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LACK OF ASSOCIATION BETWEEN *HELICOBACTER PYLORI*'S VIRULENCE AND INCREASED SERUM C-REACTIVE PROTEIN LEVELS IN FUNCTIONAL DYSPEPTIC PATIENTS

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ABSTRACT - *Background* - Recently, a great variety of studies aimed to investigate and even suggest *Helicobacter pylori* as an important key factor in gastrointestinal and non-gastrointestinal events development. The well-established relationship between bacterial virulence and increased risk for peptic ulcer or gastric carcinoma is not so clear when comparing inflammation markers alterations, such C-reactive protein, with the pathogen. ***Objective* -** The objective of this study was to evaluate the presence of *H. pylori*, bacterial virulence and C-reactive protein serum levels in individuals diagnosed with functional dyspepsia. ***Methods* -** Were prospectively included in this study 489 dyspeptic individuals. They fulfill Rome III clinical criteria for the diagnosis of functional dyspepsia with no organic disease at endoscopy. The bacterial infection was established by histology and urease rapid test. The levels of serum C-reactive protein were obtained by immunonefelometry and CagA status of *H. pylori* positive individuals was determined through an imunoenzimatic assay. ***Results* -** Prevalence rate of *H. pylori* was 66.3% and virulence factor CagA was detected in nearly 43% of positive samples. In addition, it has been noticed an association between *Ilex paraguariensis* (yerba maté) consumption and pathogen's prevalence. An important effect of bacterial infection on inflammation was only observed in gastric epithelium. ***Conclusion* -** No systemic response to the pathogen, measured through C-reactive protein levels, was observed, regardless of CagA status. Otherwise, the intake of yerba maté should be considered as a cultural factor possibly related to *H. pylori*'s transmission.

HEADINGS - *Helicobacter pylori*. Dyspepsia. C-reactive protein. Virulence factors. Inflammation.

INTRODUCTION

Since *Helicobacter pylori* (*H. pylori*) was described by Robin Warren and Barry Marshal, in 1982, this bacterium opened a new period in the gastric microbiology diagnosis and therapeutics[8,24]. *H. pylori* causes one of the most prevalent infections in human beings with a worldwide distribution that can reach relative frequencies that vary from 20% to 90% in different populations[33,35]. The microorganism exhibits a high tropism to the gastric epithelium, where can causes immune and inflammatory responses that may persist for all life if not eradicated[5,10].

This pathogen, that initially was included in the *Campylobacter* genus, is a Gram-negative curved bacillus, presenting 2-6 flagella and an ability to produce urease abundantly[16,24]. Other virulence factors such CagA protein have been studied extensively[7,23,40]. According to the recent reports, CagA positive strains can cause severe damage to the gastric epithelium, being related specially with increased levels of interleukin-8 (IL-8), gastroduodenal ulcers and gastric neoplasia occurrence[26,29,41]. In addition, this virulence factor has been associated with systemic inflammation, resulting in high serum C-reactive protein (CRP) levels and the bacteria presence being related to higher cardiovascular risks[21,37].

Some behaviors have been associated to the transmission of *H. pylori*. Studies have reported that several aspects can be related to incidence and prevalence rates like socioeconomic status, years of study, institutionalization practice or social habits[8,20]. In 2010, a Brazilian study has demonstrated an anti-*H. pylori* activity of plant extracts, like yerba maté tea (*Ilex paraguariensis*), but no association between bacteria prevalence and tea consumption has been previously described[6].

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All *H. pylori*'s infected people present histological gastritis. Moreover, the bacteria, classified as type I carcinogen[32], has been associated with the pathogenesis of gastric and duodenal peptic ulcers, with gastric carcinoma and with the gastric MALT lymphoma[11]. Some studies also suggest a bacterial role in cardiovascular disorders, referring to the inflammatory process that results in the atheroma formation and evolution[1, 17]. Additionally, one of the most challenging questions related to *H. pylori* is the bacterium association with the functional dyspepsia[12,25,27].

