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H. pylori effects on ghrelin axis: Preliminary change in gastric pathogenesis

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ABSTRACT

Ghrelin and its receptors are present in the stomach, suggesting that the ghrelin axis plays an essential role in gastrointestinal complications. This investigation aimed to explore the effects of *H. pylori* infection and gastritis on serum ghrelin and ghrelin axis gene expression. In this study, we enrolled 68 adult ambulatory people referred for upper gastrointestinal endoscopy. The individuals were classified into three groups based on *H. pylori* infection and gastritis. Total serum ghrelin and tissue gene expression were tested with ELISA and quantitative RT-PCR, respectively. Serum ghrelin and mRNA expression were significantly lower in *H. pylori*-positive with gastritis subjects compared with both *H. pylori*-negative with and without gastritis. Growth hormone secretagogue receptor1a mRNA expression was not different between groups while *GHSR1b* expression was significantly higher in patients with *H. pylori* infection and gastritis. We propose the ghrelin axis intermediaries, such as *GHSR1b,* as a potential clinical target for gastric disorders.

1. Introduction

H. pylori infection is one of the most common chronic bacterial infections in the world, particularly in developing countries [1]. This bacterium increases inflammatory cytokines. It triggers inflammatory reactions by its virulence factors such as Lipopolysaccharides (LPS), *H. pylori* neutrophil-activating protein (HP-NAP), and cytotoxin-associated gene A (CagA) [2]. In general, primary *H. pylori* infection causes mild chronic gastritis [3]. It has been published that between 1 and 3% of affected individuals would be involved by the presence of gastric adenocarcinoma [4]. Gastric cancer is the third leading cause of cancer deaths worldwide [5]. The importance of *H. pylori* in the development of this cancer is such that it is considered the most crucial risk factor for the development of gastric cancer. Therefore, *H. pylori* has been identified as a class 1 carcinogen by the World Health Organization [6].

Ghrelin, a 28-amino acid peptide, was first introduced by Kojima in 1999 as the Growth hormone secretagogue receptor *(GHSR)* endogenous ligand. Ghrelin-O-Acyltransferase (GOAT) enzyme adds an acyl group to the third residue of serine amino acid after translating ghrelin peptide. Only the acylated form of ghrelin is active and can bind to its receptor with high affinity [7,8]. Ghrelin is mainly produced by X/A-like cells in the stomach [9]; however, other organs also have little ability to create this hormone [10].

The ghrelin receptor belongs to G-protein-coupled receptors, which was identified by Howard in 1996 [11]. This receptor has two known isoforms, including *GHSR1a,* containing seven transmembrane domains (7α), and a truncated isoform known as *GHSR1b*, consisting of five transmembrane domains (5α) [12]. The *GHSR1a* is a functional isoform of ghrelin receptor that can bind to acylated ghrelin and activate it. However, several studies have reported changes in the expression and importance of *GHSR1b* in various pathological conditions [13–15]. Due to the point that ghrelin and its receptor are expressed in different tissues, this axis performs multiple physiological functions in the organs of

Abbreviations: BMI, body mass index; GHSR, growth hormone secretagogue receptor; LPS, lipopolysaccharides; CagA, cytotoxin-associated gene A; NSAIDs, nonsteroidal anti-inflammatory drugs; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RUT, rapid urease test; H&E, Hematoxylin and eosin; LSD, least significant difference test; NF-κB, nuclear factor-κB; ELP4, elongation protein 4 homolog; ANP32B, acidic nuclear phosphoprotein 32 family member B; HDAC7, histone deacetylase 7.

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the body as endocrine and paracrine, including the regulation of appetite, energy homeostasis, glucose homeostasis, bone formation, cardiovascular system, and regulation of the movements of the gastrointestinal tract [16]. Ghrelin is also an anti-inflammatory agent [17]. Local and chronic inflammation is associated with tumorigenesis and carcinogenesis and this correlation has been well established [18,19]. Therefore, ghrelin can be correlated with cancer for its anti-inflammatory role. Researchers have studied changes in ghrelin and its receptors in various cancers, including gastric cancer [20]. Some studies have also suggested ghrelin as a marker for the prognosis of gastric cancer [21].

