ORIGINAL ARTICLE

UDC: 616.3-07
DOI: https://doi.org/10.2298/VSP200720134M

Seroreactivity against *Helicobacter pylori* VacA, 50 kDa, and 30 kDa along with alarm features may improve the diagnostic approach to uninvestigated dyspepsia – a pilot study

Seroreaktivnost protiv *Helicobacter pylori* VacA, 50 kDa i 30 kDa zajedno sa simptomima i znacima alarma može unaprediti dijagnostički pristup neistraženoj dispepsiji – pilot studija

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Abstract

Background/Aim. Alarm features (AF) are of limited utility in predicting endoscopic findings, and the majority of patients with uninvestigated dyspepsia will have no organic pathology identified at upper gastrointestinal endoscopy. In our previous study, we highlighted seroreactivity against *Helicobacter pylori* (HP) antigens VacA, 50 kDa, and 30 kDa as biomarkers for gastric cancer, peptic ulcers, and functional dyspepsia. We designed and conducted this pilot study in order to compare the diagnostic utility of seroreactivity against HP VacA, 50 kDa, and 30 kDa with AF and investigate the possibility and adequacy of its synchronous application. **Method.** A careful history and physical examination with special attention to AF, esophagogastroduodenoscopy with biopsy, abdominal ultrasound or computer tomography, complete blood count (CBC) and blood biochemistry, a Western Blot IgG against HP antigens VacA, 50 kDa, and 30 kDa, were performed in 123 patients with dyspepsia: 31 with gastric cancer, 31 with duodenal ulcer, 31 with gastric ulcer, and 30 with gastritis and functional dyspepsia. AF *vs* various combinations of seroreactivity against HP VacA, 50 kDa, and 30 kDa in patients with functional dyspepsia and others were analyzed in this study. Synchronous and alternative seroreactivity against VacA, 50 kDa, and 30 kDa, along with/without AF in patients with functional dyspepsia and other groups of patients were also analyzed. **Results.** VacA and 50 kDa seropositivity or AF had excellent case-findings clinical utility index for investigating dyspepsia. The absence of AF and seroreactivity against VacA either with: 50 kDa or 30 kDa seropositivity or 50 kDa and 30 kDa seropositivity had an excellent screening clinical utility index for investigating dyspepsia. **Conclusion.** Seroreactivity against HP antigens VacA, 50 kDa, and 30 kDa might improve our approach to patients in investigating dyspepsia if used along with AF.

Key words:
antigens; biomarkers; duodenal neoplasms; duodenal ulcers; gastroscopy; helicobacter pylori; stomach neoplasms; stomach ulcer.

Apstrakt

Uvod/Cilj. Simptomi i znaci alarma (SZA) su od ograničene koristi u predikciji endoskopskih nalaza i većina pacijenata sa neistraženom dispepsijom neće imati nalaz organske patologije tokom gornje gastrointestinalne endoskopije. U našoj prethodnoj studiji istakli smo seroreaktivnost protiv *Helicobacter pylori* (HP) antigena VacA, 50 kDa i 30 kDa kao biomarkere karcinoma želuca, peptičkih ulkusa i funkcionalne dispepsije. Ova pilot studija je dizajnirana i sprovedena sa ciljem da se uporedi dijagnostička korist seroreaktivnosti protiv HP VacA, 50 kDa i 30 kDa sa onom od SZA i da se istraži mogućnost i adekvatnost njihovog zajedničkog korišćenja. **Metode.** Od 123 bolesnika sa dispepsijom među kojima je bilo 31 sa karcinomom želuca, 31 sa ulkusom duodenuma, 31 sa ulkusom želuca i 30 sa gastritisom i funkcionalnom dispepsijom uzeta je pažljiva anamneza i obavljen fizikalni pregled sa posebnim osvrtom na SZA. Svim bolesnicima je urađena ezofagogastroduodenoskopija sa biopsijom, ultrazvuk abdomena ili kompjuterizovana tomografija, kompletna krvna slika (KKS) i biohemijske analize, *Western*

