Serum Calprotectin and B-cell activating factor are potential biomarkers for *Helicobacter pylori* infection

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Abstract

Humans always mount a robust immune response to the bacterial infection caused by *Helicobacter pylori*, which causes various gastrointestinal tract infections. Calprotectin (CALP) and B-Cell Activating Factor (BAFF) are inflammatory biomarkers having a role in the gastrointestinal neutrophilic response to bacterial infection. The study was designed to assess serum CALP and BAFF as inflammatory biomarkers in *H. pylori* infection and peptic ulcer patients. The current study comprised 112 people, including 62 *H. pylori*-infected patients (34 men and 28 women) who were clinically diagnosed with *H. pylori* infection via testing positive for the *H. pylori* stool antigen test; they were compared to a control group of 50 healthy people (34 men and 16 women) who were age and gender-matched to *H. pylori*-infected patients. The serum level of CALP and BAFF were assayed using the ELISA technique. The biochemical parameters were statistically compared between patients and controls by unpaired Man-Whitney U *t*-test and Receiver Operating Characteristic (ROC) curve analysis. There was a significant elevation of serum CALP in *H. pylori*-infected patients [116.4(120.7), p=0.0132] in comparison to healthy controls [99.50(115.8)]. Similarly, there was a significant elevation of serum BAFF concentration in *H. pylori*-infected patients [485.7(367.1), p=0.0014] in comparison to healthy controls [444.5(513.0)]. The ROC curve analysis suggests serum CALP and BAFF as reasonable inflammatory biomarkers for *H. pylori* infection with statistically significant (p=0.0135, p=0.0015) area under the ROC curve of (0.6361, 0.6748), respectively. CALP and BAFF are potent inflammatory biomarkers involved in the development and etiology of *H. pylori* infection. Serum CALP and BAFF levels could be used as biomarkers for chronic inflammation induced by *H. pylori*. CALP and BAFF biomarkers can be combined to diagnose and predict the prognosis of *H. pylori* infection.

Introduction

Worldwide, infection with *Helicobacter pylori* (*H. pylori*) is common; however, the frequency varies significantly between nations and populations, even within one country. The luminal surface of the gastric epithelium harbors *H. pylori* which is a gram-negative spiral-shaped bacterium.[1] Several effector toxins, primarily Vacuolating cytotoxin A (VacA) and Cytotoxin-associated gene A (CagA), released by *H. pylori* cause host stomach lining tissue damage. Furthermore, the gastric epithelium layer acts as a major coop-

erator between the *H. pylori* and stomach host cells that secretes various chemokines to initiate neutrophilic immunity. *H. pylori* infection is the leading cause of gastritis, peptic ulcers, as well as gastric cancers worldwide.[2] Typically, gastritis, peptic ulcer, and gastric cancers are complications of chronic *H. pylori* infection. Peptic ulcers are a nonfatal (curable) disease that results from an *H. pylori* infection that breaks the mucosa lining of the stomach and sometimes proximal intestine, which expands through the muscularis mucosa and then causes exposure to gastric acid and pepsin enzyme. Several epidemiologic investigations have reported a substantial link between chronic *H. pylori* infection with stomach malignancies; therefore, the World Health Organization (WHO) has classified *H. pylori* as a human bacterial carcinogen.[3]

Socioeconomic variables considerably influence the overall prevalence of *H. pylori* infection.[4] *H. pylori* colonization increases the likelihood of developing a variety of upper gastrointestinal tract clinical illnesses but is not a disease in and of itself. To identify the cause of underlying problems, such as peptic ulcers, or to prevent disease, such as in persons with a family history of stomach cancer, an accurate diagnosis of *H. pylori* infection via sensitive markers is necessary. Therefore, it is essential to have a thorough grasp of the clinical etiology of *H. pylori*-related diseases and the impact of *H. pylori* abolition. A robust immune reaction from the host against the infecting strain is typically the result of *H. pylori* infection; however, the infection is seldom cleared due to this immunological response. Studies revealed that the host's immune system, rather than actual bacterial activity, is primarily to be blamed for the pathology related to *H. pylori* infection. Polymorphisms in inflammatory-related genes, such as those that encode for inflammatory cytokines, including Interleukin-10 (IL-10) as well as Tumor Necrosis Factor-α (TNF-α), have been linked to the pathology of *H. pylori* infection.[5]