Numerous studies have explored the serum and tissue ghrelin levels in *H. pylori*-infected subjects. However, the results were inconsistent. Given the importance of *H. pylori* infection and its associated inflammation in gastric cancer development [22], the examination of the ghrelin axis in people at risk of gastric cancer can be critical. No analysis has yet been performed on ghrelin receptors' expression in the gastric tissue of patients with *H. pylori* infection. This investigation aimed to explore the effects of *H. pylori* infection and gastritis on serum ghrelin and ghrelin axis gene expression.

2. Material and methods

2.1. Patients

The study population included 68 adults referred for upper gastrointestinal endoscopy at Kosar Hospital in Semnan, Iran between February 2018 and January 2019. The individuals were classified into three groups based on two factors: *H. pylori* infection and gastritis. Out of the 68 subjects, 31, 28, and nine subjects respectively were *H. pylori*-positive with gastritis, *H. pylori*-negative with gastritis, and *H. pylori*-negative without gastritis. We explained the research process to all the participants in the study and those taking part in the study signed an informed consent form. Research Ethics Committee of the Semnan University of Medical Sciences reviewed the protocol of this research project and approved it with the code IR. SEMUMS. REC 1397.313 and all the stages of study have been carried out following the ethical guidelines in the research method. All the participants signed the informed consent form. The groups studied were homogenous in terms of age, gender, and Body Mass Index (BMI).

Exclusion criteria were smoking and alcohol consumption, pregnancy, BMI over 30 and under 20, diabetes mellitus, cancer, systemic infection, thyroid dysfunction, liver, kidney, and heart failures, and history of gastric surgery, history of *H. pylori* eradication, and use of any NSAIDs, antibiotic or acid suppressants treatment during the three months before the study enrollment.

2.2. Biopsy and serum samples

Blood Sampling and endoscopy operations were performed between 8 and 10 a.m. after a 12-h fasting. Initially, 5 ml of venous blood were taken from the subjects. Blood samples were centrifuged at 3000×*g* for 15 min and the isolated serum was stored at −80 °C until examined. Six biopsies were taken from all the subjects during endoscopy. Two samples were utilized for RNA isolation and gene expression experiments in the antrum. The Samples were immediately immersed in liquid nitrogen and then held at −80 °C until RNA extraction and evaluation of mRNA expression. Other specimens were employed for rapid urease test (RUT) and Hematoxylin and eosin (H&E) staining.

2.3. Diagnosis of H. pylori and gastritis

RUT and microscopic observation of bacteria in stained slides were applied to diagnose *H. pylori-positive* persons. *H. pylori*-positive individuals were determined with a positive RUT and/or H&E stained sections prepared from two specimens of antrum and corpus [23]. We considered patients *H. pylori*-positive when at least one of two diagnostic test results was positive. It classified the microscopic assessment to determine the gastritis status according to the updated Sydney System [24] by a specialized pathologist.

2.4. Measurement of serum ghrelin concentration

The total serum ghrelin level was determined applying the sandwich ELISA method by ZellBio commercial kit. All the reactions were performed in duplicate.

2.5. RNA isolation and cDNA synthesis

Total RNA was extracted from the specimens using TRIzol (GeneAll Co.) according to the relevant protocol. A total of 1 μg of the extracted RNA was treated with DNase I (Sinacolon Co.). Afterwards, 6 μl of DNase-treated RNA samples were used in a commercial cDNA synthesis kit (Yekta Tajhiz Azma Co.) to perform the reverse transcription reaction. In this reaction, cDNA was synthesized from the extracted RNA utilizing the M-MLV reverse transcriptase and random hexamer and oligo (dT) primers according to the manufacturer's instructions.

