Facultat de ciències

Memòria del Treball de Fi de Grau

# Immunoinflammatory profiling of mesenteric adipose tissue: neutrophil-induced metalloproteinase 9 expression in creeping fat in Cronh's disease


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# RESUM

La malaltia de Crohn (CD) és una malaltia inflamatòria intestinal complexa influenciada per la predisposició genètica, factors ambientals i respostes immunitàries. Malgrat els avenços significatius en els tractaments mèdics que han millorat les taxes de remissió, una proporció substancial dels pacients encara s'enfronten a la realitat de requerir intervenció quirúrgica durant la seva vida. A més, un alt percentatge d'aquests pacients experimentarà recurrència postquirúrgica (POR) de la malaltia, la qual continua sent un repte important en el maneig de la malaltia de Crohn. La naturalesa crònica d'aquesta malaltia posa de manifest la necessitat d'investigar el curs de la seva patogènesi. Una àrea d'interès és el paper del *Creeping Fat (CrF),* que és teixit adipós mesentèric hiperplàsic, en la progressió del CD. Se sap que el CrF contribueix a la resposta immunoinflamatòria aberrant, jugant un paper important en el POR. Aquest projecte se centra en la metal·loproteinasa-9 de matriu (MMP-9), una proteïna relacionada amb la infiltració de neutròfils i degradació de la matriu extracel·lular (ECM), que contribueix a la remodelació dels teixits i a la inflamació, i també, facilita l'alliberament de múltiples molècules que amplifiquen la resposta immunitària. L'estudi ha consistit en l'anàlisi de mostres de teixit de 42 pacients amb malaltia de Crohn; 10 pacients amb càncer colorectal i 11 mostres de pacients amb obesitat, servint com a controls. L'extracció de proteïnes es va realitzar meticulosament per al seu ús posterior. La distribució ponderada dels grups d'estudi i les mostres corresponents asseguren resultats imparcials. La immunofluorescència es va utilitzar per detectar aquesta proteïna en algunes de les mostres de teixit, revelant informació sobre els patrons d'expressió de MMP-9. A més, l'anàlisi de Western Blot es va optimitzar per validar els resultats de la immunofluorescència. Es van dur a terme assajos repetits per abordar els reptes inicials i millorar la qualitat de les dades. Els resultats llancen llum sobre la intricada interacció entre neutròfils, MMP-9 i el metabolisme dels lípids en aquesta malaltia. En destacar el paper del MMP-9, la recerca obre el camí per a futurs estudis i el possible desenvolupament de teràpia dirigida. Aquest estudi contribueix a dibuixar un perfil immunoinflamatori més detallat del CrF en la malaltia de Crohn, obrint el camí per a futures investigacions i avenços terapèutics.

# RESUMEN

La enfermedad de Crohn (CD) es una enfermedad inflamatoria intestinal compleja influenciada por la predisposición genética, factores ambientales y respuestas inmunitarias. A pesar de los adelantos significativos en los tratamientos médicos que han mejorado las tasas de remisión, una proporción sustancial de pacientes todavía se enfrentan a la realidad de requerir intervención quirúrgica durante su vida. Además, un alto porcentaje de estos pacientes experimentará recurrencia postquirúrgica (POR) de la enfermedad, la cual continúa siendo un reto importante en el manejo de la enfermedad de Crohn. La naturaleza crónica de esta enfermedad pone de manifiesto la necesidad de investigar el curso de su patogénesis. Un área de interés es el papel del *Creeping fat* (CrF), que es tejido adiposo mesentérico hiperplásico, en la progresión del CD. Se sabe que el CrF contribuye a la respuesta inmunoinflamatoria aberrante, jugando un papel importante en el POR. Este proyecto se centra en la metaloproteinasa-9 de matriz (MMP-9), una proteína relacionada con la infiltración de neutrófilos y la degradación de la matriz extracelular (ECM), que contribuye a la remodelación de los tejidos y a la inflamación, pero también, facilita la liberación de múltiples moléculas que amplifican la respuesta inmunitaria. El estudio ha consistido en el análisis de muestras de tejido de 42 pacientes con enfermedad de Crohn; 10 pacientes con cáncer colorrectal y 11 muestras de pacientes con obesidad, sirviendo como controles. La extracción de proteínas se realizó meticulosamente para su posterior uso. La distribución ponderada de los grupos de estudio y las muestras correspondientes aseguran resultados imparciales. La inmunofluorescencia se utilizó para detectar esta proteína en algunas de las muestras de tejido, revelando información sobre los patrones de expresión de MMP-9. Además, el análisis de Western Blot se optimizó para validar los resultados de la inmunofluorescencia. Se llevaron a cabo ensayos repetidos para abordar los retos iniciales y mejorar la calidad de los datos. Los resultados lanzan luz sobre la intrincada interacción entre neutrófilos, MMP-9 y el metabolismo de los lípidos en esta enfermedad. Al destacar el papel del MMP-9, la investigación abre el camino para futuros estudios y el posible desarrollo de terapia dirigida. Este estudio contribuye a dibujar un perfil inmunoinflamatorio más detallado del CrF en la enfermedad de Crohn, abriendo el camino para futuras investigaciones y avances terapéuticos.

# ABSTRACT

Crohn’s disease (CD) is a complex inflammatory bowel disease influenced by genetic predisposition, environmental factors, and immune responses. Despite significant advancements in medical treatments that have improved remission rates, a substantial proportion of patients still face the reality of requiring surgical intervention in the lifetime. Additionally, a high rate of these patients will experience post-surgical recurrence of the disease, which remains a significant challenge in the management of Crohn’s disease. The chronic nature of this disease underscores the necessity for ongoing research into its pathogenesis. One area of interest is the role of creeping fat, which is hyperplasic mesenteric adipose tissue, in the progression of CD. Creeping fat is known to contribute to the aberrant immunoinflammatory responses, playing an important role in POR. This project focuses on matrix metalloproteinase-9 (MMP-9), a protein related to neutrophil infiltration and degradation of the extracellular matrix (ECM) contributing to tissue remodelling and inflammation. But also facilitating the release of multiple molecules that amplify immune response. The study involved an analysis of tissue samples from 42 Crohn’s disease patients, 10 colorectal cancer patients, and 11 patients with obesity samples, serving as controls. Protein extraction was meticulously performed for subsequent use. The weighted distribution of the study groups and the corresponding samples ensure unbiased results. Immunofluorescence was employed to detect this protein in some tissue samples, revealing insights into MMP-9 expression patterns. Additionally, Western Blot analysis was optimized to validate the findings from immunofluorescence. Repeated trials were conducted to address initial challenges and improve data quality. Results shed light on the intricate interplay between neutrophils, MMP-9 and lipid metabolism in this disease. By highlighting the role of MMP-9, research paves a way for future studies and possible target therapy development. This study contributes into drawing a more detailed immunoinflammatory profile of creeping fat in Crohn’s disease, paving the way for future research and therapeutic advancements.

Reflection on Gender Perspective

The IBD group (Inflammatory Bowel Disease) has proved excellence in clinical and translational research with women engaging key roles in this success. However, global data highlighting gender inequalities in science is still reported every day. Structural and cultural barriers constrain women's visibility in leadership positions, that is why implementing feminist ethics principles and gender policies is crucial for equal opportunities in research.

As previously detailed, the IBD group's significant female representation shows it is possible to overcome these obstacles. Maintaining proactive strategies to strengthen women's leadership is crucial. The group's gender dynamics serves as an example of equal opportunities and active recognition of female contributions, being a clear inspiration for inclusion and diversity in research.

Reflection on Ethics

Equity in resource assignment is essential for effective collaboration in research. The IBD group’s success brings up ethical questions about resource distribution and authorship policies, existing an unequal perception in recognition, were medical research tends to surpass translational research.

Securing public funds is challenging in our country, and lack of recognition can impact funding competitiveness. It is imperative to implement measures that can ensure equitable distribution of funds, recognizing both contributions. Hiring an external expert group could help to avoid possible internal conflicts, enhancing the group’s image and ensuring a successful path in research.

Reflection on Sustainability

Embracing sustainable research practices such as waste reduction, resource optimization and consideration of environmental impact, ensures a promising future for research. Sustainability in the IBD research group means securing long-term viability and impact while striving to minimise negative effects.

Collaboration and knowledge sharing not only within the group, but also with IGTP, the research centre, lead to innovative and sustainable solutions. IGTP is consistently involved in efficient management of resources, including equipment, materials and waste conducting a community engagement. This philosophy enhances the research centre’s impact and relevance, promoting not just a remarkable reputation in research but also in progression and sustainability

# 1. Introduction

Inflammatory bowel disease (IBD), a chronic and challenging condition characterised by immune-mediated inflammation of the gastrointestinal tract, affects millions of individuals worldwide. This complex disorder, often manifested as either ulcerative colitis or Crohn's disease (CD), is distinguished by the intricate interplay between host genetic factors, environmental influences, and gut dysbiosis (1), resulting in a multifaceted disease process that still remains incompletely understood.

A defining feature of IBD is the disruption of intestinal barrier function, leading to permeability defects marked by alterations in tight junction structure, protein composition, and functionality. These barrier irregularities facilitate the entry of luminal microbes, toxins, and allergens into the gut, triggering immune cell infiltration and promoting an inflammatory response within the intestinal tissue (2).

The human gut, which is now recognised as unique in every individual, harbours a diverse microbial community dominated by Firmicutes and Bacteroidetes phyla in healthy adults. Significant alterations are observed In IBD patients, particularly in Crohn’s disease, where approximately 30% of the dominant bacteria belong to novel phylogenetic groups not commonly prevalent in healthy individuals. Additionally, a loss of bacterial diversity is typically observed in these patients (3).

In light of this context, the aim of this project is to enhance the comprehension of IBD, especially Crohn’s disease (CD), which is distinguished by its unique trait, creeping fat (CrF) , by investigating the immunoinflammatory profile of mesenteric adipose tissue (MAT), with a specific emphasis on the expression of neutrophil-induced metalloproteinase 9 (MMP-9) in adipose tissue infiltration.

## 1.1. Crohn's disease and post-surgical recurrence

Despite significant advancements in medical treatments, a substantial proportion of IBD patients continue to require surgical intervention. Data collected from diverse sources indicate that the risk of surgery within 10 years of diagnosis is 44.6% for CD and 15.6% for UC. This highlights the urgent need for innovative therapeutic strategies that can effectively manage the disease and improve patient outcomes (4).

