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THE ROLE OF DIPEPTIDYL PEPTIDASE-4 IN INFLAMMATORY BOWEL DISEASE

Pedro Pinto Lopes

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**THE ROLE OF DIPEPTIDYL PEPTIDASE-4 IN
INFLAMMATORY BOWEL DISEASE**

**O PAPEL DA DIPEPTIDIL PEPTIDASE-4 NA
DOENÇA INFLAMATÓRIA INTESTINAL**

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Professor Doutor Fernando Magro

Co-supervisors:

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Ever tried.
Ever failed.
No matter.
Try again.
Fail again.
Fail better.

Samuel Beckett
“Worstward Ho”

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ABBREVIATIONS

a.a.	Amino acids
ADA	Adenosine deaminase
APN	Aminopeptidase N
AUC	Area under the curve
CARD15	Caspase-activating recruitment domain family, member 15
CD	Crohn's disease
CD26	Cluster of differentiation 26
CDAI	Crohn's disease activity index
CRP	C-reactive protein
DPP-4/-6/-8/-9/-10	Dipeptidyl peptidase-4/-6/-8/-9/10
ECM	Extracellular matrix
ESR	Erythrocyte sedimentation rate
FAP-α	Fibroblast activation protein α
FC	Fecal calprotectin
fDPP-4	Fecal dipeptidyl peptidase-4
FL	Fecal lactoferrin
GLP-1/2	Glucagon-like peptide-1/-2
HBI	Harvey-Bradshaw index
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IgA/G/M	Immunoglobulin A/G/M
IL	Interleukin
JAK	Janus kinase
MMPs	Matrix metalloproteinases
NK	Natural killer
NOD2	Nucleotide-binding oligomerization domain containing 2
NSAIDs	Non-steroidal anti-inflammatory drugs
ROC	Receiver operating characteristic
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SARS-CoV-2	Severe acute respiratory syndrome coronavirus-2
sDPP-4	Serum dipeptidyl peptidase-4
TGF-β	Transforming growth factor- β
Th	T helper
TNF	Tumor necrosis factor
UC	Ulcerative colitis
VIP	Vasointestinal peptide

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RESUMO / ABSTRACT

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RESUMO

Introdução

A doença inflamatória intestinal (DII) é uma doença crónica que compreende dois subtipos: colite ulcerosa (CU) e doença de Crohn (DC). Enquanto a primeira está restrita ao cólon, caracterizando-se por inflamação superficial da mucosa, a DC pode afetar qualquer parte do trato digestivo, sendo caracterizada por inflamação transmural. Embora tenham sido tradicionalmente estudadas em conjunto, porque partilham características comuns, sabemos atualmente que representam duas entidades fisiopatológicas distintas. Estas doenças estão associadas a múltiplos fatores patogénicos, incluindo uma série de variantes genéticas de suscetibilidade, uma resposta imunitária largamente desregulada, uma microbiota intestinal anormal e causas ambientais.

A progressão da DII e a resposta ao tratamento são extremamente variáveis, com alguns doentes a apresentarem doença ligeira, enquanto outros evoluem com doença grave ou complicada. Desta forma, os biomarcadores minimamente invasivos surgiram como importantes indicadores de atividade da doença, risco de recidiva, necessidade de escalada terapêutica e/ou resposta ao tratamento. No entanto, apesar dos grandes avanços nesta área, ainda escasseiam biomarcadores capazes de uma gestão personalizada e eficaz.

A dipeptidil peptidase-4 (DPP-4) é uma glicoproteína ligada à membrana que atua como recetor, mas também existe na forma solúvel, atuando através da inativação de vários peptídeos que participam da cascata inflamatória. Esta protease da serina atraiu particular interesse devido às observações de perfis de expressão alterados em doentes com DII, emergindo assim como um potencial biomarcador.

Objetivos

Este trabalho teve como objetivo explorar a patogénese da DII, possibilitando a identificação de potenciais biomarcadores de atividade e prognóstico de doença. Os objetivos específicos foram: (a) avaliar o papel da DPP-4 sérica (sDPP-4) como preditora

da atividade de doença e prognóstico na DII; (b) avaliar o potencial da DPP-4 fecal (fDPP-4) como biomarcador de atividade de doença na DII; (c) analisar os níveis de expressão de DPP-4 em amostras de tecido intestinal de indivíduos com DII; (d) analisar a evidência disponível através de uma revisão sistemática sobre DPP-4 e outras ectopeptidases.

População e Métodos

Para avaliar a capacidade preditora da sDPP-4, relativamente à atividade de doença e prognóstico na DII, procedemos a um estudo de coorte prospetivo multicêntrico, envolvendo 4 centros terciários, que incluiu 195 doentes com DII (94 com CU e 101 com DC). Para a sua avaliação recorreu-se a índices clínicos, sendo que 68 foram submetidos a avaliação endoscópica no início do estudo. Os doentes foram acompanhados durante o período de seguimento para avaliar a necessidade de escalada terapêutica. Um subgrupo de 46 doentes com DII, da coorte previamente referida, recrutados nas fases de indução e manutenção da terapêutica biológica, foi avaliado em vários momentos ao longo do tempo de seguimento, tendo como objetivo determinar a resposta ao tratamento nesta segunda coorte. Os níveis de sDPP-4, proteína C reativa (PCR) e calprotectina fecal (CF) foram quantificados no início do estudo e durante o seguimento.

Para esclarecer o potencial papel da fDPP-4 como biomarcador de atividade na DII, avaliamos uma coorte recrutada prospetivamente, composta por 101 doentes (51 com CU e 50 com DC), do estudo acima mencionado. Estes foram avaliados através de índices clínicos validados, biomarcadores sanguíneos e fecais (sDPP-4, PCR, CF e fDPP-4), sendo que 22 doentes com CU também apresentaram avaliação endoscópica. Para analisar os níveis de expressão de DPP-4 em amostras intestinais de indivíduos com DII foi também realizado estudo imunohistoquímico.

Por fim, com o objetivo de se analisar a evidência científica disponível, realizamos uma revisão sistemática sobre DPP-4 e DII, estabelecendo uma ligação entre dados pré-clínicos e clínicos, através do uso de modelos animais de inflamação intestinal e estudos em doentes com esta patologia. Foram avaliados no total 45 estudos, permitindo compreender o papel da DPP-4 na patogénese desta doença, assim como potencial alvo terapêutico.

Principais resultados

No primeiro artigo incluído nesta tese, mostramos que os níveis de sDPP-4 eram significativamente mais baixos em indivíduos com DII ativa em comparação com aqueles em remissão e controlos saudáveis, sendo capazes de discriminar a atividade clínica e endoscópica da remissão, assim como a necessidade de escalada terapêutica. Aliás, o uso simultâneo de três biomarcadores – sDPP-4, CF e PCR – aumentou significativamente o poder preditivo, denotando uma abordagem “multibiomarcador” promissora, tendo em conta a natureza complexa e heterogênea desta doença. Além disso, a utilidade da sDPP-4 na monitorização da resposta ao tratamento de doentes sob terapêutica biológica foi também investigada, ajudando potencialmente na adaptação de estratégias de tratamento. Os nossos resultados revelaram níveis mais baixos de sDPP-4 em “não respondedores”, particularmente em doentes com CU.

No segundo artigo, evidenciamos que os níveis de fDPP-4 foram significativamente mais elevados em doentes com CU em remissão comparativamente com aqueles em atividade. Pelo contrário, no grupo de doentes com DC ileal, aqueles que apresentaram doença ativa exibiram níveis mais elevados de fDPP-4. Demonstramos igualmente que a fDPP-4 discrimina atividade *versus* remissão clínica em ambos os grupos de doença, bem como atividade endoscópica em doentes com CU, com elevada precisão diagnóstica. A análise imunohistoquímica revelou padrões de expressão distintos no tecido intestinal de indivíduos com DII, com expressão aberrante de DPP-4 apical em doentes com CU em remissão, enquanto aqueles com doença ativa não apresentaram tal expressão. Pelo contrário, indivíduos com DC ileal em remissão apresentaram elevada expressão apical de DPP-4, com tendência à redução nos casos de doença ativa, embora não tenham sido encontradas diferenças estatisticamente significativas.

Por fim, a revisão sistemática (terceiro artigo), incluiu 45 estudos, 36 dos quais foram realizados em modelos animais e 12 em humanos (3 incluíram ambos). Em modelos animais, o uso de inibidores de DPP-4 melhorou significativamente a inflamação intestinal, ao reduzir a atividade catalítica desta protease da serina sobre os seus substratos. Os análogos do peptídeo semelhante ao glucagon (GLP) - 2 mostraram benefícios consideráveis na recuperação do dano inflamatório, reportando melhorias significativas nos índices histológicos e clínicos, bem como redução dos níveis de

citocinas pró-inflamatórias e uma resposta intestinoatrófica. O único estudo de intervenção em humanos, com análogo do GLP-2 (teduglutida) mostrou uma tendência para a remissão.

Conclusões

Neste trabalho propomos uma perspectiva atualizada sobre biomarcadores na DII, com foco particular na DPP-4. Os resultados desta investigação sugerem que a sDPP-4 pode ser usada nesta patologia como um biomarcador não invasivo de atividade clínica e endoscópica, um preditor de escalada terapêutica (particularmente no contexto de uma estratégia de vários biomarcadores) e também como um marcador de resposta à terapêutica biológica. Além disso, os nossos resultados indicam que a fDPP-4 pode ser usada como um biomarcador não invasivo de atividade na DII, particularmente em pacientes com CU. Pela primeira vez, comprovamos que doentes com CU em remissão apresentam expressão imunohistoquímica aberrante de DPP-4, sugerindo que esta poderá ser importante para alcançar e manter a remissão. Estes estudos tornaram evidente que os doentes com CU e DC têm perfis distintos de expressão de DPP-4, o que pode representar uma compartimentação funcional.

O uso de inibidores de DPP-4 e análogos de GLP-2 mostrou claros benefícios no tratamento da inflamação intestinal induzida experimentalmente. No entanto, a extrapolação destes resultados para seres humanos constitui um desafio e será necessária investigação adicional para validar as suas potenciais aplicações diagnósticas e terapêuticas na DII.

Palavras-chave:

Dipeptidil Peptidase-4; Doença Inflamatória Intestinal; Doença de Crohn; Colite Ulcerosa; Biomarcadores.

ABSTRACT

Background

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder encompassing two subtypes: ulcerative colitis (UC) and Crohn's disease (CD). While UC is limited to the colon, involving superficial mucosal inflammation, CD can affect any part of the digestive tract, being characterized by transmural inflammation. Although they have traditionally been studied together because they share common characteristics, it is now evident that they represent two different pathophysiological entities. These diseases are associated with multiple pathogenic features, including several susceptibility gene variants, a largely dysregulated immune response, an abnormal intestinal microbiota, and environmental triggers.

IBD progression and treatment response are highly variable, with some patients experiencing mild disease course, whereas others have severe or complicated disease. Therefore, minimally invasive biomarkers have emerged as important indicators of disease activity, risk of relapse, need for treatment escalation, and/or response to therapy. Nevertheless, despite great advances in this area, biomarkers capable of personalized and effective management are still lacking.

Dipeptidyl peptidase-4 (DPP-4) is a membrane-bound glycoprotein that acts as a receptor but also exists in a soluble form, operating by inactivating peptides that participate in the inflammatory cascade. This serine protease has attracted particular interest due to observations of altered expression profiles in IBD patients, emerging as a potential biomarker.

Purpose

This research project aimed to explore the pathogenesis of IBD, enabling the identification of potential biomarkers of disease activity and prognosis. The specific aims were: (a) to assess the role of serum DPP-4 (sDPP-4) as a predictor of disease activity and prognosis in IBD; (b) to address the potential use of fecal DPP-4 (fDPP-4) as a biomarker of disease activity in IBD; (c) to analyze the DPP-4 expression levels in

intestinal tissue samples of IBD patients; (d) to evaluate the evidence available in this field through a systematic review about DPP-4 and other ectopeptidases.

Populations and Methods

For the assessment of serum DPP-4 as a predictor of disease activity and prognosis in IBD, we conducted a multicentric, prospective cohort, involving 4 tertiary care centers, that included 195 IBD patients (94 had UC and 101 had CD). These were evaluated using clinical indexes, and 68 underwent endoscopic evaluation at baseline. Patients were followed up to assess treatment escalation. A subgroup of 46 biologically treated IBD patients of the above-described cohort, were enrolled in the induction and maintenance phases of biological treatment and evaluated at several time points. Treatment response was assessed in this second cohort. Serum DPP-4, C-reactive protein (CRP), and fecal calprotectin (FC) levels were quantified at baseline and during follow-up.

To clarify the potential role of fDPP-4 as a biomarker of IBD activity, we evaluated a prospectively recruited cohort, consisting of 101 patients with IBD (51 had UC and 50 had CD), from the above-mentioned study. These were evaluated using validated clinical indexes, blood and fecal biomarkers (sDPP-4, CRP, FC, and fDPP-4), and 22 UC patients also had endoscopic evaluation. In order to analyze the DPP-4 expression levels in intestinal samples of IBD patients, immunohistochemical staining was also performed.

Finally, to evaluate the available evidence, we conducted a systematic review of DPP-4 and IBD, establishing a link between preclinical and clinical data, using validated animal models of intestinal inflammation and studies on IBD patients. We analyzed 45 studies, allowing us to better understand the specific role of DPP-4 in IBD pathogenesis and also as a therapeutic target.

Main results

In the first paper included in this thesis, we showed that sDPP-4 levels were significantly lower in active IBD patients compared to those in remission and healthy controls, being able to distinguish clinical and endoscopic activity from remission, along with the need for treatment escalation. The simultaneous use of three biomarkers — sDPP-4, FC, and CRP — significantly enhanced the predictive power, suggesting a

promising multibiomarker approach for the complex and heterogeneous nature of IBD. Furthermore, the utility of sDPP-4 in monitoring treatment response among biologically treated patients was also investigated, helping potentially in tailoring treatment strategies. Our results revealed lower sDPP-4 levels in nonresponders, particularly in UC patients.

In the second paper, we evidenced that fDPP-4 levels were significantly higher in UC patients in remission compared to those with active disease. On the contrary, in the group of CD patients with ileal involvement, those with active disease exhibited higher levels of fDPP-4. Fecal DPP-4 discriminated with high diagnostic accuracy, remitters from patients with active disease, in both disease groups; it was also able to differentiate endoscopic activity in UC patients. Immunohistochemical analysis revealed distinct expression patterns in the intestinal tissue of IBD patients, with aberrant expression of apical DPP-4 in UC patients in remission, while those with active disease did not show such expression. In contrast, ileal CD patients in remission exhibited high expression of apical DPP-4, with a trend toward reduced expression in cases of active disease, although no statistically significant differences were found.

Finally, the systematic review (third paper), included 45 studies, 36 of which were carried out in animal models and 12 in humans (3 included both). In animal models, the use of DPP-4 inhibitors significantly improved intestinal inflammation, by reducing the catalytic activity of this serine protease over its substrates. Glucagon-like peptide (GLP) - 2 analogs showed substantial benefits in recovery from inflammatory damage, reporting significative improvements in histological and clinical scores, as well as a reduction in pro-inflammatory cytokine levels and an intestinotrophic response. The sole interventional human study with a GLP-2 analog (teduglutide) demonstrated a tendency to remission.

Conclusions

In this project, we propose an updated perspective on biomarkers in IBD, with a particular focus on DPP-4. The results from this investigation suggest that sDPP-4 can be used in IBD as a noninvasive biomarker of clinical and endoscopic activity, a predictor of treatment escalation (especially in the context of a multi-biomarker strategy), and also

as a marker of biological treatment response. In addition, our results suggest that fDPP-4 can be used as a noninvasive biomarker of IBD activity, particularly in UC patients. For the first time, we showed that UC patients in remission present aberrant immunohistochemical expression of DPP-4, suggesting it can be important to achieve and maintain remission in UC. It is now clear that UC and CD patients have distinct DPP-4 expression profiles, which might represent functional compartmentalization.

The use of DPP-4 inhibitors and GLP-2 analogs showed clear benefits in the treatment of experimentally induced intestinal inflammation. However, extrapolating these results to humans is challenging and more research is needed to validate their potential diagnostic and therapeutic applications in IBD.

Keywords:

Dipeptidyl Peptidase-4; Inflammatory Bowel Disease; Crohn's Disease; Ulcerative Colitis; Biomarkers.

INTRODUCTION

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INTRODUCTION

1. INFLAMMATORY BOWEL DISEASE

1.1. Definition

Inflammatory bowel disease (IBD) is a chronic disorder, typically classified as one of two subtypes: ulcerative colitis (UC) and Crohn's disease (CD). These are separate conditions with several clinical, endoscopic, and histologic features that are critical for their diagnosis, some of which are overlapping.

UC is restricted to the colon, usually starting in the rectum and extending proximally continuously. It is characterized by superficial mucosal inflammation, leading ultimately to ulcerations, severe hemorrhage, toxic megacolon, and fulminant colitis. In contrast, in CD, any part of the digestive tract can be affected, although the distal ileum and proximal colon are more commonly afflicted, frequently in a noncontinuous way. It is characterized by transmural inflammation, leading eventually to complications such as fibrotic strictures, fistulas, and abscesses (**Figure 1**). Nearly 10% of IBD patients have indeterminate colitis, which is when Crohn's colitis cannot be distinguished from UC.¹

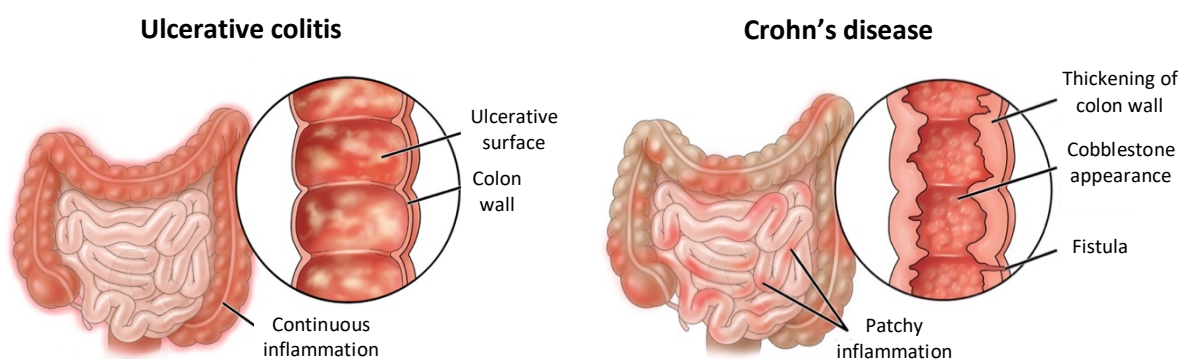


Figure 1. Differences between ulcerative colitis and Crohn's disease. Adapted with permission from the American Gastroenterological Association.²

Between 25-40% of IBD patients experience various extra-intestinal manifestations, such as: anemia, arthritis, osteopenia/osteoporosis, erythema nodosum, pyoderma

gangrenosum, aphthous stomatitis, uveitis/episcleritis, urolithiasis, kidney amyloidosis, hepatitis, primary sclerosing cholangitis, and others (**Figure 2**).³

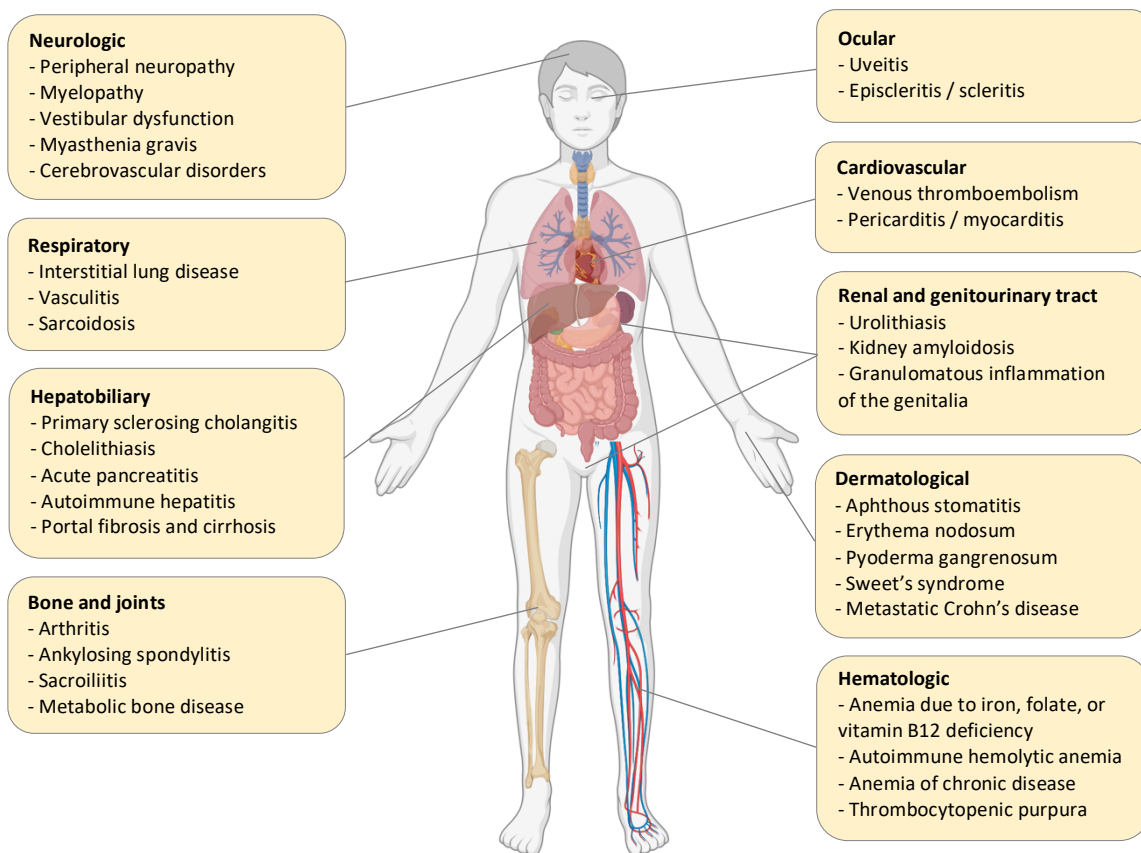


Figure 2. Extraintestinal manifestations of inflammatory bowel disease. Adapted from Das, 1999.³

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1.2. Epidemiology

IBD occurs worldwide, although North America and Northern Europe have the highest incidence, suggesting a north-south gradient. There is evidence of an increasing incidence and prevalence over time and in different regions around the world, with UC remaining slightly more prevalent than CD.⁴

The prevalence of IBD has been increasing in Portugal, and the last national available data from 2007 estimates a prevalence of 71/100 000 inhabitants and 73/100 000 inhabitants for UC and CD, respectively. In terms of IBD prevalence in Europe, Portugal is ranked average, but its incidence is increasing toward the highest-ranked group.⁵

These diseases occur in all socioeconomic groups, but their incidence is particularly high in Caucasians and Ashkenazi Jewish descendants.¹

IBD has traditionally been considered a disease of the West, nevertheless, incidences in the Eastern hemisphere are growing and follow geographical patterns of Westernization (**Figure 3**).⁶ Research on migrant populations moving from low- to high-prevalence areas of IBD and the subsequent increase of the disease prevalence in future generations, highlight the influence of new environmental and lifestyle factors, suggesting that their impact is most pronounced during the initial phases of life when the immune system is still developing and the microbiota is becoming established.⁷

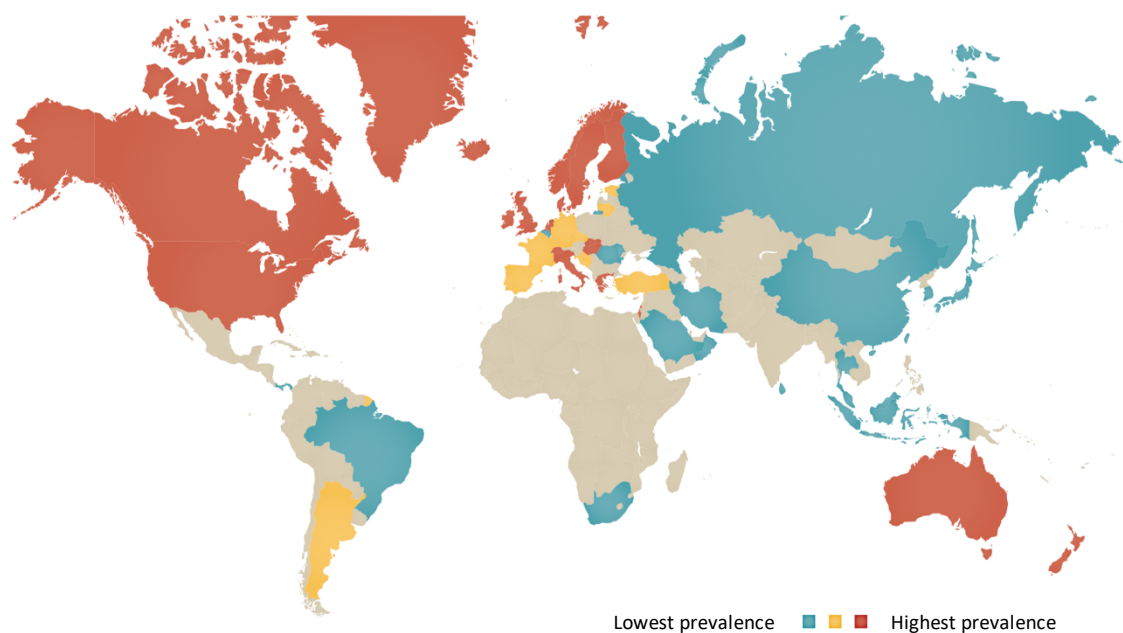


Figure 3. Inflammatory bowel disease global prevalence, in 2015. Adapted with permission from Springer Nature. Kaplan, 2015.⁸

Although both of these diseases can manifest at any stage of life, their incidence is highest between the second and fourth decade, with a subsequent peak observed in the seventh decade.⁷

1.3. Etiopathogenesis

Although the trigger for IBD is unknown, four major pathways might initiate the disease: genetic susceptibility, environmental trigger, gut microbiota, and immune response (**Figure 4**).⁹

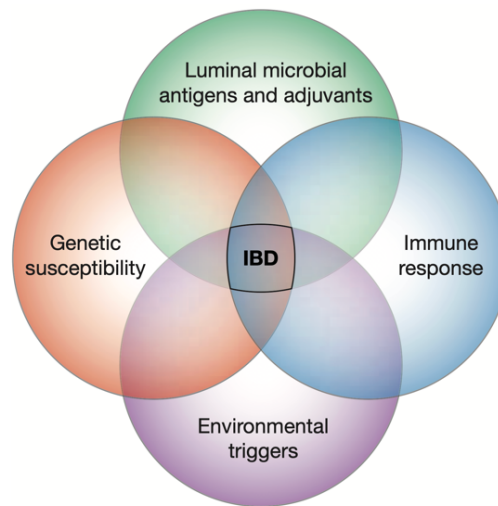


Figure 4. Interaction of different factors contributing to the onset of IBD. Reproduced with permission from Springer Nature. Sartor, 2006.¹⁰

Host and microbial factors have been implicated in the pathogenesis of IBD, which ultimately results in inappropriate immune responses against microorganisms residing in the intestinal lumen.¹¹ Therefore, immune events must be integrated and interpreted in the context of a larger scenario in which genetics, the environment, and the microbiota have significant roles in IBD pathogenesis.⁹

1.3.1. Genetics

Multiple studies have demonstrated that first-degree relatives of IBD patients have a higher incidence of the disease compared to the general population, as well as a greater genetic risk, that is particularly higher for those with CD.¹²⁻¹⁴ In fact, research conducted on monozygotic twins with IBD revealed that the concordance rate for CD was significantly greater than that of UC (50% *versus* 19%), and continued to rise over time.¹⁴ CD patients also demonstrate a genetic clinical pattern, with concordance in disease location (e.g., ileal *versus* colonic) and behavior (e.g., stricturing *versus* penetrating).¹⁵ Furthermore, another factor suggesting a genetic cause is the presence of genetic anticipation, when the subsequent generations develop earlier onset and more severe disease.¹⁶

The first susceptibility gene for CD, *NOD2* (nucleotide-binding oligomerization domain containing 2), also known as *CARD15* (caspase-activating recruitment domain family,

member 15), was identified in 2001 and drove further research for additional genes.¹⁷ Genome-wide association studies have been notably successful at implicating IBD risk loci and more than 240 risk variants have been identified. These can affect pathways responsible for regulating adaptive immunity (e.g., *IL23R*); genes controlling epithelial barrier function (e.g., *ECM1*); intracellular pathways recognizing microbial products (e.g., *NOD2*); and the autophagy pathway, which regulates the recycling of intracellular organelles and elimination of intracellular microorganisms (e.g., *ATG16L1*, *IRGM*, and *NOD2*).^{6,18} These loci account for only a small fraction of total variance in disease risk (8-13% in CD and 4-7% in UC), demonstrating that no single susceptibility gene is sufficient to cause IBD.¹⁹

1.3.2. Environmental factors

It is possible that dietary factors influence the risk of developing IBD. Food antigens are thought to induce an immunologic response, leading ultimately to the development of IBD, but no particular pathogenic antigens have yet been identified.²⁰ Individuals who consumed excessive amounts of meat, total fats, and polyunsaturated/omega-6 fatty acids were associated with an increased risk of CD and UC. Conversely, a decreased CD risk was reported in people with substantial intake of dietary fiber, specifically from fruits and cruciferous vegetables.^{20,21}

Cigarette smoking is correlated with an increased risk of CD and its complications (e.g., strictures, fistulae), as well as the need for surgery.^{22,23} In patients with ulcerative colitis, quitting smoking is linked to an increase in disease activity and a higher probability of requiring hospitalization.²⁴ While the underlying cause of this paradox remains unknown, it is possible that nicotine and smoking metabolites have direct effects on blood flow, microbiota composition, as well as immune and barrier function.²⁵

Some medications can act as risk factors for IBD, in particular nonsteroidal anti-inflammatory drugs (NSAIDs), oral contraceptives, and hormone replacement therapy. These appear to be associated with an increased incidence of IBD, although the risk is small.^{26,27} While IBD has been linked to antibiotic exposure, the causality of this association remains unknown.²⁸

1.3.3. Microbiota

A substantial microbial community, consisting of millions of bacteria, viruses, fungi, and eukaryotic microorganisms, colonizes the gastrointestinal tract, especially the colon and distal ileum. This collection of organisms is known collectively as the microbiome.^{19,29}

The intestinal microbiota is acquired at birth and undergoes significant transformations during the first year of life. Several environmental exposures (e.g., food, hygiene, antibiotics) exert a major influence on the intestinal microbiota in childhood. On the contrary, in adults, it is moderately stable over time, although variations can occur due to environmental factors and diseases.³⁰

The intestinal microbial colonization is essential to the development and maturation of the immune system. These commensal bacteria are usually innocuous and exist in a mutually beneficial relationship with the host, interfering with the ability of pathogens to colonize and invade, due to competition for space and nutrients. When the integrity of the mucosal barrier is compromised, they can become pathogenic by crossing the epithelium, stimulating an immune response, leading ultimately to intestinal inflammation.^{18,31} The disruption of the normal balance between the intestinal microbiota and host, known as dysbiosis, has been associated with IBD.^{32,33}

In conclusion, the microbiota is protective for most individuals, may be a permissive bystander in some IBD patients, or may have a more significant role in others, depending on the interactions with genes and environment, especially in childhood.⁷

1.3.4. Immune dysregulation

A dysregulated response with excessive and inadequate immune reactivity to the microbiota of the intestinal lumen is implicated in the pathogenesis of IBD, highlighting the importance of a balanced immune response.

In the healthy state, macrophages engulf and destroy pathogens and infected cells. The dendritic cells initiate immune responses in mucosal tissues by acquiring antigens and interacting with naive B and T lymphocytes. Naive B cells become activated and differentiate into plasma cells, which undergo IgM-to-IgA isotype switching. IgA is secreted and transported across the epithelium and neutralizes toxins and pathogens

without causing inflammation, due to its incapacity to fix complement. Therefore, the mucosal immune system promotes an anti-inflammatory environment due to active downregulation of immune responses.¹⁸ However, in IBD patients, activated macrophages produce increased levels of interleukin (IL)-6, IL-12, IL-23, and tumor necrosis factor (TNF), which are proinflammatory cytokines. After the presentation of antigens by dendritic cells, CD4+ T lymphocytes proliferate and differentiate into type 1 T helper (Th) and type 17 Th cells, upregulating chemokine receptors and integrins, enabling them to enter the systemic circulation and redistribute to mucosal surfaces, where they carry out inflammatory functions.^{7,18} Therefore, these patients present increased levels of mucosal and circulating B lymphocytes, plasma cells, autoantibodies, and increased antibodies to microbial antigens, pointing to an abnormal B-cell regulation. In fact, IgG-secreting plasma cells are increased in IBD patients, leading to inflammation through recruitment of immune cells and activation of complement, in contrast to the IgA predominance in the healthy state.^{34,35} Other inflammatory cells, such as neutrophils, found within the mucosa in IBD patients, must be recruited from the blood vessels through an enhanced expression of adhesion molecules on leukocytes and endothelial cells and increased chemokines, a process called "mucosal homing".³⁶ Therapeutic approaches aimed at inhibiting mucosal homing of leukocytes are already in use in clinical practice.

