent predictor of GC (OR=2.9; 95% CI=1.01-8.0). Conclusion: For the first time in a Caucasian population, we demonstrated that PGI, PGII and PGI/PGII ratio are reliable longitudinal predictors of incident GC.*

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Gastric cancer (GC) remains the fifth most frequent malignancy worldwide, with close to one million new cases annually (1). Russia is among the high-risk countries, with 45,000 cases and 35,000 annual deaths from GC (1, 2). The ominous prognosis of GC is due to the fact that in most settings, the disease is diagnosed at advanced stages. This applies *e.g.* to Novosibirsk, a Central Siberian city in Russia (with predominantly Caucasian population), where stage III and IV GC represent 70% of all newly-diagnosed cases (3), making early detection of the disease mandatory. Recent experience suggests that a systematic screening of the risk groups by stomach-specific biomarkers might provide a potential solution (4), but additional studies are required prior to implementation of population-based screening programs (5, 6).

Atrophic gastritis (AG) associated with *Helicobacter pylori* (HP) infection is a well-established cancer precursor lesion (7) in the cascade of distal gastric adenocarcinoma, as described by Correa (8). However, population-based screening by endoscopy for detection of these GC precursors is hardly acceptable outside some rare countries in Asia. Therefore, a non-invasive diagnostic test for detection of patients at-risk for GC (those with AG or HP) would be urgently needed (4, 6, 7). Serum pepsinogen (PG) tests have been applied for non-

invasive screening of GC risk for some time now (9, 10), alone or in combination with HP antibody measurement.

To fulfil the unmet demand, a panel of four stomach-specific biomarkers known as GastroPanel was developed (Biohit Oyj, Helsinki, Finland) in the late 1990's as the first non-invasive diagnostic test for stomach health (structure and function) (6). This 4-biomarker panel is based on stomach physiology, including three markers of atrophy in different topographic locations (PGI and PGII for the corpus; G-17 for antrum), combined with testing IgG antibodies to HP. This marker panel allows identifying AG with high sensitivity (71%-83%) and specificity (95-98%) when endoscopic biopsy is used as the gold standard (6, 10-13).

So far, GastroPanel has been extensively tested in screening of subjects at-risk for GC, *i.e.*, those with HP infection and AG, in different populations (6, 13-15). To date, practically all biomarker studies with GC as their end-point have originated from Japan (16, 17). As repeatedly emphasized in Europe, GastroPanel is not a GC test but is designed for detection of the subjects at-risk for GC (*i.e.*, HP-infection, AG) (6, 11, 13-15). Because of this, there are no previous studies performed in a Caucasian population to corroborate these Japanese data on serum biomarker screening of GC (16, 17). This is the first study designed to assess the value of GastroPanel test as a longitudinal predictor of GC, using a matched case-control setting nested within a Caucasian population-based cohort in Russian Siberia.

Patients and Methods

Study design. This longitudinal (prospective) case-control study using GC as end-point was based on clinical and follow-up data collected from the HAPIEE (Health, Alcohol and Psychosocial Factors In Eastern Europe) cohort of 9,360 subjects enrolled in a Siberian city Novosibirsk between 2003-2005. HAPIEE is a cross-sectional epidemiological study targeted to local Caucasian population aging between 45 and 69 years at enrollment. All individuals with any malignant disease diagnosed prior to enrollment were excluded. Altogether, 9,360 individuals were enrolled from two areas of the Novosibirsk city, reflecting typical demographical structure, social infrastructure and potential industrial impact. The overall response rate was 76%. The design of the HAPIEE study has been detailed elsewhere (18). Apart from detailed interviews, baseline visits included serum sampling of all HAPIEE cohort members, stored at –70˚C for future analyses (18).

All GC cases diagnosed during the prospective follow-up until 2012 were identified among the HAPIEE cohort members by linkage to the local Cancer Registry that covers the entire population and maintained by the Institute of Internal and Preventive Medicine. Each identified GC case (n=52) was randomly matched with two controls (n=104) from the same HAPIEE cohort, using (i) the area of residence, (ii) gender, and (iii) age as matching variables, and excluding all cases with any malignancy reported in the Cancer Registry. Thus, a matched case-control setting of 156 subjects was created, consisting of 52 cases (GC) and 104 healthy controls, comprising the material of the present analysis.