2.6. Quantitative real-time PCR

The real-time PCR method was used to compare the relative gene expression in gastric samples. We designed the real-time PCR primer by the use of Allele ID 7.5 primer design software (Table 1). The reaction mixture comprised 10 μl of 2x Master Mix, 0.5 μl of each primer (10 μM), and 1 μl of cDNA and the final reaction volume increased to 20 μl by adding water. The initial denaturation was set at 95 °C for 10 min and 40 cycles of 95 °C for 15 s and annealing/polymerization temperature was set at 60 °C for 30 s and melting curve analysis was performed.

2.7. Statistical analysis

Statistical analysis and diagram plotting were carried out through the use of GraphPad Prism version 8.0 and IBM SPSS version 23.0. Shapiro-Wilk test was first utilized to examine the normality of data distribution and P less than 0.05 was considered as an abnormally distributed variable. Where the variable distribution was normal, the parametric *t*-test was employed to compare the independent two groups and one-way ANOVA was applied for over two independents groups. If the data distribution was not normal, the non-parametric Mann-Whitney test was used to compare the two groups and the Kruskal-Wallis test was employed for over two groups. A posthoc Fisher's Least Significant Difference (LSD) test was also applied to compare the groups. We evaluated the correlations between variables in multivariate linear regression calculating P based on the regression Pearson correlation. The confidence interval was considered 95% in all the tests and $P < 0.05$ was considered significant.

3. Results

Table 2 summarizes the information about 68 subjects. The studied

Table 1
Primer sequences.

Symbol	Primers sequences[a]	Amplicon size (base pair)
GAPDH	GGAAGGACTCATGACCACAG CACGTTGGCAGTGGGGAC	**208**
GHRL	GGGAGAAAGGGCGAGTAAGC GTTGGCGAGGGAAGAAGCAT	**162**
GHSR1a	TCCAGCATCTTCTTCTTCCTTC CACTACAGCCAGCATTTTCAC	**156**
GHSR1b	ACGGTCCTCTACAGTCTCATC ATAGGACCCGCGAGAGAAA	**155**

[a] Upper sequences are Forward primers.

Table 2
Characteristics of subjects.

	H + G+ N = 31	*H* –G+ N = 28	*H* -G-N = 9	*Total N = 68*	*P*
Female/Male	20/11	16/12	4/5	40/28	**0.54**[a]
Age (years)	38.58 ± 10.65	43.54 ± 10.26	38.89 ± 13.34	40.66 ± 10.97	**0.19**[b]
BMI (kg/m^2)	24.66 ± 3.56	26.36 ± 2.86	25.37 ± 3.16	25.46 ± 3.29	**0.16**[c]
Ghrelin concentration (ng/ml)	0.56 ± 0.16	0.67 ± 0.12	0.71 ± 0.1	0.63 ± 0.15	**0.001**[b]

Values are presented as mean ± SD. P values are based on.
[a] Chi-square.
[b] One-way ANOVA or.
[c] Kruskal-Wallis tests. N: Number, BMI: Body Mass Index, H-G-: *H. pylori*-negative without gastritis, H-G+: *H. pylori*-negative with gastritis, H + G+: *H. pylori*-positive with gastritis.

groups did not differ in terms of age, gender, and BMI of participants. Serum ghrelin concentration in the patients with *H. pylori,* significantly decreased compared to those not infected with this bacterium either with ($P = 0.002$) or without ($P = 0.004$) gastritis. In the subjects who were not infected with *H. pylori*, no statistically significant differences were observed in serum ghrelin levels between those with and without gastritis ($P = 0.43$) (Fig. 1). There was no statistically significant difference in serum ghrelin levels between men and women ($P = 0.9$) (Table 3). Serum ghrelin was negatively and moderately associated with BMI in both groups with and without *H. pylori* infection (Fig. 2).