In conclusion, mucosal immunity must maintain a delicate balance between regulating tolerance to benign antigens (e.g., food and commensal microorganisms) and conducting surveillance for pathogens, in order to elicit an appropriate immune response to benefit the host, without causing damage.³⁷

1.4. Therapy

There are several drugs used in the treatment of IBD, including untargeted therapies (corticosteroids, aminosalicylates, and immunomodulators), and targeted biological therapies (anti-TNF- α antibodies, anti- $\alpha 4\beta 7$ integrin antibodies, anti-interleukin-12/23 antibodies, and Janus kinase - JAK - inhibitors).^{1,7}

Corticosteroids are the most important treatment to induce remission in IBD patients with active disease. However, they must be used in conjunction with other treatments, as long-term steroid use is not recommended for the management of IBD.^{7,38,39}

Mesalazine and sulfasalazine, which are 5-aminosalicylic acid-based medications, are the cornerstone of treatment in mild to moderate UC, due to their efficacy in inducing and maintaining remission. On the contrary, these therapies have limited value for the treatment of CD.^{7,38,39}

Thiopurines (azathioprine and 6-mercaptopurine) are immunomodulators recognized as steroid-sparing agents in IBD patients and are indicated for maintenance therapy of moderate to severe CD and also for UC that remains active despite optimally dosed 5-aminosalicylic acid therapy. Methotrexate, another immunomodulator, is usually reserved for CD patients who fail to respond or are intolerant to thiopurines.^{7,38,39}

When an immunomodulator therapy alone has failed to achieve satisfactory control over a patient's disease, biological therapy is indicated for the induction and maintenance of remission. Despite the good response to these drugs, almost 30% of IBD patients do not achieve a satisfactory clinical response and those who do, will suffer a loss of response of 5-10%, each year, commonly related to immunogenicity.³⁸ The anti-TNF- α monoclonal antibodies are efficacious and have revolutionized the treatment of IBD. They are used in moderate to severe luminal CD (infliximab and adalimumab) and UC (infliximab, adalimumab and golimumab). Vedolizumab is a monoclonal antibody that inhibits leukocyte trafficking in the intestinal mucosa, by blocking the $\alpha 4 \beta 7$ integrin, resulting in intestinal selective anti-inflammatory activity, and is approved for induction and maintenance therapy for CD and UC. Ustekinumab is an anti-IL-12/23 monoclonal antibody with efficacy in the treatment of moderately to severely active CD.³⁹ Tofacitinib, an oral small molecule JAK inhibitor, is used for treating patients with moderate to severe UC, who have failed or are intolerant to anti-TNF agent-based therapy.⁴⁰

The main target of IBD treatment is to induce and maintain disease remission, which means control of intestinal inflammation, improvement of symptoms, and the prevention of long-term damage.^{1,7}

2. BIOMARKERS IN INFLAMMATORY BOWEL DISEASE

A biomarker has been previously defined as “*a characteristic that is measured as an indicator of normal biological or pathogenic processes, and biological responses to an exposure or intervention, including therapeutic interventions*”.^{41,42} Biomarkers comprise a wide variety of molecular, physiologic, histologic, endoscopic, and radiographic indicators, being the basis of precision medicine.⁴²

Over the past few decades, researchers have focused on identifying reliable biomarkers of disease burden that can be easily tested in biological samples, without affecting the quality of patients' life. Yet, in the IBD field, we lack minimally invasive validated biomarkers to accurately diagnose and prognosticate disease course, as well as to predict and monitor response to treatment.⁴³

The ideal biomarker should fulfill three distinct domains: (a) reliability for the disease; (b) patient compliance; (c) kinetic stability. First, the ideal biomarker should have the capability to accurately measure the true concentration of a marker, being able to distinguish between different levels of severity, with high specificity and reproducibility. Second, it should be harmless for the patient (i.e., have reduced invasiveness), rapid (with quick results and no need to follow a preparation), and also cost-effective. Finally, it should be stable with long-term storage or shipment and also invariable (not influenced by systemic inflammation, for instance).^{44,45}

So far, the ideal biomarker that possesses all the above-mentioned qualities does not exist and the discovery of an ideal biomarker seems unattainable. Therefore, the combination of distinct biomarkers can prove to be advantageous.⁴⁵

Serum inflammatory markers are useful for identifying active inflammation, although they are not specific and may be elevated in conditions other than gastrointestinal disorders. In contrast, fecal biomarkers are typically more accurate due to their direct contact with the intestinal mucosa.⁴⁶ Next, we will address several biomarkers in use in clinical practice.

Orosomucoid is an acute-phase protein, predominantly produced in the liver as a result of tissue damage, inflammation, or infection. There is a correlation between circulating orosomucoid levels and the disease activity of IBD, as assessed by standard

disease activity indices. However, its extended half-life of 5 days limits its utility as an indicator of disease activity improvement.⁴⁷

Serum C-reactive protein (CRP) is generally recognized as an inflammatory marker in the IBD field. Produced by hepatocytes in response to stimulation from inflammatory cytokines (IL-1, IL-1 β , and TNF- α), this acute-phase reactant has a relatively short half-life of about 19h.⁴⁸ Increased levels of CRP help differentiate mucosal active disease from quiescent IBD. However, due to its non-specific nature as a biomarker, CRP levels may rise in the presence of infections and extraintestinal inflammation, among other conditions besides IBD.⁴⁹ On the other hand, normal CRP levels can be seen in IBD patients with active disease, in particular those who are asymptomatic and have mild mucosal lesions, namely with ileal disease.⁴⁹ The contexts in which CRP has been shown to be most reliable are the distinction between IBD and irritable bowel syndrome (IBS)⁵⁰, CD patients with transmural inflammation, UC patients with extensive colitis⁵¹, and the monitoring of disease relapse and therapy failure^{52,53}.

The erythrocyte sedimentation rate (ESR) is an indirect measure of inflammation, largely through an increase in plasma viscosity, due to the production of acute phase response proteins.⁵⁴ However, ESR rises slowly compared with CRP during inflammation and declines even more slowly after the improvement of this state.⁵⁵ It is also affected by the hematocrit, both in anemia and polycythemia, as well as in other diseases and physiologic states such as aging and pregnancy, reducing its accuracy and specificity in IBD.⁵⁴

Calprotectin is one of the most important biomarkers in IBD, currently used to evaluate diagnosis and monitor response to treatment.⁵⁶ It is a zinc and calcium-binding protein, primarily existing in neutrophil granules, presenting an important antimicrobial function. When compared to the blood levels of healthy people, the concentration in feces is six times higher.⁵⁷ Patients with active IBD have increased levels of fecal calprotectin (FC) and this is due to neutrophil margination in the bowel mucosa.⁵⁸ FC lacks specificity and elevated levels have been found in a range of other conditions such as celiac disease, colon cancer, intestinal infections, and with the use of NSAIDs.⁵⁹ However, elevations of FC levels in these situations are typically much lower than those found in active IBD, so they are still helpful, particularly when considering their absolute levels.⁶⁰ FC showed to be an important tool for differentiating between IBD and IBS⁵⁰, as

well as a biomarker of endoscopic activity in IBD (mainly UC)⁶¹, a predictor of clinical and endoscopic relapse⁶²⁻⁶⁴, and also a predictor of response to treatment⁶⁵⁻⁶⁸.

Fecal lactoferrin (FL) is a fecal biomarker with similar results compared with FC. It is an iron-binding glycoprotein that is primarily released by neutrophils after margination in the bowel mucosa. Data do not differ from the results already observed for FC, as a consequence, FL does not provide any additional clinical utility when considered independently.⁶⁹

In the future, advanced biomarkers such as microRNAs, metabolomics, and genetic testing could enable precise screening of susceptible individuals before disease onset, as well as the diagnosis of symptomatic patients.⁷⁰

Biomarkers testing significantly assist in the management of IBD patients and gained particular importance as less invasive and cost-effective modalities for a curative approach in IBD.^{44,71}

3. DIPEPTIDYL PEPTIDASE-4

Dipeptidyl peptidase (DPP)-4, also known as cluster of differentiation 26 (CD26), is a 110-kDa glycoprotein and was first reported by Hopsu-Havu and Glenner in 1966.⁷² It is a multifunctional protein, with several biological actions: (a) acts as a surface antigen to interact with other molecules, to mediate the interaction between cells-cells and cells-matrix; (b) acts as a cell surface coreceptor mediating viral entry (e.g., severe acute respiratory syndrome coronavirus-2 [SARS-CoV-2]); (c) plays several regulatory roles in immune activation/inflammatory response, and tumorigenesis; (d) regulates the activities of hormones, cytokines, growth factors, and neuropeptides.⁷³

DPP-4 is a member of the serine peptidase/prolyl oligopeptidase gene family, subclassified partly by structure and function, which includes: (a) the membrane-bound peptidases DPP-4 and fibroblast activation protein α (FAP- α); (b) the resident cytoplasmic enzymes DPP-8 and DPP-9; (c) the nonenzymatic members DPP-6 and DPP-10.⁷⁴

Human DPP-4 is composed of 766 amino acids (a.a.), including a short cytoplasmic N-terminal tail (a.a. 1-6), a hydrophobic transmembrane segment (a.a. 7-28), a flexible

stalk (a.a. 29-48), and a large extracellular part (a.a. 49-766) that can be further divided by a heavily glycosylated region (a.a. 49-289), a cysteine-rich region (a.a. 290-510), and a catalytic region (a.a. 511-766), which selectively removes the N-terminal dipeptide from peptides with proline or alanine at the penultimate position (**Figure 5**).^{75,76}

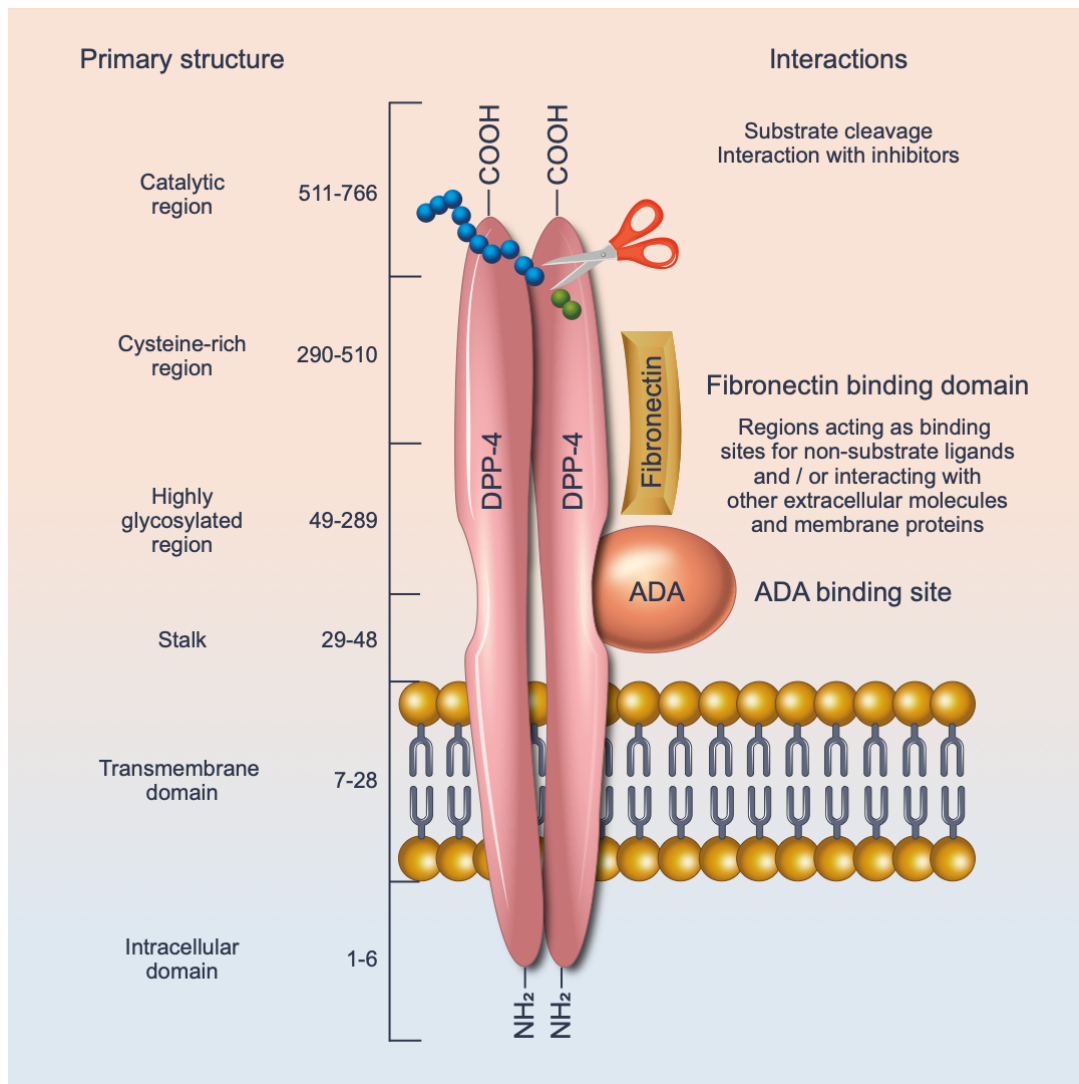


Figure 5. Schematic representation of the DPP-4 dimer on the cell surface. The vertical bar at the left represents the primary structure with the delineation of the different regions (not to scale). Sites within the glycosylated and cysteine-rich regions serve as a receptor or ligand for different molecules, including adenosine deaminase (ADA) and fibronectin, in order to mediate its non-enzymatic functions. The enzymatic activity resides in the catalytic pocket, formed by residues located in the C-terminal portion of the protein. Adapted with permission from Springer Nature. Razavi, 2022.⁷⁷ Image created in Adobe Photoshop CS6.

DPP-4 is present on the cell surface in various forms (monomer, dimer, tetramer), and this process is initiated upon interaction at the cysteine-rich region level, with DPP-4 itself or with other binding partners.^{78,79} Apparently, DPP-4 needs dimerization for enzymatic activity, however, Chien et al. demonstrated that the monomers of DPP-4 are not completely devoid of activity, as previously speculated, having much lower activities than the dimeric DPP-4.⁷⁸ Although dimerization is not entirely required to complete the active site architecture of DPP-4, it may affect the efficiency of cleavage of substrates by the catalytic active site or allow intercellular communication.⁷⁶

DPP-4 is ubiquitously expressed on the surface of many cell types, among which are epithelial cells, fibroblasts, and lymphocytes. The expression level of this protein varies considerably across tissues and has a widespread organ distribution. It can be found in the intestinal and renal brush border membranes, glandular epithelial cells, liver and pancreas, vascular endothelium, and cells of the immune system.⁸⁰

Particularly abundant on the brush-border membranes of the small intestine, DPP-4 expression increases distally, being high in the jejunum and ileum, and low in the duodenum.^{79,81} On the contrary, DPP-4 is not expressed on normal colon epithelium, although aberrantly present in colon tumors.⁸¹ The surface-expressed DPP-4/CD26 is also considered a general marker of cellular activation in the immune system and its expression levels increase on T, NK, and B cells, after stimulation.⁸²

The precise mechanisms controlling DPP-4 gene transcription and its enzymatic activity remain unclear and may be potentially cell type-dependent. So far, it is well known that interferons (α , β , and γ) and IL-12 are capable of upregulating the DPP-4 expression, supporting its role in immune cells activation.^{83,84}

Additionally, DPP-4 can be found in a soluble form, which is devoid of the transmembrane region and the intracellular segment, retaining its enzymatic properties due to the intact catalytic center. Accordingly, serum DPP-4 (sDPP-4) is responsible for 95% of all dipeptidyl peptidase activity in human serum.⁸⁵ It is believed to result from specific proteolytic cleavage of the membrane-anchored form, through a process called shedding, by a group of enzymes collectively referred to as “sheddases”. Several studies reported that matrix metalloproteinases (MMPs) are involved in this process: MMP1, MMP2, and MMP14 have been implicated in the release of DPP-4 from smooth muscle cells, MMP14 from adipocytes, and kallikrein 5 from Th17 cells.^{86,87}

The shedding process occurs due to a complex interplay between different proteases (in a cell type-specific manner), and an anomalous shedding could be a potential reason for the altered sDPP-4 concentrations, in some diseases.^{86,87} Since DPP-4 is shedded with intact enzymatic function, it can exert biological functions in a paracrine or endocrine fashion and participate in intracellular signaling events in targeted cells. Nonetheless, the precise mechanisms by which sDPP-4 induces signal transduction remain obscure and no specific receptors have been identified so far.⁸⁶

Besides shedding, DPP-4 might also be processed within intracellular storage granules to allow for its rapid release, as Lettau et al. demonstrated in a previous study. The authors showed that DPP-4 can be stored in secretory granules of human cytotoxic lymphocyte populations, and upon stimulation, vesicular DPP-4 is rapidly translocated to the cell surface, leading to a substantial discharge of proteolytically active DPP-4. Consequently, the observed changes in serum DPP-4 concentrations in several diseases might be explained by the fact that activated effector lymphocytes are a significant source of this peptidase.⁸⁸

DPP-4 transmits signals across cell membranes through interactions with adenosine deaminase (ADA), collagen, fibronectin, caveolin-1, T-cell antigen CD45, and components of the extracellular matrix (ECM) (**Figure 5**).⁸⁹⁻⁹¹ A study by Tanaka et al. assessed whether the enzymatic activity of DPP-4 is necessary or not for its co-stimulatory action, suggesting that while enzymatic activity might contribute to signal transduction, it is not essential for this process.⁹²

Many bioactive or inactive precursor peptides have been identified as potential substrates of DPP-4 action, such as: peptide hormones (glucagon-like peptide [GLP] - 1/2), neuropeptides (substance P, neuropeptide Y), chemokines, and growth factors. Nevertheless, it is important to highlight that, even if they are good substrates *in vitro*, additional clearance pathways may be more significant in humans or simply have no effect on their biological activity.^{93,94}

The relative importance of an enzymatic *versus* non-enzymatic role of DPP-4 for normal physiological regulation, as well as for the pathogenesis of various diseases, is still largely unknown. Due to its implication in immunoregulation and fibrotic response, increasing studies are focusing on the potential role of DPP-4 in inflammatory disorders, such as IBD.

3.1. DPP-4 and inflammatory bowel disease

The expression and activity of DPP-4 have been the focus of research as a possible biomarker for many diseases, including IBD. It is extensively glycosylated, which protects it from proteolytic attack and contributes to its increased stability, making it an interesting biomarker in IBD patients.

Xiao et al. reported decreased sDPP-4 enzymatic activity (by 3.5-fold) in IBD patients compared to healthy individuals.⁹⁵ Hildebrandt et al. and Varljen et al. found that sDPP-4 activity correlates negatively with disease activity scores (Crohn's Disease Activity Index [CDAI], Rachmilewitz index, and Truelove and Witts' classification) and with inflammatory markers (orosomucoid and CRP), in IBD patients.^{96,97} Additionally, these patients presented a higher surface expression of DPP-4/CD26 as well as a proliferation of CD26-positive T cells co-expressing CD25 (IL-2 receptor α chain), potentially promoting a systemic T cell activation toward a Th1 immune response.⁹⁶ Moran et al. also reported lower sDPP-4 levels in CD patients (1.5 times lower) than in healthy controls, as well as a 2.7-fold reduction in DPP-4 protein expression in intestinal tissue from CD patients, when compared to healthy tissue controls (assessed by western blotting). The sDPP-4 levels described had a significant inverse correlation to disease activity as measured by the CRP, but not the CDAI.⁹⁸

The pathophysiological mechanisms underlying the association between decreased sDPP-4 levels and active inflammation in IBD remain largely unknown.

3.2. DPP-4 as a regulator of extracellular matrix and fibrosis

All tissues contain a highly dynamic ECM, consisting of fibrillar macromolecules (collagens, elastin, fibronectin), and non-fibrillar components (glycoproteins, proteoglycans, hyaluronan), forming a complex network, that undergoes controlled remodeling.⁹⁹ In addition to serving as a framework for cells, within tissues, the ECM also participates in several molecular pathways and activities, including cellular proliferation, migration, and adhesion.¹⁰⁰

ECM remodeling is a continuous process involving deposition, degradation, and modification of its structure, generated by several enzymes such as MMPs, neutrophil elastases, and meprins.¹⁰¹ In normal circumstances, the ECM continuously remodels itself, and throughout this process, the synthesis of new matrix elements coexists with matrix degradation, being determinant in the preservation of tissue homeostasis. Consequently, aberrant ECM remodeling can accelerate disease progression by modulating cell-ECM interactions and intracellular signaling pathways.¹⁰²

The increased remodeling of the ECM is one of the defining characteristics of IBD, as the structure and function of the intestinal tissue matrix undergo progressive modifications, as a result of an excessive and protracted inflammatory response.¹⁰⁰ The continuous cycle of fibroblast activation and ECM deposition ultimately leads to tissue fibrosis and functional damage.¹⁰³

DPP-4/CD26 is expressed in fibroblasts and increases the production of matrix components and profibrotic mediators, by enhancing intracellular transforming growth factor-beta (TGF- β) signaling. It has been shown to bind to collagen and fibronectin, facilitating cell migration, and contributing to the formation of the fibrotic microenvironment. DPP-4/CD26 also forms a complex with Seprase/fibroblast activation protein- α (FAP- α), assisting in the degradation of matrix proteins, which further facilitates cell migration (**Figure 6**).^{104,105}

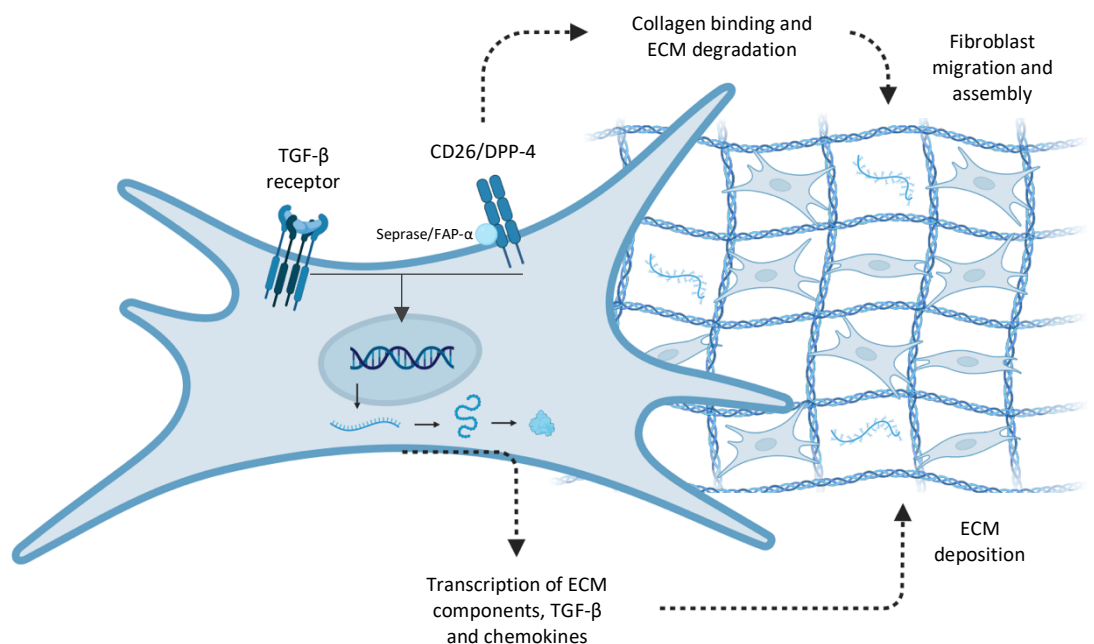


Figure 6. Impact of DPP-4/CD26 on fibroblasts. Adapted with permission from John Wiley & Sons, Inc. Ohm, 2023.¹⁰⁶ Image created in BioRender.

As a consequence, in addition to its enzymatic and costimulatory functions, DPP-4/CD26 may play a role in the pathogenesis of several chronic fibrotic diseases.

Growing evidence suggests that DPP-4/CD26 inhibition may be able to prevent excessive matrix deposition while promoting physiological tissue repair. Therefore, it could be a potential target for antifibrotic therapy.¹⁰⁶

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PURPOSE

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PURPOSE

The general aim of this investigation presented hereby in this thesis was to contribute to the comprehension of IBD pathogenesis and identify potential biomarkers of disease activity and prognosis.

For this purpose, three main objectives were defined, as follows:

1 – Evaluation of the role of serum DPP-4 (sDPP-4) as a predictor of disease activity and prognosis in IBD (Paper I)

- Clarify the role of sDPP-4 as a biomarker of IBD activity.
- Address its potential in predicting the need for treatment escalation and monitoring response to biological therapy.

2 – Address the potential use of fecal DPP-4 (fDPP-4) as an emergent biomarker and analyze the DPP-4 expression levels in intestinal samples of IBD patients (Paper II)

- Evaluate the potential use of fDPP-4 as a biomarker of IBD activity.
- Assess the mechanisms underlying the variations of DPP-4 immunohistochemical expression profiles, in intestinal tissue samples of IBD patients.

3 – Systematic review on the importance of sDPP-4 and other ectopeptidases in IBD (Paper III)

- Evaluate the evidence available in this field through a systematic review about sDPP-4 and other ectopeptidases.
- Understand their specific role in IBD pathogenesis.
- Provide a link between preclinical and clinical data on their role in IBD, through the use of validated animal models of intestinal inflammation and studies on IBD patients.
- Discuss the potential role of DPP-4 as a therapeutic target.

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MATERIAL AND METHODS

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MATERIAL AND METHODS

The approaches followed for each study and detailed methodologies are indicated in the articles belonging to the thesis.

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RESULTS & PUBLICATIONS

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RESULTS & PUBLICATIONS

Paper I

“Serum Dipeptidyl Peptidase 4: A Predictor of Disease Activity and Prognosis in Inflammatory Bowel Disease”

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ORIGINAL RESEARCH ARTICLE—CLINICAL

Serum Dipeptidyl Peptidase 4: A Predictor of Disease Activity and Prognosis in Inflammatory Bowel Disease

Pedro Pinto-Lopes, MD, MSc,^{*,†,‡} Joana Afonso, MSc,^{*,§} Rui Pinto-Lopes, MD, MSc,[¶] Cátia Rocha, MSc,^{*,‡} Paula Lago, MD,^{**,††} Raquel Gonçalves, MD,^{††} Helena Tavares de Sousa, MD,^{††,§§} Guilherme Macedo, MD, PhD,^{¶¶} Cláudia Camila Dias, PhD,^{‡‡,***} and Fernando Magro, MD, PhD,^{*,¶¶}; for the Portuguese IBD Study Group (GEDII)

Background: Serum dipeptidyl peptidase 4 (DPP-4) has drawn particular interest as a biomarker in inflammatory bowel disease (IBD), as this protease inactivates several peptides that participate in the inflammatory cascade.

Methods: Two prospectively recruited cohorts consisting of 195 patients (101 had Crohn's disease [CD] and 94 had ulcerative colitis [UC]) were evaluated using clinical indexes and followed up to assess for treatment escalation. Sixty-eight patients underwent endoscopic evaluation at baseline. In the second cohort of 46 biologically treated patients, treatment response was assessed. Serum DPP-4, C-reactive protein (CRP), and fecal calprotectin levels were quantified at baseline and during follow-up.

Results: Median DPP-4 levels were significantly lower in active IBD patients when compared with remitters (CD: 1043 [831–1412] vs 1589 [1255–1956] ng/mL; $P < 0.001$; UC: 1317 [1058–1718] vs 1798 [1329–2305] ng/mL; $P = 0.001$) and healthy controls (2175 [1875–3371] ng/mL). In fact, DPP-4 was able to distinguish clinical and endoscopic activity from remission, with areas under the curve (AUC) of 0.81/0.93 (CD) and 0.71/0.79 (UC), along with the need for treatment escalation, with comparable AUCs of 0.79 (CD) and 0.77 (UC). Furthermore, DPP-4 levels were higher in responders to treatment and more pronounced among UC (1467 [1301–1641] vs 1211 [1011–1448] ng/mL; $P < 0.001$) than CD patients (1385 [1185–1592] vs 1134 [975–1469] ng/mL; $P = 0.015$).

Conclusions: Our results suggest that serum DPP-4 can be used as a noninvasive biomarker of IBD activity and biological treatment response and a predictor of treatment escalation, particularly when combined with other biomarkers.

Key Words: biomarkers, dipeptidyl peptidase 4, inflammatory bowel disease

INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are part of a group of idiopathic autoimmune inflammatory bowel diseases (IBDs) characterized by disease flares followed by periods of remission. Disease flares usually involve episodes of mucosal inflammation, which ultimately result in

structural and functional alterations in the intestinal epithelium.¹ Thus, the main goals of IBD treatment are to induce and maintain clinical remission. Still, even in cases of effective treatment, subclinical inflammation may persist, contributing to the risk of relapse and the need to escalate treatment to immunomodulators, biological therapies, or even surgery.²

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From the *Department of Internal Medicine, Faculty of Medicine, Centro Hospitalar São João, Porto, Portugal; †Department of Internal Medicine, Centro Hospitalar Tâmega e Sousa – Hospital Padre Américo, Penafiel, Portugal; ‡Unit of Pharmacology and Therapeutics, Department of Biomedicine, Faculty of Medicine, University of Porto, Porto, Portugal; §MedInUP, Centre for Drug Discovery and Innovative Medicines, University of Porto, Porto, Portugal; ¶Department of Gastroenterology, Mid Essex Hospital Services NHS Trust, Broomfield, Essex, United Kingdom; ††Instituto de Saúde Ambiental, Faculty of Medicine, University of Lisbon, Lisbon, Portugal; **Department of Gastroenterology, Centro Hospitalar do Porto, Porto, Portugal; ††Department of Gastroenterology, Hospital de Braga, Braga, Portugal; ‡‡Department of Gastroenterology, Centro Hospitalar e Universitário do Algarve – Portimão Unit, Portimão, Portugal; §Department of Medicine and Medical Biosciences, University of Algarve, Faro, Portugal; ¶Department of Gastroenterology, Faculty of Medicine, Centro Hospitalar São João, Porto, Portugal; ¶¶Department of Community Medicine, Information and Health Decision Sciences, Faculty of Medicine, University of Porto, Porto, Portugal; ***CINTESIS – Centre for Health Technology and Services Research, Porto, Portugal

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Address correspondence to: Fernando Magro, MD, PhD, Unit of Pharmacology and Therapeutics, Department of Biomedicine, Faculty of Medicine, University of Porto, Alameda Prof. Hernâni Monteiro 420-319 Porto, Portugal (fm@med.up.pt).

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Accurate detection of inflammation in the intestinal mucosa is challenging, which is reflected by the fact that most clinical scores correlate poorly with disease activity.³ The Harvey-Bradshaw Index (HBI) for CD and the partial Mayo Score (pMS) for UC are commonly used indexes; however, they tend to reflect patients' well-being rather than the degree of intestinal inflammation.⁴ Examination of biopsy samples collected during endoscopic procedures remains the gold standard for assessing intestinal inflammation, but it is rather invasive and might lead to misleading results if the endoscope fails to reach the lesion site.⁵ In this context, biomarkers have emerged as important indicators of disease activity, risk of relapse, need for treatment escalation, and/or response to therapy. Among the panel of biomarkers available, those measurable in serum samples have the greatest potential to provide a timely and convenient assessment of inflammation in routine clinical practice settings.⁶

Over the past few years, several studies have focused on identifying and characterizing the key molecules involved in the regulation of mucosal immunity and IBD pathogenesis. Dipeptidyl peptidase 4 (DPP-4) has drawn particular interest in this context due to observations of altered expression profiles in patients with IBD.⁷⁻⁹ DPP-4 has a widespread distribution, being expressed in numerous tissues, such as intestine, kidney, liver, lung, prostate, adipocytes, thymus, spleen, lymph nodes, and on activated lymphocytes (B and T cells), monocytes, and endothelial cells.¹⁰ This enzyme is active as a cell surface protein (CD26) but also exists in a soluble form, which is thought to result from proteolytic cleavage of the membrane-bound form into the circulation in a process called "shedding," acting as a protease that cleaves and inactivates several peptides like neuro-peptides, chemokines, and growth factors.⁹

This study aims to clarify the role of serum DPP-4 as a biomarker of IBD activity, specifically addressing its potential in predicting the need for treatment escalation and monitoring response to biological therapy.

METHODS

Study Design and Population

This article reports on 2 cohorts and was designed to meet the Standards for Reporting Diagnostic Accuracy requirements (Supplementary Table 1). The first was a multicentric, prospective cohort involving 4 tertiary care centers (Centro Hospitalar São João, Porto; Centro Hospitalar do Porto, Porto; Hospital de Braga, Braga; Centro Hospitalar Universitário do Algarve, Faro, Portugal) that included active and remitting IBD patients attending routine outpatient consultations or who were admitted to the hospital due to an IBD flare. Between October 2014 and December 2017, 195 consecutive patients were invited to participate in this study; follow-up was carried out until May 2018. All patients with IBD were eligible to participate.

Patients were excluded from the study if they were younger than 18 years, had an unclear IBD diagnosis (indeterminate colitis), or had other autoimmune diseases.

The second cohort was a subgroup of 46 biologically treated IBD patients of the above-described cohort attending outpatient consultations between July 2016 and December 2017. Follow-up was carried out until May 2018. Patients were enrolled in the induction and maintenance phases of biological treatment (namely infliximab, adalimumab, or vedolizumab) and evaluated at several time points (5 per patient). These assessments were scheduled according to routine practice, taking into account the condition being studied (CD or UC) and the treatment received. Patients were excluded if they did not comply with these routine clinical assessments. A control group of 52 healthy volunteers was enrolled as part of this study. Figure 1 provides an illustration of the study design.

Ethical Considerations

The local research ethics committees for each study center granted approval for this study. All patients provided their written informed consent before enrollment.

Assessments

For both cohorts, demographic and clinical data were collected at baseline. Disease location and clinical phenotypes were evaluated with the Montreal classification¹¹; disease activity was assessed using the HBI¹² (clinical remission ≤ 4) and the pMS¹³ (clinical remission ≤ 1) for CD and UC patients, respectively. For the second cohort, physicians reported clinical outcomes at several data collection time points, according to HBI and pMS. The investigators were blinded to fecal calprotectin (FC) and C-reactive protein (CRP) levels during assessment.

Treatment Escalation

Treatment escalation was evaluated in the first cohort. A therapeutic adjustment was considered treatment escalation in the following situations: (a) introduction of a new drug due to flare, including a new immunomodulator or biological; (b) switching between immunomodulators or biologicals; (c) increasing dosage or shortening the infusion interval in biologically treated patients; and (d) need to undergo bowel-related surgery.¹⁴ Therapy changes due to adverse reactions or interruptions due to the occurrence of infections were not considered treatment escalation.

Treatment Response

Response to biological treatment was evaluated in the second cohort, after induction and during the maintenance phase of infliximab, adalimumab, and vedolizumab, both for CD and UC patients. Response was defined as a decrease in

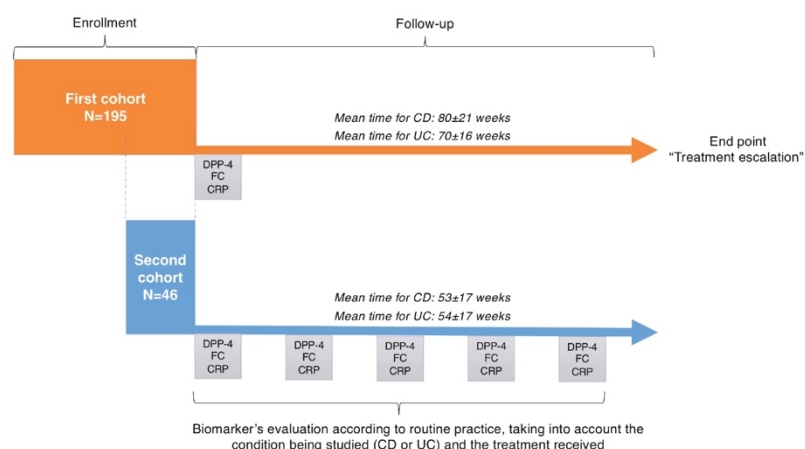


FIGURE 1. Study selection and follow-up scheme. The first cohort included 195 IBD patients between October 2014 and December 2017, and follow-up was carried out until May 2018 to evaluate treatment escalation. Blood and stool samples for DPP-4, CRP, and FC were collected at baseline (1 determination only). The second cohort, a subgroup of the first, which was composed of biologically treated IBD patients in the induction and maintenance phases, included 46 patients between July 2016 and December 2017, followed until May 2018. Stool and blood samples were collected at baseline and at evaluations (5 determinations per patient) at several time points to evaluate treatment response.