Sample collection. Serum samples from all 156 members of the study group were identified and retrieved from the serum bio-bank of the HAPIEE study, stored at –70˚C since sampling (2003-2005). Importantly, the collection of all serum samples from the HAPIEE cohort was conducted at a fasting state, thus excluding the analysis of stimulated G-17 in the GastroPanel testing (11, 13).

Measurement of serum biomarkers by the GastroPanel test. Biomarker concentrations were assessed in serum samples by using the enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions (6, 12-14). The manufacturer-recommended cut-off values were used for all 4 biomarkers as follows: pepsinogen I (PGI) <30 μg/l, pepsinogen II (PGII) <3 μg/l, the PGI/PGII ratio <3.0, and fasting G-17 <1 pmol/l (G-17b). Values below these cut-off levels implicate AG of the corpus (PGI, PGII, PGI/PGII) and AG of the antrum (G-17b), respectively. *H. pylori* IgG antibody levels above 30 EIU (enzyme-immunoassay units) were considered an indicator of HP infection (ongoing or recent HP exposure).

Risk stratification by the ABCD system. The patients were also stratified into the ABC(D) categories as recently described by Miki (10). In this system, Group "A" denotes individuals with normal pepsinogens (PGI, PGII and PGI/PGII ratio) but negative HP Ab; Group "B" includes those with normal Pepsinogens, but testing HP Ab-positive; Group "C" represents individuals with below-cut-off PG values (and PGI/PGII ratio) and, testing HP Ab-positive result; Group "D" subjects show below-cut-off levels of pepsinogens (PGI/PGII), but test HP Ab-negative (<30 EIU cut-off) result (10).

Atrophy index (AI). In addition, the authors created a novel scoring system: Atrophy index (AI), consisting of 3 parameters (PGI, PGI/II, and G-17b). Each normal value (see above) was scored as 0, while abnormal (below cut-off) values as 1. Therefore, the total score could obtain values ranging between 0 and 3.

Statistical analysis. Statistical analysis was performed using the SPSS 11.0 software package (SPSS Inc. Chicago, IL, USA) and STATA 11.0 software (Stata Corp LP, College Station, TX, USA). Data were expressed as means±SD. The differences between groups were analyzed by the Student's *t*-test (for normally-distributed variables). For variables not fulfilling normal distribution the differences between the groups were analyzed with the Mann-Whitney U-test and multiple comparisons with the Kruskal-Wallis test. Significance of differences between means was estimated with ANOVA, and between proportions using χ^2 test. The criterion for statistical significance level was set as $p<0.05$. The odds ratio (OR) with confidence intervals (95% CI) was calculated by contingency tables. In order to control for the eventual confounders, a conditional (fixed effects) logistic regression analysis was used for this matched case-control setting. Results of the logistic regression analyses are presented as crude and adjusted OR and their 95%CI. For some parameters, positive predictive value (PPV), negative predictive value (NPV), sensitivity (SE) and specificity (SP) were also calculated.

Ethical considerations. The HAPIEE cohort study was approved by the Human Ethics Review Committee, the Institute of Internal and Preventive Medicine (Novosibirsk, Russia) before study onset. Written informed consent was obtained from each participant prior to inclusion. The study was conducted in accordance with the Helsinki Declaration.

Results

Characteristics of the study subjects. During the prospective follow-up between 2003-2012, 60 patients with GC were identified from the Cancer Registry. Out of these 60, the baseline serum samples of 8 patients were not available in the bio-bank. This leaves 52 GC patients in the present analysis, being matched (1:2) with 104 control subjects with no malignancy. Key characteristics of the cases and controls are summarized in Table I.

Characteristics of the gastric cancer (GC) cases. According to histological confirmation, adenocarcinoma (AC) was seen in 87.2% of GC cases, 12.8% consisting of other histological types: signet-ring cell carcinoma - 8.6%, papillary cancer - 2.1%, and non-differentiated cancer - 2.1%. The clinical stage of the GC was available for 37 patients only with advanced stages (stages III and IV) being confirmed in 59.5% of the cases.