Crohn's disease, which we will address in this project, presents a diverse range of clinical manifestations marked by variations in age of onset, disease location, and behaviour. While this condition can affect the entire gastrointestinal tract, approximately half of patients primarily experience active ileocolonic disease. Small bowel involvement is observed in about 30% of patients, with isolated colonic involvement occurring in the remaining 20% (3).

Several studies conducted in the 1980s have indicated a tendency for intestinal lesions to occur in the distal bowel, where the intestinal microbiome is more abundant. This is significant because bacteria found in faecal matter have been implicated as factors that contribute to the recurrence of intestinal lesions after surgery (5). Recent research has emphasised the correlation between specific gut microbiota profiles at the time of surgery and during postoperative follow-up with disease recurrence in Crohn's disease patients.

## 1.2. Creeping fat implication in post-surgical recurrence

As mentioned previously, an exclusive and pathological feature of Crohn's disease is creeping fat which entails hypertrophy (an enlargement of the existing adipocytes) and hyperplasia (the formation of new adipocytes through the differentiation of preadipocytes) of MAT (6). This wrapping fat, graphically described in Figure 1, was first described by Burril Crohn in 1932 (7) and nowadays it is known to be predictive of early clinical recurrence after surgery in patients with CD.

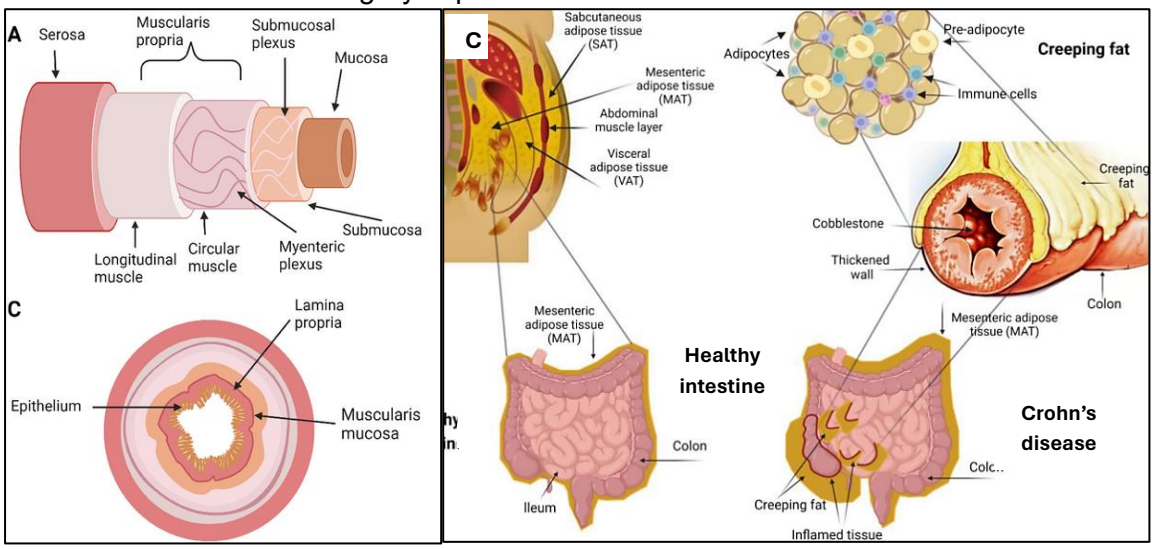



Figure 1. Structure of the small intestine: A) Lateral view of the layers. B) Cross sectional view of the layers. C) Transformation of MAT to CrF, adipose tissue located around the inflamed intestinal segments. Both figures were generated by BioRender and sourced from: McCoy, R. et.al.(2023) and Aggeletopoulou I. et.al. (2023) (8).

As proven, the inclusion of the mesentery in ileocolic resection for Crohn's disease has been associated with reduced surgical recurrence, suggesting that the MAT plays a crucial role as a source of immune cells and soluble factors that contribute to the pathogenesis of CD. This is further underscored by the diverse cellular composition of the adipose tissue, with adipocytes constituting approximately 20-40% of the cellular content. The remaining cells include endothelial cells, fibroblasts, stem cells, immune cells and pre-adipocytes. In CD patients an increase in the cellular content is a common observation (9).

The main characteristic of CD is the overgrowth and inflammation of MAT due to transmural lesions and bacterial translocation that results in the formation of CrF surrounding the lesion. Alterations in nuclear receptors as well as dysregulation of adipokines are also implicated in the dysfunction of creeping fat (6). It is important to recognise that creeping fat exhibits a microbiome signature enriched in *Proteobacteria* and *C.innocuum*, and the abundance of these bacteria varies depending on the clinical status of the disease (9).
All these alterations not only worsen the inflammatory course occurring in CD but also favour intestinal fibrosis and fistulizing complication, all leading to an increased risk of postoperative recurrence (POR).

### 1.3. Role of neutrophils in pathological transformation of mesenteric adipose tissue

Recent research has shed light on the important role of neutrophils in orchestrating the inflammatory cascade that drives the pathological transformation from mesenteric adipose tissue, as a protective barrier, to this compact mass known as creeping fat.

Neutrophils are recognized as the frontline defenders against microbial invasion, but in CD assume a dual role, both as defenders and initiators of aberrant inflammatory activity within the MAT.

Infiltration by neutrophils is one of the first actions that take place in response to an infection leading to an inflammatory process (10). In this tissue, the presence of lipids, particularly fatty acids, sets the stage for an inflammatory neutrophil activation as they act as potent stimuli, releasing proinflammatory mediators like cytokines, matrix metalloproteinases and radical oxygen species, amplifying the inflammatory response and perpetuating tissue damage (11).

The intensity of the inflammatory response produced by immune cells induces injury to the epithelial barrier, allowing antigens such as bacteria enter causing the activation of an adaptive response. This mechanism involves T-helper, mostly Th17 (higher in mucosa) responsible for granuloma formation, and Th1 (higher in CrF), that secretes interleukin, such as IL-12, IL-1, IL-2; interferon (IFN)-α and (IFN)-γ; tumour necrosis factor (TNF) and more. (6,9)

Clearly, there is an intricate interplay between neutrophils, lipids and innate immunity. This interaction not only contributes significantly to the progression of this pathology but also serves as a bridge between the innate and adaptive immune systems, highlighting the complexity of the immune response in this context.

### 1.4. Previous background of the group

The IBD research group has been dedicating several years to investigating CrF. As delineated by Suau et al. (2022), CrF is characterised as a hyperplasia of MAT perilesional to the inflamed ileum in Crohn's disease (CD) patients.

The complex immune microenvironment in CrF and the crosstalk with the inflamed intestine underscore the necessity of identifying markers to facilitate the study of disease progression. Currently, numerous markers are under investigation.

The IBD group from the IGTP research centre has, in the present, focused its efforts on two distinct markers that show a subtle interconnection and are both modulated by the activity of neutrophils: Free fatty acid receptor-1 and metalloproteinase-9.

Continuing along this investigative path, recent months have seen our group's focus on refining methodologies such as immunofluorescence and Western Blot. For instance, the team explored the free fatty acid receptor 1, FFAR1, also known as GPR40.

This receptor is part of the rhodopsin family of G-protein coupled receptors and is primarily found in pancreatic β-cells, where it enhances glucose-stimulated insulin secretion, and in enteroendocrine cells, where it stimulates the secretion of incretins (12).

In Crohn's disease, short and long-chain fatty acids, also secreted by adipocytes, interact with FFAR, particularly with FFAR1, modulating immune functions and contributing to intestinal homeostasis. Subsequently, they regulate pro- and anti-inflammatory mediators that can directly impact CD pathophysiology (13).

Additionally, studies show that the activation of FFAR1 can specifically stimulate the proliferation of intestinal muscularis propria muscle cells, contributing to stricture development. This is because FFAR1 regulates matrix metalloproteinases expression, including MMP-9 - the focus of our study - which are involved in extracellular matrix degradation and the progression of fibrosis, as will be elaborated upon later (14).

Besides this, histological analyses were conducted on adipose tissue samples from CD patients, employing haematoxylin and eosin staining, alongside Masson's trichrome stain. The objective of this step of the research was to assess adipocyte size in CD patients and to evaluate CrF prevalence in conjunction with intestinal fibrotic stricture.

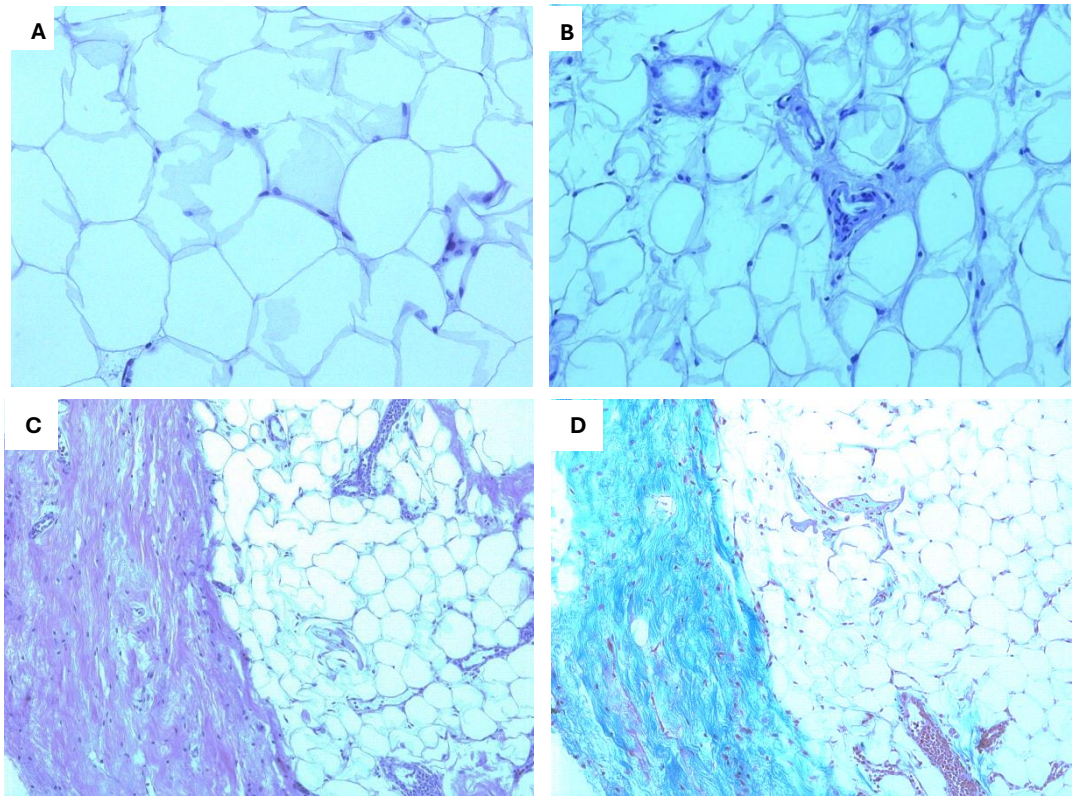



Figure 2. Representative histological images of mesenteric peri-lesional adipose tissue samples stained with Haematoxylin & Eosin (A-C) and Masson's Trichrome (D). A) Adipocytes from patients with mild Crohn's disease progression, displaying large lipid vesicles and healthy morphology. (B) Adipocytes from patients with moderately severe Crohn's disease progression, showing a reduction in size, the presence of connective tissue, and a few inflammatory cells. C) Adipocytes from patients with severe Crohn's disease progression, characterized by significant reduction in size, inflammatory infiltrate, and extensive connective tissue. D) Masson's Trichrome staining highlighting the fibrosis in the sample shown in (C). Magnification: 20x.The images were captured using the inverted microscope Leica DMI 6000B. The samples were sourced from the *"Treball de fi de grau de Carol Pérez".*