HBI of ≥ 2 points from baseline in CD patients and as a decrease in pMS ≥ 3 points and $\geq 30\%$ from baseline and a decrease in the rectal bleeding subscore ≥ 1 or an absolute rectal bleeding subscore of 0 or 1 in UC patients.^{15, 16}

Nonresponse to biological treatment was defined as failure to achieve those objectives (ie, primary nonresponse) or loss of response, with relapse of clinical symptoms while on treatment after an initial favorable response to induction and maintenance therapy (ie, secondary loss of response), requiring drug optimization including switch to another therapy or the addition of corticosteroids or immunomodulators.^{17, 18}

Biomarkers

Stool and blood samples were collected to quantify FC, serum DPP-4, and CRP. For the first cohort, they were collected at baseline; for the second cohort, 5 samples were collected per patient at the time of infusion during evaluation at several time points. Collected stool samples were kept at 4°C (for a maximum of 48 hours) until shipment to the central laboratory (Department of Pharmacology and Therapeutics, Faculty of Medicine of University of Porto, Porto, Portugal). Fecal calprotectin was extracted from stools within 7 days after collection using the Fecal Sample Preparation Kit (Roche Diagnostics, Mannheim, Germany) following the manufacturers' instructions and then stored at -80°C until quantification. Fecal calprotectin concentrations were determined using the automated fluorimmunoassay-EliA test (Phadia, ThermoFisher, Sweden). Blood samples were taken by venipuncture after a minimum fasting of 6 hours. Blood samples were processed within an hour of sampling (using

centrifugation at 2500g for 15 minutes at 4°C) and stored at -80°C until further analysis. Serum DPP-4 levels were measured using a quantitative enzyme-linked immunosorbent assay (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturers' instructions. Serum CRP levels were determined by standard techniques. Laboratory investigators were blinded to any clinical information about the patients.

Endoscopic Assessment

Endoscopic procedures (ileocolonoscopy, full colonoscopy, or flexible sigmoidoscopy) were performed for some of the patients of the first cohort at baseline in the setting of routine clinical practice, with the proceduralist blinded from the biomarker's results. The endoscopic reports (including photographs) were reviewed by an IBD physician (F.M.) to grade endoscopic activity.

In CD patients, endoscopic evaluation was based on the presence or absence of ulceration, and endoscopic remission was defined as the resolution of ulceration at colonoscopy.¹⁹ Rutgeerts score was used as the standard evaluation of postsurgical recurrences at the ileocolic anastomosis level in CD patients. Scores of i0 or i1 were regarded as postsurgery remission, and scores of i2, i3, or i4 were considered postsurgery recurrence.²⁰ In UC patients, endoscopic activity was assessed according to the Mayo endoscopic score, which reflects the severity of intestinal inflammation on colonoscopy as follows: 0 points, normal or inactive disease; 1 point, mild disease (erythema, decreased vascular pattern, and mild friability); 2 points, moderate disease (marked erythema, absent vascular pattern, friability, and erosions); and 3 points, severe disease

(spontaneous bleeding and ulcerations). Endoscopic remission was defined as a Mayo endoscopic score of 0.¹⁹

Statistical Analyses

Categorical variables are described as absolute and relative frequencies and were analyzed using the Fisher exact test. Continuous variables are presented as mean and standard deviation (for parametric data) or median and interquartile range (in nonparametric distributions). Missing data (a few FC and CRP determinations) were handled by exclusion.

Hypothesis testing was performed using unpaired *t* tests or Mann-Whitney tests, as appropriate, taking into account normality assumptions and the number of groups compared. Correlation between variables was assessed using the Spearman rank-order correlation test. Receiver operating characteristics (ROC) curves were generated by plotting sensitivity against specificity, and areas under the curve (AUC) were calculated to determine the optimal cutoff points for the biomarkers of interest. Kaplan-Meier curves of time to treatment escalation were generated for all patients, and the log-rank test was used for comparisons between groups. Patients who had no need for treatment escalation during the study were censored at the end of follow-up.

Statistical Package for Social Sciences, version 25.0 (IBM SPSS Statistics for MacOS), and GraphPad Prism, version 7.00 for Mac OS X (GraphPad Software, La Jolla, CA, USA), were used for the statistical analysis and plot design. In all cases, significance was set at $P < 0.05$.

RESULTS

Cohorts' Characterization and Disease Outcomes

The first study cohort consisted of 195 patients, of whom 101 had CD and 94 had UC (Table 1). Of these, 56.4% and 63.8% were in clinical remission, respectively. Age, sex, disease duration, age at diagnosis, and the need for a previous surgical resection were similarly distributed between patients in clinical remission and those with clinical activity, except for the UC patients in clinical remission, who had significantly longer disease duration and were mainly female. Interestingly, the Montreal classification was similarly distributed between CD patients in remission and those with clinical activity, but not between UC patients. In the UC subgroup, the E3 category was more frequent in clinically active patients, whereas the E1 and E2 categories were more frequent among remitters. Medication was not significantly different between CD or UC patients. As expected, the mean clinical activity score was higher in patients with active disease when compared with those in clinical remission (HBI in CD patients: 7.6 ± 3.2 vs 1.4 ± 1.3 ; $P < 0.001$; pMS in UC patients: 4.7 ± 2.3 vs 0.1 ± 0.3 ; $P < 0.001$). Similarly, therapeutic escalation during follow-up was more frequent in patients with clinical activity at baseline (CD patients: 72.7% vs 17.5%; $P < 0.001$; UC patients: 47.1% vs 6.7%; $P < 0.001$).

The mean follow-up time (range) was 80 ± 21 (25–102) weeks among CD patients and 70 ± 16 (29–92) weeks among UC patients. The 52 healthy controls recruited in the scope of this study had an average (SD) age of 46.4 (18.6) years, the majority of them were female (57.7%), and a single healthy control (1.9%) had undergone a previous surgical resection.

As mentioned before, the second cohort consisted of a subgroup of the first cohort composed of patients enrolled in the induction and maintenance phases of biological treatment, in order to prospectively conduct a clinical and analytical (DPP-4, FC, CRP) evaluation, including 5 assessments per patient. This cohort consisted of 46 patients, 24 with CD and 22 with UC; of these, 75.0% and 77.3% were in clinical remission at baseline, respectively. Age, sex, disease duration, age at diagnosis, previous surgical resection, Montreal classification, Montreal behavior, and medication at baseline were similarly distributed between patients in clinical remission and those with clinical activity (Supplementary Table 2). Crohn's disease patients in clinical remission were significantly younger at diagnosis (25.1 ± 9.6 vs 38.5 ± 18.1 ; $P = 0.027$). The mean clinical activity score was higher in active disease patients (HBI in CD patients: 5.8 ± 1.0 vs 1.6 ± 1.3 ; $P < 0.001$; pMS in UC patients: 3.2 ± 1.6 vs 0.3 ± 0.5 ; $P = 0.016$), as was the occurrence of the need to escalate treatment (CD patients: 42.9% vs 18.9%; $P = 0.022$; UC patients: 48.1% vs 15.2%; $P = 0.001$).

Serum DPP-4 as a Marker of Disease Activity

Levels of blood and fecal biomarkers in the first cohort are presented in Table 2. In both diseases, patients with clinical activity had significantly higher serum CRP and FC levels and lower serum DPP-4 when compared with those in clinical remission. Healthy controls had a median (interquartile range [IQR]) FC concentration of 30 (16–43) $\mu\text{g/g}$, which was lower than that of CD and UC patients in clinical remission ($P = 0.002$ and $P = 0.014$, respectively) and that of clinical activity ($P < 0.001$ and $P < 0.001$, respectively). Moreover, these control individuals had a DPP-4 median concentration of 2175 (1875–3371) ng/mL, which was higher than that of CD and UC patients in clinical remission ($P < 0.001$ and $P = 0.006$, respectively) and that of those with clinical activity ($P < 0.001$ and $P < 0.001$, respectively). For both CD and UC patients, serum DPP-4 levels were negatively correlated with FC, serum CRP, and the activity index, whereas FC was positively correlated with serum CRP and the activity index (Supplementary Table 3).

ROC curves computed using the data from the first cohort showed that serum DPP-4 is able to differentiate between patients with clinical activity and those in clinical remission, with AUCs of 0.81 (95% confidence interval [CI], 0.72–0.90; $P < 0.001$) for CD and 0.71 (95% CI, 0.60–0.82; $P = 0.001$) for UC (Fig. 2). These AUCs were identical to or lower than those calculated for FC (CD: 0.84; 95% CI, 0.75–0.92; $P < 0.001$; UC: 0.91; 95% CI, 0.86–0.97; $P < 0.001$) and CRP (CD: 0.90; 95% CI, 0.83–0.97; $P < 0.001$; UC: 0.84; 95% CI, 0.75–0.93;

TABLE 1. Demographic and Clinical Characteristics of the Study Population in the First Cohort (n = 195)

	Crohn's Disease			Ulcerative Colitis		
	Clinical Remission (n = 57)	Clinical Activity (n = 44)	P	Clinical Remission (n = 60)	Clinical Activity (n = 34)	P
Age, mean $\pm$ SD, y	38.5 $\pm$ 12.2	40.3 $\pm$ 13.5	0.485	44.1 $\pm$ 15.2	44.4 $\pm$ 15.5	0.940
Female, No. (%)	30 (52.6)	24 (54.5)	1.000	36 (60.0)	12 (35.3)	0.031
Disease duration, mean $\pm$ SD, y	10.0 $\pm$ 8.2	8.0 $\pm$ 9.9	0.273	9.3 $\pm$ 6.0	6.4 $\pm$ 6.6	0.033
Age at diagnosis, mean $\pm$ SD, y	27.9 $\pm$ 10.2	32.0 $\pm$ 13.3	0.097	34.4 $\pm$ 14.6	37.5 $\pm$ 15.7	0.338
Previous surgical resection, No. (%)	20 (35.7)	13 (29.5)	0.530	2 (3.3)	1 (2.9)	1.000
Activity score (HBI), mean $\pm$ SD	1.4 $\pm$ 1.3	7.6 $\pm$ 3.2	<0.001	0.1 $\pm$ 0.3	4.7 $\pm$ 2.3	<0.001
Montreal location ^a						
L1 $\pm$ L4, No. (%)	31 (54.4)	22 (50.0)	0.877	5 (8.3)	0	0.019
L2 $\pm$ L4, No. (%)	8 (14.0)	6 (13.6)		31 (51.7)	11 (32.4)	
L3 $\pm$ L4, No. (%)	18 (31.6)	16 (36.4)		24 (40.0)	23 (67.6)	
Montreal behavior ^c						
B1, No. (%)	28 (49.1)	13 (29.5)	0.139			
B2, No. (%)	13 (22.8)	14 (31.8)				
B3, No. (%)	16 (28.1)	17 (38.6)				
P, No. (%)	22 (38.6)	19 (43.2)	0.686			
Medications at baseline						
5-ASA, No. (%)	14 (24.6)	4 (9.1)	0.065	41 (68.3)	18 (52.9)	0.183
Azathioprine, No. (%)	32 (56.1)	18 (40.9)	0.161	27 (45.0)	10 (29.4)	0.188
Methotrexate, No. (%)	6 (10.5)	4 (9.1)	1.000	1 (1.7)	0	1.000
Biological treatment, No. (%)	37 (64.9)	25 (56.8)	0.419	31 (51.7)	14 (41.2)	0.393
Therapeutic escalation, No. (%)	10 (17.5)	32 (72.7)	<0.001	4 (6.7)	16 (47.1)	<0.001

^aL1: ileal; L2: colonic; L3: ileocolonic; L4: upper disease.^bE1: proctitis; E2: left-sided colitis; E3: pancolitis.^cB1: nonstricturing, nonpenetrating; B2: stricturing; B3: penetrating; P: perianal disease.

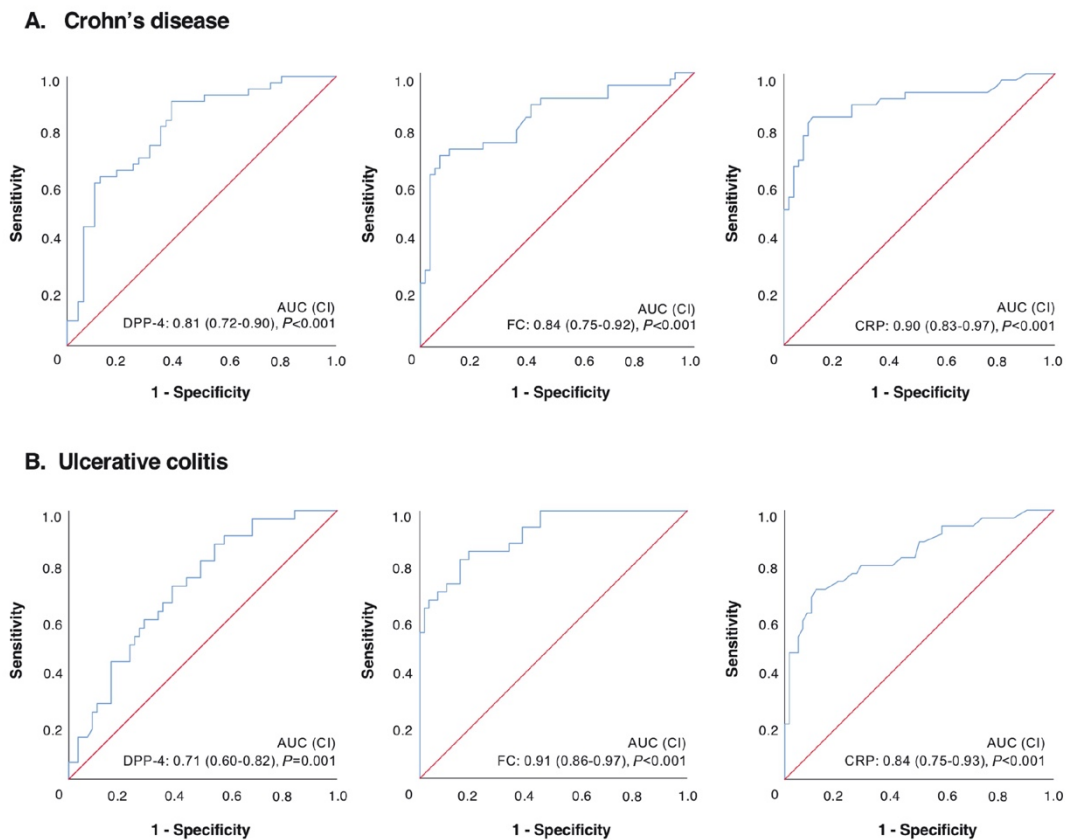


FIGURE 2. Receiver operating characteristics curve analysis of serum DPP-4, FC, and serum CRP in predicting clinical activity in CD patients (A) and UC patients (B) (first cohort, $n = 195$).

$P < 0.001$). Serum DPP-4 performs equally well in postoperative CD patients, as evidenced by an identical AUC of 0.81 (95% CI, 0.65–0.97; $P = 0.005$).

Correlation of Serum DPP-4 With Endoscopic Activity

During the recruitment period, 34 CD patients underwent ileocolonoscopy or full colonoscopy; of these, 11 (32%) had endoscopic remission and 23 (68%) had endoscopic activity. Regarding UC patients, 34 underwent colonoscopy or flexible sigmoidoscopy during the recruitment period. According to Mayo endoscopic score, 14 patients (41%) had endoscopic remission and 20 (59%) had endoscopic activity. In both diseases, patients in the endoscopic activity group had significantly higher serum CRP and FC levels and lower serum DPP-4 when compared with those in the endoscopic remission group (Table 2). Biomarker levels and clinical activity scores

(HBI and pMS) strongly correlated with the endoscopic activity group (Supplementary Table 3).

ROC curves confirmed that serum DPP-4 could help clinicians discriminate between patients with endoscopic activity and those in remission, with high AUCs of 0.93 (95% CI, 0.84–1.00; $P < 0.001$) for CD and 0.79 (95% CI, 0.59–0.98; $P = 0.010$) for UC. These were generally higher than those calculated for the clinical activity and remission groups.

The definition of optimal cutoff points (highest sum of sensitivity and specificity) based on ROC curves (Fig. 3) showed that CD patients, with serum DPP-4 levels >1534 ng/mL, FC levels ≤ 146 μ g/g, and serum CRP levels ≤ 4.3 mg/L, were in endoscopic remission in 35.9%, 39.0%, and 47.0% of the cases, respectively. The proportion of UC patients in endoscopic remission was 39.3% when serum DPP-4 levels were >1786 ng/mL, 35.5% when FC levels were ≤ 106 μ g/g, and 53.2% when serum CRP levels were ≤ 3.5 mg/L.

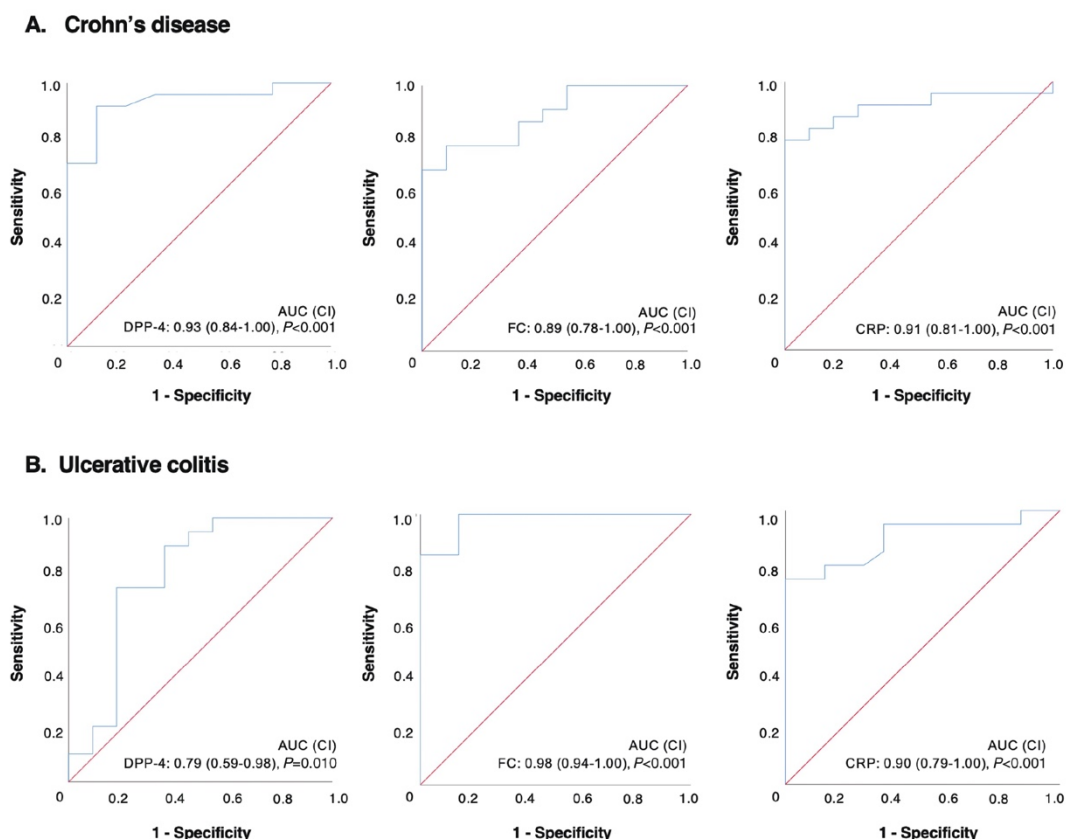


FIGURE 3. Receiver operating characteristics curve analysis of serum DPP-4, FC, and serum CRP in predicting endoscopic activity in CD patients (A) and UC patients (B) (first cohort, $n = 195$).

Table 3 summarizes the sensitivity, specificity, negative/positive predictive values (NPV/PPV), and negative/positive likelihood ratios (NLR/PLR) to evaluate the performance of the biomarkers of interest in the assessment of endoscopic activity in the first cohort. These tests presented high sensitivity (CD patients: DPP-4 [91.3%]; FC [86.4%]; CRP [91.3%]; UC patients: DPP-4 [89.5%]; FC [100.0%]; CRP [80.0%]).

A major proportion of IBD patients with active disease did not present CRP elevation. In this population, it was evidenced that serum DPP-4 had a slightly better performance than FC in the discrimination of endoscopic activity (AUC, 0.82; 95% CI, 0.70–0.94; $P = 0.002$; vs AUC, 0.77; 95% CI, 0.61–0.94; $P = 0.009$) (Supplementary Fig. 1).

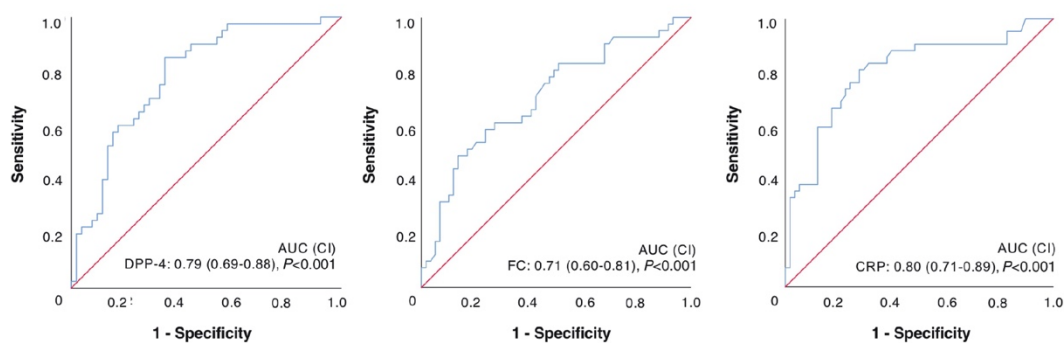
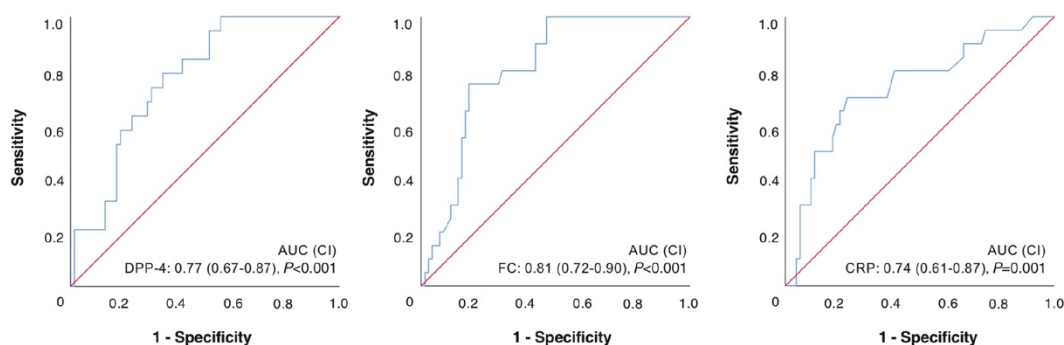
Serum DPP-4 as a Predictor of the Need for Therapeutic Escalation

ROC analysis from the first cohort showed that serum DPP-4 at baseline may help clinicians discriminate between

patients who require treatment escalation and those who do not require it, with AUCs of 0.79 (95% CI, 0.69–0.88; $P < 0.001$) for CD and 0.77 (95% CI, 0.67–0.87; $P < 0.001$) for UC (Fig. 4). These AUCs were comparable to those calculated for FC (CD: 0.71; 95% CI, 0.60–0.81; $P < 0.001$; UC: 0.81; 95% CI, 0.72–0.90; $P < 0.001$) and CRP (CD: 0.80; 95% CI, 0.71–0.89; $P < 0.001$; UC: 0.74; 95% CI, 0.61–0.87; $P = 0.001$). These ROC curves were used to define the optimal cutoff points (highest sum of sensitivity and specificity). The time to treatment escalation in CD patients, stratified according to these biomarker cutoff points, is shown in Figure 5. This analysis showed that CD patients with serum DPP-4 levels ≤ 1452 ng/mL, FC > 840 μ g/g, or serum CRP > 5.0 mg/L had a significantly shorter time to treatment escalation ($P < 0.001$). After 1 year, 62.2% of CD patients with DPP-4 levels below the cutoff (ie, 1452 ng/mL) had escalated treatment, whereas only 7.8% of those with DPP-4 levels above the cutoff had done so. Moreover, the simultaneous presence of 3 positive biomarkers (ie, indicative of worse outcome according to

TABLE 3. Blood and Fecal Biomarker Performance (95% CI) in the Assessment of Endoscopic Activity (First Cohort, n = 195)

	Crohn's Disease					
	Sensitivity, %	Specificity, %	PPV, %	NPV, %	PLR	NLR
DPP-4 ≤ 1534 ng/mL	91.3 (72.0–98.9)	88.9 (51.8–99.7)	95.5 (76.7–99.3)	80.0 (51.1–93.9)	8.2 (1.3–52.4)	0.1 (0.0–0.4)
FC > 146 µg/g	86.4 (65.1–97.1)	63.6 (30.8–89.1)	82.6 (68.1–91.4)	70.0 (42.7–88.0)	2.4 (1.1–5.3)	0.2 (0.1–0.7)
CRP > 4.3 mg/L	91.3 (72.0–98.9)	72.7 (39.0–94.0)	87.5 (72.6–94.9)	80.0 (50.3–94.0)	3.4 (1.3–8.9)	0.1 (0.0–0.5)
	Ulcerative Colitis					
	Sensitivity, %	Specificity, %	PPV, %	NPV, %	PLR	NLR
DPP-4 ≤ 1786 ng/mL	89.5 (66.9–98.7)	63.6 (30.8–89.1)	81.0 (65.7–90.4)	77.8 (46.7–93.3)	2.5 (1.1–5.5)	0.2 (0.0–0.7)
FC > 106 µg/g	100.0 (83.2–100.0)	85.7 (57.2–98.2)	90.9 (73.5–97.3)	100	7.0 (1.9–25.3)	0
CRP > 3.5 mg/L	80.0 (56.3–94.3)	85.7 (57.2–98.2)	88.9 (68.5–96.7)	75.0 (54.9–88.1)	5.6 (1.5–20.6)	0.2 (0.1–0.6)

A. Crohn's disease**B. Ulcerative colitis****FIGURE 4.** Receiver operating characteristics curve analysis of serum DPP-4, FC, and serum CRP in predicting treatment escalation in CD patients (A) and UC patients (B) (first cohort, n = 195).

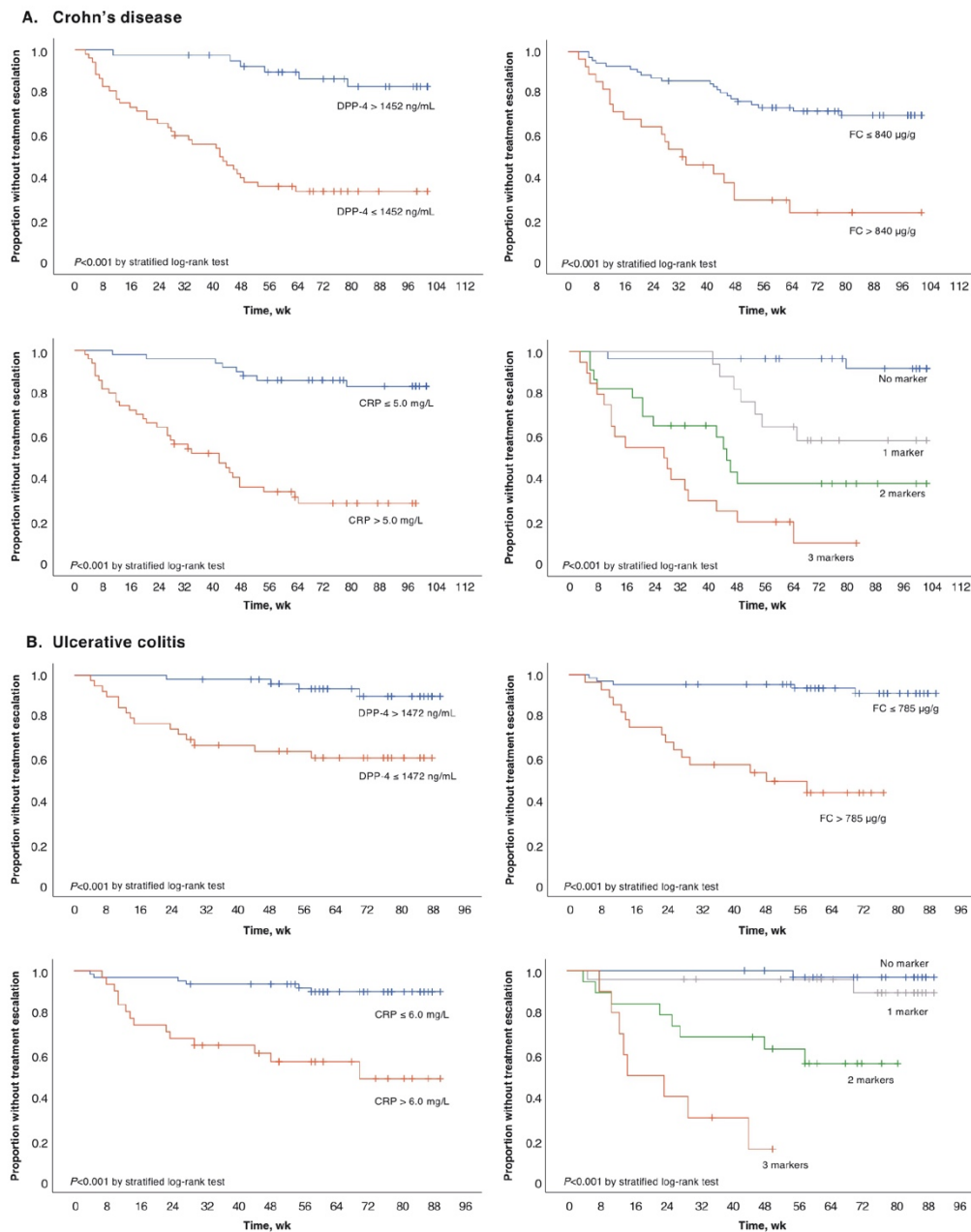


FIGURE 5. Kaplan-Meier cumulative event curves of time to predict treatment escalation in CD patients (A) and UC patients (B) (first cohort, $n = 195$). In CD patients (A), a single marker represents either DPP-4 ≤ 1452 ng/mL or FC > 840 $\mu\text{g/g}$ or CRP > 5.0 mg/L. Two markers represent a combination of 2 of the categorical variables as a combined biomarker. Three markers represent DPP-4 ≤ 1452 ng/mL and FC > 840 $\mu\text{g/g}$ and CRP > 5.0 mg/L as a combined biomarker. In UC patients (B), a single marker represents either DPP-4 ≤ 1472 ng/mL or FC > 785 $\mu\text{g/g}$ or CRP > 6.0 mg/L. Two markers represent a combination of 2 of the categorical variables as a combined biomarker. Three markers represent DPP-4 ≤ 1472 ng/mL and FC > 785 $\mu\text{g/g}$ and CRP > 6.0 mg/L as a combined biomarker.

the cutoffs) was also associated with a significantly shorter time to treatment escalation: 80.0% of patients with 3 positive biomarkers had escalated treatment after 1 year, whereas only 3.3% of patients with 3 negative biomarkers had done so.

Patients with UC showed similar results (Fig. 5), and those with serum DPP-4 levels ≤ 1472 ng/mL, FC > 785 μ g/g, or serum CRP > 6.0 mg/L showed a significantly shorter time to treatment escalation ($P < 0.001$). After 1 year, 36.3% of all patients with DPP-4 levels below the cutoff had escalated treatment vs only 4.1% of all patients with DPP-4 levels above the cutoff. Furthermore, 85.0% of patients with all 3 positive biomarkers had escalated treatment after 1 year, whereas none of the patients with negative biomarker levels had done so. These tests displayed high NPV, which was more pronounced among UC patients (DPP-4 [92.0%]; FC [92.3%]; CRP [90.5%]) than CD patients (DPP-4 [85.0%]; FC [70.8%]; CRP [84.0%]), demonstrating that a large proportion of patients with negative tests did not need to escalate therapy (Table 4).

Serum DPP-4 as a Marker of Response to Biological Treatment

The second cohort, consisting of biologically treated patients in the induction and maintenance phases, was stratified into responders/nonresponders to determine the presence of significant differences in the levels of the biomarkers of interest (Supplementary Table 4). All patients had 5 measurements of biomarkers; whenever the first determination was before the beginning of induction, the assessment was not considered for analysis. Overall, the median serum DPP-4 levels were significantly higher in responders; this difference was more pronounced among UC (1467 ng/mL vs 1211 ng/mL; $P < 0.001$) than CD patients (1385 ng/mL vs 1134 ng/mL; $P = 0.015$). Fecal calprotectin levels were also significantly different between IBD responders and nonresponders, whereas CRP levels were significantly higher

in CD nonresponders (compared with responders) but not in UC nonresponders. ROC analysis of DPP-4, FC, and CRP in predicting response to treatment in IBD patients showed that the 3 biomarkers have a similar ability to distinguish between IBD responders and nonresponders, with AUCs of 0.68 (0.61–0.75; $P < 0.001$) for DPP-4, 0.76 (0.70–0.83; $P < 0.001$) for FC, and 0.67 (0.60–0.74; $P < 0.001$) for CRP (Supplementary Fig. 2).

