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Unravelling the role of beta-CGRP in inflammatory bowel disease and its potential role in gastrointestinal homeostasis

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Abstract

Background The role of beta calcitonin gene-related peptide (beta-CGRP) in gastrointestinal tract is obscure, but experimental models suggest an effect on the homeostasis of the intestinal mucosa. We measured beta-CGRP circulating levels in a large series of subjects with a recent diagnosis of inflammatory bowel disease (IBD), in order to assess the potential role of this neuropeptide in IBD pathogenesis.

Methods Morning serum beta-CGRP levels were measured by ELISA (CUSABIO, China) in 96 patients recently diagnosed of IBD and compared with those belonging from 50 matched healthy controls (HC) and 50 chronic migraine (CM) patients.

Results Beta-CGRP levels were lower in patients with IBD (3.1 ± 1.9 pg/mL; 2.9 [2.4-3.4] pg/mL) as compared to HC (4.7 ± 2.6; 4.9 [4.0-5.8] pg/mL; $p < 0.001$) and to CM patients (4.6 ± 2.6; 4.7 [3.3-6.2] pg/mL; $p < 0.001$). Beta-CGRP levels in CM were not significantly different to those of HC ($p = 0.92$). Regarding IBD diagnostic subtypes, beta-CGRP levels for ulcerative colitis (3.0 ± 1.9pg/mL; 2.5 [2.1-3.4] pg/mL) and Crohn's disease (3.3 ± 2.0 pg/mL; 3.2 [2.4-3.9] pg/mL) were significantly lower to those of HC ($p < 0.01$ and $p < 0.05$, respectively) and CM ($p < 0.01$ and $p < 0.05$, respectively).

Conclusions We have found a significant reduction in serum beta-CGRP levels in patients with a recent diagnosis of all kinds of IBD as compared to two control groups without active intestinal disease, HC and CM, which may suggest a role for this neuropeptide in the pathophysiology of IBD. Our data indicate a protective role of beta-CGRP in the homeostasis of the alimentary tract.

Keywords Alpha-CGRP, Beta-CGRP, CGRP, Crohn's disease, Inflammatory bowel disease, Ulcerative colitis

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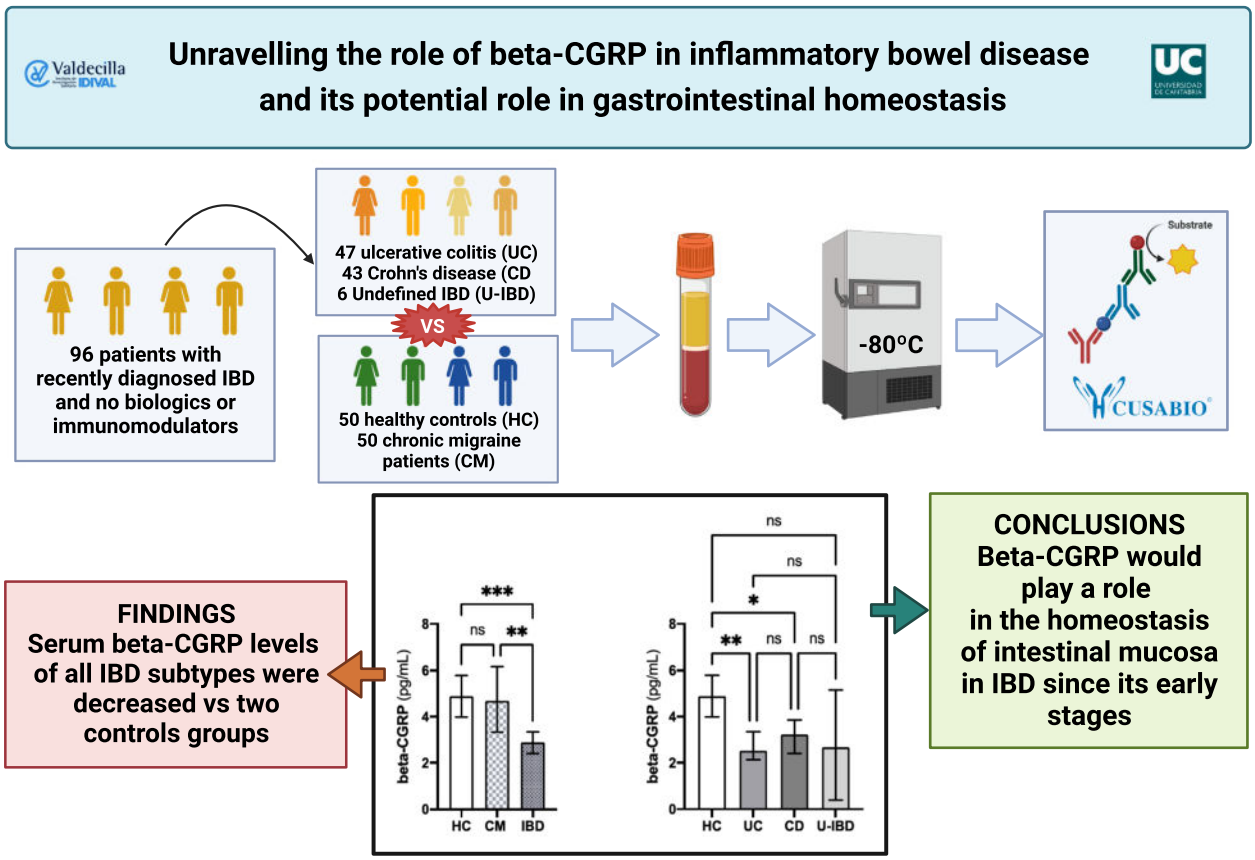
Graphical Abstract



