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μ-opioid receptor, β-endorphin and cannabinoid receptor-2 are increased in the colonic mucosa of irritable bowel syndrome patients

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Abstract

Background and Aims: The gut immune, cannabinoid and opioid systems constitute an integrated network contributing to visceral sensation and pain modulation. We aimed to assess the expression of the μ opioid receptor (MOR), its ligand β-endorphin (β-END), and cannabinoid receptor-2 (CB_2) in patients with irritable bowel syndrome (IBS) and asymptomatic controls (AC) and their correlation with sex and symptom perception.

Methods: Mucosal biopsies were obtained from the left colon of 31 IBS patients (45% women) with predominant constipation (IBS-C, 9) or diarrhea (IBS-D, 10) or with mixed bowel habits (IBS-M, 12) and 32 AC (44% women) and processed for qRT-PCR, Western blotting and immunohistochemistry.

Key Results: MOR and CB_2 mRNA and protein expression and β-END protein levels were increased in patients with IBS compared to AC (all *Ps*=.021). A significant sex by IBS interaction

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CONTRIBUTIONS

GD and GB conceived the study; GD, GB, CS, FDP designed the research study; LC, EAM, VS, CC, and GB were responsible for recruitment of the participants involved in the study; LC and MRB performed the endoscopic procedures and tissue sampling; GD performed imaging experiments and molecular assays; WS performed statistical analysis; GD, GB, LC and CS wrote the manuscript. All authors reviewed and approved the manuscript. FDP, GB, LC, EAM and CS provided funding for the study.

DISCLOSURE

The authors disclose the following competing interests: GB has received consultancy fees and/or speaker fees from Alfasigma, Allergan, Cadigroup, Danone, Yakult, Ironwood, Malesci, Nestlè, Noos, Shire Sofar, Synergy. LC has served on advisory boards for Ardelyx, Alnylam, Arena, Bioamerica, IM HealthSciences, Ironwood, Ritter, Salix, and Synergy. She has received grant funding from Ardelyx and Allergan and speaker honorarium from Allergan.

was found in relation to CB_2 mRNA expression (*P*=.003) with women showing a markedly higher expression to men (*P*=.035). In contrast, in AC, men had higher expression than women *(P*=.033). β-END, MOR and CB_2 immunoreactvities (IR) were localized to CD4+ T cells including EMR-1+ eosinophils and CD31+ T cells but not to mast cells.

Conclusions: The increased expression of MOR, β-END and CB_2 in the mucosa of IBS patients, where they are localized to immune cells, suggests that opioid and cannabinoid systems play an immune-related compensatory role in visceral pain in IBS patients. Further work is necessary to support this hypothesis.

Abbreviated abstract:

The purpose of this study was to determine whether Mu opioid receptor (MOR), its ligand β-endorphin (β-END) and the cannabinoid receptor-2 (CB2) were altered in the colonic mucosa of patients with irritable bowel syndrome (IBS) vs. asymptomatic controls (AC) using qRT PCR, Western Blot and immunohistochemistry with confocal microscopy. We found that MOR, β-END and CB2 expression was increased in IBS vs. AC subjects and that MOR, β-END and CB2 immunoreactivity was localized to immune cells. These findings suggest an involvement of the opioid and cannabinoid systems in the immune response that might affect visceral sensation in IBS either through neuronal or alternative pathways.

Keywords

opioid; cannabinoid; irritable bowel syndrome; immune system; neuro-immune crosstalk

3. INTRODUCTION

Irritable bowel syndrome (IBS) is a chronic functional bowel disorder characterized by abdominal pain associated with changes in bowel habits [1]. Patients with IBS are subtyped according to their bowel habit in IBS with predominant constipation (IBS-C), with predominant diarrhea (IBS-D) or with mixed bowel habits (IBS-M) [2]. Notably, these features are defined as predominant, since they may vary over time [3]. With a global prevalence of 11.2%, IBS is one of the most common gastrointestinal (GI) disorders. IBS markedly affects the quality of life of patients and is associated with a major socio-economic burden [4]. IBS is considered a multifactorial disorder with many contributing factors, including psychosocial and environmental stressors, previous infection gastroenteritis, diet, intestinal microbiota, serotonin, and immunological factors [5].

The opioid system regulates a variety of biological functions within the GI tract, including motility and secretion [6], visceral sensitivity [7] and immune response [8]. β-endorphin (β-END), which couples with high affinity the μ opioid receptor (MOR), mediates analgesic response in central and peripheral nervous system [9] but is also expressed by immune cells, and immune-derived β-END secretion is altered in IBS patients [10]. Beside the well-known function in opioid-induced constipation [11], MOR and β-END exert a prominent role in the modulation of immune mechanisms in intestinal inflammation [9,12,13]. In the gut, MOR is expressed by enteric neurons and immune cells [14,15]. Moreover, MOR expression is increased in patients with inflammatory bowel disease (IBD) [15], and MOR activation

ameliorates inflammation in acute animal models of inflammation [12,16,17] and showed beneficial effect on pain perception in a mouse model of dysbiosis [18].

The endocannabinoid system (including receptors, endogenous ligands and ligand-degrading enzymes) is also involved in intestinal sensory perception, motility and secretion [19,20]. Cannabinoid $(CB)_1$, and to a lesser extent, CB_2 receptors are present in both submucosal and myenteric plexuses [19,21]. CB_2 is prominently expressed by leukocytes [22,23] in the *lamina propria* of the intestinal mucosa [24] and plays an important role in the modulation of immune response and in mechanisms of leukocyte recruitment [19,25]. Compared with healthy controls, patients with IBD showed enhanced CB_2 immunoreactivity in the intestinal epithelium[26].

Changes in the concentrations of the endocannabinoid-like molecules palmitoylethanolamide (PEA), oleoylethanolamide (OEA) and 2-arachidonyloglycerol, were detected in the serum of IBS patients. Interestingly, low serum concentration of PEA was inversely correlated to frequency of abdominal pain in IBS [27]. As activation of CB receptors leads to analgesic activity, modulates gastrointestinal motility and downregulates inflammation, CB_1 and CB_2 and their endogenous ligands have been increasingly studied as possible therapeutic targets for IBS [28,29].

Taken together, these findings prompted us to investigate the expression of MOR, β-END and CB_2 in the colonic mucosa of IBS patients and their correlation with clinical parameters.

4. MATERIALS & METHODS

Study Participants

The study was conducted in two centers, both recruiting IBS patients and asymptomatic controls (AC), namely, the Department of Surgical and Medical Science, Unit of Gastroenterology, University of Bologna, Italy (Italy cohort) and the G. Oppenheimer Center for Neurobiology of Stress and Resilience, David Geffen School of Medicine at UCLA, Los Angeles (CA), USA (US cohort; recruiting predominantly via community advertisements). Participants from the US cohort were age and sex matched with the Italy cohort in order to minimize cohort-related confounding factors. All participants with IBS were diagnosed according to the Rome III criteria [1]. AC included participants who underwent colonoscopy for screening of colorectal carcinoma or polypectomy follow-up (Italy cohort) or sigmoidoscopy (US cohort); GI symptoms were excluded in all AC subjects.

All participants with IBS completed a modified version of the Bowel Disease Questionnaire for symptom assessment. Exclusion criteria comprised organic intestinal diseases, including celiac disease, diverticular disease, Crohn's disease, microscopic and ulcerative colitis; ongoing treatment with NSAIDs, tricyclic antidepressant or serotonin selective reuptake inhibitors, mast cell stabilizers and corticosteroids; allergic diseases determined by family and personal history and specific anti-IgE antibodies and other organic or severe psychiatric disorders as assessed by history taking, appropriate consultations and laboratory tests. IBS and AC participants gave written informed consent and the study protocol was approved by the local Ethic Committees (for the Italian patients, approval identification no: 23/2012/U/TESS; for the U.S. patients, approval identification no: IRB# 08-03-019) and conducted in

accordance with the Declaration of Helsinki. Severity of symptoms were scored by means of a 5-point Likert scale (none to very severe). Abdominal pain severity was rated over the past two weeks in the Italy cohort and usual severity of IBS symptoms was rated in the U.S. cohort.

