

# Can serum histone H4 levels predict mucosal healing in Crohn's disease?

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Gastroenterology Rev 2021; 16 (2): 127–131  
DOI: <https://doi.org/10.5114/pg.2021.106663>

**Key words:** Crohn's disease, mucosal healing, histone H4.

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## Abstract

**Introduction:** Mucosal healing (MH) has been a treatment target with the introduction of biological agents in Crohn's disease (CD). Histone H4 increases in chronic inflammation.

**Aim:** Our goal was to investigate the role of serum histone H4 in predicting MH.

**Material and methods:** The study included 44 patients who applied to the endoscopy unit for ileocolonoscopy evaluation with the diagnosis of ileocecal CD and 26 healthy controls. After ileocolonoscopy evaluation, we divided the patients into 2 groups: those with and those without MH, according to the presence of endoscopic ulcer or erosion findings. Blood samples were taken from these patients to analyse serum histone H4 before the endoscopic procedure. We first compared serum histone H4 levels between CD patients and the healthy control group and then between those with and those without MH among the CD patients. Finally, we compared CRP, ESR, and serum histone H4 levels in patients with CD according to the presence of MH and symptoms.

**Results:** Serum histone H4 levels were significantly higher in ileocolonic CD patients compared to the healthy control group ( $p = 0.002$ ). Also, serum histone H4 levels were significantly higher in CD patients with no MH ( $p = 0.028$ ) or symptomatic patients ( $p = 0.033$ ). We did not find a significant difference in C-reactive protein and erythrocyte sedimentation rate levels between CD patients in the presence of MH ( $p = 0.281$  and  $p = 0.203$ , respectively) or symptoms (0.779 and 0.652, respectively).

**Conclusions:** Serum histone H4 might be a useful biomarker for MH prediction in ileocolonoscopy CD patients. Validation is needed for large numbers of patients.

## Introduction

In the evaluation of the effectiveness of anti-inflammatory therapy in Crohn's disease (CD) in recent years, mucosal healing (MH) has emerged as an increasingly important therapeutic goal in reducing clinical relapse rates, hospitalization rates, and surgical need [1]. Mucosal healing, a concept introduced especially after the emergence of biologicals, is an important endpoint in sustained clinical remission of CD, and therefore might be accepted as a goal for CD treatment [2–4].

Histones H3 and H4 are nuclear proteins with antimicrobial activities and are considered as a component of the innate immune system [5]. Serum levels of histones are significantly elevated and possibly correlate with the severity or poor prognosis of several disorders such as acute bacterial infection, sepsis, autoimmune

diseases (e.g systemic lupus erythematosus), cerebral stroke, trauma, and cancer. It has been described that histone H4 can activate Toll-like receptors 2 and 4 resulting in downstream cytokine production, leukocyte recruitment, microvascular leakage, inflammation, and tissue injury in murine models [6–8]. Therefore, extracellular histones are thought to be used as biomarkers [9]. It is stated that the level of circulating histones can be a novel biomarker in predicting disease severity and mortality in acute liver failure and in acute pancreatitis [10, 11]. However, the severity of pancreatitis was not found to be related with serum H3 and H4 [12].

Currently, a noninvasive biomarker of MH in CD is needed. In previous studies, histones have been reported to increase in inflammatory bowel diseases. Tsaprouni *et al.* studied acetylated histone H4 levels in animal models and human tissues taken by ileocolono-

scopic biopsy in patients with CD. They found that histone H4 acetylation on CD was associated with inflammation, and they stated that histone H4 may constitute a therapeutic target for mucosal inflammation [13].

## Aim

Thus, we aimed to investigate the role of serum histone H4 in distinguishing between those with and those without MH in ileocolonic CD patients.

## Material and methods

### Patients

Our study was an observational, preliminary study and was conducted in the Department of Gastroenterology at Bezmialem Vakıf University Faculty of Medicine between January 2017 and February 2018.