Background

Inflammatory bowel disease (IBD), including mainly Crohn's disease (CD) and ulcerative colitis (UC), involves chronic inflammation and disturbance of the gut immune system. In IBD the epithelial barrier is breached, which allows the entry of luminal microflora that stimulate a proinflammatory immune response. The mucosal injury, entry of luminal factors, dysbiosis and cytokine release overwhelms tissue protection and repair. The cause of IBD remains unknown and its pathogenesis is complex, encompassing genetic and epigenetic factors, microbiota and immunological abnormalities [1].

The neuronal influence on the inflammatory state of gastrointestinal tract in IBD is well demonstrated (Fig. 1) [2, 3]. In addition, gut immune cells express receptors for a range of neurotransmitters, including receptors for neuropeptides released by the intrinsic neurons of the nervous system, such as neurokinins, vasoactive intestinal peptide, neuromedin or calcitonin gene-related peptide (CGRP) [4, 5]. CGRP is a 37 amino acid peptide discovered in 1982 with its gene located on chromosome 11 belonging to the calcitonin family, which is comprised by calcitonin, amylin, adrenomedullin, adrenomedullin 2/intermedin and CGRP [6–8]. CGRP has two isoforms (alpha and beta), which differ by only three amino acids. However, they are encoded by different genes and their location is different [9, 10]. While alpha-CGRP is expressed in areas of the central nervous system (dorsal root, autonomic and trigeminal ganglia and primary sensory neurons), which explains its key role in migraine pathophysiology [11, 12], beta-CGRP is mainly found within myenteric neurons in the small bowel and colon [13–15]. The role of beta-CGRP in gastrointestinal tract is obscure, but experimental data would suggest a protective effect on intestinal mucosa and therefore a role in the pathophysiology of diseases such as IBD or diverticulitis [16, 17]. Therefore, we measured beta-CGRP serum levels in a series of subjects with a recent diagnosis of IBD.