Calprotectin (CALP), according to the available literature, has many alternative names (Calgranulin A/B, Complex S100A9 and S100A8 proteins, 27E10 antigen, MRP8/14, L1L & L1H proteins); it is a 24 kilodalton antibacterial heterodimer polypeptide made up of heavy Myeloid Related Protein 14 (MRP14) and light Myeloid Related Protein 8 (MRP8) chains (14 and 8 kDa). CALP is a zinc- and calcium-binding peptide consisting of two small anionic domains, S100A9 and S100A8, that constitute about 45% of the cytosolic proteins in neutrophils and only 1% in monocytes.[6,7] Neutrophils and endothelial cells mainly release CALP in response to neutrophil inflammation and monocyte activation or adhesion to endothelial cells. Therefore, CALP is an antibacterial inflammatory factor that is also part of the host's innate immune system. Studies report CALP as a potent inflammation-related peptide that competes with bacteria for zinc, killing the bacteria in the process. The reason for this is that CALP, a biomarker of gastrointestinal tract inflammation, is susceptible to intestinal protease and bacterial enzymes.[8] CALP synthesis and release in pathological processes are induced by several immunomodulatory mediators, including TNF-α, IL-6, and IL-1β.[9]

The B cell-Activating Factor (BAFF) is a recent biomarker for intestinal inflammation. BAFF, also referred as BAFF/B lymphocyte stimulator (BLyS), TNFSF13B, THANK, and TALL1, is a cytokine and a member of the Tumor Necrosis Factor (TNF) superfamily which is primarily synthesized and released by myeloid lymphocytes (macrophages, monocytes, and dendritic cells). BAFF is required for normal B lymphocytes growth, survival, and homeostasis.[10] As a critical cytokine controlling adaptive responses, BAFF, produced mainly by innate immune cells, controls B lymphocyte survival and differentiation into antibody-producing plasma B cells.[11] BAFF can bind to three receptors belonging to the TNF receptor superfamily, each with distinctive expression patterns centered on B lymphocyte development stages and immunomodulatory functions. The receptors are: BR3 BAFF-receptor (BAFF-R/BR3), B Cell Maturation Antigen (BCMA), as well as transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI). These receptors are primarily expressed in B cells and a subset of T cells.[12,13] Several studies showed a potential role of BAFF in the pathogenesis of Inflammatory Bowel Disease (IBD), which looks to be related to the BAFF's ability to trigger multiple immune pathways, including B cell differentiation and survival, expression of proinflammatory cytokines (IL-6, TNF-α), as well as activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) signaling cascade in macrophages and epithelial cells.[10] These evidences show a correlation between BAFF activity and clinical disease conditions and suggest BAFF as a potential clinical biomarker for IBD.[14]

Various clinical diagnostic tests are available for *H. pylori* infection and related diseases. However, each diagnostic procedure has advantages, drawbacks, and limits. Although numerous studies have identified BAFF and Fecal CALP as key biomarkers of gastrointestinal inflammation, none of them has studied their blood levels as inflammatory markers to *H. pylori* infection and Peptic ulcers. Furthermore, based on our understanding and review of the available literature, no local or foreign authors have investigated serum CALP and BAFF as inflammatory markers in *H. pylori*-infected individuals. Therefore, this study aims to evaluate serum CALP and BAFF as inflammatory biomarkers in patients with *H. pylori* infection and peptic ulcers.