Biomarker levels in cases and controls. The levels of the GastroPanel biomarkers in cases and controls are depicted in Table II. The two groups were significantly different in their expression of PGI and PGI/PGII ratio, both being significantly lower among the GC group. However, no difference was revealed in the levels of PGII or HP Ab titers.

Frequency of abnormal biomarker levels in cases and controls. Table III summarizes the frequency of the out-of-range (abnormal) biomarker values in cases and controls, stratified according to the manufacturer-defined cut-off values for PGI, PGII, PGI/PGII and HP Ab titers. The below-cut-off levels of PGI, PGII and PGI/PGII ratio were significantly more frequent among GC cases than in controls, using univariate conditional logistic regression, being most marked for PGII (OR=9.0, 95%CI=1.8-44.3). However, no difference between the two groups was found in the frequency of abnormal G-17 levels and HP Ab-positivity. In the multivariate model, adjusted for sex, age, and all GastroPanel biomarkers, the low PGI/PGII ratio was the only significant independent predictor of GC, with OR=2.9, 95%CI=1.01-8.0) (Table III). In the model, where all biomarkers were included as continuous variables, PGI (p=0.0001, OR=0.99; 95% CI=0.98-0.99) and PGI/PGII (OR=0.70; 95% CI: 0.5-0.8 p=0.0001) were significant predictors of GC, *i.e.*, normal values being "protective".

Patient distribution into the four groups of the ABCD classification is summarized in Table IV. A significantly higher proportion of individuals (19.4%) who subsequently developed GC were allocated to Group D at study enrollment (p=0.02), whereas the cases and controls were equally distributed into categories A, B and C. Subjects in group D were at significantly increased risk for developing incident GC, compared to subjects in group A (OR=28.0; 95%CI=1.4-580.6; p=0.015) or those in group B (OR=13.7; 95% CI: 1.6-117.5; p=0.003).

Table I. *Key demographics of cases and controls included in the study.*

	GC group (n=52)	Control group (n=104)	Total (%)
Males n (%)	31 (59.6%)	65 (62.5%)	96 (61.5%)
Females n (%)	21 (40.4%)	39 (37.5%)	60 (38.5%)
Mean age (±SD)	60.2±7.5	59.8±7.4	

Table II. *Mean levels of pepsinogens and G-17 in cases and controls.*

Group	Biomarkers of Gastro Panel (M±SD)			
	PGI (μg/l)	PGII (μg/l)	PGI/PGII	G-17 (pmol/l)
Gastric cancer	65.5±8.8	15.3±1.5	3.7±0.4	12.8±2.2
Control	94.5±5.7	16.4±0.9	6.3±0.3	9.3±1.3
p-value	0.005	0.499	0.0001	0.158

p, Significance after Student's t-test; PGI, pepsinogen I; PGI/PGII, the ratio between PGI and PGII; G-17, gastrin-17.

Performance indicators of the biomarkers in predicting GC. PPV for PGI is 53% and NPV is 72%, with SE 34.6% and SP 84.6%. For PGI/PGII ratio, PPV is 55.5% and NPV is 72.8%, with SE 39.2% and SP 83.8%.

Atrophy Index (AI) in cases and controls. Distribution of the AI in cases and controls is depicted in Table V. At baseline, AI 3 (*i.e.*, all biomarkers below the normal range) was more common among those who subsequently developed GC (14.0%) than in healthy controls (2.2%) (p=0.006). In contrast, AI score 0 (normal stomach) was more common among controls in their baseline sample (p=0.019).

Figure 1 illustrates the distribution of the AI index categories (0-3) among cases and controls. The severity of atrophy at baseline closely parallels the subsequent development of GC. Among the subjects with AI 3 at baseline, GC was detected in 77.8% of cases, only 22.2% remaining healthy during the follow-up (p<0.03). This is in contrast to the group with no gastric atrophy at baseline (AI 0), among which GC was found only in 30%, the remaining 70% remaining cancer-free (p<0.0001).