Four mucosal biopsies were obtained from the descending (Italy cohort) or sigmoid (U.S. cohort) colon, 2 of these were fixed in 4% paraformaldehyde in 0.1 M saline phosphate buffer (PBS) and processed for H&E histology to exclude microscopic colitis and for immunohistochemistry (see below); 2 biopsies were snap frozen in liquid nitrogen and kept at −80°C until use for quantitative Real Time-PCR (qRT-PCR) and Western blot.

qRT-PCR analysis

Tissues were processed with Illustra Triple Prep™ kit (GE Healthcare, Pittsburgh, PA, USA) following manufacture indications. RNA concentration was evaluated through a Nanodrop spectrophotometer (Nanodrop 1000; Thermo Scientific, Waltham, MA, USA). Samples with a concentration higher than 1.8 μg/μl, a purity index within 1.8 and 2.1 (260/280 ratio) and within 1.9 and 2.2 (260/230 ratio) were utilized for qRT-PCR analysis. The corresponding volume of 1 μg was converted to cDNA through SuperScript III reverse transcriptase (Superscript III™ Life Technologies, Carlsbad, CA, USA). Target sequences were amplified adding SYBR Green I Master Mix (Roche, Applied Biosystem, Penzberg, Germany), primer oligos and DEPC water to a volume of 25 μl per well. Each sample was assayed in duplicate in a LightCycler ® 480 (Roche, Applied Biosystem). Primer sequences were as follows: μOR F: 5'-ATGCCAGTGCTCATCATTAC-3', R: 5' -GATCCTTCGAAGATTCCTGTCCT-3' and CB_2 F: 5'-CGCCGGAAGCCCTCATACC-3', R-CCTCATTCGGGCCATTCCTG −3'), β-actin F: CCATCATGAAGTGTGACGTGG, R: GTCCGCCTAGAAGCATTTGCG). β-actin was used as housekeeping gene. Since β-END is one of 5 products derived from the same precursor proopiomelanocortin [30], we only quantified the corresponding protein expression to ensure we did not measure other proopiomelanocortin derivatives.

Western Blot

Concentration of protein fractions was determined by spectrophotometry with a bicinchoninic acid-based assay (BCA, Thermo Fisher Scientific, Waltham, MA, USA). Samples were diluted in Laemmli buffer (pH 6.8) and boiled 5 minutes before use. 45 μg of total proteins from each sample were loaded in 10% acrylamide or, in case of β-END, 30% acrylamide tricine-buffered gels and separated in an electrophoresis chamber (52V), overnight at room temperature (RT); 2 μl of pre-stained marker proteins were used as molecular mass standards (LI-COR, Lincoln, NE, USA). Gels were blotted on PVDF Immobilon-FL membranes (Millipore, Temecula, CA, USA) in a transfer chamber (140V) for 2 h and 30 min at 4 °C. Membranes were blocked with LI-COR Blocking buffer (LI-COR Biosciences) for 1 h at RT and incubated overnight at 4°C with rabbit anti-GAPDH, used as reference protein (Abcam, Cambridge, MA, USA); rabbit anti-μOR (1:2000, Immunostar, Hudson, WI, USA); rabbit anti-β-END (1:1500; kindly provided by Dr. Niall Murphy); or goat anti-CB_2 (1:500, Santa Cruz Biotechnologies, Dallas, TX, USA).

Immunohistochemistry

Colonic biopsies were fixed in cold 4% paraformaldehyde/PBS pH 7.4 and embedded in paraffin. Four μm-thick sections were cut by microtome and serially mounted on slides; sections were deparaffinized (twice, in Xylene for 7 min each), rehydrated in graded ethanol (100% twice, 95% and 70%, for 5 min each) and washed 5 min in distilled water. Slides were kept in Citrate Buffer (10 mM Citric Acid, 0.05% Tween 20, pH 6.0) for 20 min at 100°C for antigen retrieval, washed three times with PBS and incubated with 5% normal donkey serum in PBS containing 0.1% Triton® X-100 to reduce non-specific binding. Sections were incubated in a humid chamber at 4 °C overnight with the following primary antibodies (for single or double labeling): rabbit anti-MOR (1:200; Immunostar, Hudson, WI), rabbit anti-β-END (1:100, kindly provided by Dr. Niall Murphy), goat anti-CB_2 (1:50; Santa Cruz Biotechnologies, Dallas, TX), mouse anti-EMR-1 (1:100; Santa Cruz Biotechnologies), mouse anti-CD4 (1:50; Santa Cruz Biotechnologies), mouse anti-CD31 (1:1000; Millipore). The following day, slides were rinsed with PBS, and incubated at RT with goat anti-mouse Alexa-Fluor© 555- (1:1000; Life Technolgies, Carlsbad, CA) or goat anti-rabbit Alexa-Fluor© 488-antibodies (1:1000; Life Technologies) for 2 h. Immunoblocking experiments for MOR, β-END and CB_2 antibodies were performed at the beginning of the study to assure antigen-specificity of the antibodies used (Suppl. Figure 1). Briefly, 1μg of antibody was incubated with 10 μg of the relative peptide for 2h hours at RT (MORp 384-398 Immunostar; β-END, Abbiotech, San Diego, CA; CB_2 p, Santa Cruz Biotechnologies; Suppl. Figure 1, A–D). There was no immunostaining in tissue sections incubated with each antibody-preabsorbed with its corresponding peptide compared to those incubated with antibody only and processed simultaneously, indicating specificity of the tissue immunostaining. In addition, the immunostaining with the goat anti-CB2 from Santa Cruz Biotechnologies was comparable to the immunostaining obtained using a different CB2 antibody (Novus Biological, Centennial, CO; Suppl. Figure 1). Negative controls (i.e. omission of the primary antibodies) were included in each experiment. Immunostained sections were analyzed with a LSM Zeiss 710 META confocal microscope and the supplied software ZEN (Carl Zeiss, Thornwood,NY, USA).

Preliminary Power/Sample Size Calculations

Our preliminary data obtained in a pilot trial showed that the effect sizes for MOR, CB_2, and β-END protein expression ranged from 0.72-1.8 respectively. In order to detect similar effect sizes, 64 participants (32 IBS and 32 AC) were needed to achieve 80% power for the current study. In addition, preliminary data indicated that the Italy cohort was on average older than the US cohort. Hence, the current study purposely recruited older US participants in order to match the Italy cohort.

Statistical Analysis

Regression analyses were used to evaluate differences in mRNA relative expression and protein abundance between IBS and AC and among bowel habits subtypes. Significant bowel habits subtypes were further investigated using pairwise contrasts. The effect of sex, interactions between sex with IBS status, and association between severity scores with the expression of MOR, β-END, and CB_2 were also analyzed using regression analyses. The

associations between the expression of MOR, β-END, CB_2 and symptom severity scores were controlled for cohort (Italy vs. U.S) as the severity questions were not identical. Differences in mRNA relative expression and protein abundance in the two cohorts were assessed. They were similar with the exception of significantly higher CB2 protein levels in IBS and AC in the Italy cohort compared to the US cohort (P<.001). All statistical analyses included the main effect of cohort to control for potential confounding, were performed using R version 3.5.1 (http://cran.r-project.org/) and were two-tailed.

5. RESULTS

Clinical characteristics of study participants

A total of 63 participants were included in the study for final analysis (31 patients with IBS and 32 AC). The Italy cohort was comprised of 16 participants, 10 IBS and 6 AC. The U.S. cohort included 47 participants, 32 IBS and 17 AC (Suppl. Table 1). IBS symptom severity was significantly higher in the U.S cohort compared to the Italy cohort (P<0.001). Cannabis use was only available for 24 (12 IBS and 12 AC) out of the 47 U.S. participants. Of these 24 participants, only one IBS patient used cannabis more than 6 years prior to study entry. Information about cannabis use was not collected from the Italy cohort.