Materials and Methods

Study participants

The current study comprised 112 people, including 62 *H. pylori*-infected patients (34 women and 28 men) who were clinically diagnosed with *H. pylori* infection and a subset of them having peptic ulcers. Patients were screened using a brief medical history to rule out any existing systemic disease or drugs that would alter the biochemical parameters to be investigated. The *H. pylori* stool antigen test was positive for all selected patients (mean value: 17.94 COI). They were compared to a control group of 50 healthy people (34 men and 16 women) who were age and gender-matched to *H. pylori*-infected patients.

Sample collection

The samples were collected at Asia Polyclinics in Soran, Iraq, under the supervision of an internist physician. An aliquot of 4 to 5 millilitres of venous blood specimens was collected from each subject, placed in Gold-top serum separator tubes (Gold top-SST), left to stand at room temperature for 15 minutes, and subsequently centrifuged at 3500 rpm for 15 minutes. The serum samples were immediately transferred to Eppendorf tubes that had been pre-labelled and coded. These samples were preserved at –20°C for later examination. Hemolyzed serum specimens were discarded.

Inclusion and exclusion criteria

Patients enrolled in the study had been attending the health clinic for at least six months and had a positive *H. pylori* stool antigen test (results greater than >10 COI). Patients and controls above 45 years were excluded. Participants having diabetes, uncontrolled blood pressure, untreated stress, depression, cigarette smokers, Shisha smokers, alcoholism, or longtime users of non-steroidal anti-inflammatory drugs were excluded from the study since their conditions or lifestyle would alter the biochemical parameters under evaluation.

Research purpose kits

The ELISA kits used throughout this work were Human Calprotectin (CALP) and Human B-Cell Activating Factor (BAFF) (SunLong Biotech, China). *H. pylori* stool antigen test was performed by iChroma™ *H. pylori* SA (Boditech Med Inc., South Korea). The research parameters were analyzed following the manufacturer's provided protocols.

ELISA kit protocols

The Sandwich ELISA method was used in the ELISA kits. The ELISA microplate supplied with the kits has been pre-coated with an anti-CALP and anti-BAFF antibody. Standards or samples were placed in the relevant microplate ELISA wells and mixed with the appropriate antibody. Then, in each microplate well, a Horseradish Peroxidase (HRP)-conjugated antibody specific for CALP and BAFF was added and incubated for 30 minutes. A wash buffer was used to remove free and unbound components (3 wash cycles). Each well received the TMB (3,3', 5,5;-tetramethylbenzidine) chromogen substrate solution. Only wells containing Horseradish Peroxidase (HRP)-conjugated CALP and BAFF antibodies will appear blue and turn yellow after the stop solution (0.16M H_2SO_4) was added. At 450 nm, the Optical Density (OD) was determined spectrophotometrically. The OD value in both kits was consistent to the analyte concentration. The concentrations of CALP and BAFF in the samples were then calculated by comparing the OD values of the samples to the calibration curve created automatically by the ELISA analyzer software (ELISA Microplate Reader BK-EL10C, Biobase, China).

Statistical analysis

The study data was statistically analyzed via the GraphPad Prism 8 computer program. Results of statistical analyses and bar graphs were presented as the median (range). The median values of the study parameters among the patient and control groups were compared using the unpaired Students' *t*-test (Man-Whitney U) test. Additionally, the research parameters' Receiver Operating Characteristic curve (ROC) was analysed. Considering that the Confidence Interval (CI) of choice was 95%, p-values ≤0.05 were considered significant.

Results

The results in Table 1 demonstrate the comparison of serum CALP and BAFF concentration between *H. pylori*-infected patients and healthy control groups.

Serum level of Calprotectin

The results in Table 1 and the bar graph in Figure 1 show a significant elevation of serum CALP in *H. pylori* infection and peptic ulcer patients compared to healthy controls. The ROC curve analysis suggests CALP as a reasonable and valid biomarker for *H. pylori* infection and *H. pylori*-induced peptic ulcers.