## 1.5. Crohn's disease markers

Crohn's disease markers are biological indicators used to diagnose and understand the progression of this disorder, as well as measure the activity and severity of the IBD. Some of them are commonly used, such as C-reactive protein (CRP) and faecal calprotectin, which indicate inflammation in the organism. Other markers are related to the immune system: antibodies and cytokines, to help understand the underlying mechanism and develop targeted treatment. Genetic studies for specific mutations that are related with an increased risk of developing Crohn's disease are also crucial for the management and research of this complex inflammatory condition. (15,16)

Creeping fat, has been elucidated as a source rich of pro-inflammatory and pro-fibrotic cytokines, conducting a downstream cascade of soluble cytokines that are well known to be a hallmark of CD and play an important role against bacterial infection (9).

In addition, adipocytes have also an important capacity of exerting effect on neighbouring cells and regulating immune response through molecules named adipokines: adiponectin, leptin, apelin… that have been demonstrated to modulate the immune system in CD. These cells sense antigens via pattern recognition receptors (PRRs) developing the innate immune response, by recognizing common microbial molecules known as pathogen-associated molecular patterns (PAMPs), especially toll-like receptors (TLRs) and nucleotide-oligomerization domain (NOD)-like receptors, that are key to IBD. These receptors initiate the transformation of adipocytes into hypertrophic proinflammatory cells that produce, as explained, cytokines, adipokines and chemokines (6).

### 1.5.1. Metalloproteinase-9

Intestinal fibrosis is a common complication of IBD, which is characterised by the excessive deposition of extracellular matrix (ECM) proteins, like collagen. In CD the abnormal deposition of ECM during the inflammatory response leads to hyperplasia of connective tissue, tissue remodelling, and promotes fibrogenesis, concluding in intestinal fibrosis (17).

Matrix metalloproteinases (MMPs) have a key role in fibrosis, thus turning them as potential targets. They regulate fibrosis by degrading the ECM, their function is precisely regulated by tissue inhibitors of matrix metalloproteinases (TIMPs), which inhibit MMP activity. MMPs belong to a family of endopeptidases that are normally expressed at very-low levels, they are normally synthesised as latent precursors that have to be proteolytically activated to form the fully mature enzyme (18).

This project focuses on metalloproteinase-9 (MMP-9), also known as gelatinase B. This protein is initially secreted as an inactive zymogen (92 kDa) and gets activated by various proteinases, such as MMP-3. Its expression spans various cell types, including immune cells like monocytes, macrophages, neutrophils; and non-immune cells such as keratinocytes, fibroblasts, osteoclasts, chondrocytes, endothelial cells, and tumour cells, and is modulated by cytokines like IL-1b, TGFb1, PDGF, and TNFα (10).

Inflammation in Crohn's disease leads to an increase in MMP-9 signalling, which triggers fibrotic processes and strictures. Neutrophils to be able to digest the basement membrane and the surrounding ECM, they secrete MMPs from the granules that help contribute to damage the tissue thanks to their proteolytic activity. MMP9 compared with other metalloproteases has a wide range of capacity to degrade most of the components of the membrane, giving this protein a huge role in neutrophil migration (10).

Neutrophils from these patients show an elevated expression of genes related to granule formation, including MMP-9. This metalloprotease interacts with various ECM proteins like cytokines, chemokines, that can influence the inflammation process.

MMP-9 not only has a role in ECM degradation, but also generates MHC bounded peptides such as autoimmune neo-epitopes, activating interleukins and further stimulating chemokines. Nowadays various mediators are known to stimulate MMP-9 release : IL-8, TNF- α, fMLP (19).

Understanding MMP-9's role in CD, its correlation with neutrophil activity, and lipid metabolism receptors in creeping fat offer insights into CD immunological mechanisms and potential therapeutic targets.

## 2. Hypothesis

Neutrophil infiltration and activation in creeping fat of Crohn's Disease patients contribute to immunometabolic dysregulation, characterised by altered lipid metabolism receptors and upregulation of matrix metalloproteinase-9 (MMP-9) expression, leading to increased post-surgical complications and disease progression.

The study of neutrophil infiltration associated with MMP-9, could provide insights into the pathogenesis of creeping fat and potential therapeutic targets for Crohn's Disease management.

### Objectives

The aim of this project is to determine the cellular and subcellular localization of MMP9 in creeping fat samples at the time of surgery. We aim to determine the specific location of this metalloproteinase and its crosstalk with neutrophil infiltration. The specific objectives are:

- Developing a robust IF protocol to visualise the expression and localization of MMP-9 in the target tissues in paraffin-embedded creeping fat samples.
- Verifying findings through Western Blot: validate the expression levels of MMP-9 and observed in the IF assays. This step will confirm the presence of this marker and provide quantitative data.
- Co-localize, as a long-term objective, both MMP-9 and FFAR1 in the tissues: investigate the correlation between neutrophil activity and creeping fat by assessing the levels of neutrophil infiltration and lipid metabolism in tissues exhibiting creeping fat.

# 3. Materials and methods

## 3.1. Patients and tissue samples

A total of 63 tissue samples donated from the Germans Trias i Pujol University Hospital (HUGTP) were collected from patients undergoing surgery due to uncontrolled intestinal conditions. The intestinal resections included mucosa and mesenchymal adipose tissue adhered to the perilesional area. These samples entered a standardised and validated protocol at the BioBank, ensuring sample traceability, preservation, and ethical controls (approved by the clinical research ethics committee of HUGTP) and patient data protection (all patients signed the data protection document).

Among these, 42 samples were obtained from patients diagnosed with Crohn's disease, including both discovery and replicate cases. Additionally, 10 samples were sourced from patients with colorectal cancer, and 11 samples were collected from patients with obesity. Both of these groups served as control groups for the project.

## 3.2. Protein extraction and quantification

The samples were subjected to the extraction of total RNA and protein using QIAzol. A crucial procedure in the project, as it serves to collect the needed protein for the next approach: Western Blot. The protocol involves several steps to ensure the successful isolation of RNA and protein from tissue samples.

Firstly, the homogenization of the tissue is carried out using QIAzol in a gentleMACS Dissociator with several cycles of centrifugation, followed by the addition of chloroform to separate the phases. After another centrifugation at 4ºC, the aqueous phase containing RNA is carefully collected, while the interphase and organic phase containing DNA and proteins, respectively, are preserved.

For RNA extraction, the aqueous phase is transferred to RNeasy columns and processed according to the manufacturer's protocol.

On the other hand, the protein extraction process begins by recovering the two phases present at 4°C and thoroughly mixing them. Ethanol is then added to the mixture, followed by inversion and incubation at room temperature (RT). Subsequent centrifugation at 2000xg and 4°C for 20 minutes allows for the collection of the supernatant in a clean tube.

Further protein precipitation is achieved by adding four volumes of cold acetone to the supernatant, followed by incubation at -20°C (2h30min). After centrifugation at maximum speed and 4°C, the supernatant is discarded, and the protein pellet is air-dried for 7 minutes. The pellet is then resuspended in a 10M Urea/ 5mM DTT solution helped by Pellet Tester, incubated at RT for 1h, and subsequently heated in a thermoblock at 95°C and a last cycle of centrifugation at RT.

When the supernatant is recovered, the protein sample is ready to be divided into 4 aliquots: 3 of 90 µl and a 30 µl aliquot, for next stored at -80°C until further use.

The protein quantification assay was conducted using the DC Protein Assay protocol. To prepare the BSA dilution bench, a stock solution of 2.0 mg/mL BSA was diluted in water

and 10% lysis buffer at room temperature, the lysis buffer contained 10M Urea/ 5mM DTT.

For sample preparation, it was crucial to maintain a consistent lysis buffer concentration in both standard tubes and samples, with a recommended maximum of 10%. Samples were diluted 1/10 (3 µL of sample + 27 µL of ddH2O), 1/20 (15 µL of sample + 15 µL of ddH2O) and 1/40 (15 µL of sample + 15 µL of ddH2O) as shown in Figure 3 and kept on ice.

