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ARE CROHN'S DISEASE PHENOTYPES A MYTH?

A HISTOPATHOLOGICAL AND MOLECULAR STUDY ON TRANSMURAL FIBROSIS AND INFLAMMATION IN ILEAL CROHN'S DISEASE

MARIA HELENA PEREIRA TAVARES DE SOUSA

TESE DE DOUTORAMENTO EM MEDICINA APRESENTADA
À FACULDADE DE MEDICINA DA UNIVERSIDADE DO PORTO

Maria Helena Pereira Tavares de Sousa

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Dissertação de Candidatura
ao Grau de Doutor em Medicina
apresentada à Faculdade De Medicina
da Universidade do Porto

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Doutora Isadora Alexandra da Luz Rosa, individualidade de reconhecida competência do Instituto Português de Oncologia (IPO) de Lisboa Francisco Gentil, EPE

Doutora Maria de Fátima Machado Henriques Carneiro, Professora Catedrática da Faculdade de Medicina da Universidade do Porto

Doutor José Carlos Lemos Machado, Professor Catedrático da Faculdade de Medicina da Universidade do Porto

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Science is not only a disciple of reason but,

also,

one of romance and passion.

Stephen Hawking

Ao Jorge Bruno,

à Teresa e à Alice.

AGRADECIMENTOS

A todos os que, de forma direta ou indireta, científica ou pessoal, presencial ou à distância, contribuíram para que esta tese fosse possível, o meu sincero agradecimento.

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Foi extremamente difícil e desafiante desenvolver uma tese de doutoramento em simultâneo com uma atividade assistencial hospitalar intensa e uma família onde "brilham" 2 filhas em fases opostas da adolescência. Por isso, agradeço aos Diretores de Serviço por a nunca nada se terem oposto nas necessárias ausências; às minhas colegas da Unidade de Portimão do Serviço de Gastrenterologia, pela substituição nas minhas indisponibilidades e pela compreensão e incentivo nas frustrações e alegria nos sucessos; e aos internos pela partilha de alegrias e tristezas, por serem exemplos de esforço e dedicação recordando-me que, afinal, os nossos caminhos não são assim tão distantes.

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For the molecular biology study (Objective 3) additional funding was required, namely through the “Best Poster Presentation” and “Best Oral Presentation” awards at the 2021 and 2024 GEDII Annual Meetings, respectively; part of the Doctoral Program fees upon authorization of the Program Director, as anticipated in its regulation; and from an additional contribution from GEDII.

LIST OF PUBLICATIONS

I. Core Publications

The core structure of this thesis is composed of **three research papers**, as follows.

(Ao Abrigo do Art.º 8º do Decreto-Lei n.º 388/70, fazem parte desta dissertação os seguintes trabalhos publicados)

1. Tavares de Sousa H, Estevinho MM, Peyrin-Biroulet L, Danese S, Dias CC, Carneiro F, Magro F. **Transmural Histological Scoring Systems in Crohn's Disease: A Systematic Review with Assessment of Methodological Quality and Operating Properties**. J Crohns Colitis. 2020 Jul 9;14(6):743-756. doi: 10.1093/ecco-jcc/jjz178.

   Journal Impact Factor (2020): 9.071[1]

   JCR Gastroenterology & Hepatology – ranking 14/92; Q1 Percentile 85.33

   Number of citations: 10 (Google Scholar); 7 (ResearchGate)

2. Tavares de Sousa H, Gullo I, Castelli C, Dias CC, Rieder F, Carneiro F, Magro F**. Ileal Crohn's disease exhibits similar transmural fibrosis irrespective of phenotype**.

   Clin Transl Gastroenterol. 2021;12:e00330.

   doi.org/10.14309/ctg.0000000000000330

   Journal Impact Factor (2021): 4.396[2]

   JCR Gastroenterology & Hepatology – ranking 42/93; Q2 Percentile 55.38

   Number of citations: 11 (Google Scholar); 9 (ResearchGate)

[1] *Journal Citation Reports™ from Clarivate/Web of Science, 2020.* The *Journal Impact Factor* from*™ from Clarivate/Web of Science* is defined as all citations to the journal in the current JCR year to items published in the previous two years, divided by the total number of scholarly items (these comprise articles, reviews, and proceedings papers) published in the journal in the previous two years (in: https://authorservices.wiley.com/author-resources/Journal-Authors/find-a-journal/journalmetrics.html)

[2] *Journal Citation Reports™ from Clarivate/Web of Science, 2021*

3. Tavares de Sousa H, Ferreira M, Gullo I, Rocha AM, Oliveira C, Carneiro F, Magro F. **Fibrosis-related transcriptome unveils a distinctive matrix remodelling pattern in penetrating ileal Crohn's disease.** J Crohns Colitis. 2024 May 3:jjae064. doi: 10.1093/ecco-jcc/jjae064. Online ahead of print.

Journal Impact Factor (2023): 8.0[3]

JCR Gastroenterology & Hepatology – ranking 14/93; Q1 Percentile 85.5

Number of citations: 0 (Google Scholar); 0 (ResearchGate)

[3] *Journal Citation Reports™ from Clarivate/Web of Science, 2023*

II. Other Publications Related to the Thesis Theme

These papers are not part of the core structure of this thesis. However, they were written by the candidate during the course of the program and are herein presented due to their close relation to the thesis theme, as follows.

a. Tavares de Sousa H, Carneiro F. **Understanding progression of strictures in ileal Crohn's disease - The importance of setting methodological standards.** *United European Gastroenterol J*. 2022 Nov;10(9):915-916. doi: 10.1002/ueg2.12327. Epub 2022 Oct 17. PMID: 36251489; PMCID: PMC9731658

   Editorial.[4]

   Journal Impact Factor (2022): 6.866[5] *(Q1 Gastroenterology)*[6]

b. Tavares de Sousa H, Gullo I, Magro F**. Fibromuscular expansion in Crohn's disease ileal strictures: an open issue.** *Clin Gastroenterol Hepatol*. 2023 May;21(5):1378-1380. doi: 10.1016/j.cgh.2022.06.023. Epub 2022 Jul 15. PMID: 35850408.

   Letter to the Editor.[7]

   Journal Impact Factor (2022): 11.382[8] *(Q1 Gastroenterology)*[9]

c. Tavares de Sousa H, Magro F. **How to Evaluate Fibrosis in IBD?** *Diagnostics*. 2023; 13(13):2188. https://doi.org/10.3390/diagnostics13132188. PMID: 37443582. PMCID: PMC10341182

   Review.

   CiteScore (2023): 3.992[10] *(Q2 Clinical Biochemistry)*[11]

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[4] ***Vide*** **APPENDICES, section III**. Concerning the paper by El Ouali S, Baker ME, Lyu R et al. Validation of stricture length, duration and obstructive symptoms as predictors for intervention in ileal stricturing Crohn's disease. United European Gastroenterol J. 2022 Nov;10(9):958-972. doi: 10.1002/ueg2.12314.– *vide* abstract in APPENDICES, section IV.

[5] *Journal Citation Reports™ from Clarivate/Web of Science, 2022*

[6] From https://www.scimagojr.com/journalrank.php?category=2715&year=2022

[7] ***Vide*** **APPENDICES, section III**. Concerning the paper by Liu Q, Zhang X, Ko HM et al. Constrictive and Hypertrophic Strictures in Ileal Crohn's Disease. Clin Gastroenterol Hepatol. 2022 Jun;20(6):e1292-e1304. doi: 10.1016/j.cgh.2021.08.012. – *vide* abstract in APPENDICES, section IV.

[8] *Journal Citation Reports™ from Clarivate/Web of Science, 2022*

[9] *Journal Citation Reports™ from Clarivate/Web of Science, 2022*

[10] *CiteScore from Scopus, 2023.* The CiteScore is calculated by dividing the number of citations to documents published in a 4-year period by the number of documents in same 4-year period (in: https://authorservices.wiley.com/author-resources/Journal-Authors/find-a-journal/journalmetrics.html)

[11] From https://www.scimagojr.com/journalsearch.php?q=21100852989&tip=sid&clean=0

OTHER THESIS-RELATED ACTIVITIES WITHIN THE PROGRAM TIMEFRAME

I. Presentations and Awards in Scientific Meetings

During the thesis timeframe the candidate **presented**:

- 4 oral communications related to the thesis (one international);
- 3 posters related to the thesis (two internationals).

During the thesis timeframe the candidate was **awarded** with:

- Best Poster Presentation at the Annual GEDII Meeting 2021[12]
- 1st Prize at the Janssen Immunology RWE Award IBD 2022[15]
- Best Oral Presentation at the Annual GEDII Meeting 2024[13]

[12] Sousa HT, Gullo I, Castelli C, Dias CC, Carneiro F, Magro F. Ileal Crohn's disease exhibits similar transmural fibrosis irrespective of phenotype

[13] Tavares de Sousa H, Ferreira M, Gullo I, Rocha AM, Oliveira C, Carneiro F, Magro F. Fibrosis-related transcriptome unveils a distinctive matrix remodelling pattern in penetrating ileal Crohn's disease.

II. Pathology Apprenticeship

During this thesis timeframe the candidate undertook activities aiming to increase her knowledge on IBD histopathology. The most important was a one-week Pathology Short Internship at the Department of Pathology of São João University Hospital Centre, for which the candidate presented a written report, as follows.

a. Pathology Short Internship Report

Location: Department of Pathology of São João University Hospital Centre

Date: 22-26.11.2021

Duration: 1 week

Tutoring: Fátima Carneiro, MD, PhD and Irene Gullo, MD, PhD

Aims:

1. To understand basic procedures in preparing surgical specimens for histopathological study; to be familiarized with routine histopathological study of digestive slides, types of staining and immunohistochemistry processing, their indications, limitations and specificities.
2. Together with the tutoring pathologists, to select the samples for Objective 3 study.

REPORT

A. Tissue processing for histopathological analysis

1. Grossing of pathological specimens

I was introduced to the concept of grossing of biopsies and surgical specimens. Grossing procedures depend on the type of specimen, location and clinical information and follow standard protocols which can be found in "Manual de Macroscopia – Versão II, Serviço de Anatomia Patológica, Centro Hospitalar São João, Porto, 2013", available online at the hospital. Macroscopic description of specimens is assigned to residents or pathology assistants and should include:

a. Identification of the patient.
b. Double-checking of the number of containers sent and received in the laboratory
c. Type of product (biopsy, excisional biopsy, surgical specimen, …) and origin.
d. Number of fragments per container.
e. Shape, weight and size.
f. Location and size of the lesion in the specimen.
g. Colour and consistency of the lesion.
h. Relationships of the lesion with adjacent tissues and surgical margins, if pertinent.

i. Schemes or photographic registry of complex specimens.

j. Procedures for sectioning pathological specimens can be summarized as follows:

   i. Whenever indicated by protocol, the surgical margins should be marked with different coloured inks. Fragments of the margins should be perpendicular to the specimen surface.

   ii. Metal, suture wires and other synthetic materials, as well as bone (except for bone lesions) should be removed but retained in the specimen container.

   iii. Fragments to be placed in the cassette should be under 2,0x2,0x0,3cm to allow an adequate penetration of the fixing agent.

   iv. Fragments of the lesion and adjacent to apparently normal tissue should be obtained.

   v. All remaining tissue should be maintained in the identified container, which should contain an adequate amount of formalin, according the general rule of tissue: formalin of 1:10. By department regulation, this material is saved for 15 days after a definitive diagnosis is provided, then is discarded.

I observed the macroscopic registry of the following surgical specimens: Superficial parotidectomy for tumour; sigmoidectomy for colonic adenoma; splenectomy for tumour; cholecystectomy; brain excisional biopsy; cutaneous excisional biopsy; endometrial biopsy; pancreaticoduodenectomy (Whipple) specimen.

2. Histological processing

After observation of macroscopic registry, I witnessed the sequential procedures of histologic processing, which can be resumed as follows:

a. Cassette inclusion in liquid paraffin
b. Paraffinized block
c. Paraffinized block 3μm-sectioning (microtome)
d. Sections laying in slides
e. Automated haematoxylin and eosin staining and final slide assembly
f. Histochemistry staining
g. Immunohistochemistry staining (automatic protocol)

3. Cytological processing

The origin of cytologic material can be:

a. Exfoliative – cells are suspended in an organic liquid, e.g urine, pleural or peritoneal fluid, etc.
b. Aspirative – cells are aspirated from their location – fine needle aspiration (FNA) biopsies.

The sequential procedures of cytological processing can be summarized as follows:

   i. Liquid-based cytology: “Thin-prep” or “cytospin” preparations
   ii. Smears: staining of alcohol-fixed or air-dried slides from FNA biopsies

b. Slides undergo automated staining as described above.

4. **OSNA** (One-step nucleic acid amplification)

OSNA is a molecular technique for quantitative measurement of the target mRNA of a metastatic lymph node or other tissues. In the department it is routinely done in an automated way for targeting mRNA of cytokeratin CK 19 in the sentinel node of breast cancer patients.

B. Tutored slides evaluation

During the internship I had the opportunity to observe histological slides guided mainly by Prof. Irene Gullo but also, for liver biopsies, Dra. Joanne Lopes.

a. Liver biopsies – I observed a total of 10 liver biopsies in slides with the following stains: chromotrope aniline blue (CAB), haematoxylin and eosin (HE), Periodic acid-Schiff (PAS; for glycogen), PAS after diastase (glycogen-digesting enzyme), Hall (for bilirubin), Pearls (for iron), rhodamine (for copper), silver (for reticulin). I learned how to identify portal spaces, centrolobular vein, normal hepatocytes, macro and microvesicular steatosis, ballooning degeneration of hepatocytes, fibrosis and granulomas. Final diagnosis included 6 cases of metabolic associated fatty liver disease (MAFLD), of which 5 with non-alcoholic steatohepatitis (NASH) - graded according the Steatosis-Adipose-Fibrosis (SAF) score; one case of neonatal biliary atresia; one case of toxic hepatitis; one case of unspecific chronic hepatitis; and one case of hepatic B virus (HBV)-related cirrhosis.
b. Gastric biopsies – 4 cases. I learned to recognize pyloric and oxyntic glands, hyperplastic foveolar epithelium, intestinal metaplasia, acute and chronic inflammatory infiltrates, neuroendocrine cells hyperplasia (in autoimmune gastritis context). I observed slides stained with HE and with immunohistochemistry (IHC) for gastrin and synaptophysin.
c. Colon biopsies (ulcerative colitis case) – features of chronicity included architectural changes of intestinal crypts; features of acute inflammation included polymorphic inflammatory infiltrate and ulceration. IHC for cytomegalovirus (CMV) was requested.
d. Colon biopsies (chronic diarrhoea) – the intestinal architecture was normal; no inflammatory permeation of crypts; a mild unspecific chronic inflammatory infiltrate was noted. The hypothesis of microscopic colitis was not supported.
e. Peritoneal fluid cytology (gastric cancer staging) – numerous signet-ring cells from gastric cancer were found mixed with epithelial and inflammatory cells. The diagnosis was supported by IHC: CK5-, EMA+, CDX2+. The tumour was thus staged as advanced (M1).
f. Ileal Crohn's disease (CD) slides – vide D.; 42 slides stained with HE and the corresponding slides stained with Masson Trichomic were evaluated. I learned about fibromuscular changes – muscularis mucosae hyperplasia and splay, muscularis propria hypertrophy and hyperplasia, muscularization of submucosa and adipose metaplasia of the submucosa; acute and chronic inflammatory changes – erosion, ulcerations, fissures, architectural disarray of crypts, fistulae, serositis and lymphoid aggregates; epithelioid granulomata. We also assessed CD associated small bowel adenocarcinoma slides, in which I observed 1 case of pTis adenocarcinoma (confined to lamina propria), 1 case of

pT1 carcinoma (invasion of muscularis mucosae) and the difference between pT3 and pT4 cases (invasion of serosa with peritoneal implants in the latter).

C. Pathology department weekly meeting

On the first day of the internship the weekly meeting of the department occurred at 12.00H. It was conducted by the head of the department, Prof. Fátima Carneiro, and several matters were discussed. I was presented and the purpose of my internship was explained to the department. Also, Prof. Miriam Cuatrecasas from Hospital Clinic from Barcelona was introduced and explained that she would stay in the service for 3 weeks.

D. Research

As a part of my PhD dissertation, a molecular study with nCounter® Fibrosis Consortium Panel will be conducted in Crohn's disease (CD) patients. The aim of this study is to profile and compare the tissue transcriptome of the 3 groups studied in "Ileal Crohn's disease exhibits similar transmural fibrosis irrespective of phenotype. Sousa HT, Gullo I, Castelli C, Dias CC, Rieder F, Carneiro F, Magro F. Clin Transl Gastroenterol. 2021;12:e00330. https://doi.org/10.14309/ctg.0000000000000330". During this internship a meeting with both my PhD tutors took place on 21.11.2021, with discussion of the methodology to be used and the number and type of patients to be included was determined. From the 103 cases studied in the above-mentioned paper, 36 were selected according to clinical phenotype: 12 B2, 12 B3s and 12 B3o (as defined in the paper). The confirmation of adequacy of the 36 cases selected for molecular study was made through tutored slides assessment guided by Prof. Irene Gullo, in order to assure that enough quality material was available in corresponding FFPE block. From the archived slides, only the "most affected area" slides were retrieved and assessed in microscopy for adequacy for molecular study (only sections with well-preserved, non-necrotic tissue and representative of the whole thickness of the ileal wall were considered) marking the slide for areas to exclude (e.g. necrosis and debris). A few additional cases of each phenotype were selected for safekeeping, in case of potential problems with the original 36 cases.

In addition, a group of 6 patients with CD-related small bowel adenocarcinoma will be studied using the same methodology and compared with the above-described cases. For each case, we selected one slide representative of the tumor and one slide with non-tumoral CD lesions according to "most affected area" criteria. In the latter, inflammation and fibrosis were graded in the same way as the previously studied cases. Patients' clinical data will be retrieved from the same data bases.

CONCLUSION

This Pathology Internship was short but intense and noteworthy for the amount (although general) knowledge gained concerning the processing of pathological material from macroscopy to histological assessment. I learned on the possibilities and limitations of Pathology in achieving a final diagnosis in gastroenterological diseases and on the main tools that may be requested to complement the first evaluation.

A huge step forward in my PhD dissertation was made during this time, through the decision of an innovative approach to the investigation of fibrosis and inflammation and the selection of the adequate histological material for the future molecular studies.

COMMENTARY

The friendly environment and the availability of all the department personnel, especially technician Susana, all the residents and Prof.s Irene Gullo, Joanne Lopes and Fatima Carneiro was touching and memorable. The modern multidisciplinary medical practice is reflected in the way I was welcomed, taught and listened during this internship.

Porto, 26.11.2021

Helena Tavares de Sousa

b. H-ECCO – ECCO IBD Pathology Masterclasses

As a complementary education in IBD Pathology, the candidate attended the 6th, 7th and 8th Histopathology Masterclasses of the European Crohn's and Colitis Organization (ECCO) meetings in 2021, 2022 and 2023, respectively, as follows.

CERTIFICATE
OF ATTENDANCE

6th H-ECCO IBD Masterclass

16th Congress of ECCO
European Crohn's and Colitis Organisation
Inflammatory Bowel Diseases 2021
Virtual
July 2-3 & 8-10, 2021

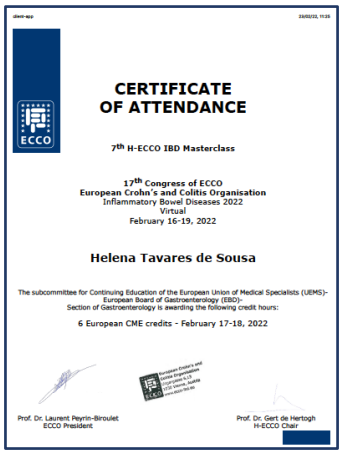
Helena Tavares de Sousa

The subcommittee for Continuing Education of the European Union of Medical Specialists (UEMS)-
European Board of Gastroenterology (EBD)-
Section of Gastroenterology is awarding the following credit hours:

5 European CME credits - July 3, 2021

Prof. Dr. Laurent Peyrin-Biroulet
ECCO President

Prof. Dr. Gert De Hertogh
H-ECCO Chair



CERTIFICATE
OF ATTENDANCE

7th H-ECCO IBD Masterclass

17th Congress of ECCO
European Crohn's and Colitis Organisation
Inflammatory Bowel Diseases 2022
Virtual
February 16-19, 2022

Helena Tavares de Sousa

The subcommittee for Continuing Education of the European Union of Medical Specialists (UEMS)-
European Board of Gastroenterology (EBD)-
Section of Gastroenterology is awarding the following credit hours:

6 European CME credits - February 17-18, 2022

Prof. Dr. Laurent Peyrin-Biroulet
ECCO President

Prof. Dr. Gert de Hertogh
H-ECCO Chair

CERTIFICATE OF ATTENDANCE

8th H-ECCO IBD Masterclass
18th Congress of ECCO
European Crohn's and Colitis Organisation
Inflammatory Bowel Diseases 2023
Copenhagen, Denmark
March 1-4, 2023

Helena Tavares de Sousa

The subcommittee for Continuing Education of the European Union of Medical Specialists (UEMS)
European Board of Gastroenterology (EBD)
Section of Gastroenterology is awarding the following credit hours:

6 European CME credits – March 1-4, 2023

Prof. Dr. Laurent Peyrin-Biroulet
President of ECCO

Prof. Dr. Gert De Hertogh
Chair of H-ECCO

LIST OF ABBREVIATIONS

ADAM17 - A-disintegrin and metalloprotease (MMP) 17 gene

CD – Crohn's disease

COSMIN – [COnsensus-based Standards for the selection of health Measurement INstruments]

CEACAM-3 - Carcinoembryonic antigen cellular adhesion molecule gene

CTGF – Connective tissue growth factor gene

DC – Doença de Crohn

DEGs – Differentially expressed genes

ECM – Extracellular matrix

FFPE - Formalin-fixed and paraffin-embedded

GDEs – Genes diferencialmente expressos

GEDII – Grupo de Estudos da Doença Inflamatória Intestinal

IBD – Inflammatory Bowel Disease

qPCR – real-time Polymerase Chain Reaction

PRISMA – Preferred Reporting Items for Systematic Reviews and Meta-Analyses

RNA – Ribonucleic acid

TGFβ1 – Transforming growth factor 1 gene

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ABSTRACT

Background

Crohn's disease (CD) is a chronic inflammatory disorder of the digestive tract, with transmural inflammation, submucosal fibrosis and muscular expansion being the disease hallmarks. Intestinal fibrosis is defined by excessive extracellular matrix (ECM) accumulation and mesenchymal cell expansion in response to chronic inflammation, but that may progress after inflammation subsides. Fibrosis in CD underlies most of the disease complications that require surgery, such as strictures and/or penetrating events. Bearing this in mind, a CD patient may be perceived as bearing early / non-complicated or late-stage / complicated disease. In this way the phenotype Montreal classification [inflammatory (B1), stricturing (B2) and penetrating (B3)] may be considered imperfect. While transmural intestinal inflammation in CD can be non-invasively and accurately estimated by cross-sectional imaging, fibrosis cannot and still require surgical specimens' histopathological analysis for an accurate assessment. However, an objective evaluation of inflammation and fibrosis in CD surgical specimens warrants the use of a validated transmural histopathological scoring system, which is not available. Importantly, most of the fibrosis-related histopathological and molecular research has been done on stricturing CD, leaving the pathogenesis of penetrating CD still poorly defined.

Aims

The global aim of this thesis was to add knowledge on the similarities and differences of inflammatory and fibrosis changes in stricturing and penetrating CD. For this purpose, three objectives were defined, as follows:

- Objective 1 – To uncover a liable transmural histopathological scoring system for the study of inflammation and fibrosis in stricturing and penetrating CD. To propose the best score(s) to be used in clinical practice and/or translational research.
- Objective 2 - To characterize and quantify inflammation and fibrosis in ileal CD resection specimens, according to a CD transmural histopathological scoring system, in stricturing and penetrating CD (Montreal phenotype classification). To correlate inflammation and fibrosis scorings with previously described clinical outcomes.
- Objective 3 – To investigate and compare the fibrosis-related transcriptome profiles in stricturing and penetrating CD full-thickness ileal sections.

Methods

- Objective 1 – For this objective, it was conducted a systematic review of all existing transmural histopathological scoring systems evaluating inflammation and/or fibrosis in CD, focusing on the originally developed scores. Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines were applied. A meta-analysis on the reviewed scores was not feasible due to high heterogeneity. Hence, the methodological quality of the studies reporting an original score was assessed using the 10-items´ COSMIN [COnsensus-based Standards for the selection of health Measurement INstruments] checklist. Analysis of the four operating properties and of the variables included in each original score was additionally performed.
- Objective 2 – To accomplish this objective a double-blinded histopathologic analysis of 103 archived penetrating and stricturing CD ileal surgical specimens was conducted. Phenotype Montreal classification was applied for the 29 stricturing (B2) and 74 penetrating (B3) cases – the latter including 54 cases with associated stricture(s) (B3s)

and 20 without associated stricture(s) (B3o). Per specimen, three sections (ileal proximal margin, inflamed area, most affected area) were examined and graded for inflammation and fibrosis based on a histopathological score, adopted after Objective 1 conclusions. Clinical data were retrieved from GEDII prospective national registry.

- Objective 3 – To answer this innovative objective, the expression of 787 genes covering the core pathways and processes involved in fibrosis was analyzed after RNA extraction from full-thickness intestinal sections. These were retrieved from the most affected area (as defined in Objective 2 study) of 36 ileal surgical specimens – 12 B2 and 24 B3, according to Montreal phenotype classification; B3 cases included 12 B3s and 12 B3o, as defined in Objective 2. Nanostring technology and comparative bioinformatics were used for the transcriptome analyses and data interpretation. qPCR and immunohistochemistry analyses were used to validate selected differentially expressed genes.

Results

- Objective 1 – A total of 29 original CD transmural scoring systems were included in the systematic review, none of which was validated. Three scores emerged as the most widely reproduced, probably due to ease of application in clinical studies. Of these, two included both inflammation and fibrosis scores and could be applied in clinical studies and practice. Two highly comprehensive scores were identified, showing good operating properties and high methodological quality, as well as the lowest risk of bias, which might be suitable for basic research and clinical trials.
- Objective 2 - In diseased areas, penetrating (B3) CD exhibited significantly higher inflammation compared with stricturing (B2) disease (score 3: 96% vs. 76%, p=0.005

in inflamed areas; 78% vs. 55%, *p*=0.019 in most affected areas). This was also observed for the two B3 subgroups (B3s vs. B2: 81% vs. 55%, *p*=0.033 in most affected areas; B3o vs. B2, score 3: 100% vs. 76%, *p*=0.006 in inflamed areas and 70% vs. 55%, *p*=0.039 in most affected areas). No differences were found regarding fibrosis scores and fibromuscular changes between the three groups. Regarding postoperative outcomes, new penetrating events occurred only in B3s (n=6, 11%, *p*=0.043) patients. Postoperative change of biologic therapy correlated with severe inflammation at the proximal ileal margin (55% changed vs. 25% did not, *p*=0.035).

- Objective 3 – The study included 34 patients with B2 and B3 phenotypes, balanced for age at diagnosis, age at surgery, gender, CD localization, perianal disease and therapy. Inflammation and fibrosis histopathological scorings were similar – median of 3 and 1, respectively - in all cases. B2 and B3 groups showed a very good clustering regarding 30 significantly differentially expressed genes (DEGs), all being remarkably upregulated in B3. More than half of the genes were involved in CD fibrogenesis, while 8 DEGs were so in other organs. The most significantly active biologic pathways in penetrating disease were response to TGFβ and matrix organization and degradation, as validated by immunohistochemistry.

Conclusions

When using an objective and widely reproduced transmural scoring system, there were no histopathological differences on transmural fibrosis and fibromuscular changes between penetrating and stricturing ileal CD. However, their fibrosis-related transcriptomic profiles were distinct, with penetrating CD being remarkable for the activation of all DEGs and of biologic pathways concerning permanent matrix remodeling.

RESUMO

Introdução

A doença de Crohn (DC) é uma doença inflamatória crónica do tubo digestivo em que a inflamação transmural, a fibrose da submucosa e a expansão das camadas musculares constituem as características distintivas da doença. A fibrose intestinal é definida como a deposição excessiva de matriz extracelular e expansão de células mesenquimatosas em resposta à inflamação crónica, podendo, contudo, progredir após redução da inflamação. A fibrose está subjacente à maioria das complicações da DC que requerem cirurgia, como estenoses e/ou eventos penetrantes, podendo o doente ser encarado como portador de doença em fase inicial/não complicada versus avançada/complicada. Assim, a classificação de Montreal dos fenótipos da DC em doença inflamatória (B1), estenosante (B2) ou penetrante (B3) pode ser considerada imperfeita. Embora a inflamação intestinal transmural na DC possa ser estimada de forma não invasiva e com elevada acuidade por imagiologia seccional, tal não é possível para a fibrose, que requer ainda o estudo histopatológico de peças operatórias. Contudo, uma apreciação objetiva da inflamação e fibrose na DC em peças de ressecção cirúrgica, exige o uso de um sistema de pontuação histopatológica transmural validado, que não está disponível. A maioria da investigação histopatológica e molecular relacionada com fibrose na DC tem sido realizada na doença estenosante, pelo que a patogénese da DC penetrante permanece mal definida.

Objetivos

O propósito global desta tese foi acrescentar conhecimento sobre as semelhanças e diferenças das alterações inflamatórias e fibróticas na DC estenosante e penetrante. Para tal foram definidos três objetivos, a saber:

- Objetivo 1 – Discernir um sistema de pontuação histopatológica transmural fidedigno para o estudo da inflamação e fibrose na DC estenosante e penetrante. Propor o(s) melhor(es) sistema(s) para uso na prática clínica e/ou investigação translacional.
- Objetivo 2 - Caracterizar e quantificar a inflamação e fibrose em peças de ressecção ileal, de acordo com um sistema de pontuação histopatológica transmural específico para a doença, em doentes com DC com fenótipo estenosante ou penetrante, de acordo com a classificação de Montreal. Correlacionar os graus de inflamação e fibrose com resultados clínicos previamente descritos.
- Objetivo 3 – Investigar e comparar os perfis de transcriptoma relacionados com fibrose em secções de parede intestinal ileal com DC estenosante e penetrante.

Métodos

- Objetivo 1 – Para este objetivo, procedeu-se à realização de uma revisão sistemática de todos os sistemas existentes de pontuação histopatológica transmural que avaliassem a inflamação e/ou a fibrose na DC, com foco nos sistemas originais. Foram aplicadas as diretrizes PRISMA [Preferred Reporting Items for Systematic Reviews and Meta-Analyses; Itens descritivos preferenciais para revisões sistemáticas e meta-análises). Não foi possível a realização de uma meta-análise dos sistemas de pontuação revistos devido à elevada heterogeneidade. Em alternativa, procedeu-se à avaliação da qualidade metodológica dos estudos originais que descreveram os sistemas de pontuação, através da aplicação da lista de verificação com 10 itens, COSMIN [COnsensus-based Standards for the selection of health Measurement INstruments; Padrões baseados em consenso para a seleção de instrumentos de

medição de saúde]. Adicionalmente, realizou-se a análise das quatro propriedades operacionais e das variáveis incluídas em cada sistema de pontuação original.