DISCUSSION

The use of clinical parameters to ascertain remission in IBD patients is challenging, as they tend to be more of a subjective indicator of patient well-being than an objective assessment of intestinal inflammation and response to treatment. Nonetheless, early detection of persistent activity is of the utmost importance in clinical practice, as this often predicts the need to escalate therapy and/or to undergo a surgical resection.¹⁷ Such prediction may allow clinicians to optimize their therapeutic approach in a preventive fashion, thereby avoiding disease progression.¹⁸

The gold standard procedure used to evaluate mucosal inflammatory levels—endoscopic analysis—is invasive. Therefore, there is an urgent need to find and validate noninvasive biomarkers that can be easily extracted and quantified from blood or fecal samples, along with which levels can accurately indicate the degree of disease activity. This study is, to the best of our knowledge, the first to explore the potential of serum DPP-4 as a biomarker of IBD disease activity and prognosis. Our results are promising: Lower levels of serum DPP-4 were significantly associated with disease activity in both CD and UC patients, as were higher levels of 2 other currently known biomarkers (FC and CRP). Moreover, serum DPP-4 was inversely correlated both with disease activity scores (HBI and pMS) and endoscopic activity groups, a correlation that was shown to be stronger in CD patients than in UC patients. The

TABLE 4. Blood and Fecal Biomarker Performance (95% CI) in the Assessment of Treatment Escalation (First Cohort, n = 195)

	Crohn's Disease					
	Sensitivity, %	Specificity, %	PPV, %	NPV, %	PLR	NLR
DPP-4 ≤ 1452 ng/mL	85.0 (70.2–94.3)	65.4 (50.9–78.0)	65.4 (56.0–73.7)	85.0 (72.5–92.4)	2.5 (1.7–3.6)	0.2 (0.1–0.5)
FC > 840 μ g/g	48.8 (32.9–64.9)	86.4 (75.0–94.0)	71.4 (55.0–83.7)	70.8 (63.9–76.9)	3.6 (1.8–7.4)	0.6 (0.4–0.8)
CRP > 5.0 mg/L	81.0 (65.9–91.4)	72.4 (59.1–83.3)	68.0 (57.7–76.8)	84.0 (73.4–90.9)	2.9 (1.9–4.6)	0.3 (0.1–0.5)
	Ulcerative Colitis					
	Sensitivity, %	Specificity, %	PPV, %	NPV, %	PLR	NLR
DPP-4 ≤ 1472 ng/mL	79.0 (54.4–93.9)	65.7 (53.4–76.7)	38.5 (29.5–48.2)	92.0 (82.6–96.5)	2.3 (1.5–3.4)	0.3 (0.1–0.8)
FC > 785 μ g/g	75.0 (50.9–91.3)	82.2 (71.5–90.2)	53.6 (39.9–66.8)	92.3 (84.8–96.3)	4.2 (2.4–7.3)	0.3 (0.1–0.7)
CRP > 6.0 mg/L	70.0 (45.7–88.1)	77.0 (65.8–86.0)	45.2 (33.2–57.7)	90.5 (82.8–94.9)	3.1 (1.8–5.1)	0.4 (0.2–0.8)

ROC curves confirmed that serum DPP-4 is able to distinguish endoscopic activity scores better than clinical activity indices (HBI and pMS).

Although published data regarding serum DPP-4 levels among IBD patients are scarce, the few existing studies are consistent with our results. In fact, Xiao et al. compared a small number of IBD patients (5 with CD and 1 with UC) with otherwise healthy subjects ($n = 6$) and reported significantly lower serum DPP-4 activity among IBD patients.⁷ In another study, Hildebrandt et al. showed that DPP-4 activity was significantly lower in 110 patients with IBD (63 with CD and 47 with UC) when compared with 28 control subjects.⁸ Moreover, the same authors reported an inverse correlation between serum DPP-4 activity and clinical activity scores, such as the Crohn's Disease Activity Index (CDAI) and the Rachmilewitz score. Finally, Moran et al. reported the existence of significantly lower serum DPP-4 levels in 23 CD patients when compared with 17 otherwise healthy controls and a negative correlation between serum DPP-4 levels and CRP.⁹ A recent observational study by Abrahimi et al. showed that the use of DPP-4 inhibitors is associated with increased risk of IBD in patients with type 2 diabetes, although the absolute risk was small. The authors advised physicians to be aware of this association and avoid using DPP-4 inhibitors in patients with elevated risk of IBD.²¹ In contrast, an observational study by Kim et al. concluded that DPP-4 inhibitor therapy results in decreased risk of autoimmune diseases, including IBD.²² Because of conflicting studies, a recent meta-analysis of clinical trials and observational studies was conducted to analyze the potential risk of IBD when comparing DPP-4 inhibitor therapy with controls in adult diabetes patients. Based on a conservative random-effects analysis, DPP-4 inhibitors do not appear to increase the risk of IBD, but long-term postmarketing surveillance is warranted.²³

Further studies are needed to understand the pathophysiological process(es) sustaining the association between decreased serum DPP-4 levels and active inflammation in IBD. Two hypotheses can be outlined: Either reduced DPP-4 levels are directly implicated in IBD pathogenesis, or their decrease is part of a counter-regulatory mechanism that reduces the activation of the peptide hormones that participate in the inflammatory cascade, thereby limiting the systemic immune response.

Our results show that serum DPP-4 is able to differentiate between IBD patients in remission and those with active disease (clinical and endoscopic activity), with low levels indicating mucosal inflammation. Serum DPP-4 performed equally well in postoperative CD patients. This marker also revealed added benefit in IBD patients with normal CRP, as it was capable of distinguishing endoscopic activity in this population (slightly better than FC).

Baseline DPP-4 levels also proved to be important predictors of the need for treatment escalation in both CD and UC. Moreover, these levels can be combined with the 2 known

IBD biomarkers, CRP and FC, improving the power of this prediction. Therefore, DPP-4 assessment can potentially be used to predict disease course at an early stage to identify patients who would benefit from more aggressive therapy.

Finally, serum DPP-4 levels are also different between responders and nonresponders to biological therapy, both in CD and UC patients, and these could be used to monitor response to treatment, avoiding unnecessary exposure to futile treatments and lowering the risk of adverse events.

Our study was prospectively designed, included a multicentric cohort, and was, to the best of our knowledge, the first to assess the potential of serum DPP-4 as a biomarker of treatment escalation and biological treatment response in IBD patients. It should be acknowledged that both cohorts were recruited from tertiary hospitals, and therefore our findings may not be applicable to the overall IBD community.

CONCLUSIONS

Serum DPP-4 is a promising noninvasive biomarker of IBD activity and biological treatment response and a predictor of the need to escalate the therapeutic approach. As a blood-based marker, DPP-4 (and CRP) offers a few advantages over FC, as not all patients are able or willing to provide fecal samples. Blood samples, on the other hand, are relatively easy to obtain and process.

Pending further exploration and validation in larger multicentric cohorts, our results suggest that serum DPP-4 can be used to monitor IBD patients and to anticipate therapeutic adjustments, reducing the frequency of invasive endoscopic procedures and contributing to a preventive and targeted therapeutic approach.

SUPPLEMENTARY DATA

Supplementary data are available at *Inflammatory Bowel Diseases* online.

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Supplementary Table 1. STARD 2015: An updated list of essential items for reporting diagnostic accuracy studies

Section & Topic		
TITLE OR ABSTRACT		
	1	Identification as a study of diagnostic accuracy using at least one measure of accuracy (such as sensitivity, specificity, predictive values, or AUC)
ABSTRACT		
	2	Structured summary of study design, methods, results, and conclusions (for specific guidance, see STARD for Abstracts)
INTRODUCTION		
	3	Scientific and clinical background, including the intended use and clinical role of the index test
	4	Study objectives and hypotheses
METHODS		
<i>Study design</i>	5	Whether data collection was planned before the index test and reference standard were performed (prospective study) or after (retrospective study)
<i>Participants</i>	6	Eligibility criteria
	7	On what basis potentially eligible participants were identified (such as symptoms, results from previous tests, inclusion in registry)
	8	Where and when potentially eligible participants were identified (setting, location and dates)
	9	Whether participants formed a consecutive, random or convenience series
<i>Test methods</i>	10a	Index test, in sufficient detail to allow replication
	10b	Reference standard, in sufficient detail to allow replication
	11	Rationale for choosing the reference standard (if alternatives exist)
	12a	Definition of and rationale for test positivity cut-offs or result categories of the index test, distinguishing pre-specified from exploratory
	12b	Definition of and rationale for test positivity cut-offs or result categories of the reference standard, distinguishing pre-specified from exploratory
	13a	Whether clinical information and reference standard results were available to the performers/readers of the index test
	13b	Whether clinical information and index test results were available to the assessors of the reference standard
<i>Analysis</i>	14	Methods for estimating or comparing measures of diagnostic accuracy
	15	How indeterminate index test or reference standard results were handled
	16	How missing data on the index test and reference standard were handled
	17	Any analyses of variability in diagnostic accuracy, distinguishing pre-specified from exploratory
	18	Intended sample size and how it was determined
RESULTS		
<i>Participants</i>	19	Flow of participants, using a diagram
	20	Baseline demographic and clinical characteristics of participants
	21a	Distribution of severity of disease in those with the target condition
	21b	Distribution of alternative diagnoses in those without the target condition
	22	Time interval and any clinical interventions between index test and reference standard
<i>Test results</i>	23	Cross tabulation of the index test results (or their distribution) by the results of the reference standard
	24	Estimates of diagnostic accuracy and their precision (such as 95% confidence intervals)
	25	Any adverse events from performing the index test or the reference standard
DISCUSSION		
	26	Study limitations, including sources of potential bias, statistical uncertainty, and generalizability
	27	Implications for practice, including the intended use and clinical role of the index test
OTHER INFORMATION		
	28	Registration number and name of registry
	29	Where the full study protocol can be accessed
	30	Sources of funding and other support; role of funders

Supplementary Table 2. Demographic and clinical characteristics of the study population in the 2nd cohort (N=46).

	Crohn's disease				Ulcerative colitis		
	Clinical remission (n=18)	Clinical activity (n=6)	P-value		Clinical remission (n=17)	Clinical activity (n=5)	P-value
Age, yr, mean $\pm$ SD	34.4 $\pm$ 8.3	54.0 $\pm$ 9.8	<0.001	Age, yr, mean $\pm$ SD	42.4 $\pm$ 16.7	43.0 $\pm$ 10.5	0.936
Female, n (%)	11 (61.1)	2 (33.3)	0.357	Female, n (%)	10 (58.8)	1 (20.0)	0.311
Disease duration, yr, mean $\pm$ SD	8.6 $\pm$ 6.7	15.2 $\pm$ 12.1	0.248	Disease duration, yr, mean $\pm$ SD	8.2 $\pm$ 5.6	7.2 $\pm$ 4.0	0.707
Age at diagnosis, yr, mean $\pm$ SD	25.1 $\pm$ 9.6	38.5 $\pm$ 18.1	0.027	Age at diagnosis, yr, mean $\pm$ SD	33.7 $\pm$ 15.2	35.0 $\pm$ 10.8	0.855
Previous surgical resection, n (%)	4 (22.2)	3 (50.0)	0.318	Previous surgical resection, n (%)	1 (5.9)	0	1.000
Activity score (HBI), mean $\pm$ SD	1.6 $\pm$ 1.3	5.8 $\pm$ 1.0	<0.001	Activity score (pMS), mean $\pm$ SD	0.3 $\pm$ 0.5	3.2 $\pm$ 1.6	0.016
Montreal location ^a				Montreal extent ^b			
<i>L1 $\pm$ L4, n (%)</i>	7 (38.9)	3 (50.0)	0.436	<i>E1, n (%)</i>	0	0	0.323
<i>L2 $\pm$ L4, n (%)</i>	3 (16.7)	2 (33.3)		<i>E2, n (%)</i>	8 (47.1)	4 (80.0)	
<i>L3 $\pm$ L4, n (%)</i>	8 (44.4)	1 (16.7)		<i>E3, n (%)</i>	9 (52.9)	1 (20.0)	
<i>L4, n (%)</i>	0	0					
Montreal behavior ^c							
<i>B1, n (%)</i>	7 (38.9)	2 (33.3)	0.744				
<i>B2, n (%)</i>	6 (33.3)	3 (50.0)					
<i>B3, n (%)</i>	5 (27.8)	1 (16.7)					
<i>Perianal disease, n (%)</i>	7 (38.9)	4 (66.7)	0.357				
Medications at baseline							
<i>5-ASA, n (%)</i>	1 (5.6)	0	1.000	<i>5-ASA, n (%)</i>	6 (35.3)	1 (20.0)	1.000
<i>Azathioprine, n (%)</i>	8 (44.4)	4 (66.7)	0.640	<i>Azathioprine, n (%)</i>	9 (52.9)	2 (40.0)	1.000
<i>Methotrexate, n (%)</i>	1 (5.6)	1 (16.7)	0.446	<i>Methotrexate, n (%)</i>	1 (5.9)	0	1.000
<i>Biologic treatment, n (%)</i>	18 (100)	6 (100)	-	<i>Biologic treatment, n (%)</i>	17 (100)	5 (100)	-
Therapeutic escalation, n (%)	4 (22.2)	3 (50.0)	0.307	Therapeutic escalation, n (%)	3 (17.6)	3 (60.0)	0.100

^a L1 – ileal; L2 – colonic; L3 – ileocolonic; L4 – upper disease^b E1 – proctitis; E2 – left sided colitis; E3 – pancolitis^c B1 – non-stricturing, non-penetrating; B2 – stricturing; B3 – penetrating; P – perianal disease; 5-ASA – 5 aminosalicylic acid; HBI – Harvey-Bradshaw index; pMS – partial Mayo Score; SD – Standard deviation

Supplementary Table 3. Correlation coefficient (R Spearman) between biomarkers, clinical indexes and endoscopic activity (1st cohort, N=195).

	Parameters	Correlation (R spearman)	P-value
Crohn's disease	DPP-4 vs FC (n=91)	- 0.55	<0.001
	DPP-4 vs CRP (n=91)	- 0.54	<0.001
	DPP-4 vs HBI (n=92)	- 0.47	<0.001
	FC vs HBI (n=100)	0.57	<0.001
	CRP vs HBI (n=100)	0.67	<0.001
	DPP-4 vs Endoscopic activity (n=32)	- 0.67	<0.001
	FC vs Endoscopic activity (n=33)	0.64	<0.001
	CRP vs Endoscopic activity (n=34)	0.66	<0.001
	HBI vs Endoscopic activity (n=34)	0.72	<0.001
Ulcerative colitis	DPP-4 vs FC (n=88)	- 0.36	0.001
	DPP-4 vs CRP (n=89)	- 0.25	0.017
	DPP-4 vs pMS (n=89)	- 0.43	<0.001
	FC vs pMS (n=93)	0.72	<0.001
	CRP vs pMS (n=94)	0.60	<0.001
	DPP-4 vs Endoscopic activity (n=30)	- 0.48	0.008
	FC vs Endoscopic activity (n=34)	0.82	<0.001
	CRP vs Endoscopic activity (n=34)	0.68	<0.001
	pMS vs Endoscopic activity (n=34)	0.83	<0.001

CRP – C-reactive protein; DPP-4 – Dipeptidyl peptidase 4; FC – Fecal calprotectin; HBI – Harvey-Bradshaw index; pMS – partial Mayo Score

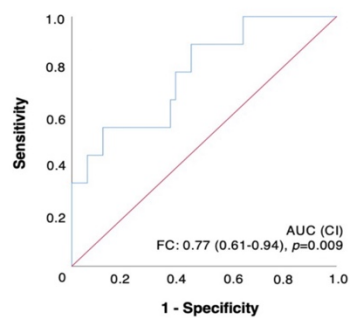
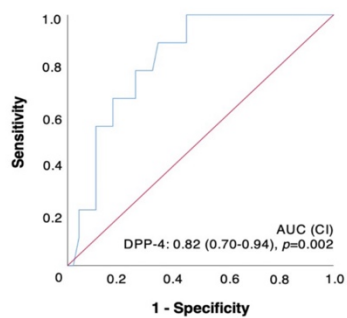
Supplementary Table 4. Levels of biomarkers during the study, stratified by response to biological treatment (2nd cohort, N=46).

	Crohn's disease			Ulcerative colitis		
	Responders (n=9)	Non-responders (n=15)	<i>P-value</i>	Responders (n=8)	Non-responders (n=14)	<i>P-value</i>
DPP-4 (ng/mL), median (IQR)	1385 (1185-1592)	1134 (975-1469)	0.015	1467 (1301-1641)	1211 (1011-1448)	<0.001
FC (µg/g), median (IQR)	90 (34-278)	510 (229-832)	<0.001	178 (51-560)	675 (271-923)	<0.001
CRP (mg/L), median (IQR)	1.1 (0.6-3.1)	5.2 (2.9-10.1)	<0.001	2.7 (1.6-4.3)	2.6 (1.3-10.1)	0.698

CRP – C-reactive protein; DPP-4 – Dipeptidyl peptidase 4; FC – Fecal calprotectin; IQR – Interquartile range

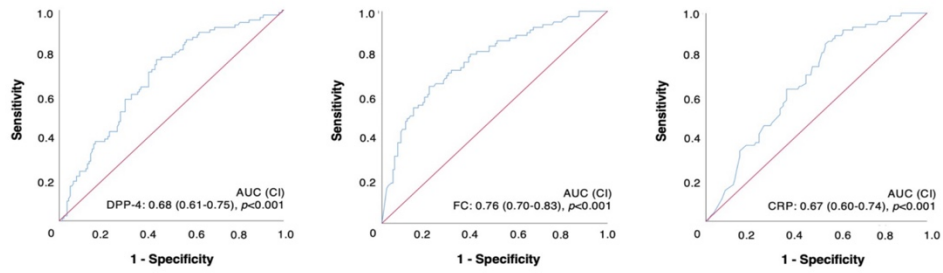
Supplementary figure 1. Receiver operating curve (ROC) analysis of serum dipeptidyl peptidase 4 (DPP-4) and fecal calprotectin (FC) in predicting endoscopic activity in IBD patients with normal CRP (1st cohort, N=195).

AUC – Area under the Curve; CI – Confidence interval



Supplementary figure 2. Receiver operating curve (ROC) analysis of serum dipeptidyl peptidase 4 (DPP-4), fecal calprotectin (FC) and serum C-reactive protein (CRP) in predicting response to treatment in IBD patients (2nd cohort, N=46).

AUC – Area under the Curve; CI – Confidence interval



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Paper II

“Fecal Dipeptidyl Peptidase-4: An Emergent Biomarker in Inflammatory Bowel Disease”

Clin Transl Gastroenterol. 2021;12(3):e00320.

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Fecal Dipeptidyl Peptidase-4: An Emergent Biomarker in Inflammatory Bowel Disease

Pedro Pinto-Lopes, MD, MSc^{1,2,3}, Francisco Melo, MD, MSc³, Joana Afonso, PhD^{3,4}, Rui Pinto-Lopes, MD, MSc⁵, Cátia Rocha, MSc^{3,6}, Daniel Melo, MD, MSc⁷, Guilherme Macedo, MD, PhD⁸, Cláudia Camila Dias, PhD^{9,10}, Fátima Carneiro, MD, PhD^{7,11} and Fernando Magro, MD, PhD^{3,4,8,12} for the Portuguese IBD Study Group (GEDII)

INTRODUCTION: Dipeptidyl peptidase-4 (DPP-4) is a membrane-bound glycoprotein that acts as a receptor but also exists in a soluble form. It has been recognized as a mediator of inflammation and considered a biomarker in inflammatory bowel disease (IBD).

METHODS: We evaluated a prospectively recruited cohort, consisting of 101 patients with IBD, using validated clinical indexes; 22 patients with ulcerative colitis (UC) underwent endoscopic evaluation. Fecal DPP-4 (fDPP-4) levels were analyzed and correlated with clinical scores, Mayo endoscopic score (in UC patients), serum DPP-4, C-reactive protein, and fecal calprotectin. Immunohistochemical staining for DPP-4 in intestinal biopsies was also performed.

RESULTS: When compared with remitters, median fDPP-4 levels were higher in patients with ileal Crohn's disease (CD) (7,584 [1,464–7,816] vs 2,104 [630–2,676] ng/mL, $P = 0.015$) and lower in patients with UC exhibiting clinical activity (1,213 [559–1,682] vs 7,814 [2,555–7,985] ng/mL, $P < 0.001$). Patients with UC presenting endoscopic activity also had lower levels than remitters (939 [559–1,420] vs 7,544 [4,531–7,940] ng/mL, $P = 0.006$). Fecal DPP-4 discriminated clinical activity from remission with areas under the curve of 0.76 (95% confidence interval [CI] 0.58–0.94, $P = 0.015$) and 0.80 (95% CI 0.68–0.93, $P < 0.001$) in CD and UC, respectively; it allowed to differentiate endoscopic activity in patients with UC, with areas under the curve of 0.84 (95% CI 0.63–1.00, $P = 0.009$). Immunohistochemical analysis revealed higher DPP-4 apical expression in UC remitters, but no statistically significant differences were revealed between patients with ileal CD.

DISCUSSION: Our results suggest that fDPP-4 can be used as a biomarker of IBD activity, particularly in UC. The expression profiles in intestinal tissue might represent a functional compartmentalization of DPP-4 expression.

SUPPLEMENTARY MATERIAL accompanies this paper at <http://links.lww.com/CTG/A521>

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INTRODUCTION

Inflammatory bowel disease (IBD) encompasses 2 main distinct forms, Crohn's disease (CD) and ulcerative colitis (UC). These conditions are characterized by repeated episodes of mucosal inflammation and result in marked alterations in intestinal structure and function (1). Although the etiology of IBD is still

unknown, the most accepted hypothesis states that the disease is initiated and maintained by an inappropriate immune response to unknown environmental or microbial antigen(s) in genetically susceptible individuals (1).

Although the diagnosis of IBD is still a challenge, over the past few years, several studies managed to identify key molecules

¹Department of Internal Medicine, Faculty of Medicine, Centro Hospitalar Universitário São João, Porto, Portugal; ²Department of Internal Medicine, Centro Hospitalar Tâmega e Sousa—Hospital Padre Américo, Penafiel, Portugal; ³Unit of Pharmacology and Therapeutics, Department of Biomedicine, Faculty of Medicine, University of Porto, Porto, Portugal; ⁴MedInUP, Centre for Drug Discovery and Innovative Medicines, University of Porto, Porto, Portugal; ⁵Department of Gastroenterology, Mid Essex Hospital Services NHS Trust, Broomfield, Essex, UK; ⁶Instituto de Saúde Ambiental, Faculty of Medicine, University of Lisbon, Lisbon, Portugal; ⁷Department of Pathology, Faculty of Medicine, Centro Hospitalar Universitário São João, Porto, Portugal; ⁸Department of Gastroenterology, Faculty of Medicine, Centro Hospitalar Universitário São João, Porto, Portugal; ⁹Department of Community Medicine, Information and Health Decision Sciences, Faculty of Medicine, University of Porto, Porto, Portugal; ¹⁰CINTESIS—Centre for Health Technology and Services Research, Porto, Portugal; ¹¹Institute of Molecular Pathology and Immunology of the University of Porto (Ipatimup), Instituto de Investigação e Inovação em Saúde (i3S), University of Porto, Porto, Portugal; ¹²Clinical Pharmacology Unit, Centro Hospitalar Universitário São João, Porto, Portugal. **Correspondence:** Fernando Magro, MD, PhD. E-mail: fm@med.up.pt.

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involved in IBD pathogenesis. In this context, dipeptidyl peptidase-4 (DPP-4) emerged as a potential noninvasive biomarker of IBD activity and as a monitoring tool of the response to biological treatments (2). DPP-4, also known as the cell surface antigen CD26, is a transmembrane glycoprotein expressed in numerous tissues, including intestinal brush border membrane and cells of the immune system (3,4). Its activity and expression have been studied in epithelial cells from different regions of the human small intestine and colon. Protein activity and expression levels are high in the ileum and jejunum, low in the duodenum, and not detectable in the colon (5). DPP-4 is also present in a soluble form believed to result from specific proteolytic cleavage of the membrane-bound form, through a process called shedding, mediated by various enzymes in a cell type-specific manner (6). Potent cytokines such as RANTES (regulated on activation, normal T cell expressed, and secreted) and tumor necrosis factor- α (TNF- α) are some of the substrates described for DPP-4, suggesting a complex immunomodulatory role of DPP-4 in serum (7–10).

Although published data regarding DPP-4 expression among patients with IBD are scarce, a few studies demonstrated that when compared with healthy controls, patients with IBD present lower levels of circulating/serum DPP-4 and higher membrane expression of CD26 in T lymphocytes (11–13). DPP-4/CD26 was associated to the pathogenesis of IBD because of its involvement in immune regulation, extracellular matrix formation, and tissue remodeling. Our group also demonstrated that serum DPP-4 (sDPP-4) is able to discriminate patients with IBD in remission from those with active disease (clinical and endoscopic activity), with low levels indicating mucosal inflammation. Baseline sDPP-4 levels proved to be important predictors for treatment escalation and showed to be useful to monitoring response to IBD treatment, thus avoiding unnecessary treatments and lowering the risk of adverse events (2).

In this context and considering the demonstrated data about the different expression profiles of DPP-4 in patients with IBD, the study of the correlation of fecal DPP-4 (fDPP-4) with IBD can contribute to further explain the role of this protein in this disease.

This prospective study aims to clarify the role of fDPP-4 as a biomarker of IBD activity and to correlate it with clinical parameters and other biomarkers, namely: sDPP-4, fecal calprotectin (FC), and C-reactive protein (CRP). In addition, our study intends to assess the mechanisms underlying the variations of DPP-4 expression levels in IBD, through the evaluation of the expression of DPP-4/CD26, in intestinal samples of patients with IBD.

MATERIAL AND METHODS

Patients and study design

This study included 101 patients diagnosed with CD and UC previously recruited for another study performed by our research group (2). The cohort included active and remitting patients with IBD attending routine outpatient consultations or who were admitted to the hospital because of an IBD flare. A control group of 40 healthy volunteers was recruited from phlebotomy clinics for blood and fecal samples collection purposes. These were randomly selected after screening with a standardized questionnaire to ensure no medical history of IBD. An additional 10 individuals who underwent ileocolonoscopy for non-IBD/noninflammatory diseases were included for immunohistochemical analysis. Inclusion and exclusion criteria have been previously described (2). The local Research Ethics

Committee granted approval for this study. All patients signed an informed written consent.

Demographic and clinical data were collected at baseline. Disease location and clinical phenotypes were evaluated with the Montreal classification (14). Disease activity was assessed using the Harvey-Bradshaw index (HBI) (15) (clinical remission ≤ 4) for patients with CD and the partial Mayo Score (pMS) (16) (clinical remission ≤ 1) for patients with UC.

This article was designed to meet the Standards for Reporting Diagnostic Accuracy requirements (see Supplementary Table 1, Supplementary Digital Content 1, <http://links.lww.com/CTG/A521>).

Biomarkers assessment

Stool samples were collected and kept at 4 °C, for a maximum of 48 hours, until shipment to the laboratory. Fecal DPP-4 was extracted from stool samples, within a maximum of 7 days after collection using the Fecal Sample Preparation kit (Roche Diagnostics, Mannheim, Germany) and stored at -80 °C until quantification. These fecal samples underwent only one freeze-thaw cycle. DPP-4 levels were measured using a quantitative enzyme-linked immunosorbent assay (Sigma-Aldrich, St. Louis, MO), according to the manufacturers' instructions.

The protocols for the measurement of fecal calprotectin, sDPP-4, and serum C-reactive protein were followed as described in a previous study (2).

Endoscopic assessment

Endoscopic procedures were performed on some of the patients in the context of a previous study, with the endoscopist blinded for the biomarker results (2). The endoscopic reports (including photographs) were reviewed by an IBD physician (F.M.) to grade endoscopic activity. Because of the low number of patients with ileal CD with endoscopic evaluation, the authors used these data only for patients with UC. In these patients, endoscopic activity was assessed according to the Mayo endoscopic score, which reflects the severity of intestinal inflammation on colonoscopy as follows: 0 points, normal or inactive disease; 1 point, mild disease (erythema, decreased vascular pattern, and mild friability); 2 points, moderate disease (marked erythema, absent vascular pattern, friability, and erosions); and 3 points, severe disease (spontaneous bleeding and ulcerations). Endoscopic remission was defined as a Mayo endoscopic score of 0 (17).

Immunohistochemical assessment

Biopsies and surgical resections of small intestine and colon from 25 patients with IBD (UC: 13 colon samples; CD: 12 ileum samples) and 10 healthy volunteers (5 ileum and 5 colon samples) were managed following conventional protocols. Immunohistochemical staining for DPP-4/CD26 was carried out in formalin-fixed and paraffin-embedded tissues. These were immunostained with a monoclonal antibody specific for DPP-4/CD26 (Novus Biologicals NB 100-59021, rabbit anti-human, working dilution 1:800) using the BenchMark ULTRA automatic system (Ventana Medical Systems, Oro Valley, AZ). Reactions were revealed using the UltraView Universal DAB (Ventana Medical Systems). Immunohistochemical expression of DPP-4/CD26 was evaluated in the epithelial apical side, cytoplasm, and remaining membrane. Membranous and cytoplasmic expression of DPP-4 was semi-quantitatively analyzed. The scoring system was mainly based on membranous staining: 0, no membranous staining of epithelial cells; 1+, membrane staining in up to 25% of epithelial cells; +2,

Fecal Dipeptidyl Peptidase-4

Table 1. Demographic and clinical characteristics of the study population

	UC			CD		
	Clinical remission (n = 29)	Clinical activity (n = 22)	P	Clinical remission (n = 23)	Clinical activity (n = 27)	P
Male, n (%)	10 (35)	13 (59)	0.096	14 (61)	13 (48)	0.407
Age, yr, median (IQR)	45 (35–55)	43 (30–59)	0.849	40 (34–46)	36 (26–49)	0.386
Disease duration, yr, median (IQR)	9 (5–14)	5 (0–15)	0.083	11 (5–17)	5 (1–7)	0.011
Age at diagnosis, yr, median (IQR)	32 (25–39)	31 (23–50)	0.879	29 (24–36)	28 (20–42)	0.845
Previous surgical resection, n (%)	0	1 (5)	0.431	10 (44)	6 (22)	0.136
Montreal extent, ^a n (%)						
E1	4 (14)	0		15 (65)	15 (56)	
E2	17 (59)	5 (23)	0.001	1 (4)	1 (4)	
E3	8 (28)	17 (77)		7 (30)	10 (37)	0.211
				0	1 (4)	
Montreal behavior, ^c n (%)						
B1				12 (52)	7 (26)	
B2				3 (13)	9 (33)	
B3				8 (35)	11 (41)	0.105
p				8 (35)	9 (33)	>0.999
Medication						
5-ASA, n (%)	25 (86)	12 (55)	0.025	7 (30)	1 (4)	0.017
Thiopurine, n (%)	9 (31)	6 (27)	>0.999	15 (65)	10 (37)	0.088
Methotrexate, n (%)	0	0	—	2 (9)	2 (7)	>0.999
Biological treatment, n (%)	12 (41)	7 (32)	0.566	12 (52)	14 (52)	>0.999

CD, Crohn's disease; IQR, interquartile range; UC, ulcerative colitis.
^aE1—proctitis; E2—left sided colitis; E3—pancolitis.
^bL1—ileal; L2—colon; L3—ileocolonic; L4—upper disease.
^cB1—non-stricturing, non-penetrating; B2—stricturing; B3—penetrating; p—perianal disease.
 5-ASA, 5-aminosalicylic acid.

Table 2. Clinical scores and fDPP-4 levels in IBD patients

	UC				CD		
	Clinical remission (n = 29)	Clinical activity (n = 22)	P		Clinical remission (n = 23)	Clinical activity (n = 27)	P
Clinical activity score (pMS), median (IQR)	0 (0–0)	5 (3–7)	<0.001	Clinical activity score (HBI), median (IQR)	1 (0–2)	7 (6–9)	<0.001
fDPP-4 (ng/mL), median (IQR)	7,814 (2,555–7,985)	1,213 (559–1,682)	<0.001	fDPP-4 (ng/mL), median (IQR)	2,104 (630–3,269)	2,964 (811–7,782)	0.142
E2	7,791 (2,880–7,981)	894 (564–1,682)	0.039	L1	2,104 (630–2,676)	7,584 (1,464–7,816)	0.015
E3	7,824 (4,623–7,992)	1,235 (559–1,514)	0.009	L3	1,393 (434–5,290)	900 (347–7,645)	0.669
	UC				CD		
	Endoscopic remission (n = 8)	Endoscopic activity (n = 14)	P				
fDPP-4 (ng/mL), median (IQR)	7,544 (4,531–7,940)	939 (559–1,420)	0.006				

E1, L2 and L4 patients were excluded from subgroup analysis because of a low number of patients in these categories.

E2—left sided colitis; E3—pancolitis; L1—ileal; L3—ileocolonic.

CD, Crohn's disease; fDPP-4, fecal dipeptidyl peptidase-4; HBI, Harvey-Bradshaw index; IQR, interquartile range; pMS, partial Mayo score; UC, Ulcerative colitis.

membrane staining observed in 26%–50% of epithelial cells; and +3, membrane staining in >50% of epithelial cells.

Statistical analyses

Categorical variables are described as absolute and relative frequencies and analyzed using Fisher exact tests. Continuous variables are presented as median and interquartile range (for asymmetrical distributions). When testing hypothesis concerning continuous variables, the nonparametric Mann-Whitney test was used as appropriate, taking into account normality assumptions and the number of compared groups. Correlation between variables was assessed using the Spearman rank-order correlation test. Receiver operating characteristics (ROC) curves were generated by plotting sensitivity against specificity, and the areas under the curve (AUC) were calculated. The level of statistical significance was set at 0.05. Statistical Package for Social Sciences V.25.0 (IBM SPSS Statistics for MacOS, Armonk, NY) and GraphPad Prism V.8.00 for Mac OS X (GraphPad Software, La Jolla, CA) were used for the statistical analysis and plot design.

RESULTS

Cohort characterization and disease outcomes

The cohort enrolled in this study included 101 patients with IBD (50 with CD and 51 with UC), and 40 healthy controls; of the former, 46% of the patients with CD and 57% of the patients with UC were in clinical remission (Table 1). Age, sex, disease duration, age at diagnosis, and need for previous surgical resection were similarly distributed among the groups, except for patients with CD in clinical remission, who had significantly longer disease duration. Montreal classification was equally balanced between patients with CD in remission and with active disease, but not between patients with UC: the E3 category was more frequent in patients with active disease (77%), whereas E1 and E2 categories were more frequent among remitters (14% and 59%, respectively).

As for medication, remitters were mainly patients with CD and UC on 5-amino-salicylic acid (30% and 86%, respectively).

The main outcomes of the study are described in Table 2. Twenty-two patients with UC underwent colonoscopy, and according to the Mayo endoscopic score, 8 patients (36%) were in endoscopic remission and 14 (64%) had endoscopic activity.

Fecal DPP-4 levels were lower in patients with UC with clinical activity comparing with remitters (1,213 [559–1,682] vs 7,814 [2,555–7,985] ng/mL, $P < 0.001$) and healthy controls (1,213 [559–1,682] vs 2,842 [1,570–5,712] ng/mL, $P = 0.019$). However, remitters presented higher levels of fDPP-4 than healthy controls (7,814 [2,555–7,985] vs 2,842 [1,570–5,712] ng/mL, $P = 0.006$). Patients with UC exhibiting endoscopic activity also had lower levels than remitters (939 [559–1,420] vs 7,544 [4,531–7,940] ng/mL, $P = 0.006$) and healthy controls (939 [559–1,420] vs 2,842 [1,570–5,712] ng/mL, $P = 0.004$). CD patients with active ileal disease showed higher fDPP-4 than those in remission (7,584 [1,464–7,816] vs 2,104 [630–2,676] ng/mL, $P = 0.015$). No statistically significant differences were found among ileocolonic patients with CD (clinical remission: 1,393 [434–5,290] ng/mL vs clinical activity: 900 [347–7,645] ng/mL, $P = 0.669$), between patients with active ileal CD and healthy controls (7,584 [1,464–7,816] vs 2,842 [1,570–5,712] ng/mL, $P = 0.086$), and between patients with ileal CD in clinical remission and healthy controls (2,104 [630–2,676] ng/mL vs 2,842 [1,570–5,712] ng/mL, $P = 0.061$) (Table 2 and Figure 1).