A total of 44 patients with a diagnosis of ileocolonic CD, who were followed up at the IBH outpatient clinic of our hospital and who applied to the endoscopy unit for ileocolonoscopy evaluation, were included in the study. Exclusion criteria included infectious or other inflammatory diseases, and heart, liver, and kidney failure. In addition, 26 age- and sex-matched control patients who were admitted to the outpatient clinic with nonspecific complaints but no prominent pathology were enrolled. The study protocol was approved by the Bezmialem Vakıf University local Ethics Committee (71306642-050.01.04). Written informed consent was obtained from the enrolled patients.

Peripheral blood samples (3 ml) were taken from the ileocolonic CD patients and from the healthy control group. The blood samples were centrifuged in sodium citrate tubes at 3000 rpm for 20 min. Then the separated serums were kept at  $-80^{\circ}\text{C}$  until analysis time. Clinical features, demographic data, biochemistry, and haemogram results of the patients were recorded from the electronic database.

### Blood assay

Histone H4 was measured with a sandwich enzyme-linked immunosorbent assay (ELISA) using commercial kits (lot no: 71135C-H1658C094; MyBioSource, Inc., San Diego, CA, USA) and an ELISA microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). The assay was performed in 96-well plates pre-coated with anti-human histone H4 acetylated on lysine 16 (HIST1H4) antibodies according to the manufacturer's recommendations. Biotin-conjugated HIST1H4A antibodies were used as detection antibodies. Standards, test samples, and biotin conjugated detection antibodies were added to the wells subsequently and washed with wash buffer. Horseradish peroxidase (HRP)-Strepta-

vidin was then added, and unbound conjugates were washed away with wash buffer. 3,3',5,5'-Tetramethylbenzidine (TMB) was used to visualize the HRP enzymatic reaction. In the presence of HRP, TMB generates a blue product, converted into a yellow compound after the addition of an acidic stop solution. The intensity of the yellow was proportional to the amount of HIST1H4A in the plate. The absorbance was read at 450 nm in a microplate reader, and the concentration of HIST1H4A in the samples was calculated using a standard curve. The results were expressed in ng/ml.

### Mucosal healing

Mucosal healing is simply defined as the absence of ulceration and erosions [14, 15]. Also, the largest trials that used MH as the primary or secondary endpoint, have used the definition of absence of ulcers instead of the prespecified CDEIS or SES-CD cut-off values [14]. In this study, as reported by Baert *et al.*, we used the definition of MH as the absence of mucosal erosions or ulcers (a SES-CD score of 0), and we defined the absence of MH as the presence of any size of superficial or deep ulcers or erosions on the mucosal surface (a SES-CD score of  $\geq 1$ ) [16].

In addition, patients were questioned about abdominal cramping pain, diarrhoea, and weight loss, which are among the characteristic features of CD before ileocolonoscopy evaluation [17]. Accordingly, those with the above complaints were recorded as "symptomatic" and those without were recorded as "asymptomatic".

### Statistical analysis

IBM SPSS Statistics 25.0 package 25.0 (IBM Corp., Armonk, NY, USA) was used. The Kolmogorov-Smirnov test was used to assess the normal distribution of the parameters. Quantitative variables were calculated as mean (interval) or median (interquartile range), and the qualitative variables were calculated as a percentage. Comparison of the quantitative variables with a nonparametric distribution was performed by the Mann-Whitney test. The Mann-Whitney test was used to compare the averages since the distribution was compatible with nonparametric variables other than FC. Comparison of qualitative data was done with the  $\chi^2$  test. Spearman's rho correlation analysis was used to examine the relationship between parameters with non-normal distribution. Values below  $p < 0.05$  were considered statistically significant.

## Results

Forty-four consecutive patients with ileocolonic CD and 26 age- and gender-matched healthy control group's demographic details are shown in Table I.

**Table I.** Demographic features of ileocolonic Crohn's disease (CD) patients and healthy control group

Parameter	Crohn's disease (n = 44)	Healthy control group (n = 26)	P-value
Age [years] mean ± SD	41.9 ±13.8	40.4 ±13.1	0.663
Gender (F), n (%)	22 (50)	13 (50)	1.0
Disease duration [months] median (IQR)	24 (12–72)	NA	NA
CRP [mg/dl] median (IQR)	0.46 (0.07–1.32)	0.02 (0.02–0.56)	0.024
ESR [mm/h] median (IQR)	9.5 (4.0–18.7)	12 (4.0–20.0)	0.836
CD behaviour, n, %:			
Inflammatory	27, 61.4	NA	NA
Fistulizing	11, 25		
Stenosing	6, 13.6		

CRP – C-reactive protein, ESR – erythrocyte sedimentation rate. FC data excluded from this table.