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Blot IgG prema HP antigenima VacA, 50 kDa i 30 kDa. Analizirana je pojava SZA naspram različitih kombinacija seroreaktivnosti protiv HP VacA, 50 kDa i 30 kDa kod bolesnika sa funkcionalnom dispepsijom i ostalih bolesnika. Analizirana je sinhrona i alternativna seroreaktivnost protiv VacA, 50 kDa i 30 kDa sa/bez SZA kod funkcionalne dispepsije i drugih grupa. **Rezultati.** Seropozitivnost VacA i 50 kDa ili SZA imaju odličan dijagnostički klinički indeks korisnosti kod neistražene dispepsije. Odsustvo SZA i seroreaktivnosti protiv VacA bilo sa 50 kDa ili 30 kDa seropozitivnosti ili 50 kDa i 30 kDa seropozitivnosti imaju odličan klinički indeks korisnosti za skrining kod neistražene dispepsije. **Zaključak.** Seroreaktivnost protiv HP antigena VacA, 50 kDa i 30 kDa može unaprediti naš pristup bolesnicima sa neistraženom dispepsijom ukoliko se koristi zajedno sa SZA.

Ključne reči:
antigeni; biološki pokazatelji; duodenum, neoplazme; duodenum, ulkus; gastroskopija; helicobacter pylori; želudac, neoplazme; želudac, ulkus.

Introduction

Dyspepsia is a complex condition comprising chronic and recurrent symptoms related to the upper gastrointestinal tract. The cardinal symptoms are epigastric pain and discomfort, including postprandial fullness and early satiety, which may overlap with heartburn and regurgitation. Around 25–40% of adults in the general population have dyspepsia, and dyspepsia accounts for 2–5% of all consultations in primary care [1]. Although several guidelines have been published [1–6], the management of patients with uninvestigated dyspepsia is still controversial [2]. Individual dyspeptic symptoms or subgroups of symptoms, such as predominant epigastric pain (ulcer-like) or discomfort (dysmotility-like), poorly predict the presence of underlying organic lesions. A systematic review found that neither primary care doctors nor gastroenterologists could distinguish patients with organic lesions from those with functional dyspepsia (FD) on the basis of symptom evaluation. Dyspeptic symptoms can have several organic causes, but in many patients, no obvious cause is identified. Extragastrointestinal causes, such as hepatobiliary and pancreatic diseases, are infrequent but important and should always be considered. However, most cases of dyspepsia can be ascribed to one of four causes – gastro-oesophageal reflux disease with or without oesophagitis, peptic ulcer disease, malignancy, and FD [3]. Traditionally, clinicians are alerted to the possibility of malignancy by the history and physical examination; alarm features (AF) on the clinical evaluation that include chronic gastrointestinal bleeding, progressive unintentional weight loss, progressive difficulty swallowing, persistent vomiting, iron deficiency anemia, or epigastric mass. AF, also called alert features, red flags, or warning signs, are specific features thought to be associated with serious gastroenterological diseases such as underlying malignancy or significant pathologies such as a stricture or ulcer.

Current guidelines recommend that, in the initial evaluation of patients with dyspepsia, the decision to perform endoscopy should be based on older age and AF because it is generally believed that these factors indicate a higher probability of malignancy being present [4].

Although AF are used to help prioritize access to upper gastrointestinal endoscopy, they are of limited utility in predicting endoscopic findings, and the majority of patients with dyspepsia will have no organic pathology identified at upper gastrointestinal endoscopy. This has led to the investigation of alternative diagnostic approaches, including whether a capsaicin pill or combined serum biomarkers can accurately identify patients with FD. However, there is insufficient evidence to support either of these approaches at present [5].

Our previous publications suggested that Western Blot IgG against *Helicobacter (H) pylori* antigens VacA, 50 kDa, and 30 kDa may be a biomarker for a specific outcome of an infection encompassing gastric cancer (GC), duodenal and gastric ulcer (DU and GU, respectively), and FD, as the most important cause of uninvestigated dyspepsia [5, 6].

Therefore, we designed and conducted an additional pilot analysis on the material of our previous study in order to investigate the diagnostic value of seroreactivity against *H. pylori* VacA, 50 kDa, and 30 kDa *versus* and along with AF, as a non-invasive predictor for GC and peptic ulcers (PU) as well FD.