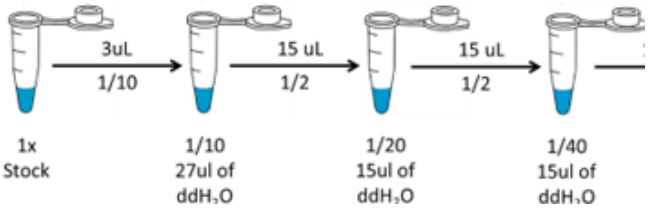



Figure 3. Example of dilution bench preparation for samples at 1/10, 1/20 and 1/40 dilutions

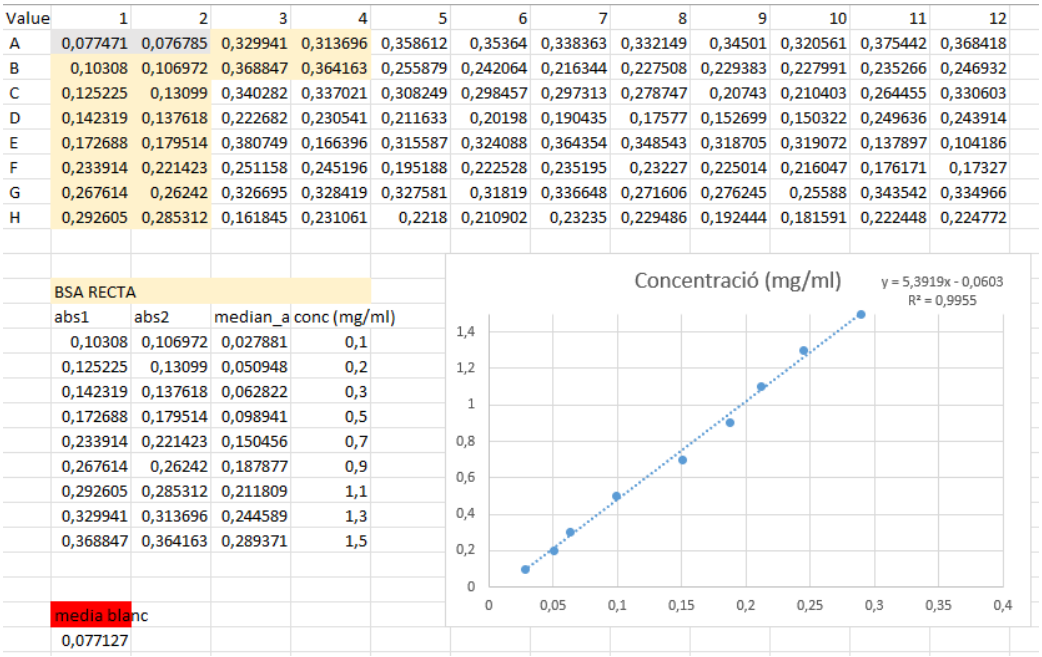
Subsequently, a dilution bench was prepared for each sample, with 5 µL of each sample dilution and standard added to individual wells in replicates. The blank solution comprised 4 µL of lysis buffer, 36 µL of ddH2O, and no BSA stock. Standard solutions included concentrations of 0.1, 0.2, 0.3, 0.5, 0.7, 0.9, 1.1, 1.3, and 1.5 mg/mL, each prepared with varying proportions of lysis buffer, ddH2O, and BSA stock as indicated in Table 1.

**Table 1.** Schematic representation of the dilution bench setup for BSA quantification.

| | | | | |
|---|---|---|---|---|
| i. | Blank solution: | 4ul of lysis buffer + | 36ul of $ddH_2O$ + | 00ul of stock of BSA |
| ii. | 0,1 mg/mL: | 4ul of lysis buffer + | 34ul of $ddH_2O$ + | 02ul of stock of BSA |
| iii. | 0,2 mg/mL: | 4ul of lysis buffer + | 32ul of $ddH_2O$ + | 04ul of stock of BSA |
| iv. | 0,3 mg/mL: | 4ul of lysis buffer + | 30ul of $ddH_2O$ + | 06ul of stock of BSA |
| v. | 0,5 mg/mL: | 4ul of lysis buffer + | 26ul of $ddH_2O$ + | 10ul of stock of BSA |
| vi. | 0,7 mg/mL: | 4ul of lysis buffer + | 22ul of $ddH_2O$ + | 14ul of stock of BSA |
| vii. | 0,9 mg/mL: | 4ul of lysis buffer + | 18ul of $ddH_2O$ + | 18ul of stock of BSA |
| viii. | 1,1 mg/mL: | 4ul of lysis buffer + | 14ul of $ddH_2O$ + | 22ul of stock of BSA |
| ix. | 1,3 mg/mL: | 4ul of lysis buffer + | 10ul of $ddH_2O$ + | 26ul of stock of BSA |
| x. | 1,5 mg/mL: | 4ul of lysis buffer + | 06ul of $ddH_2O$ + | 30ul of stock of BSA |

Following the sample and standard additions, the A' solution (A + S in a 1S:50A ratio) was prepared and 25 µL was added to each well without mixing. Subsequently, 200 µL of B solution was added to each well, and the plate was incubated for 15 minutes. The absorbance was then measured at 750 nm using a *Varioskan*, following the specified protocol for data acquisition and analysis.

This protocol was followed for the 63 samples with a total of 7 plates analysed, the results of which we will see in the next part of the project. The process for determining the original concentration involved several steps. Initially, the regression line was calculated using the obtained absorbances from the dilution bench, and the provided concentrations (Figure 5). Subsequently, the mean of the two replicates for each concentration was computed and the sample blank absorbance was subtracted. Plotting the data and fitting a regression line yielded a graphical representation along with the associated equation and coefficient of determination (R). This equation allowed for the calculation of sample concentrations based on absorbance readings. Finally, the applied dilutions were reversed, and the dilutions that best fit the regression line were assessed.



| Value | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 0,077471 | 0,076785 | 0,329941 | 0,313696 | 0,358612 | 0,35364 | 0,338363 | 0,332149 | 0,34501 | 0,320561 | 0,375442 | 0,368418 |
| B | 0,10308 | 0,106972 | 0,368847 | 0,364163 | 0,255879 | 0,242064 | 0,216344 | 0,227508 | 0,229383 | 0,227991 | 0,235266 | 0,246932 |
| C | 0,125225 | 0,13099 | 0,340282 | 0,337021 | 0,308249 | 0,298457 | 0,297313 | 0,278747 | 0,20743 | 0,210403 | 0,264455 | 0,330603 |
| D | 0,142319 | 0,137618 | 0,222682 | 0,230541 | 0,211633 | 0,20198 | 0,190435 | 0,17577 | 0,152699 | 0,150322 | 0,249636 | 0,243914 |
| E | 0,172688 | 0,179514 | 0,380749 | 0,166396 | 0,315587 | 0,324088 | 0,364354 | 0,348543 | 0,318705 | 0,319072 | 0,137897 | 0,104186 |
| F | 0,233914 | 0,221423 | 0,251158 | 0,245196 | 0,195188 | 0,222528 | 0,235195 | 0,23227 | 0,225014 | 0,216047 | 0,176171 | 0,17327 |
| G | 0,267614 | 0,26242 | 0,326695 | 0,328419 | 0,327581 | 0,31819 | 0,336648 | 0,271606 | 0,276245 | 0,25588 | 0,343542 | 0,334966 |
| H | 0,292605 | 0,285312 | 0,161845 | 0,231061 | 0,2218 | 0,210902 | 0,23235 | 0,229486 | 0,192444 | 0,181591 | 0,222448 | 0,224772 |

BSA RECTA

| abs1 | abs2 | median_a | conc (mg/ml) |
|---|---|---|---|
| 0,10308 | 0,106972 | 0,027881 | 0,1 |
| 0,125225 | 0,13099 | 0,050948 | 0,2 |
| 0,142319 | 0,137618 | 0,062822 | 0,3 |
| 0,172688 | 0,179514 | 0,098941 | 0,5 |
| 0,233914 | 0,221423 | 0,150456 | 0,7 |
| 0,267614 | 0,26242 | 0,187877 | 0,9 |
| 0,292605 | 0,285312 | 0,211809 | 1,1 |
| 0,329941 | 0,313696 | 0,244589 | 1,3 |
| 0,368847 | 0,364163 | 0,289371 | 1,5 |

media blanc
0,077127



Figure 5. Linear regression analysis in Excel to determine original concentrations of the samples based on absorbance reading.

### 3.3. Immunofluorescence

The immunofluorescence protocol for paraffin-embedded tissue sections, which were 5 microns thick, was conducted following a standardised procedure. Firstly, deparaffinization and hydration were carried out in a fume hood using a series of containers containing *Clearene*, followed by varying concentrations of ethanol and distilled water (Figure 6).

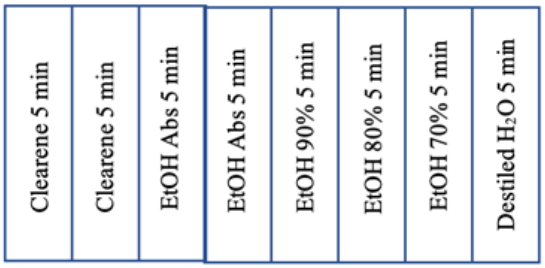



Figure 6. Deparaffinization and hydration process of the tissue samples

Subsequently, antigen unmasking was performed by immersing the slides in a commercial Citrate Buffer for Heat-Induced Epitope Retrieval at pH 6.0, utilising microwave treatment for 5 minutes at high power followed by cooling for a minimum of 20 minutes. This step was crucial for epitope exposure. The sections were then washed in a PBS buffer. Following antigen retrieval, nonspecific binding sites were blocked using SuperBlock (PBS) Blocking Buffer for 1 hour at room temperature.

For primary Mouse Anti-MMP9 monoclonal antibody (NBP2-59699; *Novus Biologicals*, LLC; US) staining, concrete concentrations were prepared using Lab Vision™ Antibody Diluent OP Quanto-*Thermo Fisher* Scientific. Four slides were utilised, each containing two slices of fat from the same patient. 100 µL were dispensed for each section. Two sections underwent incubation with the primary antibody MA5-15886, while two were treated with NBP2-59699 (both 1/50 and 1/100) (Figure 7). The sections were then incubated overnight at 4ºC.

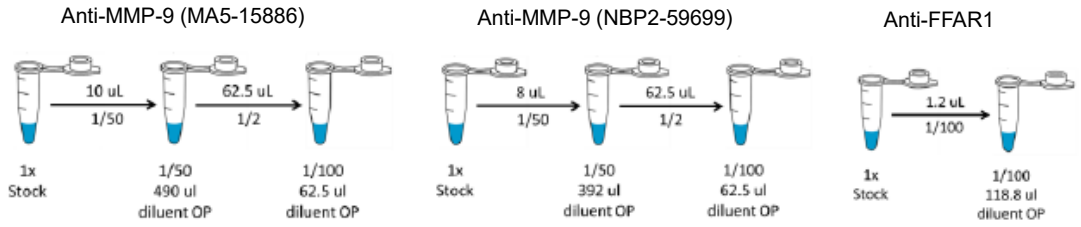



Figure 7. Dilution bench scheme representation for the two primary antibodies: anti-MMP-9 (MA5-15886) and (NBP2-59699); and as positive control: anti-FFAR1.

After several PBS washes, incubation was followed by a 1-hour room temperature with also two secondary antibodies: Anti-Ms (mouse) STAR Orange (*Abberior*, Germany) 1/100 for MMP-9 and 1/200; and Anti-Rb (rabbit) STAR Red (*Abberior, Germany*) 1/500 for FFAR1 (Figure 8).