MOR and β-END expression and localization

MOR mRNA expression in colonic mucosal biopsies of patients with IBS was significantly higher than in AC (Figure 1A; P=.045). In IBS patients, MOR mRNA expression was not significantly different according to bowel habit or sex (P=.080 and P=.446, respectively). However, within AC, men had higher MOR mRNA expression compared to women (Figure 1B; P=.034). A sex difference was not found within IBS (P=.427). Similarly, MOR protein levels were significantly increased in IBS patients compared to AC (Figure 2; P=.044), but there were no significant bowel habit differences (P=.222) or a sex by IBS status interaction (P=.570). In addition, there were no sex differences within IBS (P=.373) or within AC (P=.804).

β-END protein expression was significantly higher in patients with IBS compared to AC (Figure 3A *; P*=.021). There was a trend for bowel habit difference (Figure 3D ; p=0.06). IBS-M, but not IBS-C or IBS-D, showed significantly higher β-END levels compared to AC participants (P=.016). Among AC, but not IBS, men had significantly higher β-END protein levels expression compared to women (Figure 3B; P=.019). Finally, there was no significant sex by IBS status interaction (P=.106).

Confocal imaging analysis of colonic mucosal sections showed MOR or β-END immunoreactive cells in the lamina propria (Figure 4 a, d, g, j; Figure 5 a, d, g, j). Double immunostaining identified the presence of MOR and β-END immunoreactivity in CD4+ cells (Figure 4 a–f; Figure 5 a–f), which is in line with previous studies [10,31]. Notably, several cells showed an irregular shape rather than the lymphocyte's typical spherical one. Therefore, we performed double labeling of MOR and β-END with EMR-1, a specific marker for eosinophils, CD31, a marker for transmigrating leukocytes, and tryptase, a marker for mast cells. This analysis showed the expression of both MOR and β-END

immunoreactivities in EMR-1+ eosinophils (Figure 4 g–l; Figure 5 g–l) and CD31+ leukocytes (Suppl. Figure 2 MOR: a–f; β-END: g–l), but not in mast-cells (Suppl. Figure 3; MOR: a–f; β-END: g–l).

CB_2 expression and localization

CB_2 mRNA expression was significantly higher in IBS patients compared to AC participants (Figure 6 A; P<.001). All three IBS bowel habit subtypes had significantly higher expression compared to AC (Figure 6 B; Ps<.001). There was also a significant sex x IBS interaction with CB_2 mRNA expression (P=.003). IBS women had significantly higher CB_2 mRNA expression compared to IBS men (P=.035). The association was reversed in the AC group; AC men had significantly higher CB_2 mRNA expression compared to AC women (P=.033).

Conversely, CB_2 protein expression was higher in patients with IBS vs. AC (Figure 7A*; P*=. 091), but there was no statistically significant difference. No significant bowel habit difference or sex by IBS interaction was found for CB_2 protein expression (P=.940 and P=. 322 respectively).

CB_2 immunostaining was observed in CD4+ (Figure 8 a–f), but also in some EMR-1+ (Figure 8 g–l) and CD31+ (Suppl. Fig 2 m–r) cells scattered in the *lamina propria* but not in tryptase+ mast cells (Suppl. Figure 3 m–r).

Association between the level of expression of MOR, β-END and CB_2 in the colonic mucosa of participants with IBS with GI symptom severity.

There were no significant associations between level of expression of MOR, β-END and CB_2 in the colonic mucosa of patients with IBS with GI symptom severity (P-value range: . 064 – .875). There was a marginal positive association between GI symptom severity with CB_2 mRNA expression (P=.064).

6. DISCUSSION

This study compared the level of expression and cellular localization of MOR, β-END and CB_2 in the colonic mucosa of patients with IBS and AC. mRNA quantification analysis showed significantly higher MOR and CB_2 expression (β-END was measured only at the protein level) in IBS compared to AC participants. Furthermore, there were statistically significant increases in β-END and MOR protein levels in IBS vs. AC groups, whereas CB_2 protein levels were higher in IBS vs. AC but did not reach statistically significance. One explanation for the discrepancy between CB_2 mRNA and protein levels is that mRNA levels do not necessary predict protein levels [32], though we cannot exclude that it could be due to a low antibody sensitivity for Western blot. However, CB2 protein levels were higher in both IBS and AC participants in the Italy vs. U.S. cohorts, which could have also contributed to the lack of significant differences.

MOR and its ligand β-END and CB_2 were prominently localized to CD4+ immune cells but also to EMR-1+ and CD31+ cells, indicating their expression by mucosal T-helper lymphocytes, eosinophils and leukocytes, respectively. Moreover, in contrast with previous studies [33], we did not detect CB_2, MOR or β-END immunoreactivity in mucosal mast cells.

The higher expression of MOR and β-END in IBS patients observed in our study could be related to immune activation and higher infiltration of immune cells in IBS patients, which in turn could indirectly modulate intestinal nerves [34–36]. MOR agonists such as loperamide [37] and eluxadoline [38] have proven to be effective over placebo in treating symptoms in patients with IBS-D, but whether their efficacy or adverse effects (e.g. constipation) can be predicted by the mucosal level of MOR expression remains to be established.

The localization of β-END in CD4+ T cells in colonic mucosal sections of IBS patients is in line with previous findings by Hughes et al [10] suggesting that β-END positive immune cells are involved in eliciting an anti-nociceptive response perhaps through the activation of mucosal nerve fibers. β-END released by immune cells might also exert antinociceptive and anti-inflammatory effects through modulation of pro-inflammatory cytokines, including Tumor Necrosis Factor (TNF) α, and sensitization of T-lymphocytes and macrophages to pro-inflammatory stimuli [16] by activating MOR on immune cells. Moreover, TNFα induces MOR expression in primary T cells by a Nf-κB-mediated pathway [39]. Our study shows upregulation of β-END immunoreactivity in IBS patients vs. AC participants as opposed to downregulation of β-END reported by Hughes et al [10]. This discrepancy could be explained by methodological differences, though it could also be attributed to the smaller sample size and lower number of IBS-M patients in the Hughes et al. study [10], since we found that β-END expression was highest in IBS-M patients.

Several lines of evidence indicate changes in the cannabinoid system during intestinal inflammation and visceral sensitivity [19,40]. In this study, we focused on CB_2, because it is expressed peripherally, is an established constituent of immune system cells and is up-regulated during inflammation [41]. The fact that the increase in CB_2 expression was not specific to any IBS bowel habit subgroup is in line with a recent study by Cremon et al. [42], showing an increased expression of CB_2 receptors in mucosal biopsies of IBS patients regardless of bowel habit. This could reflect a link to a particular immune reaction occurring in IBS with a similar degree of immune cell activation irrespective of bowel habit. In line with this, the observed expression of CB_2 by CD31-positive cells suggests a role of the cannabinoid receptor in modulating immune cell recruitment [25]. Moreover, different factors associated with the altered intestinal milieu characterizing IBS patients [43], such as TNF α [44] and histamine [45], might concur to the induction of CB_2. Surprisingly, we did not detect CB_2 receptors on the surface of mast cells, implying that this cell type might mediate its response to cannabinoids [46] through other receptors than CB_2, such as CB_1, transient potential vanilloid receptor-1 or the recently described GPR55 [47]. CB_2 might exert a modulatory role in intestinal inflammation and visceral pain as suggested by the observation that CB_2 activation on immune cells suppresses the immune response through the inhibition of cAMP and reduces secretion of proinflammatory cytokines [48,49]. Evidence for CB_2 involvement in visceral hypersensitivity derives from the reduction of visceromotor response to colorectal distension induced by CB_2 agonists [40], the increased sensitivity of the endocannabinoid system in the setting of hyperalgesia [50] and the inhibition of afferent nerve activity by CB_2 activation [51]. Several studies have shown an interaction between opioid and cannabinoid on immune cells [52] and functional [53,54] and structural affinity [55] between these systems. Furthermore, a CB_2-dependent β-END release by keratinocytes has been described in a rat model of peripheral hypersensitivity [56]. Moreover, experimental evidence suggests an

interplay of opioid and cannabinoid systems associated with IBS pathophysiology. Interferon γ (IFNγ) can be released by β-END-stimulated NK-cells [57] and has been indicated as a fundamental player in CB_2 signaling in a murine model of neuropathic pain [58]. Interestingly, IFNγ is associated with an increased expression of MOR [31] and was found increased in mucosal biopsies of patients with IBS [59]. Finally, it is unlikely that the increased CB_2 expression is due to cannabis since most of our participants did not report using it regularly. Notably, two previous studies reported no changes of CB_2 expression in immune cells in cannabis users compared to non users [60,61].