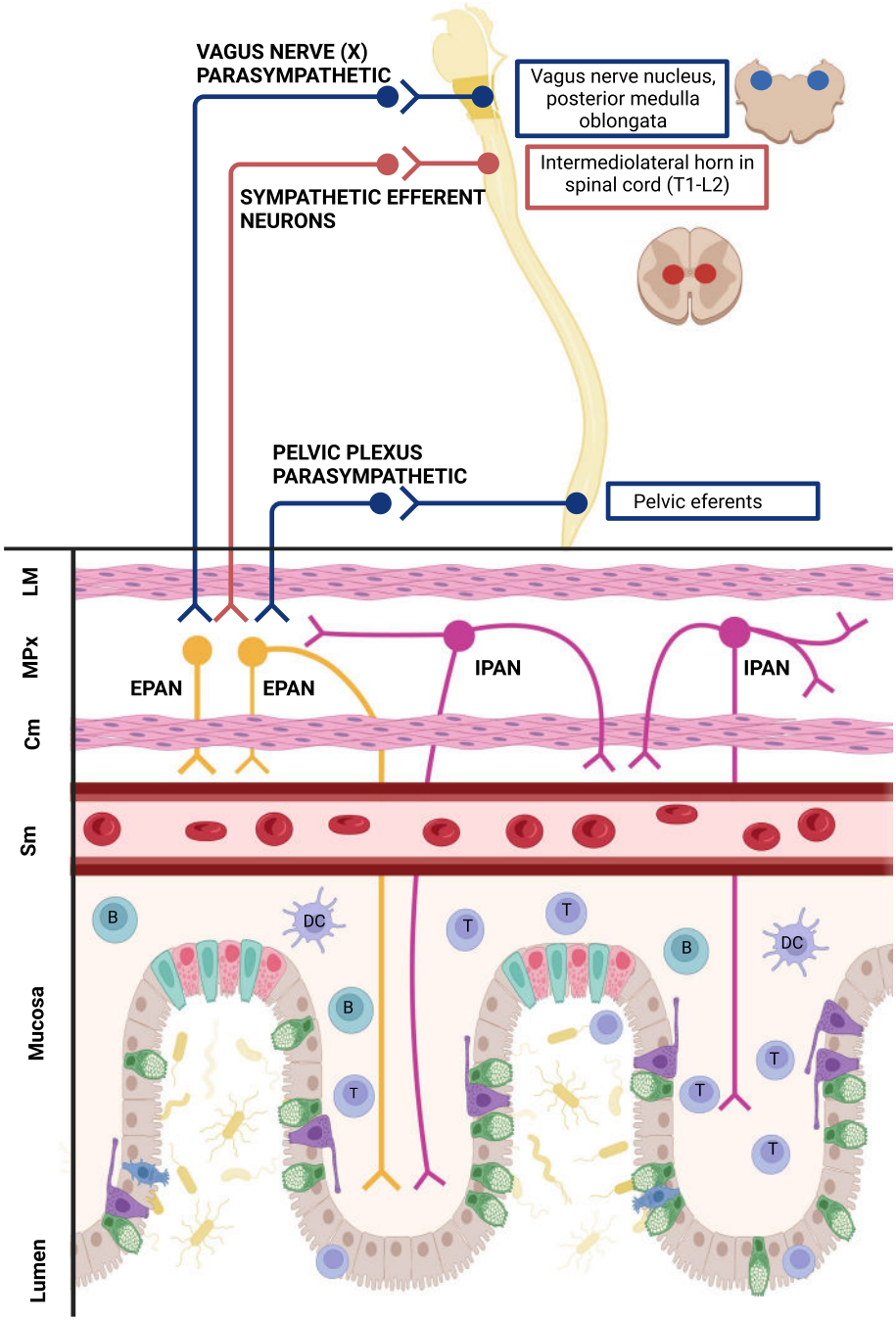


Fig. 1 Gastro-intestinal tract innervation with emphasis on efferent neuronal pathways involved in CGRP release. Alpha-CGRP is liberated by EPANs, which are modulated by sympathetic and parasympathetic system. Beta-CGRP is released by Dogiel type II neurons or IPAN. B, B lymphocytes; T, T lymphocytes; DC, dendritic cell. Created with BioRender.com

Methods

Study design and selection of study participants

Consecutive subjects with a recent diagnosis of IBD and classified according to the Montreal Classification [18] were recruited from our IBD Unit from January 2021 to March 2023. To be included, IBD patients had to be > 17 years and have a new, recent (< 1 year) IBD diagnosis. Patients on biologics or immunosuppressants were excluded. We allowed only treatment with mesalazine or steroids. We registered autoimmune comorbidities and/or extraintestinal manifestations, and cardiovascular risk factors. We considered as a significant cardiovascular risk

factor the presence of active smoking, arterial hypertension, dyslipemia, diabetes mellitus and body mass index (BMI) > 30 kg/m^2.

We included two control populations with similar age and sex. As healthy controls (HC), volunteers with no history of active medical or psychiatric disease, with absence of gastrointestinal or headache symptoms and taking no medication. The second control group was composed by patients who met current chronic migraine (CM) criteria attending our Headache Unit [19]. We included a group of CM patients as this is the disease for which the value of CGRP as a biomarker has been well-established and as gastrointestinal symptoms are a part of the migraine clinical spectrum. CM patients were not on any anti-CGRP treatment and had not taken acute antimigraine treatment for the previous 24 h to blood extraction. Recruiting process was performed simultaneously for the three groups.

Sample size and data collection

Based on our data from previous studies measuring beta-CGRP in patients with diarrhea due to COVID-19 and HC and in CM patients [20, 21], we calculated that with an expected change of 33% between the IBD and the HC groups, alpha equal to 0.05 and a power of 80%, we had to include a minimum of 47 subjects per group.

Detailed clinical data were available for all the participants. The study received IRB approval by the Ethics Committee of Cantabria (28/2020). All participants gave written informed consent.

Laboratory testing

Blood samples were extracted in our outpatient clinic in fasting condition between 9 and 12 am. The blood was left to clot for 10 min prior to the centrifugation, at 3500 rpm and 4°C for another 10 min. The obtained serum was then immediately transferred into sterile tubes and stored at -80°C. All samples were frozen within the first 30 min since extraction and all were assayed before reaching 6 months of cryopreservation.

For the determination of beta-CGRP levels we employed a commercially available ELISA kit specifically designed for the detection of this isoform (CUSABIO, China) as described previously [20, 21]. We proceed by strictly following the manufacturer's instructions. For last step of the ELISA process, in which the user must determine the optimal time for incubation of the plate with the substrate from a given window by the manufacturers, we carried out incubations of 20 min after internal validation. All samples were measured in duplicate, and all measurements had an intra-assay coefficient of variation below 8%, therefor meeting the quality criteria set by manufacturers. A standard curve was generated for every single bath, and these were calculated using a 4-parameter logistic (4-PL) regression with $r^2 > 0.999$. For ensuring the reproducibility of results, every batch included at least 10 samples from the HC group assayed in previous plates, obtaining an inter-assay variability < 10%, also below the threshold set by the manufacturer for this criterium.