**Table II.** Comparison of inflammatory markers according to MH

Parameter	MH	No MH	P-value
CRP [mg/dl]* n, median (IQR)	10, 0.11 (0.02–3.27)	32, 0.52 (0.12–1.34)	0.281
ESR [mm/h]* n, median (IQR)	10, 10 (7.7–32.2)	30, 8 (4–18.2)	0.203
Serum histone H4 [ng/ml] n, median (IQR)	10, 0.010 (0.006–0.017)	34, 0.021 (0.009–0.039)	0.028

\*Available recorded markers as found in the recording system. CRP – C-reactive protein, ESR – erythrocyte sedimentation rate, MH – mucosal healing. FC data excluded from this table.

**Table III.** Comparison of inflammatory markers according to the presence of symptoms

Parameter	Asymptomatic	Symptomatic	P-value
CRP [mg/d] n, median (IQR)*	14, 0.2 (0.03–1.8)	28, 0.58 (0.08–1.29)	0.779
ESR [mm/h] n, median (IQR)*	13, 9 (4.5–25.5)	27, 10 (4–19)	0.652
Serum histone H4 [ng/ml] n, median (IQR)	14, 0.01 (0.008–0.019)	30, 0.02 (0.01–0.039)	0.033

\*Available recorded markers as found in the recording system. CRP – C-reactive protein, ESR – erythrocyte sedimentation rate. FC data excluded from this table.

### Endoscopic activity assessment

We compared serum histone H4 levels as well as available recorded surveillance markers as found in the recording system such as C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) between groups with and without MH in patients with ileocolonic CD (Table II).

In Table III, we compared CRP, ESR, and histone H4 levels between patients with abdominal pain or bowel movement disorder and those who were symptom free.

Serum histone H4 levels were significantly different between those with and without MH, and between those with and without symptoms. However, when we compared those with MH with respect to the presence of symptoms, the *p*-value was close to the limit of significance, but not significant (*p* = 0.051) (Table IV).

According to our data MH cannot be expected in all asymptomatic patients. Therefore, it can be thought

that some of the patients who do not have MH continue to have the disease without symptoms.

### Serum histone H4 levels according to CD behaviours, MH, and symptoms

Comparison of serum histone H4 levels between patients diagnosed with ileocolonic CD and the healthy control group are shown in Table V. Also, the comparisons between CD behaviour, MH, and symptoms are shown in Table V.

**Table IV.** Comparison of mucosal healing and symptoms in ileocolonic CD

Mucosal healing	Asymptomatic	Symptomatic	P-value
Positive, n (%)	6 (60)	4 (40)	0.051
Negative, n (%)	8 (23.5)	26, (76.5)	

Fisher's Exact Test.

**Table V.** Comparison of serum histone H4 with healthy control group and CD behaviour in ileocolonic CD patients

Variable	Serum histone H4 [ng/ml] median, IQR	P-value
CD (n = 44) vs. healthy control (n = 26)	0.017 (0.009-0.030)/ 0.000 (0-0.079)	0.002
CD behaviour:		0.749
Inflammatory (n = 27)	0.018 (0.009–0.031)	
Fistulizing (n = 11)	0.016 (0.007–0.023)	
Stenosing (n = 6)	0.014 (0.007–0.040)	

CD – Crohn's disease.

Serum Histone H4 levels were significantly higher in patients diagnosed with ileocolonic CD compared to the control group ( $p = 0.002$ ). Also, serum histone H4 levels were significantly lower in asymptomatic patients ( $p = 0.033$ ) and in patients with MH ( $p = 0.028$ ).

#### Serum histone H4 levels do not correlate with CRP and ESR

We did not find a significant correlation between serum histone H4 and CRP ( $r = 0.118$ ,  $p = 0.456$ ) or ESR ( $r = 0.113$ ,  $p = 0.480$ ).