Methods

The study was conducted and performed in 2009 at the Clinic for Gastroenterology and Hepatology, Institute of Pathology and Institute of Microbiology of the Military Medical Academy (MMA), Belgrade, Serbia. We selected and enrolled patients with upper dyspeptic symptoms, different underlying diseases [gastric cancer (GC), duodenal ulcer (DU), gastric ulcer (GU), and gastritis), and actual *H. pylori* infection confirmed by histopathological examination and anti-*H. pylori* IgG positive Vira Blot.

We have taken a careful history from all patients and performed a physical examination, abdominal ultrasonography (US) or computed tomography (CT), esophagogastroduodenoscopy (EGDS), complete blood count (CBC), and liver and renal chemistry. Inclusion criteria were as follows: 1) presence of upper dyspepsia symptoms; 2) previously untreated patients due to *H. pylori* infection; 3) without proton pump inhibitors and H_2 blockers use in the last two weeks; 4) absence of malignancy except for GC; 5) absence of any immunological disorder; 6) informed consent of the patients for: EGDS and biopsy, taking the blood samples for analyses, participation in the study; endoscopic and histopathological diagnosis of one of the following diseases: GC, DU, GU, gastritis; 8) confirmed histopathological diagnosis of *H. pylori* infection; 9) Western blot (ViraBlot) IgG positive for *H. pylori* infection.

EGDS was performed in all our patients in the endoscopy section using Olympus (GIFQ165, SN: 2207997,

Olympus Corporation, Tokyo) forward-viewing EGD under local application of Xylocaine spray. A minimum of four gastric mucosal tissue biopsies (2 each from the antrum and corpus) were taken, including additional biopsies from every endoscopically visible lesion. All patients were examined for findings suggestive of endoscopic gastritis, such as erythema, hyperemia, atrophy, and mucosal nodularity according to the criteria of the Sydney grading system, and for gastric tumor, duodenal and gastric ulcer [7, 8]. All the obtained biopsies were collected, placed on filter paper, fixed in 10% neutral formalin, and sent for preparation of formalin-fixed, paraffin-embedded tissue blocks. Three-micrometer-thick sections were prepared. One set of tissue sections was stained with hematoxylin and eosin and the other with Giemsa stain for histopathological examination, including detection of *H. pylori* in the gastric mucosa. The biopsies were evaluated for the intensity of mononuclear inflammatory cellular infiltrates, inflammatory activity (neutrophilic infiltrations), glandular atrophy, metaplasia, and *H. pylori* colonization [8]. Additionally, the cases were graded according to the Houston-updated Sydney system [7], which was graded according to the intensity of mononuclear inflammatory cellular infiltrates within the lamina propria: absent inflammation (Grade 0), mild inflammation (Grade 1), moderate inflammation (Grade 2), and severe inflammation (Grade 3). Grading was done for activity, atrophy, intestinal metaplasia, and degree of *H. pylori* colonization. Additional immunohistochemistry staining was performed in the case of a tumor.

The blood samples were obtained from all of them and frozen at -20 °C degrees. Using the Western Blot detection system (ViraBlot), IgG anti Vacuolating cytotoxin A (VacA) 87 kDa, Cytotoxin associated with gene A (CagA) 136 kDa, urease B 66 kDa (UreB 66), heat shock protein 60 kDa (Hsp60), flagellin 55 kDa (Fla 55), 50 kDa, 30 kDa, urease A 26 kDa (UreA 26), and 24 kDa *H. pylori* antigens were identified. *H. pylori* antigens of ViraBlot represent a combination of German patient isolates of highly antigenic *H. pylori* strains. Bands for diagnosing *H. pylori* infection are divided into highly specific as CagA 136 kDa, VacA 87 kDa, 30 kDa, UreA 26 kDa, 24 kDa, and less specific as Hsp 60 kDa and 50 kDa. According to the manufacturer guidelines for use, the test was negative if: there were no bands or if there were nonspecific bands, one or more UreB 66 kDa, Hsp 66 kDa, Fla 55 kDa, 50 kDa. The test was suspected positive: if one clear of a specific band of 30 kDa, UreaA 26 kDa, 24 kDa was present. The test was positive if: at least one band of the following two specific CagA 136 kDa or VacA 87 kDa or at least one clear band of 30 kDa, Urea A 26, 24 kDa or one clear band of 30 kDa, UreA 26 kDa, 24 kDa and one clear band of Hsp60 kDa, 50 kDa was present.