Statistical analysis

Categorical variables are reported as percentages, whereas continuous variables are displayed as mean ± SD for normally distributed data and together with median (with 95% confidence interval [CI] of median) for non-normally distributed data unless stated differently in the text. For normality testing of quantitative variables we carried out the D'Agostino & Pearson test ($p < 0.05$ to refuse Ho). To assess the statistical differences between groups of continuous variables following normal distribution (age) we employed the student's t test. For non-normally distributed data (beta-CGRP), Mann-Whitney U test was carried out. For group comparison of categorical variables, the chosen prove was the Fisher's exact test. For multiple group comparisons of sub-groups created upon post-hoc division, we performed Kruskal-Wallis test followed by Dunn's test. The evaluation of correlation relationships was done by Pearson's test.

The p values presented are for two- tailed testing, and we considered a $p < 0.05$ to prove statistical significance. All analyses were performed using GraphPad Prism version 9.4.1 (GraphPad Software).

Results

Baseline characteristics of study participants

We included 96 IBD cases (age 47.8 ± 16.5 years, range 18-82 years; 62.5% women); 50 HC (age 47.9 ± 16.3 years, range 23-77 years; 62.5% women) and 50 CM patients (age 47.9 ± 12.0 years, range 21-69 years; 76% women). Among IBD subjects, 47 (49.0%) met diagnostic criteria of UC, 43 (44.8%) of CD and 6 (6.2%) of unclassified IBD (U-IBD). The mean time from diagnosis to blood extraction was 74.9 ± 64.5 days (median 55.5 days; range between 0-250 days). Regarding treatments, 75% of patients were with either steroids, mesalazine or a combination of the two of them. Fourteen patients (14.6%) had at least one associated autoimmune disorder or extraintestinal manifestation of IBD. 45 patients (46.9%) had one vascular risk factor (Table 1).

Beta-CGRP levels

Beta-CGRP circulating levels were lower in patients with IBD (mean ± SD 3.1 ± 1.9 pg/mL; median [range] 2.9 [2.4-3.4] pg/mL) as compared to HC (4.7 ± 2.6; 4.9 [4.0–5.8] pg/mL; $p < 0.001$) and to CM patients

Table 1 Main characteristics of IBD patients

	Crohn's disease	Ulcerative colitis	Unclassified-IBD
Total, n	43	47	6
Sex, women	26 (60.5)	32 (68.1)	1 (16.7)
Age, y	51.25 ± 15.19, (22-82)	45.3 ± 18.05, (18-74)	44.5 ± 10.62, (27-56)
Days from diagnosis to serum extraction	56.9 ± 56.5, 0-213	87.2 ± 67.5, 0-250	106.6 ± 69.2, 0-193
Disease distribution (Montreal's classification)	A2: 14 (32.6)	E1: 24 (51.1)	
	A3: 29 (67.4)	E2: 16 (34.0)	
	L1: 24 (55.8)	E3: 7 (14.9)	
	L2: 8 (18.6)		
	L3: 8 (18.6)		
	L4: 3 (7.0)		
	B1: 38 (88.4)		
	B2: 3 (7.0)		
	B3: 2 (4.7)		
Perianal disease	5 (11.6)	1 (2.1)	0
Cardiovascular risk factors,			
≥ 1	27 (62.8)	13 (27.7)	5 (83.3)
Active smoking	18 (41.86)	5 (10.6)	2 (33.3)
Arterial hypertension	8 (18.6)	2 (4.3)	1 (16.7)
Dyslipemia	8 (18.6)	6 (12.8)	1 (16.7)
Diabetes mellitus	2 (4.7)	0	1 (16.7)
Body mass index, kg/m^2	25.3 (17.2–40.1)	23.5 (17.5–32.3)	26.5(21.9–31.5)
BMI > 30	5 (9.3)	2 (4.3)	2 (4.3)
Immune disorders			
≥ 1	7 (16.3)	7 (12.8)	0
Rheumatoid arthritis	1 (2.3)	0	0
Multiple sclerosis	1 (2.3)	0	0
Asthma	1 (2.3)	2 (4.3)	0
Spondylitis	3 (7)	0	0
Uveitis	1 (2.3)	0	0
Coeliac disease	1 (2.3)	0	0
Psoriasis	0	2 (4.3)	0
Erythema nodosum	0	1 (2.1)	0
Dermatitis herpetiformis	0	1 (2.1)	0
Urticaria-vasculitis	0	1 (2.1)	0
Treatment			
Any	31 (72.1)	36 (75.6)	5 (83.3)
Mesalazine	21 (48.8)	33 (70.2)	5 (83.3)
Steroids	11 (25.6%)	7 (14.9)	1 (16.7)
Other comorbidities			
Hypothyroidism	5 (11.5)	2 (4.3)	0
Chronic pancreatitis	1 (2.3)	0	0

BMI body mass index

Values are reported as number (percentage), means ± SD (range), or mean (range)

(4.6 ± 2.6; 4.7 [3.3–6.2] pg/mL; $p < 0.001$). Beta-CGRP levels in CM were not significantly different to those of HC ($p = 0.92$). Regarding IBD subtypes, beta-CGRP levels for UC (3.0 ± 1.9pg/mL; 2.5 [2.1-3.4] pg/mL) and CD (3.3 ± 2.0 pg/mL; 3.2 [2.4-3.9] pg/mL) were significantly lower to those of HC ($p < 0.01$ and $p < 0.05$, respectively, while beta-CGRP for U-IBD remained numerically lower (3.0 ± 1.8pg/mL, 2.7 [0.4–5.2] pg/mL). When compared to CM, beta-CGRP content in UC and CD patients remained significantly lower ($p < 0.01$ and $p < 0.05$

respectively) but not U-IBD patients, although the comparison showed a numerical decrease (Fig. 2).