- Objetivo 2 – Para atingir este objetivo, foi realizada uma análise histopatológica duplamente-cega de 103 peças operatórias de resseção ileal arquivadas pertencentes a doentes com DC penetrante e estenosante. A classificação de Montreal foi aplicada para os 29 casos de doença estenosante (B2) e 74 de doença penetrante (B3) – este grupo incluiu 54 casos com estenose(s) associada(s) (B3s) e 20 casos sem estenose(s) associada(s) (B3o). Para cada peça, foram analisadas três secções de diferentes zonas (margem ileal proximal, área inflamada, área mais afetada), tendo a inflamação e fibrose sido classificadas de acordo com um sistema de pontuação histopatológica transmural adotado após as conclusões do Objetivo 1. Os dados clínicos foram obtidos a partir do registo prospetivo nacional do GEDII.
- Objetivo 3 – Para responder a este objetivo inovador, procedeu-se ao estudo da expressão de 787 genes que cobrem as principais vias e processos envolvidos na fibrogénese, após a extração de RNA de secções de parede intestinal. Estas foram obtidas a partir da área mais afetada (definida no estudo do Objetivo 2) de 36 peças de resseção ileal – 12 B2 e 24 B3, de acordo com a classificação de Montreal; os casos B3 incluíram 12 B3s e 12 B3o, conforme definidos no Objetivo 2. Utilizou-se tecnologia Nanostring e bioinformática comparativa no estudo do transcriptoma e na análise e interpretação dos dados. A validação de genes selecionados a partir dos genes diferencialmente expressos foi realizada mediante técnicas de qPCR e de imunohistoquímica.

Resultados

- **Objetivo 1** – Um total de 29 sistemas originais de pontuação histopatológica transmural em DC foram incluídos na revisão sistemática, nenhum dos quais se encontrava validado. Destacaram-se três sistemas como sendo os mais reproduzidos, provavelmente pela facilidade de aplicação em estudos clínicos. Destes, dois incluem sistemas de pontuação para inflamação e fibrose e poderão ser aplicáveis em investigação e prática clínicas. Foram ainda identificados dois sistemas altamente abrangentes e completos, os quais apresentaram boas propriedades operacionais e alta qualidade metodológica, bem como menor risco de viés, sendo passíveis de aplicação em investigação básica e em ensaios clínicos, a merecer validação futura.
- **Objetivo 2** - Nas áreas atingidas, a DC penetrante (B3) apresentou graus de inflamação significativamente superiores quando comparada com a doença estenosante (B2) (grau 3: 96% vs. 76%, *p*=0.005 nas áreas inflamadas; 78% vs. 55%, *p*=0.019 nas áreas mais afetadas). Isto foi também observado para os dois subgrupos de B3 (B3s vs. B2, grau 3: 81% vs. 55%, *p*=0.033 nas áreas mais afetadas; B3o vs. B2, grau 3: 100% vs. 76%, *p*=0.006 nas áreas inflamadas e 70% vs. 55%, *p*=0.039 nas áreas mais afetadas). Salientou-se, de forma importante, a ausência de diferenças nas pontuações de fibrose e nas alterações fibromusculares entre os três grupos. Em relação aos resultados pós-operatórios, observou-se que os novos eventos penetrantes ocorreram apenas em doentes B3s (n=6, 11%, *p*=0.043) e que a alteração de terapêutica biológica se relacionou com a existência de inflamação grave na margem ileal proximal (55% mudaram vs. 25% não mudaram, *p*=0.035).
- **Objetivo 3** – O estudo incluiu 34 doentes com fenótipos B2 e B3, numa amostra equilibrada quanto à idade ao diagnóstico, idade no momento da cirurgia, sexo,

localização da DC, doença perianal e terapêutica. A pontuação histopatológica de inflamação e fibrose foi semelhante (mediana de 3 e 1, respetivamente) em todos os casos. Observou-se um muito bom agrupamento dos grupos B2 e B3 relativamente aos 30 genes diferencialmente expressos (GDE), a totalidade dos quais se encontrava positivamente regulada nos doentes B3. Mais de metade destes genes foram envolvidos na fibrogénese da DC, enquanto 8 foram implicados na fibrose em outros órgãos. Os processos e vias biológicas mais significativamente ativas na doença penetrante estavam relacionadas com a resposta ao TGFβ e organização e degradação da matriz extracelular, como demonstrado na validação por imunohistoquímica.

Conclusões

Recorrendo a um sistema de pontuação histopatológica transmural específico para a DC, objetivo e amplamente reproduzido, não se observaram diferenças histopatológicas na fibrose transmural e nas alterações fibromusculares entre a DC ileal penetrante e estenosante. No entanto, o perfil transcriptómico relacionado com fibrose foi distinto nos dois fenótipos, com a DC penetrante exibindo uma ativação de todos os genes diferencialmente expressos e particularmente de vias biológicas relacionadas com a remodelação da matriz extracelular.

THESIS OUTLINE

This thesis is organized as follows:

- CHAPTER 1 – Introduction, presents an introduction to the importance of fibrosis in IBD, especially in CD, brings a summary of the existing knowledge of fibrogenesis, discusses on current and future sectional imaging and biomarker technologies aiming to non-invasively assess intestinal fibrosis in CD, and briefly addresses the status of anti-fibrotic therapy in IBD. For this purpose, the non-core review article How to Evaluate Fibrosis In IBD? published by the candidate in 2023, is herein presented.

- CHAPTER 2 – Aims, summarizes the main and specific objectives of this thesis, which concern the three core papers included in the respective following chapters.

- CHAPTER 3 – Transmural Histological Scoring Systems in Crohn's Disease: A Systematic Review with Assessment of Methodological Quality and Operating Properties, presents the paper that fulfilled this thesis' Objective 1.

- CHAPTER 4 – Ileal Crohn's Disease Exhibits Similar Transmural Fibrosis Irrespective of Phenotype, presents the paper that fulfilled this thesis' Objective 2.

- CHAPTER 5 – Fibrosis-Related Transcriptome Unveils a Distinctive Matrix Remodelling Pattern in Stricturing Ileal Crohn's Disease, presents the paper that fulfilled this thesis' Objective 3.

- CHAPTER 6 – General Discussion, summarizes the main findings of the three core papers, presents a general discussion on this thesis, addresses its limitations and points directions for future research.

- CHAPTER 7 – Conclusions, presents the main conclusions of this thesis and addresses the answer to the thesis' title.

CHAPTER 1 – Introduction

1. Introduction

An introduction to the importance of fibrosis in IBD, especially in CD, is presented, together with a summary of the existing knowledge of fibrogenesis, an extended discussion on current and future sectional imaging and biomarker technologies aiming to non-invasively assess intestinal fibrosis in CD and a brief word on the status of anti-fibrotic therapy in IBD.

For this purpose, the candidate presents the non-core review article **How to Evaluate Fibrosis In IBD?**.

Tavares de Sousa H, Magro F. **How to Evaluate Fibrosis in IBD?** *Diagnostics*. 2023; 13(13):2188. https://doi.org/10.3390/diagnostics13132188. PMID: 37443582.

diagnostics

MDPI

Review

How to Evaluate Fibrosis in IBD?

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Abstract: In this review, we will describe the importance of fibrosis in inflammatory bowel disease (IBD) by discussing its distinct impact on Crohn's disease (CD) and ulcerative colitis (UC) through their translation to histopathology. We will address the existing knowledge on the correlation between inflammation and fibrosis and the still not fully explained inflammation-independent fibrogenesis. Finally, we will compile and discuss the recent advances in the noninvasive assessment of intestinal fibrosis, including imaging and biomarkers. Based on the available data, none of the available cross-sectional imaging (CSI) techniques has proved to be capable of measuring CD fibrosis accurately, with MRE showing the most promising performance along with elastography. Very recent research with radiomics showed encouraging results, but further validation with reliable radiomic biomarkers is warranted. Despite the interesting results with micro-RNAs, further advances on the topic of fibrosis biomarkers depend on the development of robust clinical trials based on solid and validated endpoints. We conclude that it seems very likely that radiomics and AI will participate in the future non-invasive fibrosis assessment by CSI techniques in IBD. However, as of today, surgical pathology remains the gold standard for the diagnosis and quantification of intestinal fibrosis in IBD.

Keywords: inflammatory bowel disease; fibrosis; inflammation; imaging; biomarkers

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1. The Importance of Fibrosis in IBD

Inflammatory bowel disease (IBD), which includes Crohn's disease (CD) and ulcerative colitis (UC), is characterized by chronic intestinal inflammation mediated by dysregulated immune responses to such factors as diet and microbiota [1–4].

In both CD and UC, chronic inflammation causes disruption of the epithelial barrier and tissue destruction. Fibrosis, which is a healing mechanism, becomes progressive and damaging in the scope of long-lasting IBD, in which persistent tissue damage and healing result in scar tissue formation [5,6]. At tissue and cellular levels, fibrosis is an amplified response characterized by the accumulation of collagen-rich extracellular matrix (ECM) produced by an increased number of mesenchymal cells, including fibroblasts, myofibroblasts, and smooth muscle cells (SMCs) [7]. The proliferation of fibroblastic cells, along with the accumulation of ECM, are the hallmarks of intestinal strictures in IBD [8]. Fibrosis is a frequent outcome in the natural history of IBD and is the background for most of the IBD complications, such as strictures, bowel penetration, and obstruction, often demanding surgery [5,6,9]. It has been estimated that about 30% to 50% of CD patients and 1% to 12% of UC patients would suffer from fibrosis complications during the disease course [6,10,11]. Until recently, intestinal fibrosis was considered an unavoidable complication of IBD in patients that did not respond to anti-inflammatory therapy, often requiring surgical intervention [6]. The emergence of the possibility of an anti-fibrotic approach changed this paradigm, creating challenges in terms of diagnosis and treatment

of bowel fibrosis [6,12]. As such, understanding the molecular and cellular mechanisms underpinning fibrosis and improving techniques for the assessment of fibrosis in IBD patients are still relevant research topics.

1.1. Fibrosis in CD

In CD, both inflammation and fibromuscular changes are transmural, leading to progressive thickening of the bowel wall and stricture development, even in the absence of inflammation. Pathologically, intestinal fibrosis in CD is characterized by ECM accumulation and mesenchymal cell expansion affecting all layers of the bowel wall along the intestinal tract [13]. In addition, recent pathologic consensus defined small bowel strictures in CD as a combination of decreased luminal diameter and increased thickness of all layers of the intestinal wall, including expansion of the muscularis mucosae (MM) and inner muscularis propria (MP), muscularization of the submucosa, and fibrosis of the submucosa and intestinal wall [14]. Notwithstanding, the universality of this concept was recently challenged by the description of a non-hypertrophic, constrictive type of stricture in CD [15]. Regardless of the type of stricture, these remain common complications of CD with serious clinical relevance and impact on the patients' quality of life [12,15,16].

Aside from fibrosis in strictures, it has been proposed that a certain degree of fibrosis would exist in nearly all CD phenotypes, even from early onset. In addition, it has been demonstrated that the degree of fibrosis may be similar in both stricturing and penetrating CD, with differences regarding the degree of transmural inflammation [17].

Though still used in clinical practice, the classification of CD in three-category phenotypes, as inflammatory or non-stricturing, non-penetrating (B1), stricturing (B2), and penetrating (B3) disease, is now considered too rigid [18,19]. As an alternative, CD shall be viewed through the lens of a progressive accumulation of intestinal fibrosis and damage over the course of the disease, leading to stricturing and/or penetrating complications, as supported by epidemiological natural history studies [16,20–26]. This progressive and cumulative structural bowel damage would occur irrespective of symptoms and, considering current fibrogenesis knowledge, of the degree of intestinal inflammation [6,27,28]. Hence, clinical symptoms, disease activity [29,30], and progression of bowel damage [4,31] are not totally correlated.

Considering that population-based studies have shown a 10-year cumulative risk of surgery between 40% and 71% [32–34] and that fibrosis is a marker of advanced disease, its importance is central in the setting of CD, as it underlies the need for surgical resection in stricturing disease and, maybe, also in penetrating complications, as strictures coexist in over 85% of penetrating CD [4,30–35].

In this context, to further understand pathology changes in CD, deep research on the basic cellular and molecular mechanisms of fibrogenesis is warranted.

1.2. Fibrosis in UC

In UC patients, fibrosis is characterized by a thickening of MM and excessive ECM deposition in the submucosa, affecting deeper layers only after profound ulceration of the submucosa [36–38]. Strictures are uncommon in UC, and the majority are benign and reversible [39]. However, in UC, fibrosis originates the increased wall stiffness, which may result in motility abnormalities, anorectal dysfunction, rectal urgency, and incontinence [10,38].

The evidence regarding fibrosis in UC is limited and controversial, but a comprehensive assessment performed by Gordon et al. in 2018 demonstrated that UC is characterized by progressive fibrosis and MM thickening in correlation with the severity and chronicity of inflammation. Hence, deep remission, including histological remission, should be a priority and a therapeutic target [40]. Recent research in mice with dextran sulfate sodium (DSS)-induced colitis has shown that, in UC, changes in motility may also be related to neuronal modification. The study highlighted that UC does not promote neuron death but induces changes in the chemical code of myenteric neurons [41]. A better comprehension of these data and the translation of these results depend on studies on human tissue.

2. Is There Fibrosis without Inflammation?

During physiological tissue repair, collagen substitutes the temporary fibrin plug to create a permanent sealant of the injured tissue. Briefly, in response to injury signals, fibroblasts activate, proliferate, expand, and transform into myofibroblasts, which have the innate contractile ability and produce higher levels of ECM components. Under normal circumstances, once the healing process is accomplished, the fibrotic matrix is degraded by matrix metalloproteases (MMPs), and both fibroblasts and myofibroblasts undergo apoptosis or revert to a non-activated state [42,43]. However, in the setting of recurrent or persistent epithelial injury, intestinal inflammation initiates and sustains fibrogenesis, which can progress even after the inflammatory trigger has subsided [6]. Chronic epithelial and endothelial injury release chemotactic factors that promote recruitment and constitutional activation of immune and mesenchymal cells, leading to inflammation-dependent and -independent progression of fibrosis with progressive organ dysfunction [43].

2.1. Inflammation-Dependent Fibrogenesis

In CD patients, research has highlighted a strong connection between inflammation and fibrosis (Figure 1). In fact, in intestinal resection specimens, both components generally overlap and share a similar distribution [6,44–46].

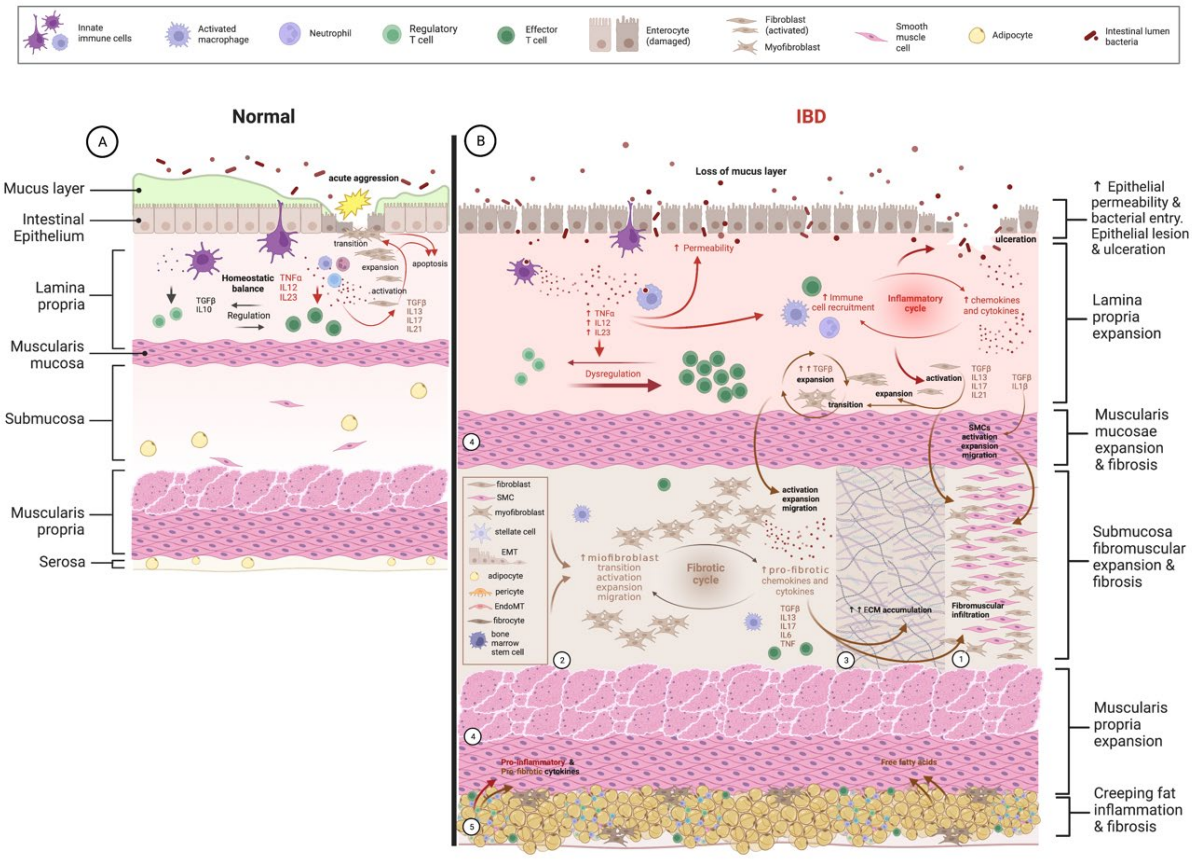

Figure 1. Inflammation-dependent fibrogenesis. (**A**) Left panel. Schematic representation of homeostatic balance between innate and adaptative immune cells in the intestinal lamina propria. Acute aggression to the intestinal epithelium (yellow star) leads to physiological inflammation in view of removing aggression and allowing tissue repair through activation and expansion of local fibroblasts. Part of these will undergo transition to active myofibroblasts, which finalize the restoration of the ECM. When healing is complete, both fibroblasts and myofibroblasts suffer apoptosis. (**B**) Right panel. Schematic representation of dysregulated chronic inflammation occurring in intestinal lamina propria due (among other causes) to increased permeability of the intestinal epithelium, allowing penetration

of microbiota perpetuating inflammatory cascades (both cellular and humoral), which also cause local tissue injury (ulceration) with further increase in microbiota access and inflammation on the lamina propria. Chronic inflammation will eventually activate local fibroblasts in the lamina propria, which will expand and migrate to other locations of the intestinal wall, namely, the submucosa. 1. Fibromuscular expansion of the submucosa—due to massive infiltration of activated and expanded fibroblasts, smooth muscle cells (SMCs), and myofibroblasts. 2. Sources of recruitment of activated myofibroblasts driven by intense production of pro-fibrotic mediators by activated myofibroblasts in a vicious-cycle way. 3. Activation of fibroblasts, SMCs, and mostly myofibroblasts leads to chronic and intense production and accumulation of ECM components, mostly on the submucosa, but that may transverse the whole intestinal wall. 4. Activation and expansion of SMCs lead to the thickening of all muscular layers, being disproportional on the muscularis mucosae where fibrosis splaying is usually more common. 5. Creeping fat has recently been demonstrated as a source of both pro-inflammatory and pro-fibrotic mediators, including free fatty acids, which will target both locally, leading to inflammation and fibrosis through creeping fat and on adjacent layers of the intestinal wall. ECM: extracellular matrix; EMT: epithelial-to-mesenchymal transition; endoMT: endothelial-to-mesenchymal transition; IL: interleukin; SMCs: smooth muscle cells; TGFβ: transforming growth factor β.

Inflammation has been established as the most potent activator of mesenchymal cells, initiating fibrogenesis, both in the early stages of CD and over the course of the disease, eventually leading to a fibrotic scar that may permeate the whole tissue architecture [47].

The physiopathological process of inflammation-dependent fibrogenesis is complex and involves a variety of such molecules and cells as immune cells, ECM-producing cells, and intestinal microbiota [48]. Without exhausting all involved mechanisms, it is known that Th17 and Th2 cells play a central role in IBD inflammation and fibrosis through the secretion of interleukins (ILs) involved in intestinal myofibroblasts' activation, migration, and ECM production (IL17, IL21) [11]; epithelial to mesenchymal transition (EMT, IL17) [11,45]; collagen deposition by fibroblasts (IL13); secretion of latent transforming growth factor (TGF)-β and MMP-9 by macrophages; and TGF-β activation by cleaving its latency-associated peptide (LAP) [11,43,45,48]. TGF-β is considered the major cytokine in intestinal fibrosis [43,48,49], mediating the differentiation of fibroblasts into myofibroblasts and promoting myofibroblasts' proliferation, migration, contraction, and resistance to apoptosis, while increasing the production of ECM components and tissue inhibitor of MMP (TIMP)-1 [11,50,51]. Importantly, TGF-β has an anti-inflammatory role, including the promotion of class-switching immunoglobulin A, inhibition of antibody production, and downregulation of inflammatory cytokine production by monocytes and macrophages through inhibition of nuclear factor (NF)-B. Overall, TGF-β is key in keeping the immune balance of the intestine through enhancing mucosal defense and tissue healing, promoting immune tolerance, and suppressing anti-inflammatory responses [52]. Apart from TGF-β, many other soluble factors produced by immune cells during inflammatory responses can promote fibrogenesis, such as transforming necrosis factor (TNF), IL-23, IL-36, activins, connective tissue growth factor (CTGF), epidermal growth factor (EGF), insulin-like growth factor (IGF)-1 and -2, platelet-derived growth factor (PDGF), vascular growth factor (VGF), galectin-3, endothelins (ET-1, -2 and -3), products of oxidative stress, components of the renin-angiotensin system (RAS), and mammalian target of rapamycin (mTOR) [6,11,43,45,48–51].

After T cells, macrophages are also expanded in IBD, although their specific role in intestinal fibrosis is only partly explained [45]. It has been described that IL-36α secreted from M1 macrophages locally induces myofibroblasts proliferation and collagen VI production [53], while an increase in M2 macrophages was demonstrated in creeping fat [54]. However, it was shown that, in certain situations, M2 cells could inhibit ECM synthesis; thus, their definitive role in fibrosis requires further investigation [48]. Neutrophils, mast cells, and eosinophils can all promote fibrosis through the release of pro-fibrotic cytokines,

chemokines, and, for neutrophils, reactive oxygen and nitrogen species, while basophils have a less clear role in fibrogenesis [43].

Considering that the hallmark of CD strictures includes excessive secretion of ECM and increased numbers of mesenchymal cells in distinct locations of the bowel wall, the importance of ECM-producing cells, namely, fibroblasts, myofibroblasts, and SMCs in fibrosis is crucial [6]. Histopathologically, these cells can be distinguished according to the expression of vimentin (V), α-smooth muscle actin (α-SMA), and desmin (D): fibroblasts exhibit a V(+), α-SMA (−), D (−) pattern, myofibroblasts a V(+), α-SMA (+), D (−) one, and SMCs are V(− or low), α-SMA (+), D (+) [45]. Fibroblasts are the primary effector cell in CD fibrosis. As referred, several mediators (the strongest being IL13 and TGF-β) drive the fibrotic response of intestinal fibroblasts in IBD [11,48,55]. Once activated, fibroblasts proliferate, migrate, increase the secretion of ECM, and can transform into myofibroblasts [56]. The same occurs with activated myofibroblasts, which can produce much higher levels of ECM than intestinal fibroblasts [45,57]. Moreover, if dysregulated, activated myofibroblasts may shift collagen type IV synthesis to collagens type I and III, which will be gradually deposited into fibrillar ECM, distort normal architecture, and increase tissue stiffness and scarring [58]. In an inflammatory environment, they can differentiate from fibroblasts and SMCs, but also from other cell types, such as fibrocytes, pericytes (blood vessels walls' fibroblasts), and epithelial, endothelial, stellate, or bone-marrow-derived stem cells [6,11,43,47,48,58]. Moreover, under chronic inflammation, SMCs can transdifferentiate into myofibroblasts and vice-versa [43]. Once activated by TGF-β1 and IL-1β, SMCs increase IL-6 production, thus further contributing to intestinal inflammation [59]. Apart from originating myofibroblasts, contributing directly to fibrogenesis, SMCs are the major contributors to intestinal wall thickening in CD strictures, both by hyperplasia and/or hypertrophy of muscular layers and by undergoing fibromuscular hyperplasia in the submucosa [47,60–62]. To finalize, a reference to creeping fat is mandatory. Creeping fat, defined as a pathologically increased fat tissue adjacent to areas of the intestine affected by CD, has been shown to correlate both to strictures and degree of inflammation [63] and, more recently, to MP hyperplasia through free fatty acids-mediated intestinal SMCs hyperplasia [8,64]. Conversely, Ren Mao et al. demonstrated that activated MP SMCs interact with creeping fat preadipocytes through the production of a specific ECM scaffold able to induce preadipocytes migration out of mesenteric fat into de novo creeping fat [64]. Creeping fat directly promotes inflammation-dependent fibrosis in the adjacent intestine through large amounts of pro-fibrotic cytokines, adipokines, growth factors, and fatty acids produced by both innate and adaptive immune cells as well as adipocytes [65]. On the other hand, creeping fat fibrosis is a well-known histopathological feature of CD, which was described by Karl Geboes as "fibrous strands are present in the mesenteric fat, irradiating from the intestine and surrounding thickened, hypertrophied fat lobules" [66]. The mechanisms underlying the concept of penetrating fibrosis and creeping fat fibrosis are complex and not fully understood, involving fibroblasts, SMCs, preadipocytes, and macrophages (specifically M2-subtype [67,68]. Serum markers for microbiota were also associated with complicated and stricturing CD [69]. In addition, intestinal dysbiosis and its secondary products have been shown to be able to induce fibrosis in the gut of CD patients [70]. However, it is not clear if dysbiosis affects only inflammation-dependent fibrogenesis or also the -independent one, and, if so, which microbiota components promote fibrosis without inflammation [48].

2.2. Inflammation-Independent Fibrogenesis

Despite our increasing capacity to control intestinal inflammation through such drugs as biologics and new small-molecule drugs, the progress in preventing progression to fibrosis and stricture development is minimal [43]. Moreover, while suppressing inflammation waives inflammatory markers, it does not reduce the expression of profibrotic mediators, suggesting the existence of inflammation-independent mechanisms mediating self-perpetuating fibrogenesis [6].

In the absence of inflammatory stimuli, ECM stiffness and mechanotransduction by fibroblasts (Figure 2) should be considered the central inflammation-independent mechanisms of intestinal fibrosis [71]. In fact, even though ECM is accountable for keeping tissue integrity, it is a dynamic structure able to communicate with a variety of cells, including those involved in the production of its own constituents. The interaction of ECM with fibroblasts includes multi-protein assemblies at the cell membrane, called focal adhesions (or focal adhesion complexes). ECM stiffness is determined by the abundance of fibrillary collagens and their degree of cross-linking, as well as the degree of hydration of the matrix, determined by the concentration of proteoglycans and hyaluronic acid and also the coexistence of inflammatory edema [71]. As referred, through the course of IBD, cytokines and fibrotic growth factors mediate the deposition and crosslinking of ECM components, making ECM stiffer with changes in its mechanical properties. These changes appear to activate pro-fibrotic signaling cascades in fibroblasts that only recently began to be explored. Briefly, ECM stiffness perpetuates fibrogenesis through the activation of mesenchymal cells, which, in turn, can further increase stiffness and regulate contraction in an inflammation-independent way [6,72]. It has been shown that in the absence of inflammation, tissue stiffness alone can lead to the progression of fibrosis in CD by inducing a morphological transformation of intestinal myofibroblasts from round to stellate shape, cellular proliferation, collagen and αSMA production and development of focal adhesions [73]. The mechanical properties of ECM stiffness are able to induce profibrotic signaling cascades in fibroblasts at least by two concurrent mechanisms [71]. First, the increased stiffness of the regenerated ECM drives fibroblast differentiation through the focal adhesion-mediated translation of ECM mechanical forces to biochemical activity within the cell, mediated by a dynamic cellular cytoskeleton. Cells adhered to ECM respond to resistance changes by increasing both the number and size of focal adhesions and the cytoskeletal pre-stress, increasing F-actin/myosin stress fibers and downstream intracellular pro-fibrotic signaling to increase ECM deposition. Second, through mechanotransduction by fibroblasts—a process by which fibroblasts convert mechanical signals into biochemical signals—ECM stiffness can also lead to the release of the potent pro-fibrotic and anti-apoptotic TGF-β "stored" in the ECM, creating a positive feedback loop crucial for sustained myofibroblast function [71,74].

The process is complex and involves a variety of structures and molecules; integrins have been demonstrated to play an important role as components of focal adhesion complexes [71]. The most important integrins involved in mechanotransduction are α5β1, which is expressed in fibroblasts [73], and $\alpha\gamma$ class integrins [71]. Herein, it is worthwhile to recall the role α5β1integrin in mediating the ability of fibronectin of the SMCs-derived matrisome adjacent to the outer aspect of the MP to induce preadipocytes migration out of mesenteric fat leading to de novo formation of creeping fat. Moreover, no proinflammatory cytokine could promote this migration [64], reinforcing the importance of this molecule in inflammation-independent fibrogenesis in CD.

In the ECM, TGF-β is kept inactive through its inclusion in the so-called TGF-β-large latent complex, composed of a latency-associated peptide (LAP) bounded to a latent TGF-β binding protein (LTBP), which, in turn, is linked to collagen and proteoglycans (as decorin, thrombospondin, and fibronectin), determining its bioavailability [53,75].

In resting conditions, fibroblasts form only loose contacts with ECM through weak and short-lived integrin adhesions. Upon tissue injury, fibroblasts rapidly transform into an activated state, secreting mainly fibronectin and fibronectin ED-A and migrating over the injured tissue to restore tissue integrity. Only in the late contracting phase of wound healing, by means of a combination of mechanical stimulations through focal adhesions and TGF-β presence, will fibroblasts terminate their differentiation into myofibroblasts by expressing αSMA [51,76]. Importantly, after wound healing, the contracting ECM is always less organized and more rigid than the original one [6,71].

Hence, the ECM functions as a reservoir of both pro-fibrotic and pro-inflammatory mediators, ready to be released upon mechanical stretching of the stiff ECM, leading to

fibrosis either directly (without inflammation drivers) or indirectly through the initiation of inflammatory cascades [71]. How cells, namely, fibroblasts and myofibroblasts, integrate the mechanical and biochemical information present in the ECM to impact cellular functions is still not understood.