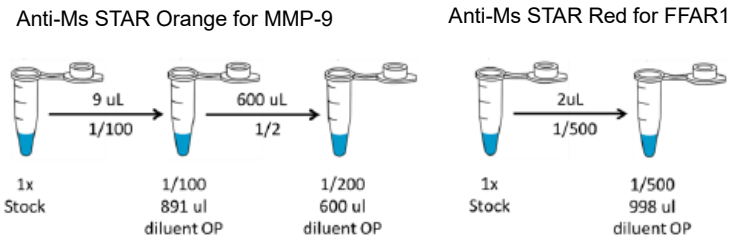



Figure 8. Dilution bench scheme representation for the two secondary antibodies: Anti-Ms STAR Orange for MMP-9 and Anti-Rb STAR Red for FFAR1.

One of the 8 sections will be subjected to incubation with polyclonal anti-FFAR1 (*Thermo Fisher*; US) 1/100 as a positive control for the technique. Finally, one of the sections will be exposed to 1/100 of the secondary antibody along with 1/50 of MA5-15886 (provided by the IGTP Microscopy Unit). Two sections will serve as negative controls with 1/100 and 1/200 of the secondary antibody. The negative control procedure entails exclusion of primary antibody incubation, employing only diluent OP.

Subsequently, three additional slides of intestinal tissue, each comprising two sections (healthy and inflamed mucosa), will be processed as a positive control. Incubation will

be conducted with 1/50 of both MMP-9 primaries in healthy and inflamed tissue, along with 1/200 of the anti-Ms STAR Orange secondary antibody. A negative control will be prepared for each zone: inflamed and healthy.

Fluorescent secondary antibody staining was conducted in darkness, with appropriate concentrations of secondary antibody diluted in Lab Vision™ Antibody Diluent OP Quanto. Subsequent washes with PBS were performed in darkness to minimise background fluorescence.

DAPI staining was carried out using a diluted DAPI stock solution for 15 minutes at room temperature (Figure 9). The final step involves a dehydration process (opposite to Fig. 7). Finally, the samples were mounted in the appropriate mounting media for subsequent visualisation under the microscope.

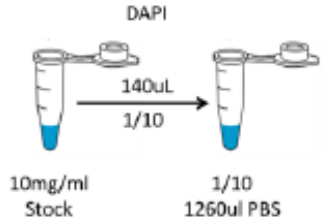



Figure 9. Dilution bench scheme representation for DAPI staining.

### 3.4. Western Blot

In the Western blot analysis of creeping fat samples using the Odyssey system, a meticulous protocol was followed to ensure accurate and reproducible results. The starting material consisted of samples: 60 ug and 80 ug respectively. The materials utilised included 1.5 mL microcentrifuge tubes, a Thermoblock, XCell SureLock Mini-Cell, 10-well NuPAGE Novex Bis-Tris Gel, Biorad transfer chamber, Whatman paper, nitrocellulose membrane, sponges, and a sandwich cassette.

The reagents essential for this analysis comprised deionized water, 4x NuPAGE LDS Sample Buffer, 10x NuPAGE Reducing Agent, 20x MES/MOPS SDS Running Buffer, NuPAGE Antioxidant, SeeBlue Plus 2 prestained Standard 1x, methanol, TRIS, glycine, Li-cor Intercept Blocking Buffer PBS, Tween-20, and IRDye 800Cw Conjugated Goat Anti Mouse IgG 0.5 mg Li-Cor.

The buffer preparation consisted of 1x Running Buffer Lower Chamber by combining 20x MES/MOPS SDS Running Buffer with deionized water. For the 1x Running Buffer Upper Chamber, the MES/MOPS SDS Running Buffer was mixed with an antioxidant. Additionally, a transfer buffer was prepared using methanol, TRIS, glycine, and deionized water.

The electrophoresis procedure started with the preparation of samples with 10% Tween-20, followed by incubation on ice for 1 hour and subsequent incubation at 70°C for 10 minutes. The NuPAGE Gel was then prepared by rinsing the gel cassette, removing the tape and comb, and rinsing the wells with 1x Running Buffer Lower Chamber. The gel

was loaded with samples and a standard, and electrophoresis was conducted at 200V for 35 minutes approximate, until the Coomassie blue stain disappeared.

After electrophoresis, the gel was separated, and the blotting process was initiated by transferring the gel onto a nitrocellulose membrane *(LI-COR Odyssey*; US) in a transfer buffer, being the mount transfer this order: White part- Sponge- Whatman - Membrane - Gel- Whatman- Sponge - Black part. Once the cassette was inside the chamber with ice, it ran for 1h at 100V (Figure 10).

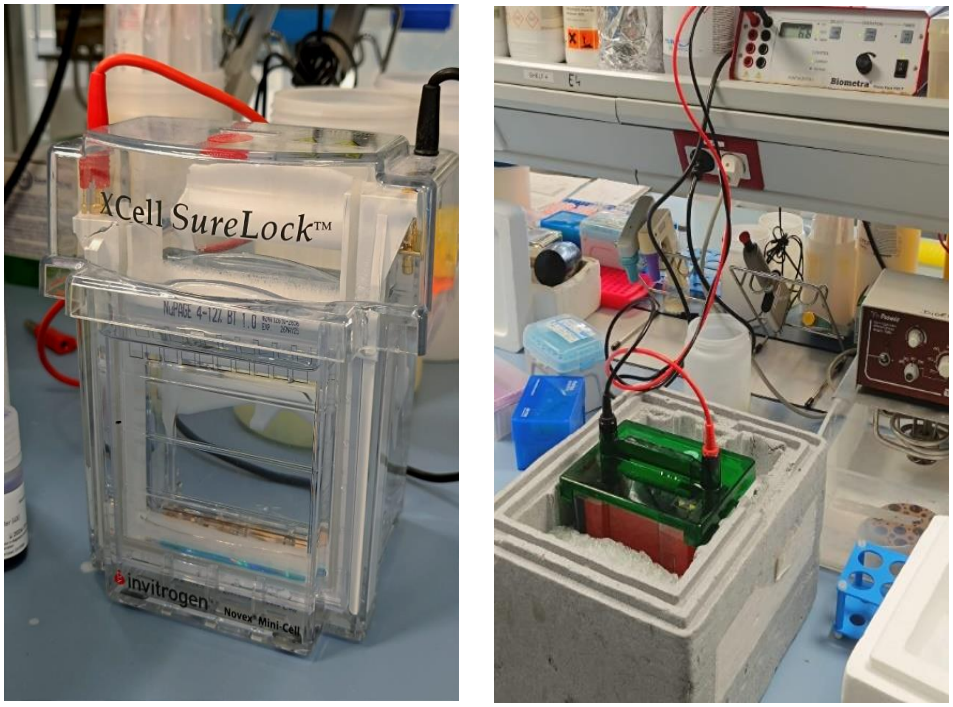



Figure 10. SDS-PAGE electrophoresis process (left panel), and blotting process (right panel).

The membrane was then incubated for 1h at room temperature with Li-COR Intercept Blocking Buffer PBS, followed by primary Mouse Anti-MMP9 monoclonal antibody (NBP2-59699; *Novus Biologicals*, LLC; US) buffer overnight at 4ºC in the roller. And ending with the IRDye® 800CW secondary Donkey anti-mouse IgG Secondary Antibody (*LI-COR Bio*; US) buffer 1h in darkness at room temperature in the orbital shaker. Washing steps were performed with T-PBS and PBS, and the membrane was finally placed in PBS for analysis at both wet and dry conditions in the Odyssey M fluorescence scanner *(LI-COR Bio*; US). The employed ladder was the following one indicated in Figure 11.

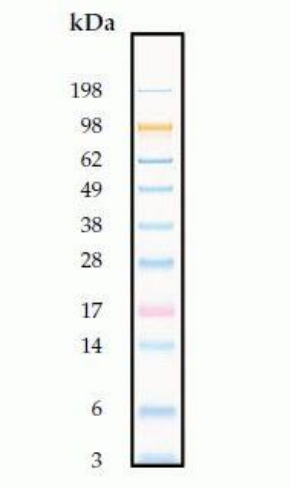



Figure 11. Apparent molecular weights of the SeeBlue® Pre-Stained Standard employed as ladder on a NuPAGE® Novex® 4-12% Bis-Tris Gel with MES

### 3.5. Statistical Analysis

The statistical analysis was conducted using Microsoft Excel to analyse the total samples collected from the study groups. The data were compared using appropriate statistical tests to assess differences between groups. The comparison was made between Crohn's disease patients and control groups. Specifically, comparisons were conducted between Crohn's disease patients and colorectal cancer patients, as well as between Crohn's disease patients and obese individuals. Moreover, the analysis was done within Crohn's disease patients based on the recurrence: non-recurrence, mild-recurrence and severe-recurrence.

For continuous variables such as age, the student's t-test was employed to assess differences between groups. The null hypothesis (H0) of this test states that there is no significant difference between the means of two independent groups. Otherwise, the alternative hypothesis (H1) suggests that there is a significant difference between the means (20). A significance level (p-value) of ≤ 0.05 was considered statistically significant. Therefore, a lower p-value indicates stronger evidence against the null hypothesis, confirming a significant association between the variables.

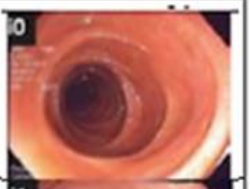
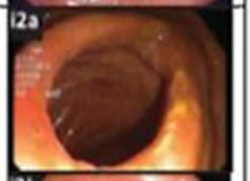
On the other hand, the chi-square test was used to examine the association between two categorical variables, that is to say, data counted and divided into categories. This assessment evaluates the size of any differences between the expected results and the real obtained results, considering both the sample size and the number of variables involved in the analysis. P-Value > 0.05 was considered as not statistically significant.

# 4. Results

## 4.1. Patients and tissue samples

Patients diagnosed with Crohn's disease were subjected to a rigorous follow-up protocol post-surgery, with assessments conducted at six-month intervals for a duration of two years. These patients were further categorised, Table 2, into two groups based on the *Rutgeerts* score: Non-Responsive (NR; i0 and i1) and Responsive (R; i2, i3, and i4) (21). However, for a more precise classification regarding disease recurrence, three distinct groups were stablished: non-recurrent patients (i0 and i1), mild-recurrent patients (i2) and severe-recurrent patients (i3 and i4).

**Table 2.** Classification of Crohn's Disease lesions following Rugeerts score on mucosa phenotype. Images are sourced from De Cruz et. al. (2022).

| | Rutgeerts Score | Phenotype | Colonoscopy |
|---|---|---|---|
| Responsive | Grade i0 | No lesions in the distal ileum |  |
| | Grade i1 | <5 aphthous lesions |  |
| Non-responsive | Grade i2 | <5 aphthous lesions with normal mucosa between them in the neo-terminal ileon (i2b) or confined to the anastomosis zone (i2a) |   |
| | Grade i3 | Diffusely inflamed mucosa with aphthous ileitis |  |
| | Grade i4 | Diffuse inflammation, large ulcers/nodules/stenosis |  |

Moreover, a set of control samples comprised ileal tissue obtained from individuals free from inflammatory bowel disease (IBD), who had undergone surgical procedures for colorectal carcinoma an bariatric surgery. Criteria for selecting these controls included the absence of familial history of IBD and normal histological examination of the ileal samples. All tissue samples were meticulously preserved at -80ºC in RNAlater (Sigma-Aldrich, St. Louis, MO) until required for analysis.