In this study, we also evaluated the gastric ghrelin mRNA expression in the subjects with and without *H. pylori* infection. The gastric ghrelin mRNA expression in the patients with *H. pylori* infection was significantly reduced compared to those not infected with *H. pylori* either with ($P = 0.0004$) or without ($P = 0.009$) gastritis. Meanwhile, in the subjects not infected with *H. pylori,* no significant differences were observed in ghrelin expression between those with and without gastritis ($P = 0.9$) (Fig. 3). Ghrelin expression was directly and moderately related to serum ghrelin concentrations in both groups with and without *H. pylori*

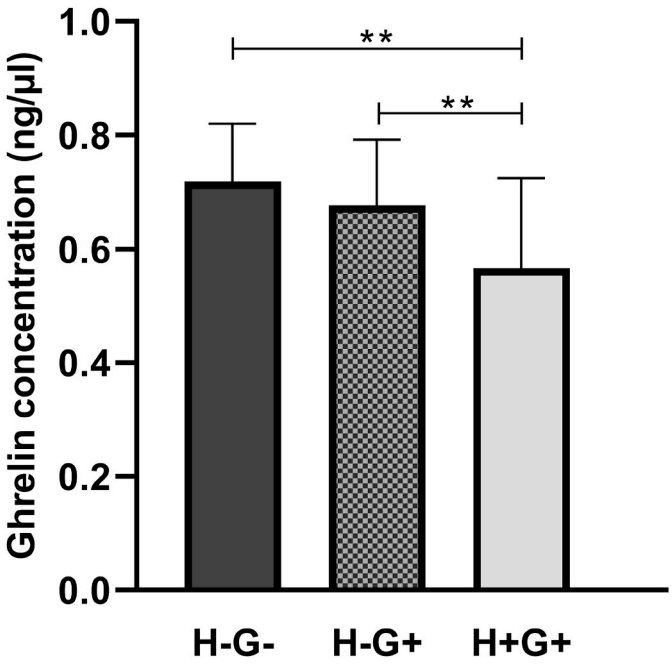

Fig. 1. Serum ghrelin levels. **P < 0.01, Fisher's LSD multiple comparison test, H-G-: *H. pylori*-negative without gastritis, H-G+: *H. pylori-negative* with gastritis, H + G+: *H. pylori*-positive with gastritis.

Table 3
Ghrelin serum levels in male and female subjects.

	***H. pylori* +**	***H. pylori* -**	**Total**
Females	0.56 ± 0.16 N = 20	0.71 ± 0.11 N = 20	**0.63 ± 0.16 N = 40**
Males	0.58 ± 0.16 N = 11	0.66 ± 0.09 N = 17	**0.63 ± 0.13 N = 28**
P	0.7[a]	0.23[a]	**0.9**[a]

Values are presented as mean ± SD.
[a] Independent sample *t*-test. N: Number.

infection (Fig. 4).

Herein, the expression of ghrelin receptors in gastric tissue was also examined. There were no significant differences in *GHSR1a* receptor expression between the groups ($P = 0.23$). However, the *GHSR1b* expression in *H. pylori*-infected patients differed significantly from those who were uninfected either with ($P = 0.04$) or without ($P = 0.0001$) gastritis. Furthermore, the expression of this receptor is affected by gastritis. As we showed that in *H. pylori*-negative subjects, *GHSR1b* expression was increased in the subjects with gastritis, rather than the people without gastritis ($P = 0.01$) (Fig. 5).

4. Discussion

Several key findings emerged from a review of results: the serum ghrelin concentration and gastric ghrelin expression in the patients with *H. pylori* infection were reduced compared to the uninfected individuals (Figs. 1 and 3). Another promising finding was that *GHSR1b* receptor expression increased over four times for *H. pylori* infection and gastritis (Fig. 5).

It is by now generally accepted that *H. pylori* and its subsequent inflammation are the primary risk factors for gastric cancer. On account of the association of inflammatory conditions with the incidence of cancers [25,26], it is essential to know more about the mechanisms that make the inflammation progress [27]. Various studies have investigated the effect of *H. pylori* and gastritis on ghrelin levels; however, the results have been contradictory. Different factors would be considered to achieve inconsistent results, such as the *H. pylori* strain [28,29], the presence or absence of atrophy, the occurrence of the peptic ulcer, and inflammation able to affect the ghrelin level [30,31]. By considering this issue in the present study, a pathologist examined all the subjects in the group with *H. pylori* and diagnosed them with gastritis. The non-infected subjects were also divided into two groups based on gastritis. Atrophy and peptic ulcer was not detected in any of the subjects. It matched all the participants in terms of the grade of gastritis. However, when comparing our results to those of previous studies, it must be pointed out *H. pylori* infection and inflammation can reduce ghrelin expression independent of tissue damage and gastric atrophy (Fig. 1).