Serum level of BAFF

The results in Table 1 and the bar graph in Figure 2 show the significant elevation of serum BAFF concentration in *H. pylori* patients compared to healthy controls. The ROC curve analysis suggests BAFF as a reasonable and valid biomarker for *H. pylori* infection and *H. pylori*-induced peptic ulcers.

Discussion

The serum Calprotectin (CALP) level was assessed as an inflammatory marker for *H. pylori* infection and *H. pylori*-induced

Table 1. Median(range) value of serum CALP and BAFF concentration in control and patient groups.

Biochemical parameters	Healthy controls	*H. pylori*-infected patients	P-values
CALP (ng/mL)	99.50 (115.8)	116.4 (120.7)	0.0132
BAFF (pg/mL)	444.5 (513.0)	485.7 (367.1)	0.0014

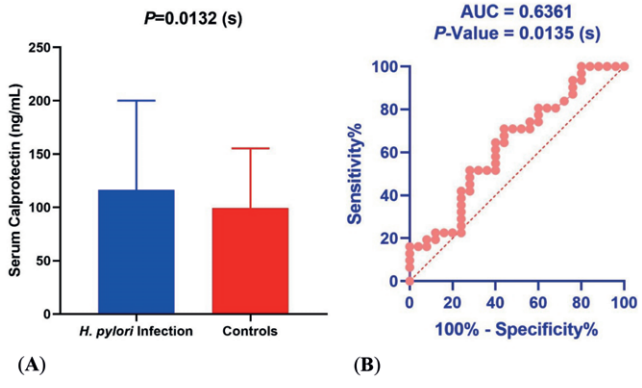

Figure 1. Comparison of CALP concentrations between sera samples of control and *H. pylori*-induced peptic ulcer patients. A show the Bar graph, and B shows the ROC curve.

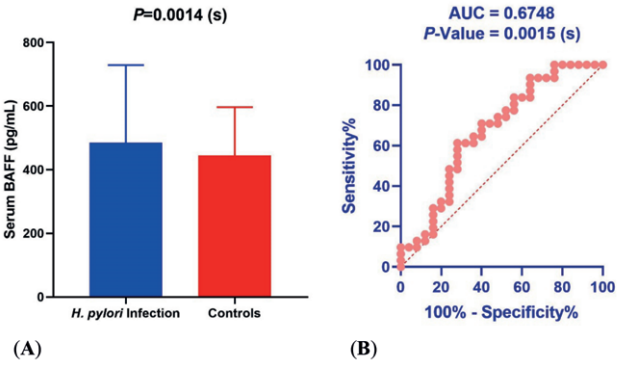

Figure 2. Comparison of BAFF between sera samples of Control and *H. pylori*-induced peptic ulcer patients. A shows the Bar Graph, and B shows the ROC curve.

peptic ulcers. In the current study, the sera CALP concentration increased compared to the healthy control group. There have only been a few published research that explains the link between CALP and *H. pylori* infection. Several studies have looked at fecal CALP as a good inflammatory marker for Inflammatory Bowel Disease (IBD).[6,7,15,16] In addition, several researchers discovered a link between fecal CALP and *H. pylori* infection.[17,18,19] However, no studies have reported the relationship between serum CALP and *H. pylori* infection.

Studies proved that CALP expression increases in many inflammatory processes as a sign of neutrophil chemotaxis.[20] CALP, as an inflammatory phase protein, can exist in the blood, urine, feces, saliva, and cerebrospinal fluid in the human body that plays a compelling role in various biological activities, such as inflammation as well as immunoregulation. During inflammation, CALP functions as an alarm by indicating inflammatory activities mainly induced by neutrophils. CALP transduces its inflammatory signaling and plays its role by binding to its receptors on the cell surface, triggering signal transductions via activation of the nuclear factor-κB (NF-kB) transcription factor and Mitogen-Activated Protein Kinases (MAPKs) pathway that induces leukocyte migration and inflammatory cytokine production in inflammatory regions.[6,15]