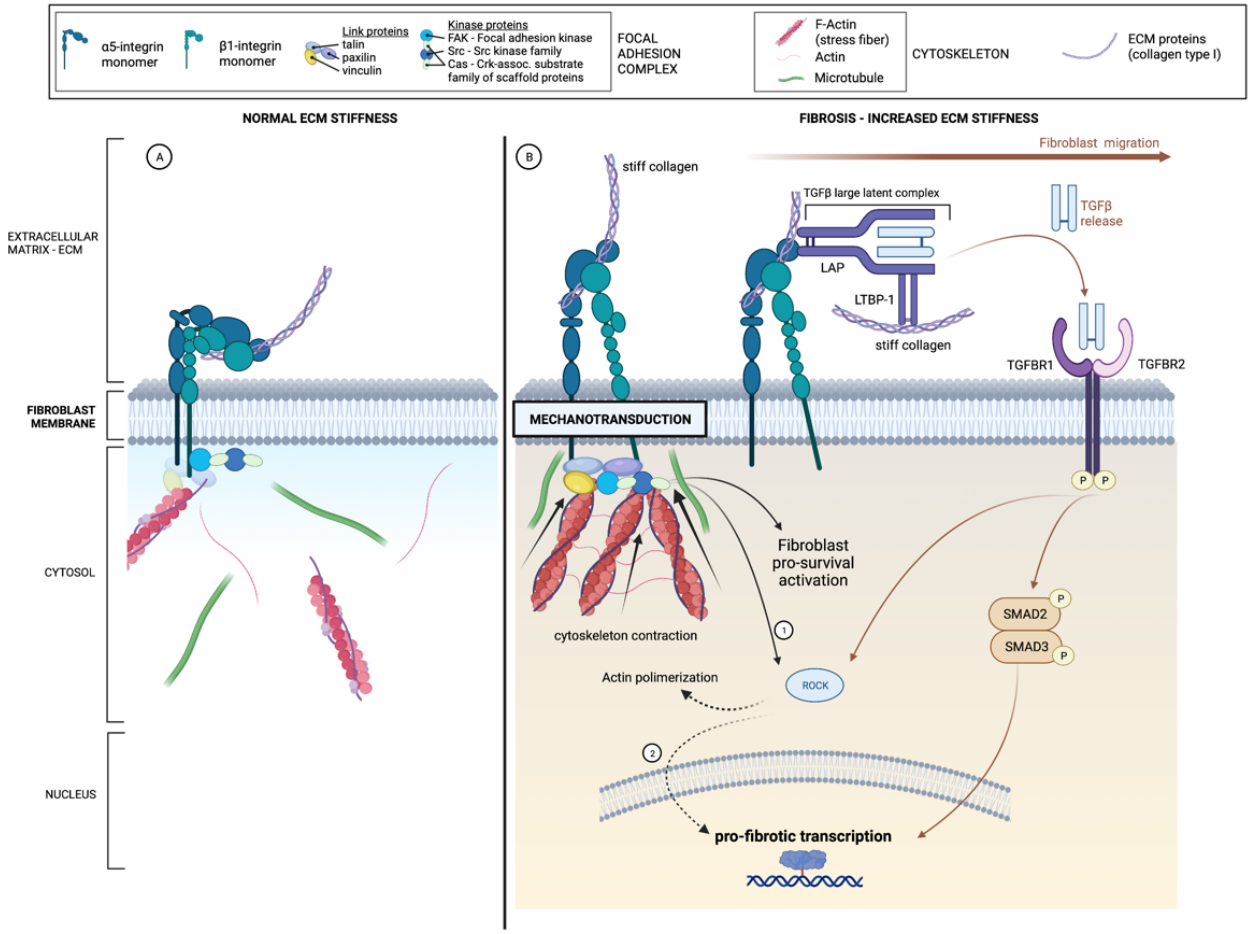

Figure 2. Inflammation-independent fibrogenesis. Schematic representation of mechanotransduction of fibroblasts and latent-TGFβ liberation in response to ECM stiffness. (**A**) Left panel. "Resting state" of focal adhesion complex in normal ECM stiffness. (**B**) Right panel. Mechanotransduction of fibroblasts and latent-TGFβ liberation in response to ECM stiffness. 1. ROCK activation through PI3K and AKT serine/threonine kinase (phosphorylation). 2. ROCK promotes G-actin binding to myocardin-related transcription factor (MRTF), which then migrates to the nucleus. LAP: latency-associated peptide; LTBP-1: latent transforming growth factor β-binding protein; P: phosphorylation; PI3K: phosphatidylinositol 3-kinase ROCK: rho kinase; SMAD: suppressor of mothers against decapentaplegic; TGFβ: transforming growth factor β; TGFBR: TGFβ receptor.

2.3. Unmet Needs

Considering that inflammation is the most potent trigger for fibrogenesis in CD, the early control of inflammation should reduce the incidence of strictures in the long term. Although this was not proven before the biologic era [68,69], it has been shown that early biologic therapy may achieve this purpose [77–79], leading to a reduction in hospitalizations and surgery [80]. However, as stated before, current anti-inflammatory and immunosuppressive strategies have not yet been capable of fully controlling tissue remodeling and fibrosis progression nor eliminating established complications.

In this setting, preventing intestinal fibrogenesis or reversing already established bowel strictures in patients with IBD should remain the ultimate goal of disease management [35]. For the end purpose of developing clinical trials for anti-fibrotic agents

that would change the IBD treatment paradigm, the Stenosis Therapy, and Anti-fibrosis Research (STAR) consortium has been dedicated to the revision of the available knowledge on the identification and characterization of strictures in CD, in which fibrosis is a major issue [81–83]. Based on the collected data, the STAR consortium has been working on definite standards to measure response to anti-fibrotic agents by developing endpoints and standard methodology for clinical, radiological, and histopathologic scoring systems essential for the design of reliable anti-fibrotic clinical trials [83–86].

3. Non-Invasive Techniques to Access Fibrosis

Several clinical, genetic, and serological risk factors for complicated or disabling disease and/or surgery have been identified in the setting of IBD. Among these, only clinical factors are being used in clinical practice to select patients who would benefit from early medical aggressive therapy [31]. However, none of these risk factors has been undoubtedly associated with stricturing disease or proved to predict fibrosis development [11,87].

Considering that, at this point, fibrosis cannot be predicted, IBD management would benefit from an accurate and non-invasive assessment of intestinal fibrosis, as patients with predominant inflammatory strictures are likely to respond better to current therapies, whereas those with established fibrotic ones will probably require surgery [6,88,89]. However, as stated, pure fibrotic or inflammatory strictures in surgical specimens are rare, with inflammation and fibrosis usually co-existing in varying degrees [6].

Broadly, fibrosis assessment in IBD is a challenging and non-invasive evaluation of fibrosis remains elusive. Despite the advances in imaging and molecular technologies, definitive identification, characterization, and quantification of intestinal fibrosis in CD still depend on the histopathological evaluation of surgical specimens [87], even though no histopathological scoring system has been validated or widely accepted for this purpose [14,82,85,90–92]. To some extent, the same applies to UC, in which fibrosis affects mostly MM but can sometimes extend to the submucosa. Thus, its complete quantification also cannot rely solely on endoscopic biopsies or imaging [10,38].

While some imaging techniques have been intensively investigated through the last decade, mainly ultrasound elastography [93–95] and specific magnetic resonance techniques, such as diffusion-weighted imaging (DWI) [96–101], diffusion kurtosis imaging (DKI) [102,103], magnetization transfer (MT) [104–109], and intravoxel incoherent motion imaging (IVIM) [110,111], none has been definitively established as reliable for this objective [112,113]. Very recently, some promising developments arose through artificial intelligence (AI) techniques in cross-sectional imaging, such as radiomics [114–117]. In addition, biomarkers remain a field of intense investigation and deserve proper discussion [42,83,118,119].

Imaging Techniques

Even though imaging techniques have been used for several decades to identify and measure the severity of IBD, only in recent years has research explored methods with valuable prognostic value [120].

Cross-Sectional Imaging

Among imaging techniques, cross-sectional imaging (CSI) is an essential tool for IBD characterization in all disease stages [81,106,121–124]. Traditionally, CSI has been used to evaluate the extent and activity of CD and to detect such complications as abscesses or fistulae, but it is also being used for the assessment of treatment response and prediction of outcomes and post-surgery recurrence [106,121].

It is recommended that such CSI techniques as ultrasonography (US), computed tomography (CT), enterography (CTE), or magnetic resonance (MR) enterography (MRE) should be performed at the time of diagnosis of CD to complement endoscopy by assessing stricturing and penetrating complications [110,125–129]. These techniques are all able to detect strictures with high accuracy, with the selection of the best approach depending

on availability, cost, patient clinical status (including comorbidities), and radiation concerns [124]. Their ability to identify and quantify fibrosis has been variably studied mostly in CD (Table 1).

Table 1. Comparison of the available cross-sectional imaging techniques for the assessment of fibrosis in CD.

Cross Sectional Imaging	Features	Limitations	Future Perspectives
MRE	No radiation High contrast resolution Possibility of performing fluoro-magnetic resonance Can be combined with perianal imaging High accuracy for severe fibrosis identification	Time consuming Intravenous and oral contrast agents Longer scanning time than CTE Less robust than CTE Lower patient compliance than CTE Availability	Validation in more robust clinical trials Combination with radiomics
DWI-MR	Short-time Possible with standard MR scanners No intravenous contrast Qualitative and quantitative analysis High accuracy for inflammation and penetrating complications in IBD High accuracy for severe fibrosis identification	Lack of anatomic details Low reproducibility of ADC Availability	Promising results to be confirmed in more robust clinical trials
DKI-MR	More physiologic imaging No intravenous contrast High accuracy for inflammation Correlation with different fibrosis grades	Few data	Promising results to be confirmed in more robust clinical trials
MT-MR	No intravenous contrast agent Correlation with different fibrosis grades Higher accuracy for fibrosis than MRE with or without DWI	Few data	Promising results to be confirmed in more robust clinical trials
CTE	Accessible Fast Robust Better spatial resolution than MRE	Radiation	Combination with radiomics Reduction in the radiation dose with high-standard dual-source or ultra-high-pitch CT scanners and iterative reconstruction systems
PET/CTE PET/MRE	In combination with CTE or MRE adds functional data	Radiation (labeled marker; CTE) High cost Limited availability Lack of anatomic details	The disadvantages and lack of advantages when compared to CTE and MRE may hinder further developments
USE US-SWI	Real-time visualization of tissue stiffness	Operator dependent Not easy to interpret More difficult to compare current examination with previous studies Heterogeneous data	Promising results to be confirmed in more robust clinical trials
CEUS	Severe fibrosis identification when associated to elastography techniques	Operator dependent Not easy to interpret More difficult to compare current examination with previous studies Heterogeneous data	Promising results to be confirmed in more robust clinical trials

CTE: computed tomography enterography; CEUS: contrast-enhanced ultrasonography; DKI-MR: diffusion kurtosis imaging–magnetic resonance; DWI-MR: diffusion-weighted imaging–magnetic resonance; MRE: magnetic resonance enterography; MT-MR: magnetization transfer–magnetic resonance; PET/CTE: positron emission tomography/CTE; PET/MRE: positron emission tomography/MRE; USE: ultrasound strain elastography; US-SWI: ultrasound–shear wave imaging.

Magnetic Resonance

MRE is considered the most advanced technique for imaging fibrosis in CD strictures. Its accuracy and lack of radiation exposure are the most attractive features. Overall, the

sensitivity of MRE for stricture detection ranges from 75% to 100%, with an estimated specificity between 91% and 96% [81,124,130–132]. As for fibrosis assessment, MRE has been described to accurately differentiate between severe and mild–moderate fibrosis, with a sensitivity of 0.94 and a specificity of 0.89 ($p < 0.01$), irrespective of the degree of inflammation [133]. In addition, MRE could also distinguish severe fibrosis from severe muscle hypertrophy in ileal CD [134]. Recently, an MRE-based composite score was shown to be a very good predictor of histologic fibrosis ($ROC_{AUC} = 0.910$) [128].

Concerns related to the intravenous administration of gadolinium justify the efforts to replace MRE with other MR techniques that do not demand intravenous contrast [106]. Even though diffusion-weighted imaging (DWI) has been used to detect inflammatory activity in CD [97], its utility for fibrosis assessment based on the assumption that the presence of fibrotic tissue is related to the restricted diffusion of water molecules, is still not defined [81,106,124,135]. Initial research showed that fibrosis was associated with low attenuated diffusion coefficient (ADC) values, presumably due to the reduction in extracellular space in fibrotic tissue leading to a restriction in diffusion [96,98,99,136]. However, more recent data evidenced constraints while distinguishing severe fibrosis from severe muscle hypertrophy in ileal CD [134], while others showed that the accuracy of DWI in detecting fibrosis varies with the degree of bowel inflammation. Since the available reports on the use of DWI on IBD included a wide range of ADC values, threshold values have not yet been defined to differentiate between active inflammatory, non-active, and fibrotic disease [98,136–138]. Mainenti et al. associated this variability with technical aspects, such as differences in MR equipment concerning magnetic field strengths, lack of reproducibility, and absence of standardized sequence parameters [100,101]. Still, even though DWI is not validated as a reliable quantitative biomarker for fibrosis, its short analysis time, absence of contrast, ability to provide qualitative and quantitative data, and high accuracy for inflammation and penetrating complications in IBD support the continuous research on its utility in the setting of fibrosis assessment in IBD.

However, researchers have highlighted relevant inconsistencies in DWI, such as the concept that, in this method, ADC calculation assumes that water distribution obeys a Gaussian model, not reflecting the impact of cell structures and biophysical properties on water displacement [139]. In response to this inconsistency, MR scanners evolved to consider non-Gaussian diffusion, demanding distinct analysis models. In this setting, diffusion kurtosis imaging (DKI) emerged as a more robust analysis model, providing a more precise display of water diffusion in the human body than conventional DWI [139,140]. DKI was first applied in the context of IBD to evaluate CD activity, providing values of K_{app} (apparent diffusional kurtosis) and D_{app} (diffusional coefficient) corrected for non-Gaussian behavior, which could distinguish between inactive, mild, and moderate–severe CD ($p < 0.05$) with better accuracy than DWI [139]. Concerning fibrosis, DKI has been considered useful for staging liver fibrosis in a rabbit model [141]. In the scope of IBD, it has been shown that K_{app} was significantly correlated to fibrosis grades and allowed to distinguish between the absence of fibrosis or mild fibrosis and moderate to severe fibrosis (sensitivity of 95.9% and specificity of 78.1%), evidencing its potential for the assessment of bowel fibrosis [102]. However, further studies are warranted to validate this data.

Based on previous data on animal models [105,108], magnetization transfer–magnetic resonance (MT-MR) was explored in a cohort of 31 CD patients. The results showed that magnetization transfer ratio (MTR) values correlated with fibrosis ($p < 0.0001$) [96], confirming that MT-MR may be of value for fibrosis identification in CD with only a small increase in the analysis time. In 2018, Li et al. confirmed that MTR was strongly correlated with fibrosis scores ($r = 0.769$, $p = 0.000$) but not with inflammation scores ($r = -0.034$, $p = 0.740$) and could differentiate moderately–severely fibrotic from non-fibrotic and mildly fibrotic bowel walls. This study showed its superiority when compared to DWI-MR and contrast-enhanced MR [107].

In recent years, the concept of textural analysis (TA) was introduced in MR imaging, namely, for fibrosis detection purposes. In this line, MR elastography was presented by

Avila F and colleagues in a pilot study in which the tissular stiffness value, measured by MR elastography, correlated with the degree of fibrosis ($p < 0.001$), according to an MR-based score [142]. No pathological correlation was undertaken; hence, the true value of this technique remains unproven. Very recently, TA of T2-weighted MR imaging (T2WI) was used to assess intestinal fibrosis in a dextran sodium sulfate (DSS) murine model and, as a proof-of-concept in 5 CD patients, against MT-MR and histopathology. TA features included skewness, kurtosis, and entropy. Both entropy and MT ratio correlated with histopathological fibrosis (r = 0.85 and r = 0.81, respectively); MT was superior in monitoring bowel fibrosis when coexisting with inflammation (linear regression $R^2 = 0.93$ vs. $R^2 = 0.01$, respectively) as well as in assessing antifibrotic response in mice. As entropy increased with fibrosis accumulation in human CD strictures, TA was capable of quantifying fibrosis in mixed inflammatory–fibrotic strictures. Considering that TA of T2WI is an accessible post-processing technique, the authors conclude that it deserves further research and validation both for clinical practice and antifibrotic trial design [143].

Computer Tomography

CTE has proved to be adequate, in terms of sensitivity and specificity, for the detection of features suggestive of CD and its complications [106,121,127]. In addition, it is widely available, fast, has relatively low cost, and enables the analysis of longer portions of the gastrointestinal tract than MR and the detection of extraintestinal manifestations [124,127,144]. The accuracy of CTE for the detection of strictures in CD ranges from 78.7% to 83% [145,146]. However, its use is limited by radiation exposure, mainly in the pediatric population [121,126], and previous studies suggest that CTE findings do not correlate with intestinal fibrosis [147]. Meng et al. considered later that this alleged lack of correlation could be related to the focus of the study on the diseased bowel, with reduced attention to the potential value of mesenteric abnormalities on CTE, which, when integrated into a nomogram, could differentiate between non-mild and moderate-to-severe fibrosis in CD patients [148].

In this context of risks and doubts, research was directed to safer and more effective approaches within CT. One of the most valuable explored strategies was the reduction in radiation dose, resorting to high-standard dual-source or ultra-high-pitch CT scanners and iterative reconstruction systems [144,149–152]. These strategies maintain or improve the quality of CT images and signal-to-noise ratio with lower doses of radiation.

Positron Emission Tomography

Even though positron emission tomography (PET) is not considered in current IBD guidelines [153], its ability to detect inflammation in IBD and to add functional data to the structural abnormalities found with MR and CT has supported the research of hybrid techniques, such as PET/MR and PET/CT, in the setting of IBD [136,154,155]. Although promising, their performances in detecting and quantifying fibrosis are globally modest when compared with the results from their single counterparts, especially MR.

Furthermore, high costs and radiation exposure may hinder the wide applicability of these techniques.

Ultrasonography

Based on the ability of US elastography to measure tissue stiffness, this technique has been used to evaluate fibrosis in IBD [93,95,113,120]. Several variations of this technique have been studied in the following settings: ultrasound strain elastography (USE) [156–161]; shear wave imaging (SWI) [162–164]; and contrast-enhanced ultrasonography (CEUS) [160,161,165,166], which have been showing different performances across studies in small cohorts. USE is a non-invasive, innovative technique, in which quantitative measurements of strain ratio can be obtained through the ratio between the strain of a reference region and the strain of the pathologic region, with values above 1 indicating higher stiffness [167]. In the context of IBD, USE showed promising results in animal models [156] and has proven to be able to differentiate between normal and strictured bowel

segments [156]. Importantly, it showed to correlate with the severity of fibrosis [157–159]. A recent systematic review showed that, in comparison to histopathological assessment, USE showed moderate-to-good accuracy in detecting histological fibrosis [95]. However, based on the available data, the authors considered that USE could not replace the tissue specimen yet, and its applicability must be validated in randomized clinical trials with proper design.

SWI is based on the measurement of shear wave vibration upon application of a pulse wave to tissues by means of an ultrasound probe. The applicability of SWI to fibrosis measurements relies on the fact that transmission of shear waves is faster in stiffer tissues than in softer ones [120]. In CD, SWI was able to distinguish between inflammation from fibrosis in a rodent model [168] but also in human bowel resected from CD patients immediately after surgery [163] and to discriminate between distinct levels of fibrosis in a pilot study with 35 CD patients ($p = 0.002$) [162]. Furthermore, in a comparative study, including three ultrasound techniques, SWI proved to be superior to USE and acoustic radiation force impulse (ARFI) in evaluating and differentiating intestinal stenosis in CD [169]. However, these features could not be confirmed in a pediatric CD cohort [170], and SWI showed no correlation with fibrosis scores in a population of 105 ileal CD patients [164].

Three studies based on the combination of CEUS and USE [160,161] or SWI [171] in the setting of CD showed that combined techniques present an increased ability to differentiate inflammation from fibrosis. The utility of CEUS for fibrosis assessment had been suggested before in a quantitative study in CD patients in which fibrosis seemed to be associated with reduced blood volume and blood flow [172]. However, CEUS did not show similar performance in a 2018 study with 25 CD patients who were evaluated before elective surgery [166]. Despite these conflicting results and the need to recur to an intravenous contrast agent, CEUS seems to add diagnosis value to other imaging techniques. Still, more studies are warranted to explore its full potential.

Even though only a few studies have resorted to Doppler-ultrasonography for the assessment of IBD patients, this technique also deserves mention in this section. In 2013, a study designed to assess the accuracy of US parameters for the evaluation of mural inflammation in CD revealed a significantly negative association between color Doppler grade and fibrosis score ($r = -0.584$, $p = 0.001$) [165]. Later, Sasaki and colleagues demonstrated that color Doppler was able to predict tissue inflammation and fibrosis in small-intestinal CD lesions ($p < 0.05$) [173].

At this point, CSI techniques have proven to be valuable tools in the attempt to measure fibrosis non-invasively, and ongoing research will be pivotal to defining validated measurement protocols with high accuracy and specificity while guaranteeing minimal risks for patients. In fact, from all the available data and clinical evidence, even though none of the available methodologies is capable of defining the fibrotic component of a CD stricture accurately, MRE-based modalities have proven to be the more advanced for the non-invasive assessment of severe fibrosis in stricturing CD, followed by US-based techniques.

Despite the uncertainties regarding future advances in this field, it is undebatable that the evaluation and diagnosis of fibrosis in IBD requires a multidisciplinary approach involving gastroenterologists, radiologists, pathologists, surgeons, and nurses, among others. Overall, patients benefit from regular monitoring with biomarkers and imaging techniques and from deep clinical discussions in a multidisciplinary setting. In addition, the development of effective referral processes, improved access, and departmental guidelines/pathways with the identification of quantifiable quality indicators creates conditions to provide patients with the best possible diagnosis, treatment, and follow-up [174].

4. What Is the Future Holding for Fibrosis?

4.1. Radiomics

In a 2020 commentary, Lin and colleagues discussed the concept of computer-assisted image analysis in the context of IBD and suggested radiomics as a tool to transform

qualitative fibrosis evaluations on quantitative data [114]. The path to this discussion was opened by a study on the applicability of semi-automated analysis to the measurement of bowel structural damage, with evidence of high consistency with measurements performed by experienced radiologists [117]. In line with data from other diseases, at this point, a few studies support that radiomics of MRE and CTE, consisting of the extraction of high-dimensional data from CSI images, are viewed as a potential source of valuable data for the assessment of IBD fibrosis [115–117]. In 2021, Li and colleagues developed a novel CTE-radiomic model for the characterization of intestinal fibrosis in CD, which distinguished the histological non-mild from moderate–severe fibrosis with an AUC of 0.888 on the training cohort and AUCs between 0.724 and 0.816 (95% CI) in the three test cohorts. Moreover, the model performed better than visual interpretations by two experienced radiologists ($p < 0.001$) [116]. The potential of radiomics in this setting was also evidenced in a recent study that reported the integration of CTE on a deep-learning model based on a 3D deep convolutional neural network with 10-fold cross-validation. This model also presented higher accuracy for the assessment of fibrosis severity than CTE evaluation by two radiologists, with the advantage of having a shorter processing time [115]. Despite the robustness of these data, in a letter to the Editor of Gastroenterology, Zhang considered that the validation of these results depends on the development of reliable radiomic biomarkers and criteria to evaluate the design and report of radiomic studies in prospective cohorts [175]. Very recently, the STAR consortium presented the results of a machine-reader evaluation of severe inflammation and fibrosis in CD strictures through quantitative radiomic features and expert radiologist scoring on CTE [176]. Based on the evidenced association of two distinct sets of radiomic features for severe inflammation and fibrosis ($p < 0.01$), the authors considered that the combination of quantitative radiomics with radiological visual assessment might favor more personalized treatments by providing more accurate phenotyping of CD strictures. In the validation study, however, while confirming the value of radiomics in the identification of fibrosis but not inflammation in stricturing CD, the same group did not find advantages in combining radiomic features with the radiologist's visual assessment [177]. Hence, it seems very likely that radiomics and AI will set the path for the future in the scope of fibrosis assessment by the CSI techniques in CD. However, it is still not clear whether AI alone (without concomitant human intervention) will be able to accomplish this purpose.

4.2. Others

Even though imaging techniques have been the focus of most of the developed research regarding fibrosis assessment, studies have diverted to other approaches, such as biochemical and genetic markers, including proteomics, genomics, metabolomics, and transcriptomics. The efforts are supported by the assumption that fibrosis biomarkers would provide useful data for risk stratification and treatment optimization of IBD patients. However, the available evidence includes conflicting data and is focused on markers with low diagnostic and prognostic value. Still, considering that past and ongoing research has provided promising data, an update of the most relevant candidate biomarkers seems appropriate in the context of this revision.

In 2014, the fourth scientific workshop of the European Crohn's and Colitis Organization (ECCO) focused on understanding basic mechanisms and markers of intestinal fibrosis and considered that, as none of the available biomarkers were able to accurately assess fibrosis, research for novel targets should proceed, as it is pivotal for the development of novel therapeutic options for intestinal fibrosis [119].

In 2012, based on previous findings in the scope of renal disease, Chen and coworkers explored the role of miR-200a and miR-200b in intestinal fibrosis in a colorectal adenocarcinoma epithelial cell line [178]. The results showed that miR-200b was overexpressed in the serum of the fibrosis group and could have diagnostic and therapeutic applications for CD patients with fibrosis. This study leveraged further research on this topic, including a revision on the emerging role of micro-RNAs (miRNAs) in IBD [179], exploring their

involvement in the pathways of inflammation and fibrosis in IBD. At that point, the authors considered that miR-200 [178,180] and miR-29b [181] seemed to deserve further research due to evidence of their potential as IBD biomarkers. The importance of these two miRNA families was addressed in two 2015 and 2023 reviews [182,183]. In both, it is stated that the research performed so far still warrants confirmation in more robust studies due to small sample sizes, lack of control of patients' heterogeneity, and absence of a standard protocol to assess miRNAs.

Other studied candidate biomarkers include serum and plasma proteins, such as collagen [184], ECM [185], pentraxin-2 [186], serum glycoproteins [187], enzymes, such as metalloproteinases [188], antimicrobial antibodies [189,190], and serum growth factors, such as YKL-40 [191,192] and gene variants [193]. Similar to miRNAs, data on these topics are conflicting and do not support their utility in the scope of fibrosis measurement.

In conclusion, considering the vast evidence on this topic, it seems that the efforts on the discovery of novel biomarkers to assess fibrosis would be more consequent through the development of more robust clinical trials based on solid and validated endpoints.

4.3. Anti-Fibrotic Therapy

The discussion of fibrosis in the context of IBD can only be completed by addressing the current therapeutic challenges and perspectives toward fibrosis. In the scope of CD, ECCO recommends endoscopic balloon dilatation (EBD) or surgery for patients with short strictures (<5 cm), and strictureplasty for the resection of long segments of the bowel; strictureplasty of the colon is not recommended [194]. Regarding EBD, the PRODILAT study—an RCT with CD patients with the obstructive disease and predominantly fibrotic strictures of less than 10 cm—showed that 80% of the patients approached with this technique were free of a new therapeutic intervention at 1 year; compared with fully covered self-expandable metal stents, EBD proved to be more effective for CD strictures [195].

So far, past and ongoing research did not generate evidence to support the approval of any anti-fibrotic agent. Considering that the fibrosis process is similar in IBD and in systemic and pulmonary fibrosis, several drugs are under investigation as anti-fibrotic agents, in a pre-clinical setting resorting mainly to UC animal models, with promising results in the TGF-β [196–204], TNF [205], IL-36 [206], rho-kinase [207], peroxisome-proliferator activated receptor (PPAR) [208], HMG-CoA reductase [209] pathways, among others (Table 2) [5,210]. Table 2 includes the most promising targets and molecules and is not an exhaustive description of all the ongoing research in this field. Regarding phase 2 studies, spesolimab proved to be well tolerated with an adverse event rate similar to placebo (without meeting efficacy criteria) [206], and PF-06480605 demonstrated an acceptable safety profile with concomitant endoscopic improvement (week 14) in patients with moderate to severe UC [205].

Table 2. Potential anti-fibrotic agents under research.

Agent	Pathway	Model	Research Status	Reference
Pirfenidone	TGFβ	Human cells	Pre-clinical	[196–201]
		Murine models		
		Mice		
Tranilast	TGFβ	Rats	Pre-clinical	[202,203]
		Rat models		
		Patients with CD		
EW-7197	TGFβ	Murine model	Pre-clinical	[204]
PF-06480605	TNF	Patients with UC	Phase 2	[205]
Spesolimab	IL-36	Patients with UC	Phase 2	[206]

Table 2. *Cont.*

Agent	Pathway	Model	Research Status	Reference
AMA0825	Rho-kinase inhibitor	Mice models Cells CD biopsies	Pre-clinical	[207]
GED-0507-34	PPARγa agonist	Mice	Pre-clinical	[208]
Statins	HMG-CoA reductase inhibitors	Human intestinal fibroblasts	Pre-clinical	[209]

CD: Chron's disease; IL-36: interleukin 36; HMG-CoA: 3-hydroxy-3-methylglutaryl-CoA; PPARγa: peroxisome proliferator-activated receptor-γ; TGFβ: transforming growth factor β; TNF: tumor necrosis factor; UC: ulcerative colitis.

Several molecules are now awaiting clinical trials in humans, and in the near future, new therapeutic agents may be approved. Further improvements in this field have been hindered by the reduced research in CD models and by the lack of research standards. The intensive work of the STAR consortium regarding the standardization of the conditions to measure response to anti-fibrotic agents will be determinant for the success of these processes.

5. Conclusions

Intestinal fibrosis is a serious complication of IBD with relevant clinical implications that determine treatment selection, prognosis, and quality of life. Currently, available data support the concomitant influence of inflammation-dependent and -independent mechanisms on the induction and progression of fibrosis.

In this review, we highlighted the importance of the development of accurate non-invasive methodologies for the assessment of fibrosis and discussed their strengths, limitations, and future perspectives. In the setting of CSI, MRE advanced modalities seem to be the most robust techniques to measure fibrosis. However, the subjectivity of the visual analysis and interpretation of the images has been hindering the endorsement of CSI in this field. In fact, from the available recent data on radiomics, we believe that imaging techniques will only reach their full potential in terms of accuracy through the combination with artificial intelligence systems. Data in the setting of biomarkers research lack consistency and warrant deeper and more structured trials.

At the end of the day, surgical pathology remains the definitive modality for diagnosing and quantifying intestinal fibrosis in IBD, with the unavoidable disadvantages of being invasive and limiting this study of intestinal damage to "end-of-stage" disease.

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CHAPTER 2 – Aims

2. Aims

This thesis aimed to add knowledge regarding inflammatory and fibrosis changes in stricturing and penetrating CD.

For this purpose, three objectives were defined, as follows and is outlined in **Figure 1**.

- Objective 1 [CHAPTER 3]

  To uncover a liable transmural histopathological scoring system for the study of inflammation and fibrosis both in stricturing and penetrating CD.

- Objective 2 [CHAPTER 4]

  To grade inflammation and fibrosis in ileal CD resection specimens, according to a liable CD transmural histopathological scoring system in stricturing or penetrating CD.

- Objective 3 [CHAPTER 5]

  To compare the fibrosis-related transcriptome profiles in stricturing and penetrating CD.