All patients included in our cross-sectional study were classified and analyzed in several ways. In the first analysis we compared frequency of AF and six in previously work selected synchronous/alternative combinations [3] of VacA seroreactivity, 50 and 30 kDa seroreactivity: VacA+ or 50 kDa-; VacA+ or (50 kDa- 30 kDa-); VacA- 50 kDa+; VacA- 30 kDa+; VacA- 50 kDa+ 30 kDa+; VacA- (50 kDa+ or 30 kDa+) in GC, DU and GU and FD, and the fraction correct (FC) in all groups.

In the second analysis, the frequency and FC of the following seroreactivity combinations and AF were investigated in GC, DU, GU, and FD groups and in all patients: I. VacA+ or AF+ either with 50 kDa- or (50 kDa-, 30 kDa-); II. VacA- and AF- either with (50 kDa+ or 30 kDa+), 50 kDa+, 30 kDa+, (50 kDa+, 30 kDa+). They were also compared with the features of the AF group alone.

Statistical analysis

Complete statistical data analysis was done with the statistical software package SPSS Statistics 18.

Most of the variables were presented as a frequency of certain categories; hence, the *t*-test of proportion or cross-tabulation analysis (odds ratio, confidence intervals) was done to calculate the statistical significance of differences between groups.

In the case of continuous data, variables were presented as median, minimal, and maximal values (range).

Accuracy and discriminative ability of the seroreactivity combinations against *H. pylori* VacA, 50 kDa and 30 kDa, and AF, in predicting the outcome of infection was estimated with Sensitivity (Se), Specificity (Sp), Positive Predictive Value (PPV), Negative Predictive Value (NPV), FC and Clinical Utility Index (CUI) in the form of Case-finding Utility or Positive Clinical Utility Index (CUI+) and Screening Utility or Negative Clinical Utility Index (CUI -). CUI + = Se × PPV and CUI Ve- = Sp × NPV represent important indices for clinicians, estimating together the accuracy and discriminative ability of the test [9, 10].

All analyses were estimated at a minimal level of statistical significance of $p < 0.05$.

Results

Four groups of patients with GC, DU, GU, and upper FD were comparable in gender and age (Table 1). The pa-

Table 1

Demographic and clinical characteristics of the patients

Parameters	Groups				Total (n = 123)
	GC (n = 31)	DU (n = 31)	GU (n = 31)	FD (n = 30)	
Gender, n					
male	10	13	12	13	48
female	21	18	19	17	75
Age (years), median (range)	65 (40–85)	54 (21–87)	67.5 (34–81)	63.5 (21–80)	63 (21–87)

GC – gastric cancer; DU – duodenal ulcer; GU – gastric ulcer; FD – functional dyspepsia.

tients with GCr were represented with advanced GC and none with early GC.

At least one of the AF was present in 81 of 123 participants, with an FC of 71% (Table 2). The GC group had the highest FC (98%) and the lowest FD (60%).

VacA+ 50 kDa- had higher FC (74%), with better results in GU and DU, but lower FC in GC and FD compared with AF.

VacA+ or (50 kDa- 30 kDa-) had a slightly higher FC (72%), higher in DU, GU, and FD, but lower in GC compared with AF.

VacA- (50 kDa+ or 30 kDa+) had the same FC as AF, and in the group with FD, lower in the GC group but higher in DU and GU groups compared with AF.

VacA- 50 kDa+ had higher FC (74%) than AF, lower in GC, and FD, but higher in DU and GU compared with AF.

VacA- 30 kDa+ had a higher total FC (73%), lower in GC and FD, but higher in DU and GU compared with AF.

VacA- (50 kDa+ 30 kDa+) had the highest total FC (76%), slightly lower FC in GC, lower in FD, and higher in DU and GU compared with the results of AF.

There is no significant difference in total FC between groups with different criteria and AF (Table 2).