Discussion

Screening for GC remains under debate outside East Asian countries. Non-invasive tests including PGI, PGII, G-17 and HP Ab biomarkers are attractive (6, 11-14), but still further

Table III. *Frequency (%) of out-of-range (abnormal) biomarker values in cases and controls.*

Group	Indicators of AG and HP infection (%)				
	PGI <30 μg/l	PGII <3 μg/l	PGI/PGII <3	G-17 <1 pmol/l	*H. pylori* IgG (>30 EIU)
Gastric cancer	34.6	15.7	39.2	19.6	80.0
Control	15.4	2.0	16.2	11.5	90.1
p-Value	0.006	0.001	0.002	0.179	0.134
OR (95% CI)	2.9 (1.3-6.4)	9.0 (1.8-44.3)	3.3 (1.5-7.3)	1.8 (0.7-4.8)	0.4 (0.1-1.3)
Conditional logistic regression analysis (multivariate) Adjusted OR (95% CI) (gender, age and all GastroPanel markers in the model)	NS	NS	2.9 (1.01-8.0)	NS	NS

p, Significance after Chi-square test. NS, Not significant. OR, Odds Ratio; CI, confidence intervals. PGI, Pepsinogen I; PGI/PGII, the ratio between pepsinogen I and II; G-17, gastrin-17 (fasting level).

confirmatory studies are required before biomarker tests (*e.g.* GastroPanel) can be recommended for implementation in population-based GC screening programs (24). Most importantly, the encouraging results reported from Asia on the value of PG testing as a predictor of GC (16, 17) have not been reproduced in a Caucasian population to date. The present study is the first to provide such evidence, by demonstrating that testing for PGI, PGII and PGI/PGII ratio is helpful in predicting the development of incident GC in a Caucasian population, living in the high-risk territory for GC (18).

In the present longitudinal matched case-control setting, we provided evidence that the baseline PGI and PGI/PGII levels below the agreed cut-off values (6, 11-15) were associated with a significantly increased risk of developing GC among 45-69-year-old individuals prospectively followed-up of for 7 to 10 years (2003-2012). In conditional logistic regression, the OR for developing GC was 2.9 (95%CI=1.3-6.4) for decreased PGI and OR=3.3 (95%CI=1.5-7.3) for PGI/PGII ratio (Table III). These findings are consistent with the results obtained in similar type of follow-up studies originating from Japan (17, 19).

Accordingly, Yanaoka *et al.* conducted a 10-year follow-up of 5,209 asymptomatic middle-aged Japanese subjects, showing that the risk of incident GC increased in the presence of elevated HP antibodies (HR=3.48, 95% CI=1.26-9.64) and below cut-off levels of PGI (HR=3.54, 95% CI=1.95-6.40) or PGI/PGII ratio (HR=4.25, 95% CI=2.47-7.32) (17). In a more recent cohort study in rural villages of the Kyoto prefecture, the baseline examination of 2,859 subjects included testing of HP Ab and PGI and PGII levels (19). During a 10-year follow-up, a multivariate proportional hazards (Cox) regression model, adjusted for age and sex, HR for GC was 4.2 (95% CI=0.96-18.4) for HP Ab even without mucosal atrophy. The risk increased markedly for cases with HP Ab was associated with AG, HR=11.2; 95%CI=2.71-46.51. The subjects with AG but without elevated HP Ab showed the highest risk for incident GC: HR=14.81; 95%CI=2.47-88.8 (19). The present study is fully comparable with the Japanese studies regarding its cohort size (n=9,360) as well as the number of incident GC cases diagnosed during the follow-up that extends to an almost 10-year period (2003-2012) (18).

Despite the fact that PG testing has been considered a useful non-invasive test in identification of populations at-risk for developing GC (6,12-14) either by Asian-Pacific (15, 20) or European guidelines (11, 21-22) biomarker

Table IV. *Risk stratification of cases and controls by the ABCD system.*

Category	ABCD grading		Gastric cancer %	Control %	*p*-Value
	*PG Status (+/–)	HP Ab Status (+/–)			
A	PG (–)	HP Ab (–)	2.8	6.5	0.43
B	PG (–)	HP Ab (+)	75.0	85.5	0.20
C	PG (+)	HP Ab (+)	2.8	6.5	0.43
D	PG (+)	HP Ab (–)	19.4	1.6	0.02

*PGI and PG/PGII included; GC, gastric cancer; PG (–), normal PGI and PGI/PGII ratio (>3.0); PG (+), decreased PGI and PGI/PGII ratio (<3.0).