Influence of clinical factors in beta-CGRP levels

No significant differences arose when patients were classified by presence/absence of cardiovascular risk factors (yes: 3.1 ± 1.7 pg/mL; no: 3.2 ± 2.1 pg/mL; $p = 0.93$); autoimmune comorbidities (yes: 3.4 ± 1.4 pg/mL; no: 3.1 ± 2.0 pg/mL; $p = 0.24$); active mesalazine treatment (yes: 3.1 ± 1.9 pg/mL; no: 3.2 ± 1.9 pg/mL; $p = 0.91$); or active steroid treatment (yes: 3.0 ± 1.9 pg/mL; 3.2 ± 1.9 pg/mL; $p = 0.52$).

Discussion

We have found a significant reduction in serum beta-CGRP levels in patients with a recent diagnosis of IBD versus two control groups without intestinal disease, HC and CM. The decrease in beta-CGRP levels was uniform for the three kinds of IBD: CD, UC and U-IBD. To our knowledge, this is the first study analyzing specifically beta-CGRP levels in IBD patients.

Beta-CGRP has a predominant gut location and its concentration in the intestine is seven times higher than that of alpha-CGRP; therefore, though most studies do not differentiate between alpha and beta isoforms, it can be assumed that data about gut CGRP are mostly referring to beta-CGRP [13–15]. CGRP has been shown to be anti-inflammatory in many tissues [22], including the gut [23–25]. In experimental models, CGRP knockout mice are more susceptible to develop colitis and spontaneous lymphoid hyperplasia [26], which might indicate a protective role in bowel inflammation. Concurring with our data, Li et al. found that CGRP and CGRP mRNA expression were decreased in the intestinal mucosa of UC patients. As the magnitude of this decrease correlated with the severity of IBD, they proposed CGRP as a UC biomarker [27]. We show here that beta-CGRP levels are decreased already in many patients on their first stages of the disease, which suggests that this reduction is not secondary to an established chronic damage of the mucosa, but that could have a key role in the maintenance of wall homeostasis in the initial IBD stages.

Considering all these data, it is tempting to propose a protective role of beta-CGRP in keeping gastrointestinal homeostasis in IBD. In the enteric nervous system, beta-CGRP is expressed in a subset of Dogiel type II, intrinsic primary afferent neurons, while alpha-CGRP is expressed in the afferent neurons of the extrinsic nervous system originating from the dorsal root and vagal ganglia [14, 28, 29]. CGRP has been shown to serve as a protective factor in experimental models of colitis, such as those induced by trinitrobenzenesulfonic acid or dextran sulfate sodium [16, 30, 31]. Although the mechanisms underlying this mucosal protection are largely unknown, there are multiple ways by which beta-CGRP might manifest its effects (Fig. 3). CGRP is a potent vasodilator and mediator of localized blood flow [32], which could minimize damage by promoting tissue repair. CGRP liberation increases colon chloride secretion [33], which would aid in the clearance of toxics agents. Experimentally induced colitis

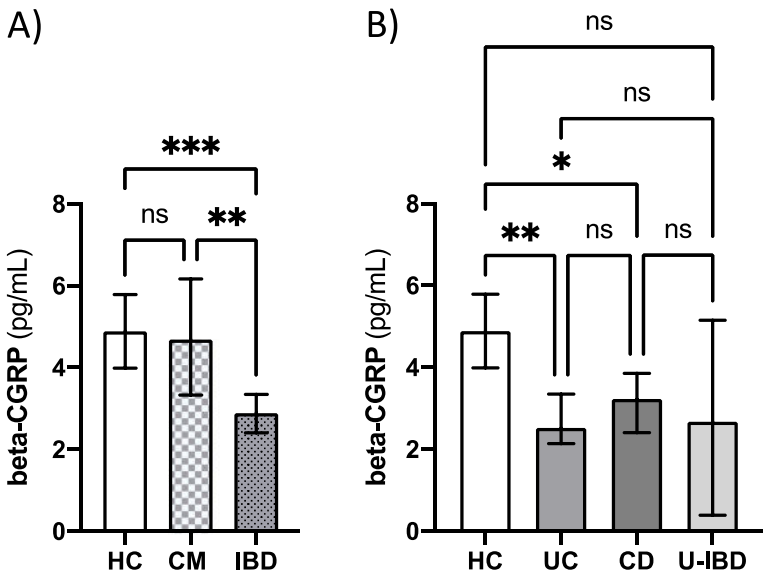


Fig. 2 Serum beta-CGRP levels in IBD vs HC and CM patients (median; 95% CI). **A** Significant decrease in IBD patients as compared to HC and CM subjects. **B** This decrease in beta-CGRP levels versus HC is uniform in UC, CD and U-IBD. ns: $p > 0.05$;*: $p < 0.05$; ** $p < 0.01$; ***: $p < 0.001$. HC, healthy controls; CD, Crohn's disease; UC, ulcerative colitis; U-IBD, unclassified inflammatory intestinal disease