AF alternatively with VacA+ or 50 kDa, were present in all GC patients, FC (100%), in almost all of DU (97%) and GU (97%), but undesirable in more FD (FC = 40%) (Table 3). Total FC was high (84%), significantly higher than in AF alone ($p = 0.015$). AF approach missed indicating EGDS in 23/93 (25%) patients with GC or PU, with the performance of unnecessary EGDS in 12/31 (39%) patients with FD. The combined approach failed to indicate EGDS in only 2% of patients with a serious disease (no one with cancer, two with PU) but indicated 18/31 (60%) unnecessary EGDS in FD. If we analyzed all unnecessary endoscopies indicated with the combined approach, we would find 7 patients with gastric atrophy of different degrees. Reasons for EGDS would be AF and VacA+ in 4 patients, AF and 50 kDa- in 1, VacA+ in 2, and AF in only 1 patient. After correcting the number of unnecessary EGDS with findings of gastric atrophy, 11 (37%) EGDS were left as "true unnecessary". It is lower than in the AF approach (40%). Total FC with this correction raised to 89%, and, therefore, to a highly statistically significant difference compared with the AF approach alone (71%) ($p = 0.0008$).

VacA+ or (50 kDa- 30 kDa-) or AF+ is present in all patients with GC and 97% of PU but also in 87% of FD. Due to a very high percent of positivity in FD, FC was only 77%, and the difference compared with AF was not significant nor with/without correction for atrophy in FD.

VacA- and (50 kDa or 30 kDa+), along with AF-, were absent in GC and present in 3% and 6% of DU and GU and 47% of FD. FC was 85% and it was significantly better than in AF ($p < 0.01$), and with the correction of atrophy findings in FD, the difference was also statistically significant. With this approach, we will improve proper indication in GC by 3% and will miss proper indication in DU by 3% (39% in

Table 2

The number of confirmatory results regarding alarm feature and seroreactivity against *Helicobacter pylori* VacA, 50 kDa, and 30 kDa in four groups of patients

Parameters	Number (%) of confirmatory results (fraction correct)				
	GC (n = 31)	DU (n = 31)	GU (n = 31)	FD (n = 30)	Total (n = 123)
Alarm feature	30 (97)	19 (61)	20 (65)	12 (60)	81 (71)
VacA+ 50 kDa-	24 (77)	25 (81)	26 (84)	14 (53)	89 (74)
VacA + or (50 kDa- 30 kDa-)	24 (77)	20 (65)	24 (77)	10 (67)	78 (72)
VacA- 50 kDa+	6 (81)	5 (84)	6 (81)	15 (50)	32 (74)
VacA- 30 kDa+	5 (84)	10 (65)	4 (87)	16 (53)	35 (73)
VacA- (50 kDa+ or 30 kDa+)	8 (74)	10 (67)	6 (81)	18 (60)	42 (71)
VacA- (50 kDa + 30 kDa+)	2 (94)	5 (84)	3 (90)	11 (37)	21 (76)

GC – gastric cancer; DU – duodenal ulcer; GU – gastric ulcer; FD – functional dyspepsia.

Table 3

The number of confirmatory results regarding alarm features (AF) alone and/or seroreactivity against *Helicobacter pylori* VacA, 50 kDa, and 30 kDa in four groups of patients

Parameters		Number (%) of confirmatory results (fraction correct)				
		GC (n = 31)	DU (n = 31)	GU (n = 31)	FD (n = 30)	Total (n = 123)
AF		30 (97)	19 (61)	20 (65)	12 (60)	81 (71)
AF or	VacA+ 50 kDa-	31 (100)	30 (97)	30 (97)	18 (40)	103 (84)*
AF or	VacA + or (50 kDa- 30 kDa-)	31 (100)	30 (97)	30 (97)	26 (13)	95 (77)
AF- and	VacA- 50 kDa+	0 (100)	1 (97)	1 (97)	10 (33)	101 (82)*
AF- and	VacA- 30 kDa+	0 (100)	5 (84)	2 (94)	14 (47)	100 (82)
AF- and	VacA- (50 kDa+ or 30 kDa+)	0 (100)	1 (97)	2 (94)	14 (47)	104 (85)**
AF- and	VacA- (50 kDa+ 30 kDa+)	0 (100)	1 (97)	1 (97)	14 (47)	105 (85)**

GC – gastric cancer; DU – duodenal ulcer; GU – gastric ulcer; FD – functional dyspepsia.
AF *vs* other AF and/or seroreactivity combinations: * $p < 0.05$; ** $p < 0.01$.

AF) and in GU by 6% (35% in AF) with failure to help us in FD by 53% (60% for AF).