MOR and CB_2 agonists have been shown to reduce visceral pain in a rat model of chronic colonic hypersensitivity [62] supporting the notion that activation of these receptors might be beneficial in IBS patients, though the mechanism remains to be elucidated. Changes in the CB_2 and MOR expression in intestinal mucosa have been reported in a mouse model of dysbiosis induced by 7 days of antibiotic administration [18]. In addition, *Lactobacillus acidophilus* NCFM has been shown to induce MOR and CB_2 expression in epithelial cells that was associated to a visceral pain reduction through the activation of NF-kB pathways in chronic colonic hypersensitivity [62]. Nf-κB-mediated pathway has also been shown to be responsible for TNFα-induced MOR expression in primary T cells [39]. Furthermore, *Lactobacillus acidophilus* NCFM has been reported to affect MOR but not CB_2 expression and to evoke a higher tolerance to bowel distention in a recent clinical trial [63]. All together, these findings suggest a possible association between microbiota diversity and the expression of these receptors and the existence of different mechanisms involved in visceral sensation on IBS. However, the role of microbiota in regulating MOR and CB2 expression is unknown and whether changes in microbiota composition affect symptoms in IBS patients remains to be established.

Our study was powered to examine the differences in colonic mucosal MOR, β-END and CB_2 expression between IBS and AC participants, however we explored both bowel habit and sex differences. Interestingly, whereas CB_2 mRNA expression was significantly increased regardless of the IBS bowel habit subtype compared to AC participants, β-END expression was predominantly higher in participants with IBS-M vs. AC. While we cannot exclude that the lack of statistical significance may be due to the small numbers of patients within each IBS bowel habit subtype, it is possible that the molecular mechanisms involved in the regulation of these components of the opioid and cannabinoid systems differ between bowel habit subgroups.

There was differential expression of MOR, β-END and CB_2 with respect to sex. In particular, MOR mRNA and β-END protein levels were higher in AC men compared to AC women, but there was no difference between men and women with IBS. These findings are consistent with previous evidence reporting a sexually dimorphic response of opioidergic system in animal models treated with morphine and a markedly lower expression of MOR in male rodents' central nervous system [64]. Furthermore, women with IBS showed a higher CB_2 mRNA levels compared to IBS men, whereas the difference was in the opposite direction in AC. Also in this case, a number of studies report differential response to cannabinoid activity associated with sex differences [65]. It is not known if there will be differences in the response to a CB_2 agonist in women and men with IBS.

Finally, we showed that the intensity of IBS symptoms had a marginal positive correlation with CB_2 mRNA levels. However, there were differences in how IBS symptoms were assessed in the two cohorts. Abdominal pain severity over the past two weeks was measured in the Italy cohort, while usual IBS severity was measured in the U.S cohort, although both used a similar Likert scale of none to very severe. Future studies are needed to determine if MOR, β-END and CB_2 expression correlates with IBS symptoms.

In summary, the increased expression of MOR, β-END and CB2 in IBS patients and their localization to immune cells suggest an involvement of the opioid and cannabinoid systems in the immune response that might affect visceral sensation in IBS either through neuronal or alternative pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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KEY POINTS

- Opioid and cannabinoid systems in the gut have been implicated in visceral perception, intestinal secretion and immunity. Alterations in these systems have been associated with irritable bowel syndrome (IBS) pathophysiology.
- Compared to asymptomatic controls, IBS patients showed an increased colonic mucosal expression of μ opioid receptor (MOR), β-endorphin (β-END) and cannabinoid receptor 2 (CB_2), which were localized to immune cells.
- The observed upregulation of the opioid and cannabinoid systems represent a neuro-immune response that may affect bowel secretory and sensory-motor function in patients with IBS.

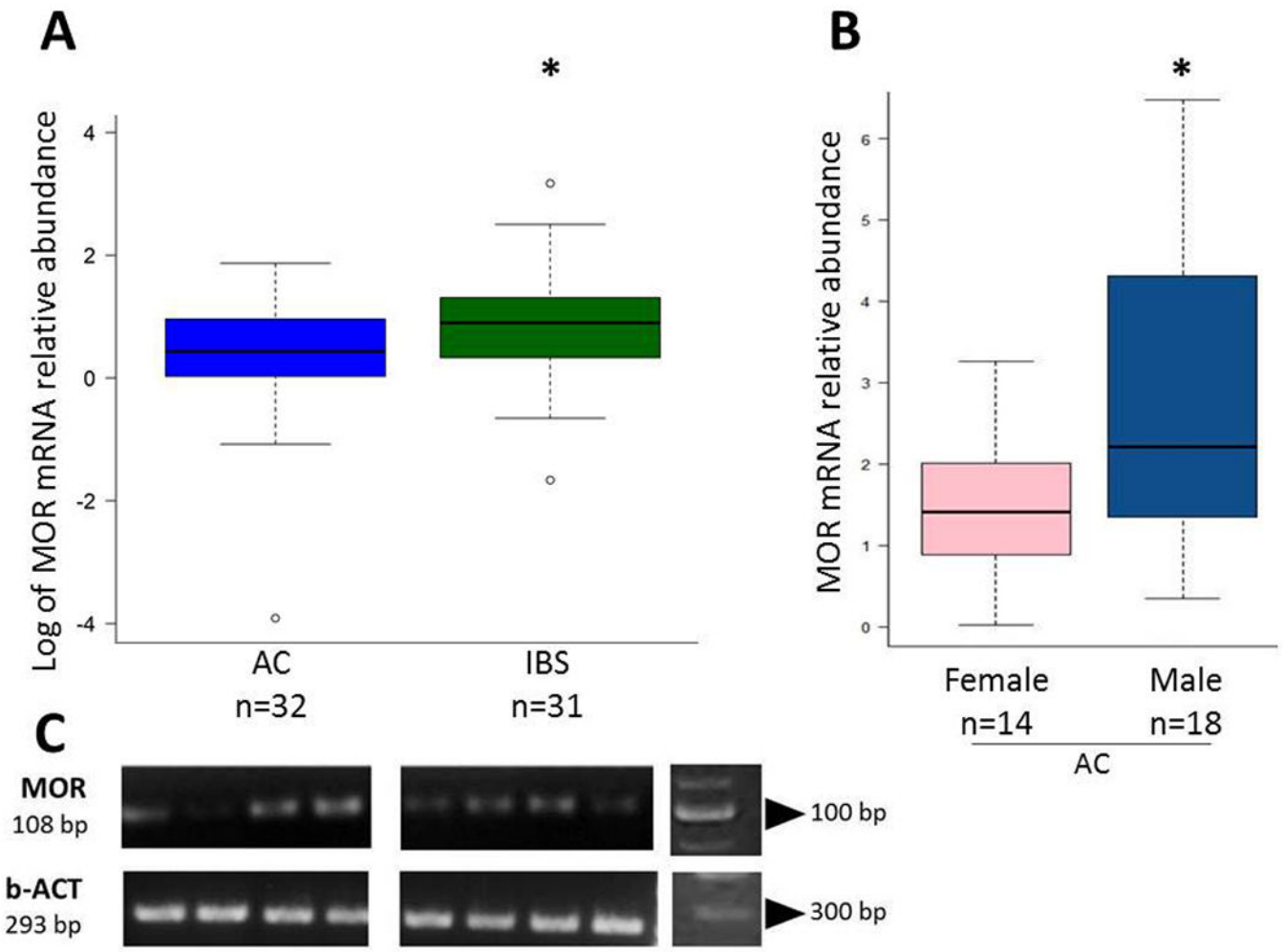

Figure 1 –.