Because serum CALP is a viable blood-based biomarker that is more practical in everyday clinical practice and more acceptable for patients, it has attracted increased interest as a biomarker for IBD.[6] Studies accomplished by Kalla *et al.*[7] and Daniluk *et al.*[21] on IBD patients (ulcerative colitis and Crohn's disease) have revealed that serum CALP levels were positively correlated with fecal CALP and were better for IBD diagnosis in comparison to serum Albumin, CRP, and ESR. In a noteworthy study, Hare *et al*. assessed the diagnostic value of serum and fecal CALP in patients with Acute Severe Ulcerative Colitis (ASUC).[22] The study results were in agreement with the present study as they reported fecal CALP did not substantially correlate with serum CALP, but it did with albumin and CRP. Meuwis *et al.*[23] discovered that Crohn's disease patients had higher serum CALP levels than healthy controls in another study similar to ours and that serum CALP was significantly higher in active illness than in inactive disease. Recently, several studies reported a statistically significant elevation of fecal CALP levels in numerous gastrointestinal disorders, including: gastritis, peptic ulcers, ulcerative colitis, Crohn's disease, stomach carcinoma, colorectal carcinoma, colorectal polyps, duodenitis, and other bowel diseases when compared to healthy controls.[16] However, there is a scarcity of data to analyze and illustrate the diagnostic potential of the CALP in blood specimens, which is the purpose of this work. Because of the possible role of *H. pylori* infection in the pathogenesis of IBD, the current study's results are consistent with studies evaluating serum CALP in IBD. Aksoy *et al.*[20] and Wang *et al.*[24] assessed fecal CALP in *H. pylori*-infected patients, illustrating that fecal CALP significantly increased in patients related to healthy controls. Wang *et al.*,[24] Özşeker *et al.*,[25] and Ataee *et al.*[26] found relatively comparable results to the current study, concluding that fecal CALP levels were higher in individuals with active *H. pylori* colonization as well as other gastrointestinal inflammatory disorders.

Various studies concluded that *H. pylori* use the cagType 4 secretion system (cagT4SS) to introduce the cytotoxin-associated antigen A (CagA) oncoprotein into the host epithelial cells, stimulating the production of proinflammatory chemokines and expression of CALP via activation of inflammatory signaling cascades.[27] According to the current study results and available literature, we can postulate that Calprotectin is released as an inflammatory reaction based on the activity and accumulation of neutrophils in the infection microenvironment generated by *H. pylori*. As a result, the serum CALP concentration rises and binds Zn and Mn with high affinity, depriving *H. pylori* of these important micronutrient transition metals and generating a Zn- and Mn-limited environment.[28] According to the findings of Gaddy *et al.* [28] studies, Zinc homeostasis plays a vital role in regulating the cag T4SS during *H. pylori* infection. In response to *H. pylori* infection, enterocytes are recruited to the infection microenvironment, CALP is deposited, and micronutrients Mn and Zn are sequestered, which ultimately represses CagA translocation and cag T4SS pilus formation.[28,29] Taking all of the existing pieces of evidence and the results of the current study, we can infer that serum CALP plays a critical role in the etiology of *H. pylori* infection along with peptic ulcers, as well as its serum level can be employed as a potential diagnostic and prognostic inflammatory marker for *H. pylori* infection and peptic ulcers.

In the present study, the serum BAFF levels were assessed in *H. pylori* infection and *H. pylori*-induced peptic ulcers by comparison with the healthy controls. The serum BAFF concentrations in the *H. pylori*-infected individuals and peptic ulcer patients were significantly higher than in the healthy controls. The present study's findings consider serum BAFF as a promising and new inflammatory biomarker for *H. pylori* infection supported by the ROC curve analysis. The area under the ROC curve indicates that serum BAFF concentration can be used as a reasonable diagnostic and a prognostic marker in monitoring and appointment of *H. pylori* infection.