VacA-, along with 50 kDa+ and AF- was absent in the group with GC and present in 3% of groups with GU and DU. It was also present in 33% in FD. FC was 82% and it was significantly better than in FD, but with a correction for atrophy findings in FD, significance disappeared.

VacA- and 30 kDa+ AF- were absent from the GC group but present in 16% of DU, 6% with GU, and 47% of FD, making FC 82%, which was not significantly different from AF with atrophy correction.

VacA- (50 kDa-, 30 kDa-) AF- was completely absent in GC, present in 3% of DU and GU, and in 47% of FD. FC was 85%. It was statistically significant ($p < 0.01$) better than AF, being significantly different even after correction for atrophy in FD ($p < 0.05$).

The four combinations, which were significantly better than AF, were analyzed for their diagnostic characteristics (Se, Sp, PPV and NPV, CUI+ and CUI-) (Table 4).

Alarm features alone had moderate sensitivity, low specificity, high PPV, low NPV, satisfactory CUI+ (0.63), and very poor CUI- (0.26), making overall utility poor. After correction for atrophy findings in FD CUI+ become good 0.67 (0.58–0.77), and overall utility satisfactory (FC 75%).

AF or VacA+ 50 kDa- had high sensitivity (98%), very low specificity, high PPV and NPV, excellent CUI+ (0.82), very poor CUI- (0.34), with overall utility satisfactory (FC 84%). After correction for atrophy in FD, Sp rised to a modest 63%, CUI- (0.57) to the satisfactory and overall utility at good test (FC 89%).

AF- along with VacA- (50 kDa or 30 kDa+) had low sensitivity, but high specificity, PPV and NPV, poor CUI+ (0.38) but excellent CUI- (0.82), with overall utility as satisfactory (FC 85%). After correction for atrophy findings in FD, Sp rose to 64%, CUI+ became satisfactory (0.57), and overall utility became good (FC 89%).

AF- VacA- 50 kDa+ had very low sensitivity, but high Sp, PPV and NPV, very poor CUI+ (0.28), good CUI- (0.8) and overall utility satisfactory (FC 82%). After correction for atrophy in FD, Se rose to 40%, CUI- became excellent (0.82), and overall utility became good (FC 84%).

AF- VacA- (50 kDa+ 30 kDa+) had low sensitivity, high Sp, PPV and NPV, poor CUI+ (0.41), and excellent CUI- (0.83), making overall utility satisfactory (FC 85%) (Table 4). After correction for atrophy in FD, overall utility became good (FC 86%).

Discussion

The approach to the patient with uninvestigated dyspepsia based on the AF represents a standard with unsatisfactory results. AF approach has high Sp and NPV but low Se and PPV in diagnosing gastroesophageal malignancy. There was a positive correlation between AF and the diagnosis of advanced gastreoesophageal cancer, with the exception of gastrointestinal bleeding signs [11, 12].

The value of individual AF varies widely. Dysphagia, significant weight loss, and age ≥ 55 were found to be significant predictors for GC, but the value of other accepted AF was more limited. Fast track endoscopy in patients with AF suspected of upper gastric malignancy results in a significant yield of cancer (~ 4%) and serious benign diseases such as severe oesophagitis, peptic stricture, or PU [13]. In Western countries, 40–60% of patients with dyspepsia have normal findings of upper gastrointestinal endoscopy, with findings of oesophageal and gastric cancer in less than 1%. A meta-analysis of the value of AF in the Asian population finds overall malignancy detection of 1.3%, with calculated all organic disease detection of 26.4%, and among them PU 11.9% and oesophageal disease 5.5%. AF yield moderate diagnostic accuracy for predicting malignancy, with an area of 0.74 under curve [13, 14]. A Scandinavian study reported significant endoscopic findings in 32–40% of patients with AF (erosive oesophagitis 15–23%, PU 13–16%, erosive duodenitis 2%, gastric neoplasia 1–2%) [15]. Defining serious endoscopic findings (SEF) as gastric malignancy, PU, stricture, and erosive oesophagitis in 650 patients, Abdeljawad et al. [16] reported only 10.2% of patients had SEFs. AF were more

Table 4

Diagnostics characteristics of alarm feature (AF), and AF or VacA+ 50 kDa-, AF along with VacA- and 50 kDa+, 50 kDa or 30 kDa+, 50 kDa+ 30 kDa+