Table V. *Atrophy Index (AI) in cases and controls.*

Group	Severity of gastric mucosal atrophy (AI score) (%)			
	3	2	1	0
Gastric cancer	14.0**	20.0	10.0	56.0
Control	2.2	10.8	11.8	75.3*

*$p<0.019$; **$p<0.006$.

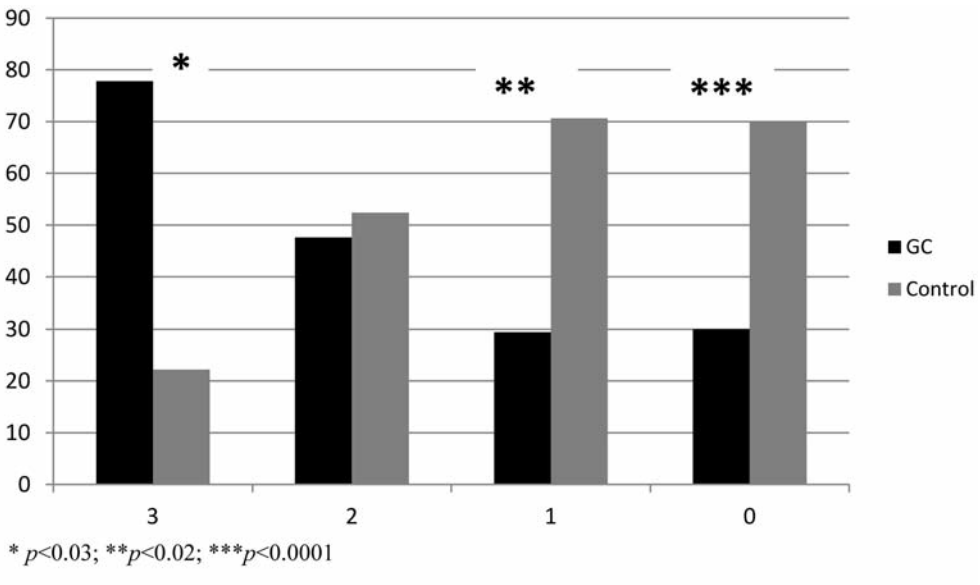

Figure 1. *Cases and controls classified according to their Atrophy Index (AI).*

testing is not yet implemented in organized screening for GC in any geographic region (23). So far, the key limiting step has been the low sensitivity of PGs in GC prediction (24). Although PGI and PGI/PGII levels are usually clearly decreased in subjects who were diagnosed with GC during the follow-up compared to the group of controls, only the minority of GC cases usually present with decreased levels of these biomarkers at baseline, *i.e.*, 34.6% had decreased PGI levels, and 39.2% showed below-cut-off PGI/PGII levels (24). Therefore, if used as a diagnostic test in population-based screening, there is a danger that a substantial proportion of individuals at increased GC risk would be missed (23, 24).

In contrast to the two Japanese studies (17,19), the present analysis did not disclose any longitudinal predictive value of GC for HP Ab-positivity, other than a non-significant trend of the GC group being HP-infected less frequently than the controls. The failure to establish HP Ab status as a predictive factor for incident GC is most likely due to the fact that the prevalence of the HP-infection was very high in both study groups, *i.e.*, 80.0% in the GC group and 90.1% in the control group, being substantially higher than even in the high-risk GC areas in Asia (25-27).

It is well-established that in the late stages of AG development, HP-infection may disappear spontaneously (6-8, 12, 14). This does not, however, decrease the risk of GC, but vice versa, this risk is even increased, making these people the group at the highest risk of all. Indeed, this was convincingly demonstrated in the present study as well (Table IV). When study subjects were stratified by the ABCD classification (10) on the basis of their baseline samples, 19.4% of the subjects in the GC group fell into Group D (low PG levels but testing HP Ab-negative). This is in perfect alignment with the results from Japan, confirming Group D subjects being the group at the highest risk for incident GC (28). In the present study, however, the data were missing whether the subjects in Group D have undergone previous HP eradication therapy, which might cause some inherent bias in their stratification between Group C and D (23).