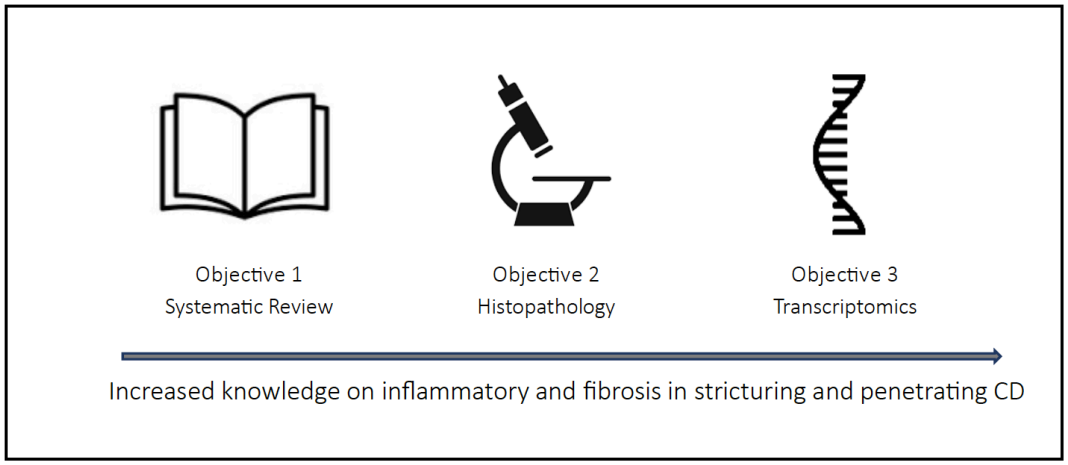

Figure 1 – Objectives and key methodology addressed in chapters 1 to 3 of this thesis.

CHAPTER 3 – Transmural Histological Scoring Systems in Crohn's Disease: A Systematic Review with Assessment of Methodological Quality and Operating Properties

3. Transmural Histological Scoring Systems in Crohn's Disease: A Systematic Review with Assessment of Methodological Quality and Operating Properties

The first main objective of this thesis was to uncover a liable transmural histopathological scoring system in order to accurately study inflammation and fibrosis in stricturing and penetrating CD. Also, to propose the best score(s) to be used in clinical practice and/or translational research. For this purpose, a systematic review of all existing transmural histopathological scoring systems evaluating inflammation and/or fibrosis in CD, focusing on the originally developed scores, was conducted. Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines were applied. As a meta-analysis on the reviewed scores was not feasible due to high heterogeneity, an assessment of the methodological quality of the studies reporting an original score was assessed using the 10-items COSMIN [COnsensus-based Standards for the selection of health Measurement INstruments] checklist, which was complemented with an analysis of the four operating properties of each original score.

This study produced a **core scientific paper** published in a **Q1** Gastroenterology journal, with a 2020 Clarivate/Web of Science Journal Impact Factor of 9.071.

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Transmural Histological Scoring Systems in Crohn's Disease: A Systematic Review With Assessment of Methodological Quality and Operating Properties

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Abstract

Background: The relative proportion of inflammation and fibrosis in a stricture is highly relevant in defining the clinical approach for Crohn's disease [CD] patients. Whereas transmural inflammation in CD can be accurately estimated by cross-sectional imaging, evaluating the extent and severity of fibrosis still requires surgical pathology of intestinal resection specimens. This study systematically reviewed all existing transmural histopathological scoring systems developed for the assessment of inflammation and/or fibrosis in CD.

Methods: A systematic review of histopathological scoring systems for the assessment of transmural inflammation and/or fibrosis in CD, focusing on originally developed scoring systems. Risk of bias, methodological quality, and operating or psychometric properties [validity, reliability, responsiveness, and feasibility] of each histological scoring system were analysed.

Results: A total of 29 original scoring systems were included in this review. Three scoring systems were highlighted as the most widely reproduced, one aimed at assessing inflammation only and two aimed at assessing inflammation and fibrosis. These scores were more widely reproduced probably due to their ease of application in clinical studies. Two highly comprehensive scores were identified, showing good operating properties and high methodological quality, as well as the lowest risk of bias; these should, therefore, be further validated in clinical research studies.

Conclusions: This study reviewed all existing transmural histopathological scoring systems for the assessment of inflammation and/or fibrosis in CD and identified the most reliable and accurate scores for clinical research and clinical practice settings.

Key Words: Crohn; transmural; inflammation; fibrosis; score

1. Introduction

Crohn's disease [CD] is a chronic inflammatory bowel disease characterised by remitting and relapsing episodes that can involve any location within the gastrointestinal tract, although most frequently affecting the terminal ileum and right colon. The pattern of CD varies over time, with 45.9% of patients progressing from non-stricturing non-penetrating to stricturing [27.1%] or penetrating [29.4%] phenotype during the first 10 years of disease,[1] with a lifetime risk of developing a stricture of approximately 50%.[2,3] Despite therapeutic advances, the incidence of intestinal strictures in CD has remained stable, no specific anti-fibrotic therapy exists,[4] and surgery rates have not declined,[5] reaching 30% even after 2 years of infliximab treatment.[6]

In patients with stricturing CD, transmural fibrosis occurs due to collagen deposition and proliferation of fibroblasts and smooth muscle cells in the submucosa and muscularis propria; fibromuscular obliteration of the submucosa is particularly associated with small bowel strictures.[3,7] Pure fibrotic or inflammatory strictures are, however, rare, with inflammation and fibrosis usually co-existing to varying degrees.[8–10] Patients with predominantly inflammatory lesions are likely to respond better to therapy, whereas patients with established fibrostenotic lesions are more likely to need surgery.[11,12]

Transmural inflammatory healing (inactive magnetic resonance [MR] enterography and colonoscopy) was associated with better outcomes [lower rates of hospital admission, therapy escalation, and surgery] than endoscopic healing alone over a period of 12 months.[13] Thus, it is crucial to non-invasively evaluate and quantify the relative proportion of inflammation and fibrosis in a stricture, in order to choose the best treatment for an individual patient and to assess response to treatment. Intestinal inflammation can be evaluated with high accuracy by non-invasive means using cross-sectional imaging, namely MR, computed tomography [CT], and intestinal ultrasound [US].[14] Non-invasive quantification of fibrosis is much more challenging, as cross-sectional imaging cannot accurately quantify the fibrotic component of an otherwise easy-to-diagnose stricture.[4,14–16] Similarly, fibrosis cannot be adequately assessed by biomarkers, nor by endoscopy or biopsy-based histology,[4,14] since tissue remodelling occurs primarily in the submucosa and muscularis propria, which are not amenable to endoscopic biopsies.[11] This was acknowledged in two systematic reviews of biopsy-based histopathological scoring systems for CD activity,[17,18] none of which is validated for use in clinical practice.[19] Hence, whereas transmural inflammation in CD can be accurately estimated by cross-sectional imaging, evaluating the extent and severity of fibrosis still requires surgical pathology of intestinal resection specimens.

Several transmural histopathological scoring systems have been developed since the 1980s to assess inflammation and/or fibrosis in CD. These systems show, however, highly diverse characteristics, depending on the aim and type of study in which they are based [e.g. molecular, surgical, pathological, pathology-imaging correlation, etc]. The transmural assessment of fibrosis in CD still requires surgical pathology, and these scoring systems should be thoroughly analysed to select those best suited for use either in clinical studies and in clinical practice.

This study systematically reviewed all existing transmural histopathological scoring systems developed for the assessment of inflammation and/or fibrosis in CD. Furthermore, we assessed the methodological quality of each study reporting an original score and the operating properties [validity, reliability, responsiveness and feasibility] of each original scoring system, in order to identify the most reliable and accurate indexes.

2. Methods

2.1. Search strategy

This study was performed as recommended by the Preferred Reporting Items for Systematic Reviews and Meta-Analyses [PRISMA] Guidelines.[20,21] The published studies were retrieved after a search performed on four electronic databases: PubMed [https://www.ncbi.nlm.nih.gov/pubmed/], Web of Science [http://www.isiwebofknowledge.com], Science Direct [www.sciencedirect.com], and CENTRAL - Cochrane Central Register of Controlled Trials [http://www.mrw.interscience.wiley.com/cochrane/cochrane_clcentral_articles_fs.htm]. The search was carried out in January 2019 using the following terms: ['Crohn's disease'] and ['histology' or 'pathology' or 'index' or 'indices' or 'scale' or 'score' or 'grade'] and ['surgery' or 'surgical specimens' or 'resection']. In addition, the bibliographies of all included studies, as well as excluded reviews, were manually reviewed to ensure that all pertinent articles were identified.

2.2. Eligibility and inclusion/exclusion criteria

Studies were considered eligible if fulfilling the following inclusion criteria: i] performing the transmural histological assessment of surgical intestinal resection specimens of human patients with CD; ii] describing the use of a transmural inflammation and/or fibrosis histopathological scoring system, irrespective of being numerical or stepwise; and iii] written in English language. No restriction was applied regarding the publication date. All studies that fulfilled at least one of the following conditions were excluded: i] review papers, guidelines, consensus papers, editorials, case reports, or case series; ii] studies involving patients with diseases other than CD; iii] studies using animal models; iv] studies that did not present histological data; and v] studies that did not describe a specific transmural inflammation and/or fibrosis histological index or that described an index specifically devoted to a unique aspect of CD pathology [for example, quantification of myenteric plexitis alone].

2.3. Study selection and data collection process

The studies identified through the electronic databases or by manual search were independently screened by two reviewers [HTS and MME] after removal of duplicate records. Any study whose title and abstract indicated that it did not meet the eligibility criteria was excluded from further analysis. In all other studies, the full text was

considered in order to determine its inclusion or exclusion. The following information was collected from the selected studies: journal and authors' names; publication year; article type; number of patients enrolled; type of score [inflammation and/or fibrosis; numerical and/or stepwise], categories/scoring, and key features of the score; anatomical origin of the surgical specimen; number of gastrointestinal pathologists involved in developing score and their blindness to clinical and/or imaging and/or laboratorial findings; which stains were used (haematoxylin & eosin [H&E]; special stains for fibrosis assessment: Masson Trichrome, Sirius Red or others) if immunohistochemistry stains were applied and if morphometry was used.

2.4. Evaluation of the risk of bias of the included studies

The risk of bias was evaluated through the declaration of blindness of histopathologists to clinical, endoscopic, and imaging information and of independent observation by more than one histopathologist.

2.5. Analysis of the methodological quality of the studies reporting an original score

The methodological quality of the studies reporting an original score was assessed using the COSMIN [COnsensus-based Standards for the selection of health Measurement INstruments] checklist, which consists of 10 properties: internal consistency, reliability, measurement error, content validity, structural validity [factor analysis], hypothesis testing, cross-cultural validity, criterion validity, responsiveness to change, and interpretability. Each property is rated on a four-point scale [1 = poor, 2 = fair, 3 = good, 4 = excellent], with overall score for each individual property being taken by the lowest score in the box.[22–24]

2.6. Analysis of the operating properties of the original scores

The operating or psychometric properties [validity, reliability, responsiveness, and feasibility] of each histological scoring index were analysed. Each parameter was scored as follows: unknown [?], moderately assessed [+/-], and well assessed [+]. Validity includes content validity [whether the elements of the scoring index are sufficient to measure disease activity in Crohn's disease], criterion validity [the degree to which the index scores adequately reflect CD disease activity as compared twith gold standard], and construct validity [whether the index is consistent with other available markers of disease activity]. Content validity is generally evaluated qualitatively, for example through the systematic review of the literature supporting the development of the index.

Regarding criterion validity, the lack of a single gold standard for measuring Crohn's disease activity limited its assessment. In this context, criterion validity was assessed by comparing whether the score actually predicted disease activity as measured by available objective measures of inflammation including C-reactive protein, erythrocyte sedimentation rate, faecal calprotectin, and/or disease sequelae like surgery or disability. For this purpose, the statistical parameters reported on each study regarding the agreement between the histological index and other disease activity gold standards were assessed (i.e., sensitivity, specificity, receiver operating characteristic [ROC] curve, area under the curve, mean difference, weighted kappa, Spearman's r squared, among others).

Construct validity was assessed by checking whether the index was consistent with other available markers of disease activity, like clinical disease activity indices and quality of life measures.

Reliability is the ability to reproduce a consistent result in time and space [intra-rater], or from different observers [inter-rater], including aspects on coherence, stability, equivalence, and homogeneity. Each study was screened for the presence of reliability measures, including the interclass correlation coefficient, Pearson's r, or kappa statistic.

Responsiveness refers to the ability of a given index to detect changes, for example following a treatment of known efficacy. Responsiveness was quantified using indicators of effect size, t test for related data, and ROC curves and regression models to describe how well various score changes could be used to distinguish between patients who improved and patients who did not improved.

Each study was screened for the presence of rater evaluations regarding the feasibility of the histological index, meaning the ease with which an instrument may be used.

3. Results

3.1. Bibliographic search and study selection

Figure 1 summarises the study selection process. The search of the four electronic databases yielded 4330 records, and 744 studies were immediately excluded: 372 were duplicates and 372 were not written in English. After title and abstract review of the remaining 3586 studies, 187 papers were considered for full text analysis and 143 were excluded, mainly for not describing a specific transmural inflammation and/or fibrosis histological index. Overall, 53 studies [44 obtained from database search and nine selected by manual search] were included in this systematic review. Throughout the review of the 53 studies, we recognised that 29 studies reported original scores and the remainder used previously described ones, thus we focused on the first group, leading to a sample of 29 original scoring systems.

3.2. Risk of bias of the included studies

3.2.1. Blindness

Of the 29 studies developing original scores, 14 reported blinding of histopathologists to clinical, endoscopic, or imaging information,[8,9,25–35] three declared the unblindness of histopathologists to these data,[36–38] and for remaining 12 studies no information was provided regarding this aspect.[39–47]

3.2.2. Independency of observation

Only five of the 29 studies reporting original scores stated that samples were assessed by two histopathologists.[9,29,33,34,44] Of these, all but one[44] declared blinding to clinical, endoscopic, or imaging information. The histopathological observation was reported as independent in two studies[33,34] and as joint in one study,[29] being omitted in the remaining studies.

3.3. Methodological quality of the included studies

The assessment of the 29 studies reporting an original score using the COSMIN checklist [Figure 2] revealed that the overall methodological quality of the included studies is moderate, with a mean percentage of compliance with all checklist parameters of 62.24 ± 10.37. The lowest score [47.5%] was obtained for the studies by Heuman *et al.*[36] and Smedh *et al.*,[28] which reported two of the oldest histopathological scoring systems. The highest scores were obtained for the studies by Schaeffer *et al.*[33] [85%], Chiorean *et al.*[31] [80%], Adler *et al.*[9] [77.5%], and Chen *et al.*[34] [75%].

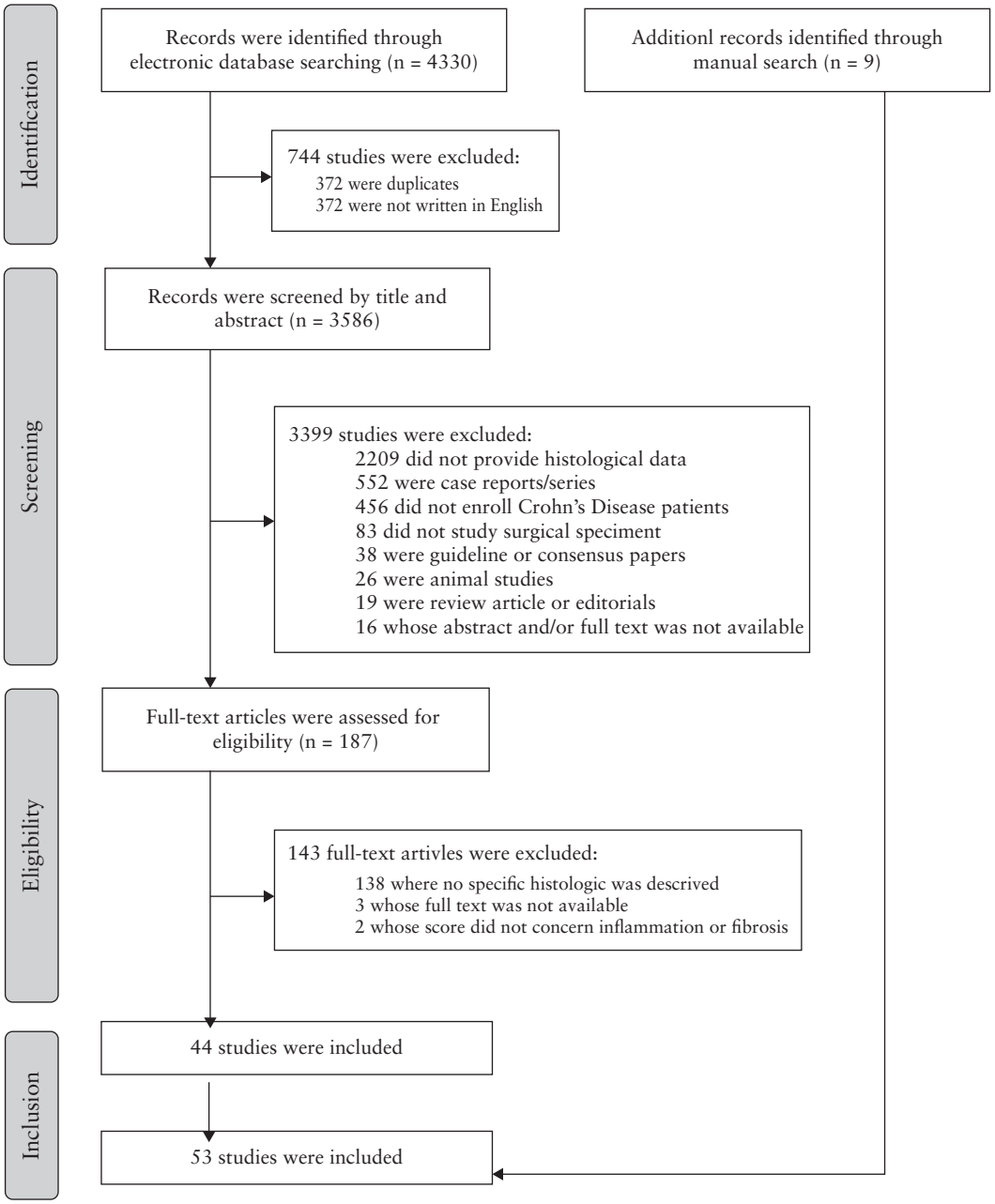

Figure 1. Selection of studies.

3.4. Analysis of the operating properties of the original scores

Figure 3 summarises the psychometric properties [validity, reliability, responsiveness, and feasibility] of the 29 original scoring systems. Most of the histological indexes provided no data for the majority of operating properties. Validity was the property more commonly reported, still only eight studies [Adler *et al.*,[9] Baumgart *et al.*,[25] Chiorean *et al.*,[31] Havre *et al.*,[38] Knod *et al.*,[47] Pellino *et al.*,[48] Schaeffer *et al.*,[33] and Zappa *et al.*[8]] had objectively evaluated this property. Reliability was objectively evaluated in five histological scores [Adler *et al.*,[9] Borley *et al.*,[49] Chen *et al.*,[34] Pellino *et al.*,[48] and Zappa *et al.*[8]]. On the other hand, feasibility [ease of application] was not formally assessed in any study.

3.5. Transmural histological scoring systems in CD

The 29 studies reporting original scoring systems are described on Supplementary Tables 1–3 [available as Supplementary data at *ECCO-JCC* online] according to the histological features included in the score: both inflammation and fibrosis [Supplementary Table 1], fibrosis only [Supplementary Table 2], or inflammation only [Supplementary Table 3]. The first scores date back to the last two decades of the 20th century and were mostly dedicated to studying the prognostic impact of microscopic inflammation at the margins of intestinal resection.[36,37,39,45] Most scoring systems were developed in pathology-imaging correlation studies,[8,9,25,26,29,31,32,38,41–44,46] a few were especially devoted to the description of the histopathologic bowel changes due to CD or therapy,[28,33–35,40,49] and others were integrated in the study of specific molecular aspects of these

	Internal consistency	Reliability	Measurement error	Content validity	Structural validity	Hypothesis testing	Cross-culture validity	Criterion validity	Responsiveness	Interpretability	Total score (%)
Adler et al., 2012	3	3	3	4	3	3	3	3	3	3	77.5
Baumgart et al., 2015	2	3	3	2	3	2	3	2	2	3	62.5
Borley et al., 2000	3	3	3	4	3	3	2	3	3	3	75.0
Catalano et al., 2016	3	3	3	3	3	3	2	2	2	3	67.5
Chen et al., 2017	2	3	4	4	3	3	2	3	3	3	75.0
Chiorean et al., 2007	3	3	3	4	3	3	3	3	3	4	80.0
Cooper and Williams, 1986	2	2	2	3	2	2	2	2	2	2	52.5
de Bruyn et al., 2018	2	2	2	3	3	3	2	2	2	3	60.0
Dillman et al., 2014	3	3	3	3	3	3	2	2	2	3	65.0
Girlich et al., 2011	2	2	2	2	2	2	2	2	2	3	52.5
Han et al., 2017	2	3	2	2	2	2	2	3	3	3	60.0
Havre et al., 2014	3	2	2	2	2	2	2	2	2	3	55.0
Heuman et al., 1983	2	2	1	2	2	2	2	2	2	2	47.5
Jacene et al., 2009	2	2	2	2	2	2	1	2	2	2	47.5
Knod et al., 2015	2	3	2	3	2	2	2	2	2	2	55.0
Kotanagi et al., 2001	2	2	2	2	2	2	2	2	2	3	52.5
Lasocki et al., 2011	3	3	3	3	3	2	2	3	2	3	67.5
Lawrance et al., 2009	2	2	2	3	3	2	2	2	2	3	57.5
Maconi et al., 2003	3	3	3	3	3	3	2	3	3	3	72.5
Nylund et al., 2008	2	2	3	3	3	3	2	3	2	3	65.0
Paquet et al., 2016	2	2	2	3	3	2	2	2	2	3	57.5
Pellino et al., 2016	3	3	3	3	3	3	2	3	2	4	72.5
Pennington et al., 1980	2	2	2	3	3	2	2	2	2	2	55.0
Pucilowska et al., 2000	2	2	2	3	2	2	2	2	2	2	52.5
Schaeffer et al., 2014	4	3	3	4	4	3	3	3	3	4	85.0
Shen et al., 2004	2	2	2	3	2	2	2	2	2	2	52.5
Smedh et al., 1995	2	2	1	2	2	2	2	2	2	2	47.5
Wagner et al., 2018	2	3	2	3	3	2	2	3	2	3	62.5
Zappa et al., 2011	3	3	3	3	3	3	2	3	3	3	72.5

Figure 2. Assessment of the methodological quality of the studies reporting original histopathological scoring systems. Each parameter of the COSMIN checklist was scored as follows: poor [1], fair [2], good [3], and excellent [4]; global percentage mean ± standard deviation = 62.24 ± 10.37.

	Validity	Reliability	Responsiveness	Feasibility
Adler et al., 2012	+	+	+	?
Baumgart et al., 2015	+	?	?	?
Borley et al., 2000	+/–	+	+	?
Catalano et al., 2016	?	?	?	?
Chen et al., 2017	+/–	+	+/–	?
Chiorean et al., 2007	+	+/–	+/–	?
Cooper and Williams, 1986	?	?	?	?
de Bruyn et al., 2018	+/–	?	?	?
Dillman et al., 2014	+/–	+/–	?	?
Girlich et al., 2011	?	?	?	?
Han et al., 2017	+/–	?	?	?
Havre et al., 2014	+	?	?	?
Heuman et al., 1983	?	?	?	?
Jacene et al., 2009	?	+/–	+/–	?
Knod et al., 2015	+	?	+	?
Kotanagi etal., 2001	?	?	+/–	?
Lasocki et al., 2011	?	?	?	?
Lawrance et al., 2009	+/–	?	?	?
Maconi et al., 2003	+/–	?	?	?
Nylund et al., 2008	?	?	?	?
Paquet et al., 2016	?	+/–	?	?
Pellino et al., 2016	+	+	?	?
Pennington et al., 1980	?	?	?	?
Pucilowska et al., 2000	?	?	?	?
Schaeffer et al., 2014		+/–	+	?
Shen et al., 2004	?	?	?	?
Smedh et al., 1995	+/–	?	?	?
Wagner et al., 2018	+/–	?	+	?
Zappa et al., 2011	+	+	+/–	?

Figure 3. Assessment of the operating characteristics of the original histopathological scoring systems. Each parameter was scored as follows: unknown [?], moderately assessed [+/-], and well assessed [+].

physiological changes.[27,47] The surgical specimens used for the development of the score were both small bowel and colon in 15 studies,[25,27,28,31,33–37,39,40,45,46,65,68] only small bowel in nine,[8,9,29,41,43,47–49,64] and colon in one[30]; they were not reported in four studies.[26,32,38,42]

Given the highly heterogeneous approaches of assessing and grading the multiple histological features displayed in Supplementary Tables 1–3, we deconstructed each original scoring system according to its ability to assess the different histopathological variables of transmural inflammation and fibrosis in CD [Tables 1 and 2, respectively].

From a histopathological point of view, the more detailed and complete scoring systems derive from studies specifically designed to describe the pathological changes occurring in the bowel as a result of CD [namely the numerical systems from Chen *et al.*,[34] Schaeffer *et al.*,[33] and Borley *et al.*,[49]]. Chen *et al.*[34] developed a semiquantitative grading system covering not only inflammation and fibrosis, but also the full spectrum of CD pathology, as they aimed to better understand the pathogenesis of fibrostenosis. Each layer of the bowel wall was categorised for: active and chronic inflammation features; fibrosis and smooth muscle changes [this score is unique in distinguishing muscular hyperplasia in the submucosa from muscular hypertrophy of the muscularis propria Table 2]; neuronal hypertrophy; and adipocyte hyperplasia [being the only score describing it in intermediate layers—submucosa and muscularis propria]. This score is also singular in accounting for the presence of abscesses as a sign of acute inflammation in all layers except the mucosa, and is one of the two systems looking at chronic eosinophilic infiltrates [Table 1], although the presence of granulomas is not addressed. Fibromuscular assessment included H&E, Masson's Trichrome, and immunohistochemistry stain for smooth muscle

Table 1. Histopathologic transmural inflammation assessment.

Layer	Type of inflammation	Histologic features	Scoring systems including each feature
Mucosa	Acute inflammation	Erosion/superficial ulcer	Pennington *et al.*,[39] Heuman *et al.*,[36] Kotanagi *et al.*,[37] Smedh *et al.*,[28] Pucilowska *et al.*,[40] Maconi *et al.*,[29] Chiorean *et al.*,[31] Lawrance *et al.*,[41] Girlich *et al.*,[32] Schaeffer *et al.*,[33] Catalano *et al.*,[42] Chen *et al.*,[34] deBruyin *et al.*,[35] Cooper *et al.*,[45] Borley *et al.*,[49] Lasocki *et al.*,[46] Knod *et al.*,[47] Han *et al.*[27]
		Fissuring ulcer	Heuman *et al.*,[36] Kotanagi *et al.*,[37] Smedh *et al.*,[28] Maconi *et al.*,[29] Chiorean *et al.*,[31] Schaeffer *et al.*,[33] Chen *et al.*,[34] Cooper *et al.*,[45] Lasocki *et al.*[46]
		Cryptitis or crypt abscesses	Pennington *et al.*,[39] Heuman *et al.*,[36] Kotanagi *et al.*,[37] Maconi *et al.*,[29] Chiorean *et al.*,[31] Lawrance *et al.*,[41] Girlich *et al.*,[32] Chen *et al.*,[34] deBruyin *et al.*[35]
		Oedema	Kotanagi *et al.*,[37] Maconi *et al.*,[29] Girlich *et al.*,[32] Borley *et al.*[49]
		Epithelial neutrophil infiltrates	Smedh *et al.*,[28] Girlich *et al.*,[32] Schaeffer *et al.*,[33] deBruyin *et al.*,[35] Knod *et al.*,[47] Han *et al.*,[27]
		Lamina propria neutrophil infiltrates	Pennington *et al.*,[39] Kotanagi *et al.*,[37] Smedh *et al.*,[28] Pucilowska *et al.*,[40] Maconi *et al.*,[29] Shen *et al.*,[30] Chiorean *et al.*,[31] Jacene *et al.*,[64] Lawrance *et al.*,[41] Zappa *et al.*,[8] Adler *et al.*,[9] Catalano *et al.*,[42] Chen *et al.*,[34] deBruyin *et al.*,[35] Wagner *et al.*,[43] Borley *et al.*,[49] Knod *et al.*,[47] Paquet *et al.*,[26] Han *et al.*[27]
	Chronic inflammation	Crypt architectural distortion	Kotanagi *et al.*,[37] Smedh *et al.*,[28] Lawrance *et al.*,[41] Girlich *et al.*,[32] Adler *et al.*,[9] Schaeffer *et al.*,[33] Chen *et al.*,[34] deBruyin *et al.*,[35] Knod *et al.*,[47] Han *et al.*[27]
		Pyloric metaplasia	Pennington *et al.*,[39] Kotanagi *et al.*,[37] Smedh *et al.*,[28] Schaeffer *et al.*[33]
		Depletion of intracellular mucin	Kotanagi *et al.*,[37] Lawrance *et al.*,[41] Girlich *et al.*,[32]
		Epithelial lymphoplasmacytic infiltrates	Smedh *et al.*,[28] Girlich *et al.*[32]
		Epithelial eosinophilic infiltrates	Girlich *et al.*[32]
		Lamina propria lymphoplasmacytic infiltrates	Pennington *et al.*,[39] Heuman *et al.*,[36] Kotanagi *et al.*,[37] Pucilowska *et al.*,[40] Maconi *et al.*,[29] Shen *et al.*,[30] Chiorean *et al.*,[31] Jacene *et al.*,[65] Lawrance *et al.*,[41] Havre *et al.*,[38] Adler *et al.*,[9] Schaeffer *et al.*,[33] Dillman *et al.*,[65] Chen *et al.*,[34] deBruyin *et al.*,[35] Cooper *et al.*,[45] Knod *et al.*,[47] Han *et al.*[27]
		Lamina propria eosinophilic infiltrates	Girlich *et al.*,[32] Chen *et al.*[34]
		Macrophage infiltrates	Borley *et al.*[49]
		Lymphoid follicles/ aggregates/ hyperplasia	Pennington *et al.*,[39] Kotanagi *et al.*,[37] Maconi *et al.*,[29] Shen *et al.*,[30] Schaeffer *et al.*,[33] Chen *et al.*,[34] Borley *et al.*[49]
		Granulomas	Pennington *et al.*,[39] Heuman *et al.*,[36] Kotanagi *et al.*,[37] Smedh *et al.*,[28] Maconi *et al.*,[29] Shen *et al.*,[30] Girlich *et al.*,[32] Schaeffer *et al.*,[33] deBruyin *et al.*,[35] Cooper *et al.*,[45] Borley *et al.*,[49] Knod *et al.*,[47] Han *et al.*[27]
	Extent of inflammation		Schaeffer *et al.*[33]
	Haemorrhage		Girlich *et al.*[32]
	Epithelial changes		Girlich *et al.*,[32] Schaeffer *et al.*,[33] deBruyin *et al.*,[35] Knod *et al.*,[47] Han *et al.*[27]
	Pseudopolyps		Girlich *et al.*[32]
Submucosa	Acute inflammation	Fissuring ulcer	Kotanagi *et al.*,[37] Smedh *et al.*,[28] Maconi *et al.*,[29] Chiorean *et al.*,[31] Schaeffer *et al.*,[33] Chen *et al.*,[34] Cooper *et al.*,[45] Lasocki *et al.*[46]
		Abscess	Chen *et al.*[34]
		Oedema	Kotanagi *et al.*,[37] Smedh *et al.*,[28] Maconi *et al.*,[29] Girlich *et al.*[32]
		PMNs infiltrates [non-ulcer/ abscess area]	Pennington *et al.*,[39] Kotanagi *et al.*,[37] Smedh *et al.*,[28] Maconi *et al.*,[29] Shen *et al.*,[30] Chiorean *et al.*,[31] Jacene *et al.*,[64] Lawrance *et al.*,[41] Zappa *et al.*,[8] Girlich *et al.*,[32] Adler *et al.*,[9] Schaeffer *et al.*,[33] Chen *et al.*,[34] Wagner *et al.*,[43] Borley *et al.*,[49] Lasocki *et al.*,[46] Paquet *et al.*[26]