We know that cytokine profiles will be different before and after the treatment of *H. pylori* infection and the expression of inflammatory cytokines will decrease following treatment [32]. Stengel et al. reported that intraperitoneal injection of LPS in rats reduced plasma ghrelin levels during the first three injection hours [33]. Various studies have examined the interaction between ghrelin and inflammatory cytokines and shown that ghrelin reduces the expression of inflammatory cytokines and inflammatory cytokines also reduce the expression of ghrelin [34]. Another study revealed that LPS increased NF-κB expression by binding to Toll-like 4 receptors [35]. Comparison of gene expression profiles in gastric antrum with chronic gastritis of three *H. pylori*-infected and three uninfected patients employing cDNA microarray and real-time PCR demonstrated a distinct pattern of gene expression. They found it in response to *H. pylori* alters the expression of genes that directly or indirectly activate the NF-κB pathway [36]. Analysis of the ghrelin gene promoter has also shown that NF-κB can reduce its expression by binding to the ghrelin promoter [37]. Although

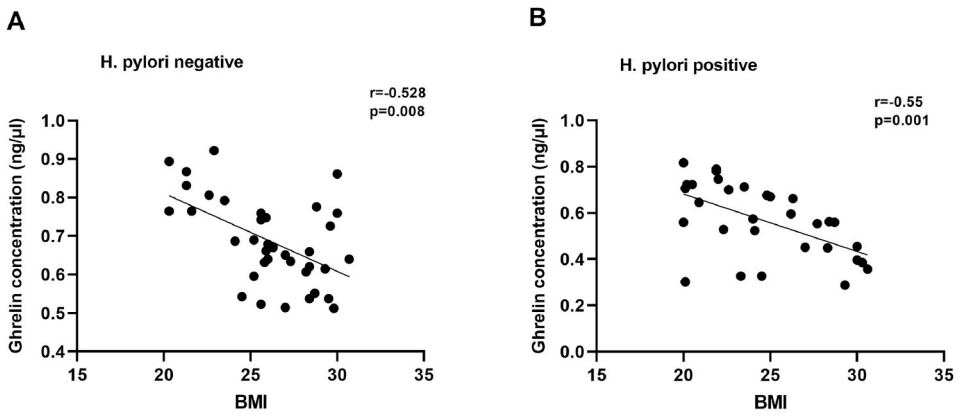

Fig. 2. The correlation between ghrelin concentration and BMI in *H. pylori*-negative (A) and *H. pylori*-positive (B) subjects. R-Value represents Pearson Rank coefficients in multivariate linear regression and *P* was calculated based on regressions Pearson correlation, BMI: Body Mass Index.