ROC curves confirmed that in UC (Figure 2a), fDPP-4 is able to discriminate between (i) healthy controls and patients in clinical remission and with clinical activity, with an AUC of 0.70 (95% confidence interval [CI] 0.56–0.84, $P = 0.006$) and 0.68 (95% CI 0.53–0.84, $P = 0.019$), respectively (Figure 2, 1.2 and 1.3); (ii) healthy controls and patients with endoscopic activity, with an AUC of 0.76 (95% CI 0.61–0.91, $P = 0.004$) (Figure 2, 2.3); (iii) patients with clinical activity and remitters, with an AUC of 0.80

Fecal Dipeptidyl Peptidase-4

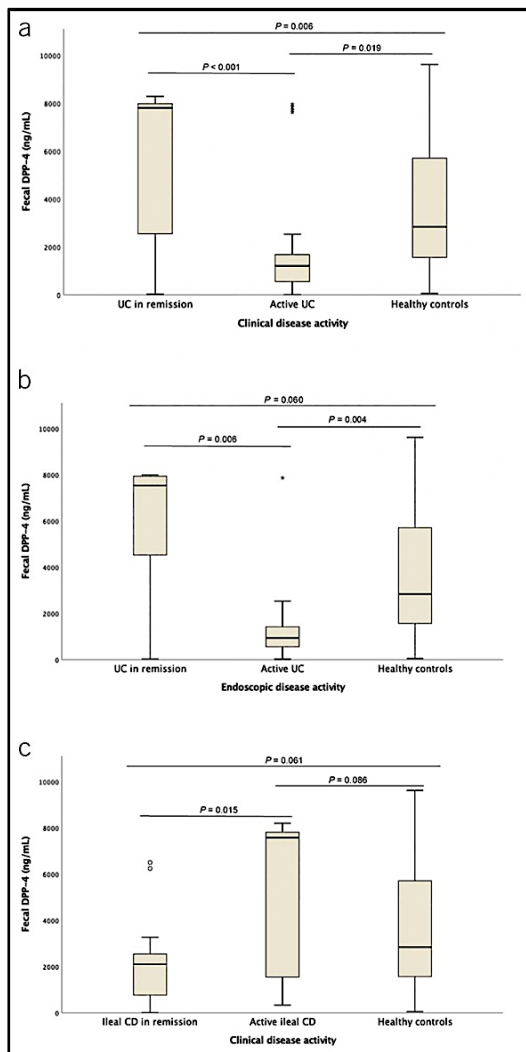


Figure 1. Comparison of median fecal DPP-4 levels in healthy controls and patients with UC regarding clinical activity (a) and endoscopic activity (b) and in healthy controls and patients with ileal CD regarding clinical activity (c). CD, Crohn's disease; DPP-4, dipeptidyl peptidase-4; UC, ulcerative colitis.

(95% CI 0.68–0.93, $P < 0.001$) (Figure 2, 1.1); and (iv) patients with endoscopic activity and those in endoscopic remission, with an AUC of 0.84 (95% CI 0.63–1.00, $P = 0.009$) (Figure 2, 2.1). ROC curves also showed that in patients with ileal CD (Figure 2b), fDPP-4 can discriminate remitters from patients with clinical activity, with an AUC of 0.76 (95% CI 0.58–0.94, $P = 0.015$) (Figure 2, 3.1).

In patients with UC, fDPP-4 correlated positively with sDPP-4 ($r = 0.52$, $P < 0.001$) and CD26 intestinal immunostaining ($r = 0.54$, $P = 0.049$) and inversely correlated with other biomarkers such as FC ($r = -0.43$, $P = 0.001$) and serum CRP ($r = -0.32$,

$P = 0.022$), as well as with pMS ($r = -0.51$, $P < 0.001$) and Mayo endoscopic score ($r = -0.57$, $P = 0.005$). In this group of patients, sDPP-4 correlated directly with CD26 intestinal expression ($r = 0.72$, $P = 0.006$). Patients with CD, with ileal involvement, presented a direct correlation between fDPP-4 and CRP ($r = 0.43$, $P = 0.018$) and between sDPP-4 and CD26 intestinal expression ($r = 0.64$, $P = 0.025$) (Table 3).

DPP-4/CD26 immunohistochemical evaluation

Figure 3 shows an example of the immunohistochemical expression of DPP-4/CD26 in healthy volunteers and in patients with IBD. Normal colon mucosa (Figure 3, 1A) did not express DPP-4/CD26, whereas the ileum (Figure 3, 2A) displayed positive luminal membrane reinforcement ($P = 0.002$). Sixty percent of the patients with UC in clinical remission ($n = 5$) presented some degree of apical DPP-4/CD26 expression (Figure 3, 1B) compared with none of those with clinical activity ($n = 8$) ($P = 0.012$) (Figure 3, 1C). All patients with CD with ileal disease in clinical remission ($n = 5$) showed high apical DPP-4/CD26 expression, in contrast to 29% of the patients with CD with clinical activity ($n = 7$) (Figure 3, 2B and 2C); nevertheless, these differences did not reach statistical significance ($P = 0.190$). Although cytoplasm and basolateral membrane were always negative for DPP-4/CD26 expression, lymphocytes of lamina propria were positive for this marker in all cases, as expected (see Supplementary Table 2, Supplementary Digital Content, <http://links.lww.com/CTG/A521>).

DISCUSSION

In this prospective study, we explored, for the first time, the potential of fDPP-4 as a biomarker of IBD activity. To further understand the role of DPP-4 in IBD, we evaluated the expression of DPP-4/CD26 in biopsies and surgical resections of small intestine and colon from patients with IBD and healthy volunteers.

Our study evidenced that fDPP-4 levels were lower in patients with UC with clinical and endoscopic activity compared with those in remission. Conversely, ileal patients with CD with clinical activity presented higher fDPP-4 levels than remitters. ROC curves confirmed that fDPP-4 is a biomarker able to discriminate clinical activity in patients with IBD (UC and CD) and endoscopic activity in patients with UC. It correlated well with other biomarkers such as sDPP-4, FC, and serum CRP and with clinical and endoscopic activity scores in patients with UC. These results resemble, in some way, those obtained in a previous study of our group in which we proved that sDPP-4 was able to discriminate IBD remitters from patients with active disease (clinical and endoscopic activity), with low levels indicating mucosal inflammation (2). In fact, in that study, lower levels of sDPP-4 were associated with disease activity, in both patients with CD and UC, and sDPP-4 was inversely correlated with both disease activity scores (HBI and pMS) and endoscopic activity groups. Experimental and clinical studies on other diseases such as systemic lupus erythematosus (18), rheumatoid arthritis (19), and colorectal cancer (20) also reported changes in sDPP-4 activity.

Our study also aimed to contribute toward a deeper knowledge on the mechanisms underlying the changes in DPP-4 levels in patients with IBD and on the origin of fDPP-4. Because free DPP-4 is originated from the membrane bounded form, the study of the shedding process is of utmost importance to clarify these aspects (21). Although DPP-4 shedding is poorly understood, recent studies attempted to unravel the molecular basis of this

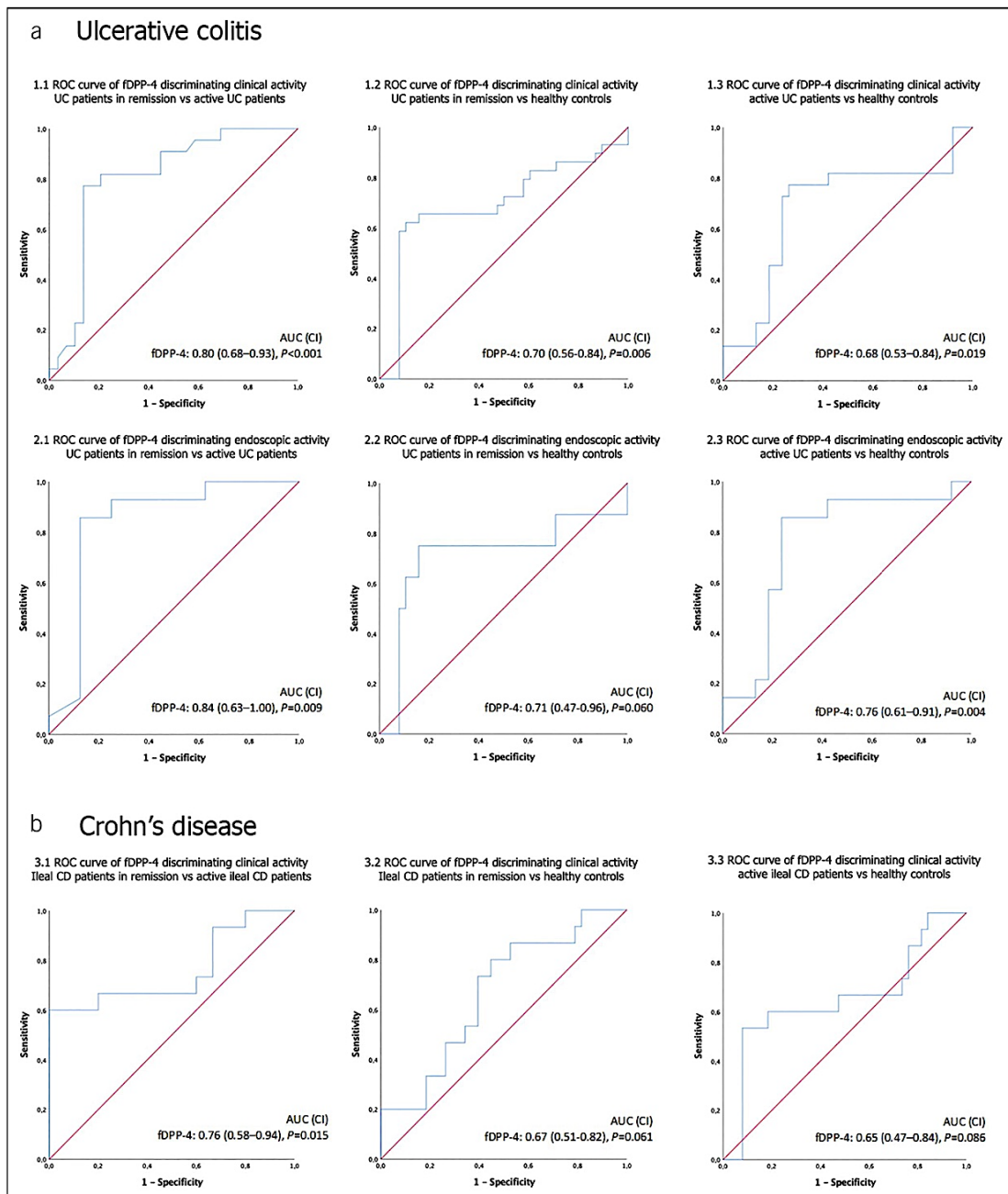


Figure 2. Receiver operating characteristics (ROC) curve analysis of fecal DPP-4 in predicting clinical and endoscopic activity in patients with UC (a) and clinical activity in patients with ileal CD (b). 1.1—patients with UC in clinical remission vs patients with UC with clinical activity; 1.2—healthy controls vs patients with UC in clinical remission; 1.3—healthy controls vs patients with UC with clinical activity; 2.1—patients with UC in endoscopic remission vs patients with UC with endoscopic activity; 2.2—healthy controls vs patients with UC in endoscopic remission; 2.3—healthy controls vs patients with UC with endoscopic activity; 3.1—patients with ileal CD in clinical remission vs patients with ileal CD with clinical activity; 3.2—healthy controls vs patients with ileal CD in clinical remission; and 3.3—healthy controls vs patients with ileal CD with clinical activity. AUC, areas under the curve; CD, Crohn's disease; CI, confidence interval; DPP-4, dipeptidyl peptidase-4; fDPP-4, fecal DPP-4; UC, ulcerative colitis.

Fecal Dipeptidyl Peptidase-4

Table 3. Correlation coefficients (R Spearman) between DPP-4 and other biomarkers, clinical indexes (HBI and pMS), Mayo endoscopic score, and immunohistochemical expression in IBD patients

Parameters		Correlation (r spearman)	P
UC	fDPP-4 vs sDPP-4 (n = 51)	0.52	<0.001
	fDPP-4 vs FC (n = 51)	-0.43	0.001
	fDPP-4 vs CRP (n = 51)	-0.32	0.022
	fDPP-4 vs pMS (n = 51)	-0.51	<0.001
	fDPP-4 vs Mayo endoscopic score (n = 22)	-0.57	0.005
	fDPP-4 vs DPP-4 intestinal expression (n = 13)	0.54	0.049
	sDPP-4 vs DPP-4 intestinal expression (n = 13)	0.72	0.006
Ileal CD	fDPP-4 vs sDPP-4 (n = 30)	-0.18	0.356
	fDPP-4 vs FC (n = 30)	0.32	0.081
	fDPP-4 vs CRP (n = 30)	0.43	0.018
	fDPP-4 vs HBI (n = 30)	0.23	0.228
	fDPP-4 vs DPP-4 intestinal expression (n = 10)	-0.06	0.873
	sDPP-4 vs DPP-4 intestinal expression (n = 12)	0.64	0.025

CD, Crohn's disease; CRP, C-reactive protein; DPP-4, dipeptidyl peptidase-4; FC, fecal calprotectin; fDPP-4, fecal dipeptidyl peptidase-4; HBI, Harvey-Bradshaw index; pMS, partial Mayo Score; sDPP-4, serum dipeptidyl peptidase-4; UC, ulcerative colitis.

phenomenon. Röhrborn et al. (6) showed that DPP-4 shedding is based on a complex interplay between different matrix

metalloproteases in a cell type-specific manner: MMP1, MMP2, and MMP14 are involved in DPP-4 shedding from human vascular smooth muscle cells and MMP9 from adipocytes. Nargis et al. verified that in patients with type 2 diabetes mellitus, circulatory Th17 cells express increased amounts of kallikrein-related peptidase 5 that consequently cleaves DPP-4 from the surface of Th17 cells, shedding them into circulation (22).

Although the knowledge on the tissues and cell types involved in the generation of the soluble pool of DPP-4 is limited, bone marrow derived cells, endothelium, adipocytes, enterocytes, and hepatocytes have been identified as important sources (23). Despite these advances, the origin and regulation of the circulating DPP-4 in various inflammatory diseases, including IBD, has not yet been clarified.

In this context, our immunohistochemical assays, implemented to evaluate the location and expression of DPP-4/CD26, lead to improved knowledge on the origin of serum and fecal DPP-4. The results obtained with healthy volunteers confirmed those from previous studies: ileal mucosa, unlike colon mucosa, expresses DPP-4/CD26 (4,5). We evidenced that DPP-4/CD26 is expressed in the lymphocytes of lamina propria in normal mucosa and in the mucosa of patients with IBD. With these results, we hypothesize that fDPP-4 is originated mainly by shedding from small intestine enterocytes because epithelial cells from normal colon mucosa did not express it. However, in patients with IBD DPP-4/CD26 was expressed in colon mucosa which also contributed to the pool of fDPP-4. UC and CD patients showed distinct immunohistochemical patterns. Patients with UC in remission presented higher immunohistochemical expression of DPP-4/CD26 and higher levels of both fDPP-4 and sDPP-4. These results suggest that this receptor/enzyme can be important to achieve and maintain remission in these

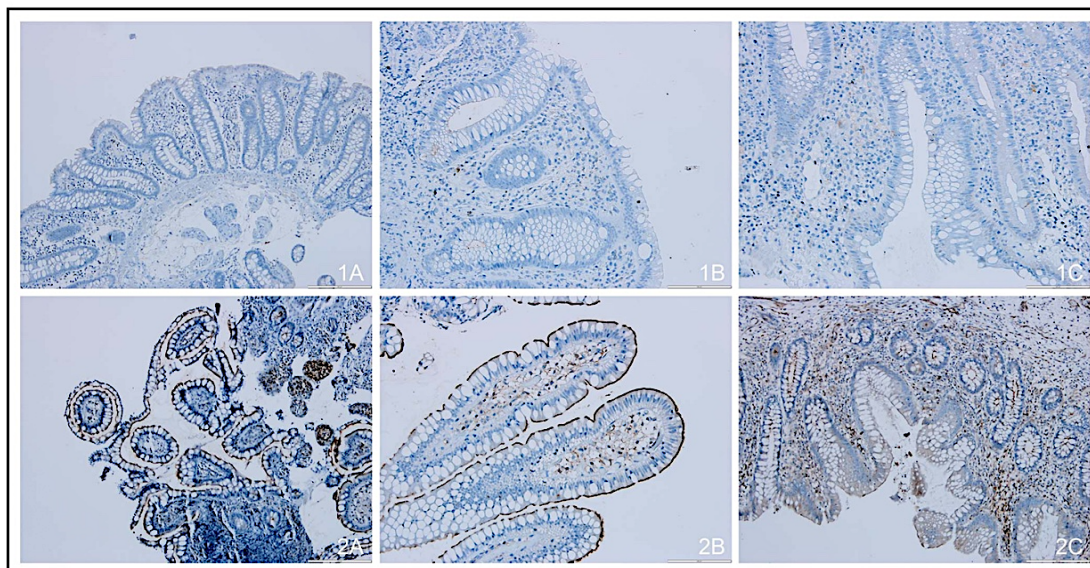


Figure 3. Immunohistochemical expression of DPP-4/CD26 in normal colon mucosa (1A, original magnification $\times 40$), UC in clinical remission (1B, original magnification $\times 100$), UC with clinical activity (1C, original magnification $\times 100$), normal ileum mucosa (2A, original magnification $\times 40$), ileal CD in clinical remission (2B, original magnification $\times 100$), and ileal CD with clinical activity (2C, original magnification $\times 40$). CD, Crohn's disease; DPP-4, dipeptidyl peptidase-4; UC, ulcerative colitis.

patients, with a protective role against relapses. On the contrary, patients with CD with active ileitis presented lower sDPP-4 but higher levels of fDPP-4, without differences in the intestinal expression of DPP-4/CD26. We believe that this phenomenon can be associated to a loss of the receptor/enzyme, from the epithelium to the intestinal lumen. These observations might be associated with increased levels and activity of the MMPs responsible for the shedding of biologically active proteins from their membrane-anchored proforms. In fact, several animal model experiments and analysis of IBD patient biopsies demonstrated that the expression and activity of the MMPs is upregulated in this disease, especially MMP1, MMP3, MMP9, and MMP26 (24). Therefore, the different patterns of fecal and serum DPP-4 expression might be reflecting such activity in patients with IBD.

Through its enzymatic function, DPP-4 can modulate the activity of several mediators of immunity and inflammation. Therefore, we hypothesize that DPP-4 may be implicated in IBD pathogenesis being responsible for activation of proinflammatory or inactivation of anti-inflammatory mediators. Low levels of sDPP-4, as evidenced in patients with active IBD, are believed to be part of a counter-regulatory mechanism that could prevent the degradation of anti-inflammatory peptides, promoting mucosal healing (13).

This study has a few limitations: (i) patients were recruited from tertiary-level hospitals, and therefore, our findings may not be applicable to the overall IBD community; (ii) only patients with UC underwent endoscopic assessment; and (iii) the endoscopic evaluations and immunohistochemical assays were performed in a small population, and thus, we anticipate that larger prospective studies must be carried out to validate these results.

In conclusion, our results suggest that fDPP-4 can be used as a noninvasive biomarker of IBD activity, particularly in patients with UC. We believe that the expression profiles of DPP-4, previously described in other processes, are unlikely to represent simple up/downregulations induced by inflammation. In fact, the DPP-4 variations herein evidenced are governed by more complex mechanisms, suggesting the existence of a functional compartmentalization of DPP-4 expression.

CONFLICTS OF INTEREST

Guarantor of the article: Fernando Magro, MD, PhD.

Specific author contributions: P.P.-L.: study concept and design, acquisition of data, recruitment of patients and collection of samples, analysis and interpretation of data, DPP-4 assays, drafting of the manuscript, study supervision, and critical revision of the manuscript. F.M.: DPP-4 assays and analysis and interpretation of data. J.A.: DPP-4 and fecal calprotectin assays and analysis and interpretation of data. R.P.-L.: recruitment of patients and collection of samples, acquisition of data, analysis and interpretation of data, and drafting of the manuscript. C.R.: DPP-4 and fecal calprotectin assays. D.M.: histological analysis. G.M.: critical revision of the manuscript. C.C.D.: analysis and interpretation of data and statistical analysis. F.C.: histological analysis and critical revision of the manuscript. F.M.: study concept and design, acquisition of data, analysis and interpretation of data, study supervision, and critical revision of the manuscript. All authors read and approved the final version of the manuscript.

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Potential competing interests: F.M. served as speaker and received honoraria from Merck Sharp & Dohme, AbbVie, Vifor, Falk, Laboratórios Vitoria, Ferring, Hospira, and Biogen. All other authors have nothing to declare.

Data sharing and data accessibility: All data relevant to the study are included in the article. Further information will be shared on reasonable request to the corresponding author.

Study Highlights

WHAT IS KNOWN

- ✓ Dipeptidyl peptidase-4 (DPP-4) exhibit altered expression profiles in patients with inflammatory bowel disease (IBD).
- ✓ Serum DPP-4 is a biomarker of IBD activity and biological treatment response and predictor of treatment escalation.
- ✓ There are no published data about fecal DPP-4 or immunohistochemical assays of DPP-4/CD26 in patients with IBD.

WHAT IS NEW HERE

- ✓ Fecal DPP-4 levels were higher in ileal Crohn's disease and lower in patients with UC exhibiting clinical activity.
- ✓ Patients with ulcerative colitis (UC) with endoscopic activity had lower fecal DPP-4 levels than remitters.
- ✓ Immunohistochemical analysis revealed aberrant DPP-4 apical expression in patients with UC in remission.

TRANSLATION IMPACT

- ✓ Fecal DPP-4 can be used as a noninvasive biomarker of IBD activity, particularly in patients with UC.
- ✓ Therapeutic decisions may be implemented early enough for patients at higher risk of disease progression.

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Supplementary Table 1. STARD 2015: An updated list of essential items for reporting diagnostic accuracy studies

Section & Topic		
TITLE OR ABSTRACT		
	1	Identification as a study of diagnostic accuracy using at least one measure of accuracy (such as sensitivity, specificity, predictive values, or AUC)
ABSTRACT		
	2	Structured summary of study design, methods, results, and conclusions (for specific guidance, see STARD for Abstracts)
INTRODUCTION		
	3	Scientific and clinical background, including the intended use and clinical role of the index test
	4	Study objectives and hypotheses
METHODS		
<i>Study design</i>	5	Whether data collection was planned before the index test and reference standard were performed (prospective study) or after (retrospective study)
<i>Participants</i>	6	Eligibility criteria
	7	On what basis potentially eligible participants were identified (such as symptoms, results from previous tests, inclusion in registry)
	8	Where and when potentially eligible participants were identified (setting, location and dates)
	9	Whether participants formed a consecutive, random or convenience series
<i>Test methods</i>	10a	Index test, in sufficient detail to allow replication
	10b	Reference standard, in sufficient detail to allow replication
	11	Rationale for choosing the reference standard (if alternatives exist)
	12a	Definition of and rationale for test positivity cut-offs or result categories of the index test, distinguishing pre-specified from exploratory
	12b	Definition of and rationale for test positivity cut-offs or result categories of the reference standard, distinguishing pre-specified from exploratory
	13a	Whether clinical information and reference standard results were available to the performers/readers of the index test
	13b	Whether clinical information and index test results were available to the assessors of the reference standard
<i>Analysis</i>	14	Methods for estimating or comparing measures of diagnostic accuracy
	15	How indeterminate index test or reference standard results were handled
	16	How missing data on the index test and reference standard were handled
	17	Any analyses of variability in diagnostic accuracy, distinguishing pre-specified from exploratory
	18	Intended sample size and how it was determined
RESULTS		
<i>Participants</i>	19	Flow of participants, using a diagram
	20	Baseline demographic and clinical characteristics of participants
	21a	Distribution of severity of disease in those with the target condition
	21b	Distribution of alternative diagnoses in those without the target condition
	22	Time interval and any clinical interventions between index test and reference standard
<i>Test results</i>	23	Cross tabulation of the index test results (or their distribution) by the results of the reference standard
	24	Estimates of diagnostic accuracy and their precision (such as 95% confidence intervals)
	25	Any adverse events from performing the index test or the reference standard
DISCUSSION		
	26	Study limitations, including sources of potential bias, statistical uncertainty, and generalizability
	27	Implications for practice, including the intended use and clinical role of the index test
OTHER INFORMATION		
	28	Registration number and name of registry
	29	Where the full study protocol can be accessed
	30	Sources of funding and other support; role of funders

Supplementary table 2. Immunohistochemical expression in intestinal tissue of DPP-4/CD26.

	UC		<i>P-value</i>
	Clinical remission (n=5)	Clinical activity (n=8)	
DPP-4 / CD26 (no IHC expression), n (%)	2 (40)	8 (100)	0.012
DPP-4 / CD26 (IHC expression), n (%)	3 (60)	0	
	Ileal CD		<i>P-value</i>
	Clinical remission (n=5)	Clinical activity (n=7)	
DPP-4 / CD26 (no IHC expression), n (%)	0	2 (29)	0.190
DPP-4 / CD26 (IHC expression), n (%)	5 (100)	5 (71)	
	Healthy controls		<i>P-value</i>
	Ileum (n=5)	Colon (n=5)	
DPP-4 / CD26 (no IHC expression), n (%)	0	5 (100)	0.002
DPP-4 / CD26 (IHC expression), n (%)	5 (100)	0	

IHC - Immunohistochemical

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Paper III

“The Role of Dipeptidyl Peptidase 4 as a Therapeutic Target and Serum Biomarker in Inflammatory Bowel Disease: A Systematic Review.”

Inflamm Bowel Dis. 2020;27(7):1153-1165.

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BASIC SCIENCE REVIEW ARTICLE

The Role of Dipeptidyl Peptidase 4 as a Therapeutic Target and Serum Biomarker in Inflammatory Bowel Disease: A Systematic Review

Francisco Jorge Melo, MD,* Pedro Pinto-Lopes, MD,*† Maria Manuela Estevinho, MD,*‡ and Fernando Magro, MD, PhD*§

Background: The roles dipeptidyl peptidase 4 (DPP4), aminopeptidase N (APN), and their substrates in autoimmune diseases are being increasingly recognized. However, their significance in inflammatory bowel diseases (IBD) is not entirely understood. This systematic review aims to discuss the pathophysiological processes related to these ectopeptidases while comparing findings from preclinical and clinical settings.

Methods: This review was conducted according to the PRISMA guidelines. We performed a literature search in PubMed, SCOPUS, and Web of Science to identify all reports from inception until February 2020. The search included validated animal models of intestinal inflammation and studies in IBD patients. Quality assessment was performed using SYRCLE's risk of bias tool and CASP qualitative and cohort checklists.

Results: From the 45 included studies, 36 were performed in animal models and 12 in humans (3 reports included both). Overall, the methodological quality of preclinical studies was acceptable. In animal models, DPP4 and APN inhibition significantly improved intestinal inflammation. Glucagon-like peptide (GLP)-1 and GLP-2 analogs and GLP-2-release-inducing drugs also showed significant benefits in recovery from inflammatory damage. A nonsignificant trend toward disease remission with the GLP-2 analog teduglutide was observed in the sole interventional human study. All human studies reported an inverse correlation between soluble DPP4/CD26 levels and disease severity, in accordance with the proposal of DPP4 as a biomarker for IBD.

Conclusions: The use of DPP4 inhibitors and analogs of its substrates has clear benefits in the treatment of experimentally induced intestinal inflammation. Further research is warranted to validate their potential diagnostic and therapeutic applications in IBD patients.

Key Words: pathogenesis, inflammation, translational, biomarker, ectopeptidase

INTRODUCTION

Inflammatory bowel diseases (IBDs) are a group of chronic relapsing autoimmune disorders, comprising Crohn's disease (CD),¹ ulcerative colitis (UC),² and an intermediate spectrum of unclassifiable conditions designated as indeterminate colitis.³ Inflammatory bowel disease present many extraintestinal manifestations and may pertain to a cluster of autoimmune diseases affecting the same patient.⁴ Left untreated, these conditions are highly debilitating and potentially life-threatening and represent a high economic burden.^{1, 2, 4} In recent decades, IBD has been the target of intensive

research, with considerable progress in the therapeutic management of the disease and related pathologies. However, most available treatment strategies have a significant amount of nonresponders and a wide range of adverse effects.^{1, 2} Thus, the development of new and optimized therapeutic weapons is now supported by research on the underlying pathophysiological mechanisms of IBD.

Dipeptidyl peptidase 4 (DPP4), homologous to cell-surface marker CD26 (cluster of differentiation 26), is a near-ubiquitous membrane protease that cleaves N-terminal dipeptides from many endogenous and exogenous peptides.⁵

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From the *Department of Biomedicine, Unity of Pharmacology and Therapeutics, Faculty of Medicine of the University of Porto, Porto, Portugal; †Department of Internal Medicine, Tâmega e Sousa Hospital Center, Padre Américo Hospital, Penafiel, Portugal; ‡Department of Gastroenterology, Vila Nova de Gaia/Espinho Hospital Center, Vila Nova de Gaia, Portugal; §Unit of Clinical Pharmacology, São João Hospital Center, Porto, Portugal

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Conflicts of Interest: FM served as speaker and received honoraria from Merck Sharp & Dohme, Abbvie, Vifor, Falk, Laboratórios Vitoria, Ferring, Hospira and Biogen. All other authors have nothing to declare.

Address correspondence to: Fernando Magro, MD, PhD, Department of Biomedicine, Unity of Pharmacology and Therapeutics, Faculty of Medicine of the University of Porto, Rua Plácido da Costa, 4200-450 Porto, Portugal, E-mail: fm@med.up.pt.

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of interleukin (IL)-2 and ultimately contributes to differentiation into a T helper (Th) 1 phenotype.^{12,13} Adenosine deaminase binding to CD26 colocalizes ADA to the cell surface, allowing local degradation of adenosine (a known inhibitor of T-cell activation) to inosine, hence controlling its extracellular levels at the immunological synapse. Furthermore, ADA binding to CD26 was found to produce a direct costimulatory response in T-cell activation.^{14,15}

Many bioactive or inactive precursor peptides are substrates of DPP4, including peptide hormones (GLP-1, GLP-2), neuropeptides (neuropeptide Y, substance P), and many chemokines and growth factors. Additionally, this enzyme acts as long as the penultimate amino acid is either proline or alanine. It is also capable of cleaving N-terminal X-glycine/serine/valine/leucine, albeit at a slower rate. Cleavage is hindered by the presence of proline in NH₂-Xaa-Xaa-Pro position.⁵ In inflammatory settings, the most relevant substrates of DPP4 are GLP-1, GLP-2, and vasoactive intestinal peptide (VIP).

In addition to its insulinotropic action, GLP-1, acting through GLP-1R, stimulates protein kinase A (PKA) and leads to the inhibition of the T-cell costimulatory CD28/CD86 signal.¹⁶ It is rapidly inactivated by DPP4 into GLP-1_{9-36/7}, with a short half-life of around 1 to 4 minutes.¹⁷ The GLP-2 is a sister molecule of GLP-1 that is co-expressed and coreleased from enteroendocrine L cells, after processing of their common precursor, proglucagon. Through its receptor, GLP-2R, localized in enteric nervous plexuses (myenteric, submucosal)¹⁸ and subepithelial myofibroblasts,¹⁹ stimulates the release of key intestinal growth factors such as KGF, IGF-1, and EGF; this mechanism reverts inflammatory changes in the intestinal epithelium.^{20,21} As with GLP-1, GLP-2 is rapidly inactivated by local DPP4 to GLP-2₃₋₃₃, with a half-life of 7 minutes; however its metabolite has a half-life of around 27 minutes.²² Additionally, GLP-2₃₋₃₃ acts as a competitive inhibitor of GLP-2 at GLP-2R.²² Teduglutide, a DPP4-resistant long half-life GLP-2 analog, is approved for use in short bowel syndrome and is under investigation for its applicability in IBD.²³ In addition, GLP-2 stimulates the release of VIP from enteric neurons.²⁴ Vasoactive intestinal peptide is a 28-amino acid peptide with a short half-life of around 1 minute and a wide range of effects, including neurotransmitter, immunomodulatory, and secretagogue activities.^{25,26} It inhibits TNF- α production by macrophages²⁷ and promotes T_H cell differentiation toward a Th2 phenotype.²⁸

Aminopeptidase N (APN) is an ectopeptidase homologous to CD13.²⁹ It is being studied in the setting of hematological disorders and gained interest as a potential co-effector of DPP4/CD26 in immune regulation.³⁰ Aminopeptidase N is involved in antigen processing and interaction with extracellular matrix proteins.³⁰ Its substrates include several immunoregulatory molecules.³⁰ It cleaves off N-terminal neutral amino acids of oligopeptides but stops if proline is in the penultimate position.³¹ These catalytic specificities, the

subcellular localization similar to CD26, and increased expression in activated T cells point to a potential role of APN/CD13 as a DPP4/CD26-substrate generator and vice versa, acting in tandem as regulators of immune responses. This is further supported by an improved anti-inflammatory response of the dual APN/DPP4 inhibition compared with antagonism of only one of either of these proteins.³² For this reason, we decided to extend the scope of this review to also include reports on the role of APN/CD13 in intestinal inflammation.

Although some reviews on the importance of DPP4 and APN on AIDs and inflammation have been published, the knowledge on their specific role in IBD pathogenesis is limited. This systematic review aims to fill this gap and provide a link between preclinical and clinical data on the role of these ectopeptidases in IBD (as well as other molecules of their related axes), through the use of validated animal models of intestinal inflammation³³ and studies on IBD patients.

METHODS

This review was conducted following the recommendations of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses Statement (PRISMA 2009).³⁴ Study screening was conducted in 3 electronic databases: PubMed, Web of Science, and SCOPUS, covering all reports published through February 4, 2020. The query used for PubMed was as follows: “DPPR OR DPPIV OR Dipeptidyl peptidase 4 OR Dipeptidyl peptidase IV OR CD26 OR ADCP2 OR Adenosine deaminase complexing protein 2 OR Aminopeptidase N OR Alanyl aminopeptidase OR Alanine aminopeptidase OR CD13 OR AAP OR APN AND inflammatory bowel disease OR crohn's disease OR ulcerative colitis.” Similar queries were used for the other 2 databases, after syntax adaptation. To ensure the inclusion of all pertinent studies, the reference lists of the included reports were reviewed by 2 independent researchers.