MOR mRNA levels were higher in mucosal biopsies of patients with IBS compared to AC (1A); **P*=.045, note y axis in logarithmic scale, but there were no statistically significant differences among IBS subgroups (not shown). Asymptomatic male participants showed a higher MOR mRNA expression compared to females (1B; **P*=.03), whereas there was no sex-related difference in the IBS group (not shown). The bold line in the boxplot indicates median values, the bottom and top of the box are the 25th and 75th percentile, and the upper and lower brackets represent the 75th percentile + 1.5 interquartile range (IQR) and the 25th percentile −1.5 IQR respectively. The data points outside this range are indicated as open circle (possible outliers). MOR and β-actin (b-ACT) representative gels and molecular weights are shown in Figure 1C.

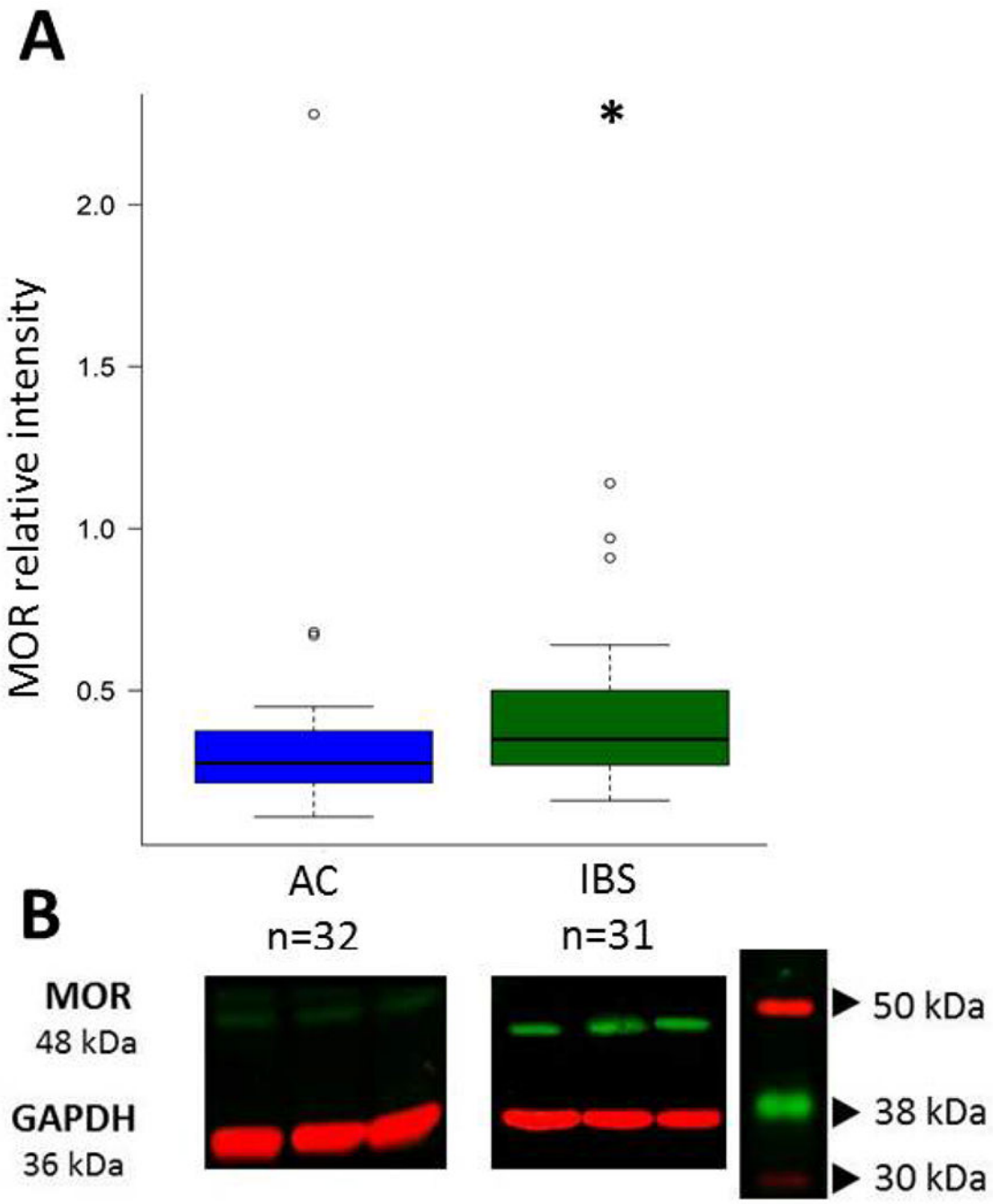

Figure 2 –.

MOR protein levels in overall patients with IBS were increased compared to AC (2A; **P*=. 044), but no significant differences were detected between IBS subgroups (not shown). No significant differences between women and men were found within each group investigated (data not shown). Representative images of MOR and GAPDH immunoreactive bands are shown in Figure 2B.