Table 1. Continued

Layer	Type of inflammation	Histologic features	Scoring systems including each feature
	Chronic inflammation	Lymphoplasmacytic infiltrates	Pennington *et al.*,[39] Kotanagi *et al.*,[37] Smedh *et al.*,[28] Maconi *et al.*,[29] Shen *et al.*,[30] Chiorean *et al.*,[31] Jacene *et al.*,[65] Lawrance *et al.*,[41] Girlich *et al.*,[32] Havre *et al.*,[38] Adler *et al.*,[9] Schaeffer *et al.*,[33] Dillman *et al.*,[65] Chen *et al.*,[34] Cooper *et al.*,[45] Lasocki *et al.*[46]
		Eosinophilic infiltrates	Girlich *et al.*,[32] Chen *et al.*[34]
		Macrophage infiltrates	Borley *et al.*[49]
		Lymphoid follicles/ aggregates/ hyperplasia	Pennington *et al.*,[39] Kotanagi *et al.*,[37] Smedh *et al.*,[28] Maconi *et al.*,[29] Shen *et al.*,[30] Schaeffer *et al.*,[33] Chen *et al.*,[34] Borley *et al.*[49]
		Lymphangitis/dilated lymph vessels	Heuman *et al.*,[36] Smedh *et al.*[28]
		Granulomas	Pennington *et al.*,[39] Heuman *et al.*,[36] Kotanagi *et al.*,[37] Smedh *et al.*,[28] Maconi *et al.*,[29] Shen *et al.*,[30] Girlich *et al.*,[32] Schaeffer *et al.*,[33] Cooper *et al.*,[45] Borley *et al.*,[49] Lasocki *et al.*[46]
	Haemorrhage		Girlich *et al.*[32]
Muscularis propria	Acute inflammation	Fissuring ulcer	Kotanagi *et al.*,[37] Smedh *et al.*,[28] Maconi *et al.*,[29] Chiorean *et al.*,[31] Schaeffer *et al.*,[33] Chen *et al.*,[34] Cooper *et al.*,[45] Lasocki *et al.*[46]
		Abscess	Chen *et al.*[34]
		PMNs infiltrates [non-ulcer/abscess area]	Kotanagi *et al.*,[37] Smedh *et al.*,[28] Maconi *et al.*,[29] Shen *et al.*,[30] Jacene *et al.*,[65] Chiorean *et al.*,[31] Lawrance *et al.*,[41] Zappa *et al.*,[8] Girlich *et al.*,[32] Adler *et al.*,[9] Schaeffer *et al.*,[33] Chen *et al.*,[34] Wagner *et al.*,[43] Borley *et al.*,[49] Lasocki *et al.*,[46] Paquet *et al.*[26]
	Chronic inflammation	Lymphoplasmacytic infiltrates	Kotanagi *et al.*,[37] Smedh *et al.*,[28] Maconi *et al.*,[29] Shen *et al.*,[30] Chiorean *et al.*,[31] Jacene *et al.*,[65] Lawrance *et al.*,[41] Girlich *et al.*,[32] Havre *et al.*,[38] Adler *et al.*,[9] Schaeffer *et al.*,[33] Dillman *et al.*,[66] Chen *et al.*,[34] Cooper *et al.*,[45] Lasocki *et al.*[46]
		Eosinophilic infiltrates	Girlich *et al.*,[32] Chen *et al.*[34]
		Macrophage infiltrates	Borley *et al.*[49]
		Lymphoid follicles/aggregates/ hyperplasia	Kotanagi *et al.*,[37] Maconi *et al.*,[29] Shen *et al.*,[30] Schaeffer *et al.*,[33] Chen *et al.*,[34] Nylund *et al.*,[44] Borley *et al.*[49]
		Perineural chronic inflammation	Borley *et al.*[49]
		Granulomas	Heuman *et al.*,[36] Kotanagi *et al.*,[37] Smedh *et al.*,[28] Maconi *et al.*,[29] Shen *et al.*,[30] Girlich *et al.*,[32] Schaeffer *et al.*,[33] Cooper *et al.*,[45] Borley *et al.*,[49] Lasocki *et al.*[46]
	Haemorrhage		Girlich *et al.*[32]
Serosa/ subserosal adventitia	Active inflammation	Fissuring ulcer	Kotanagi *et al.*,[37] Smedh *et al.*,[28] Maconi *et al.*,[29] Chiorean *et al.*,[31] Schaeffer *et al.*,[33] Chen *et al.*,[34] Cooper *et al.*,[45] Lasocki *et al.*[46]
		Abscess/fistula	Zappa *et al.*,[8] Chen *et al.*,[34] Lasocki *et al.*,[46] Paquet *et al.*[26]
		PMNs infiltrates [non-ulcer/abscess area]	Kotanagi *et al.*,[37] Smedh *et al.*,[28] Maconi *et al.*,[29] Shen *et al.*,[30] Jacene *et al.*,[64] Chiorean *et al.*,[31] Lawrance *et al.*,[41] Zappa *et al.*,[8] Girlich *et al.*,[32] Adler *et al.*,[9] Chen *et al.*,[34] Wagner *et al.*,[43] Borley *et al.*,[49] Paquet *et al.*[26]
	Chronic inflammation	Lymphoplasmacytic infiltrates	Kotanagi *et al.*,[37] Smedh *et al.*,[28] Maconi *et al.*,[29] Shen *et al.*,[30] Jacene *et al.*,[65] Chiorean *et al.*,[31] Lawrance *et al.*,[41] Girlich *et al.*,[32] Havre *et al.*,[38] Adler *et al.*,[9] Schaeffer *et al.*,[33] Dillman *et al.*,[65] Chen *et al.*,[34] Cooper *et al.*[45]
		Eosinophilic infiltrates	Girlich *et al.*,[32] Chen *et al.*[34]
		Macrophage infiltrates	Borley *et al.*[49]
		Lymphoid follicles/aggregates/ hyperplasia	Kotanagi *et al.*,[37] Maconi *et al.*,[29] Shen *et al.*,[30] Schaeffer *et al.*,[33] Chen *et al.*,[34] Borley *et al.*[49]
		Granulomas	Heuman *et al.*,[36] Kotanagi *et al.*,[37] Smedh *et al.*,[28] Maconi *et al.*,[29] Shen *et al.*,[30] Girlich *et al.*,[32] Schaeffer *et al.*,[33] Cooper *et al.*,[45] Borley *et al.*,[49] Lasocki *et al.*[46]
	Peritonitis		Schaeffer *et al.*[33]
	Haemorrhage		Girlich *et al.*[32]

Table 1. Continued

Layer	Type of inflammation	Histologic features	Scoring systems including each feature
Mesentery	Neutrophil infiltrates in extramural fat		Borley *et al.*[49]
	Lymphoid aggregates in extramural fat		Borley *et al.*[49]
	Hyperaemia		Lawrance *et al.*[41]
	Haemorrhage		Girlich *et al.*[32]
	Adenopathy		Chiorean *et al.*,[31] Lawrance *et al.*[41]
	Fatty proliferation		Lawrance *et al.*[41]

The work from Pellino *et al.*[48] is not included in Tables 1 and 2 as no histological features or variables are described. The score by Pucilowska *et al.*[40] does not discriminate acute from chronic inflammation in the lamina propria; thus we considered both neutrophilic and lymphoplasmacytic infiltration in this layer. Similarly, some scores use unspecific terms for inflammation—Shen *et al.*[30] [leucocyte infiltration], Chiorean *et al.*,[31] and Lasocki *et al.*[46] [inflammation] —in these cases, we considered both neutrophilic and lymphoplasmacytic infiltration in each layer. When 'transmural inflammation' is used—Chiorean *et al.*,[31] Maconi *et al.*,[29] Kotanagi *et al.*,[37] Lawrance *et al.*,[41] Adler *et al.*,[9] Dillman *et al.*[65]—we considered neutrophilic and lymphoplasmacytic infiltration assessment in all layers. Cooper *et al.*[45] refer to 'chronic inflammation within the bowel wall;, thus we considered lymphoplasmacytic infiltration assessment in all layers Of note, in the mucosal layer we only considered neutrophilic infiltration of the epithelium if specifically stated. Otherwise, only the lamina propria infiltration was considered. Jacene *et al.*[64] consider both acute and chronic inflammatory infiltrates which are graded according to 'extent and depth'; thus we considered both infiltrates in each layer. Similarly, Havre *et al.*[38] account for lymphoplasmacytic inflammation according to a visual semiquantitative scale; thus we considered it in each layer.

PMN, polymorphonuclear neutrophil.

actin [SMA], with identical grading of fibrosis and smooth muscle components being achieved for all three methods. The most relevant finding of this study was that muscularisation [mainly hypertrophy of the muscularis propria but also hyperplasia of smooth muscle of the submucosa] was the most prevalent histological change of the bowel wall in CD with fibrostenosis, although the magnitude of fibrosis had a less relevant impact on the stenosis itself. Moreover, such changes were also present, although to a lesser extent, in the inflamed tissue adjacent to the stenosis, suggesting that they already exist before the fibrostenotic phenotype appears. The authors further noticed that ileal lesions seem to have a higher degree of muscular changes but less fibrosis than colonic lesions.

In a case-control study, Schaeffer *et al.*[33] aimed to describe the bowel changes in resection specimens of CD patients according to their exposure to anti-tumour necrosis factor alpha [TNFα] agents. A previously described biopsy-based histological system[50] was greatly modified to allow qualitative and semiquantitative histological characterisation of both inflammatory and fibrosis components in each layer of the bowel wall [Tables 1 and 2, and Supplementary Table 1]. This scoring system showed good characteristics in distinguishing smooth muscle changes from fibrosis [Table 2]. It goes into detail in describing most of the acute inflammation features [being one of the few attentive to the presence of neutrophils in the epithelium, though not referring them in the lamina propria], as well as most of the chronic inflammation aspects, including pyloric metaplasia of the mucosa and presence of granulomas in each layer [Table 1].

Borley *et al.*[49] conducted the first specifically designed study to describe the transmural inflammatory changes of the bowel in CD; fibromuscular features were not considered in this score [Table 1]. Both macroscopic, morphometric, and microscopic items were incorporated in a dual-component inflammation scoring system, including an acute histopathological inflammation index (AHDI; or Acute Inflammation Score [AIS]) and a chronic histopathological disease index [CHDI] [Supplementary Table 3]. The first accounts for the presence of mucosal ulcerations [but not of fissuring ulcers], mucosal oedema [but not submucosal], and depth of polymorphonuclear neutrophil infiltrates from mucosa to serosa or extramural fat; however, it does not include mucosal cryptitis or crypt abscesses, nor the presence of abscesses in deeper layers [Supplementary Table 3 and Table 1]. The chronic inflammation component is unique in accounting for macrophage and lymphoplasmacytic infiltration [as lymphoid aggregates] through all the layers up to extramural fat and in assessing perineural chronic inflammation in the muscularis propria. However, it does not consider crypt architectural changes, depletion of intracellular mucin, or pyloric metaplasia of the mucosa, as it does not value eosinophilic infiltration of the different layers [Table 1 and Supplementary Table 3].

Chiorean *et al.*[31] and Adler *et al.*[9] then introduced other scoring systems that have been widely used [Supplementary Tables 1–3], both resulting from cross-imaging-pathological correlation studies. Chiorean *et al.*[31] was the first study to correlate CT enterography [CTE] findings with inflammatory activity in CD, using surgical pathology as gold standard. It included both macroscopic (adapted from the Simple Endoscopic Score for CD [SES[51]]) and microscopic features, resulting in a numerical dual scoring system with an inflammation and a fibrostenosis component [Supplementary Table 1]. The inflammation score includes both acute and chronic inflammation features, which are graded according to the size [ulcerations], extension [under or over 50%; ulcerations, cryptitis, affected surface], or depth of inflammatory infiltration [mucosa, submucosa, or transmural]. However, it does not consider the presence of granulomas.

Table 2. Histopathological transmural fibromuscular assessment

Layer	Fibromuscular feature	Scoring systems including each feature
Mucosa	Fibrosis/increased collagen deposition	Pennington *et al.*,[39] Kotanagi *et al.*,[37] Pucilowska *et al.*,[40] Maconi *et al.*,[29] Shen *et al.*,[30] Chiorean *et al.*,[31] Lawrance *et al.*,[41] Zappa *et al.*,[8] Girlich *et al.*,[32] Havre *et al.*,[38] Dillman *et al.*,[65] Chen *et al.*,[34] deBruyin *et al.*,[35] Wagner *et al.*,[43] Baumgart *et al.*[25]
	Muscularis mucosae hyperplasia	Smedh *et al.*,[28] Schaeffer *et al.*,[33] Chen *et al.*,[34] Wagner *et al.*,[43] Nylund *et al.*[44]
	Space volume expansion	Lawrance *et al.*,[41] Chen *et al.*[34]
Submucosa	Fibrosis/increased collagen deposition	Pennington *et al.*,[39] Heuman *et al.*,[36] Kotanagi *et al.*,[37] Smedh *et al.*,[28] Pucilowska *et al.*,[40] Maconi *et al.*,[29] Shen *et al.*,[30] Chiorean *et al.*,[31] Lawrance *et al.*,[41] Zappa *et al.*,[8] Girlich *et al.*,[32] Havre *et al.*,[38] Adler *et al.*,[9] Schaeffer *et al.*,[33] Dillman *et al.*,[66] Catalano *et al.*,[42] Chen *et al.*,[34] deBruyin *et al.*,[35] Wagner *et al.*,[43] Nylund *et al.*,[44] Baumgart *et al.*[25]
	Muscular hyperplasia	Chiorean *et al.*,[31] Chen *et al.*,[34] Wagner *et al.*[43]
	Fibromuscular obliteration	Schaeffer *et al.*[33]
	Neuronal hypertrophy/hyperplasia	Kotanagi *et al.*,[37] Schaeffer *et al.*,[33] Chen *et al.*[34]
	Adipocyte proliferation	Chen *et al.*[34]
	Space volume expansion	Lawrance *et al.*,[41] Chen *et al.*,[34]
Muscularis propria	Fibrosis/increased collagen deposition	Smedh *et al.*,[28] Pucilowska *et al.*,[40] Shen *et al.*,[30] Jacene *et al.*,[64] Lawrance *et al.*,[41] Girlich *et al.*,[32] Havre *et al.*,[38] Schaeffer *et al.*,[33] Catalano *et al.*,[42] Chen *et al.*,[34] deBruyin *et al.*,[35] Wagner *et al.*,[43] Nylund *et al.*,[44] Baumgart *et al.*[25]
	Loss of muscular bundles	Schaeffer *et al.*[33]
	Muscular hypertrophy	Pucilowska *et al.*,[40] Chiorean *et al.*,[31] Jacene *et al.*,[64] Adler *et al.*,[9] Chen *et al.*,[34] Wagner *et al.*,[43] Baumgart *et al.*[25]
	Neuronal hypertrophy/hyperplasia	Kotanagi *et al.*,[37] Chen *et al.*[34]
	Adipocyte proliferation	Chen *et al.*[34]
	Space volume expansion	Lawrance *et al.*,[41] Adler *et al.*,[9] Chen *et al.*,[34] Baumgart *et al.*[25]
Serosa/subserosal adventitia	Fibrosis/increased collagen deposition	Smedh *et al.*,[28] Pucilowska *et al.*,[40] Shen *et al.*,[30] Lawrance *et al.*,[41] Girlich *et al.*,[32] Havre *et al.*,[38] Adler *et al.*,[9] Catalano *et al.*,[42] Chen *et al.*,[34] Wagner *et al.*,[43] Baumgart *et al.*[25]
	Muscular hyperplasia	Chen *et al.*,[34] Wagner *et al.*[43]
	Neuronal hypertrophy/hyperplasia	Kotanagi *et al.*,[37] Chen *et al.*[34]
	Space volume expansion	Lawrance *et al.*,[41] Chen *et al.*[34]
Transmural fibrosis		Chiorean *et al.*,[31] Zappa *et al.*,[8] Adler *et al.*,[9] Dillman *et al.*,[65]
Effacement of normal layers		Chiorean *et al.*,[31] Zappa *et al.*,[8] Adler *et al.*[9]
Presence of stricture		Chiorean *et al.*,[31] Lawrance *et al.*,[41] Lasocki *et al.*[46]

The work from Pellino *et al.*[48] is not included in Tables 1 and 2 as no histological features or variables are described. The score by Pucilowska *et al.*[40] does not discriminate acute from chronic inflammation in the lamina propria; thus we considered both neutrophilic and lymphoplasmacytic infiltration in this layer. Similarly, some scores use unspecific terms for inflammation—Shen *et al.*[30] [leucocyte infiltration], Chiorean *et al.*,[31] and Lasocki *et al.*[46] [inflammation]—in these cases, we considered both neutrophilic and lymphoplasmacytic infiltration in each layer. When 'transmural inflammation' is used—Chiorean *et al.*,[31] Maconi *et al.*,[29] Kotanagi *et al.*,[37] Lawrance *et al.*,[41] Adler *et al.*,[9] Dillman *et al.*[65]—we considered neutrophilic and lymphoplasmacytic infiltration assessment in all layers. Cooper *et al.*[45] refer to 'chronic inflammation within the bowel wall'; thus we considered lymphoplasmacytic infiltration assessment in all layers Of notice, in the mucosal layer we only considered neutrophilic infiltration of the epithelium if specifically stated,; otherwise, only the lamina propria infiltration was considered. Jacene *et al.*l[64] consider both acute and chronic inflammatory infiltrates which are graded according to 'extent and depth'; thus we considered both infiltrates in each layer. Similarly, Havre *et al.*[38] account for lymphoplasmacytic inflammation according to a visual semiquantitative scale; thus we considered it in each layer. The scores by Lawrance *et al.*,[41] Girlich *et al.*,[32] Havre *et al.*,[38] deBruyin *et al.*,[35] and Wagner *et al.*[43] do not specify deposition of collagen per layer, but vaguely refer to its deposition in the bowel wall. For analysis purposes we considered collagen deposition in each of the bowel wall layers.

The fibrostenosis score accounts for submucosal fibrosis and muscular hyperplasia [under or over 25% of the submucosa], up to stricture formation. Indeed, it is one of the few referring to fibrotic effacement of normal layers leading to severe stricture [Table 1 and Supplementary Table 1]. Unfortunately, no information is given on the stains used for the histopathological studies, which are crucial when assessing fibrosis. Nevertheless, its ease of application and the ability of the numerical components to be converted into a stepwise categorisation of strictures [predominantly inflammatory, fibrostenotic, or compound] made this system the most widely used in cross-imaging-pathological correlation studies conducted afterwards [used either fully[52–55] or partially[8,9,56–58]].

Adler *et al.*[9] developed a numerical tri-component scoring system for inflammation, fibrosis, and muscle hypertrophy [the latter only at the muscularis propria]. This system is original in describing the overtaking of the deeper layers by fibrotic septa up to the effacement of normal layers [Table 2 and Supplementary Table 1]. However, its inflammation component only considers crypt distortion and an undefined 'inflammation' at the lamina propria, submucosa, or transmurally. Despite this ambiguity in defining inflammation, this system has also been used in several cross-imaging-pathological correlation studies.[10,59–61]

Some studies used or adapted previously described biopsy-based scores for the assessment of full thickness bowel wall changes due to CD, which inevitably led to incomplete study of the deeper layers. Catalano *et al.*[42] adapted an ulcerative colitis histological activity index[62] accounting only for superficial acute inflammation. Girlich *et al.*[32] developed an inflammation and fibrosis scoring system after a modification of the biopsy-based score by Bataille *et al.*,[63] where superficial inflammation features were extended to deeper layers [wall infiltration by neutrophils, lymphocytes, and eosinophils] and fibrosis was indicated as absent/present with no information

regarding its layered or transmural deposition. Finally, deBruyin *et al.*,[35] Knod *et al.*,[47] and Han *et al.*[27] all used the original biopsy-based activity score by D'Haens *et al.*[50] and thus, with the exception of granulomas, they limit inflammation assessment to the mucosal layer.

The remaining 19 systems described in Supplementary Tables 1–3 are not described in detail here, since they overlook important histological features of CD. In terms of inflammation features, some systems do not rigorously define histological acute,[28,30,40,45,46,64,65] chronic,[28–30,36,40,41,46,64,65] or transmural[29,37,65] inflammation, or only considered acute[8,26,43] or chronic[38] inflammation. As for fibromuscular changes, some scores do not include any muscular[36,37,39] or serosal changes,[36,39] and many omit smooth muscle changes[8,29,32,33,38,39,41,42,48,65] or limit muscular thickening to the muscularis mucosa[25] or muscularis propria.[64] Other systems limit the identification of fibrosis to muscularis mucosa and submucosa[29,36,37,65] or to submucosa and muscularis propria,[28,44,64] or do not address fibrous deposition by layer.[38–41] Finally, Pellino *et al.*[48] do not define any histological features to assess when scoring inflammation and fibrosis, and Lasocki *et al.*[46] include some features not related to CD.

4. Discussion

Currently, there is no transmural histological grading system for CD that is widely accepted as the best choice to grade the severity of intestinal fibrosis or inflammation.[4] The main reason to develop a transmural histopathological scoring system is to reduce subjectivity in the analysis, and although many histological scoring indices have been created, there are still limited data to validate their use. Therefore, before applying such scoring systems in clinical trials or in routine practice, it is crucial to assess each measuring instrument in detail in terms of the included items and domains, assessment forms, and operating properties of the scoring system.[18]

We agree with Chen *et al.*[34] in that most of existing transmural histopathological scores for fibrostenotic CD were developed as a part of imaging-pathology correlation studies, using exceedingly variable degrees of histological detail. The most comprehensive histopathological systems for the assessment of both transmural inflammation and fibromuscular changes due to CD were developed in purpose-built studies, such as Chen *et al.*[34] and Schaeffer *et al.*[33] This is naturally reflected in their good operating properties and also in the high methodological quality of the corresponding studies (75% and 85%, respectively[Figure 2]) which, notably, were the only ones using two independent and blinded pathologists. Although neither of these scores was used in studies conducted afterwards, it is our opinion that validation studies should be implemented. Interestingly, the system by Chen *et al.*[34] attempted to cover the full spectrum of CD pathology, ultimately identifying the relative importance of muscularisation over fibrosis in the stenosis structure, but also highlighting that such changes already exist, to a lesser extent, before stricture development. This study illustrates the physicians' still unmet need for information regarding early bowel changes due to CD, mainly concerning fibrostenosis.

Indeed, transmural studies of resected bowel strictures merely provide a snapshot of an 'end-of-the-line' phase of the fibrogenic process[40]; even the evaluation of tissue adjacent to the stricture cannot define the role of mesenchymal cells in initiation or progression of fibrosis.[40] To gain a better understanding of the cellular basis of inflammation-induced fibrosis in the bowel, a number of animal models have been developed.[66] Nevertheless, the pattern of disease in these models is more homogeneous and the phenotype of mesenchymal cells may be better correlated with the onset and progression of fibrosis[40] and, therefore, they may not entirely mimic the process leading to fibrosis in humans.[3,66] Hence, for clinical research purposes, histopathological transmural characterisation is still needed and has to be systematised through the development of adequate scoring systems.

Our systematic review revealed that the most reproduced transmural histopathological scores for CD were the ones developed by Borley *et al.*[49] for inflammation [particularly its AIS component] and Chiorean *et al.*,[31] followed by Adler *et al.*[9] for both inflammation and fibrosis. The high methodological quality of the studies which led to the development of those three scores [75% for Borley *et al.*,[49] 80% for Chiorean *et al.*,[31] and 77.5% for Adler *et al.*[9]] and the evidence of adequate assessment of validity [the extent to which a score truly measures the outcome that it is intended to assess], responsiveness [the ability to detect a meaningful change in health status], and reliability [the consistency or reproducibility] of the indexes, support their application both in future clinical trials and in routine practice. Importantly, although none of the studies formally assessed the ease with which a scoring system may be applied in a given setting [e.g., feasibility], the fact that all three scores were the most used by other authors indirectly suggests that this operating property should be high.

We recognise that using either small bowel or colon, or both, surgical specimens as a base for the development of the score may bring some inaccuracy to the score itself, as there are some histological differences between small and large bowel [for instance, pyloric metaplasia occurs mainly in the small bowel]. In our analysis, we tried to overcome this heterogeneity by deconstructing the scores into their histopathological variables [Tables 1 and 2]. However, we propose that future studies using these scoring systems should score small and large bowel separately.

One limitation of this review is the fact that it included only studies using surgical specimens as standard of reference, which has the potential to introduce spectrum bias [e.g., patients represented either 'end stage' fibrostenosing disease or suffered from penetrating complications].[67] However, this may not be regarded as a weakness if we consider that, currently, studies of surgical specimens remain the only way of assessing the entirety of transmural changes occurring in the human bowel with CD. The systematisation of the histopathological findings into a score is crucial for non-invasive assessment of fibrosis, especially if the score allows for accurate distinction between different degrees of inflammation and fibrosis which can be correlated to preoperative non-invasive means of bowel evaluation.

The strength of our systematic review relies on the objective assessment of the 29 original transmural histopathological scoring systems, through both the operating properties of each score and the assessment of the methodological quality according to the COSMIN checklist.[22–24] Furthermore, we conducted a detailed analysis of each score according to the histopathological variables included [Tables 1 and 2]. Using this triple appraisal methodology, we were able to demonstrate which of the existing scoring systems perform best and which might be more suitable for research studies or clinical practice.

5. Summary and perspectives

This study aimed to systematically review all existing transmural histopathological scoring systems for the assessment of inflammation and/or fibrosis in CD and, through the assessment of the

methodological quality of the underlying studies together with the evaluation of the operating properties of these original scores, to identify the most reliable and accurate score[s] for clinical research and for clinical practice settings.

We included 53 studies and focused on those 29 that originally described a transmural histopathological score. We further deconstructed each score according to its inflammatory or fibromuscular variables. Among the 29 original scoring systems, the most reproduced transmural histopathological scores were the scores by Borley *et al.*[49] for inflammation [namely its AIS component], and Chiorean *et al.*[31] and Adler *et al.*[9] for both inflammation and fibrosis, probably due to their ease of application in clinical studies. The high methodological quality of the studies [75%, 80%, and 77.5%, respectively] and adequate operating properties [validity, reliability, and responsiveness] of the scoring systems make them suitable for use in clinical practice and also in population-based observational studies or in interventional studies. There are, to our knowledge, no clinical trials in which a transmural histopathological scoring system is used as a means of evaluating an outcome [e.g., intestinal resection]. However, the arrival of specific anti-fibrotic agents will demand a high-quality scoring system, sensitive to fine inflammation and fibromuscular changes, to be integrated in the near future into clinical trials. Either the scoring system by Chen *et al.*[34] or that by Schaeffer *et al.*[33] could fulfill these requirements.

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Conflict of Interest

The authors have no competing interests to report.

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Author Contributions

HTS: planned and conducted the study, collected and critically interpreted the data, drafted the manuscript, and approved the final draft submitted. MME: planned and conducted the study, collected and critically interpreted the data, drafted the manuscript, and approved the final draft submitted. LP-B: critically interpreted the data and approved the final draft submitted. SD: critically interpreted the data and approved the final draft submitted. CCD: collected and critically interpreted the data, drafted the manuscript, and approved the final draft submitted. FC: planned the study, critically interpreted the data, drafted the manuscript, and approved the final draft submitted. FM: planned and conducted the study, critically interpreted the data, drafted the manuscript, and approved the final draft submitted.

Supplementary Data

Supplementary data are available at *ECCO-JCC* online.

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Supplementary Table 1. Inflammation and Fibrosis Scoring Systems.