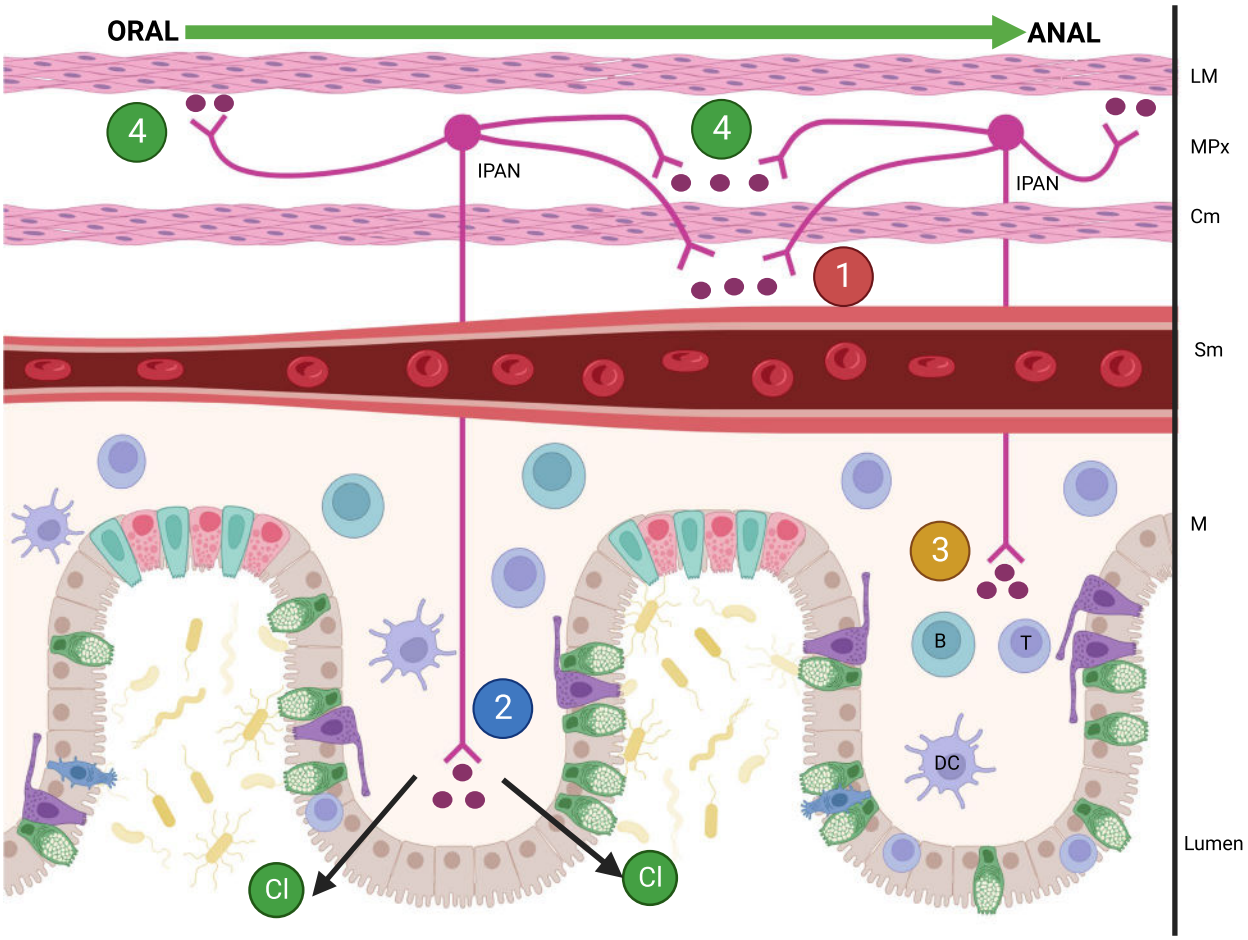


Fig. 3 Proposed physiology of beta-CGRP. Beta-CGRP (violet circles) is released by intrinsic primary afferent neurons (IPAN), located in the myenteric plexus. Four main functions have been associated with beta-CGRP secretion: 1) vasodilation of enteric blood vessels; 2) secretion of chloride in the enteric cells; 3) regulation of proliferation and activation of lymphocytes and dendritic cells; and 4) activation of gut muscle and oral/anal propulsion. Cl, chloride; Cm, circular muscle layer; LM, longitudinal muscle layer; M, mucosa; MPx, myenteric plexus; Sm, submucosal layer. Created with BioRender.com