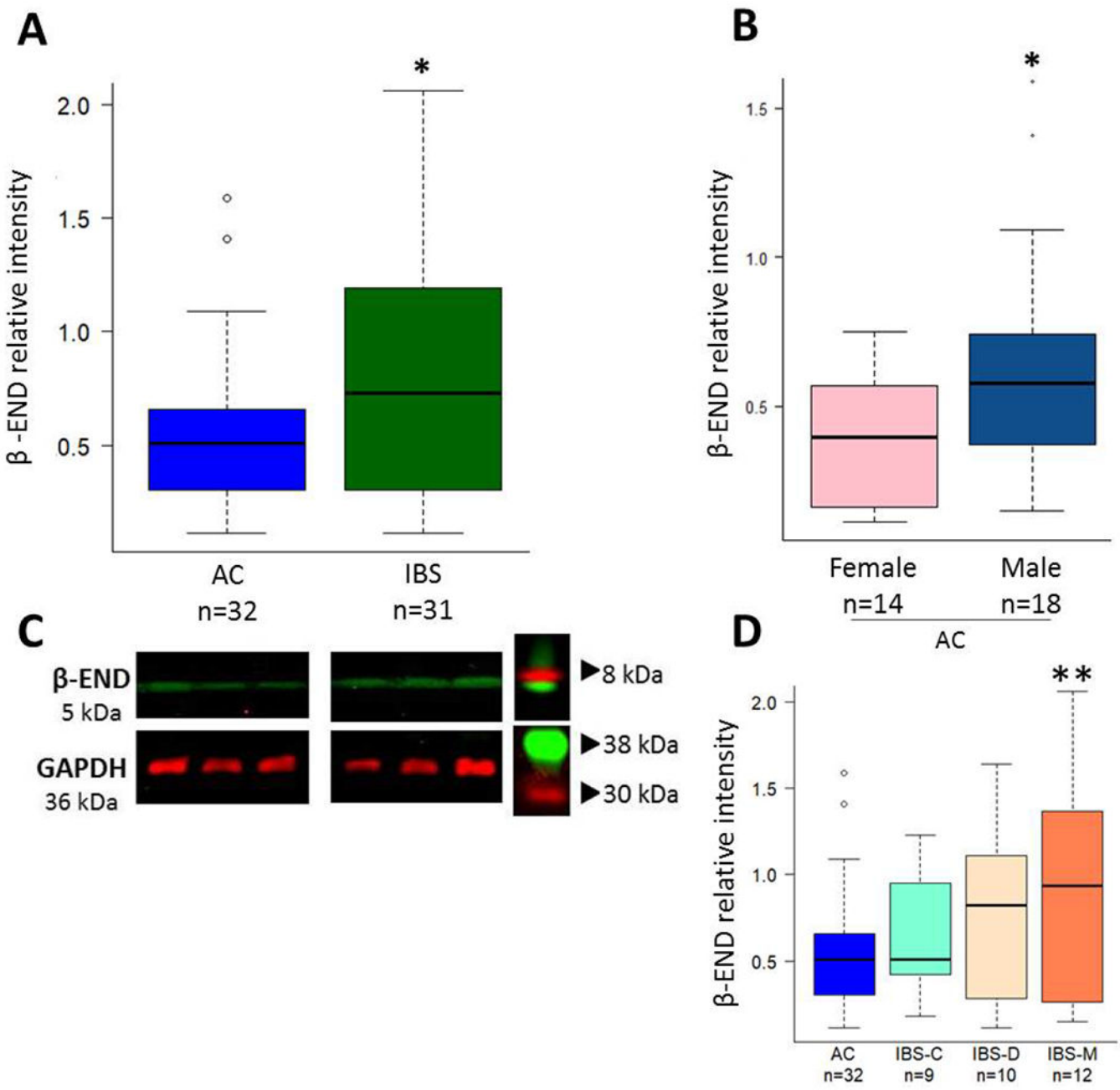

Figure 3 –.

β-END protein levels in mucosal biopsies of patients with IBS were higher compared to AC (3A). Within the AC group, men showed a higher protein expression than women (3B; *P*=.019). Representative imagines of protein gels are shown (3C). With respect to bowel habit differences, IBS-M showed significantly higher levels of β-END compared to AC (3D; ** *P*=.016).

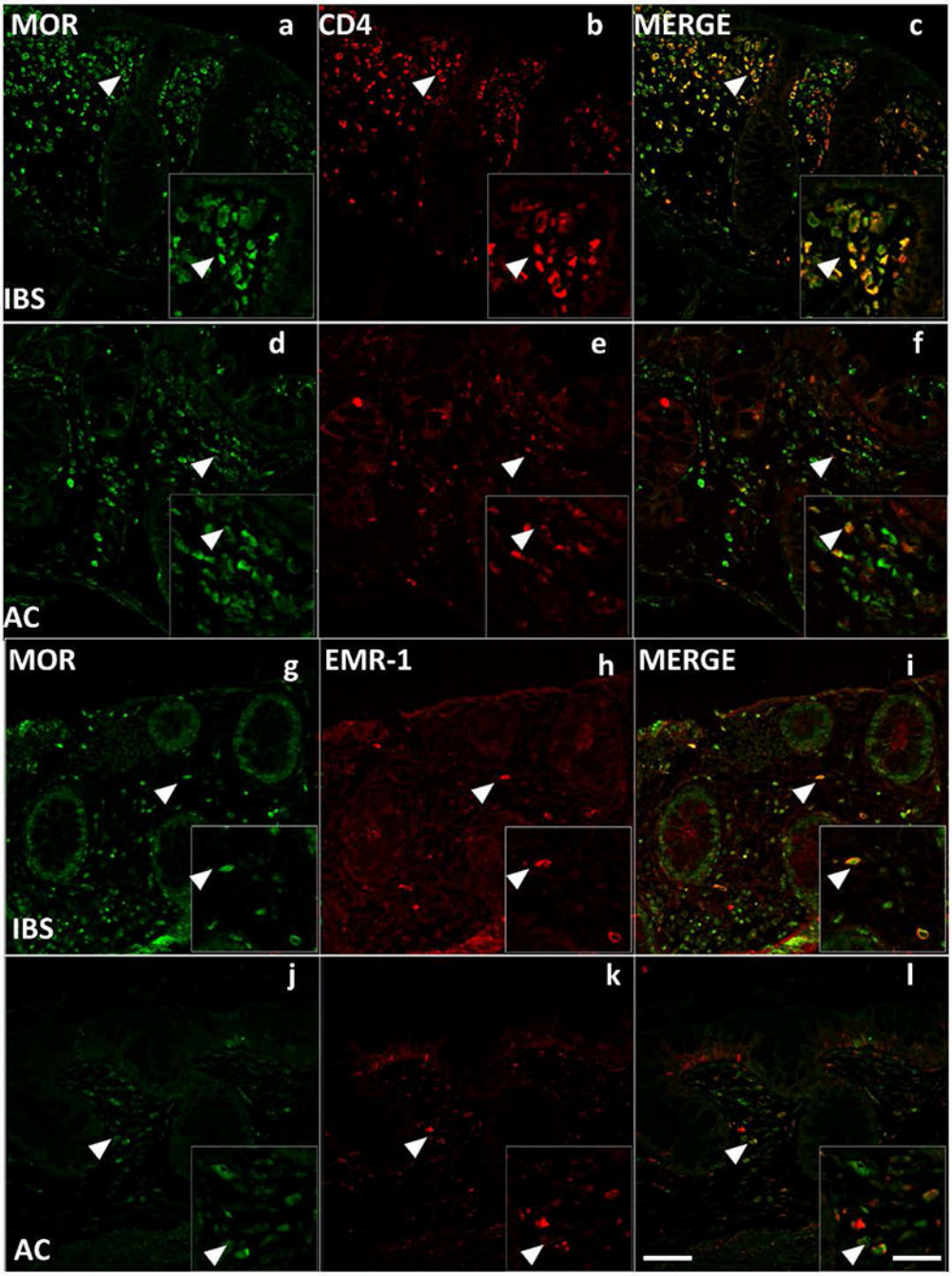

Figure 4 –.

Representative photomicrographs of colocalization of MOR (green; 4a, d) and CD4 (red; 4b, e), MOR (green; 4g, j) and EMR1 (red; 4h, k), in sections of colonic mucosa of participants with IBS (4a-c; g-i) or AC (4d-f; j-l). Third column shows merge stainings of MOR and CD4 (4c, f), MOR and EMR1 (4i, l); scale bar: 50 μm. Arrowheads indicate areas that are shown at higher magnification in the insets, small arrowheads in the insets point to examples of cell bodies containing immunoreactivity for MOR and β-END and immune cell markers (scale bar: 25 μm).