Recent investigations have shown that the BAFF is a reliable biomarker of gastrointestinal inflammation. BAFF is a cytokine member of the TNF superfamily that exists as a type II membrane protein (mBAFF/BLyS) and a soluble protein (sBAFF/BLyS).[30] The 17 kilodaltons soluble protein BAFF is released into circulation after being broken down by Furin proteases. Therefore, it appears that in inflammatory conditions, cytokines cause non-hematopoietic cells to express BAFF, creating a local microenvironment that may facilitate the initiation of inflammation or reduce its persistence.[13,14] Several clinical experiments reported increased BAFF levels in particular tissues linked to the pathogenesis of inflammatory diseases, autoimmune diseases, and some malignancies, in addition to a significant amount of data from preclinical experiments.[30] The serum levels of BAFF appear to be increased in various autoimmune diseases,[31,32] such as rheumatoid arthritis,[33] Sjogren's syndrome,[34] systemic lupus erythematosus,[35] as well as IBD.[13] Furthermore, some researchers validated that the increased serum BAFF levels were interconnected with increased autoantibodies in autoimmune diseases and correlated with disease activity in most cases.[30]

This case-control study is the first to investigate the biochemical role of BAFF in *H. pylori* infection, demonstrating a probable correlation between serum BAFF level and *H. pylori* infection and peptic ulcers. This conclusion is based on the present study results and a review of the literature on the biological role of BAFF.

No studies have been published that examine serum and fecal BAFF levels as a possible inflammatory marker for *H. pylori* infection. In order to compare the serum BAFF results of the current investigation with recent studies on IBD, we contemplated the possibility that BAFF might become a candidate IBD inflammatory biomarker for the non-invasive diagnosis and prognosis of *H. pylori* infection in the future. Although fecal CALP, the standard *in vitro* diagnostic marker for IBD, is being employed, recent studies have indicated that BAFF can be used as a sensitive biomarker for IBD.[10,13,30]

The present study result was conformable with the findings of Zhang *et al.*[13] and Fu *et al.*,[36] also in agreement with the metanalysis of Kumric *et al.*,[30] Striz *et al.*,[14] and Uzzan *et al.*,[37] as they reported a significant increase of serum and fecal BAFF level in IBD patients.

Contrary to the findings of the current study, the study led by Fodor *et al.*[10] on children found that children with Irritable Bowel Syndrome (IBS) and/or IBD had insignificant differences in their serum BAFF levels but not their fecal BAFF levels. In an interesting study using mice, Wu *et al.*[38] showed that BAFF can activate the T helper 17 cells response by encouraging the production of the proinflammatory cytokines IL-6, IL-1β, and TGF-β, which are closely linked to chronic gastritis brought on by the *H. pylori* bacteria. We may conclude from the available data and the results of the current investigation that BAFF is a key player in the development of *H. pylori* infection and peptic ulcers and in the neutrophilic response. BAFF serum level may be used as a reasonable non-invasive inflammatory marker for diagnosis and prognosis of *H. pylori* infection. If further studies are conducted on more individuals and if better research funding is allocated, the outcomes of the current study will be substantially better. As a result, additional studies are needed to better investigate the diagnostic and prognostic value of serum BAFF for patients with *H. pylori* infection and peptic ulcers.

Conclusions

CALP and BAFF are potent inflammatory biomarkers involved in the development as well as etiology of *H. pylori* infection and *H. pylori*-induced peptic ulcers. BAFF is a novel and potential inflammatory biomarker for IBD and peptic ulcers induced by *H. pylori* infection. The blood levels of CALP and BAFF could be biomarkers for chronic inflammation caused by *H. pylori*. CALP and BAFF biomarkers can be combined to diagnose and predict the prognosis of *H. pylori*-infected individuals. Additional research studies with a systematic methodology and a larger participant pool are required to better assess and grasp the biochemical functions and correlations of CALP and BAFF in *H. pylori* infection and other gastrointestinal tract diseases.

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