

Plasma ghrelin and leptin in patients with inflammatory bowel disease and its association with nutritional status

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Abstract

Background/Aims: Ghrelin and leptin are thought to play a role in the loss of appetite in active inflammatory bowel disease (IBD). This study seeks to probe into the association of these markers with regards to IBD and the nutritional status of these patients. A case-control study was conducted between May 2015 and March 2016 at King Khalid University Hospital (KKUH). Thirty-one patients with IBD (both active and non-active) and forty-one healthy controls (both non-fasting and fasting) were recruited.


Patients and Methods: Plasma ghrelin and leptin levels were determined using an enzyme immunoassay (EIA) technique. The nutritional status was determined through the standardized Mini-Nutritional Assessment (MNA) questionnaire.

Results: The difference in the plasma ghrelin between active (263.7 pg/mL) and non-active (108 pg/mL) cases was significant ($P = 0.02$). The difference in mean plasma leptin level between active cases (229.4 pg/mL) vs. non-active cases (359.7 pg/mL) was insignificant ($P = 0.4$). In fasting (2028.6 pg/mL) and non-fasting controls (438.8 pg/mL), the mean plasma ghrelin values was significantly different ($P < 0.01$). In contrast, the plasma leptin level difference between fasting (727.3 pg/mL) and non-fasting (577 pg/mL) controls was insignificant ($P = 0.14$). There is a statistically significant association in mean ghrelin levels between the case group and the control group ($P < 0.01$). With regards to nutritional status, the mean MNA score of active cases compared to fasting controls was 18.8 ± 5 vs. 20.8 ± 3.8 , respectively ($P < 0.01$)

Conclusion: Ghrelin levels were lower in the active IBD cases compared to the inactive ones, signifying an underlying pathology as etiology to this phenomenon. Furthermore, ghrelin levels were significantly lower in both case groups compared to the controls. These findings, along with the disparity in the MNA scores, insinuate a possible link between hormone levels and the loss of appetite from which these patients suffer.

Keywords: Ghrelin, inflammatory bowel disease, leptin, management, nutritional status

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INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC), comprising a disease entity known as inflammatory bowel disease (IBD), are both immune-mediated diseases that affect different parts of the gastrointestinal tract. Ghrelin, a 28-amino-acid peptide, is an anabolic hormone produced in the fundus of the stomach,^[1] which stimulates hunger, acid secretion, and gut motility. In addition to being a regulator for the body's appetite, ghrelin plays a role in energy metabolism and the secretion of growth hormones.^[2] It has also been found to have an association with inflammation^[3] where there was an association with increased C-reactive protein (CRP) and fibrinogen.^[4] Increased levels of ghrelin were also discovered in those with active IBD compared to those in remission^[5] and those with IBD compared to normal controls.^[6] Other studies have reported variable serum ghrelin levels in IBD patients, rendering its role unclear and controversial.^[7] Leptin, a non-glycosylated protein, is a satiety hormone produced by adipocytes. It has metabolic and immunological effects, linking nutritional status to immune responses.^[2,8,9] Tuzun *et al.* found anorexia and weight loss in acute UC were associated with an increase in serum leptin.^[10] Both ghrelin and leptin have been implicated in studies as potential mediators of inflammation in IBD, as one study by Karmiris *et al.* shows.^[11]

The aim of this study is to determine if there is any difference between the plasma ghrelin and leptin levels in patients with IBD (active and inactive) compared to controls and their association with the nutritional status in both. This study may highlight the importance of ghrelin and leptin concentration in IBD patients as disease markers in those patients.

PATIENTS AND METHODS

This study was conducted in King Khalid University Hospital (KKUH), Riyadh, Saudi Arabia between May 2015 and March 2016. Thirty-four patients suffering from IBD were included in the study; their diagnoses were based on clinical, histopathological, and endoscopic criteria (Mayo Clinic score for UC). Those cases were subdivided into active disease ($n = 22$) and non-active disease ($n = 9$) according to the endoscopy reports and clinical assessment of the managing gastroenterologist. Exclusion criteria were patients over the age of 60 years, on hormone-altering medication (e.g., tricyclic antidepressants), or those with *H. pylori* gastritis (which were elicited based on patient history and his/her medical records).