Parameters	Diagnostics characteristics						
	Sn	Sp	PPV	NPV	CUI+	CUI-	FC
AF	74 (65–89)	66 (43–78)	85 (77–93)	43 (28–58)	0.63 (0.59–0.73)	0.26 (0.12–0.39)	71
AF or VacA+ 50kDa-	98 (95–100)	40 (25–58)	84 (77–99)	86 (67–100)	0.82** (0.75–0.89)	0.34 (0.15–0.54)	84 •
AF- VacA-50kDa+	33 (16–50)	98 (95–100)	83 (62–100)	82 (75–98)	0.28 (0.02–0.54)	0.8* (0.75–0.85)	82•
VacA- (50kDa+30kDa+)	47 (29–65)	98 (95–100)	88 (71–100)	85 (78–92)	0.41 (0.17–0.65)	0.83** (0.79–0.88)	85••
VacA- (50kDa+ or 30kDa+)	47 (28–64)	97 (93–100)	83 (64–100)	85 (78–92)	0.38 (0.15–0.62)	0.82** (0.77–0.87)	85•

Sn – sensitivity; Sp – specificity; PPV – positive predictive value; NPV – negative predictive value; CUI+ – clinical utility index positive; CUI- – clinical utility index negative; FC – fraction correct.

A qualitative interpretation of the clinical utility index: excellent (E) => 0.81; good (G) => 0.64; satisfactory/adequate (SA) => 0.49; poor (P) => 0.36; very poor (VP) < 0.36.

***good clinical utility index; ** excellent clinical utility index; • overall utility satisfactory/adequate; •• overall utility good.**

frequent in patients with SEFs (12.6% *vs* 5.4%). A Nigerian study, considering oesophagitis, gastritis, duodenitis, PU, and gastric polyp or cancer as SEFs, did not find an association between AF and SEFs. Sp, Se, and PPV and NPV of AF are 65%, 49%, 71%, and 41%, respectively [17]. A large multicenter database study encompassing 3,815 participants did not find an effective prediction of age and alarm symptoms for upper endoscopy finding, with a Se of 87% and Sp of 26% [18]. A large Chinese retrospective analysis of more than 100,000 participants reported the presence of AF in only 52% of patients with malignancy, and in patients with AF, only 14.8% were found to have gastric malignancy. The pooled Se and Sp of the AF were 96.6% and 13.4%, respectively [19]. German analysis of 215 patients with GC and AF did not find any association of AF with the stage of the disease, duration of dyspeptic symptoms, age threshold of 45 years, and AF [20].

Our pilot study in a small population with an exceptionally high percentage of SEFs in 75% of participants (Table 1) and a small percentage of non-significant findings in 25% had Se, Sp, and PPV and NPV of AF 74%, 66%, 85%, and 43%, respectively, with clinical utility indices in the range of poor to borderline with a satisfactory range for case-finding (0.63) and very poor for screening (0.26) (Table 4). When we compared FC of the AF group with FC of the investigated seroreactivity combinations, regardless of the numerically higher value of FC in 5/6 seroreactivity combinations, the difference was not significant (Table 2).

Upgrading AF with seroreactivity combinations leads to a significant increase of FC in four out of six AF and seroreactivity against *H. pylori* VacA, 50 kDa, and 30 kDa combinations. AF or VacA seropositivity and 50 kDa seronegativity had significantly better FC than AF alone and yielded a case-finding utility index in the range of excellent (0.82) and overall utility in the range of satisfactory. The absence of AF along with VacA seronegativity and 1) 50 kDa or 30 kDa seropositivity and 2) 50 kDa and 30 kDa seropositivity reached FC significantly higher compared to AF ($p < 0.01$). The absence of alarm features along with VacA seronegativity and 50 kDa seropositivity yielded significantly higher FC than AF alone. AF- VacA- (50 kDa or 30 kDa+) and AF- VacA- (50 kDa and 30 kDa+) had excellent screening utility indices with satisfactory overall utility and good overall utility, respectively. AF- VacA- 50 kDa+ had a good screening utility index and a satisfactory overall utility (Tables 3 and 4).