Author Year	Type of Score	Categories / Scoring and key features	Type of study. Aim requiring development of a score	Pathologists involved (n). Independency (if ≥2). Blindness to clinical/imaging of patients.	Patient number /Control group (n)	Histological stains. Morphometry	Surgical specimen	Subsequent applications
Pennington et al[39] 1980	Stepwise	• **Category I: No histological evidence of CD** • **Category II: changes of chronic inflammation.** Increased numbers of lymphocytes and plasma cells enlarging the lamina propria, occasionally involving the submucosa. Possible findings of lymphoid hyperplasia, foci of pyloric metaplasia of crypts and fibrosis. Possible presence of granulomas. • **Category III: findings of acute and chronic inflammation**. Polymorphonuclear leucocytes in the epithelium, lamina propria and sometimes in the submucosa. Chronic inflammation and other features present in category II. Possible presence of granulomas. • **Category IV: tissue destruction.** Multiple crypt abscesses, mucosa erosions, and ulcers – in addition to features present in category III. Possible presence of granulomas.	Retrospective. Influence of microscopic disease at the margins of resection on the incidence of suture line complications immediately following intestinal resection for CD and on long-term recurrence of disease.	1 Not reported.	97/-	Not reported. Not used.	Ileum Colon (proximal and distal margins)	
Heuman et al[36] 1983	Stepwise	• **Group I:** no histological changes or slight increase of mononuclear cells in the mucosa. • **Group II:** acute and chronic inflammation in the mucosa, crypt abscesses, lymphangitis and fibrosis in the submucosa. • **Group III:** ulceration of the mucosa and/or granulomas at different levels of the intestinal wall; chronic inflammation and fibrosis in the intestinal wall, mainly in the submucosa.	Retrospective. Influence of microscopic disease at the margins of resection on recurrence rate of CD treated with intestinal resection surgery	1 No.	67/ -	Not reported. Not used.	Ileum Colon (proximal and distal margins)	
Kotanagi et al[37] 1991	Stepwise	• **Category I: histologically normal** • **Category II: nonspecific changes** (increased mucosal chronic inflammatory cells, villous shortening, mild patchy cryptitis, rare crypt abscesses, patchy mild acute inflammatory cells in the lamina propria, mucosal oedema) • **Category III: changes suggestive of but not diagnostic for CD** (focal ulcers, erosions, pyloric gland metaplasia, multiple or extensive areas of cryptitis, crypt abscesses) • **Category IV: changes diagnostic for CD** (any of the above features plus non-necrotizing granulomas or transmural lymphoid aggregates)	Retrospective. Influence of microscopic disease at the margins of resection on anastomotic recurrence rate of CD treated with intestinal resection surgery	1 No.	100/ -	Not reported. Not used.	Ileum Colon (proximal and distal margins)	Fazio et al[69]

Author Year	Type of Score	Categories / Scoring and key features	Type of study. Aim requiring development of a score	Pathologists involved (n). Independency (if ≥2). Blindness to clinical/imaging of patients.	Patient number /Control group (n)	Histological stains. Morphometry	Surgical specimen	Subsequent applications
	Numerical	**Semi quantitative system** 0 – absent 1 – mildly or focally present 2 – diffusely or markedly present **Histologic features:** • Mucosa/submucosa oedema • Neutrophils in mucosa/submucosa • Mucosal chronic inflammatory cells • Lymphoid aggregates • Pyloric gland metaplasia • Fibrosis of muscularis mucosae/submucosa • Cryptitis/crypt abscesses • Mucosal erosions and ulcers • Fissural ulcers • Granuloma • Shortening of intestinal villi • Depletion of intracellular mucin • Neuronal Hyperplasia • Transmural inflammation						
Smedh et al[28] 1995	Stepwise and Numerical	**Categories: absent/present** Severity assessment grading: **0-100 visual analogue scale** (**mean**) **Histologic features:** • Villous atrophy • Epithelial leucocyte infiltration • Pyloric metaplasia • Ulcers • Lamina propria leucocyte infiltration • Muscularis mucosae thickened • Submucosal oedema • Submucosal leucocyte infiltration • Submucosal fibrosis • Submucosal lymphoid aggregates • Submucosal dilated lymph vessels • Muscularis propria leucocyte infiltration • Muscularis propria fibrosis • Subserosal leucocyte infiltration • Subserosal fibrosis • Granuloma • Fissures	Prospective. Correlation between intraoperative endoscopic findings and external lesions of the bowel at laparotomy with transmural histopathology. Influence of previous surgery and septic complications on external changes and transmural histopathology	1. Blinded	23/10	H&E. Not used.	Ileum Colon	
Pucilowska et al[40] 2000	Numerical	**Semiquantitative system** 0 – normal 1 – mild abnormality 2 – severe abnormality **Histologic features:**	Retrospective. To assess whether there were qualitative or quantitative differences in mesenchymal cell subtypes in diseased/fibrotic intestine of	Not reported	13/11	H&E Masson Trichrome Sirius Red Immunohistochemistry.	Ileum Colon	

Author Year	Type of Score	Categories / Scoring and key features	Type of study. Aim requiring development of a score	Pathologists involved (n). Independency (if ≥2). Blindness to clinical/imaging of patients.	Patient number /Control group (n)	Histological stains. Morphometry	Surgical specimen	Subsequent applications
		• Surface epithelial damage • Lamina propria inflammation • Thickness of muscularis propria • Fibrosis **Total histology score = sum of values per bowel layer**	patients with CD relative to uninvolved or normal intestine			Not used.		
Maconi et al[29] 2003 (modification from Fazio et al[69], which used Kotanagi et al[37] score)	Numerical	**Semiquantitative system** 0 – absent 1 – mild 2 – moderate 3 – severe **Histologic features:** • Mucosal/submucosal oedema • Chronic inflammatory infiltrate • Interstitial and intraepithelial neutrophil infiltrate • Crypt abscesses • Mucosal erosions • Wall ulcers • Lymphoid aggregates • Fibrosis of muscularis mucosae/submucosa **Binary system** 0 – absent 1 – present **Histologic features:** • Fissural ulcers • Granuloma • Transmural inflammation	Cross-sectional. To correlate the echo pattern at the stenosis with the histological features in a series of patients with CD undergoing surgery for a single ileal stenosis	2 Not independent Blinded	43/-	H&E Not used.	Ileum	
Shen et al[30] 2003	Stepwise	**Categories = absent/present per layer:** • Mucosa • Muscularis mucosa • Superficial submucosa • Deep submucosa • Muscularis propria • Serosa **Histologic features:** • Leucocyte infiltration • Lymphoid aggregates • Granulomas • Fibrosis	Cross-sectional, case-control, To establish histology correlated Optical Coherence Tomography (OCT) imaging criteria for transmural CD	1 Blinded	24/24	H&E Not used.	Colon	

Author Year	Type of Score	Categories / Scoring and key features	Type of study. Aim requiring development of a score	Pathologists involved (n). Independency (if ≥2). Blindness to clinical/imaging of patients.	Patient number /Control group (n)	Histological stains. Morphometry	Surgical specimen	Subsequent applications
Chiorean et al[31] 2007	Numerical	**Inflammation score** **0 – none** **1 – mild** (aphthous ulcers affected surface <50%; cryptitis <50%; inflammation limited to mucosa) **2 – moderate** (0.5-2cm and/or superficial ulcers; ulcerated surface <50%; affected surface 50-100%; cryptitis >50%; crypt abscess; submucosal inflammation) **3 – severe** (deep or >2cm ulcers; circumferential ulceration; transmural inflammation; deep fissures) **Inflammation features (macroscopic and microscopic)** • Erosions or ulcerations • Mucosal and submucosal inflammation • Cryptitis • Polymorphonuclear and mononuclear inflammatory infiltrate • Lymphadenopathy	Retrospective. To determine the accuracy of CT enteroclysis (CTE) compared to surgical pathology in patients with CD. To identify CTE variables that correlate with inflammatory or fibrostenotic pathological lesions	1 Blinded	44/-	Not reported# Not used.	Ileum Colon	Rimola et al[53] Pous-Serrano et al[54] Pous-Serrano et al[55] Rosenbaum et al[56] Punwani et al[57] Zappa et al[8] Quaia et al[58] Tielbeek et al[59] Ripollés et al[70] Wilkens et al[71] Fraquelli et al[72] Sinha et al[73] Serra et al[74]
		Fibrostenosis score **0 – none** (no or minimal fibrosis limited to submucosa (<25% thickness) **1 – mild/moderate** (mild stricture (>15mm) with non-dilated lumen; submucosal fibrosis and muscular hyperplasia >25% with preserved layers) **2 – severe** (Massive transmural fibrosis; effacement of normal layers; severe stricture) **Fibrostenosis features** • Fibrosis • Muscular hyperplasia • Strictures						
	Stepwise	**Categories:** • **Predominantly inflammatory:** inflammation score >1 with fibrostenosis score ≤1 • **Predominantly fibrostenotic:** fibrostenosis score >1 with inflammation score ≤1 • **Compound lesions:** difference between inflammation and fibrostenosis scores ≤1						
Jacene et al[65] 2009	Numerical	**Inflammation score** 0 – none 1 – mild 2 – moderate 3 – severe **Histologic features** • Acute neutrophilic infiltration – extent and depth • Chronic lymphoplasmacytic infiltration – extent and depth • Bowel-wall thickening	Prospective. To preoperatively determine the accuracy of 18f-FDG PET/CT for differentiating fixed muscle hypertrophy and fibrotic stenoses from acute transmural inflammatory stenoses in patients with CD	1 of 3 Not reported. Blinded to PET/CT data.	6/-	H&E Masson Trichrome	Ileon	

Author Year	Type of Score	Categories / Scoring and key features	Type of study. Aim requiring development of a score	Pathologists involved (n). Independency (if ≥2). Blindness to clinical/imaging of patients.	Patient number /Control group (n)	Histological stains. Morphometry	Surgical specimen	Subsequent applications
		Fibrosis score • % of fibrosis involving the muscularis propria - increments of 10% according to density, intensity, extent of involvement **Muscle hypertrophy score** • Ratio between the diameter of thickest hypertrophied muscularis propria and the normal muscularis propria diameter	scheduled for surgery for obstructive symptoms.					
	Stepwise	**Categories (subtypes)** Predominantly inflammation Predominantly fibrosis Predominantly hypertrophy						
Lawrance et al[41] 2009	Stepwise	**Categories:** • **Mild inflammation:** mild inflammatory infiltrates with scattered architectural distortion and crypt branching • **Severe inflammation:** mucosal ulceration with marked glandular changes, dilated complex crypts, transmural mixed cellular infiltrate and reactive changes in the mesentery • **Fibrosis:** presence of stricture, bowel wall thickness and collagen deposition in the bowel wall **Histologic features:** • Bowel wall thickness • Mesentery hyperhemia • Mesentery fatty proliferation • Mesenteric lymph nodes • Mucosal ulceration • Density of acute inflammatory cell infiltrate • Density of chronic inflammatory cell infiltrate • Loss of goblet cells • Crypt abscesses • Architectural distortion • Presence of stricture • Collagen deposition in the bowel wall	Retrospective. To correlate small bowel (SB) enhancement and wall thickness on MRI with the presence and level of inflammation and fibrosis on intestinal surgical pathology of patients with CD	Not reported	17/-	Not reported Not used.	Small bowel	
Zappa et al[8] 2011 (Inflammation score was adapted from Borley et al[49] score)	Numerical	**Inflammation score** **Grade 1 – mild or nonactive CD** – minimal neutrophil infiltrate limited to the mucosa **Grade 2 – moderately active CD -** neutrophil infiltrate limited to the mucosa and submucosa without muscular involvement **Grade 3 – severely active CD** – transmural neutrophil infiltrate through the muscularis propria and/or fistula and/or abscesses I the subserosa **Fibrosis score** **Grade 0 –** minimal fibrosis limited to submucosa **Grade 1** – massive submucosal fibrosis with preserved layers) **Grade 2** – massive transmural fibrosis with effacement of normal layers	To evaluate the value of small bowel (SB) MRI findings in CD in correlation with pathological inflammation score using surgical pathology as reference standard	1 Blinded	53/-	H&E Not used.	Small bowel	
Girlich et al[32] 2011	Numerical	**Mucosal surface** — Excess of intra-epithelial neutrophils: 0 – none; 1 – few; 2 – excessive	Prospective. To compare CEUS perfusion patterns and specific perfusion	1 Blinded.	20/-	Not reported Not used.	Not reported	Baumgart et al[25]

Author Year	Type of Score	Categories / Scoring and key features			Type of study. Aim requiring development of a score	Pathologists involved (n). Independency (if ≥2). Blindness to clinical/imaging of patients.	Patient number /Control group (n)	Histological stains. Morphometry	Surgical specimen	Subsequent applications
(adapted from biopsy-based score from Bataille et al[64])			Excess of intra-epithelial eosinophils	0 – none 1 – few 2 – excessive	quantification software with transmural histological findings in CD surgical specimen applying an advanced histopathological scoring system					
			Excess of intra-epithelial lymphocytes	0 – none 1 – few 2 – excessive						
			Reduction of goblet cells	0 – none 1 – few 2 – excessive						
			Erosion	0 – none 1 – few 2 – excessive						
		Depth of wall infiltration	Neutrophils	0 – none 1 – mucosa 2 – submucosa 3 – muscularis propria 4 – serosa/extramural fat						
			Eosinophils	0 – none 1 – mucosa 2 – submucosa 3 – muscularis propria 4 – serosa/extramural fat						
			Lymphocytes	0 – none 1 – mucosa 2 – submucosa 3 – muscularis propria 4 – serosa/extramural fat						
		Density of wall infiltration	Neutrophils	0 – none 1 – few 2 – excessive						
			Eosinophils	0 – none 1 – few 2 – excessive						
			Lymphocytes	0 – none 1 – few 2 – excessive						
		Pseudopolyps		0 – absent 1 – present						
		Regenerative epithelium		0 – absent 1 – present						
		Crypt architecture		0 – normal 1 – distorted						
		Crypt abscess		0 – absent 1 – present						
		Crypt atrophy		0 – absent 1 – present						
		Fibrosis		0 – absent 1 – present						
		Edema		0 – absent 1 – present						
		Hemorrhage		0 – absent						

Author Year	Type of Score	Categories / Scoring and key features	Type of study. Aim requiring development of a score	Pathologists involved (n). Independency (if ≥2). Blindness to clinical/imaging of patients.	Patient number /Control group (n)	Histological stains. Morphometry	Surgical specimen	Subsequent applications
		<table><tr><td></td><td>1 – present</td></tr><tr><td>Epithelioid granulomas</td><td>0 – absent 1 – present</td></tr></table>						
Havre et al[38] 2011	Numerical	**Semiquantitative system (visual evaluation)** 0 – absent 1 – slight 2 – moderate 3 – severe **Histologic features:** • Lymphoplasmacytic inflammation • Fibrosis	Prospective. To correlate elastography results to a histological semi-quantification of fibrosis, inflammation parameters and tumour description	1 No.	9/19	H&E Not used.	Not reported	
Adler et al[9] 2012 (adapted from Maconi et al[29] and Chiorean et al[31] scores, together with Theiss et al[75] murine score)	Numerical	**Inflammation grades** **0** – no inflammation or distortion **1** – lamina propria inflammation only **2** – submucosal foci of inflammation **3** – foci of transmural inflammation **4** – significant, dissecting, confluent transmural inflammation **Muscle hypertrophy** **0** – normal thickness **1** – increased thickness of muscular propria layer **Fibrosis grades** **0 –** no fibrosis **1 –** minimal fibrosis in submucosa or subserosa **2** – minimal submucosal fibrosis, septa into muscularis propria **3** – septa through muscularis propria, increase in subserosal collagen **4** – significant transmural scar, marked subserosal collagen	Retrospective. To identify the specific CTE correlates of strictures using histology as reference standard. To identify the CTE findings that distinguish inflammation from fibrosis in small bowel strictures in CD	2 Not reported. Blinded	22/-	H&E Not used.	Small bowel	Barkmeier et al[10] Huang et al[60] Xue-hua et al[76] Chen et al[62]
Schaeffer et al[33] 2014	Numerical	<table><tr><th>Layer</th><th>Abnormality</th><th>Grade</th></tr><tr><td rowspan="6"><u>Mucosa</u></td><td>Epithelial changes</td><td>0 – normal 1 – focal attenuation 2 – extensive attenuation</td></tr><tr><td>Architectural changes</td><td>0 – normal 1 – <10% disturbed 2 – 10-50% disturbed 3 - >50% disturbed</td></tr><tr><td>Mononuclear cells infiltrate in lamina propria</td><td>0 – normal 1 - moderate increase(<2x) 2 – severe increase(>2x)</td></tr><tr><td>Pyloric metaplasia</td><td>0 – absent 1 – present</td></tr><tr><td>Duplication of muscularis mucosa</td><td>0 – absent 1 – present</td></tr><tr><td>Neutrophils in epithelium</td><td>0 – in surface epithelium 1 – Cryptitis</td></tr></table>	Cross-sectional, case-control. To document the spectrum of histomorphologic changes in resection specimens of CD patients treated with anti-TNFα agents	2 Independent. Blinded.	62/80	H&E Masson Trichrome Immunohistochemistry. Not used.	Ileum Colon	

Author Year	Type of Score	Categories / Scoring and key features			Type of study. Aim requiring development of a score	Pathologists involved (n). Independency (if ≥2). Blindness to clinical/imaging of patients.	Patient number /Control group (n)	Histological stains. Morphometry	Surgical specimen	Subsequent applications
				2 – Crypt abscess						
			Extent of inflammation	0 – none 1 – ≤33% 2 – 33-66% 3 - ≥66%						
			Granulomata	0 – none 1 – Histiocytic aggregates 2 – Well formed						
			Erosions/ulcers	0 – absent 1 – present						
		Sub mucosa	**Mononuclear infiltrate**	0 – normal 1 - moderate increase(<2x) 2 – severe increase(>2x)						
			Neutrophilic infiltrate	0 – normal 1 - moderate increase(<2x) 2 – severe increase(>2x)						
			Neuronal hyperplasia	0 – none 1 – mild 2 – marked						
			Granulomata	0 – none 1 – Histiocytic aggregates 2 – Well formed						
			Fibrosis	0 – none 1 - patchy(<50%) 2 - diffuse(<50%)						
			Fibromuscular obliteration	0 – none 1 - patchy(<50%) 2 - diffuse(<50%)						
		Muscular propria	**Mononuclear infiltrate**	0 – normal 1 - moderate increase(<2x) 2 – severe increase(>2x)						
			Neutrophilic infiltrate	0 – normal 1 - moderate increase(<2x) 2 – severe increase(>2x)						
			Fibrosis	0 – none 1 - patchy(<50%) 2 - diffuse(<50%)						
			Loss of muscle bundles	0 – none 1 - patchy(<50%) 2 - diffuse(<50%)						
			Granulomata	0 – none 1 – Histiocytic aggregates 2 – Well formed						
		Serosa	**Mononuclear infiltrate**	0 – normal 1 - moderate increase(<2x) 2 – severe increase(>2x)						
			Granulomata	0 – none 1 – Histiocytic aggregates 2 – Well formed						
			Peritonitis	0 – absent 1 – present						
		All layers	**Lymphoid aggregates**	0 – absent 1 – rare (≤2/LPF/x40) 2 – frequent (>2/LPF,x40)						

Author Year	Type of Score	Categories / Scoring and key features			Type of study. Aim requiring development of a score	Pathologists involved (n). Independency (if ≥2). Blindness to clinical/imaging of patients.	Patient number /Control group (n)	Histological stains. Morphometry	Surgical specimen	Subsequent applications
			Fissuring Ulcers	0 – none 1 – into mucosa 2 – into submucosa 3 – into muscular propria 4 – into serosa 5 – fistula						
Dillman et al[66] 2014	Numerical	**Inflammation score** 0 (none) – No inflammation 1 – Low level of inflammation with scattered infiltrating mononuclear cells 2 – Moderate inflammation with multiple foci 3 – High level of inflammation with increased vascular density and marked wall thickening 4 (severe) – Maximal severity of inflammation with transmural leucocyte infiltration and loss of goblet cells **Fibrosis score** 0 (none) – No architectural distortion, no abnormal Masson trichrome staining 1 – No architectural distortion, mild abnormal Masson trichrome staining in mucosa/submucosa 2 – Substantial abnormal mucosal/submucosal Masson trichrome staining with modest distortion of architecture but without obscuration of the mucosal/submucosal border 3 (severe) – Transmural fibrosis with abnormal Masson trichrome staining in all histologic layers, transmural architectural distortion			Cross-sectional. To determine wether bowel wall fibrosis can be detected in surgically resected human intestinal specimens based on ex-vivo intramural shear wave speed measurements.	1 Not reported	12/-	H&E Masson Trichrome Not used.	Small bowel Colon	
Pellino et al[48] 2016	Numerical	**Inflammation score** 0 – none/mild 1 – moderate 2 – severe **Fibrosis score** 0 – none/mild 1 – moderate 2 – severe			Prospective. To access the effectiveness of PET/MR-enterography compared to PET/CT-enterography in evaluating small-bowel CD and determining its relevance on clinical management of patients with CD	Not reported. Not reported. Blinded.	29/-	Not reported[##]	Small bowel	
	Stepwise	**Categories (subtypes)** Prevalently inflammatory Prevalently fibrotic								
Catalano et al[42] 2016	Stepwise	**Active inflammation** • Positive – presence of one of the features • Negative – none of the features **Histologic features:** • mucosal neutrophil infiltration • erosions **Intestinal fibrosis** • Positive – at least moderate fibrosis • Negative – none of the features **Histologic features:** • Moderate fibrosis = fibrosis involving the submucosa or deeper layers			Retrospective. To evaluate the added value of FDG-PET to the ability of MRE in distinguishing fibrotic from inflammatory strictures in patients with CD	Not reported	19/-	H&E Masson Trichrome Not used.	Not reported	

Author Year	Type of Score	Categories / Scoring and key features			Type of study. Aim requiring development of a score	Pathologists involved (n). Independency (if ≥2). Blindness to clinical/imaging of patients.	Patient number /Control group (n)	Histological stains. Morphometry	Surgical specimen	Subsequent applications
Chen et al[34] 2017	Numerical	**Layer**	**Category** (number of histologic components)	**Grading** (for each histologic component) 0 normal 1 mild 2 moderate 3 severe	Retrospective. To describe the full spectrum of histological changes in each layer of bowel wall and the association between these changes. To create a histologic system to clearly illustrate the histologic-endoscopic-radiologic correlation	2 Independent. Blinded	48/-	H&E Masson Trichrome Immunohistochemistry Manual Morphometry	Ileum Colon	
		Mucosa	Active inflammation (5)							
			Chronic inflammation (4)							
			Fibrosis (extent) (1)							
			Muscular hyperplasia (2)							
			Space volume expansion (1)							
		Sub mucosa	Active inflammation (1)							
			Involved by fissuring ulcer (2)							
			Chronic inflammation (3)							
			Fibrosis (extent) (1)							
			Muscular hyperplasia (2)							
			Adipocyte proliferation (1)							
			Neuronal hypertrophy (1)							
			Space volume expansion (1)							
		Muscular propria	Active inflammation (3)							
			Chronic inflammation (3)							
			Fibrosis (extent) (1)							
			Muscular hypertrophy (2)							
			Neuronal hypertrophy (1)							
			Adipocyte proliferation (1)							
			Space volume expansion (1)							
		Subserosal adventitia	Active inflammation (3)							
			Chronic inflammation (3)							
			Fibrosis (extent) (1)							
			Muscular hyperplasia (1)							
			Neuronal hypertrophy (1)							
			Space volume expansion (1)							
deBruyin et al[35] 2017 #	Numerical and stepwise	**Inflammation variable**		**Grade**	Retrospective. To compare histologically ileal resection specimens from infliximab-naïve CD patients with those from patients operated after an inadequate response to infliximab	1 Blinded	20/20	H&E Immunohistochemistry Digital morphometry [Image J analysis software]	Ileum Colon	
		Epithelial damage		0 – normal 1 – focal 2 – extensive						
		Architectural changes		0 – normal 1 – moderate 2 – severe						
		Mononuclear cells in lamina propria		0 – normal 1 – moderate increase 2 – severe increase						
		Polymorphonuclear cells in lamina propria		0 – normal 1 – moderate increase 2 – severe increase						
		Neutrophils in epithelium		1 – surface epithelium 2 – cryptitis 3 – crypt abscess						
		Erosions or ulceration		0 – no 1 – yes						
		Granuloma		0 – no 1 – yes						

Author Year	Type of Score	Categories / Scoring and key features		Type of study. Aim requiring development of a score	Pathologists involved (n). Independency (if ≥2). Blindness to clinical/imaging of patients.	Patient number /Control group (n)	Histological stains. Morphometry	Surgical specimen	Subsequent applications
		Fibrosis grading (immunohistochemistry)							
		Minimal staining (background = normal) Moderate Strong							
Wagner et al[43] 2018	Numerical and stepwise	**Inflammation variable**	**Grade**	Retrospective. To assess the value of MRI including DWI for the characterization of histopathologic tissue composition of small bowel CD.	2 (1 reviewed sections, 1 performed morphometry) Not reported. Not reported.	35	H&E Sirius Red Immunohistochemistry Digital morphometry [HALO software]	Ileum	
		Active inflammation (depth of neutrophil infiltrate)	1 – none or mucosa only						
			2 –submucosa						
			3 – muscularis propria/ subserosa/serosa						
		Bowel wall oedema	1 – no or minimal oedema						
			2 – obvious oedema						
		Fibrosis grading (morphometry)							
		Ratio: normalized smooth muscle actin / normalized Sirius Red	< 1 = disproportionately increased fibrosis						
			> 1 = disproportionately increased muscular hypertrophy						

#The inflammation component of the score is similar to the biopsy-based previously described by D'Haens et al,[51] excluding the number of taken biopsies issue.

##H&E staining is seen on images, but stains are not reported in text.

Supplementary Table 2. Fibrosis only Scoring Systems.

Author Year	Type of Score	Categories / Scoring and key features		Type of study. Aim requiring development of a score	Pathologists involved (n). Independency (if ≥2). Blindness to clinical/imaging of patients.	Patient number /Control group (n)	Histological stains Morphometry	Surgical specimen	Subsequent applications
Nylund et al[44] 2008	Numerical	**Layer/feature**	**Grade**	Cross-sectional. To correlate *in vitro* high-frequency US findings of the muscularis mucosae, fibrosis and "Crohn's rosary" with matched surgical histology	2 Not reported. Not reported.	13/-	H&E Masson Trichrome. Digital morphometry [analySIS software]	Ileum Colon	Nylund et al[77] Nylund et al[78]
		Muscularis mucosa	0 – missing or <0.3mm 1 – thickened ≥0.3mm						
		Submucosa	0 – normal 1 – slight to moderate fibrosis - increase of loosely arrayed collagen fibres, not filling the whole SM, often with some fat tissue present 2 – severe fibrosis - densely packed collagen fibres filling the whole layer, no fatty tissue seen						
		Muscular propria	0 – normal 1 – slight to moderate fibrosis - increase deposition of collagen fibres between the muscular bundles 2 – severe fibrosis - muscular bundles reduction in size, destruction of layer's architecture, with difficult to identify borders						
		"Crohn's rosary"	0 – not present or <0.3mm 1 – present and ≥0.3mm - nodular lymphocyte aggregates with a diameter ≥0.3mm, localized at the outer border of MP						
Baumgart et al[25] 2015	Numerical	0 – no increased collagen deposition 1 – increased collagen deposition in submucosa 2 – increased collagen deposition in mucosa and submucosa 3 – increased collagen deposition in mucosa, muscularis mucosa and submucosa and thickening and disorganization of the muscularis mucosa 4 – increased collagen deposition in mucosa, muscularis mucosa, submucosa and muscularis propria 5 – increased collagen throughout all layers including serosa		Prospective. To assess if US-based real time elastography (RTE) can detect gut fibrosis	1 Blinded	10/-	H&E Masson Trichrome Bouin Solution Elastica-van Gieson Digital morphometry [Cell D software]	Ileum Colon	Wilkens et al[71]

Table 3. Inflammation only Scoring Systems.

Author Year	Type of Score	Categories / Scoring and key features	Type of study. Aim requiring development of a score	Pathologists involved (n). Independency (if ≥2). Blindness to clinical/imaging of patients.	Patient number /Control group (n)	Histological stains Morphometry	Surgical specimen	Subsequent applications
Cooper et al[45] 1986	Stepwise	• **Grade A** – normal • **Grade B** – Chronic inflammation (excessive lymphocytes, plasma cells and histiocytes within the bowel wall) • **Grade C** – More severe inflammation (fissures, ulcerations and granulomas)	Retrospective. To assess if the presence of microscopic disease at the margins of resection significantly increases the risk of recurrent CD	Not reported	142/-	H&E Not used.	Ileum Colon	
Borley et al[49] 2000	Numerical	**Grade** **Histologic feature** / **0** / **1** / **2** / **3** / **4** **AHDI** – **Mucosal ulceration**: none / aphthous ulceration only, <7mm diameter / Linear or rake ulceration / confluent or large ulceration / - **AHDI** – **Oedema**: none / mild / moderate / severe / - **AHDI** – **Depth of neutrophil penetration**: none / mucosa / submucosa / muscularis propria / serosa or extramural fat **CHDI** – **Corrected lymphoid aggregate score**: 1st-5th centile / 6th -26th centile / 26th-50th centile / 51th-75th centile / >76th centile **CHDI** – **Depth of lymphoid aggregate penetration**: none / mucosa / submucosa / muscularis propria / serosa or extramural fat **CHDI** – **Corrected granuloma score**: 1st-5th centile / 6th -26th centile / 26th-50th centile / 51th-75th centile / >76th centile **CHDI** – **Wall thickness score**: <2 SD above mean controls / 2-4 SD above mean controls / 4-6 SD above mean controls / 6-8 SD above mean controls / >8 SD above mean controls **CHDI** – **Perineural chronic**: none / mild / moderate / severe / -	Prospective case-control. To investigate in detail the patterns of and the relations between, gross serosal connective tissue changes and a range of inflammatory changes in ileal resection specimens of CD. To study the extent to which the histopathological features of individual blocks from diseased areas are representative of the resection specimen as a whole	Not reported	20/20	H&E Manual morphometry. [Adobe Photoshop v4.0 software]	Ileum	Punwani et al[57] Zappa et al[8] Tielbeek et al[59] Ripollés et al[70] Wilkens et al[71] Ziech et al[79] Steward et al[80] Makanyanga et al[81] Quaia et al[58] Borley et al[82]

Author Year	Type of Score	Categories / Scoring and key features							Type of study. Aim requiring development of a score	Pathologists involved (n). Independency (if >2). Blindness to clinical/imaging of patients.	Patient number /Control group (n)	Histological stains Morphometry	Surgical specimen	Subsequent applications
			inflammation score											
			Macrophage infiltrate score	none or occasional mucosal cells	mild-moderate, mainly mucosal	Moderate confined to submucosa	Extensive transmural	-						
Lasocki et al[46] 2011	Numerical	**Bowel is divided in 9 segments:** • Small bowel proximal to the terminal ileum • Terminal ileum (distal 10 cm of ileum) • Ileo-caecal valve • Caecum • Ascending colon • Transverse colon • Descending colon • Sigmoid colon • Rectum **Semiquantitative system per segment** 0 – normal 1 – inactive inflammation 2 – early mucosal inflammation (aphtous ulceration) 3 – established mucosal inflammation (fissuring ulceration) 4 – submucosal inflammation 5 – inflammation through muscularis propria **Other histologic features:** • Length of the diseased segment(s) • Presence of additional CD features (strictures, fistulae, granulomas) • Inflammation not related to CD • Diverticulosis (possible confounder)							Retrospective. To determine the sensitivity, specificity, PPV and NNP for a range of MRI signs for predicting the histological status of small and large bowel in CD	Not reported	12	Not reported Not used.	Ileum Colon	
Knod et al[47] 2016[#]	Numerical	**Histological variable**	**Grade**						Retrospective, case-control. To explore the role of angiogenesis (as a component of chronic inflammation) in paediatric CD	1 Not reported.	13/7	H&E Immunohistochemistry Digital morphometry [NIS-Advanced	Ileum	
		Epithelial damage	0 – normal 1 – focal 2 – extensive											
		Architectural changes	0 – normal 1 – moderate 2 – severe											
		Mononuclear cells in lamina propria	0 – normal 1 – moderate increase 2 – severe increase											
		Polymorphonuclear cells in lamina propria	0 – normal											

Author Year	Type of Score	Categories / Scoring and key features		Type of study. Aim requiring development of a score	Pathologists involved (n). Independency (if >2). Blindness to clinical/imaging of patients.	Patient number /Control group (n)	Histological stains Morphometry	Surgical specimen	Subsequent applications
			1 – moderate increase 2 – severe increase				Elements software and ImageJ software]		
		Neutrophils in epithelium	1 – surface epithelium 2 – cryptitis 3 – crypt abscess						
		Erosions or ulceration	0 – no 1 – yes						
		Granuloma	0 – no 1 – yes						
Paquet et al[26] 2016	Numerical	**0 – absence of inflammation** **1 – mild inflammation** (neutrophil infiltrate limited to mucosa) **2 – moderate inflammation** (neutrophil infiltrate limited to mucosa and submucosa) **3 – high inflammation** (transmural neutrophil infiltrate affecting the muscularis propria or fistula/abscess of the subserosa		Retrospective. To explore the potential value of CT morphologic pattern as predictor of inflammatory activity in CD	1 Blinded	42/-	H&E Not used.	Not reported	
Han et al[27] 2017#	Numerical	**Histological variable**	**Grade**	Retrospective. To correlate the level of nuclear factor (NF-kB) activation with histological changes and course and outcome of CD patients	1 Blinded	83/-	H&E Immunohistochemistry Digital Morphometry [Aperio ImageScope software and Aperio nuclear IHC algorithms]	Ileum Colon	
		Epithelial damage	0 – normal 1 – focal 2 – extensive						
		Architectural changes	0 – normal 1 – moderate 2 – severe						
		Mononuclear cells in lamina propria	0 – normal 1 – moderate increase 2 – severe increase						
		Polymorphonuclear cells in lamina propria	0 – normal 1 – moderate increase 2 – severe increase						
		Neutrophils in epithelium	1 – surface epithelium 2 – cryptitis 3 – crypt abscess						
		Erosions or ulceration	0 – no						

Author Year	Type of Score	Categories / Scoring and key features		Type of study. Aim requiring development of a score	Pathologists involved (n). Independency (if >2). Blindness to clinical/imaging of patients.	Patient number /Control group (n)	Histological stains Morphometry	Surgical specimen	Subsequent applications
			1 – yes						
		Granuloma	0 – no 1 – yes						

[#] The score used is the previously described biopsy-based score by D'Haens et al,[51].