As for the control group, forty-one healthy participants similar in demographic parameters to the cases were

recruited from the KKUH blood bank and phlebotomy lab of the primary care clinic. Those were also subdivided into fasting for more than 8 hours ($n = 21$) and non-fasting participants ($n = 20$).

The nutritional status was determined through the standardized Mini-Nutritional Assessment (MNA).^[12] This questionnaire contains 28 questions where a score of 24 to 28 was considered "normal nutritional status," while a score of 17 to 23.5 was considered "at risk of malnutrition" and less than 17 points "malnourished." The assessment included age, body mass index (BMI) in kg/m², loss of appetite, weight loss, lifestyle, and dietary habits.

The institutional review board approved the study. Each participant signed a consent form prior to participation. Participant anonymity and privacy were preserved and their blood samples were used within the confines of the objectives of this research exclusively.

Biochemical assessment

A blood sample of 10 mL was obtained from all the participants by brachial vein puncture. The blood samples of both cases and controls were collected in two plasma tubes containing sodium citrate, citric acid, and dextrose. The collected blood samples were centrifuged for 10 minutes then plasma was separated and stored at $<-20^{\circ}\text{C}$. After completing the sample collection for cases and controls, plasma ghrelin and leptin measurement was done.

Ghrelin concentrations were measured using commercially available EIA assay kits (Phoenix Pharmaceuticals, Burlingame, CA). In this assay, ghrelin in the plasma simultaneously binds to two antibodies directed against different epitopes on the ghrelin molecule. One antibody (capture antibody) coats the wells of a 96-well microplate while the other (detection antibody) is conjugated to biotin. After removal of unbound detection antibody by washing three times with tween-20 and PBS, 100 μL of streptavidin-horseradish peroxidase conjugate (streptavidin-HRP, Zymed Laboratories Inc., San Francisco, CA) was added to each well, followed by 20 minutes of incubation. Streptavidin-HRP binds to the bound detection antibody conjugate. The plate was then washed as before. A substrate specific for HRP (tetramethylbenzidine substrate solution, 100 μL) was added to each well, followed by 30 minutes of incubation in the dark to detect the amount of conjugate bound to the Ghrelin antibodies. Fifty μL of 2 N sulfuric acid diluted with double distilled water (1 mL of 2 N H₂SO₄ to 3 mL of ddH₂O) was added to each well to stop the reaction, and the plate was read at 450nm by a 96-well microplate reader.

Leptin plasma concentration was measured using commercially available ELISA kits (R&D systems, Bio-technie brand, Minnesota). In this assay, a monoclonal antibody specific for leptin is precoated onto a microplate. One hundred microliter of assay diluent RD1-19 was added to each well. After that, 100 μ L of standards and samples were pipetted into the well and covered with a strip for 2 hours. An immobilized antibody binds any leptin present. After washing away any unbound substances by adding 400 μ L of wash buffer and aspirating a total of four times, 200 μ L of an enzyme-linked monoclonal antibody specific for leptin was added to the wells and incubated for 1 hour. Following a wash to remove any unbound antibody-enzyme reagent, a 200 μ L of substrate solution was added to the wells followed by 30 minutes of incubation in the dark. That resulted in color development in proportion to the amount of leptin bound in the initial step. The color development was stopped by adding 50 μ L of stop solution to each well. The optical density of each well was measured using a microplate reader set to 450 nm with wavelength correction set to 540 nm.

Data analysis

Data analysis included descriptive statistics computed for continuous variables, including means, standard deviations (SDs), minimum and maximum values, as well as 95% confidence intervals (CIs). Frequencies were used for categorical variables. When hypothesis testing was conducted, the paired *t*-test, as well as Fisher's exact test, where appropriate, were used. When comparing more than one group, a one-way analysis of variance (ANOVA) was used to test for differences among these groups. We used the software STATA 11.2 (Stata Corp., College Station, TX, USA) for analysis. A statistical significance threshold of $P = 0.05$ was adopted. No attempt at imputation was made for missing data.