The study included three groups collected in the following Table 3: patients with Crohn's disease (n=42), patients with colorectal cancer (n=10), and obese patients (n=11).

The median age of CD patients (35 years) was significantly younger that the colorectal cancer group with a median age of 68 years, whereas no data was obtained from obese individuals. The difference between these two groups is related to the fact that colorectal cancer diagnosis tends to be more prevalent in older individuals, whereas CD is typically diagnosed in the age range of 20 to 30 years.

While the proportion of female patients did not differ among the studied groups. Smoking does show clear evidence of significance among the compared groups, with 67% identified as current smokers in the CD group, in comparison with the other two groups that do not present active smokers.

Last, BMI data exhibit notable differences between CD and obese patients, as expected. Obesity is considered when IBM>30, as we can observe, this mean is noticeably superior compared with Crohn's disease, which is positioned within the stablished healthy weight range with IBM= 22.

Focusing on Crohn's disease patients regarding the recurrence, three groups are classified in Table 4: non-recurrent, mild-recurrent and severe-recurrent. Further analysis of all the variables were conducted obtaining non statistical significance.

For instance, followed treatment before surgery was not considered into the statistical study due to clinical considerations. A more exhaustive sample size and continuous investigation are required to observe any potential impact of this parameter on the project's final outcomes.

In the context of surgical indication, data on inflammation were unavailable because of the characteristic pattern exhibited by this phenotype in patients. Furthermore, complications arising from inflammation may lead to the development of the other two analysed complications.

**Table 3.** Patients' demographics and clinical characteristics: Crohn's Disease vs Controls.

| | Crohn's Disease | Colorrectal Cancer | Crohn vs Cancer (P-value) | Obeses | Crohn vs Obeses (P-value) |
|---|---|---|---|---|---|
| n | 42 | 10 | | 11 | |
| Age, yr | 35 (29–44) | 68 (62–74) | ≤ 0.05 | | |
| Female sex; n (%) | 21 (51%) | 3 (30%) | ns | 7 (64%) | ns |
| Smoking; n (%) | | | | | |
| never | 14 (33%) | 5 (83%) | ≤ 0.05 | 6 (55%) | ≤ 0.05 |
| ex smoker | 0 | 1 (17%) | | 5 (45%) | |
| active | 28 (67%) | 0 | | 0 | |
| BMI | 22 (19–24) | | | 41 (39-48) | ≤ 0.05 |
| CD location; n (%) | | | | | |
| Ileal | 27 (64%) | | | | |
| Colic | 0 | | | | |
| Ileocolic | 15 (36%) | | | | |
| Perianal disease; n (%) | 15 (36%) | | | | |
| Time from CD diagnosis to surgery, mont | 73 (12–116) | | | | |
| Treatment before surgery; n (%) | | | | | |
| Nothing or aminosalicylates | 9 (21%) | | | | |
| IMM | 11 (26%) | | | | |
| Anti-TNF | 4 (10%) | | | | |
| CE | 0 | | | | |
| Anti-TNF+IMM | 8 (19%) | | | | |
| Anti-TNF+CE | 0 | | | | |
| IMM+CE | 6 (14%) | | | | |
| Anti-TNF+IMM+CE | 4 (10%) | | | | |
| Surgical indication; n (%) | | | | | |
| Strictures | 19 (45%) | | | | |
| Fistula/abscess | 23 (55%) | | | | |
| Inflammation | 0 | | | | |
| POR diagnosis (Rutgeerts score); n (%) | | | | | |
| i0 | 13 (31%) | | | | |
| i1 | 5 (12%) | | | | |
| i2a | 10 (24%) | | | | |
| i2b | 4 (10%) | | | | |
| i3 | 7 (17%) | | | | |
| i4 | 3 (7%) | | | | |
| undetermined | 0 | | | | |

*The table summarises the demographics and clinical characteristics of patients with Crohn's Disease (CD), as well as controls: Colorectal Cancer, and Obesity. It shows the number of patients (n), age (years), sex (F/M), smoking status (active, never, ex-smoker), Body Mass Index (BMI), CD location (ileal, colic, ileocolic), presence of perianal disease, time from CD diagnosis to surgery (months), treatment before surgery, surgical indication (strictures, fistula/abscess, inflammation), and Rutgeerts score for post-operative recurrence (POR). It also includes statistical comparison between Crohn's patients and controls using both t-student (continuous values) and chi-squared tests (discrete data) (ns; non-significant p≤0.05).*

**Table 4.** Patients' demographics and clinical characteristics: Non recurrent, Mild-Recurrent and Severe-Recurrent.

| Crohn's disease | | | | | | |
|---|---|---|---|---|---|---|
| | Non-recurrent | Mild-Recurrent | P-value (NRvsMR) | Severe-Recurrent | P-value (NRvsSR) | P-value (MRvsSR) |
| n | 18 | 14 | | 10 | | |
| Age, yr | 32 (27-43) | 36 (31-46) | ns | 38 (36-44) | ns | ns |
| Female sex; n (% | 10 (56%) | 7 (50%) | ns | 3 (10%) | ns | ns |
| Smoking; n (%) | | | | | | |
| never | 6 (33%) | 5 (36%) | ns | 3 (30%) | ns | ns |
| ex smoker | 0 | 0 | | 0 | | |
| active | 12 (67%) | 9 (64%) | | 7 (70%) | | |
| BMI | 21 (18–23) | 22 (19-24) | ns | 23 (22-24) | ns | ns |
| CD location; n (%) | | | | | | |
| Ileal | 10 (56%) | 10 (71%) | ns | 7 (70%) | ns | ns |
| Colic | 0 | 0 | | 0 | | |
| Ileocolic | 8 (44%) | 4 (29%) | | 3 (30%) | | |
| Perianal disease | 5 (28%) | 5 (36%) | ns | 5 (50%) | ns | ns |
| Time from CD dia | 61 (16–113) | 77 (12-111) | ns | 60 (10-126) | ns | ns |
| Treatment before surgery; n (%) | | | | | | |
| Nothing or ami | 4 (22%) | 2 (14%) | | 3 (30%) | | |
| IMM | 5 (28%) | 4 (29%) | | 2 (20%) | | |
| Anti-TNF | 0 | 1 (7%) | | 3 (30%) | | |
| CE | 0 | 0 | | 0 | | |
| Anti-TNF+IMM | 4 (22%) | 2 (14%) | | 2 (20%) | | |
| Anti-TNF+CE | 0 | 0 | | 0 | | |
| IMM+CE | 3 (17%) | 3 (21%) | | 0 | | |
| Anti-TNF+IMM+ | 2 (11%) | 2 (14%) | | 0 | | |
| Surgical indication; n (%) | | | | | | |
| Strictures | 8 (44%) | 8 (57%) | ns | 3 (30%) | ns | ns |
| Fistula/abscess | 10 (56%) | 6 (43%) | | 7 (70%) | | |
| Inflammation | 0 | 0 | | 0 | | |
| POR diagnosis (Rutgeerts score); n (%) | | | | | | |
| i0 | 13 (72%) | | | | | |
| i1 | 5 (28%) | | | 10 (42%) | | |
| i2a | | | | 4 (17%) | | |
| i2b | | | | | | |
| i3 | | 7 (29%) | | | | |
| i4 | | 3 (13%) | | | | |
| undetermined | 0 | 0 | | 0 | | |

*The table summarises the demographics and clinical characteristics of CD's patients subgroups regarding recurrence: non-recurrent, mild-recurrent and severe-recurrent It shows the number of patients (n), age (years), sex (F/M), smoking status (active, never, ex-smoker), Body Mass Index (BMI), CD location (ileal, colic, ileocolic), presence of perianal disease, time from CD diagnosis to surgery (months), treatment before surgery, surgical indication(strictures, fistula/abscess, inflammation), and Rutgeerts score for post-operative recurrence (POR). It also includes statistical comparison between the 3 groups as indicated: NRvsMR, NRvsSR, MRvsSR by using t-student (continuous values) and chi-squared tests (discrete data) (ns; non-significant $p \leq 0.05$).*

## 4.2. Quality control: protein extraction and quantification

Based on the provided table 5, protein concentrations are examined in creeping fat samples from different groups, as previously mentioned: Crohn's disease patients (separated into mild-recurrent, severe-recurrent and non-recurrent), and control groups (colorectal cancer and obese individuals).

The aim of this quality control is to ensure protein quantification methodology has been reproduced with robustness, accuracy and the results are reproducible to validate the findings. Is important also to evaluate that the sample sizes are large enough to detect differences between the groups, as long as a smaller population may result in non-significant findings despite true underlying results.

The table provides the mean of protein concentrations (mg/ml) of analysed CrF samples across different groups, as well as quartile 1 (Q1) and quartile 3 (Q3) values. The first section shows the results obtained for CD, colorectal cancer and obese patients, along with p-values for statistical comparisons. While the second section details instead the protein concentrations subdividing CD patients into non-recurrent, mild-recurrent and severe-recurrent, as seen in previous results.

As we can observe in Table 5 the analysis shows non-significant differences in protein concentrations across the studied groups, all p-values are greater than 0.05, suggesting no statistically significant differences between the compared groups regarding protein concentrations. The lack of significance may indicate that any observed differences are likely due to random chance rather than true differences.

**Table 5.** Quality control of protein extraction: protein concentrations of creeping fat samples.

| | Protein concentration of CrF samples | | |
|---|---|---|---|
| | CROHN | Controls | |
| | | Colorrectal cancer | Obeses |
| **Mean** (mg/ml) | 35 | 33 | 34 |
| **Quartile 1** | 26 | 31 | 27 |
| **Quartile 3** | 45 | 40 | 47 |

| | Crohn vs Cancer | Crohn vs Obeses |
|---|---|---|
| **p-value** | 0,82 | 0,87 |

| | Protein concentration of CrF samples | | |
|---|---|---|---|
| | Non-Recurrent | Mild-Recurrent | Severe-Recurrent |
| **Mean** (mg/ml) | 40 | 29 | 33 |
| **Quartile 1** | 20 | 23 | 28 |
| **Quartile 3** | 46 | 37 | 37 |

| | NR vs MR | NR vs SR | MR vs SR |
|---|---|---|---|
| **p-value** | 0,3 | 0,8 | 0,4 |

### 4.3. Immunofluorescence

IF is an important immunochemical technique that enables the detection and localization of a large variety of antigens in diverse types of cell-tissues. To visualize the results obtained from the studied creeping fat samples, we employed confocal microscopy. This approach utilized an indirect method, tagging a fluorophore to the secondary antibody, and facilitating precise observation as demonstrated in Figures 12 and 13.