In our study, we considered the presence of any of the AF as a positive finding without analyzing the specific value of each of them. A very interesting finding of our study was the fact that adding seroreactivity combinations to AF did not overlap with the disadvantages of alarming features but rather upgraded them. In patients with SEFs, particularly for PU peptic ulcers, seroreactivity combination indicated an alert only in patients without AF leading to an increase in test sensitivity. On the other hand, the absence of AF in the FD group was straightened with the presence of a "benign" seroreactivity combination and without significant deterioration of specificity.

The fact that AF did not have such high sensitivity is important, particularly for PU and early GC, which had a significantly better prognosis than the advanced disease. The survival of early GC undergoing curative endoscopic submucosal dissection was 5 years in 92.6% [21] with a significant decrease and an advancing stage of the disease both in Japan [Ia (91.5%), Ib (83.6%), II (70.6%), IIIa (53.6%), IIIb (34.8%), and IV (16.4%) [22]] and Sweden (31–44% in noncardia cancer and 21–43% in cardia cancer) [23]. An Italian report is notably interesting for finding alarm features in only 41.3% of 92 patients ≤ 45 years with GC [24].

Our approach is original for combining alarm features with serology, but it is not the first attempt to use a non-invasive approach for investigating dyspepsia. Low IgG titer against *H. pylori* was associated with GC and incorporated in GC screening ABC method [25]. Low IgG titer against *H. pylori* and anti-*H. pylori* IgG < IgA ratio was also associated with GC [26, 27]. A test with oral capsaicin (75 mg) in 224 patients without AF, investigating dyspepsia yielded a Se of the test between 0.51–0.59, with a Sp of 0.84–0.89 and the PPV for the diagnosis of FD of 70–71%. FD patients had significantly higher median delta symptom scores as compared to inflammatory bowel disease, PU, irritable bowel syndrome, and patients classified with "other disease". Patients with gastroesophageal reflux disease had significantly lower symptom scores when FD was not concomitantly diagnosed compared to the time when FD was present [28]. The combination of pepsinogen, gastrin-17, and anti-*H. pylori* antibodies serological assays (panel test) are a non-invasive tool for the diagnosis of atrophic gastritis. Twenty studies with a total of 4,241 subjects assessed the performance of the serum panel test for the diagnosis of atrophic gastritis regardless of the site in the stomach. The summary Se was 74.7% and the Sp was 95.6%. With a prevalence of atrophic gastritis of 27% (median prevalence across the studies), the NPV was 91% [29]. None of these non-invasive methods has been incorporated into clinical practice until now.

In our study, as in most other studies, we had only locally advanced GC without patients with early GC. Subtle but significant mucosal abnormalities, which usually are not present with AF, could be the target of "upgraded" alarm features within our pilot study and investigated seroreactivity combination in further studies.

AF and/or seroreactivity combination against *H. pylori* VacA, 50 kDa, and 30 kDa may improve the proportion of diagnosed PU playing the role of a "red flag" in patients without AF without a significant increase of unnecessary endoscopies in patients without AF and FD. In our study, AF had a high Se for advanced GC, and even though AF in combination with seroreactivity had maximal FC, it was not possible to reach a statistically significant difference. Therefore, it would be very interesting to test this approach in patients with early GC who, in the majority of cases, have no AF.

Our study has several limitations. First, this is a pilot study in *H. pylori*-infected individuals with a small number of participants and a different proportion of organic disease in comparison with the general population. We do not analyze the specific value of AF separately and use the German

ViraBlot test with *H. pylori* isolates from the German population. Studies about seroreactivity to different *H. pylori* antigens in specific outcomes of infection gave us significant but very variegated results regarding the value of specific *H. pylori* antigens [30–37]. In that sense, local validation of each specific *H. pylori* antigen could be considered.

Hence, the results of our pilot study justify conducting a large prospective study in patients with uninvestigated dyspepsia infected with *H. pylori*, eradication of *H. pylori* in treatment-naïve patients, considering the value of each AF separately and its upgrading with seroreactivity against *H. pylori* antigens VacA, 50 kDa, and 30 kDa in Serbia.

Conclusion

Seroreactivity against *H. pylori* antigens VacA, 50 kDa, and 30 kDa might improve our approach to patients investigating dyspepsia if we used it along with AF. Results of our pilot study demand confirmation and further exploration in a larger, well-designed study.

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Received on July 20, 2020
Accepted on December 14, 2020
Online First December 2020