RESULTS

Cases

Demographics

A total of 34 IBD cases were enrolled into the study; three were excluded due to incomplete information (response rate of 95.9%). Twenty patients had CD (64.5%) while 11 had UC (35.5%). The mean age of the participants is 32.3 ± 11.8 years and involved 21 females (67.7%) and 10 males (32.3%) [Figure 1]. When asked about the most frequently used medication, 51.6% were on azathioprine, 41.9% on infliximab/adalimumab, and 22.6% on steroids. The mean BMI was 24 ± 8.5 kg/m² [Figure 2].

The cases were then sub-divided into active disease ($n = 22$; 71%) and non-active disease ($n = 9$; 29%) according to

the endoscopy reports with the precise definitions. In the active group, 8 cases were UC (36.4%) and 14 cases were CD (63.6%), whereas in the non-active group 3 were UC (33.3%) and 6 were CD (66.7%).

Levels

The mean plasma ghrelin level in active cases was 263.7 ± 400.7 pg/mL compared to 108 ± 71.6 pg/mL in the non-active cases (P value = 0.02; Figure 3). Furthermore, the mean plasma leptin level was 229.4 ± 199 pg/mL in active cases vs. 359.7 ± 318.5 pg/mL in non-active cases ($P = 0.4$; Figures 3 and 4).

MNA

On a scale of 28 points, the mean MNA score for the active cases was 18.8 ± 5 points compared to 18.1 ± 6.3 points for the non-active cases. Participants were then classified based on their scores to malnourished, at risk of malnutrition, and normal nutritional status [Table 1]. When patients were asked about their food intake during the three months prior to their enrollment, 12.9% reported that they had a severe decrease in food intake, 41.9% reported a moderate decrease and 45.2% reported their intake as normal. While 29% reported weight loss greater than 3 kg, 19.4% reported weight loss between 1 and 3 kg, and 38.7% reported no weight loss at all. 12.9% were not aware of any weight loss during the same period.

Controls

Demographics

A total of 41 healthy controls were enrolled into the study. The mean age of the participants is 29.21 ± 10.17 years old [Figure 1] and involved 24 females (58.5%) and 17 males (41.5%). With regards to the physical measurements, the mean BMI was 28.1 ± 6.9 kg/m² [Figure 2]. The controls were then sub-divided into fasting (21; 51.2%) and non-fasting (non F) (20; 48.8%).

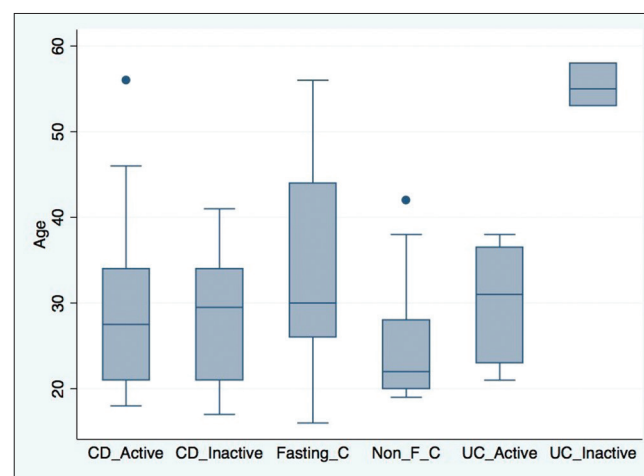


Figure 1: Age of the Crohn's Disease (CD) and Ulcerative Colitis (UC) patients in relation to disease activity

Levels

The mean plasma ghrelin level in fasting controls was 2028.6 ± 1333.1 pg/mL compared to 438.8 ± 226.6 pg/mL in the non-fasting controls ($P < 0.001$). On the other hand, the mean plasma leptin level was 727.3 ± 374.2 pg/mL in fasting controls vs. 577 ± 399.6 pg/mL in non-fasting controls ($P = 0.14$) [Figures 3 and 4].

MNA

On a scale of 28 points, the mean MNA score for the fasting was 20.8 ± 3.8 points compared to 23.4 ± 3.3 points for the non-fasting controls ($P < 0.01$). Participants were then classified based on their scores to malnourished, at risk of malnutrition, and normal nutritional status [Table 1]. When the controls were asked about their food intake during the three months prior to participating in the study, 2.44% reported that they had a severe decrease in food intake, 26.83% reported a moderate decrease, and 70.7% reported their intake as normal. With regards to weight loss

during the same period, 9.8% reported weight loss greater than 3 kg, 9.8% reported weight loss between 1 and 3 kg, and 75.7% reported no weight loss at all. Around 4.88% were not aware of any weight loss during the same period.