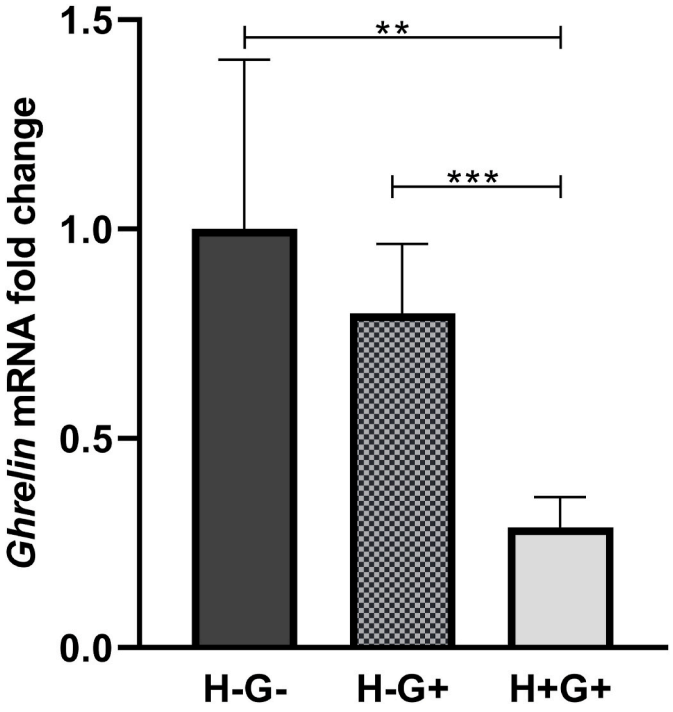

Fig. 3. The ghrelin mRNA levels of the gastric mucosa. **P < 0.01, ***P < 0.001, Fisher's LSD multiple comparison test, H-G-: *H. pylori*-negative without gastritis, H-G+: *H. pylori*-negative with gastritis, H + G+: *H. pylori*-positive with gastritis.

the exact mechanism of ghrelin reduction following LPS injection has not been determined [33], an increase in NF-κB due to *H. pylori* and its binding to the ghrelin promoter may be a possible mechanism. Therefore, the low serum ghrelin in the patients and the decrease in ghrelin expression in gastric tissue in the group of *H. pylori* patients in our study can also be described with this mechanism.

As reported in Pritchett's cohort study, low baseline serum ghrelin concentrations in the subjects were associated with an increased risk of developing gastric cancer and its progression. People in the first quartile in terms of serum ghrelin concentration have a higher risk of gastric cancer up to 10 years after the blood draw [21]. The results of *in-vitro* studies have also revealed that in the gastric cancer cell line (AGS), low ghrelin concentrations can stimulate the proliferation of these cells. On the contrary, they did not observe this effect in high ghrelin concentrations [38]. We observed the lowest ghrelin in the subjects with *H. pylori* infection and gastritis and the highest in those with no gastritis and *H. pylori* infection. These changes were also confirmed to the ghrelin mRNA expression level in gastric tissue (Figs. 1 and 3). Thus, based on the results and previous studies, it can be concluded that ghrelin reduction can be one of the first changes in the incidence of gastric pathology. However, determining this axis role in the onset and progression of gastric cancer requires further investigation.

Many studies have reported changes in the expression pattern of ghrelin receptors in various conditions and diseases; a precise mechanism for it has not yet been proposed [39,40]. Meanwhile, no researches have considered changes in these receptors in subjects with *H. pylori* infection. In the present investigation, the results indicated no significant differences in the *GHSR1a* receptor expression between the groups. However, the *GHSR1b* truncated receptor expression increased in subjects with *H. pylori* and gastritis compared to subjects without such difficulties (Fig. 5).

It has been implied that the transcription factors and chromatin remodeling gene expression are affected in the presence of *H. pylori*. For example, the ELP4 gene, a histone acetyltransferase, is downregulated while the ANP32B and HDAC7 genes which are acetylation inhibitors upregulated [37]. Hence, in *H. pylori* infection, the acetylation of histone H3 and H4 were decreased. Numerous studies have revealed that the pre-mRNA transcription and splicing takes place simultaneously while the pre-mRNA has not yet been separated from the chromatin, the spliceosome performs the alternative splicing. The study of Hnilicová et al. showed that the histone acetylation situation is critical and effectively selects the incision site. Microarray results indicated that histone deacetylation altered the splicing of over 700 genes. Histone H4 deacetylation inhibition causes a higher transcription processivity for RNA polymerase II during transcription on splicing sites [36,41]. The human ghrelin receptor has two splice variants, the primary variant being *GHSR1a*. Investigations have found that in many conditions, such as some cancers, the truncated splice variant of this receptor, *GHSR1b*, increases [42]. In the present work, although the *GHSR1a* receptor expression did not change, the *GHSR1b* receptor expression was increased in the *H. pylori*- and gastritis-positive group compared to the other two groups. According to the mentioned studies related to the effects of *H. pylori* on the Histone deacetylation, we can consider *H. pylori*-induced alternative splicing as a mechanism for higher *GHSR1b* expression. Given that high *GHSR1b* expression was also observed in the gastritis group, it may also involve other mechanisms such as promoter methylation changes [43]. In a study by Nakata et al. on schizophrenia, *GHSR1a* expression decreased and *GHSR1b* increased reciprocally, but they observed no differences in promoter methylation of the gene [15]. However, because of the distinct patterns of methylation in different tissues and conditions [44], changes in methylation patterns may be another mechanism.