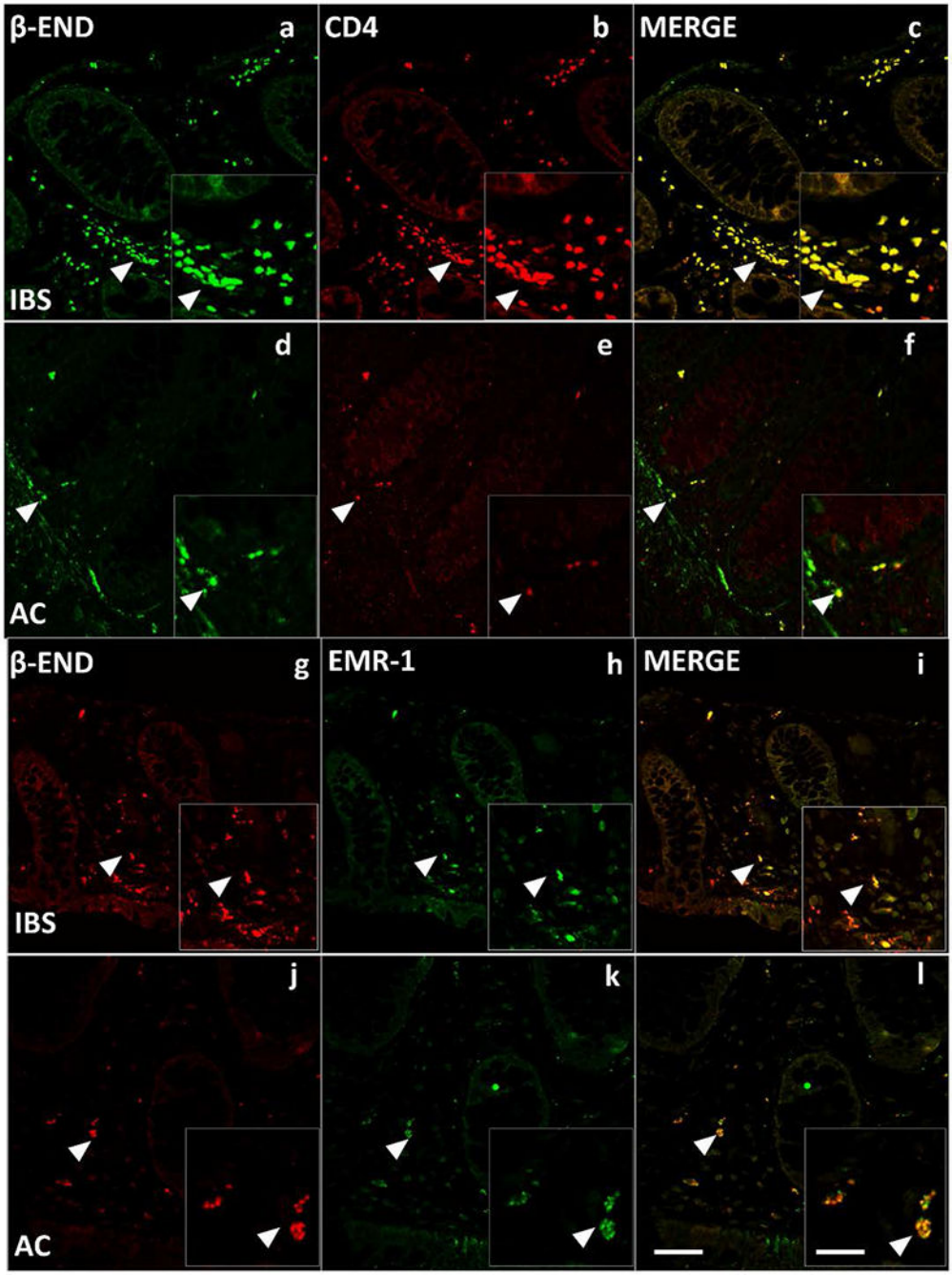

Figure 5 –.

Representative photomicrographs of colocalization of β-END (green; 4a, d) and CD4 (red; 4b, e); β-END (red; 5g, j) and EMR-1 (green; 4h, k) immunoreactivities in mucosal biopsies sections of IBS patients (5 a-c, g-i) and AC subjects (5 d-f, j-l). Right column images show merge staining of β-END and CD4 (5 c, f), and β-END and EMR-1 immunoreactivites (5i, l); scale bar: 50 μm. Large arrowheads indicate higher magnification areas (scale bar: 25 μm), small arrowheads point to some examples of immunoreactive cell bodies.

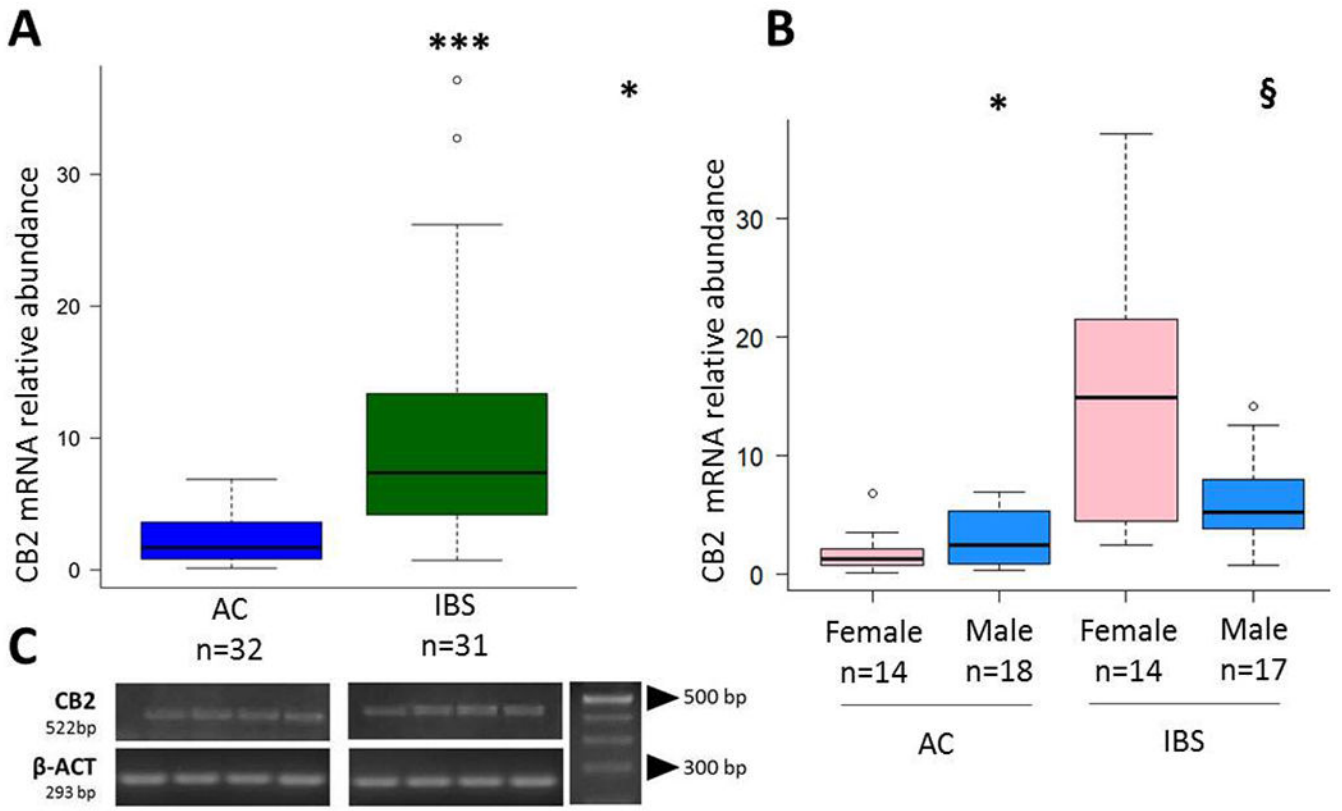

Figure 6 –.

CB_2 mRNA levels in mucosal biopsies of IBS patients and AC participants. The level of CB_2 mRNA was markedly higher in IBS patients as overall group (***P<.001, 6A). While female IBS patients showed higher CB_2 expression compared to male IBS patients (§P=.035, 6B), male AC showed higher levels of CB_2 mRNA compared to female AC (*P=.033, B). Representative images of CB_2 and β-ACT gels and relative molecular weights are shown (6C).