CHAPTER 4 – Ileal Crohn's Disease Exhibits Similar Transmural Fibrosis Irrespective of Phenotype

4. Ileal Crohn's Disease Exhibits Similar Transmural Fibrosis Irrespective of Phenotype

The second main objective of this thesis was to characterize and quantify inflammation and fibrosis in ileal CD resection specimens, according to a specific CD transmural histopathological scoring system, retrieved from this thesis' first study, presented herein on Chapter 3. The surgical resection specimens should include stricturing or penetrating CD patients, as per Montreal phenotype classification. Aditionnaly, histopathological scorings were to be correlated with previously described relevant clinical outcomes.

To accomplish this objective a double-blinded histopathologic analysis of 103 archived penetrating and stricturing CD ileal surgical specimens was conducted, on 29 stricturing (B2) and 74 penetrating (B3) cases – the latter including 54 cases with associated stricture(s) (B3s) and 20 without associated stricture(s) (B3o). Per specimen, three sections (ileal proximal margin, inflamed area, most affected area) were examined and graded for inflammation and fibrosis based on the chosen histopathological score. Clinical data and outcomes were retrieved from GEDII prospective national IBD registry.

This study produced a **core scientific paper** published in a **Q2** Gastroenterology journal, with a 2021 Clarivate/Web of Science Journal Impact Factor of 4.396.

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Ileal Crohn's Disease Exhibits Similar Transmural Fibrosis Irrespective of Phenotype

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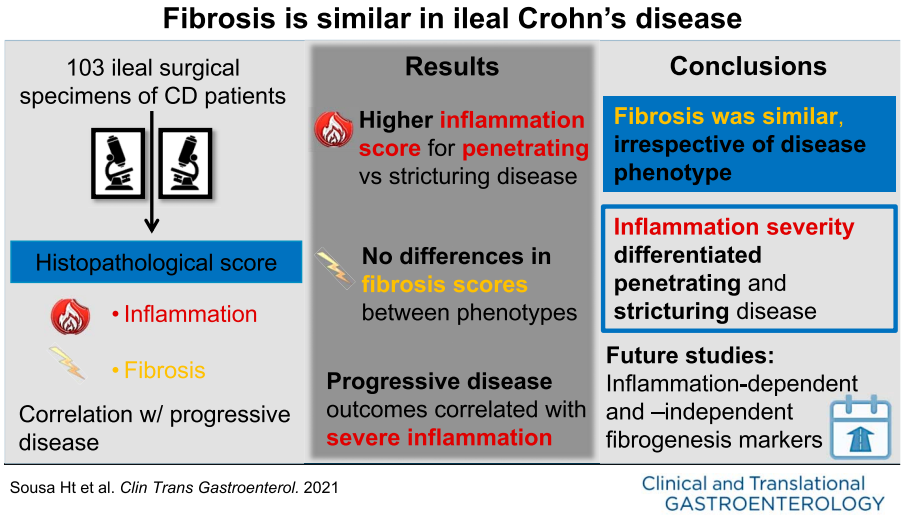
INTRODUCTION: **In Crohn's disease (CD), the assessment of transmural inflammation and fibrosis is of utmost importance. This study aimed to quantify these parameters in CD ileal specimens and correlate them with disease progression.**

METHODS: **This is a retrospective unicentric study based on the analysis of archived specimens (n = 103) of primary ileal resection. Data were retrieved from a prospective national inflammatory bowel disease registry. Two pathologists, blinded for CD phenotype and clinical indications for surgery, examined 3 sections per patient and graded inflammation and fibrosis, based on a histopathological score.**

RESULTS: **Penetrating (B3, n = 74) CD exhibited significantly higher inflammation in diseased areas, compared with stricturing (B2, n = 29) disease (score 3: 96% vs 76%, *P* = 0.005 in inflamed areas; 78% vs 55%, *P* = 0.019 in most affected areas). This was also observed for the comparison of B2 CD with B3 CD with (B3s, n = 54) and without associated stricture (B3o, n = 20): B3s vs B2: 81% vs 55%, *P* = 0.033 in most affected areas; B3o vs B2: 100% vs 76%, *P* = 0.006 in inflamed areas; 70% vs 55%, *P* = 0.039 in most affected areas. We could not show differences in fibrosis scores between the subphenotypes. Postoperative new penetrating events occurred only in B3s (n = 6, 11%, *P* = 0.043) patients. The changing of biologic therapy after surgery correlated with severe inflammation at the proximal ileal margin (55% changed vs 25% not changed, *P* = 0.035).**



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DISCUSSION: **In our cohort, fibrosis scores and fibromuscular changes were comparable, irrespective of CD phenotype. Inflammation severity was the major differentiator between penetrating and stricturing disease.**

SUPPLEMENTARY MATERIAL accompanies this paper at http://links.lww.com/CTG/A546; http://links.lww.com/CTG/A547; http://links.lww.com/CTG/A548; http://links.lww.com/CTG/A549; http://links.lww.com/CTG/A550; http://links.lww.com/CTG/A551; http://links.lww.com/CTG/A552; http://links.lww.com/CTG/A553; http://links.lww.com/CTG/A554; and http://links.lww.com/CTG/A555.

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INTRODUCTION

Transmural inflammation and submucosal fibrosis are important hallmarks of Crohn's disease (CD) (1). Intestinal fibrosis concerns extracellular matrix accumulation and mesenchymal cell expansion (2,3). In this process, inflammation is the main activator of mesenchymal cells and an essential factor to initiate fibrogenesis. Still, once fibrosis is established, it may be self-propagating (3,4). In the setting of CD, patients with inflammatory lesions are considered medical therapy-responsive, while those with more fibrotic lesions will eventually need surgery (4). Hence, despite all the available therapies targeting inflammation, intestinal fibrosis remains difficult to treat and prevent (3,4).

Strictures are subdivided in fibrotic, inflammatory, and mixed forms (5). Pure fibrotic or inflammatory strictures are rare, with both components presenting overlapped histopathology (3,6–10). In CD, transmural intestinal inflammation can be assessed by cross-sectional imaging (2,11–16). On the other hand, fibrosis cannot be measured by this technique nor through biomarkers (16,17). Endoscopy or biopsy-based histology (2,11) is not feasible as tissue remodeling occurs mostly in deeper layers (18). Thus, the extent and severity of fibrosis must be evaluated by histopathological analysis of intestinal resection specimens, resorting to several histopathological scoring systems (19,20).

The main objective of our work was to characterize and quantify inflammation and fibrosis, in ileal CD resection specimens, according to a CD transmural histopathological scoring system. We also aimed to correlate inflammation and fibrosis profiles with progressive disease.

METHODS

Patients and study design

The patients included in this retrospective, single-center study were selected as depicted in Supplementary Figure (see Supplementary Digital Content 1, http://links.lww.com/CTG/A546). Patients were retrieved from the prospective database of the Portuguese Group for the Study of Inflammatory Bowel Disease (GEDII) (gediibasedados.med.up.pt), according to the following inclusion criteria: (i) definite diagnosis of CD with stricturing (B2) or penetrating (B3) phenotypes, according to Montreal criteria (21); (ii) emergent or elective ileal resection, due to CD complications, at São João University Hospital Center (CHUSJ), Porto, Portugal; and (iii) minimum postoperative follow-up of 3 years, up to January 2018.

Patients fulfilling the inclusion criteria were crossed with the digital archive of the CHUSJ Pathology Department, available since January 1998. Because of an overrepresentation of B3 phenotype with associated ileal stricture, a portion of this group was randomly (Excel's random numbers tool) excluded, to have more balanced subgroups.

Demographical, clinical, and surgical information was retrieved from the GEDII database up to September 2019. All missing data or discrepancies were obtained from clinical files. The first ileal resection was considered the index episode (e.g., index surgery). Medical therapy data were collected for the periods before and after the index surgery and after the first subsequent surgery.

Progressive disease

Progressive disease was defined as the occurrence of at least one of the postoperative outcomes described elsewhere (22). The period from index surgery to the occurrence of each outcome was recorded.

Histopathologic workout

Two pathologists (I.G. and C.C.), blinded for CD phenotype and indications for surgery, retrieved the formalin-fixed and paraffin-embedded (FFPE) blocks of the ileal resection surgical specimens. The macroscopic report, the gross picture of the specimen (when available), and the description of the location and/or lesion represented in each block were retrieved from the files of the Department of Pathology and evaluated jointly by both pathologists. On the basis of macroscopic grounds, 3 sections were selected from each specimen (Figure 1): (i) margin: proximal ileal margin; (ii) most affected: (a) narrowest caliber area of the ileal stricture, for specimens only with strictures; (b) most severely inflamed ileal area (irrespective of having an associated stricture or not), involved by fistulas, fissures, and/or deep ulcers (defined as penetrating beyond the submucosa (23)); and (iii) inflamed: (a) area of ileal stricture outside the narrowest caliber, for specimens only with strictures; (b) area of ileum with inflammatory changes outside the most inflamed area, for specimens bearing fistulas, fissures, and/or deep ulcers. If the 3 regions were not present in the macroscopic report/picture, or if the information about the exact location of each FFPE block was missing, the cases were excluded (see Supplementary Figure, Supplementary Digital Content 1, http://links.lww.com/CTG/A546). Moreover, all the layers of the ileal wall (mucosa, submucosa, muscularis propria, and serosa) were to be adequately represented and oriented on each slide.

After a pre-evaluation of the slides to confirm the adequacy of the specimens for the study, the pathologists graded inflammation and fibrosis according to a previously described CD transmural histopathological score (23) (Table 1). Final scores were obtained by consensus. The evaluation of inflammation variables was mostly based on the histopathological analysis of hematoxylin and eosin–stained slides. Macroscopic report and

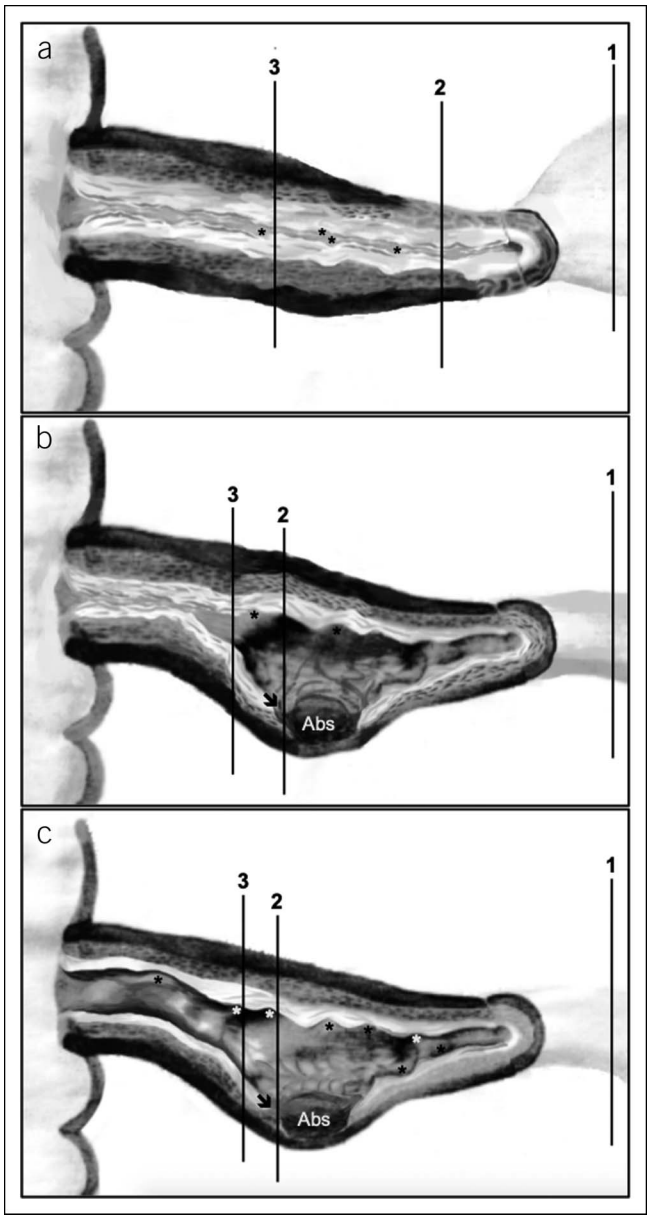

Figure 1. Schematic representation of anatomical locations of the 3 per-protocol sections for histopathological study, obtained from formalin-fixed and paraffin-embedded blocks of ileal resection surgical specimens (panels **a** to **c**). 1—Proximal ileal margin; 2—most affected area; and 3—inflamed area. (**a**) Schematic surgical specimen with strictures. (**b**) Schematic surgical specimen with fistulas, fissures, and/or deep ulcers and stricture. (**c**) Schematic surgical specimen with fistulas, fissures, and/or deep ulcers only. Abs—abscess; asterisks—superficial ulcers; arrow—deep ulcer (beyond submucosa). [1]Inflammation (1–3) and fibrosis (0–2) scoring: Higher scores indicate more severe inflammation and fibrosis, respectively (23).

pictures of the surgical specimens were considered for information on ulceration extent. Fibrosis variables were assessed on hematoxylin and eosin– and Masson trichrome–stained slides; grossing reports provided information on stricture diameter.

As the adopted score does not specify to which layer the term "muscular hyperplasia >25%" stands for, the feature was considered to be present if found either in muscularis propria or in muscularis mucosae (MM). However, in our study, MM expansion included either true cellular hyperplasia of smooth muscle cell and/or a fibrosis-splayed layer (24). The presence of smooth muscle or adipose tissue in the submucosal layer was also evaluated. A schematic representation of the main fibromuscular changes in ileal intestinal wall in CD is presented in the Supplementary Figure (see Supplementary Digital Content 2, http://links.lww.com/CTG/A547).

As the selected histopathological score does not include a 0 (zero) score for grading inflammation, cases with absence of inflammation were signaled for descriptive purposes but excluded from correlation analyses.

Table 1. Crohn's disease transmural histopathological score (23)

Score	Grade	Features	Score
Inflammation	Mild	Aphthous ulcers affected surface <50%; cryptitis <50%; inflammation limited to mucosa	1
	Moderate	Large, superficial ulcers (0.5–2 cm) Ulcerated surface <50%; affected surface 50%–100% Cryptitis >50%; crypt abscesses; submucosal inflammation	2
	Severe	Deep[a] ulcers or ulcers >2 cm in size; circumferential ulcers; deep[a] fissures; transmural inflammation	3
Fibrosis	None	None or minimal fibrosis limited to submucosa (<25% thickness)	0
	Mild/ moderate	Mild stricture (>15 mm) with nondilated lumen Submucosal fibrosis and muscular hyperplasia >25%[b] with preserved layers	1
	Severe	Massive transmural fibrosis; effacement of normal layers; severe stricture	2

[a]Deep—beyond the submucosa.
[b]Includes muscular propria hyperplasia >25%, muscularis mucosae hyperplasia >25%, and/or splayed muscularis mucosae (without cellular hyperplasia) >25%.

Statistical considerations

Categorical variables were summarized through absolute (n) and relative (%) frequencies. Continuous variables were described as mean ± SD or median (interquartile range), minimum, and maximum. Hypotheses on the distribution of continuous variables were tested using the *t* test and the nonparametric Mann-Whitney and Kruskal-Wallis tests. Associations between categorical and continuous variables were tested through χ^2 and Spearman correlation tests, respectively. For multiple comparison, Bonferroni correction was applied. IBM SPSS Statistics for Mac, Version 24.0 (IBM, Armonk, NY) was used to perform statistical analyses, adopting a 5% significance level.

Ethical considerations

Our study was exempt of patients' informed consent because of its retrospective nature based on archived pathological material. However, all patients gave consent for the collection of data from the GEDII database, which was endorsed by the Portuguese Data

Table 2. Demographic, clinical, and surgery-related variables, per phenotype

Demographical and clinical variables	Total	Phenotype at end of follow-up		
		B2 (n = 29, 28%)	B3 (n = 74, 72%)	*P*[a]
Gender, n (%)				0.390
Female	46 (45)	11 (38)	35 (47)	
Male	57 (55)	18 (62)	39 (53)	
Age at diagnosis, n (%)				**0.021**
A1: ≤16 yr old	11 (11)	1 (3)	10 (14)	
A2: 17–40 yr old	70 (68)	17 (59)	53 (72)	
A3: >40 yr old	22 (21)	11 (38)	11 (15)	
Phenotype at diagnosis, n (%)				**<0.001**
B1: nonstricturing, nonpenetrating	10 (10)	1 (3)	9 (12)	
B2: stricturing	37 (36)	28 (97)	9 (12)	
B3: penetrating	56 (54)	0 (0)	56 (76)	
CD localization, n (%)				**0.002**
L1	58 (56)	18 (62)	40 (54)	
L1+L4	8 (8)	6 (21)	2 (3)	
L3	32 (31)	3 (10)	29 (39)	
L3+L4	5 (5)	2 (7)	3 (4)	
Perianal disease, n (%)	25 (24)	5 (17)	20 (27)	0.324
Age at diagnosis, yr, mean (SD)	30 (12)	35 (13)	28 (10)	**0.001**[b]
Total follow-up, yr, median (P25–P75)	10 (7–12)	12 (7–14)	10 (7–12)	**0.024**[c]
Surgery-related variables				
Motif for first ileal surgery, n (%)				**<0.001**
Fistula/abscess	66 (64)	0 (0)	66 (89)	
Perforation	5 (5)	0 (0)	5 (7)	
Obstruction	32 (31)	29 (100)	3 (4)	
First ileal surgery, n (%)				>0.999
Segmental enterectomy	7 (7)	2 (7)	5 (7)	
Ileocecal resection	86 (83)	24 (83)	62 (84)	
Right hemicolectomy	10 (10)	3 (10)	7 (9)	
Motif of reoperation, n (%)				0.050
Abscess	3 (30)	0 (0)	3 (42)	
Stricture (primary)	4 (40)	3 (100)	1 (14)	
Stricture (anastomotic)	3 (30)	0 (0)	3 (43)	
Preoperative therapy, n (%)				
5-aminosalicylic acid	49 (48)	15 (52)	34 (46)	0.664
Steroids	70 (68)	23 (79)	47 (64)	0.122
Immunosuppressives	60 (58)	16 (55)	44 (60)	0.692
Anti-tumor necrosis factor alpha[d]	27 (26)	6 (21)	21 (28)	0.468
Postoperative therapy, n (%)				
5-aminosalicylic acid	31 (30)	11 (38)	20 (27)	0.278
Steroids	32 (31)	14 (48)	18 (24)	**0.018**
Immunosuppressives	90 (87)	27 (93)	63 (85)	0.342
Anti-tumor necrosis factor alpha[e]	61 (59)	18 (62)	43 (58)	0.713
Other biologics[f]	11 (11)	4 (14)	7 (10)	0.724

Table 2. (*continued*)

Demographical and clinical variables	Total	Phenotype at end of follow-up		
		B2 (n = 29, 28%)	B3 (n = 74, 72%)	*P*[a]
Post-re-operative therapy (n = 10), n (%)				
5-aminosalicylic acid	2 (20)	1 (33)	1 (14)	>0.999
Immunosuppressives	7 (70)	3 (100)	4 (57)	0.475
Anti-tumor necrosis factor alpha[g]	8 (80)	2 (67)	6 (86)	>0.999
Other biologics[h]	1 (10)	1 (33)	0 (0)	0.300
Age at index surgery, yr, mean (SD)	34 (13)	40 (15)	32 (11)	**0.008**[b]
Time from diagnosis to index surgery, yr, median (P25–P75)	2.0 (0.5–6.0)	3.0 (1.0–6.0)	2.0 (0.5–6.0)	0.273[c]

Bold entries indicate significant *P* values ($P < 0.05$).
CD, Crohn's disease; L1, terminal ileum; L1+L4, terminal ileum + upper gastrointestinal tract; L3, ileum and colon; L3+L4, ileocolonic + upper gastrointestinal tract.
[a]χ^2 test.
[b]*t* test for independent samples.
[c]Mann-Whitney test.
[d]Infliximab (n = 22) and adalimumab (n = 2).
[e]Infliximab (n = 51) and adalimumab (n = 10).
[f]Vedolizumab (n = 7) and ustekinumab (n = 4).
[g]Infliximab (n = 7) and adalimumab (n = 1).
[h]Vedolizumab (n = 1).

Protection Committee, authorization number 2868/2013. The study protocol conforms to the ethical guidelines of Declaration of Helsinki and was approved by the CHUSJ Ethic Committee on July 2018. Confidentiality of data was ensured.

RESULTS

Study population

From a total of 103 patients, 29 were diagnosed with B2 CD (stricturing disease) and 74 with B3 CD (penetrating disease). In the B3 subgroup, 54 patients had at least 1 associated stricture (B3s), while 20 had not (B3o). At diagnosis, B2 patients were, on average, older than B3 ones (mean age: 35 vs 28 years old, $P = 0.001$; age over 40 years old: 38% in B2 vs 15% in B3, $P = 0.021$). Regardless isolated ileal location predominating in both phenotypes, B2 CD affected more frequently the ileojejunal area when compared with B3 CD (B2 21% vs B3 3%, $P = 0.002$). By contrast, B3 CD involved more frequently the ileocolonic area, when compared with B3 CD (B3 39% vs B2 21%, $P = 0.002$) (Table 2).

Surgery-related variables and disease outcomes

The most common indication for first ileal surgery was fistula/abscess (64%); ileal resection was performed in 83% of patients. B2 patients were, on average, older at the moment of index surgery (mean age: 40 vs 32 years old, $P = 0.008$) (Table 2). After surgery, more B2 patients were treated with steroids than B3 ones (48% vs 24%, $P = 0.018$), with no differences in the number of steroid courses needed or time from surgery to the first course (Tables 2 and 3). The proportion of patients who started immunosuppressives after surgery was similar between phenotypes (41% in B2, 48% in B3). However, these were started significantly earlier in B2 patients (median: 5.8 vs 0.5 years, $P = 0.028$). Most patients (63%) started (39%) or changed (24%) biologic therapy (BT) (Table 3). Disease progression occurred in 75 (73%) patients, and 10 (10%) patients were reoperated at least once during follow-up, after a median period of 6.7 years (1.8–10.4). Postoperative stricturing events were reported in 23 (22%) patients. Time from index surgery to each outcome, i.e., time-to-event analysis, is displayed through Kaplan-Meier curves (see Supplementary Figure, Supplementary Digital Content 3, http://links.lww.com/CTG/A548).

Histopathological scoring according to section location

Overall, 10 patients (10%) showed no signs of inflammation on proximal ileal margins, and 74 (72%) showed a score of 3 in most affected regions. Histopathological scoring person can be found in Supplementary Table (see Supplementary Digital Content 4, http://links.lww.com/CTG/A549).

When comparing proximal ileal margins with inflamed areas, the inflammation scores increased in 70 (68%) patients, while fibrosis scores did not change (n = 65; 63%). Regarding inflamed and most affected areas, both inflammation and fibrosis scores remained unchanged (n = 100; 97% and n = 98; 95%, respectively). Histopathological scoring variation according to section location can be seen in Supplementary Table (see Supplementary Digital Content 5, http://links.lww.com/CTG/A550). Our study also evaluated the correlation between inflammation and fibrosis scores (see Supplementary Table, Supplementary Digital Content 6, http://links.lww.com/CTG/A551), which was weak and only in inflamed areas ($r = 0.198$, $P = 0.045$).

Histopathological scoring in B2 and B3 CD

B2 vs B3 phenotypes. Three (15%) B2 patients and 7 (10%) B3 patients had no signs of histological inflammation in proximal ileal margins (data not shown). B3 patients had significantly higher inflammation score than B2 patients in inflamed and most affected areas (score 3: inflamed: 96% vs 76%, $P = 0.005$; most affected: 78% vs 55%, $P = 0.019$) (Figure 2a, 2b). This tendency was also observed for the total score, in both regions (score 4–5: inflamed: 93% vs 72%, $P = 0.008$; most affected: 79% vs 55%, $P = 0.043$). In terms of fibrosis, no significant differences were

Table 3. Postoperative outcomes variables, per phenotype

	Total (n = 103)	B2 (n = 29, 28%)	B3 (n = 74, 72%)	P[a]
Reoperation, n (%)	10 (10)	3 (10)	7 (10)	>0.999[a]
Time from index to subsequent surgery, yr, median (P25–P75)[b]	6.7 (1.8–10.4)	6.0 (1.2–7.5)	7.8 (2.0–11.5)	0.425[c]
Hospitalization, n (%)	30 (29)	10 (35)	20 (27)	0.454[a]
Time from index to subsequent hospitalization, yr, median (P25–P75)[b]	2.0 (0.8–3.5)	7.8 (1.2–3.0)	2.0 (0.7–4.2)	0.947[c]
Steroids, n (%)	32 (31)	14 (48)	18 (24)	**0.018[a]**
No. of postoperative steroid courses, median (min–max)	1 (1–2)	1 (1–4)	1 (1–2)	0.185[c]
Time from index surgery to first steroid course, yr, median (P25–P75)[b]	2.5 (0.5–5.1)	3.5 (0.8–7.8)	2.3 (0.2–3.5)	0.171[c]
Immunosuppressive (IS), n (%)				
Start IS, n (%)	40 (39)	12 (41)	28 (38)	0.740[a]
Time from index surgery to IS, yr, median (P25–P75)[b]	1.0 (0.1–5.3)	5.8 (0.6–7.7)	0.5 (0.1–3.3)	**0.028**[c]
Change IS, n (%)	7 (7)	2 (7)	5 (7)	>0.999[a]
Time from index surgery to change IS, yr, median (P25–P75)[b]	3.6 (1.5–4.2)	4.3 (4.0–4.5)	2.0 (1.5–3.6)	0.121[c]
BT, n (%)				
Start BT, n (%)	40 (39)	14 (48)	26 (35)	0.218[a]
Time from index surgery to BT, yr, median (P25–P75)[b]	5.7 (2.1–8.5)	8.6 (1.5–12.0)	5.3 (2.5–7.5)	0.187[c]
Change BT, n (%)	25 (24)	7 (24)	18 (24)	0.984[a]
Time from index surgery to change BT, yr, median (P25-P75)[b]	4.0 (2.4–8.7)	8.5 (4.0–13.2)	4 (1.5–7.6)	0.079[c]
New event, n (%)				
Stricturing, n (%)	23 (22)	10 (35)	13 (18)	0.072[a]
Time from index surgery to new stricturing, yr, median (P25–P75)[b]	3.0 (1.0–6.0)	4.2 (2.5–7.2)	2.2 (1.0–5.0)	>0.999[c]
Penetrating, n (%)	6 (6)	0 (0)	6 (8)	0.181[a]
Time from index surgery to new penetrating, yr, median (P25–P75)[b]	2.2 (0.6–3.5)	—	2.2 (0.6–3.5)	—
Perianal, n (%)	2 (2)	1 (3)	1 (1)	>0.999[a]
Time from index surgery to new perianal, yr, median (P25–P50)[b]	2.2 (2.0–2.5)	2.5 (2.5–2.5)	2.0 (2.0–2.0)	>0.999[a]
Progressive disease, n (%)	75 (73)	23 (79)	52 (70)	0.354[a]

Bold entries indicate significant *P* values ($P < 0.05$).
BT, biologic therapy; IS, immunosuppressive.
[a]χ^2 test.
[b]Time from index surgery to each outcome is considered only for patients presenting the outcome.
[c]Mann-Whitney test.

observed between the 2 phenotypes, in the 3 studied areas (Table 4; Figure 2c,d).

B2 phenotype vs B3s and B3o subphenotypes. Three (15%) B2, 2 (10%) B3o, and 5 (9%) B3s patients did not present inflammation in proximal ileal margins (data not shown). Results for inflammation, fibrosis, and total scores for the B2 phenotype and B3o and B3s penetrating subphenotypes are listed in the Supplementary Data (Table 5 and see Supplementary Figure, Supplementary Digital Content 7, http://links.lww.com/CTG/A552).

When compared with B2 patients, B3s patients presented with significantly higher inflammation score in most affected areas (inflammation score 3: 81% vs 55%, $P = 0.033$), whereas total score was significantly higher in inflamed areas (total score 4–5:

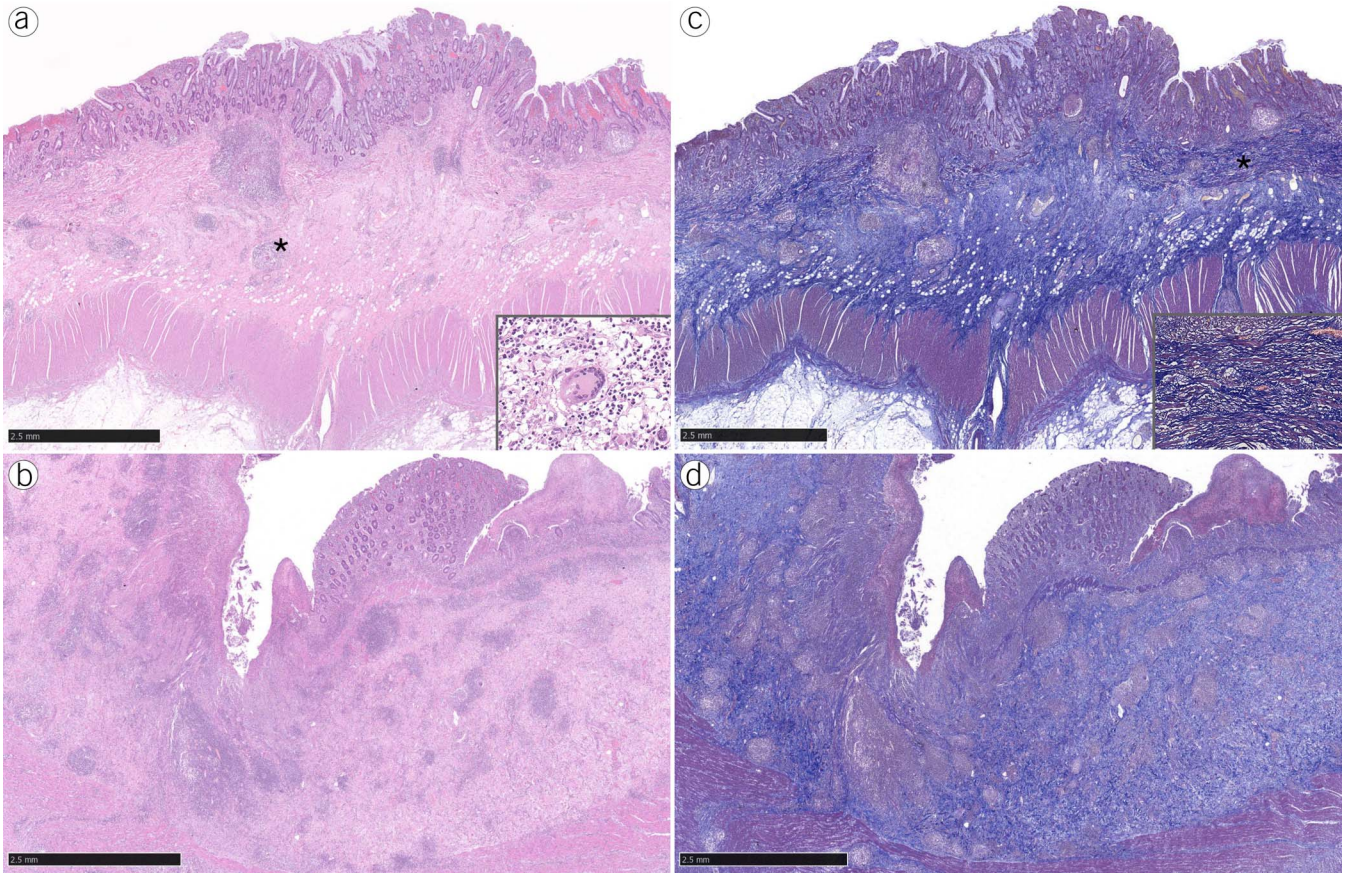

Figure 2. Ileal resections from B2 patients (**a**, hematoxylin and eosin [H&E]) showed lesser inflammation when compared with B3 patients (**b**, H&E; large superficial ulcer). Both specimens from B2 (**c**, Masson trichrome [MT]) and B3 (**d**, MT) patients showed prominent fibrosis. Granulomatous inflammation with giant cell (**a**, asterisk and inset). Hyperplastic and splayed muscularis mucosae, dissected by fibrosis (**c**, asterisk and inset).