It has recently been illustrated that the *GHSR1b* isoform can form

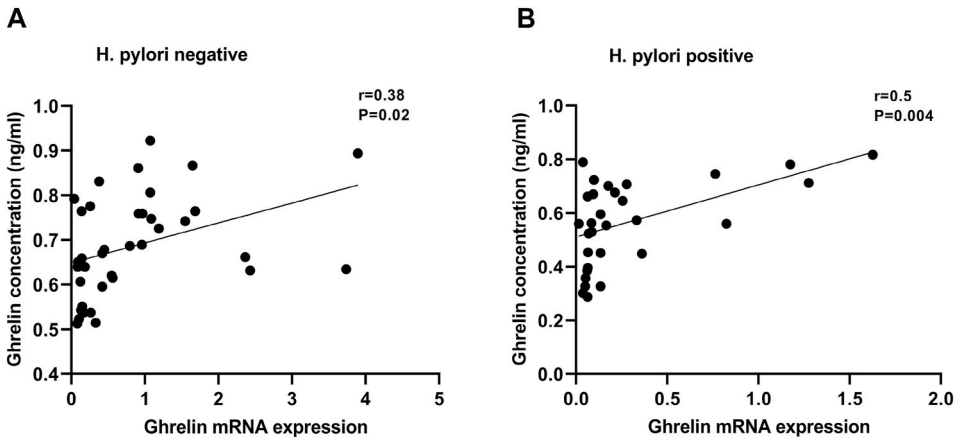

Fig. 4. Correlation between ghrelin concentration and ghrelin's gastric expression in *H. pylori*-negative (A) and *H. pylori*-positive (B) subjects. R-value represents Pearson Rank coefficients in multivariate linear regression, and *P* were calculated based on regressions Pearson correlation.

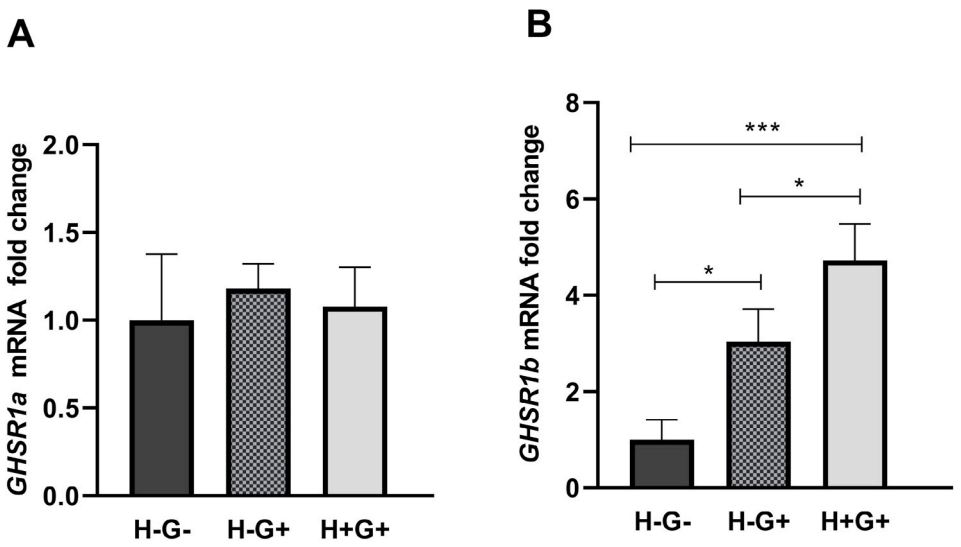

Fig. 5. The *GHSR1a* (A) and *GHSR1b* (B) mRNA expression level of the gastric mucosa. *P < 0.05, ***P < 0.001, Fisher's LSD multiple comparison test, H-G-: *H. pylori*-negative without gastritis, H-G+: *H. pylori*-negative with gastritis, H + G+: *H. pylori*-positive with gastritis.