All preclinical reports emphasizing the pathophysiological role of DPP4/CD23 and APN/CD13 in intestinal inflammation models, in addition to clinical reports demonstrating a link between DPP4/APN and IBD, were assessed for inclusion. Reports concerning other molecules pertaining to the physiological axes of these factors were also considered, as long as there was at least an indirect link to DPP4 or APN.

The inclusion criteria were (1) articles studying the association between DPP4/CD26 and IBD in animal models of intestinal inflammation and human patients; (2) articles studying the association between APN/CD13 and IBD in animal models of intestinal inflammation; and (3) articles studying the association between related molecules of the DPP4 axis with known therapeutic potential, specifically GLP-1 and GLP-2 (and molecules that influenced GLP-1 or GLP-2 levels, such as TGR5, G-protein coupled receptor [GPR]-40, and GPR120), and IBD in animal models of intestinal inflammation and human patients. No restrictions on publication language were applied.

The exclusion criteria were (1) review papers, metaanalyses, letters, commentaries, guidelines, editorials, meeting abstracts, and case reports; (2) studies with no relation to IBD or related animal models of colitis (intestinal cancer studies, radiation-induced injury, etc.); (3) studies with no pathophysiological association with DPP4 or APN; (4) studies including other substrates of DPP4 and APN (setting a limit to study screening and to avoid an overreaching and unfocused review of all possible substrates and their influence in DPP4-dependent inflammatory pathways); and (5) studies without available abstract.

The risk of bias in individual studies was assessed using quality evaluation tools/scales adapted to study type. For pre-clinical animal studies, SYRCLE's risk of bias (RoB) tool was used.³⁵ For studies in humans, CASP checklists were used for qualitative³⁶ and cohort³⁷ reports. These tools were applied by 2 independent reviewers, and discrepancies were solved by consensus.

RESULTS

Study Selection and Characteristics

Study selection, following the PRISMA 2009 Flow Diagram,³⁴ is outlined in Figure 2. Of the 45 studies selected for review, 36 used animal models of intestinal inflammation,^{38–73} and 12 concerned human IBD patients^{50, 66, 67, 74–82} (3 reports included both^{50, 66, 67}). The characteristics and main results of

animal studies are described in [Supplementary Table 1](#) and those of human studies in [Table 1](#). For animal studies, disease and animal models, with respective variations, are compiled in [Supplementary Table 2](#).

Most animal studies consisted of interventional protocols of varying durations that tested the effects of DPP4i, APNi, GLP-1, or GLP-2 analogs (mostly long-acting, DPP4-resistant, or by continuous infusion) and related drugs on the recovery from experimentally induced intestinal lesions.

The most used model of colitis was the dextran sulfate sodium (DSS) model ($n = 25$), followed by 2,4,6-trinitrobenzenesulfonic acid (TNBS, $n = 7$), indomethacin ($n = 5$), HLA-B27 ($n = 2$), CD4+ transfer ($n = 1$), and irinotecan ($n = 1$) models. The most commonly used strains of mice were Balb/c and C57BL/6 mice. Seven reports utilized knock-out mice for CD26/DPP4, and 1 study used *Glp2r*^{-/-} mice.

Despite wide variations in protocol, we decided to interpret each model's group as a whole, inasmuch as all experiments were able to elicit an inflammatory response to some extent and were therefore deemed internally valid.

With a single exception of an interventional study,⁷⁴ all studies in human IBD patients were observational, focusing on serum activity, levels of DPP4, APN, GLP-2, GLP-2R, and others, in addition to associated clinical and endoscopic findings. None of the included studies in IBD patients used DPP4 inhibitors.

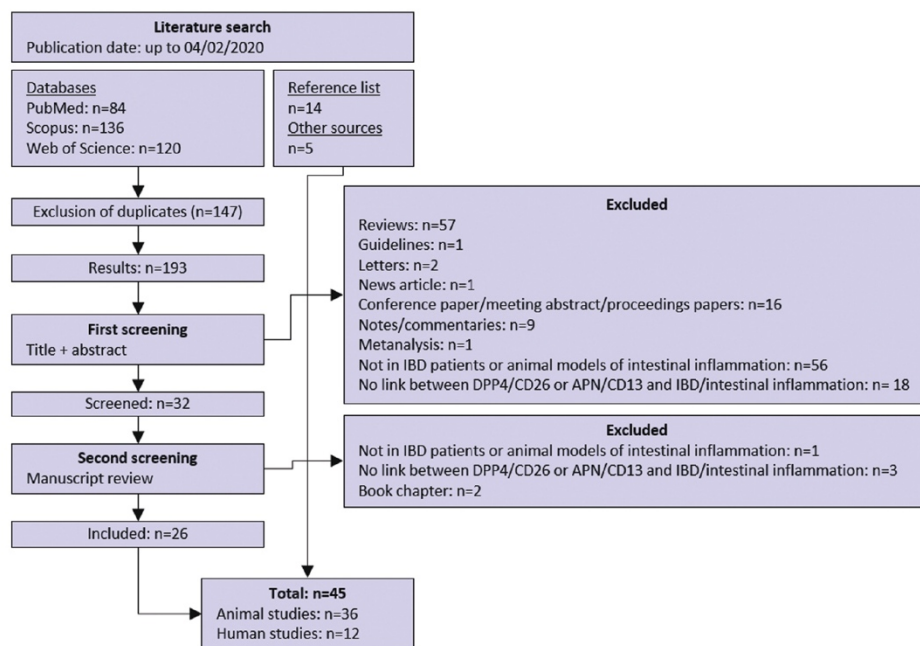


FIGURE 2. Study screening and selection process.

TABLE 1. Characteristics and Main Results of Human Studies

Authors (Year)	Study Origin	Study Type	IBD Assessment	Disease Characteristics	Intervention	Outcomes Assessed	Results
Buchman AL et al (2010)	USA and Canada (multicenter)	Prospective CD, moderate-to-severe (pilot) (n = 100) (Extension: n = 48)	CDAI	CDAI 220–450 Mean disease duration 11.1–13.7 ± 9.5–10.9 years 24% concomitant therapy with immunomodulators	Teduglutide 0.05/0.10/0.20 mg/kg/d, sc, 8w (Extension: teduglutide 0.10 mg/kg/d, sc, lid, for 12 additional weeks)	Primary: response (100pts CDAI) or remission (CDAI ≤ 150) at week 8; Secondary: response and remission at weeks 2 and 4, mean changes in disease severity (CDAI), mean decrease in # of liquid bowel movements, mean decrease in CRP.	No statistically significant differences from placebo. In the 0.2 mg/kg/d group, 40% achieved remission at 8 weeks. No difference was observed in serum CRP at any timepoint. Plasma citrulline substantially increased over time in all teduglutide groups.
El-Jamal N et al (2014)	Not reported	Cross-sectional CD (n = 19) UC (n = 15)	Not reported	Insufficient data	—	GLP-2R expression in human intestine of IBD patients.	Significantly higher expression of GLP-2R mRNA in the colon of healthy and IBD patients, vs in the ileum. Significantly lower levels of GLP-2R mRNA in healthy colon and ileal samples from CD patients, vs controls; and further significant reduction in inflamed samples, vs healthy samples; Significantly lower levels of GLP-2R mRNA in healthy ileal samples from UC patients, vs controls; and further significant reduction in inflamed samples, vs healthy samples; Significantly lower levels of GLP-2R mRNA in inflamed colon samples from UC patients, vs controls, but no difference in healthy colon samples of UC patients, vs controls.
Hildebrandt M et al (2001)	Germany, single center	Cross-sectional CD (n = 63) UC (n = 47) Controls (n = 28)	CDAI Rachmilewitz score (UC)	Mean disease duration— CD: 10.8 ± 8.1 s years UC: 11.9 ± 8.5 s years	—	sDPP4 activity and lymphocyte CD26/DPP4 expression in IBD patients.	Similar percentages of CD2+/CD26+ cells in peripheral blood between patients and healthy controls. Higher number of CD25+/CD26+ and CD2+/CD25+ cells in IBD patients. DPP4 activity was inversely correlated with disease activity, orosomucoid concentrations and CRP.

TABLE 1. Continued

Authors (Year)	Study Origin	Study Type	IBD Assessment	Disease Characteristics	Intervention	Outcomes Assessed	Results
Magro D et al (2017)	Brazil, single center	Cross-sectional	CD (n = 20) CDAI (active: >150; remission: ≤150) Montreal Classification	Active CD: n = 10 Remitting CD: n = 10	—	Correlation between serum levels of LPS and CD26, and serum levels of CRP, interleukins, TNF-α and CDAI.	Significantly higher levels of serum LPS and CRP in active and inactive CD group, vs controls. Significantly lower levels of IL-1β, IL-6, IL-17 and CD26 in CD groups, vs controls. Nonsignificant higher level of TNF-α in active CD group, vs control (p = 0.056). Negative correlation between CRP and LPS in CD groups.
Moran GW et al (2012)	Not reported	Cross-sectional	CD (n = 26) CDAI CRP	CDAI = 174.5 ± 14.26 CRP = 20.4 ± 5.4 mg/L	—	DDP4 expression and correlation to underlying intestinal inflammation (CDAI, CRP).	Significantly lower levels of plasma and tissue (terminal ileum) DDP4 in CD patients, vs controls. Significant negative correlation between plasma DDP4 and CRP.
Pinto-Lopes P et al (2020)	Portugal, multicenter	Prospective cohorts	CD (n = 101) UC (n = 94) Controls (n = 52) CD: HBI (clinical remission: ≤4) UC: pMS (clinical remission: ≤1) Montreal Classification	Active CD: HBI = 7.6 ± 3.2 Remitting CD: HBI = 1.4 ± 1.3 Active UC: pMS = 4.7 ± 2.3 Remitting UC: pMS = 0.1 ± 0.3	—	Role of sDPP4 as a biomarker in predicting the need for treatment escalation and monitoring response to biological therapy; correlation between sDPP4 levels and endoscopic activity and clinical activity scores.	Patients with active IBD had significantly higher serum CRP and FC levels and lower sDPP4, vs those in clinical remission. sDPP4 levels were negatively correlated with FC, serum CRP and DAI. FC was positively correlated with serum CRP and DAI. sDPP4 was inversely correlated with both disease activity scores (HBI and pMS) and endoscopic activity groups (stronger in CD, vs UC). sDPP4 activity was significantly higher in responders (stronger in UC, vs CD).
Salaga M, Mokrowiecka A, Zielinska M et al (2017)	Not reported	Cross-sectional	CD (n = 9) UC (n = 12) Controls (n = 8) 13 colon biopsy samples 29 serum samples CD: 5-ASA (n = 5) Anti-TNF-α (n = 4) UC: 5-ASA (n = 12)	CD: 5-ASA (n = 5) Anti-TNF-α (n = 4) UC: 5-ASA (n = 12)	—	Expression of GLP-2 and GLP-2R in the serum and colon of IBD patients.	Significant decrease in the expression of serum GLP-2 in CD patients. Significant decrease in the expression of colon GLP-2R in UC patients.

TABLE 1. Continued

Authors (Year)	Study Origin	Study Type	IBD Assessment	Disease Characteristics	Intervention	Outcomes Assessed	Results
Salaga M, Mokrowiecka A, Jacenik D et al (2017)	Not reported	Cross-sectional	CD (n = 17) UC (n = 10) Controls (n = 8) 35 colon biopsies 56 serum samples	Montreal Classification CD: 5-ASA (n = 10) Anti-TNF- α (n = 7) UC: 5-ASA (n = 10)	—	Characterization of intestinal tissue and serum expression levels of APN and NEP in IBD patients.	Significantly higher expression of APN mRNA in colonic tissue of CD patients (nonsignificant increase in UC patients). Nonsignificant increase in the expression of NEP protein in colonic tissue of CD patients. No changes in serum levels.
Schmidt PT et al (2005)	Sweden, single center	Cross-sectional	CD (n = 4) UC (n = 15) Controls (n = 10)	Not reported Active disease: CD (n = 4) UC (n = 5) Chronic/no inflammation: UC (n = 10) CD: 5-ASA (n = 1) Glucocorticoids (n = 1) Metronidazole (n = 1) UC: 5-ASA (n = 1) Prednisolone (n = 1) Azathioprine (n = 1)	Meal stimulation (430 kcal)	Tissue levels and postprandial secretion of GLP-2 and PYY in IBD patients.	No significant differences in tissue content or plasma concentration after meal stimulation of GLP-2 and PYY between IBD patients and controls.
Tsukahara T et al (2015)	Japan, single center	Cross-sectional	CD (n = 16) Controls (n = 15)	Not reported Previous therapeutic exposure: Anti-TNF- α (n = 4) Immunomodulator (n = 2) Corticosteroids (n = 0)	—	Expression of GPR40 and GPR120 in the ileal mucosa of CD patients and its correlation with inflammatory parameters.	Intestinal epithelial cells express GPR40, but rarely express GPR120, in the normal ileal mucosa. Both were overexpressed in inflamed ileal mucosa. GPR40 and GPR120 are co-expressed in L cells (significant positive correlation). HBI values significantly correlated with GPR120 expression, but not GPR40. Significantly higher levels of TNF- α . Both GPR120 and GPR40 expression levels significantly correlated with levels of TNF- α , but not those of IL-6 or IL-1 β . No differences in protein and mRNA expression of proglucagon in CD patients vs controls.

TABLE 1. Continued

Authors (Year)	Study Origin	Study Type	IBD Assessment	Disease Characteristics	Intervention	Outcomes Assessed	Results
Varljen J et al (2005)	Croatia, single center	Cross-sectional	CDAI UC: Truelove and Witts' (TW) classification	Insufficient data	—	Relation between sDPP4 activity with clinical and inflammatory parameters in patients with IBD; potential of sDPP4 activity as a distinguishing marker between CD and UC.	sDPP4 activity was significantly decreased in both CD and UC patients, vs controls, although no significant differences were found between the 2 IBD groups. DPP4 activity inversely correlates with CDAI score in CD patients, and TW in UC patients. Significant difference in sDPP4 activity between male and female patients with UC. No correlation between sDPP4 activity and routine laboratory parameters in either disease, nor in relation to the location and extension of pathological lesions.
Xiao Q et al (2000)	Canada, single center	Cross-sectional	GLP-2 CD (n = 39) CD without bowel resection (n = 30) CD with bowel resection (n = 9) UC (n = 21) Healthy controls (n = 14) Immune controls (n = 38) DPP4 CD without bowel resection (n = 1) CD with bowel resection (n = 5) UC (n = 1) Healthy controls (n = 6)	Mean disease duration— GLP-2 CD without bowel resection: 4.5 ± 5.1 years CD with bowel resection: 12.9 ± 12.7 years UC: 4.3 ± 5.9 years DPP4 CD without bowel resection: 8 years CD with bowel resection: 14.3 ± 3.8 years UC: 1 year	—	Abnormalities in the levels and/or molecular forms of circulating GLP-2 in IBD patients. No differences between plasma levels of total immunoreactive (IR)-GLP-2 between normal healthy and immunocompromised control subjects, and between normal controls and UC patients. Total plasma IR-GLP-2 levels were significantly decreased in CD patients, vs controls. No differences in total IR-GLP-2 levels between CD subgroups according to disease site, but significant decrease in total IR-GLP-2 in CD patients who had a history of intestinal resection. Significantly higher bioactive GLP-2 ₁₋₃₃ levels in IBD patients (higher ratio of GLP-2 ₁₋₃₃ to GLP-2 ₃₋₃₃). Significantly lower plasma DPP4 activity in IBD patients, vs normal controls.	

Abbreviations: CD#, cluster of differentiation #; CDAI, Crohn's disease activity index; DAI, disease activity index; HB, Harvey-Bradshaw index; IFN- γ , interferon-gamma; LPS, lipopolysaccharide; pMS, partial Mayo score; Th, T helper cell; TNF- α , tumor necrosis factor alpha; 5-ASA, 5-aminosalicylic acid/mesalazine

Quality Assessment

The results of the methodological quality evaluation are summarized in [Supplementary Tables 3–5](#). Overall, preclinical studies do not report sufficient details to allow for proper risk of bias assessment, with most items being answered as “unclear,” according to SYRCLE’s RoB tool³⁵ ([Supplementary Table 3](#)). Unlike randomized clinical trials (RCTs), experimental studies in animals usually do not implement tools to assess internal validity and risk of bias in their study design. Consequently, the generalizability of their findings is compromised due to poor reporting.³⁵ In the revised studies, the most affected domains were inadequate randomization during allocation of study groups and outcome assessment and lack of blinding/concealment.

For the included reports on human patients, we used 2 separate CASP checklists, according to study design: qualitative³⁶ ([Supplementary Table 4](#)) and cohort³⁷ ([Supplementary Table 5](#)). Human qualitative studies showed a better performance under appraisal when compared with animal studies due to better reporting. Nevertheless, some doubts arise (eg, “Can’t tell”) in the items related to adequate recruitment, representativeness, and significance of the study population. Furthermore, the studies revealed a low ability to extrapolate findings to the general population, most often due to low sample size and high homogeneity (eg, only female subjects, only recruited from tertiary centers, etc.). As expected, cohort studies had better classifications, yet the generalizability of the results was also an important drawback.

DPP4/CD26 and APN/CD13

Animal studies

None of the studies in CD26^{-/-} mice with experimentally induced colitis showed significant differences in histological and clinical scores compared with wild-type strains.^{44, 46–48, 53} Conversely, Iwaya et al⁵⁶ demonstrated a significant yet transient improvement of intestinal inflammation in an early phase of colitis development in DPP4-deficient (F344/Du) rats.

Reports using DPP4 inhibitors showed either partial or significant improvement of clinical and histological scores and reduction of MPO activity and pro-inflammatory cytokine levels.^{42, 43, 51, 52, 55, 61, 65, 67, 71–73}

Bank et al⁴³ studied the effects of combined inhibition of both DPP4 and APN on colitis attenuation. The dual DPP4/APN inhibitor IP12.C6 promoted the expression of TGF- β and FOXP3 compared with separate inhibition and controls. In a similar manner, dual inhibition of APN and neprilysin (NEP, CD10) by sialorphin or a sialorphin analogue also significantly improved colitis, in part through μ - and κ -opioid receptor-dependent mechanisms.^{57, 66}

Human studies

Serum CD26/DPP4 expression and/or activity was found to be significantly lower in IBD patients compared with healthy

controls.^{75–77, 80–82} Patients with active disease showed lower levels than patients in remission, and sCD26/DPP4 levels were negatively correlated with disease severity and classical inflammatory markers, such as C-reactive protein (CRP).^{75, 77, 80, 82} In addition, Hildebrandt et al⁸² reported significant increases in CD25⁺/CD26⁺ and CD2⁺/CD25⁺ peripheral blood lymphocytes in IBD patients vs controls but no differences in the population of CD2⁺/CD26⁺ cells. Moran et al⁷⁶ showed that DPP4 expression was significantly reduced in tissue samples from the terminal ileum of IBD patients. Still, the authors reported significantly higher levels of DPP4 in a Caco-2 cell-based study after exposure to rhTNF- α .⁷⁶ As with similar reports, they showed an inverse correlation between serum DPP4 (sDPP4) levels and CRP; however, such correlation was not found for CDAI.⁷⁶

In a recent multicentric prospective cohort undertaken by our study group,⁷⁷ sDPP4 was found to have a strong inverse correlation with clinical and endoscopic activity. It performed equally well in postoperative CD patients. Optimal cutoff points were defined based on receiver operating characteristics (ROC) curve analysis of sDPP4 and 2 other biomarkers, CRP and fecal calprotectin (FC). These were used to predict clinical activity, endoscopic activity, and treatment escalation in both CD and UC patients. Stratification according to these cutoffs in a Kaplan-Meier curve showed that after 1 year, 62.2% of CD patients and 36.3% of UC patients with DPP4 levels below the cutoff (≤ 1452 ng/mL and ≤ 1472 ng/mL, respectively) had escalated treatment, as opposed to 7.8% of CD and 4.1% of UC patients with DPP4 levels above the cutoff. The use of 3 simultaneous biomarkers proved to have a higher discriminative power. Eighty percent of the CD patients with 3 positive biomarkers escalated treatment after 1 year vs 3.3% of the patients with triple negative biomarkers. Regarding UC, 85.0% of the patients with 3 positive biomarkers escalated treatment after 1 year vs none with 3 negative biomarkers. All 3 biomarkers had a similar ability to distinguish between IBD responders and nonresponders. In a subset of the IBD population with active disease but without CRP elevation, sDPP4 was able to discriminate endoscopic activity better than FC.

Aminopeptidase N mRNA expression was significantly higher in colonic tissue of CD patients (nonsignificant increases in UC patients and in the expression of NEP protein levels in CD patients), but no changes were detected in serum levels.⁶⁶

Glucagon-like Peptides

Animal studies

Thirteen reports studied the effects of GLP-2, either by continuous infusion,^{38, 41} long-acting polymer-coupled (XTEN,³⁹ PEG^{62, 63}) or microsphere-associated⁶⁹ molecules, or degradation-resistant analogs.^{45, 49, 54, 60, 70} These studies reported significant improvements in histological and clinical scores, reduction in pro-inflammatory cytokine levels and MPO activity,

and a significant intestinal growth (intestinotrophic) response. This was assessed by increased crypt cell proliferation and increased survival/reduced apoptotic rates and manifested by decreased intestinal length reduction after experimental induction of inflammation. A study conducted in *Glp2r^{-/-}* mice demonstrated a functional Paneth cell defect, with reduced bactericidal activity and reduced expression of intestinotrophic factors.⁵⁹ Furthermore, similar outcomes were obtained by activation of GPR40 (FFAR1, free fatty acid receptor 1⁸³) and TGR5 (GPBAR1, G protein-coupled bile acid receptor 1⁸⁴) in enteroendocrine L cells by specific agonists with increased expression and release of GLP-2.^{58, 64}

A report on GLP-1 nanomedicine revealed a significant decrease of pro-inflammatory cytokine IL-1 β levels and tissue damage, in addition to a partial attenuation of the diarrheal phenotype.⁴⁰

Human studies

In accordance with the results in animal models, a pilot study⁷⁴ of a marketed GLP-2 analog, teduglutide, demonstrated a trend toward an increased response and remission rate in IBD patients, although these differences were not statistically significant. A significant increase in plasma citrulline levels, an indirect marker of intestinal mucosal mass, was also reported.

Other human studies found lower expression of GLP-2R and GLP-2 in an inflammatory setting in IBD patients compared with noninflamed sites and healthy controls.^{50, 67, 78, 81} Schmidt et al⁷⁸ found no differences in tissue or plasma levels of GLP-2 or peptide YY (PYY) after meal-stimulation between IBD patients and healthy controls.

A report on CD patients showed overexpression of both GPR40 and GPR120 (FFAR4, free fatty acid receptor 4)⁸⁵ in inflamed ileal mucosa and a GPR120-dependent inhibition of GPR40-induced GLP-2 expression by L cells, promoted by upregulation of GPR120 by TNF- α .⁷⁹

DISCUSSION

Dipeptidyl peptidase 4/CD26 and its substrates have been recognized as important mediators of inflammation and immunity. However, data on the efficacy of manipulating the incretin axis as a treatment modality in IBD lack consistency.^{49, 70, 74}

Despite the success of DPP4 inhibitors as antidiabetic drugs, the use of DPP4 inhibitors raises special concerns regarding the potential short and long-term adverse effects of inhibiting a molecule with such a broad spectrum of interactions. This is conditioned by the specific cell population expressing this protein; the local availability, half-life, and bioactivity of its substrates; reaction rates; and substrate generation by other proteases (such as APN). In this context, different tissues may present different metabolic signatures resulting from DPP4 action, depending on the underlying pathophysiological conditions.

Through its enzymatic activity, DPP4 can inactivate several inflammatory mediators, such as Mig (CXCL9), IP-10 (CXCL10), and I-TAC (CXCL11), greatly reducing their chemotactic activity, although without hindering antiangiogenic activity.⁸⁶ Conversely, cleavage of chemokine LD78 β by CD26/DPP4 significantly enhanced its lymphocyte and monocyte chemotactic properties.⁸⁷ In addition, DPP4-truncated products show different receptor interaction²² and selectivity⁸⁸ compared with their noncleaved precursor peptide. Drugs acting on these pathways can, therefore, have different net effects. Further research is warranted to assess for significant differences between truncated or nontruncated forms, with a comprehensive assessment of their regulatory mechanisms and their relevance to the inflammatory milieu.

In our systematic review, all studies showed at least a moderate therapeutic benefit of DPP4i in animal models of colitis. On the one hand, these results reflect the inhibition of the catalytic activity of DPP4i over bioactive substrates such as GLP-2, GLP-1, and VIP, greatly extending their half-lives. This indirectly inhibits costimulatory signals of T-cell activation and the production of Th1-polarizing cytokines and chemokines while promoting intestinal proliferation and tissue recovery (Fig. 1). On the other hand, a more direct effect of DPP4i action cannot be excluded because CD26^{-/-} mice did not possess any inherent resistance to colitis development, nor did they display an enhanced rate of repair of the damaged mucosal tissue. The protective effect of DPP4i was only observed in the presence of DPP4/CD26, suggesting that DPP4i may have unknown mechanisms of action besides blocking catalytic activity.^{53, 72, 73}

In line with these findings, all animal studies with GLP-2 and GLP-1 analogs showed a clear benefit (Supplementary Table 1) regarding intestinal proliferation, preservation of tissue architecture, and prevention of weight loss in the setting of induced inflammatory lesions. This is supported by similarly positive findings in reports using drugs that induce the release of endogenous GLP-1 and GLP-2, such as GPR40⁸⁸ and TGR5 agonists.⁶⁴

The extrapolation of these data to humans is challenging. One study⁷⁴ enrolling patients with moderate to severe CD treated with the GLP-2 analog teduglutide failed to achieve significance from placebo, although a trend toward remission was observed (Table 1). However, this report was limited because it was based on a pilot study with a relatively small sample size. Moreover, the study had a high dropout rate, especially due to adverse effects (up to 31%) and uncomfortable posology (daily subcutaneous injections).

Lower expression of GLP-2R was reported in IBD patients vs controls, further significant reduction was reported in inflamed tissue vs healthy samples, and a significant decrease was reported in the expression of serum GLP-2.^{50, 67} One report, however, found no significant differences between IBD patients and controls in tissue content or plasma concentration

of GLP-2 or PYY after meal stimulation.⁷⁸ Whether this is a cause or a consequence of the underlying pathological process remains to be elucidated. Still, the use of GLP-2 analogs and DPP4i in IBD is a double-edged sword. Despite their benefits, these drugs promote GLP-2-dependent intestinal tumor cell proliferation and migratory activity,⁸⁹ which may be of concern in IBD patients who are already at higher risk of developing colorectal carcinoma.^{1,2} Further research is needed to assess their viability and safety as IBD drugs.

Despite their anti-inflammatory effects, DPP4i also block DPP4-mediated degradation of pro-inflammatory chemokines,⁸⁶ possibly contributing to the perpetuation of inflammatory stimuli. As such, one could argue that DPP4 inhibition enhances certain pro-inflammatory pathways and leads to either a new onset or a flare-up of the underlying IBD or other inflammatory conditions. Nevertheless, a recent meta-analysis did not find an increased risk of IBD in patients under DPP4i therapy.⁹⁰ This same mechanism can also be beneficial in specific pathologies. A recent study by Barreira da Silva et al⁹¹ showed that DPP4 inhibition prevented CXCL10 truncation (and inactivation) and enhanced CXCR3-mediated antitumor immunity and trafficking of T cells into the tumor parenchyma. Furthermore, DPP4 is cleaved by a not fully understood mechanism (eg, shedding), and its soluble form, which is excreted to plasma, may have yet uncharacterized endocrinological effects.¹⁰

Aminopeptidase N acts in synergy with DPP4 for the regulation of immune responses by cleaving peptides preprocessed by DPP4 or by generating substrates susceptible to cleavage by DPP4.⁹² Dual inhibition of APN/DPP4 had statistically significant beneficial effects in animal models of colitis and may be of clinical significance as a new direct or adjuvant treatment modality in many pathologies.^{43,93}

In recent studies, human Th17 cells were implicated in the pathogenesis of many autoimmune diseases, including IBD⁹⁴; this represents a step forward from the previous dichotomous Th1/Th2 paradigm. Bengsch et al⁹⁵ demonstrated that CD26⁺⁺ (highly expressing) cells express typical markers of type 17 differentiation, even before stimulation, suggesting that CD26⁺⁺ T cells harbor the Th17 lineage. Moreover, Th1 and Th2 cells were shown to be compatible with an intermediate CD26⁺ phenotype, whereas regulatory CD25⁺CD127⁺FOXP3⁺ IL-10-producing T cells showed an even lower expression. Patients with active IBD were found to have the highest frequency of tissue-infiltrating Th17 cells. A strong increase was observed in inflamed tissue lesions, where 25%–50% of CD26⁺⁺ T cells produced IL-17 upon stimulation, in contrast to peripheral blood, where only about 5% of CD26⁺⁺ T cells produced IL-17. These findings suggest an incomplete differentiation of Th17 cells in peripheral blood due to the lack of sufficient stimulatory signals found in the proinflammatory cytokine-rich environment of lesion sites. Thus, a link has been established between CD26 and Th17, corroborating the preponderant role of CD26 in autoimmunity.

In the dawning of the microbiome age, DPP4 gains even more interest. A recent proof-of-concept study demonstrated a DPP4-like activity of gut microbiota,⁹⁶ extending toward uncharted territories the importance of intestinal microbiome–host interactions in pathological settings.^{59,97}

Dipeptidyl peptidase 4 is also a potential serum biomarker for many diseases, including cancer and IBD, which have become an intense target of investigation in recent years.^{77,98} As evidenced in recent studies, sDPP4 levels and activity are significantly lower in IBD patients vs healthy controls, and sDPP4 correlated negatively with other disease activity markers such as C-reactive protein, orosomucoid and fecal calprotectin, and disease activity scores (Harvey-Bradshaw Index [HBI], partial Mayo Score [pMS], Crohn's Disease Activity Index [CDAI], and Truelove and Witts index [TW]) and endoscopic activity groups.^{75–77,80,82} As reported in our previous study,⁷⁷ an important benefit of the use of sDPP4 as a biomarker is the ability to predict the need for treatment escalation from baseline sDPP4 levels at an early stage of the disease process. This enables the identification of patients who would benefit a priori from a more aggressive treatment strategy, avoiding exposure to potentially ineffective drugs and the consequent risk of adverse effects. A biomarker that could direct clinicians to more effective treatment options (skipping the necessary steps of treatment escalation with safety) may, at the end of the road, prove to be more efficient and resource-sparing, and enable a faster control of the underlying disease. This translates to obvious gains for the patient in terms of time, expenses, and quality of life. Higher-powered prospective studies with larger sample sizes are needed to confirm these findings and prove their usefulness in a clinical real-world setting.⁷⁷

This review includes 45 studies and is, to the best of our knowledge, the first attempt to systematize the role of DPP4, APN, and related substrates in IBD. It also provides, for the first time, an overview of the integration of the underlying pathophysiological processes and potential applications in clinical practice. We highlight our translational approach to this subject (from preclinical animal models of disease to studies in IBD patients), which allowed to emphasize the existing knowledge gaps within and between both settings.

Nevertheless, this systematic review is hindered by some limitations. Stemming from the inherent limitations of the included reports, the overall quality of preclinical experimental studies was less than desirable. As illustrated in [Supplementary Table 2](#), protocol variations within the “same” model and the considerable variability of animal strains and species do not allow for a reliable comparison between experiments. Underreporting of the protocol execution and the subjective nature of the quality assessment tools also limit the internal validity and the extrapolation of data from animal to human subjects. Most reports with human populations suffered for having a cross-sectional design (only one study was

a prospective cohort⁷⁷) or low sample size (Table 1), and only one⁷⁴ had an interventional approach akin to animal studies.

CONCLUSION

Despite the ubiquitous position and wide spectrum of interactions of these ectopeptidases intertwining with many known—yet poorly understood—pathophysiological processes, their diagnostic and therapeutic benefits may be soon applied to the growing roster of clinical tools for the management of IBD. Still, many concerns regarding their potential for stimulating carcinogenesis and immune dysregulation and their viability as biomarkers still need to be evaluated in depth. Further research is required to achieve the necessary data robustness to introduce these new applications into clinical practice with confidence and safety.

SUPPLEMENTARY DATA

Supplementary data is available at *Inflammatory Bowel Diseases* online.

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Supplementary Table 1. Characteristics and main results of animal studies

Authors (Year)	Animal model	Disease model	Assessed outcomes	Main results
Alavi K et al. (2000)	F344 (control) F344-HLA-B27 (25w, F)	HLA-B27 spontaneous model of GI inflammation	Effects of GLP-2 (50 µg/kg/d, by jugular catheter, delivered at a constant rate of 0.5 µL/h by a subcutaneously placed osmotic pump, 14d) on intestinal inflammation (percent mucosal damage, histological lesion score). Effects of GLP-2-2G-XTEN vs GLP-2-2G on intestinal inflammation (adhesion and transulceration scores).	Significant reduction in gross mucosal damage and histological lesion score. Significantly lower TNF-α and IFN-γ expression levels.
Alters S et al. (2012)	Wistar rats (M)	Indomethacin (7 mg/kg, 1id, s.c., 2d)	GLP-2-2G 12.5 nmol/kg, 2id, 5d (d-3 to d2) GLP-2-2G-XTEN 25 nmol/kg, 1id, 5d (d-3 to d2) 25 nmol/kg, q2d, 3d (d-3, d-1, d1) 75 nmol/kg, 1id, 1d (d-3) Effects of GLP-1-SSM (15 nmol/100 µL, i.p., 1id, 7d) on colitis.	GLP-2-2G-XTEN showed a two-fold increase in net body weight gain. Small intestine in mice treated with GLP-2-2G-XTEN and GLP-2-2G was significantly longer and less ulcerated. GLP-2-2G-XTEN and GLP-2-2G significantly reduced small intestine TNF-α levels. There was a clear dose-response to GLP-2-2G-XTEN treatment.
Anbazhagan A et al. (2017)	C57BL/6J mice (6-8w, M)	DSS (3%, 7d)		A single prophylactic dose of GLP-2-2G-XTEN (75 nmol/kg, 1id, 1d, d-3), had comparable results to ten doses of GLP-2-2G (12.5 nmol/kg, 2id, 5d, d-3 to d2, total dose of 125 nmol/kg). Significant decreases in IL-1β mRNA and DRA (SLC26A3) protein levels; Anti-diarrheal effects: regulation of alkaline secretion by DRA Cl ⁻ /HCO ₃ ⁻ exchange – maintenance of proper mucus layer; partial attenuation of diarrheal phenotype. Anti-inflammatory activity: partial preservation of crypt structures; better survival or rapid regeneration of colonic epithelial crypt cells.
Arthur G et al. (2004)	F344 (control) F344-HLA-B27 (F)	HLA-B27 spontaneous model of GI inflammation	Effects of GLP-2α (50 µg/kg/d, by internal jugular catheter, delivered at a constant rate of 0.5 µL/h by a subcutaneously placed osmotic pump, 14d; 50 µg/kg/d, by terminal ileum catheter, delivered at a constant rate of 0.5 µL/h by a subcutaneously placed osmotic pump, 14d) on bowel inflammation (histologic score) and IBD symptoms (stool score); comparison between intravenous and intraluminal routes of administration.	Significant improvement in stool scores. Significantly lower number of overall bowel lesions in the intravenous group. Significant improvement in histologic score in the intraluminal group.