DISCUSSION

There is a marked disparity in the plasma hormonal assays of ghrelin and leptin between the four sub-groups of our study [Figures 5 and 6]. Starting with ghrelin, the mean plasma level is higher in the patients suffering from active IBD compared to their non-active counterparts. A similar pattern was observed in the healthy controls; those who were fasting prior to the blood sampling had higher levels than with those who were not fasting. However, when comparing between the cases and controls, the ghrelin levels are markedly reduced in the case groups compared to their respective healthy counterparts. A significant

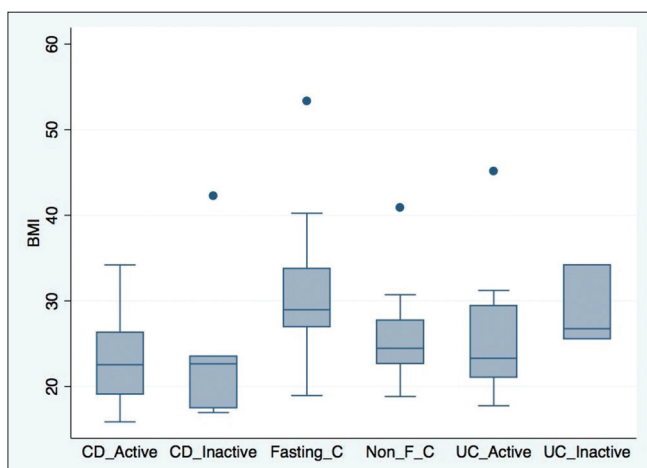


Figure 2: Body mass index of the Crohn's Disease (CD) and Ulcerative Colitis (UC) patients in relation to disease activity and fasting state

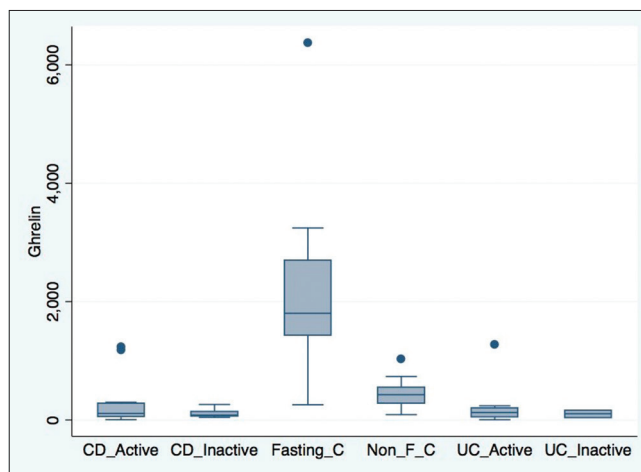


Figure 3: Plasma Ghrelin levels of the Crohn's Disease (CD) and Ulcerative Colitis (UC) patients in relation to disease activity and fasting state

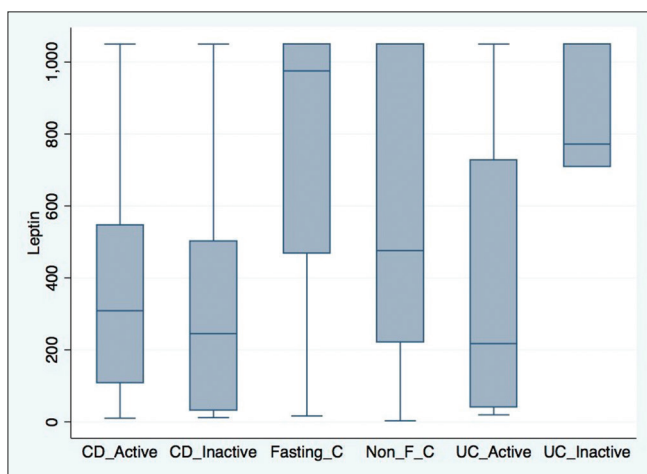


Figure 4: Plasma Leptin levels of the Crohn's Disease (CD) and Ulcerative Colitis (UC) patients in relation to disease activity and fasting state