Figure 12 illustrates the positive control sample, derived from the intestinal mucosa of a patient without recurrence. The left panel shows the overall tissue structure, while the right panel focuses on a neutrophil (blue nucleus with polymorphonuclear structure) within the mucosa. This neutrophil is observed expressing matrix metalloproteinase 9, indicated in red. This serves as a baseline for comparison with pathological samples.

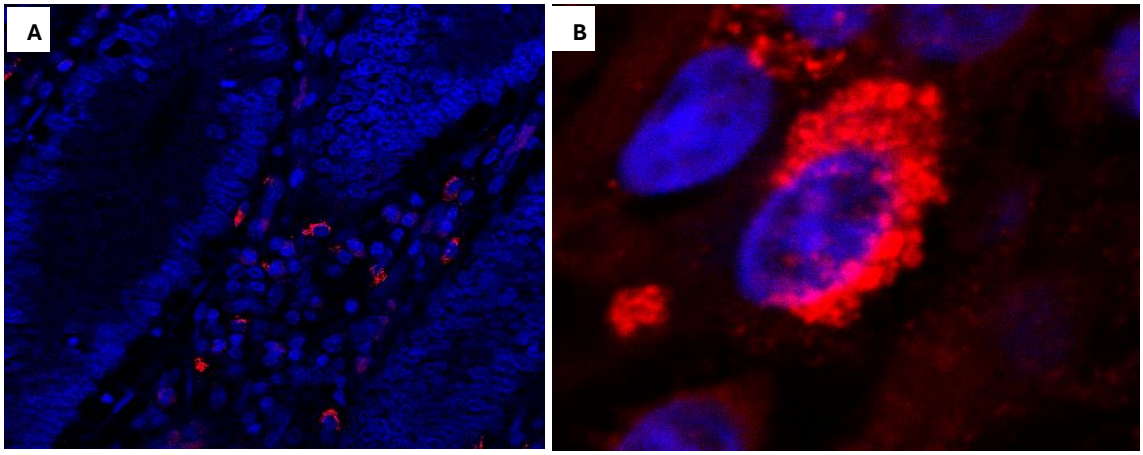



Figure 12. Fluorescence confocal microscopy image of intestinal mucosa from non-recurrent patient at 40x oil objective. Primary Anti MMP9 NBP2-59699 and 1/50 and Anti-Ms STAR Orange secondary 1/200. A) Adipocyte tissue with neutrophil infiltration. B) MMP-9 activity detected surrounding the neutrophil.

Instead, figure 13 provides a detailed view of creeping fat tissue from a CD patient exhibiting severe recurrence. The left panel (A) displays the adipocyte cell as a whole under light exposure, while the right panel (B) highlights the immune cell infiltration. These immune cells, surrounding the adipocyte, express MMP-9 within cytoplasmatic granules, evidenced by the red fluorescence. Notably, these cells are localized within a blood vessel, indicating an influx of immunoinflammatory molecules.

During the optimization process, antibody concentrations were adjusted to enhance signal detection. Primary antibody concentrations were tested at dilutions of 1/50, 1/100, 1/200, and 1/400. The 1/50 and 1/100 provided better results, leading to exclude the higher dilutions. Two primary antibodies were evaluated: NBP2-59699 and MAS-15886. For secondary antibodies, both anti-mouse and anti-rabbit options were explored.

DAPI stain was also optimized with tests conducted at 1/10 and 1/20 dilutions. The 1/10 yielded better results, and thus was chosen for the subsequent experiments.

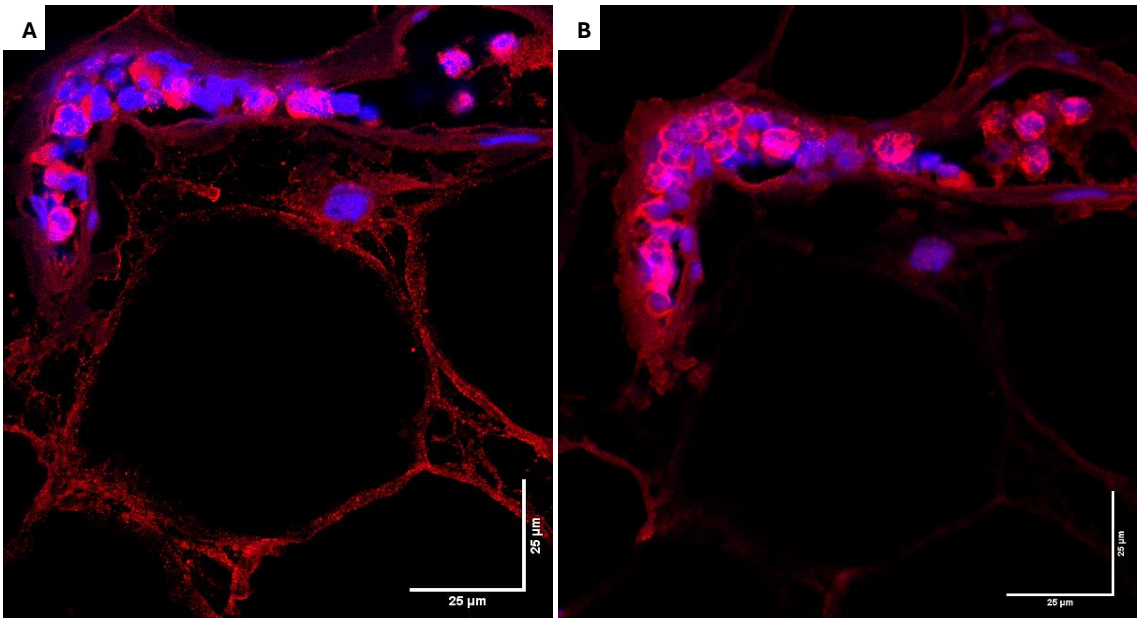



Figure 13. Fluorescence confocal microscopy image of fat tissue from patient with severe recurrence at 40x oil objective. Primary Anti MMP9 NBP2-59699 and 1/50 and Anti-Ms STAR Orange secondary 1/200. A) Adipocyte profiling surrounded by immune cells with intense luminescence exposure. B) MMP-9 exclusively localized in neutrophils.

### 4.4. Western Blot

Four creeping fat samples from Crohn's disease patients were examined by Western Blot technique: i0, i2a, i1, i0. The aim of this part of the project was to set up the technique to be ready for analysing the total number of samples and corroborate the results obtained from the immunofluorescence.

The molecular weight of metalloproteinase-9, in its inactive form (zymogen pro-MMP-9) is 92 kDa, corresponding to the one indicated in the range of 98 kDa of the ladder (Figure 14). While its activity it is associated to at least two activation forms of the protein: 82 kDa that corresponds to the N-terminal truncated form; and also, the N- and C-terminal truncated 65 kDa form, being then wider band observed in our membrane (22).

Several issues were found during the Western-blot set-up which necessitated the repetition of the membrane. This decision was made to enhance the quality of the results by improving band resolution and minimizing background interference.

Several decisions were made to address the issues encountered during the procedure: increasing the protein quantity to 60 µg and 80 µg, as well as, increasing antibody concentration: Primary Anti MMP9 NBP2-59699 was 1:500 while secondary was 1:20000. Membrane analysis was performed both wet and dry conditions, with the latter giving unclear results. Additionally, challenges with the Odyssey machine hindered our efforts. Metalloproteinase-9 was read in the 800-band range.

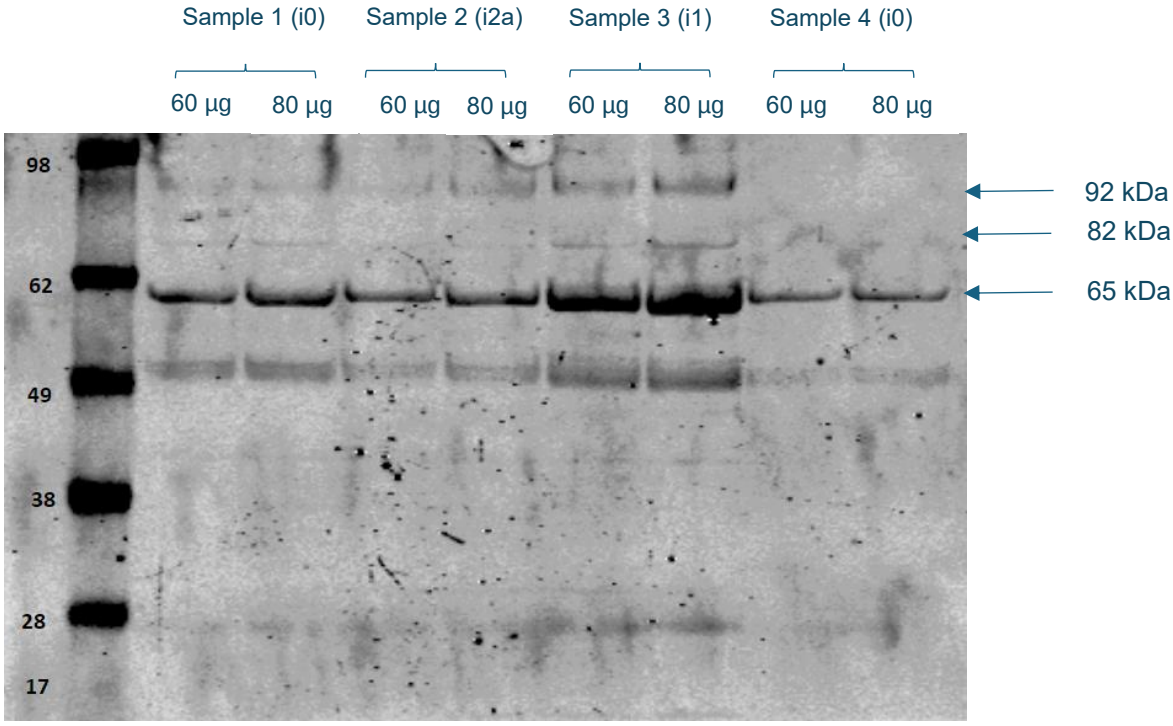



Figure 14. Detection of MMP-9 isoforms in creeping fat samples by Western Blot analysis. Three isoforms of MMP-9, with molecular weights of 92 kDa,82 kDa and 65 kDa, were identified in samples from different recurrence status (i0, i2a, i1, and i0). Each sample was loaded with 60 µg and 80 µg of protein, as indicated. Nitrocellulose membranes were incubated with anti-MMP-9 antibodies and visualized under wet conditions to ensure accurate molecular weight identification.