results in cytokine profiles characteristic of T-helper cells mediated responses [34]. Although neither T nor B cells are required for the induction of experimental colitis [35], it is possible that lymphocytes are involved in propagation of the inflammatory response [21]. Beta-CGRP has been shown to be synthesized and secreted not only by intrinsic enteric neurons but also by T lymphocytes [36]. Such secretion is able to inhibit lymphocyte proliferation, thereby providing a further possible mechanism by which beta-CGRP could act globally to limit the inflammatory process. Interestingly, further supporting a role for enteric CGRP in dysregulation of lymphocytes proliferation, CGRP inhibits interleukin-7 response of B cells through interleukin-6 and tumor necrosis factor alpha mediated pathways and CGRP-null mice both spontaneously develop colitis and significant colon lymphoid hyperplasia with aging [37, 38]. All these data support a protective role of CGRP, and specifically of beta-CGRP, against gastrointestinal mucosal damage. There are other examples of this potential protective role of CGRP. In the stomach, CGRP reverts acid-mediated healing by stimulating somatostatin and gastrin release from the antral cells [39]. CGRP levels are decreased in the enteric ganglia of patients with diverticular disease, which again suggests a role for CGRP in colonic homeostasis [22]. CGRP is involved in a variety of physiological processes throughout the alimentary tract, such as nociception, immune response, secretion and motility [40, 41]. CGRP is also a potent smooth muscle relaxant [42–44]; declined levels could induce an increase in smooth muscle tone and, as already pointed out, play a role in diverticular disease progression [17], but also contribute to some of the IBD motility-dependent symptoms (Fig. 4).