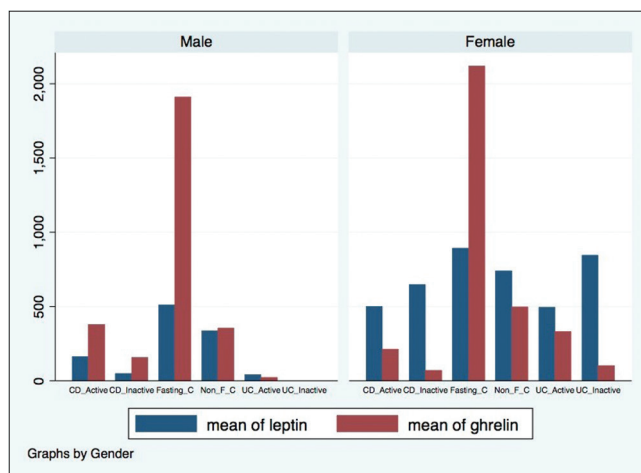
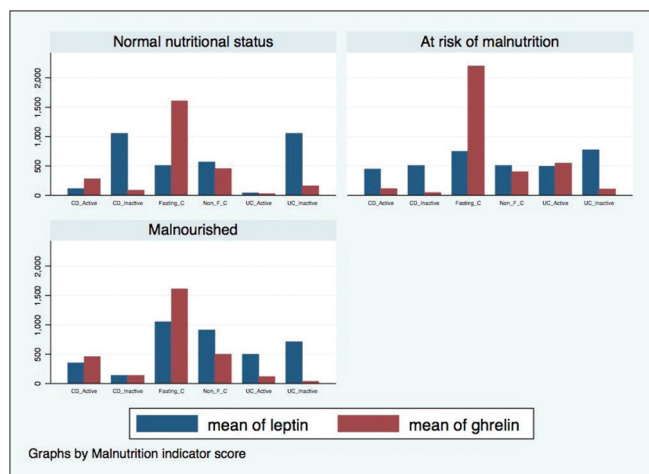


Figure 5: Mean plasma Ghrelin and Leptin levels of the Crohn's Disease (CD) and Ulcerative Colitis (UC) patients in relation to disease activity and fasting state in males and females

Table 1: Nutritional Status Distribution between Cases and Controls

MNA score	Case (active) n (%)	Case (non-active) n (%)	Control (fasting) n (%)	Control (non-fasting) n (%)	P
Malnourished (<17)	10 (45.5)	5 (55.5)	3 (0.14)	2 (10)	0.01
At Risk of Malnutrition (17-23.5)	9 (40.9)	1 (11.1)	10 (47.6)	5 (25)	0.01
Normal nutritional status (>23.5)	3 (13.6)	3 (33.3)	8 (38)	13 (65)	0.01

**Figure 6:** Mean plasma Ghrelin and Leptin levels of the Crohn's Disease (CD) and Ulcerative Colitis (UC) patients in relation to disease activity and fasting state by nutritional status

statistical association was elicited between the mean ghrelin levels comparing active cases with fasting healthy controls and non-active with non-fasting ($P < 0.01$ in both), as demonstrated in Figure 3.

There was a trend for a higher mean plasma leptin level in the non-active cases compared to the active group but was not statistically significant ($P = 0.4$). There was also a trend seen in the fasting control group when compared to the non-fasting controls but was not statistically significant ($P = 0.14$), a rather unexpected finding due to leptin's established response to satiety in normal human physiology. The lack of a significant statistical association further substantiates the lack of relationship between the cases and their healthy counterparts, regardless of disease activity ($P = 0.1$; Figure 3).