The full GastroPanel test includes G-17 as the fourth biomarker to complement the panel of PGI, PGII and HP Ab (13). G-17 is a well- established biomarker of the G-cells in the gastric antrum, providing information *e.g.* on mucosal atrophy in that topographic location (11). Used in combination, the 4-biomarker panel provides comprehensive information on the stomach health and disease (both structure and function) (6, 11-14). Compared to PGI and PGII, the physiological regulation of G-17 is more complex, however. As recently pointed out, low levels of G-17 are not exclusively inherent to antral AG, but may also reflect high gastric acid output (13). On the other way round, G-17 is up-regulated (through a negative feedback loop) by a low acid content of the corpus, caused by either (i) AG of the corpus, or (ii) more frequently, by a prolonged use of proton pump inhibitor (PPI) medication (11, 13, 14). Consequently, any biomarker being regulated by more than one trigger cannot be a highly accurate indicator of only one of these. In the case of fasting G-17, the below-cut-off values can be due to either AG of the antrum or high acid output of the corpus (11, 13). Accordingly, a distinction between antral AG and high acid

output as the cause of low (fasting) G-17 levels should always be made in GastroPanel examination by measuring the G-17 levels after a protein-rich meal stimulation (11, 13, 29). Failure to increase G-17 output after such a stimulation is a specific indicator of antral AG (11, 13). By definition, however, antral AG shall be diagnosed only in the presence of HP infection, while in HP-negative subjects, even the fasting G-17 output below cut-off level is considered as an indicator of high acid output of the corpus (11, 13).

In the present study, only the levels of basal G-17 (G-17b) were measured (18, 23), precluding the possibility for making the distinction between antral AG and high acid output as the cause of low G-17b levels. Not unexpectedly then, we failed to reveal any significant difference in G-17b levels between GC and the control groups in their baseline samples (Table II). The same was true with the frequency of the below-cut-off levels in the two study groups, being more frequent (19.6%) in the GC than (11.5%) in the control group (NS) (Table III). This situation could not be amended by using different cut-off values (2, 3 or 5 pmol/l) for G-17b, that were also tested for this.

In accordance with the above-mentioned facts on the complex physiology of G-17, several previous studies have reported low sensitivity for G-17b as a marker of antral AG. In a Caucasian population from Latvia and Lithuania, the sensitivity of G-17b to diagnose AG in the antrum was only 15.4%, but increased to 30.8% following protein stimulation (G-17s) (29). A recent study from Japan (30) suggested that adding G-17b to PG testing is a valuable approach in discriminating between multifocal AG and other types of AG, which is not possible with PG testing alone. However, also in their study, G-17b levels below 1 pmol/l were present only in 15.6% of the GC patients, which did not differ from the non-GC group (30). As recently pointed out, however, the decline of G-17b output due to antral AG is a gradual process where values below the cut-off are reached only in moderate-severe AG, and because of this, G-17b levels can remain within normal range during several years preceding the GC diagnosis (13, 15).

Finally, we also tested a novel atrophy index (AI), calculated as a sum of any abnormal values of PGI, PGI/PGII ratio and G-17. In the group with most severe atrophy (AI 3; all biomarkers below cut-off), the vast majority of subjects (77.8%) were GC patients, and only a minority were healthy controls. This suggests a "dose-dependent" relationship between AG and GC risk, also illustrated in Figure 1, where AI score is directly related to GC and inversely related to healthy stomach.

Alltogether, the present study is the first to demonstrate that low (below cut-off) PGI and PGI/PGII serum levels could serve as a predictive marker for incident GC in a Caucasian population as well, followed-up for almost 10 years. Because PGs are biomarkers of gastric mucosal atrophy, timely identification of AG by these biomarkers (GastroPanel) may allow for adequate preventive measures, *e.g. H. pylori* eradication and endoscopic surveillance to the subjects at increased risk who would clearly benefit by such interventions. Failure to accurately distinguish between antral AG and high acid output as the cause of low G-17 levels (the 4th biomarker of GastroPanel) in this study, precludes the assessment of this marker as a longitudinal predictor of GC.

Conflicts of Interest

The Authors declare no conflict of interest.

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