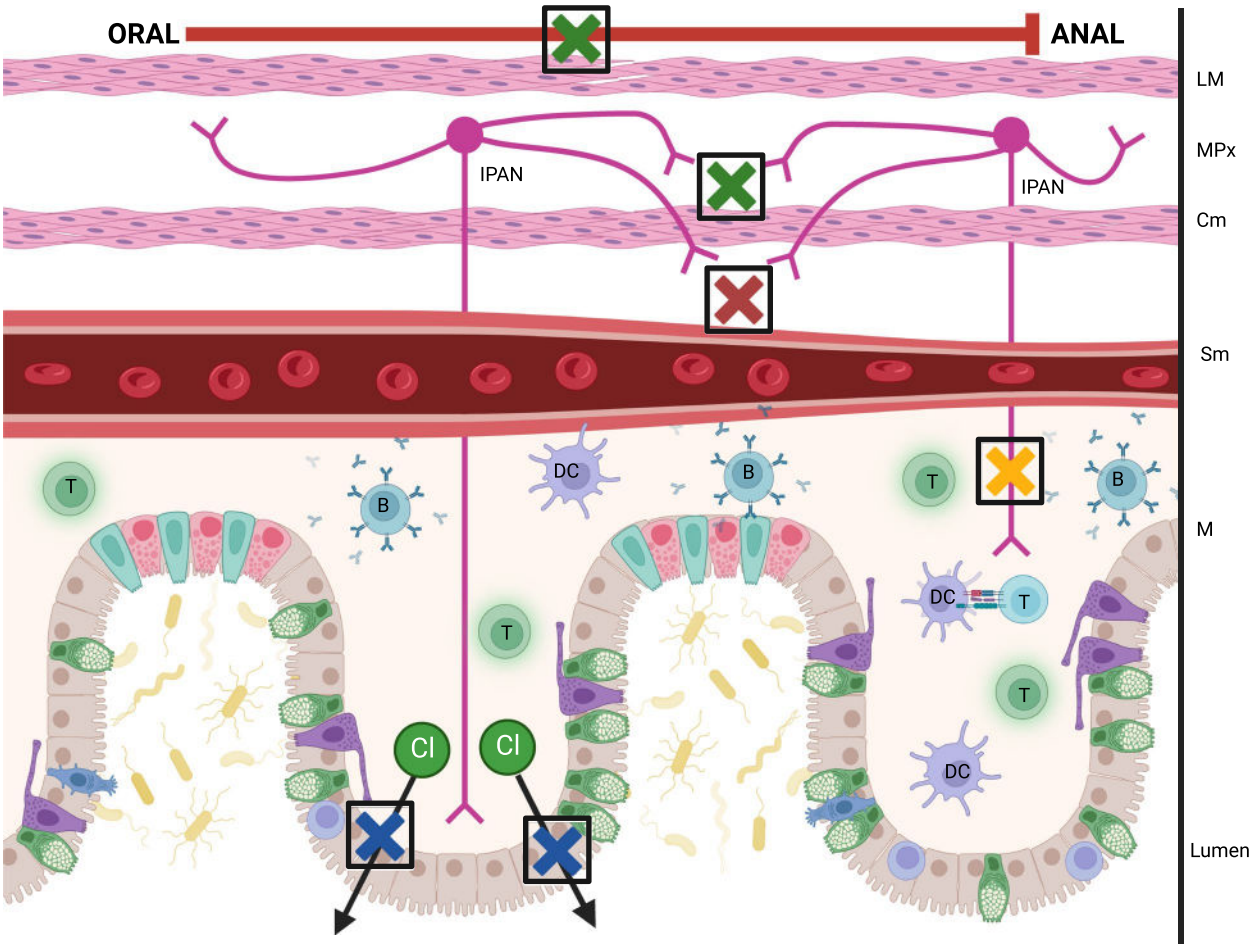


Fig. 4 Beta-CGRP reduction in IBD would induce four distinct changes, marked in this figure with different crosses: 1) red: vasoconstriction of submucosal blood vessels; 2) blue: inhibition of secretion of chloride in enteric cells, which can cause damage in the mucosal layer; 3) yellow: lymphocyte proliferation and activation, as well as cytokine release; and 4) green: inhibition of oral/anal propulsion. Cl, chloride; Cm, circular muscle layer; LM, longitudinal muscle layer; M, mucosa; MPx, myenteric plexus; Sm, submucosal layer. Created with BioRender.com

Our main limitation could be that we do not know with certainty to what extent the serum beta-CGRP reflects the enteric levels. The same happens for the alpha-CGRP isoform whose release by the cranial trigemino-vascular system plays a key role in migraine pathophysiology [11, 12]. Thinking that circulating CGRP values would be of value only if the samples were obtained locally, the first studies showing increased alpha-CGRP levels were carried out in the jugular vein ipsilateral to the pain [45]. However, subsequent studies have shown that this increase in alpha-CGRP is seen acutely within migraine attacks [45, 46], and also interictally in CM cases in cubital samples [21, 47]. Furthermore, experimental data clearly indicate that circulating levels do reflect the release of alpha-CGRP in by an activated trigemino-vascular system [48]. This increase in circulating CGRP levels in migraine is selective for the alpha-CGRP as beta-CGRP have been shown to be within HC limits [21].

One further limitation is that the ability to infer causality between low beta-CGRP levels and IBD pathogenesis is limited since our study is cross-sectional. In fact, to test a potential pathophysiological role of beta-CGRP in IBD it would be necessary to perform an intervention study, for example, to use beta-CGRP or its agonist during the induction of experimental IBD. One important point to consider here is the chameleonic behavior of circulating beta-CGRP levels: serum beta-CGRP levels are reduced here in patients with a chronic condition as IBD, but increase, for instance, in patients with diarrhea due to a COVID-19 infection [49], where a generalized neuropeptide release secondary to the cytokine storm occurs [20]. The high number of IBD cases included here, the fact that samples were obtained at the beginning of the disease -avoiding the potential influence of drugs and surgical procedures employed in IBD- and the use of an ELISA test beta-CGRP specific could be considered as strengths.

Finally, though we included only IBD patient with a recent diagnosis, there were 5 CD patients who were on B2/B3 stages and also 5 who had perianal disease, which could not be regarded as the first or initial changes of the disease. Therefore, reduced beta-CGRP levels could also be explained by an established damage of the intestinal mucosa.

Conclusion

In summary, circulating beta-CGRP levels were shown to be clearly reduced in patients with IBD, which supports its role in IBD pathophysiology since its early stages. Our data may indicate a protective role of beta-CGRP in alimentary tract homeostasis. Future studies are necessary to verify its value as an IBD biomarker; we find particularly interesting to test the relationship between beta-CGRP and disease activity. Finally, our results have translational implications: specific beta-CGRP agonists should be developed and tested in IBD and other digestive diseases and teach us that we have to pay especial attention to the course of IBD in patients who are using the new CGRP antagonists for the treatment of headache conditions.

Abbreviations

BMI	Body mass index
CD	Crohn's disease
CGRP	Calcitonin-gene related peptide
CI	Confidence interval
Cl	Chloride
CM	Chronic migraine
Cm	Circular muscle layer
DC	Dendritic cell
EPAN	Extrinsic primary afferent neuron
HC	Healthy controls
IBD	Inflammatory bowel disease
IPAN	Intrinsic primary afferent neurons
LM	Longitudinal muscle layer
M	Mucosa
MPx	Myenteric plexus
SD	Standard deviation
Sm	Submucosal layer
U-IBD	Unclassified inflammatory bowel disease
UC	Ulcerative colitis

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Authors' contributions

Study concept and design (J.P., J.C. and M.R.); data acquisition and analysis (M.P.M., G.G., V.G.Q., C.P.T., M.J.G., B.C. and J.M.); laboratory procedures (G.G.); drafting of the manuscript (M.P.P., J.P. and G.G.). All authors reviewed the manuscript.

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Availability of data and materials

The data, laboratory methods and study materials are available to other researchers upon reasonable request to the correspondence author.

Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Informed consent was obtained from all participants. All procedures involving human participants adhered to the ethical standards of the institutional and/or national research committee and with the Helsinki Declaration. The ethics research committee of Cantabria, Spain approved this study's protocol.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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