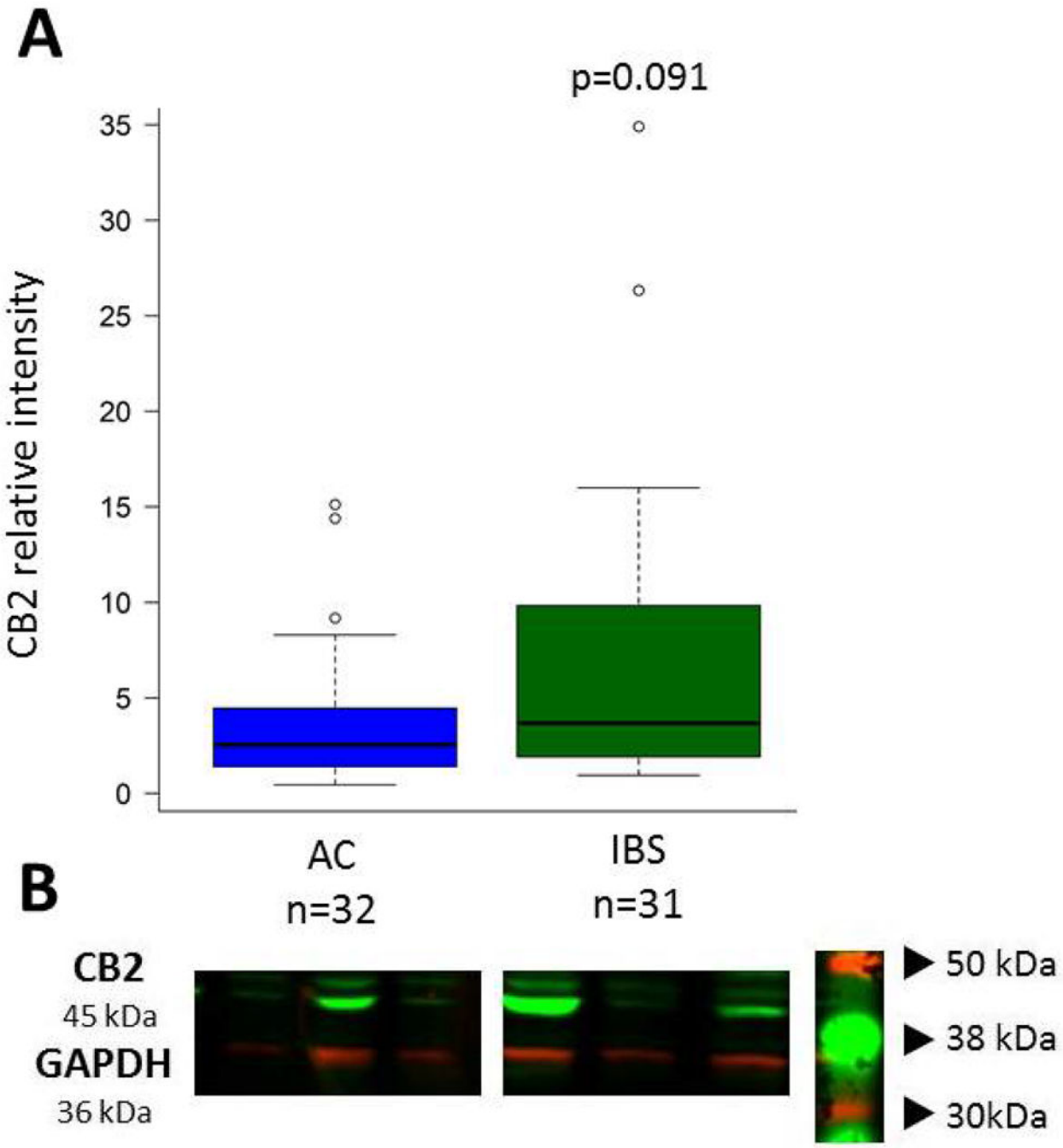

Figure 7 –.

CB_2 protein levels in overall IBS patients compared to AC participants. CB_2 protein expression was numerically higher in IBS patients compared to AC participants but did not meet statistical significance (*P*=.091, 7A). Representative images of CB_2 and GAPDH immunoreactive bands are shown in Figure 7B.

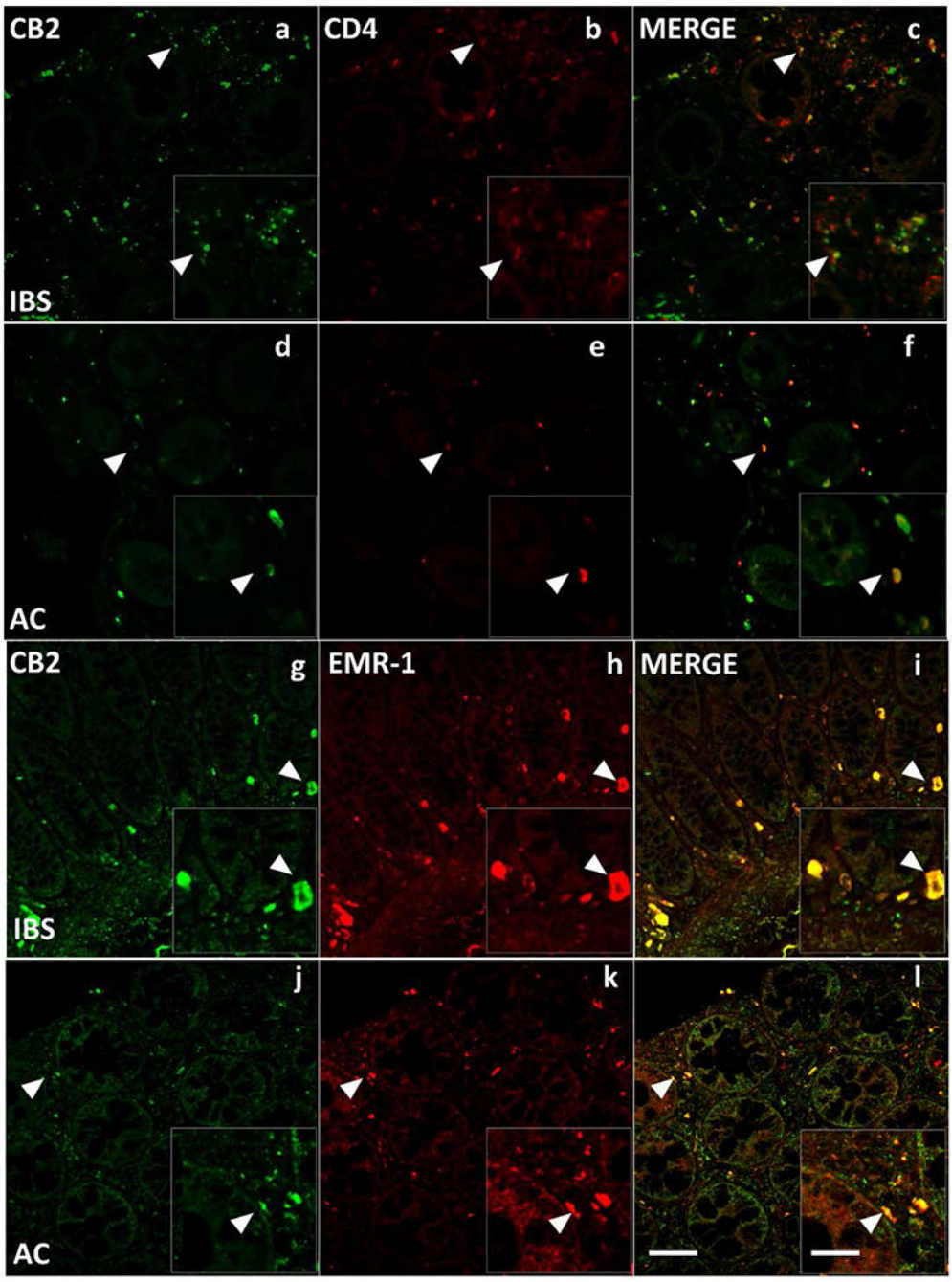

Figure 8 –.

Representative photomicrographs showing CB_2 immunoreactivity (green; 8a, d, g, j) localization in CD4+ lymphocytes (red; 8b, e) and EMR+ eosinophils (red; 8h, k) in IBS (8a-c; 8g-i) and AC (8d-f; 8j-l). Third column shows merge stainings of CB2 and CD4 (8 c, f) and CB2 and EMR-1 (8 i, l). Magnification scale bar: 50 μm. Large arrowheads indicate areas shown at higher magnification in the insets (scale bar: 25 μm); small arrowheads in the insets point immunoreactive cell bodies.