dimers with the *GHSR1a* receptor and decrease the *GHSR1a* receptor activity [45]. Therefore, in line with previous studies increased expression of *GHSR1b* isoform can be associated with the decreased physiological function of *GHSR1a.* Increased expression of *GHSR1b* in people with *H. pylori* and complications of gastritis can reduce the physiological function of *GHSR1a* to protect the gastric tissue.

In a study conducted on patients with ulcerative colitis, Hosomi et al. found changes in ghrelin axis components [46]. In another study performed on patients with colorectal cancer, these changes were more pronounced. Therein, Waseem et al. reported that ghrelin expression changes in patients with colorectal cancer were associated with tumor progression and increased at higher stages [47]. They also found that as *GHSR1b* expression increased in patients with colorectal cancer, *GHSR1a* expression in tumor tissues decreased. This change in the expression pattern of ghrelin receptors could state the role of the non-functional *GHSR1b* receptor or another unknown receptor associated with *GHSR1b* [47]. Changes of this axis in patients with ulcerative colitis and colorectal cancer reveal that this axis may play a key role in the progression of inflammation to cancer. Just like the correlation between ulcerative colitis and the incidence of colorectal cancer, there is a relationship between *H. pylori* and gastritis and the development of gastric cancer. Gastric cancer is one of the prevalent deadly cancers in the world. There are several risk factors for developing gastric cancer, the most important of which is *H. pylori* infection [48]. Unfortunately, gastric cancer is usually diagnosed at advanced stages of the disease and patients have a short lifespan after diagnosis [49]. Therefore, screening strategies for the diagnosis of gastric cancer in the early stages and even before the development of cancer contributes to a significant reduction in mortality. It can be said that initial screening is necessary to select the target population for further follow-up.

In this study, it was not possible to investigate the direct effects of *H. pylori* antigens on gastric cell biology and the ghrelin axis gene expression. However, in future studies, we will treat cultured cells with *H. pylori* antigens or co-culture cells with *H. pylori.* Therefore, the determination of changes in the expression of receptor splice variants, promoter methylation, and histone acetylation status can explain the precise mechanism of *H. pylori* effects on the ghrelin axis. Although *GHSR1b* acts as a dominant-negative transcription regulator of the *GHSR1a* receptor [42] for the small tissue biopsies in this study, we could not test the receptor property and dimerization at the protein level. It is also interesting for our research team to investigate the dimerization of ghrelin receptors and cellular localization in *H. pylori* infection.

This cross-sectional study persuades us to perform large-scale cohort

and experimental studies and perform a patient follow-up to prove gastric cancer susceptibility in those with this infection, experiencing changes in ghrelin receptor expression.

5. Conclusions

Our study showed that serum ghrelin concentration and its mRNA expression in gastric tissue were associated with *H. pylori* infection and gastritis and can be a criterion for assessing the health status of gastric tissue. To the best of our knowledge, this was the first study examining the ghrelin receptor expression. Our results declared that *H. pylori* infection and gastritis can influence the ghrelin receptor expression. We propose the ghrelin axis intermediaries, such as *GHSR1b*, as a potential clinical target for gastric disorders.

CRediT author statement

Aisa Bahar: Data curation, Writing- Original draft preparation, Investigation. **Majid Mirmohammadkhani**: Formal analysis. ***Reza Dabiri***: Methodology. **Vahid Semnani**: Methodology. **Abbas Pakdel:** Conceptualization, Writing- Reviewing and Editing, Funding acquisition, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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