Both ghrelin and leptin were lower in the IBD patients compared to the healthy controls, regardless of disease activity. This further emphasizes the possible role of inflammation in altering the plasma hormone levels, which are responsible for hunger and satiety, respectively. These aspects are in need for further research as to the real cause of these findings, since there is some controversy in the available literature regarding these biochemical findings. Osawa *et al.*, as an example, reported an increase in plasma ghrelin concentration as a response to gastric atrophy. They also found an inverse correlation between plasma ghrelin changes when compared to both body

weight change and initial plasma ghrelin levels.^[13] On the other hand, Piquer *et al.* reported higher leptin levels in quiescent IBD patients than in healthy controls and active IBD patients. The three groups showed no difference in ghrelin concentrations levels despite similar BMI levels. Leptin's association with BMI was speculated to be correlated to disease activity.^[14] Still another study by Hoppin *et al.* observed no difference in leptin levels between disease groups or controls. In this study, serum leptin varied inversely with disease activity in Crohn's patients. However, the cause of difference was considered due, again, to BMI. Since determinants of serum leptin were the same in young IBD patients as in controls, it was therefore concluded that anorexia and growth failure mediation was unlikely due to leptin levels.^[15] Our study reproduces this apparent association between the hormone levels and the participant's BMI. None of the studies mentioned above, however, link the ghrelin or leptin levels to the IBD patient's relative nutritional status (including dietary habits and recent weight loss), of which this study sheds light upon.

Continuing on the matter of disparity in hormone levels, a study done by Hosomi *et al.* observed significantly higher ghrelin mRNA in IBD patients (active and inactive) compared with controls.^[16] In a study by Valentini *et al.*, three groups were used to find an association of levels of leptin with fat mass, inflammatory parameters, actual disease activity, and relapse of disease. They found that leptin levels were similar in patient groups when compared to controls. Fat mass correlated with leptin in inactive IBD ($r 0.728$), as well as with active IBD patients ($r 0.755$), and controls ($r 0.694$) with no difference between CD and UC.^[17] A study by Nishi *et al.* has elicited contradicting findings where ghrelin and leptin levels were not altered in CD patients.^[18] Biesiada *et al.* found TNF- α administration would increase leptin release in patients with UC,^[19] which is significant given the fact that almost all of those enrolled in our study take infliximab or adalimumab as therapy, which are anti-TNF alpha agents. On the other hand, a study by Karmiris *et al.* found that leptin concentration was reduced in UC patients when compared to controls.^[20] Another study in Poland had found decreased level of leptin levels in their IBD patients. They say it might be as result of the hyperactivation of TNF-alpha. Inconsistencies found in the leptin serum

levels may be due to the fact that, as an adipokine, it has both pro and anti-inflammatory characteristics.^[21] Schouliaras *et al.* reported that increased levels of leptin were not found consistently in IBD patients.^[22] Rodrigues *et al.*, however, also did not find any substantial difference in serum leptin level among the groups.^[23]

The significant variation in ghrelin levels between the cases and controls could be explained by the underlying inflammatory process of the alimentary tract. This variation could also be explanatory to the deteriorating nutritional status as observed by the participants' mean MNA scores (18.8 in active cases compared to 20.8 in the fasting group). On this basis, one would propose using ghrelin as a non-invasive diagnostic tool for diagnosing IBD, as was suggested by a similar study by Alexandridis *et al.*^[24] Furthermore, due to the deficiency of ghrelin levels observed in the IBD group. Although there was a suggestion demonstrated that there was an association between the nutritional status, as reflected by the MNA score, and the status of disease activity in some subclasses; we could not show that statistically due to the limited power of the study with the current sample size. There has been some interest into ghrelin's possible therapeutic uses in counteracting the loss of appetite these patients suffer from. This prospect has so far been demonstrated in animal subjects only, as a study done by Gonzalez-Rey *et al.*^[25]

There are several limitations present within this study. First, the MNA scores lack a certain amount of accuracy due to the possibility of recall bias found in the method of data collection itself. However, the majority of information regarding patient histories and co-morbidities was not elicited from the patient him/herself (especially in those who suffer from active disease) and was instead elicited from their medical records and the endoscopy database. Nevertheless, the conductance of this study in this region of the world, which has never been performed prior to this study, has an additive value and replicates the findings in other patient populations.

CONCLUSION

Plasma ghrelin and leptin hormone levels were lower in patients with IBD compared to the control group and correlated with disease activity. The same, however, cannot be said about leptin due to the insignificant statistical association between the related groups. These findings support some of the prior findings in some studies. We would recommend further studies on the topic that would explore in depth the association between these hormones and the disease activity in different phenotypes of IBD and its effect on patient's nutritional status.

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Conflicts of interest

There are no conflicts of interest.

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