## Discussion

The most desired treatment endpoint is the resolution of mucosal inflammation in CD. One of the scores used to evaluate CD activity is the Crohn's Disease Activity Index (CDAI), which is considered the gold standard for clinical trials [18]. But this method was not found to be a reliable measure of the underlying inflammation [19]. Two different models are used in the severity of the endoscopic lesion scoring. The first of these is Crohn's Disease Endoscopic Index of Severity (CDEIS), which is mostly used in randomised studies [20]. The second method is the Simple Endoscopic Score of Crohn's Disease (SES-CD), which is easier to apply and more useful in routine clinical practice [21]. With the introduction of biological agents, achieving MH has become a treatment goal by decreasing the need for surgery and reducing hospitalization rates in CD [22].

According to an expert consensus report published in 2012, it is stated that MH must be regularly monitored, and endoscopy is a gold standard for assessing MH accurately [1]. These recommendations are still valid. Endoscopic assessment with objective evaluation of MH has become an essential part in tailoring the treatment [15]. Therefore, decisions regarding medical therapy can be guided by the techniques or markers showing MH, which is still under investigation. Biomarkers such as FC have yet to demonstrate sufficient specificity to

replace endoscopic evaluation for mucosal healing [23]. Also, CRP and ESR are not sufficiently specific to monitor CD activity; only endoscopic assessment was stated to be effective [24].

In this study, according to the results of ileocolonoscopy evaluation, we divided the patients into 2 groups: those who were considered to have MH and those who were not considered to have MH. First, we compared serum histone H4, CRP, and ESR levels between those with and those without MH. Accordingly, we found that serum histone H4 levels were significantly lower in patients with MH ( $p = 0.028$ ) while CRP and ESR values did not differ ( $p = 0.281$ , and  $p = 0.203$ , respectively) between the groups. In ileocolonic CD patients, CRP and serum histone H4 levels were significantly higher compared to the control group ( $p = 0.024$  and  $p = 0.002$ , respectively). Also, in terms of the presence of symptoms, there was no difference between CRP and ESR among the asymptomatic and symptomatic patients in CD patients (0.779 and 0.652, respectively), but serum histone H4 levels were significantly higher in those who were symptomatic ( $p = 0.033$ ). Finally, we compared those with MH according to the presence of symptoms; however, we could not find a significant relationship between MH and the presence of disease symptoms ( $p = 0.051$ ). According to this comparison result, a lack of symptoms does not mean that MH is present.

Therefore, as a new biomarker candidate serum, histone H4 differed significantly between those with and without MH, and those with and without symptoms in ileocolonic CD (Table IV). To the best of our knowledge, our report is the first to investigate the prediction of MH in CD patients by using serum histone H4 as a biomarker.

In earlier studies, dexamethasone was reported to suppress histone H4 acetylation induced by TNF- $\alpha$  [25]. Also, in previous studies, Tsaprouni *et al.* reported that serum histone H4 significantly increased in the disease involvement areas in animal models and CD patient biopsies, but not in areas without disease [13]. Our results are in agreement with this experimental model data. We observed that serum histone H4 levels were found to be significantly lower in patients who were found to have MH endoscopically compared to the group found to have no MH.

The limitations of the study are first that our study was a single-centre, preliminary study. Second, ethics committee approval was given only for the histone kit for this study, and no budget could be allocated for other blood tests. Therefore, other examinations related to the groups could be recorded retrospectively, as found in the recording system. Accordingly, CRP was available in 42 patients, and ESR was available in 40 patients.

However, since the main purpose of the study was to evaluate the relationship of MH with histone H4 levels, we think that the absence of CRP and ESR results in some patients will not affect the serum histone H4 –MH assessment. The third limitation of the study was that the histone H4 kits were available as an experimental kit, so cut-off values could not be calculated for MH prediction.

## Conclusions

Serum histone H4 may serve as a novel biomarker for predicting MH and may reduce the need for endoscopy in a patient evaluated for MH. Standardized validation studies involving a large number of patients are required.

## Conflict of interest

The authors declare no conflict of interest.

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Received: 17.03.2020

Accepted: 20.05.2020