As the influence of *H. pylori* CagA positive strains and potential changes in systemic inflammation remains a debatable matter. Thus, our study aimed to verify a possible relationship between bacterial virulence and systemic and/or local inflammation, through the measurement of CRP levels in serum and the comparison with the histological gastric mucosa analysis from functional dyspeptic patients.

METHODS

Patients

Between November 2006 and June 2008, 489 subjects who had undergone upper gastrointestinal endoscopic evaluation to participate in HEROES trial (*Helicobacter* Erradication Relief of Dyspeptic Symptoms), at Hospital de Clínicas de Porto Alegre, Porto Alegre, state of Rio Grande do Sul, Brazil, were prospectively and consecutively included in this study.

As inclusion criteria, the patient should meet the Rome III criteria which includes at least one of the following symptoms: a. bothersome postprandial fullness; b. early satiation; c. epigastric pain; or d. epigastric burning. Individuals presenting organic disorders diagnosed by upper gastrointestinal endoscopy, such as esophagitis, gastric or duodenal peptic ulcers, neoplasia or other conditions that could be the cause of the referred symptoms were excluded from the study. Conditions such previous treatment to *H. pylori*, clinical manifestations of organic diseases or presence of significant comorbidities and non-acceptance of the intervention by the patient were equally considered exclusion criteria.

Additionaly, after informed consent form assignment, subjects answered questions about medical history, dietary habits and quality of life.

Endoscopic procedures

It was performed with a videoendoscope (GIF-100, Olympus Co). After 8 hour fast, patients were sedated intravenously according to their age, weight and tolerance to fentanyl or meperidine plus midazolam. From each patient were collected three antral fragments, one specimen from the gastric body, and one from the *incisura angularis* region. Biopsy samples were submitted to a *H. pylori* investigation by two distinct methodologies: histology and rapid urease test.

Helicobacter pylori diagnosis

A biopsy specimen from each gastric region (antrum, body and *incisura angularis*) was placed in a 0.5 mL Christensen solution (Uretest®, Renylab) to verify the presence of bacterial urease. A positive result was reported if there was a color change from yellow to pink within 12h of incubation at room temperature according to the manufacturer's instructions.

Concurrently, paired biopsy specimens were collected from antrum, fixed with 10% formol and stained for Hematoxylin and Eosin (H&E) and Giemsa. Two independent, experienced and blinded pathologists performed the histological examination, and discordances were solved by a third expert's opinion. The inflammatory status was determined according to Sydney's Endoscopic Classification, as previously described[14]. *H. pylori*'s infection was considered present according to the positivity in both methodologies (rapid urease test and histological evaluation).

Determination of CRP levels

Approximately 10 mL of whole blood were collected from each patient and after 3000 rpm centrifugation during 15 minutes, serum samples were stored at −80°C and analyzed for high sensivity C-reactive protein (hsCRP) and anti-CagA *H. pylori* antibodies, as described below.

CRP levels were measured by a immunonephelometric assay (CardioPhase® hsCRP Dade Behring) on a Behring Nephelometer II analyzer. The detection limit for CRP was 0.17 mg/L, and the measuring range was 0.175–1100 mg/L.

Determination of cagA status

Serological search for anti-CagA was performed with a commercial available kit (CagA IgG EIA WELL®, Radim) according to manufacturer's instructions. After incubation, plates were read in a spectrophotometer at 450 nm and samples with IgG values higher than 15 RU/mL were considered reactive for anti-CagA IgG antibodies. Each sample was measured twice to ensure the precision of the method.

Ethical considerations

All study procedures were conducted in agreement with Declaration of Helsinki, with the Brazilian Federal Resolution 196/96 and were approved by our local institutional review board, inscribed as project number 07-547. All patients were informed about the study's objectives and subsequently provided written informed consent before any intervention.