Ban H et al. (2011)	C57/B6 mice (9w, F)	DSS (2%, 5d)	Effects of ER-319711 (100 mg/kg/d, 2id, 14d) on plasma GLP-2 concentration, epithelial proliferation and colitis attenuation (DAI).	Partial amelioration of colitis: increased proliferation and regeneration of epithelium, significant improvement in DAI and increases in colon weight and length.
Bank U, Heimbürg A et al. (2006)	Balb/c mice (F)	DSS (3%, 7d)	Effects of LZNP (lys[Z(NO2)]-pyrrolidide, DPP4i, 5 mg/kg, d3-7), actinonin (APNi, 5 mg/kg, d3-7) and IP12.C6 (dual DPP4i/APNi, 5 mg/kg, d3-7), on colitis attenuation (DAI) and inflammatory parameters (PMN, TGF- β , Foxp3 mRNA).	Inhibitor combination: significant reduction in DAI vs controls (p<0.01). Combined inhibition of APN and DPP4 promotes TGF- β and FOXP3 expression.
Baticic L et al. (2011)	C57BL/6 mice (WT; CD26 ^{-/-}) (8-10w, M)	TNBS (5%, dissolved in 50% ethanol, 0,1 mL)	Effects of DPP4/CD26 deficiency on colitis (histomorphometrical analysis), NPY, VIP, IL-6 and IL-10 levels.	No significant differences in analyzed histomorphometrical parameters. CD26 ^{-/-} mice constitutionally have significantly higher serum VIP, IL-6 and IL-10 concentrations, and higher brain IL-6 levels. In the setting of acute inflammation, CD26 ^{-/-} mice showed significantly higher colon and serum VIP, NPY, IL-6 and IL-10 levels, significantly higher brain VIP levels, and significantly lower brain NPY and IL-6 levels.
Boushey RP et al. (1999)	CD1 mice (7-8w, F)	Indomethacin (7 mg/kg, 1id, s.c., 2d) Indomethacin (20 mg/kg, 1id, s.c., 2d)	Effects of h[Gly ² GLP-2 (2.5 μ g, s.c., 2id, 6d, d-4 to d2) on small bowel inflammation.	h[Gly ² GLP-2 administration concomitant with, or after, indomethacin significantly increased the survival rate of indomethacin-treated mice. Significant reversion of small bowel shortening, loss in villus height and crypt depth. Significant increase in crypt cell proliferation rate (CCPR) and reduction in crypt cell apoptosis, DAI, free abdominal fluid and number of small bowel ulcerations. Significantly lower jejunal and ileal MPO activity, and TNF- α , IFN- γ and IL-10 levels. Significantly lower number of colonies in blood cultures. Significantly increased levels of jejunal GLUT-2 mRNA. No changes in jejunal or ileal proglucagon and ITF mRNAs, nor in gastric pS2 expression.
Buljevic S et al. (2018)	C57BL/6 mice (WT; CD26 ^{-/-}) (8-10w, M)	TNBS (5%, dissolved in 50% ethanol, 150 mL)	Effects of DPP4/CD26 deficiency on colitis (DAI) and comparative expression patterns and/or enzyme activity of DP8 and DP9. Presence of DP8/DP9 in mouse serum.	No differences in DAI and colon mucosa damage index values. CD26 ^{-/-} mice had significantly higher colon weight to length ratio at days 7 and 20. CD26 ^{-/-} mice constitutionally have lower levels of colon and serum DP8 concentration and higher colon levels of DP9; DP8 colon concentrations increase in inflammation, while serum concentrations decrease; DP9 is not subjected to change.

Detel D et al. (2012)	C57BL/6 mice (WT; CD26 ^{-/-}) (8-10w, M)	DSS (3%, 7d)	Effects of DPP4/CD26 deficiency on colitis (histomorphometrical analysis, microscopic damage index [MDI]) and inflammatory cell infiltration of colonic mucosa.	No significant difference in the intensity of clinical signs vs difference in the time course of clinical signs (delay of onset); KO mice had significantly higher number of macrophages (F4/80⁺) and GFAP-positive cells, and significantly lower number of dendritic cells (CD11c⁺). Possible protective mechanism of macrophages: increased TGF-β and IL-10 exposure and tissue healing as a result of DPP4/CD26 deficiency.
Detel et al. (2016)	C57BL/6 mice (WT; CD26 ^{-/-}) (8-10w, M)	DSS (3%, 7d)	Effects of DPP4/CD26 deficiency on colitis and phenotypic profile of splenic, hepatic, blood, and mucosal mononuclear lymphatic cells, and changes on NF- κ B expression.	No differences in histopathological changes. No differences in the number and distribution of CD3⁺, CD4⁺ and CD8⁺ T cells in analyzed colonic tissue sections between WT and CD26^{-/-} mice and when compared to controls. CD26^{-/-} mice showed significantly less increase in spleen weight in the acute (day 7) and recovery (days 10 and 15) phases of colitis. CD26^{-/-} mice showed significantly higher levels of NF-κB p65 subunit expression in colonic tissue during the acute phase of colitis (days 3 and 7). CD26^{-/-} mice showed significantly higher colon weight-to-length ration and MPO activity at day 10. Significantly less weight loss, greater large and small bowel weights and lengths, increase in small bowel villus height and crypt depth, increase in colonic crypt depth, increase in the number of PCNA-positive cells, higher mucosal surface area in the proximal and middle colon, and higher mucosal integrity (percentage of intact mucosa) in the proximal and distal colon vs saline-treated-mice.
Drucker D et al. (1999)	CD1 mice (6-8w, F)	DSS (5%, 10d)	Effects of h[Gly ²]GLP-2 (750 ng, 2id) on intestinal inflammation.	No evidence for h[Gly²]GLP-2-mediated induction of intestinal KGF or TGF-α gene expression. No significant change in the levels of proglucagon mRNA transcripts in the jejunum, ileum or colon of mice with DSS-induced colitis; No increase in ileal immunoreactive GLP-2 levels. Significant decrease in the levels of GLP-2 in the colon of mice with DSS-induced colitis (epithelium destruction, increased

		secretion/decreased storage of tissue GLP-2); increased levels of circulating GLP-2.	
El-Jamal N et al. (2014)	Balb/c mice (8-9w, F)	DSS (5%, 9/d)	Effects of h[Gly ²]GLP-2 (350 ng, 2id) on intestinal inflammation.
	C57BL/6 mice (9w)	TNBS (40 mL, 150mg/kg, dissolved in a 1:1 mixture of 0.9% NaCl with 100% ethanol)	Effects of GLP-2 (50 µg/kg, sc, at 36h post-TNBS) on intestinal inflammation (macroscopic – Wallace score; histologic – Ameho score); Intestinal expression of GLP-2R transcript.
	Balb/c mice (9w)	DSS (5%, 7d)	
Elkatary et al. (2015)	C57BL/6 mice (F)	DSS (3%, 7d)	Effects of different doses of sitagliptin (20 mg/kg/d, 1id, d5-14; 100 mg/kg/d, 1id, d5-14) on colitis (DAI), TNF-α concentration, and oxidative stress.
	Balb/c mice (6w, M)	—	Effects of sitagliptin on mucosal and plasma DPP activity, and GLP-1 ₇₋₃₆ and GLP-2 concentrations.
Fujiwara K et al. (2015)	SD rats (6w, M)	Indomethacin (10 mg/kg/d, s.c., 1d)	Effects of sitagliptin (STG, 1 or 3 mg/kg, p.o., 1id, 2d, at d-1 and d0) on ulcer prevention.
	Balb/c mice (6w, M)	Indomethacin (8 mg/kg/d, s.c., 2d)	Effects of sitagliptin (STG, 3 mg/kg, p.o., 1id, 7d, d2 to d8) and elemental diet (ED, 1id, 7d, d2 to d8) on ulcer healing.

Significantly less weight loss, and significantly greater large and small bowel weights and lengths.
No significant differences between the relative levels of MPO activity in the colon.
Significant decrease in the expression of GLP-2R mRNA, vs healthy controls.
Significantly greater colon length, vs TNBS+vehicle.
No significant differences in macroscopic score or GLP-2R mRNA levels, vs TNBS+vehicle.

Significant decrease in the expression of GLP-2R mRNA, vs healthy controls.

Significant decrease of serum TNF-α levels and colonic tissue malondialdehyde (MDA).
No statistically significant reduction in colonic tissue nitric oxide or increase in colonic tissue GSH.
No statistically significant reduction in colonic inflammation.
No significant increase in colon length.

Significant reduction of total ulcer score with both dosages.
Co-administration of GLP-2₃₋₃₃ (100 µg/kg, s.c., 1id, 2d, at d-1 and d0) partially reversed the preventive effects of sitagliptin on ulcer formation.
Reduced ileal expression of cleaved caspase-3.
ED: significant reduction of ulcer score.
STG: no significant changes.
ED+STG: significant reduction of ulcer score, vs ED-only.

Geier M et al. (2005)	C57BL/6 mice (WT; CD26 ^{-/-}) (6-7w, M)	DSS (2%, 6d)	Effects of DPP4/CD26 deficiency on colitis (histomorphometrical analysis, epithelial cell proliferative labeling index [L-I]).	Co-administration of GLP-2 ₃₋₃₃ (100 µg/kg, s.c., 7d, 2d, d2 to d8) partially reversed the ulcer healing effects of ED+STG. ED and ED+STG significantly increased ileal GLP-2 mucosal concentrations, whereas STG alone had no significant effect. No significant differences in most analyzed parameters (body weight, colon weight and length, DAI, histological analysis, PCNA labeling index). No significant differences in plasma DPP4-like activity at day 0. GLP-2(2): Effectively prevented the loss of mucosal architecture and inflammatory cell infiltration in mucosa and submucosa layers and inhibited inflammation-induced apoptosis <i>in vitro</i> . Significantly inhibited DSS-induced body weight loss. Significantly reduced expression of IFN-γ, TNF-α, IL-1β and IL-6, NLRP3 inflammasome, COX2, caspase-3, and significantly reduced MPO activity. Up-regulated the expression of Ki-67 in colonic tissues. GLP-2(2) (vs GLP-2): Notably dose-dependently inhibited the secretion of inflammatory cytokines stimulated by LPS. Significantly suppressed the apoptotic rates of CCD-18Co cells treated with LPS. Significantly increased colon length. Effectively reduced histological inflammation, DAI and macroscopic damage scores. Significantly ameliorated intestinal absorption and permeability. Significant amelioration of colitis development, reduction in the shortening of colon length, lower MPO activity and DAI score. Significantly lower cecal content pH in days 0 and 4 (due to differences in organic acid cecal contents and bile acid composition in feces).
	Balb/c mice (M)	DSS (3%, 7d)	Effects of GLP-2(2) (200 µg/kg/d, i.p. 7d) vs GLP-2 (200 µg/kg/d, i.p. 7d) on colitis (DAI), cytokine profiles, and regulation of apoptosis and proliferation in colonic tissues.	
Gu J et al. (2018)				
Iwaya H et al. (2013)	F344/Jcl (WT) rats	DSS (1%, 9d)	Effects of DPP4 deficiency on colitis susceptibility and severity (DAI); cecal content levels of organic acids; and fecal levels of bile acids.	At 0.3 mg/kg, K579 had no effect on ulcer formation, whereas 1 mg/kg K579, either i.g. or i.p., inhibited ulcer formation. At 3 mg/kg, the preventive effects of K579 were reversed. Exogenous GLP-2 treatment (10–100 µg/kg, i.p., 2d, at h0 and h8) dose-dependently reduced ulcer length in all segments. GLP-2 ₃₋₃₃ (1 mg/kg, i.p.) reversed K579 (1 mg/kg, i.g.) effects on ulcer formation.
	F344/Du (DPP4-deficient) (4w, M)	DSS (2%, 6d)		
Inoue T et al. (2013)	Sprague-Dawley rats (M)	Indomethacine (10 mg/kg, s.c., single-dose)	Effects of DPP4 inhibitor K579 and luminal nutrients (L-alanine, L-glutamate and inosine 5'-monophosphate [IMP]) on intestinal ulcer prevention. K579: 0.3–3 mg/kg, i.g or i.p, at d0 L-ala, L-glu: 400 µmol/kg, i.g., at d0 IMP: 4 µmol/kg, i.g., at d0	

				L-ala/IMP (400/4 $\mu\text{mol/kg}$, i.g.) significantly reduced total intestinal ulcer formation, whereas L-glu/IMP (400/4 $\mu\text{mol/kg}$, i.g.) had no effect. K579 at 1 mg/kg, but not at 0.3 mg/kg, significantly reduced the intestinal ulcer length, at day 3. Exogenous GLP-2 treatment (200 $\mu\text{g/kg/d}$, i.p.) also similarly promoted ulcer healing. GLP-2₃₋₃₃ (1 mg/kg, i.p.) reversed K579 (1 mg/kg, i.g.) effects on ulcer healing. L-ala/IMP (400/4 $\mu\text{mol/kg}$, i.g.) significantly reduced total intestinal ulcer length on day 3. Co-administration of K579 (1 mg/kg, i.g.) further decreased total intestinal ulcer length on day 3. Administration of sialorphan analog Pal-KKQHNPR significantly attenuated TNBS-induced colitis (lower macroscopic score, MPO activity and TNF-α expression in the colon). Administration of opiorphin analog Pal-KKQRFSSR did not improve any of the measured disease parameters.
Kamysz E et al. (2016)	Balb/c mice (M)	TNBS (5 mg in 0.1 mL of 30% ethanol in saline)	Effects of double NEP (neprilysin, CD10) and APN (aminopeptidase N, CD13) inhibitors, sialorphan analog Pal-KKQHNPR (2id, 1 mg/kg, i.p., 30' before TNBS) and opiorphin analog Pal-KKQRFSSR (2id, 1 mg/kg, i.p., 30' before TNBS) on colitis (macroscopic colonic damage score).	Significant attenuation of DAI, colon shortening, histological score. Significant decrease in MPO activity and TNF-α expression. Dose dependent, non-significant, tendency towards a decrease in IL-1β and IL-6 expression. Significant increase in colonic and plasma levels of GLP-2. Effects were reversed with administration of GPR40 antagonist DC260126 (20 mg/kg, 1id, d0-6, i.p.) or GLP-2 antagonist GLP-2₃₋₃₃ (100 μg, 1id, d0-6, i.p.). Functional Paneth cell defect. Paneth cell secretions in KO mice exhibited reduced bactericidal activity after stimulation with carbamylcholine and LPS <i>ex vivo</i>; Significantly reduced intestinal expression of multiple RNA transcripts encoding proteins involved in the host response to infection in Glp2^{-/-} mice, including myd88, nfkb, mip2, il-6, il-33, cxd10, il-15, mmp7, cryptidin 5 and Plag2g2a Significant reduction in the proportions of <i>Firmicutes</i> and <i>Actinobacteria</i> and a significant increase in <i>Bacteroidetes</i> and <i>Clostridiales</i>; Loss of the Glp2r does not influence the severity of or recovery from DSS-induced colonic injury.
Kato S et al. (2019)	C57BL/6 mice (9-12w, M)	DSS (2%, 7d) DSS (2%, 6d)	Effects of GPR40 activation by specific agonist AS2034178 (1/3/10 mg/kg, 1id, d0-6, p.o.) on colitis development and healing.	
Lee S et al. (2012)	C57BL/6 mice (WT; Glp2 ^{-/-}) (8-12w; M/F)	-	Effects of GLP-2R deficiency on small bowel inflammation.	
		DSS (3%, 9d)		

L'Heureux M et al. (2003)	Indomethacin (10 mg/kg, s.c., 1d, 2d)	CD1 mice (6w, F)	DSS (3.5%, 10d)	Effects of GLP-2 (h[Gly ²]GLP-2, 0.04 mg/kg, s.c., q12h, 10d), ASA (sulfasalazine, 100 mg/kg, p.o., q24h, 10d), and CS (methylprednisolone sodium succinate, 1 mg/kg, i.p., q24h, 10d), alone and in combination, on the intestinal response to inflammation (colonic damage score [CDS]).	Significantly higher morbidity and mortality in Glp2 ^{-/-} mice. The proportion of indomethacin-treated Glp2 ^{-/-} mice exhibiting positive bacterial cultures in liver, spleen, and blood trended significantly higher relative to levels in WT mice, and significantly more Glp2 ^{-/-} mice exhibited positive mesenteric lymph node bacterial cultures.
	Irinotecan (280 mg/kg, i.p., 1 d, 2d)				Jejunal crypt depth: similarly decreased in both phenotypes. Jejunal crypt density: significantly decreased in Glp2 ^{-/-} mice. Hemolysis on blood agar plates, an indirect measure of systemic bacteremia, was increased to a significantly greater extent in Glp2 ^{-/-} mice. Significantly reduced mRNA levels of <i>lgr5</i> , <i>egf</i> and <i>igf-1r</i> . Significantly increased mRNA levels of <i>il-6</i> . mRNA levels of <i>epgn</i> and <i>ErbB1</i> were not as upregulated in Glp2 ^{-/-} mice.
Mimura S et al. (2013)	DSS (1.5%, 7d)	C57BL/6 mice (7w, M)			GLP-2 and ASA, alone or in combination, significantly increased survival. CS alone did not affect survival and prevented the effects of GLP-2 in both CS+GLP-2 (no differences in survival) and CS+ASA+GLP-2 groups (blunted response to GLP-2, intermediate survival rates). GLP-2: significant reduction in body weight loss and significant increase in small intestinal weight/final body weight ratio. ASA+GLP-2: significant increase in small intestinal weight/final body weight ratio, vs ASA only. ASA+CS+GLP-2: significant reduction in body weight loss and significant increase in small intestinal weight/final body weight ratio, vs ASA+CS; GLP-2: preservation of normal colonic mucosa, significant decrease in CDS, and significant increase in colonic crypt cell proliferation. The beneficial effects of GLP-2 in CDS and colonic crypt cell proliferation were completely abrogated in mice that received concomitant CS treatment; GLP-2 treatment, alone or in combination, did not significantly decrease MPO activity, neither did it modulate the ratio of phospho- to total IκB; No significant differences in GLP-1, GLP-2 or serum glucose levels. Significantly lower body weight loss and DAI during the recovery period (no significant differences during DSS exposure).

				Significantly higher colon length, lower histological score, and increase in the Ki67-positive rate of colonic crypts during the recovery period. No significant changes in the mRNA expression of IGF-1 or KGF or tissue MPO activity. PEG-pGLP-2 significantly reduced weight loss, vs saline and vs pGLP-2. Colonic damage score (CDS) was significantly lower in PEG-pGLP-2-treated mice, vs pGLP-2. PEG-pGLP-2 and pGLP-2-treated mice had significantly lower MPO activity. Neither PEG-pGLP-2 nor pGLP-2 significantly improved colon length.
Qi KK et al. (2014)	Balb/c mice (6w, M)	DSS (3%, 7d)	Effects of PEG-p[Gly ²]GLP-2 (30 µg in 0.3 mL of saline, 1id, at d8) and pGLP-2 (5µ in 0.3 mL of saline, 1id, 7d) on colitis.	Significantly higher colonic length and percentage of body weight change, higher colonic weight/body weight ratio in DSS-PEG-pGLP-2, lower colonic damage score, higher duodenum villus height, higher duodenum villus height/crypt depth. Significantly increased expression of ZO-1 and occluding and decreased expression of claudin-1. Significantly decreased expression of IL-1, IL-7, TNF-α, IL-10 and IFN-γ.
Qi KK et al. (2017)	Sprague-Dawley rats (M)	DSS (5%, 6d)	Effects of PEG-p[Gly ²]GLP-2 (12.5/25/100 nmol/kg in 0.3 mL of saline, 1id, i.p., 6d) on growth performance, intestinal morphology, and expression levels of tight junction proteins and inflammatory cytokines in colitis (colonic damage score [CDS]).	No significant differences in the expression of IL-6. BTA (high dose) significantly improved DAI and histological scores; co-administration of sitagliptin had no additive effect; co-administration of GLP2 ₃₋₃₃ (2.5 or 25 µg/body/day, i.p., 7d) significantly increased DAI and histological score.
Sakanaka T et al. (2015)	C57BL/6 mice (6-7w, M)	DSS (5%, 7d)	Effects of selective TGR5 agonist betulinic acid (BTA, low dose: 15 mg/L in free access water, 7d; high dose: 50 mg/L in free access water, 7d) and sitagliptin (3 mg/kg, i.g., 7d) on GLP-2 release and colitis attenuation (DAI and histological score).	BTA (high dose) significantly reduced mRNA expression of TNF-α, IL-1β and IL-6; co-administration of sitagliptin potentiated the suppressive effects of BTA on the mRNA expression of IL-6, but not TNF-α or IL-1β. BTA (low and high doses) and BTA high+STG all significantly and equally lowered DPP4 intestinal mucosal activity and elevated active GLP-1 levels. BTA high+STG significantly decreased total GLP-2 mucosal concentration.
Salaga M, Mokrowiecka A.	C57BL/6 mice (6-8w, M)	TNBS (0.1 mL of 30% ethanol in saline; acute and	Effects of EMDB-1 on colitis attenuation (macro- and microscopic scores, ulcer score)	Acute model: EMDB-1 significantly improved colitis in a dose-dependent manner, for all parameters – macroscopic score, ulcer score, bowel thickness, and MPO activity.

Zielinska M et al. (2017)	semichronic models – 4 mg TNBS/animal; chronic relapsing model – 150 mg TNBS/kg + 75 mg TNBS/kg DSS (3%, 6d)	and on the expression of GLP-2, GLP-2R and DPP4. EMDB-1 acute model: 0.1/1/3 mg/kg, intracolonic (i.co.), 2id, 3d; semichronic model: 1 mg/kg, i.co., 2id, d3-6; chronic relapsing model: 1 mg/kg, i.co., 2id, d7-13	Co-administration of naloxone did not block the anti-inflammatory effect of EMDB-1. Semichronic model: EMDB-1 significantly improved colitis for all parameters – macroscopic score, ulcer score, bowel thickness, and MPO activity. Chronic relapsing model: EMDB-1 significantly improved colitis for all parameters – macroscopic score, ulcer score, bowel thickness, and MPO activity, as well as significant reduction in body weight loss at day 14, and significant reduction of TNF- α and IL-1 β expression, and microscopic total damage score. Treatment with EMDB-1 significantly elevated colonic levels of GLP-2 in healthy ($\approx$ 9-fold) and TNBS-treated mice ($\approx$ 19-fold). Treatment with EMDB-1 significantly decreased colonic levels of GLP-2R in healthy mice, with a non-significant decrease in TNBS-treated mice. Treatment with EMDB-1 did not alter the expression of DPP4 in either strain. All models: significant reduction in macroscopic score, ulcer score, bowel thickness and MPO activity. β -FNA reversed the effects of sialorphin on macroscopic score, bowel thickness and MPO activity. norBNI reversed the effects of sialorphin on macroscopic score, ulcer score, bowel thickness and MPO activity. NLTR did not reverse the anti-inflammatory effects of sialorphin.
Salaga M, Mokrowiecka A, Jacenik D et al. (2017)	TNBS (0.1 mL of 30% ethanol in saline; acute and semichronic models – 5 mg TNBS/animal; chronic relapsing model – 150 mg TNBS/kg + 75 mg TNBS/kg) DSS (4%, 6d)	Effects of NEP (nepilysin, CD10) and APN (aminopeptidase N, CD13) inhibitor sialorphin on colitis attenuation (macro- and microscopic colonic damage scores). Sialorphin Acute model: 0.3-10 mg/kg, i.p., 2id, d0-d2; Selective opioid receptor antagonists – i.p. administration, 30' before sialorphin: – MOR ant: β -funaltrexamine (β -FNA), 1mg/kg – KOR ant: nor-binaltorphimine (norBNI), 10 mg/kg – DOR ant: naltrindole (NLTR), 10 mg/kg Semichronic model: 1 mg/kg, i.p., 2id, d3-d6; Chronic relapsing model: 1 mg/kg, i.p., 2id, d7-d13	Treatment with EMDB-1 significantly elevated colonic levels of GLP-2 in healthy ($\approx$ 9-fold) and TNBS-treated mice ($\approx$ 19-fold). Treatment with EMDB-1 significantly decreased colonic levels of GLP-2R in healthy mice, with a non-significant decrease in TNBS-treated mice. Treatment with EMDB-1 did not alter the expression of DPP4 in either strain. All models: significant reduction in macroscopic score, ulcer score, bowel thickness and MPO activity. β -FNA reversed the effects of sialorphin on macroscopic score, bowel thickness and MPO activity. norBNI reversed the effects of sialorphin on macroscopic score, ulcer score, bowel thickness and MPO activity. NLTR did not reverse the anti-inflammatory effects of sialorphin.
Salaga M et al. (2018)	C57BL/6 mice (6-8w, M)	TNBS (0.1 mL of 30% ethanol in saline; acute and	Acute model: DI-1 significantly improved colitis in a dose-dependent manner, for all parameters – macroscopic score, ulcer

		semichronic models – 5 mg TNBS/animal; chronic relapsing model – 200 mg TNBS/kg + 100 mg TNBS/kg)	ulcer score) and on expression of GLP-2, GLP-2R and DPP4.	score, bowel thickness, MPO activity and microscopic total damage score.
		DSS (3%, 6d)	DL-1 acute model: 0.3/1/3 mg/kg, intracolonic (i.co.), 2id, 3d semichronic model: 1 mg/kg, i.co., 2id, d3-6 chronic relapsing model: 3 mg/kg, i.co., 2id, d7-13	Semichronic model: DL-1 significantly improved colitis for all parameters – macroscopic score, ulcer score, bowel thickness, MPO activity; as well as significant reduction in body weight loss at day 7; non-significant reduction of TNF- α and IL-1 β expression; Chronic relapsing model: DL-1 significantly improved colitis for all parameters – macroscopic score, ulcer score, bowel thickness, and MPO activity; as well as significant reduction of TNF- α and IL-1 β expression.
Schmidt PT et al. (2000)	C.B-17scid (SCID) mice Balb/c mice (controls) (6-8w, F)	Balb/c to SCID mice CD4+ Con A blast T cell transfer (300000 cells, i.p.) DSS (4%, 9d)	Effect of T-cell-induced intestinal inflammation on GLP-2 levels.	Treatment with DL-1 (3 mg/kg, i.co.) did not alter the expression of DPP4 or GLP-2R in either strain. Significantly lower levels of GLP-2, GLP-1, and glicentin in transverse and distal colon. No differences in caecum and ascending colon.
Wu J et al. (2015)	Balb/c mice (6w, M)	Therapeutic effects of porcine [Gly ²]GLP-2-loaded microspheres (10 mg in 0.3 mL of saline, i.p., at day 0) on colitis (colonic damage score [CDS]).		Significant increase in body weight, colonic length, and small intestine and colonic weights. No difference in small intestine length. Significant decrease in the histological colitis score, inflammation score, and crypt damage/regeneration score. No difference in the extent of inflammation score; Significant decrease in PCNA positive cells (colonic epithelial cell proliferation), MPO and DAO activities, and colonic expression of INF- γ , TNF- α , IL-6, IL-8, IL-10 and claudin-1. Significant increase in the expression of occludin and GLP-2R. No significant change in the expression of ZO-1.
Yang PY et al. (2018)	C57BL/6 mice (9w, M)	DSS (3%, 5d) DSS (2%, 3 cycles of 5 days, interspersed with 7-day periods of recovery)	Intestinitrophic and anti-inflammatory effects of a stapled, long-acting GLP-2 analog (peptide 10) on colitis. Peptide 10 acute model: 40 μ g/kg (7.5 nmol/kg), s.c., 2id, 12d chronic model: – 10 nmol/kg, s.c., 2id, d25-32 – 100 nmol/kg, s.c., 1id, d25-32	Peptide 10 significantly increased small intestine weight and length, and significantly reduced mRNA expression levels of TNF- α and IL-1 β . No significant differences were observed in colonic weight, or length (increasing trend-only). Chronic model: Low dose peptide 10 (65 μ g/kg or 10 nmol/kg) resulted in a significant increase in body weight. No significant effect in body weight after treatment with GLP-2G;

				GLP-2G (Tegudlutide), 15 nmol/kg, s.c., 2id	Significantly higher small intestine weight and length after treatment with GLP-2G, peptide 10 (low dose) and peptide 10 (high dose). Significant decrease in plasma DPP4 activity and lower DAI scores, vs vehicle group. P32/98 mice had significantly lower DAI scores, higher colon length at day 6, lower crypt depth and higher crypt area at day 9, and significantly and consistently higher percentage body weight vs P59/99. Significantly higher colon weight at day 6 for P59/99 and P32/98, and at day 14 for P59/99. Significantly higher colon weight corrected for body weight at day 14 for P59/99 and P32/98. Significantly higher LI in P59/99 treated mice, vs vehicle group, at day 14.
Yazbeck R et al. (2008)	C57BL/6 mice (6-7w, M)	DSS (2%, 6d)		Effects of DPP4 inhibitors isoleucyl-cyanopyrrolidine (P59/99, 10 mg/kg in 100 μ L, 2id, p.o.) and isoleucyl-thiazolidine (P32/98, 10 mg/kg in 100 μ L, 2id, p.o.) on colitis attenuation (DAI).	Plasma GLP-2 and GLP-1 concentrations only significantly increased at day 6 in WT mice treated with Ile-Pyr-(2CN)*TFA, when compared to saline or Ile-Thia treated mice. No significant changes in plasma GLP-2 and GLP-1 concentrations in CD26 ^{-/-} mice (treated only with saline or Ile-Thia; Ile-Pyr-(2CN)*TFA not tested); No significant changes in FAP, DP9, DP8 or DP2 mRNA expression in CD26 ^{-/-} mice at day 6. Significant increase in DP8 and DP2 mRNA expression in WT mice at day 6. Trend toward a decrease in membrane DPP4/8/9 activity and increase in cytosolic activity at day 6 vs day 0 in both strains, only statistically significant for cytoplasmic activity for CD26 ^{-/-} mice at day 6. No significant changes in membrane DP2 activity in either strain. Significantly higher cytoplasmic DP2 activity in WT mice and trend toward higher cytoplasmic DP2 activity in CD26 ^{-/-} mice, at day 6.
Yazbeck R et al. (2010)	C57BL/6 mice (WT; CD26 ^{-/-}) (6-7w)	DSS (2%, 6d)		Effects of DPP4 inhibitors Ile-Pyr-(2-CN)*TFA and Ile-Thia (10 mg/kg, p.o., 2id) on colitis attenuation (DAI).	In WT mice, Ile-Thia and Ile-Pyr-(2CN)*TFA treatments significantly attenuated the increase in MPO activity at day 6. In WT mice, Ile-Thia treatment significantly ablated the decrease in the number of CD4 ⁺ FOXP3 ⁺ (T _{reg}) cells at day 6. MPO activity (and neutrophil infiltration) at day 6 was significantly higher in WT+saline and both CD26 ^{-/-} groups.
Yazbeck R et al. (2011)	C57BL/6 mice (WT; CD26 ^{-/-})	DSS (2%, 6d)		Effects of DPP4 inhibitors Ile-Pyr-(2-CN)*TFA and Ile-Thia (10 mg/kg, p.o., 2id) on small	

(6-7w)	intestinal response in the setting of acute colitis.	Significantly lower jejunal DP activity in WT mice treated with Ile-Pyr-(2CN)*TFA at day 6. No changes in any parameter for ileum, except for significantly higher ileal DP activity in WT mice treated with Ile-Pyr-(2CN)*TFA at day 6.
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APN: aminopeptidase N; APN1: APN inhibitor(s); CCL/CXCL: Chemokine(s); CCR/CXCR: Chemokine receptor(s); CD: cluster of differentiation; COX2: Cyclooxygenase-2; DAI: Disease activity index; DAO: Diamine oxidase; DOR: δ -type opioid receptor; DPP4: Dipeptidyl peptidase 4; DPP4i: DPP4 inhibitor(s); DP8: dipeptidyl peptidase 8; DP9: dipeptidyl peptidase 9; DRA: down-regulated in adenoma (protein); DSS: Dextran sulfate sodium; EGF: Epidermal growth factor; F: Female; GFAP: Glial fibrillary acidic protein; GLP-1/-2/-2R: Glucagon-like peptide 1/2/2-receptor; GPR40 – G-protein coupled receptor 40; GPR120 – G-protein coupled receptor 120; GSH – glutathione; HLA – human leukocyte antigen; IBD – intestinal bowel disease(s); i.co. – intracolonic; IGF-1/-1R: Insulin-like growth factor 1/1-receptor; IL: Interleukin; IFN- γ : Interferon-gamma; i.p.: Intraperitoneal; KGF/KGFR: Keratinocyte growth factor/receptor; KO: Knock-out; KOR: κ -Type opioid receptor; LP: Lipopolysaccharide; M: Male; MMP: Matrix metalloproteinase; MOR: μ -type opioid receptor; MPO: Myeloperoxidase; NEP: Neprilysin; NLRP3: (NOD)-like receptor protein 3; nNOS: Neuronal nitric oxide synthase; NO: Nitric oxide; NPY: Neuropeptide Y; PCNA: Proliferating cell nuclear antigen; PMN: Polymorphonuclear leukocytes; p.o.: per os; s.c.: subcutaneous; sCD26/DPP4: Soluble CD26/DPP4; SSM: Sterically stabilized micelles; TGF- β : Transforming growth factor beta; TGR5: G-protein coupled bile acid receptor 1 (GPBAR1); T_H: T helper cell; TNBS: 2,4,6-Trinitrobenzenesulfonic acid; TNF- α : Tumor necrosis factor alpha; VIP: Vasoactive intestinal peptide; WT: Wild-type; Y1: NPY receptor type 1.

Supplementary Table 2. Animal models and respective variations.