## 5. Discussion

First and foremost, it is crucial to acknowledge the day-to-day difficulties that you must face in the laboratory. Research often comprises various challenges that can hinder progress. These can arise from multiples causes such as equipment malfunctions, unexpected experimental results, or material delays. It is important to highlight these difficulties because they underscore the reality that scientific research is not just dependent on the researcher's efforts. Recognising this fact emphasizes the adaptability required in this field, and it also helps to acquire a better understanding of the research process.

Crohn's disease is a chronic, relapsing inflammatory bowel disease, influenced not only by genetic factor but also by environmental factors such as microbiota and immune function. Despite several advancements in treatment that have improved patients' quality of life and reduced remission rates. It remains a reality that most will require surgical intervention during their lifetime. The importance of continuing to study CD lies in addressing, above all, postoperative recurrence. Unravelling the complex interplay

between all these factors can provide clearer insights for identifying new targets for therapeutic interventions.

In this context, neutrophils, as it has been seen, play a significant role in the pathogenesis of CD. This study aims to integrate both neutrophil infiltration and MMP-9 activity to provide a better understanding of their contributions to this condition. Creeping fat samples from CD patients and control individuals were compared, focusing on the differences between non-recurrent, mild-recurrent and severe-recurrent cases.

This project primarily focuses on establishing and optimizing immunofluorescence and western blot techniques to further study the neutrophil-mediated activity of MMP-9, with a particular emphasis on my role in meticulously setting up these techniques. These methods are critical for accurately analysing protein activity. Understanding these methods and ensuring their reliability allows to achieve precise and reproducible results.

In this study, we analysed tissue samples from 64 patients, 42 of whom were from patients diagnosed with Crohn's disease. Our aim was to study the MMP-9 marker and correlate the findings regarding this protein with clinical outcomes. Our analysis revealed that CD patients had a higher percentage of active smokers compared with controls. This habit is a well-documented risk factor strongly associated with exacerbation of the disease and increased likelihood of complications. This finding should be further studied in a larger sample in order to align our study with existing literature and underscore the necessity of implementing consciousness programs to cease this harming habit.

Another significant data was the clear difference in body mass index (BMI) between obese and CD patients. Obesity has been significantly related to an increased probability of developing IBD. The contrast between both results highlights distinct metabolic profiles. Despite being a risk factor, it is important to understand the difference between obesity related IBD risk and metabolic consequences of diagnosed IBD, such as malabsorption, chronic inflammation, and dietary restrictions (23).

Last, as documented, surgery does not cure CD, although it can provide temporary improvement. Most patients will require subsequent surgeries due to recurrence of clinical symptoms, detected endoscopically or through clinical evaluation. It is important to point out that our data did not indicate inflammation as a primary cause for surgical intervention. Instead, complications such as fistulas/abscesses (fistulizing pattern) and constriction (stenosis) were common reasons for surgery. This phenotype characterized by excessive inflammation is typically managed with to a range of medications that help control it. The issue regarding this phenotype is that often leads to either constriction or fistulas/abscesses, necessitating surgery. At present, the likelihood of requiring surgery only due to inflammation is quite low.

The distribution of data among the sample size of patients was appropriately balanced, with no other significant differences observed. This indicates good homogenization across the groups, ensuring that the observed results are not biased by variability. Data extracted from the quantification procedure was also treated under study and helped us corroborate that samples could be used in Western blot for accurate protein detection. This uniformity supports the robustness and reliability of future findings.

Immunofluorescence confirmed MMP-9 presence, as well as important neutrophil infiltration in the two shown samples. The sample corresponding to figure 13 was from a patient with severe-recurrent CD and perianal disease, who underwent surgical intervention due to fistula formation. While figure 12 corresponded from a patient with non-recurrent CD and a better prognosis. In this patient, intestinal mucosa was examined to serve as a control, as this tissue normally expresses matrix metalloproteinase 9.

In these samples neutrophils showed an elevated expression of MMP-9 granules, interacting with other immune cells to influence the inflammatory course. Neutrophils tend to accumulate this enzyme in cytoplasmatic granules, releasing them as a defensive mechanism. Other immune cells, produce this protein in response to external signals. These findings validate the data from various publications cited during the project.

It is important to recognise that MMP-9 has a complex heterogeneity of functions, implying complex regulation. Fatty acids like oleic acid and linoleic acid induce MMP-9 and ROS release through the free fatty acid receptor 1 (FFAR1). These findings establish the fundamental pillars for future colocalization studies of the two markers under study. The mechanism of gelatinase granule release is shown to be FFAR1/GPR40 and ERK1/2 dependent as represented in Figure 14. When FFAR1 is activated, it stimulates MAPK and Akt phosphorylation, crucial in gelatinase granules release. ERK1/2 activation by FFAR1 agonist is also involved in this process (14).

MMP-9 not only plays a role in ECM degradation, but also generates MHC bounded peptides, such as autoimmune neo-epitopes, activating interleukins and further stimulating chemokines. Nowadays various mediators are known to stimulate MMP-9 release, including IL-8, TNF- α, fMLP (18).

Western blot served to validate the IF results, corroborating the presence of MMP-9. This part of the study revealed the presence of diverse isoforms of the studied molecule. Initially, two bands were expected: one from the latent form of the enzyme at 92 kDa and another from the activation form after the dissociation of the N-terminal. This activation occurs due to oligomerization and post-translational processes such as glycosylation, specifically sialylation in MMP-9, and phosphorylation (24).

For both IF and WB, it was important to prevent secondary antibody cross-reaction with endogenous immunoglobulins in the tissue sample. Thus, the primary antibody should come from a different specie than the sample. Consequently, the secondary antibody must target the host species of the primary antibody.

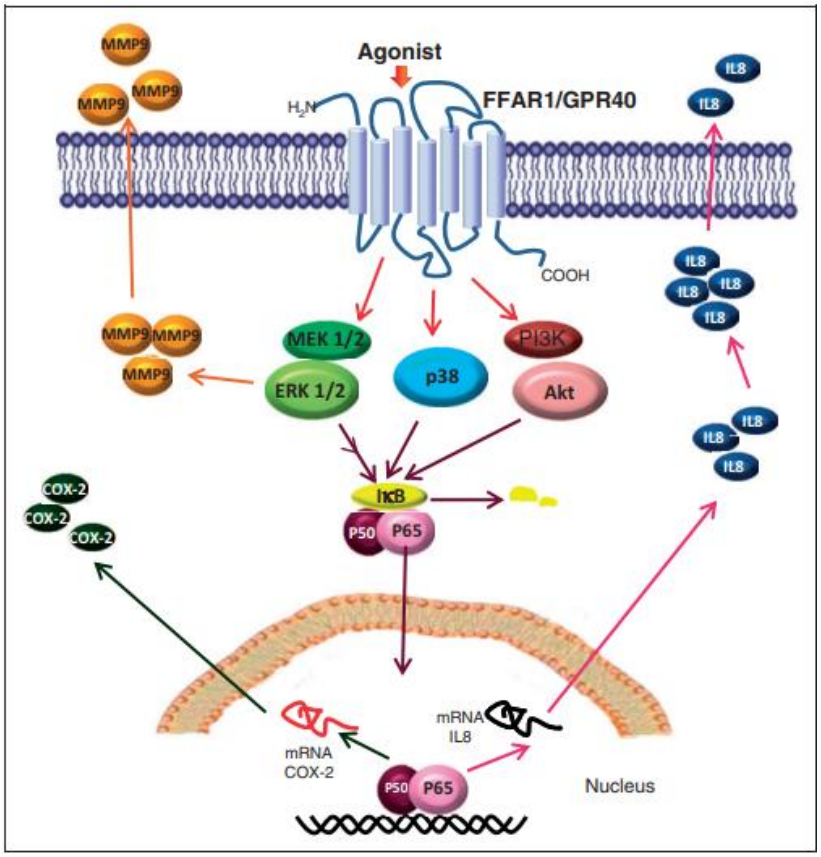



Figure 15. Summary illustration of the signalling pathways activated by FFAR1/GPR40 in neutrophils where MMP-9 release is regulated by activation of ERK1/2 and p38 MAPK. Sourced from Mena, J.S. (2016) (14).

An additional isoform was detected around 62 kDa range. Further research confirmed a 65 kDa MMP-9 isoform, which is often difficult to detect due to its migration similarity to other metalloproteinases. Studies concluded that ConA Sepharose separation can differentiate MMP-2 and MMP-9 based on their glycosylation. Notably, when truncated from the 82 kDA isoform, this 65 kDa isoform no longer responds to natural inhibitors. The fact that its activity cannot be controlled, shows the importance of study further the impact in the pathophysiology of CD. As Rossano. R. (2021) noted *"it should be noted that MMP-9 is not specific to a particular disease: in fact, MMP-9 is more a marker of inflammation, being associated with the activation of the pro-inflammatory transcription factor NF-kB. Indeed, the increase of MMP-9 expression and activity correspond to an increase of the inflammatory state in pathology"* (25).

All this accumulated data underscores the role of MMP-9 in the inflammatory process associated with CD recurrence, providing a solid foundation for our conclusions and supporting the completion of the project.

## 6. Conclusion

This project offers important insights into the role of MMP-9 and neutrophil infiltration in the development and recurrence of Crohn's disease. By optimizing immunofluorescence and western blot techniques, we successfully analysed MMP-9 in creeping fat tissue samples from CD patients.

The results obtained so far have allowed us to validate some immunoassays that we will apply to more samples to obtain statistically significant evidence of MMP-9 in creeping fat tissue, especially in cases with severe recurrence, accompanied by high neutrophil activity. The discovery of a 65 kDa isoform of MMP-9, which is normally difficult to detect and still not well understood, highlights the need for further refinement of our methods and more research into the glycosylation differences in metalloproteinases.

The improvements made in these techniques ensure that our results are reliable and can be reproduced which was one of the principal objectives of this project. These enhanced methods can now be used in larger studies to confirm our initial findings. Long-term studies could also help understand how the impact of smoking cessation and bodyweight management affect the progression and recurrence of CD.

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