Statistical analysis

Data are presented as mean (SD) or median (25th - 75th percentile), when otherwise stated. Quantitative variables were first analyzed concerning Gaussian distribution and assessed with t-test and one-way ANOVA (parametric) or Mann-Whitney and Kruskal-Wallis test (non-parametric). Categorical variables were described by absolute and relative frequencies and analyzed using chi-square test with adjusted residuals test. The analysis was performed using SPSS v. 18. A P value was considered significant if <0.05.

RESULTS

The characteristics of the subjects are shown in the Table 1. Regarding to the gastric *H. pylori* status, 66.3% of the functional dyspeptic patients were *H. pylori*-positive. Among these patients, 42.8% presented antibodies against CagA virulence protein.

Relevant findings include i. a higher prevalence of *H. pylori* in the population with less than nine years of education and ii. no association between bacterial frequency and gender, race, smoking habit or alcohol consumption was observed.

It was also asked to the patients about the habit of drinking yerba maté tea, and 45.3% of positive *H. pylori* individuals reported regular consumption of such beverage (P=0.006).

Table 2 shows local and systemic inflammatory status according to the presence of *H. pylori* and CagA virulence factor. In general, it was observed a high association between the presence of anti-CagA and a high inflammation and inflammatory activity in the gastric epithelium ($P \leq 0.001$), without significantly affect the CRP values. Regarding the systemic inflammation marker (hsCRP) and local inflammatory activity or inflammation grade in dyspeptic patients, no association was observed between the systemic inflammation marker and an inflammatory activity (P=0.339) nor between systemic inflammation marker and inflammation grade (P=0.508).

DISCUSSION

Recently, *H. pylori* and has been suggested as an important factor in extra-gastric manifestations such as increased serum CRP levels and high systemic inflammation leading to a higher potential risk factor to the development of cardiovascular diseases[17, 28]. This study was developed

TABLE 1. Sociodemographic characteristics and life style of study population according to *H. pylori* status and virulence

	N	Mean age (SD)	%Females	%White Race	% Education ≥ 9 years	% Smokers			%Alcohol drinkers
						Current	Former	Never	
H. pylori negative	165	46.4 (14.7)	83.0	80.6	68.9	16.8	23.0	60.2	6.2
H. pylori positive	324	46.1 (12.8)	82.4	77.2	57.5	19.2	20.8	60.1	8.8
P-value[a]		0.794	0.964	0.448	0.020		0.746		0.593
anti-cagA negative	111	44.3 (13.1)	83.8	79.3	59.6	18.3	22.0	59.6	7.3
anti-cagA positive	83	45.1 (12.6)	75.9	75.9	52.4	17.1	24.4	58.5	11.0
missing data	130	47.3 (13.8)	84.1	78.6	64.6	18.8	20.5	60.8	7.3
P-value[b]		0.680	0.236	0.700	0.398		0.920		0.634
P-value[c]		0.102	0.208	0.834	0.125		0.960		0.640
Total	489	46.2 (13.5)	82.6	78.3	61.4	18.4	20.5	60.1	7.9

SD: standard deviation; n: number of subjects; [a] Student's t-test used for continuous variables e Chi-square test used for categorical variables; [b] Missing data not considered (Student's t-test used for continuous variables and Chi-square test used for categorical variables); [c] Considering missing data (ANOVA one-way used to continuous variables and Chi-square test used for categorical variables).

TABLE 2. Local and systemic inflammatory status according to the presence of *H. pylori* and cagA virulence factor