Disease models	Number of articles	Model variations	Applied animal models (# of articles)
DSS	25	1%, 9d	C57BL/6 mice, WT (n=16)
		1.5%, 7d	C57BL/6 mice, CD26 ^{-/-} (n=5)
		2%, 5d	C57BL/6 mice, Gbp2 ^{-/-} (n=1)
		2%, 6d	(6-12w, M/F)
		2%, 7d	Balb/c mice (n=7)
		2%, 3 cycles of 5d, interspersed with 7-day periods of recovery	(6-9w, M/F)
		3%, 5d	CD1 mice (n=2)
		3%, 6d	(6-8w, F)
		3%, 7d	F344/JcL rats, WT (n=1)
		3%, 9d	F344/Du rats, DPP4-deficient (n=1)
		3.5%, 10d	(4w, M)
		4%, 6d	Sprague-Dawley rats (n=1)
		4%, 9d	(M)
		5%, 6d	C57BL/6 mice, WT (n=5)
		5%, 7d	C57BL/6 mice, CD26 ^{-/-} (n=2)
5%, 9 ¹ / ₂ d	(8-10w, M)		
5%, 10d	Balb/c mice (n=2)		
5%, dissolved in 50% ethanol, 100 µL	(6-8w, M)		
5%, dissolved in 50% ethanol, 150 µL	C57BL/6 mice, Gbp2 ^{-/-} (n=1)		
40 mL, 150 mg/kg, dissolved in a 1:1 mixture of 0.9% NaCl with 100% ethanol	(8-12w, M/F)		
5 mg in 0.1 mL of 30% ethanol in saline	CD1 mice (n=1)		
0.1 mL of 30% ethanol in saline, 4 mg/animal	(7-8w, F)		
0.1 mL of 30% ethanol in saline, 5 mg/animal	Wistar rats (n=1)		
0.1 mL of 30% ethanol in saline, 150 mg/kg + 75 mg/kg	(M)		
0.1 mL of 30% ethanol in saline, 200 mg/kg + 100 mg/kg	Sprague-Dawley rats (n=2)		
TNBS	7	7 mg/kg, 1id, s.c., 2d	(6w, M)
		8 mg/kg, 1id, s.c., 2d	
		10 mg/kg, 1id, s.c., 1d	
		10 mg/kg, 1id, s.c., 2d	
		20 mg/kg, 1id, s.c., 2d	
Indomethacin	5		

HLA-B27	2	HLA-B27 spontaneous model of GI inflammation	F344, WT (n=2) F344-HLA-B27 (n=2) (25w, F)
CD4+ transfer	1	Balb/c to SCID mice CD4+ Con A blast T cell transfer (300 000 cells, ip)	C.B-17scid (SCID mice) (n=1) Balb/c mice (n=1) (6-8w, F)
Irinotecan	1	280 mg/kg, ip, 1id, 2d	C57BL/6 mice, WT (n=1) C57BL/6 mice, Glp2r ^{-/-} (n=1) (8-12w, M/F)

DSS: dextran sulfate sodium; TNBS: 2,4,6-trinitrobenzenesulfonic acid.

Supplementary Table 3. Quality assessment of animal studies: SYRCLE's Risk of Bias tool.

SYRCLE's RoB tool	Alavi et al., 2000	Alters et al., 2012	Anbazhagan et al., 2017	Arthur et al., 2004	Ban et al., 2011	Bank et al., 2006	Baticic et al., 2011	Boushey et al., 1999	Buljevic et al., 2018	Detel et al., 2012	Detel et al., 2016	El-Jamal et al., 1999	Elkatar et al., 2015	Fujiwara et al., 2015	Geier et al., 2005	Gu et al., 2018	Inoue et al., 2013	Iwaya et al., 2013
1. Was the allocation sequence adequately generated and applied?	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
2. Were the groups similar at baseline or were they adjusted for confounders in the analysis?	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
3. Was the allocation adequately concealed?	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
4. Were the animals randomly housed during the experiment?	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
5. Were the caregivers and/or investigators blinded from knowledge of which intervention each animal received during treatment?	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
6. Were animals selected at random for outcome assessment?	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
7. Was the outcome assessor blinded?	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
8. Were incomplete outcome data adequately addressed?	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
9. Are reports of the study free of selective outcome reporting?	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
10. Was the study apparently free of other problems that could result in high risk of bias?	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■

■ - Yes; ■ - Unclear;

SYRCLE's RoB tool	Kamysz et al., 2016	Kato et al., 2019	Lee et al., 2012	L'Heureux et al., 2003	Mimura et al., 2013	Qi et al., 2014	Qi et al., 2017	Sakanaka et al., 2015	Salaga, Mokrowiecka, Zielińska et al., 2017	Salaga, Mokrowiecka, Jacenik et al., 2017	Salaga et al., 2018	Schmidt et al., 2000	Wu et al., 2015	Yang et al., 2018	Yazbeck et al., 2010	Yazbeck et al., 2011
1. Was the allocation sequence adequately generated and applied?	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
2. Were the groups similar at baseline or were they adjusted for confounders in the analysis?	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
3. Was the allocation adequately concealed?	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
4. Were the animals randomly housed during the experiment?	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
5. Were the caregivers and/or investigators blinded from knowledge of which intervention each animal received during treatment?	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
6. Were animals selected at random for outcome assessment?	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
7. Was the outcome assessor blinded?	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
8. Were incomplete outcome data adequately addressed?	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
9. Are reports of the study free of selective outcome reporting?	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
10. Was the study apparently free of other problems that could result in high risk of bias?	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■

■ - Yes; ■ - Unclear;

Supplementary Table 4. Quality assessment of human qualitative studies: CASP Qualitative Checklist.

CASP Qualitative Checklist	El-Jamal et al., 2014	Hildebrandt et al., 2001	Magro et al., 2017	Moran et al., 2012	Rose et al., 2002	Salaga, Mokrowiecka, Zielinska et al., 2017	Salaga, Mokrowiecka, Jacenik et al., 2017	Schmidt et al., 2005	Tsukahara et al., 2015	Varijen et al., 2005	Xiao et al., 2000
1. Was there a clear statement of the aims of the research?	■	■	■	■	■	■	■	■	■	■	■
2. Is a qualitative methodology appropriate?	■	■	■	■	■	■	■	■	■	■	■
3. Was the research design appropriate to address the aims of the research?	■	■	■	■	■	■	■	■	■	■	■
4. Was the recruitment strategy appropriate to the aims of the research?	■	■	■	■	■	■	■	■	■	■	■
5. Was the data collected in a way that addressed the research issue?	■	■	■	■	■	■	■	■	■	■	■
6. Has the relationship between researcher and participants been adequately considered?	■	■	■	■	■	■	■	■	■	■	■
7. Have ethical issues been taken into consideration?	■	■	■	■	■	■	■	■	■	■	■
8. Was the data analysis sufficiently rigorous?	■	■	■	■	■	■	■	■	■	■	■
9. Is there a clear statement of findings?	■	■	■	■	■	■	■	■	■	■	■
10. How valuable is the research?	■	■	■	■	■	■	■	■	■	■	■

■ - Yes; ■ - Can't tell;

Supplementary Table 5. Quality assessment of human cohort studies: CASP Cohort Checklist

CASP Cohort Checklist	Buchman et al., 2010	Pinto-Lopes et al., 2020
1. Did the study address a clearly focused issue?	■	■
2. Was the cohort recruited in an acceptable way?	■	■
3. Was the exposure accurately measured to minimize bias?	■	■
4. Was the outcome accurately measured to minimize bias?	■	■
5a. Have the authors identified all important confounding factors?	■	■
5b. Have they taken account of the confounding factors in the design and/or analysis?	■	■
6a. Was the follow up of subjects complete enough?	■	■
6b. Was the follow up of subjects long enough?	■	■
7. <i>What are the results of this study?</i>	—	—
8. How precise are the results?	■	■
9. Do you believe the results?	■	■
10. Can the results be applied to the local population?	■	■
11. Do the results of this study fit with other available evidence?	■	■

12. What are the implications of this study for practice?



■ - Yes; ■ - Can't tell;

DISCUSSION

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DISCUSSION

IBD is a chronic and debilitating condition, characterized by quiescent phases followed by (seemingly unprovoked) disease flares.¹⁰⁷ The goals of treatment are to improve quality of life, reduce rates of hospitalization and surgery, and achieve mucosal healing.¹⁰⁸

Although patients are commonly followed clinically, there is a limited correlation between symptomatology and the severity of mucosal damage, particularly in CD.¹⁰⁹ Currently, accurate monitoring of intestinal inflammation relies upon subjective clinical indices, in association with imaging techniques, endoscopy, and histological investigation. Nevertheless, these diagnostic tests can be time-consuming, expensive, or even invasive. Therefore, these may not be considered acceptable in situations where the patient is feeling well.⁷¹

Several biomarkers have been developed and have emerged as important indicators of disease activity, risk of relapse, need for treatment escalation, and response to therapy.¹¹⁰ Some of these are blood tests (e.g., CRP, ESR) and are already in use in clinical practice, yet they lack specificity for intestinal inflammation and may increase in systemic inflammatory states.¹¹¹ Fecal biomarkers (e.g., FC, FL) generally have better diagnostic accuracy, but are somewhat difficult in everyday practice, particularly from the patient's perspective. Individuals with smoldering disease may be reticent to comply, in the absence of clinical symptoms. Moreover, sample delivery and processing delays can hinder its clinical utility.¹¹²

In general, fecal markers are more likely than blood markers to reflect the degree of intestinal inflammation, due to direct contact with the mucosal environment, whereas blood markers often increase for different reasons (e.g., infection), and local inflammation does not always correspond to a detectable systemic inflammatory response.⁴⁵

There is an unmet need for simple, cost-effective, noninvasive, and robust biomarkers to monitor IBD patients. DPP-4 has drawn particular interest as a potential biomarker after observations of altered expression profiles in IBD. Previous studies have reported

lower sDPP-4 levels and activity in IBD patients compared to healthy controls, as well as negative correlations with disease activity scores and other biomarkers.⁹⁵⁻⁹⁸

Our first study (**Paper I**) included a prospective and multicentric cohort involving four tertiary centers nationwide (Centro Hospitalar Universitário de São João, EPE; Centro Hospitalar Universitário de Santo António, EPE; Hospital de Braga, EPE; Centro Hospitalar Universitário do Algarve, EPE), and is, to the best of our knowledge, the first to explore the potential of sDPP-4 as a biomarker of disease activity and prognosis in IBD.

There were two different cohorts: the first included 195 patients (94 had UC and 101 had CD), followed up to evaluate the endpoint “treatment escalation”; the second cohort was a subgroup of the first one, comprising 46 biologically treated IBD patients attending outpatient consultations, being enrolled in the induction and maintenance phases of biological treatment with infliximab, adalimumab, or vedolizumab. Blood and fecal samples were collected at baseline, in both cohorts and at evaluation visits, in the second cohort. Endoscopic evaluation (ileocolonoscopy, full colonoscopy, or flexible rectosigmoidoscopy) was performed, at baseline, for 68 patients of the first cohort. In symptomatic patients, infectious enteritis and colitis were ruled out with stool studies to evaluate for enteric pathogens (cultures and immunoassay for *C. difficile*), and also testing for cytomegalovirus on colon biopsies. A control group of 52 healthy people was recruited, contributing with stool and blood samples.

Our findings, in accordance with previous papers, confirmed that sDPP-4 levels were significantly lower in patients with active IBD compared to those in remission and healthy controls. No differences were seen in sDPP-4 levels between UC and CD patients, precluding its use as a discriminator between these two diseases. Serum DPP-4 correlated inversely with clinical activity scores ($r=-0.43$, $P<0.001$ and $r=-0.47$, $P<0.001$, respectively for UC and CD patients), and also with endoscopic activity ($r=-0.48$, $P=0.008$ and $r=-0.67$, $P<0.001$, respectively for UC and CD patients). Serum DPP-4 also presented significant negative correlations with other validated biomarkers – FC and CRP.

Further studies are required to understand the pathophysiological processes behind the association between decreased sDPP-4 and active inflammation in IBD, but some hypotheses can be outlined: (a) this decline is part of a counter-regulatory mechanism

that either reduces the activation of pro-inflammatory peptides (thereby limiting the systemic immune response) or decreases the degradation of some anti-inflammatory peptides (known to regenerate the intestinal mucosa, like GLP-2); (b) these reduced levels are directly implicated in IBD pathogenesis.

Receiver operating characteristic (ROC) curves analysis showed that sDPP-4 could help clinicians discriminate between IBD patients with clinical activity and those in clinical remission, with an area under the curve (AUC) of 0.71 (95% CI, 0.60–0.82; $P=0.001$) for UC and 0.81 (95% CI, 0.72–0.90; $P<0.001$) for CD. Identical results were obtained when discriminating IBD patients with endoscopic activity *versus* remitters, with high AUCs of 0.79 (95% CI, 0.59–0.98; $P=0.010$) for UC and 0.93 (95% CI, 0.84–1.00; $P<0.001$) for CD. It performed equally well in a subgroup of active IBD patients without CRP elevation (AUC 0.82; 95% CI, 0.70–0.94; $P=0.002$), as well as in postoperative CD patients (AUC 0.81; 95% CI, 0.65–0.97; $P=0.005$).

To predict treatment escalation in IBD patients, optimal cutoff points were defined based on ROC curve analysis of sDPP-4 and two other biomarkers – CRP and FC. Stratification according to these cutoffs, in a Kaplan-Meier curve, showed that after 1 year, 36% of UC patients and 62% of CD patients with sDPP-4 levels below the cutoff (1472 ng/mL and 1452 ng/mL, respectively) had escalated treatment, as opposed to 4% of UC patients and 8% of CD with sDPP-4 levels above the cutoff. The use of the three simultaneous biomarkers was advantageous and associated with a significantly shorter time to treatment escalation: 85% of the UC patients and 80% of the CD patients with 3 positive biomarkers escalated treatment after 1 year, whereas none of the UC patients and only 3% of the CD patients with triple-negative biomarkers had done so. Therefore, sDPP-4 evaluation can potentially be used to predict disease course at an early stage, identifying patients who would benefit from a more aggressive treatment strategy, as they will often go on to require further immunomodulators, biological therapies, and/or surgery. FC and CRP have already proved to be useful tests for predicting the course of the disease and the need to escalate therapy^{113,114}, and our findings indicate that the simultaneous use of three biomarkers (sDPP-4, FC, and CRP) could be an advantage, demonstrating a higher discriminative power. If we consider the heterogeneity and complexity of IBD, it becomes clear that only one biomarker cannot always predict the disease course and the need to escalate treatment in each case, therefore the

combination of biomarkers is capable of improving the predictive value of each marker taken alone.⁴⁵

Finally, we evaluated the second cohort of biologically treated patients and divided them into responders and nonresponders. Our analysis showed that sDPP-4 levels were lower in the nonresponders group, and this difference was more pronounced among UC (1211 vs. 1467 ng/mL; $P < 0.001$) than CD patients (1134 vs. 1385 ng/mL; $P = 0.015$). ROC curve analysis confirmed its ability to distinguish between IBD responders and nonresponders (AUC 0.68; 95% CI, 0.61–0.75; $P < 0.001$). According to these results, sDPP-4 could be used to monitor response to treatment, thereby avoiding unnecessary exposure to ineffective therapies and lowering the risk of adverse events. A biomarker that could guide clinicians to more effective treatment options, skipping the necessary steps of treatment escalation whilst maintaining patient safety, may prove to be more efficient, enabling faster control of the underlying disease.

In summary, the results from this investigation suggest that sDPP-4 can be used in IBD as a noninvasive biomarker of clinical and endoscopic activity, a predictor of treatment escalation, and also as a marker of biological treatment response.

In order to contribute toward a deeper knowledge of the pathophysiology of DPP-4 in IBD, we assessed the potential role of fDPP-4 as a biomarker of disease activity, as well as the expression of DPP-4/CD26 in intestinal samples (**Paper II**).

This study enrolled 101 IBD patients (51 had UC and 50 had CD), recruited for the first cohort of the previous study, as well as a group of 40 healthy volunteers, for blood and fecal samples collection purposes. Several patients underwent endoscopic procedures in the context of the previous study, but only UC patients were included for evaluation, due to the low number of ileal CD patients with endoscopic assessment. In addition, 10 healthy people who underwent ileocolonoscopy mainly for screening purposes (noninflammatory diseases), were enrolled for biopsy and immunohistochemical analysis.

Our study is the first to explore the potential of this fecal biomarker in IBD. UC patients in remission (clinical and endoscopic) presented higher fDPP-4 levels, compared to those with active disease. Conversely, active CD patients with ileal involvement (clinical activity) exhibited higher fDPP-4 levels than remitters. No statistically significant

differences were found among ileocolonic CD patients, possibly because these individuals share the features of both diseases (UC and ileal CD). Healthy controls presented fDPP-4 levels somewhat halfway of the spectrum of IBD patients' values.

In UC patients, fDPP-4 correlated inversely with clinical activity ($r=-0.51$, $P<0.001$), endoscopic activity ($r=-0.57$, $P=0.005$), and also with other biomarkers such as serum CRP ($r=-0.32$, $P=0.022$) and FC ($r=-0.43$, $P=0.001$). These results resemble, in some way, those reported for sDPP-4, although they were generally stronger. Therefore, as expected, fDPP-4 correlated positively with sDPP-4 ($r=0.52$, $P<0.001$), in this group of patients. On the contrary, ileal CD patients presented a direct correlation between fDPP-4 and serum CRP ($r=0.43$, $P=0.018$), but no statistically significant correlations were reported between fDPP-4 and the clinical activity score Harvey-Bradshaw index (HBI) or FC.

Fecal DPP-4 allowed the discrimination between clinical activity from remission, with AUCs of 0.80 (95% CI, 0.68–0.93; $P<0.001$) and 0.76 (95% CI, 0.58–0.94; $P=0.015$) in UC and ileal CD, respectively, as well as endoscopic activity from remission, with AUC of 0.84 (95% CI, 0.63–1.00; $P=0.009$) in UC patients.

In conclusion, these results suggest that DPP-4 can be used as a noninvasive stool biomarker of IBD activity, particularly in UC patients.

To further understand the mechanisms underlying the variations of DPP-4 expression levels in IBD, we implemented immunohistochemical assays to determine its location and expression. The results obtained with healthy controls showed that ileal mucosa expresses DPP-4/CD26 with high intensity, but colon mucosa doesn't, being undetectable ($P=0.002$). A previous study from Darmoul et al. demonstrated that the amount of DPP-4/CD26 was highest in the ileum and not detectable in the colon, although an RT-PCR (Reverse Transcription Polymerase Chain Reaction) analysis showed the existence of a faint signal in the colon, supporting the existence of few transcripts that had not been detected by other methods.⁷⁹ Sixty percent of the UC patients in remission presented aberrant apical DPP-4/CD26 expression. On the contrary, none of the active UC patients expressed it ($P=0.012$). All ileal CD patients in remission showed high apical DPP-4/CD26 expression, as opposed to 29% with active disease, but this difference did not reach statistical significance ($P=0.190$). DPP-4/CD26 was also

expressed in the lymphocytes of lamina propria, in the mucosa of IBD patients and healthy individuals.

DPP-4/CD26 intestinal expression presented a strong and direct correlation with sDPP-4 in UC patients ($r=0.72$, $P=0.006$) and ileal CD patients ($r=0.64$, $P=0.025$). However, the correlation with fDPP-4 in UC patients was weaker ($r=0.54$, $P=0.049$) and did not correlate at all with fDPP-4 in CD patients ($P=0.873$), showing that apical expression of DPP-4/CD26 correlates better with serum than fecal DPP-4.

In this context, our group demonstrated that UC and CD patients have distinct expression profiles of DPP-4, disclosing an apparent functional compartmentalization (Figure 7).

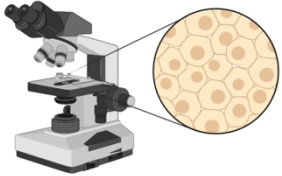
	Blood test	Fecal test	Immunohistochemical assay
			
Healthy controls	sDPP-4: +++ CRP: +	fDPP-4: ++ FC: +	DPP-4/CD26 (ileal mucosa): +++ DPP-4/CD26 (colon mucosa): -
UC in remission	sDPP-4: ++ CRP: +	fDPP-4: +++ FC: +/-	DPP-4/CD26 (colon mucosa): +
Active UC	sDPP-4: + CRP: +/-	fDPP-4: + FC: +++	DPP-4/CD26 (colon mucosa): -
CD in remission	sDPP-4: ++ CRP: +	fDPP-4: +/- ^a FC: +/-	DPP-4/CD26 (ileal mucosa): +++ ^{a, b}
Active CD	sDPP-4: + CRP: +/-	fDPP-4: +++ ^a FC: +++	DPP-4/CD26 (ileal mucosa): ++ ^{a, b}

Figure 7. Summary of blood, stool, and immunohistochemical test results.

Blood and fecal biomarker levels are classified as follows: +++ (high), ++ (moderate), +/- (moderate to low), + (low). Immunohistochemical expression is graded as: +++ (strong), ++ (moderate), + (mild), - (absent). ^a The analysis included ileal CD patients only. ^b Did not reach statistical difference

Image created in BioRender.

For the first time, we showed that UC patients in remission presented aberrant immunohistochemical expression of DPP-4/CD26 and high levels of fDPP-4 compared to patients with active UC, as well as healthy controls, suggesting it can be important to achieve and maintain remission. In fact, in this group of patients, fDPP-4 levels above 1752 ng/mL were associated with endoscopic remission in 88% of the cases. A study by Larrinaga et al. had already demonstrated an up-regulation of DPP-4/CD26 in colorectal cancer patients, advocating a role for this enzyme/receptor in the neoplastic transformation of colorectal tissue.⁸¹ Consequently, DPP-4 appears to have different functions depending on the disease, protecting against relapses in UC and facilitating the progression to colorectal cancer.

On the contrary, ileal CD patients with active disease presented higher levels of fDPP-4 and exhibited a trend toward lower enterocyte expression of DPP-4/CD26 compared to those in remission and healthy controls. This is in line with the previous observation reported by Moran et al. that DPP-4 protein expression was reduced in intestinal tissue (assessed by western blotting) from CD patients compared to healthy individuals.⁹⁸ We hypothesize that this phenomenon can be attributed to a loss of the receptor from the epithelium to the intestinal lumen, which could be associated with increased MMPs activity, responsible for the shedding of biologically active proteins from their membrane-anchored proforms (**Figure 8**).

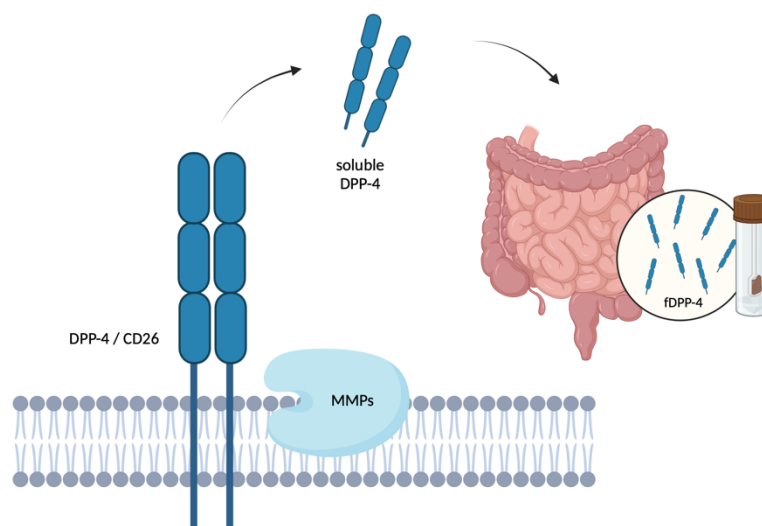


Figure 8. DPP-4 shedding mechanism hypothesis. After digestion promoted by metalloproteases (MMPs), the soluble form of DPP-4 sheds from the membrane, releasing into the intestinal lumen. Image created in BioRender.

These different patterns of serum/fecal DPP-4 levels and intestinal expression are unlikely to represent simple up/downregulations induced by inflammation, and more complex mechanisms likely exist.

As previously mentioned, the knowledge about the specific role of DPP-4 in IBD pathogenesis is limited and remains to be elucidated. **Paper III** is a systematic review that aimed to fill this gap and provide a link between preclinical and clinical data, through the use of validated animal models of intestinal inflammation and studies on patients with IBD. Its goal was to explore the role of ectopeptidases such as DPP-4 and also aminopeptidase N (APN), which gained interest as a potential co-effector of DPP-4 in immune regulation.

The inclusion criteria encompassed all preclinical studies highlighting the pathophysiological role of DPP-4 and APN in models of intestinal inflammation, in addition to clinical reports demonstrating a correlation between these ectopeptidases and IBD. Additionally, studies involving other molecules that were associated with the physiological axes of these factors were also considered as long as there was at least an indirect link to DPP-4 or APN. The risk of bias in individual studies was evaluated using quality evaluation tools/scales adapted to each study type. Of the 193 studies identified through queries, 45 were selected for subsequent analysis: 36 used animal models of intestinal inflammation, 12 concerned human patients with IBD, and 3 reports included both.

In animal models of colitis, DPP-4 inhibitors consistently shown either partial or significant therapeutic impact.¹¹⁵⁻¹²⁵ These findings may reflect, in part, the inhibition of the catalytic activity of DPP-4 inhibitors on their substrates (GLP-1, GLP-2, and vasointestinal peptide [VIP]), prolonging their half-lives. Consequently, this indirectly inhibits costimulatory signals of T-cell activation and the production of Th1-polarizing cytokines/chemokines, potentially promoting mucosal healing. Since CD26 knockout mice (CD26^{-/-}) did not have intrinsic resistance to the development of colitis or a faster rate of mucosal tissue regeneration, a more direct effect of DPP-4 inhibitor activity cannot be completely ruled out.¹²⁶⁻¹³⁰ In fact, the protective effect of DPP-4 inhibitors has only been demonstrated in the presence of this enzyme/receptor, indicating that they may work through mechanisms other than just reducing catalytic activity.^{124,125,130}

DPP-4 is also a potential serum biomarker for many diseases, including cancer and IBD, which have become an intense focus of investigation. Several human studies, including our first paper, showed that sDPP-4 levels and/or activity were significantly lower in IBD patients than in healthy individuals, and correlated inversely with clinical activity scores and other biomarkers, such as CRP and FC.^{95-98,131} The results of our first study, analyzed in this systematic review (**Paper III**), have already been mentioned.

Additionally, all animal studies that used GLP-2 analogs demonstrated a clear benefit, reporting significant reductions in pro-inflammatory cytokines and also a trophic response, with intestinal proliferation and preservation of tissue architecture.¹³²⁻¹⁴² Nevertheless, the extrapolation of these findings to humans is challenging. A study by Buchman et al. which included CD patients with moderate to severe disease treated with teduglutide (a GLP-2 analog), failed to reach statistical significance when compared to placebo, despite a tendency toward remission.¹⁴³ This trial has a few limitations: (a) it was a pilot study and was underpowered to be able to show a significant difference; (b) the use of subjective clinical indices, such as CDAI, as a primary outcome indicator; (c) compliance was quite low, due to adverse effects and uncomfortable posology.

Other human studies found lower expression of GLP-2 and its receptor in an inflammatory environment in IBD patients compared with noninflamed sites and also in healthy controls.^{95,122,144} However, one report found no differences in tissue content or serum concentration of GLP-2, not supporting the suggestion that enteroendocrine L-cell secretion is altered in IBD.¹⁴⁵

Despite the benefits of GLP-2 analogs and DPP-4 inhibitors, these drugs may increase the risk of developing colorectal cancer, and further research is required to assess their safety.¹⁴³ Furthermore, DPP-4 inhibitors may theoretically contribute to the perpetuation of inflammatory stimuli due to inhibition of DPP-4 mediated degradation of pro-inflammatory chemokines, and therefore lead to a new onset of IBD or a flare. An observational study by Abrahami et al. showed that the use of DPP-4 inhibitors was associated with an increased risk of IBD in patients with type 2 diabetes mellitus, advising physicians to be aware of this association and avoid using these drugs in patients with higher risk of IBD.¹⁴⁶ On the contrary, an observational study by Kim et al. concluded that DPP-4 inhibitor therapy results in decreased risk of IBD and other autoimmune diseases.¹⁴⁷ Due to these conflicting studies, a meta-analysis of

observational studies and clinical trials was conducted to evaluate the potential risk of IBD when comparing DPP-4 inhibitor therapy with controls¹⁴⁸ The authors concluded that based on a conservative random-effects analysis, these drugs do not appear to increase the risk of IBD, but long-term postmarketing surveillance is warranted.¹⁴⁸

This review provides an overview of the underlying pathophysiological processes and potential applications in clinical practice. We emphasize our translational approach to this subject, from preclinical animal models of disease to studies in human patients with IBD.

CONCLUSIONS & FUTURE PERSPECTIVES

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CONCLUSIONS & FUTURE PERSPECTIVES

Referring to the described aims of this Thesis, the following conclusions can be formulated:

1. Serum DPP-4 levels were significantly lower in active IBD patients compared to those in remission and healthy controls. Interestingly, no significant differences in sDPP-4 levels were demonstrated between CD and UC patients, indicating that it cannot serve as a discriminating factor between these diseases.
2. Serum DPP-4 showed an inverse correlation with clinical activity scores and endoscopic activity, as well as with other established biomarkers (e.g., FC and CRP), in both diseases.
3. ROC curve analysis confirmed that sDPP-4 can be considered a reliable surrogate marker of clinical and endoscopic activity, in IBD.
4. By defining optimal cutoff points, we have demonstrated that sDPP-4 levels can be used as a predictor of treatment escalation. Notably, the simultaneous use of three biomarkers — sDPP-4, FC, and CRP —enhanced the predictive power significantly. This multibiomarker approach holds promise, especially considering the complex and heterogeneous nature of IBD.
5. The utility of sDPP-4 in monitoring treatment response among biologically treated patients was also explored. Lower sDPP-4 levels were observed in nonresponders, particularly in UC patients. ROC curve analysis further validated sDPP-4's ability to distinguish between responders and nonresponders, indicating its potential in tailoring treatment strategies.

6. Fecal DPP-4 levels were significantly higher in UC patients in remission compared to those with active disease. On the contrary, in the group of CD patients with ileal involvement, those with active disease exhibited higher fDPP-4 levels.
7. ROC curve analysis confirmed that fDPP-4 can be considered a reliable surrogate marker of clinical and endoscopic activity, in UC patients. It appears to be a less sensitive biomarker in localized small bowel disease.
8. DPP-4/CD26 revealed distinct expression patterns in intestinal tissue of UC and CD patients. Aberrant apical DPP-4/CD26 expression was evidenced in UC patients in remission, while active UC patients showed no such expression. In contrast, ileal CD patients in remission exhibited high apical DPP-4/CD26 expression, with a tendency toward reduction in cases of active CD. We evidenced a strong and direct correlation between DPP-4/CD26 intestinal expression and sDPP-4 levels in patients with UC and ileal CD. However, the correlation with fDPP-4 was weaker, indicating that sDPP-4 may better reflect cellular expression.
9. This research reveals a potential compartmentalization of DPP-4 functions, indicating its distinct roles in different stages and types of IBD. Our study suggests that DPP-4 might have a protective function in UC, aiding in achieving and maintaining remission, while it could be associated with disease progression in CD.
10. Our systematic review concluded the following:
 - 10.1 In animal models of colitis, DPP-4 inhibitors evidenced therapeutic impact by inhibiting the catalytic activity of DPP-4 over its substrates. However, its protective properties might involve other mechanisms beyond that effect.
 - 10.2 GLP-2 analogs showed a clear benefit in animal studies, reporting significant improvements in histological and clinical scores, reduction in pro-inflammatory cytokine levels, and an intestinotrophic response. A study in humans with teduglutide (GLP-2 analog) exhibited a trend toward remission.
 - 10.3 DPP-4 inhibitors and GLP-2 analogs might increase the risk of colorectal cancer, requiring further research for safety assessment. Conflicting studies exist

regarding the association between DPP-4 inhibitors and IBD risk, suggesting the need for ongoing surveillance.

Overall, this work contributes to unraveling the role of DPP-4 in IBD and address its potential as a valuable tool for clinicians, in managing this complex and challenging condition. The integration of these findings into clinical practice has the potential to revolutionize IBD management, offering more precise diagnostics and personalized treatment strategies for patients. It emphasizes the importance of translational research, bridging the gap between preclinical models and clinical applications to improve the diagnosis and treatment of IBD.

Regarding the future and following the ideas that came up with this work, we believe it is important to investigate the underlying mechanisms behind the association of decreased sDPP-4 levels with active inflammation in IBD, and further studies could explore the molecular pathways and immune responses affected by DPP-4, shedding light on its role in disease pathogenesis. Our findings should be validated in larger and diverse patient populations to establish the clinical utility of sDPP-4 as a biomarker for disease activity, prognosis, and treatment response. Long-term studies could assess the reliability of sDPP-4 as a predictor for disease progression and the need for treatment escalation.

The potential involvement of DPP-4 in ECM remodeling and fibrosis should be further evaluated, as growing evidence suggests that DPP-4/CD26 inhibition could be a potential target for antifibrotic therapy. Knowing the shedding process of DPP-4 and the complex interplay between different proteases could also provide a more comprehensive view of their involvement in IBD pathogenesis.

The therapeutic potential of modulating DPP-4 activity in IBD could be explored, given the impact of DPP-4 inhibitors in animal models, further research could focus on developing specific drugs targeting DPP-4 for IBD treatment. However, the potential risks, such as stimulating carcinogenesis, should be carefully assessed.

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CURRICULUM VITAE

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CURRICULUM VITAE

José Pedro Reis Cabral Pinto Lopes was born on May 4th, 1986, in Penafiel, Portugal. He studied in Penafiel until attending the Faculty of Medicine of the University of Porto (FMUP), from 2004 to 2010. In the 6th year, he carried out a systematic review under the guidance of Professor Elsa Fonseca, at the Pathology Department of Centro Hospitalar Universitário São João (CHUSJ), which culminated with the master's thesis "BRAF gene mutation in the natural history of papillary thyroid carcinoma: diagnostic and prognostic implications".

After a 1-year general training at CHUSJ, he started specialization in Internal Medicine at the same institution. During this time, he began his PhD in Medicine and Molecular Oncology, which he accomplished under the supervision of Professor Fernando Magro (supervisor), Professor Guilherme Macedo and also Professor Paulo Bettencourt (co-supervisors). He participated in his supervisor's IBD consultation in July 2014, which attended until December 2016.

In October 2017 he obtained the degree of Consultant in Internal Medicine and maintained functions in the Internal Medicine Department of CHUSJ until the end of that same year. In January 2018, he began working as a Consultant at Centro Hospital do Tâmega e Sousa (CHTS), Penafiel, in the Internal Medicine Department, until December 2020. At this hospital, he was the supervisor of two internal medicine residents and tutor of several general residents, as well as medical students from FMUP, being responsible for the residents' training program. During the COVID-19 pandemic, he worked exclusively as a frontline emergency room physician.

From January 2021 he began working as an Internal Medicine consultant at Trofa Saúde Hospital Braga Sul, since then he has been the coordinator of the internal Medicine Department.

He has maintained activity as an invited assistant lecturer in the Department of Medicine from 2013 until 2018 (Medical Propedeutics lectures) and in the Biomedicine Department since 2017 (Pharmacology and Therapeutics lectures) at FMUP.

He published 16 papers in peer-reviewed journals and presented approximately 50 communications at national and international conferences.

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U. PORTO



Pedro Pinto Lopes

Porto, 2024