		H. pylori positive (%)		*H. pylori* negative (%)	*P*[a]
		cagA (-) n=111	cagA (+) n=83	N=165	
hsCRP (mg/L)	median (25th - 75th percentile)	1.65 (0.69 - 3.94)	1.46 (0.71 - 3.5)	1.39 (0.71 - 3.04)	0.817
Inflammation - n (%)	Absent	0 (0.0)	0 (0.0)	88 (54.7)[b]	< 0.001
	Mild	36 (33.0)	7 (8.5)	60 (37.3)[b]	
	Moderate	71 (65.1)[b]	70 (85.4)[b]	13 (8.1)	
	Severe	2 (1.8)	5 (6.1)[b]	0 (0.0)	
Inflammatory activity - n (%)	Absent	0 (0.0)	0 (0.0)	145 (90.1)[b]	< 0.001
	Mild	74 (67.9)[b]	16 (19.5)	9 (5.6)	
	Moderate	34 (31.2)	60 (73.2)[b]	7 (4.3)	
	Severe	1 (0.9)	6 (7.3)[b]	0 (0.0)	

[a] Kruskal-wallis test used for continuous variables and Chi-square test used for categorical variables; [b] Statistically significant association by adjusted residuals test 5% of significance. hsCRP: high sensivity C-reactive protein.

in order to verify a possible relation between bacterial virulence and systemic and/or local inflammation through the measurement of CRP levels in serum and the histological evaluation of gastric mucosa of functional dyspeptic patients.

Regarding the bacterial influence on the immune system, a study conducted by Lee et al. (2010) indicates that *H. pylori* infection or their lipopolysaccharide stimulation led to significant increased expressions of inflammatory mediators including tumor necrosis factor-alpha (TNF-α), IL-8, inducible nitric oxide synthase and cyclooxygenase- 2[22]. The mechanism of this response affects the innate immunity through the recognition of some conserved m crobial constituents by receptors expressed on host-epith lial cells as well as neutrophils. In the gut, such recognition results in the activation of conserved signaling cascades mediated by nuclear factor kB (NF-kB), mitogen-act vated protein kinases and caspase-dependent signaling pathways[30].

The main findings of our study were: 1. no association between CRP serum levels in *H. pylori* infected patients with functional dyspepsia, independently of CagA status; 2. a remarkable inflammation and inflammatory activity in gastric epithelium of patients carrying the most virulent strain; and 3. a high prevalence of *H. pylori* in patients with less than nine years of education; and, 4. in those who mentioned yerba maté tea consumption.

Regarding the first finding, our results do not agree with some reports that have shown a considerable association of *H. pylori* and increased serum CRP levels, especially in positive-CagA strains[18]. Considering the possible association between higher systemic inflammation levels and *H. pylori* infection, some data show that after the pathogen eradication, serum CRP levels can decrease significantly[2,19]. Otherwise, there is no consensus about this matter. For example, a strong study conducted by Brenner and cols (1999) involving more than 1.800 healthy subjects did not prove the relation between *H. pylori*, bacterial virulence and inflammation markers. Although it was observed an inverse relation between *H. pylori* infection and serum albumin, the bacteria presence was unrelated to C-reactive protein and the leukocyte count, regardless of CagA status[4]. Such result was also reported in several studies that showed no impact of *H. pylori*'s infection in systemic markers of inflammation[13,15,36,38].

CagA is the most extensively investigated virulence factor of *H. pylori* being encoded by cytotoxin-associated genes pathogenicity island (cagPAI)[3]. A strain expressing cagA protein, which is present in more virulent isolates, is typically associated with the production of proinflammatory cytokines, especially IL-8, and such factor has been reported as an important factor related to the peptic ulcer and gastric adenocarcinoma occurrence[41]. It was observed a remarkable inflammatory activity and inflammation in the gastric epithelium of those people with the most virulent strain, whereas, neither inflammatory activity nor inflammation were observed in more than 90% and 54% of *H. pylori*'s negative subjects, respectively.

We also found an important association between prevalence of the pathogen and years of education and yerba maté tea consumption. As previously appointed by other Brazilian researches, 66% of our study population presented *H. pylori*'s infection, among these patients, nearly 58% reported less than 9 years of study. This finding has been also observed in other studies and possibly indicates socioeconomic status and years of study as important conditions associated to the risk factors for *H. pylori*'s transmission[9,33].

According to data previously reported (Kodaira et al.), our findings support that *H. pylori* frequency is not related with smoking habit or alcohol consumption[20]. On the other hand, we report here, for the first time, the association of *H. pylori*'s infection with the cultural habit of drinking maté. Maté is an infusion of the herb *Ilex paraguariensis* that is prepared in a gourd and is drunk very hot through a metal straw. The infusion is shared by different people using the same gourd and straw. Although it has been reported that the herb presents anti-*H. pylory* activity[6], this cultural habit may provide a possible route for the bacterial transmission.

Considering our study procedures, we recognize that CRP is an unspecific marker of acute inflammation and that it can be related to a several diseases and conditions. Firstly described by Tillett e Francis (1930), this inflammatory protein has an important role to predict the cardiovascular risk, among other applications[31,39]. Although we have evaluated systemic inflammation through this analyte, we agree that other parameters should be useful and more specific to determine a possible link between *H. pylori*'s infection and systemic inflammation. In example, some studies have considered to study as inflammation markers interleukin-6 (IL-6), IL-8 and TNF-α, which seems to be specially related to bacterial infection[3,34].

As mentioned above, regarding the remarkable association between the presence of anti-CagA antibodies and higher tissue damage, although with no systemic responses through the measurement of hsCRP levels, we encourage the development of further studies involving additional inflammatory markers that could be related to higher systemic inflammation in functional dyspeptic people carrying *H. pylori* and its virulence factor.

Authors' contributions

Andreolla HF: data collection; laboratory experiments; statistical analysis; manuscript writing. Bona LR: data collection; laboratory experiments. Sander GB: study design. Mazzoleni LE: study design; critical review. Tavares RG: laboratory experiments; critical review; corrections of manuscript. Prolla JC: critical review and corrections of manuscript.

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RESUMO - ***Contexto*** **-** Recentemente, uma grande variedade de estudos tem investigado e até mesmo sugerido a presença de *Helicobacter pylori* como um importante fator no desenvolvimento de eventos restritos ou não ao trato gastrointestinal. A relação já bem estabelecida entre virulência bacteriana e risco aumentado para úlcera péptica ou adenocarcinoma gástrico não parece estar tão elucidada quando se comparam alterações de marcadores inflamatórios, como a proteína C-reativa, com a presença do patógeno. ***Objetivo*** **-** O objetivo deste estudo foi avaliar a presença da infecção por *H. pylori*, a virulência bacteriana e os níveis séricos de proteína C-reativa em indivíduos diagnosticados com dispepsia funcional. ***Métodos*** **-** Foram incluídos neste estudo, prospectivamente, 489 indivíduos dispépticos. Os pacientes deveriam preencher os critérios clínicos de Roma III para o diagnóstico de dispepsia funcional sem apresentar doença orgânica evidenciada a partir da endoscopia. A infecção bacteriana foi estabelecida por histologia e pelo teste rápido da urease. Os níveis de proteína C-reativa foram quantificados através de imunonefelometria e o status para a presença da CagA dos indivíduos infectados por *H. pylori* foi determinado por ensaio imunoenzimático. ***Resultados*** **-** A taxa de prevalência de *H. pylori* foi de 66.3% e o fator de virulência CagA foi detectado em aproximandamente 43% das amostras positivas. Adicionalmente, denotou-se uma associação entre o consumo de *Ilex paraguariensis* (chimarrão) e a prevalência do patógeno. Um importante efeito da infecção bacteriana na inflamação apenas foi observado localmente, no epitélio gástrico. ***Conclusão*** **-** Não foi evidenciada resposta sistêmica ao patógeno aferido através dos níveis de proteína C-reativa, independentemente do status para CagA. Por outro lado, o consumo de chimarrão pode ser sugerido como um fator cultural possivelmente relacionado à transmissão de *H. pylori*.

DESCRITORES - *Helicobacter pylori*. Dispepsia. Proteína C-reativa. Fatores de virulência. Inflamação.

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