94% vs 72%, $P = 0.024$). Regarding fibrosis score, we could not find differences between the 2 groups.

The comparison between B2 and B3o patients showed that B3o patients had significantly higher inflammation scores in all 3 studied areas (score 3: proximal ileal margins: 61% vs 19%, $P = 0.015$; inflamed areas: 100% vs 76%, $P = 0.006$; most affected areas: 70% vs 55%, $P = 0.039$). There were no differences between the 2 groups, in terms of fibrosis and total score.

B3o patients presented significantly higher inflammation score than B3s patients at the proximal ileal margins only (score 3: 61% vs 29%, $P = 0.044$). Regarding fibrosis and total score, no differences were found between the 2 subphenotypes, in all areas.

The study of the contribution of individual histological features to all CD subphenotypes showed that transmural inflammation was significantly more frequent in proximal ileal margins of B3o patients (59% B3o vs 26% B3s vs 17% B2, $P = 0.013$), while MM splay >25% (Figure 2c) was significantly less frequent in inflamed areas (80% B3o vs 98% B3s vs 93% B2, $P = 0.020$). No differences between subphenotypes were found for all the other histological variables (see Supplementary Table, Supplementary Digital Content 8, http://links.lww.com/CTG/A553, which presents the association of all selected histological features per CD (subphenotype). Also, we could not evidence differences between the histopathological scores of patients with and without submucosal adipose or smooth muscle tissue. The 2 histological variables according to histopathological scoring and section location can be found in the Supplementary Data (see Supplementary Table, Supplementary Digital Content 9, http://links.lww.com/CTG/A554).

Progressive disease outcomes

Severe inflammation at proximal ileal margins was associated with postoperative change of BT (score 3: 55% changed BT vs 25% not changed BT, $P = 0.035$). No differences were found between histopathological scores for the other outcomes. Also, we could not establish associations between histology and postoperative outcomes (data not shown). Postoperative outcomes in the 3 CD subphenotypes are depicted in Supplementary Table (see Supplementary Digital Content 10, http://links.lww.com/CTG/A555). New penetrating events, after the index surgery, occurred exclusively in B3s patients (n = 6, 11%, $P = 0.043$).

DISCUSSION

In this study, we quantified and characterized inflammation and fibromuscular changes in ileal CD resection specimens according to a CD histopathological score. We confirmed pure fibrotic disease may not exist as, in most patients, both components overlapped on histopathology, irrespective of disease phenotype (25,26). Importantly, our study evidenced that the major differentiator between penetrating and stricturing disease was the degree of inflammation. Patients with penetrating disease both with (B3s) or without (B3o) associated stricture exhibited higher

Table 4. Histopathological scoring per section in stricturing (B2) and penetrating (B3) Crohn's disease

	B2, n (%)	B3, n (%)	P^a
Margins			
Inflammation			0.094
1–2	21 (81)	42 (64)	
3	5 (19)	24 (36)	
Fibrosis			0.233
0	3 (10)	15 (20)	
1	26 (90)	59 (80)	
2	0 (0)	0 (0)	
Total score			0.061
≤2	20 (69)	30 (43)	
3	4 (14)	18 (26)	
4–5	5 (17)	22 (31)	
Inflamed			
Inflammation			**0.005**
1–2	7 (24)	3 (4)	
3	22 (76)	71 (96)	
Fibrosis			0.521
0	2 (7)	2 (3)	
1	22 (76)	53 (72)	
2	5 (17)	19 (26)	
Total score			**0.008**
≤2	1 (4)	0 (0)	
3	7 (24)	5 (7)	
4–5	21 (72)	69 (93)	
Most affected			
Inflammation			**0.019**
1–2	13 (45)	16 (22)	
3	16 (55)	58 (78)	
Fibrosis			0.774
0	0 (0)	1 (1)	
1	26 (90)	68 (92)	
2	3 (10)	5 (7)	
Total score			**0.043**
≤2	5 (17)	4 (5)	
3	8 (28)	12 (16)	
4–5	16 (55)	58 (79)	

Inflammation (1–3) and fibrosis (0–2) scoring: Higher scores indicate more severe inflammation and fibrosis, respectively (23).
Bold entries indicate significant *P* values ($P < 0.05$).
[a]χ^2 test.

inflammation scores in diseased areas than pure stricturing (B2) patients, with no differences in fibrosis scores. Yet, when comparing penetrating subphenotypes, B3o patients showed a significantly higher inflammation score at the proximal ileal margin only.

Penetrating disease is believed to coexist with strictures (4). Fistula formation may be guided by both intraluminal pressure and transmural inflammation-induced changes (27). This hypothesis is supported by the higher inflammation grades observed in B3s patients when compared with B2 patients and, although uncommon, by new postoperative penetrating events occurring in B3s patients.

Regarding B2 phenotype, we found inflammation and fibrosis overlap (3,6–10) in most patients without purely fibrotic stricture in all studied areas. However, we found strictures without fibrosis in inflamed areas (n = 2 of 29 patients). These might correspond to pure inflammatory strictures because inflammation seems to be required to initiate fibrogenesis (3,4).

Overall, grade 1 fibrosis was more frequent than grade 2, demonstrating the importance of submucosal fibrosis and muscular expansion not only in stricturing (1,28), but also in penetrating disease. A deeper analysis evidenced that B3o patients presented MM splay with significantly less frequency in inflamed areas. The absence of differences in other fibromuscular variables between groups suggests that these changes are important irrespective of phenotype.

The presence of adipose tissue in the submucosa could represent a potential surrogate of adjacent nonresected creeping fat (29), which was shown to correlate with chronic inflammation (30), muscle hypertrophy, fibrosis, and strictures (31,32). In our study, adipose tissue in the submucosa was more frequent in cases of higher inflammation (score 3) and of mild to moderate fibroses (score 1). However, no differences were found between CD subphenotypes, which may be due to the reduced number of patients in this subgroup.

Our secondary aim was to correlate histopathological profiles with progressive disease. New penetrating events occurred exclusively in B3s patients. Also, postoperative need of changing BT correlated with severe inflammation at the proximal ileal margin irrespective of CD phenotype. Although the literature shows that microscopic inflammation in resection margins does not affect recurrence rates (33,34), our study suggests that severe inflammation in this area may represent a red flag for nonresponse to an ongoing biologic at the time of surgery. CD subphenotypes, histopathological cores, or variables did not correlate with the other postoperative outcomes. Although most patients (73%) presented progressive disease, the 10-year reoperation rate (10%) was lower than previously reported (33%–39%) (35–37). However, relevant methodological differences hinder direct comparisons as our study is based solely on B2 and B3 surgical specimens.

This study has some limitations. First, its retrospective and single-center study design led to many case exclusions, resulting in a small B2 patients' group and somewhat underpowered the study. To avoid potential statistical constraints, we used Bonferroni correction to preserve statistical significance regardless of subgroup size. Second, the use of archived FFPE blocks could decrease the reliability of the histopathological analyses, which may be also affected by sampling error in the choice of tissue location. To overcome this limitation, we performed a double, independent, blinded pathological assessment and resorted to a systematic inflammation and fibrosis grading based on a histopathological score (23). Moreover, we choose 3 different sections per specimen to mitigate sampling error and selection biases. Third, although this score showed high methodological quality and adequate properties (20), it was not validated and no validated scores exist for this purpose. Finally, the exclusion of cases with no inflammation in the proximal ileal resection margin could potentially introduce a bias in the analysis.

Table 5. Histopathological scoring per section and Crohn's disease phenotype: stricturing (B2) vs penetrating without associated stricture (B3o) vs penetrating with associated stricture (B3s)

	B2 (n = 29), n (%)	B3s (n = 54), n (%)	B3o (n = 20), n (%)	P^a	P^b	P^c
Margins						
Inflammation[d]				>0.999	**0.015**	**0.044**
1–2	21 (81)	35 (71)	7 (39)			
3	5 (19)	14 (29)	11 (61)			
Fibrosis				0.303	>0.999	0.645
0	3 (10)	13 (24)	2 (10)			
1	26 (90)	41 (76)	18 (90)			
Total score[d]				0.627	0.081	0.792
≤2	17 (65)	22 (50)	5 (28)			
3	4 (15)	15 (28)	3 (17)			
4–5	5 (19)	12 (22)	10 (56)			
Inflamed						
Inflammation				0.067	**0.006**	>0.999
1–2	7 (24)	3 (6)	0 (0)			
3	22 (76)	51 (94)	20 (100)			
Fibrosis				0.234	>0.999	0.108
0	2 (7)	0 (0)	2 (10)			
1	22 (76)	38 (70)	15 (75)			
2	5 (17)	16 (30)	3 (15)			
Total score				**0.024**	>0.999	>0.999
≤2	1 (4)	0 (0)	0 (0)			
3	7 (24)	3 (6)	2 (10)			
4–5	21 (72)	51 (94)	18 (90)			
Most affected						
Inflammation				**0.033**	**0.039**	0.688
1–2	13 (45)	10 (19)	6 (30)			
3	16 (55)	44 (81)	14 (70)			
Fibrosis				>0.999	>0.999	>0.999
0	0 (0)	0 (0)	1 (5)			
1	26 (90)	50 (93)	18 (90)			
2	3 (10)	4 (7)	1 (5)			
Total score				0.060	>0.999	>0.999
≤2	5 (17)	2 (4)	2 (10)			
3	8 (28)	8 (15)	4 (20)			
4–5	16 (55)	44 (81)	14 (70)			

Bold entries indicate significant P values ($P < 0.05$).
Inflammation (1–3) and fibrosis (0–2) scoring: Higher scores indicate more severe inflammation and fibrosis, respectively (23).
[a] P value for B2 vs B3s with Bonferroni correction.
[b] P value for B2 vs B3o with Bonferroni correction.
[c] P value for B3o vs B3s with Bonferroni correction.
[d] Does not include patients without inflammation (n = 93).

However, this was not the case because the subgroup analyses including these 10 cases showed results consistent with those presented herein with no difference between phenotypes.

The strengths of this study rely on the large number of included patients and on the careful and well-defined histopathological exercise, designed to obviate the above-mentioned limitations.

In conclusion, our study innovatively demonstrated that the major differentiator between penetrating and stricturing disease was severity of inflammation because no differences were observed both in fibrosis scores and most of fibromuscular variables. We confirmed that pure fibrotic CD may not exist, with inflammation and fibromuscular changes overlapping in most patients irrespective of disease phenotype. Absence of inflammation was seldom found and only at the proximal ileal surgical margin. Thus, we herein propose that the designation "fibrostenosing disease" as a synonym for stricturing disease should be abandoned. In fact, CD should again be regarded as a mixture of inflammatory and fibromuscular changes irrespective of the phenotype, bearing in mind that higher degrees of inflammation are characteristic (but not exclusive) of a penetrating behavior. The focus of future studies should be on identification and therapeutic targeting of markers of inflammation-dependent and -independent fibrogenesis in view of preventing progression for both advanced phenotypes of CD.

CONFLICTS OF INTEREST

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

Specific author contributions: H.T.S. was involved in conception and design of the study, acquisition, analysis, and interpretation of data, and was responsible for manuscript drafting. I.G. was involved in study design, acquisition and interpretation of histopathology data, histopathology image selection, manuscript drafting, and critical revision of the manuscript. C.C. was involved in study design, acquisition and interpretation of histopathology data, and critical revision of the manuscript. C.C.D. was involved in statistical analysis, interpretation of data, manuscript drafting, and critical revision of the manuscript. F.C. was involved in study design, interpretation of histopathology data, and critical revision of the manuscript for important intellectual content. F.M. was involved in conception and design of the study, interpretation of data, manuscript drafting, and critical revision of the manuscript for important intellectual content. All authors revised and approved the final manuscript for submission. All authors agreed on the accountability of all aspects of the work, thereby ensuring the accuracy and integrity of all parts.

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Potential competing interests: H. Tavares de Sousa received a fee for presenting from Takeda, AbbVie, Janssen, and Pfizer. F. Magro received a fee for presenting from AbbVie, Ferring, Falk Pharma, Hospira, Pharmakern, MSD, Shering, Lab. Vitoria, Vifor, OmPharma, Janssen, Takeda, and Pfizer. F. Rieder on the advisory board or consultant for Agomab, Allergan, AbbVie, Boehringer-Ingelheim, Celgene/BMS, CDISC, Cowen, Genentech, Gilead, Gossamer, Guidepoint, Helmsley, Index Pharma, Jannsen, Koutif, Mestag, Metacrine, Morphic, Origo, Pfizer, Pliant, Prometheus, Biosciences, Receptos, RedX, Roche, Samsung, Surrozen, Takeda, Techlab, Theravance, Thetis, UCB. All other authors have no conflicts of interest to declare.

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Study Highlights

WHAT IS KNOWN

- ✓ The assessment of transmural intestinal fibrosis in Crohn's disease (CD) relies on surgical specimens' pathology.
- ✓ Inflammation and fibrosis can be quantified through a transmural histopathological score.
- ✓ Separate inflammation and fibrosis quantification in penetrating and stricturing CD has not been explored.

WHAT IS NEW HERE

- ✓ Fibrosis scores and fibromuscular changes were comparable, irrespective of CD phenotype.
- ✓ The major differentiator between penetrating and stricturing disease was the degree of inflammation.

TRANSLATIONAL IMPACT

- ✓ Therapeutic targeting of markers of inflammation-dependent and -independent fibrogenesis could prevent progression of disease both for stricturing and penetrating phenotypes of CD.

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Supplementary Digital Content

Figure, Supplemental Digital Content 1. Flowchart of patient selection.

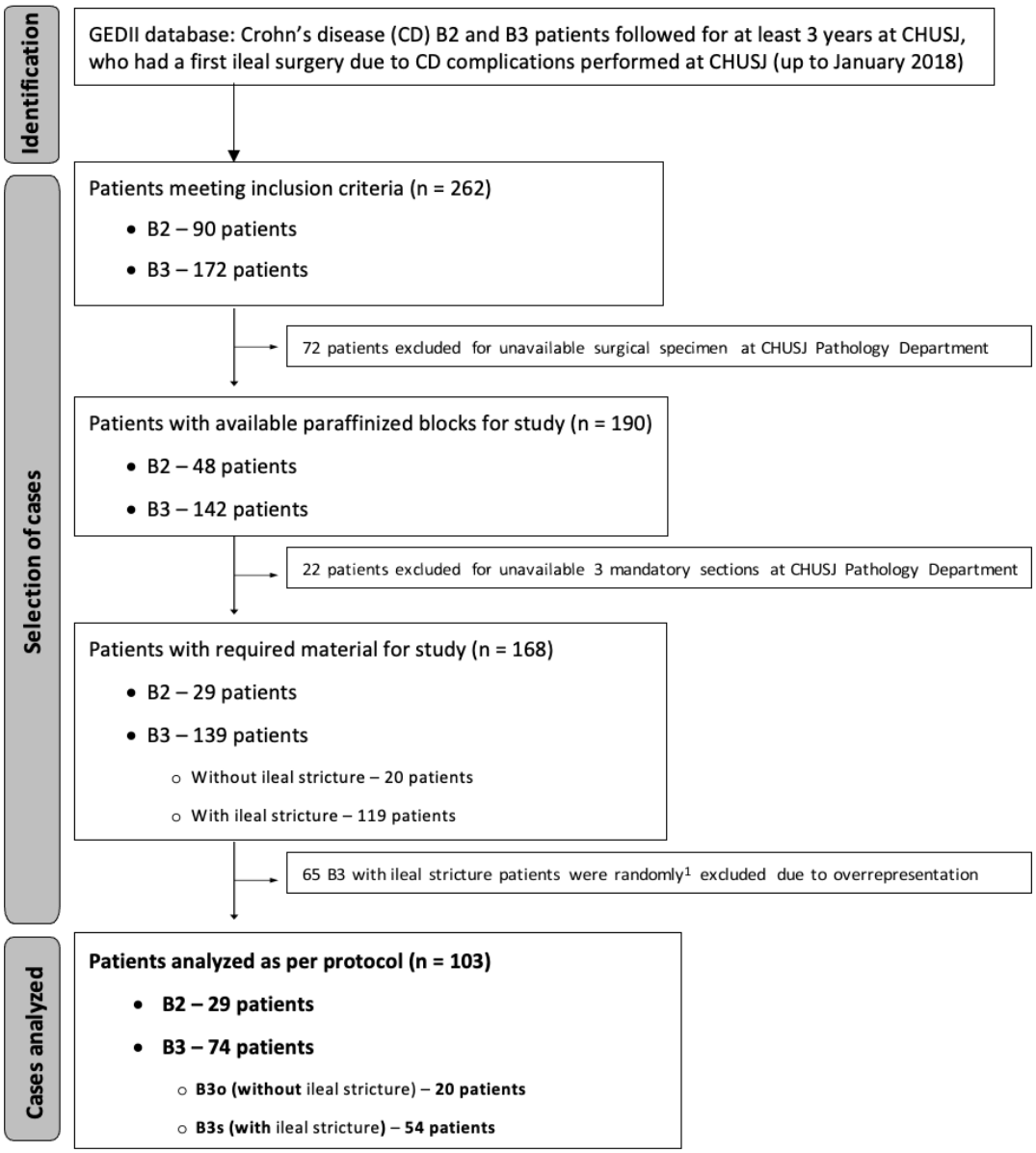
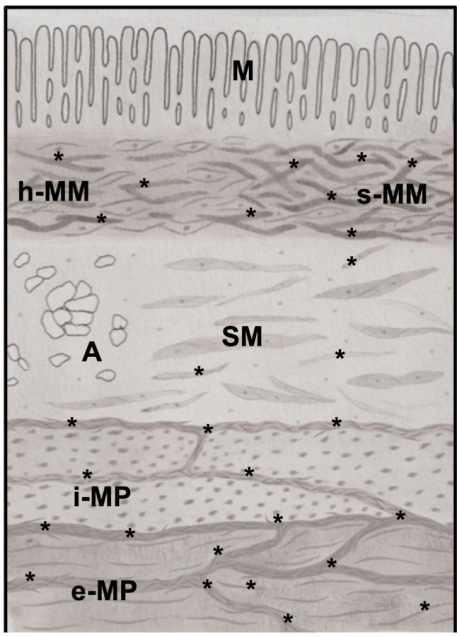


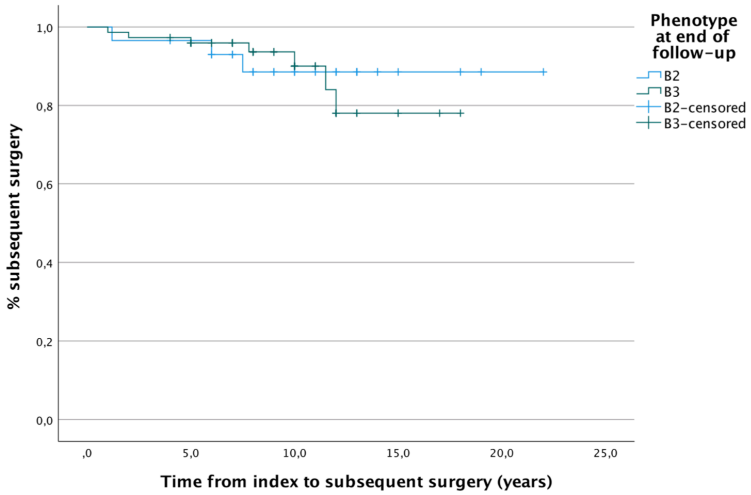
Fig.1 – Flowchart of patient selection. GEDII – Grupo de Estudo de Doença Inflamatória Intestinal (Portuguese Group for the Study of Inflammatory Bowel Disease); CHUSJ - São João University Hospital Centre; B2 – stricturing CD, B3 – penetrating CD, B3o – penetrating CD without associated stricture, B3s – penetrating CD with associated stricture; [1] Excel's random numbers tool (RAND function)

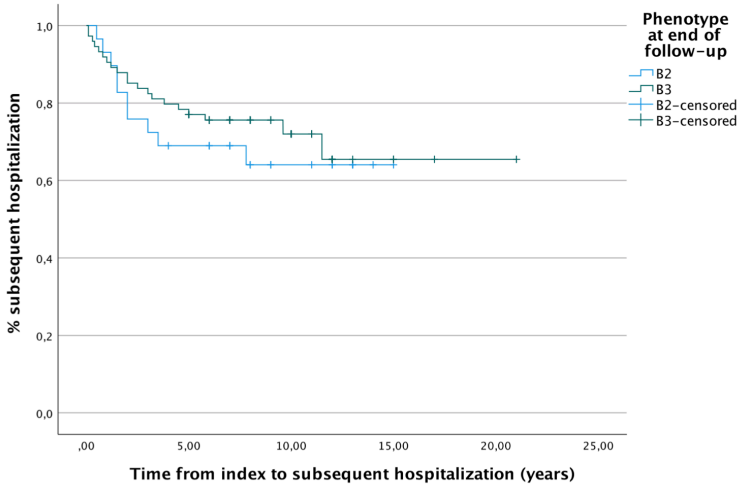
Figure, Supplemental Digital Content 2. Schematic representation of ileal intestinal wall bearing main Crohn's disease fibromuscular changes.

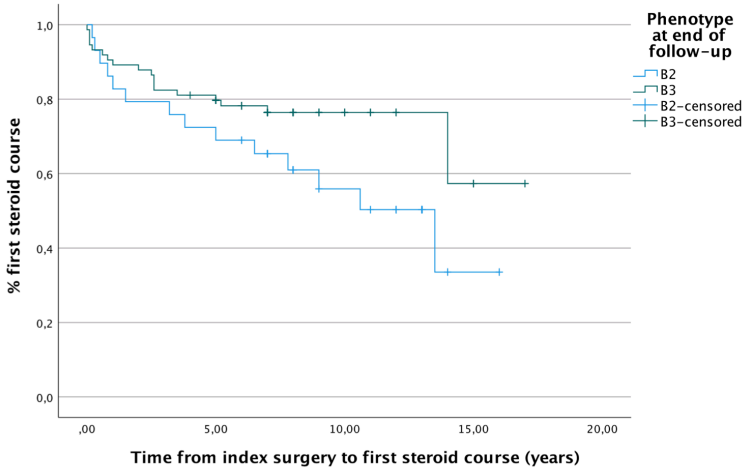


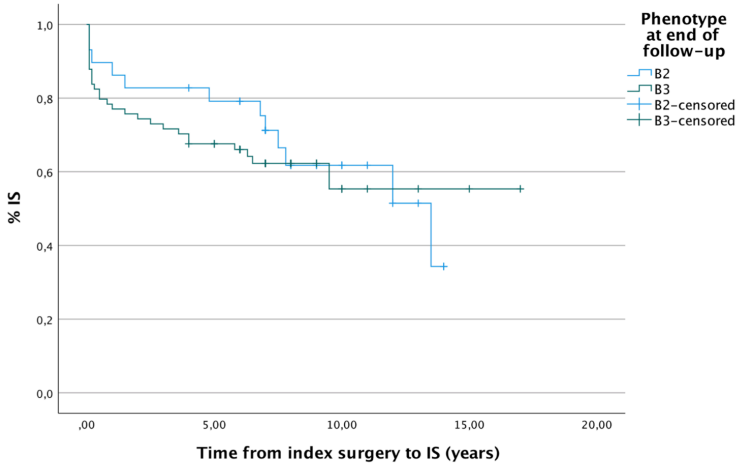
Figure, Supplemental Digital Content 2. Schematic representation of ileal intestinal wall bearing main Crohn's disease fibromuscular changes. Caption: M: mucosa; h-MM: hyperplastic expanded muscularis mucosae; s-MM: fibrosis-splayed expanded muscularis mucosae; SM: expanded submucosa with scattered smooth muscle cells; A: adipose tissue in the submucosa; i-MP: expanded internal muscularis propria; e-MP: expanded external muscularis propria; asterisks: fibrosis stripes.

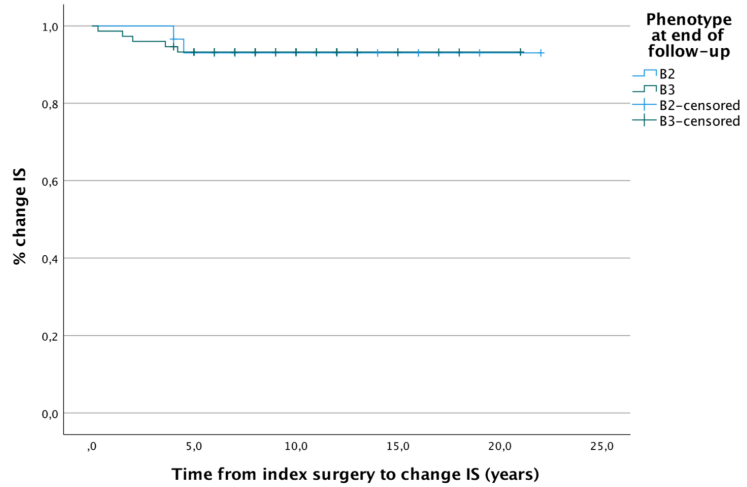
Figure, Supplemental Digital Content 3. Kaplan-Meier curves displaying time from index surgery to each outcome per phenotype. Time from index surgery to each outcome is considered only for patients presenting the outcome.

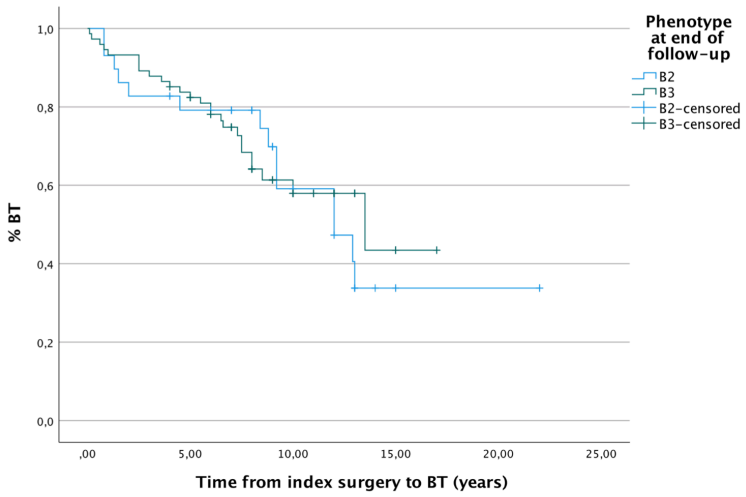


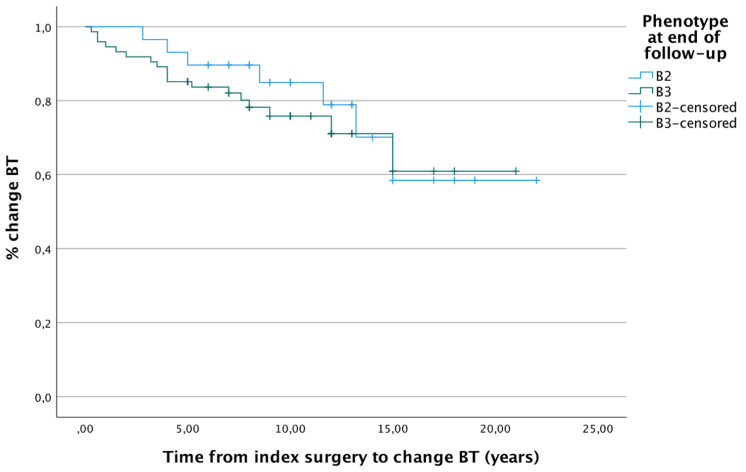


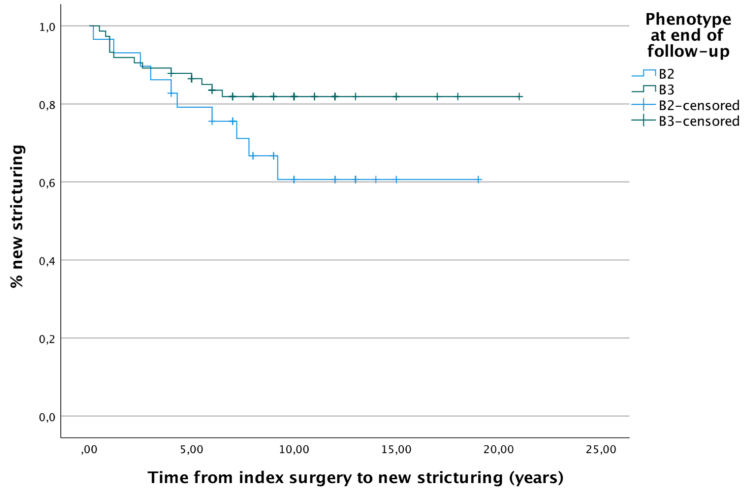

Phenotype at end of follow-up
B2
B3
B2-censored
B3-censored
% first steroid course
1,0
0,8
0,6
0,4
0,2
0,0
,00
5,00
10,00
15,00
20,00
Time from index surgery to first steroid course (years)

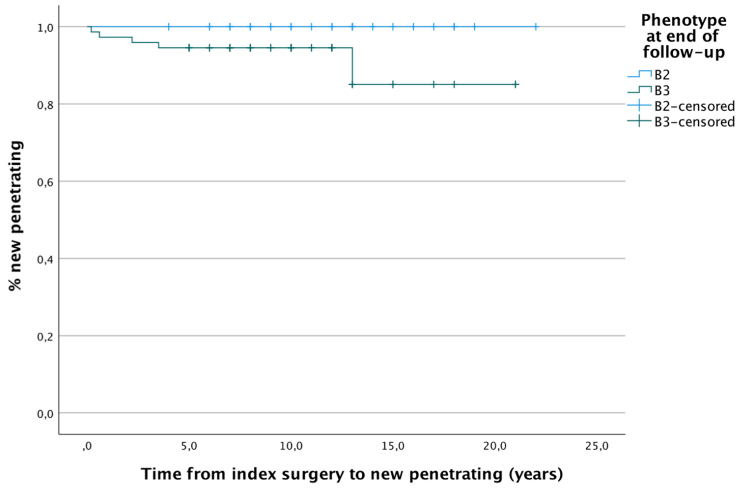

Phenotype at end of follow-up
B2
B3
B2-censored
B3-censored
% IS
1,0
0,8
0,6
0,4
0,2
0,0
,00
5,00
10,00
15,00
20,00
Time from index surgery to IS (years)

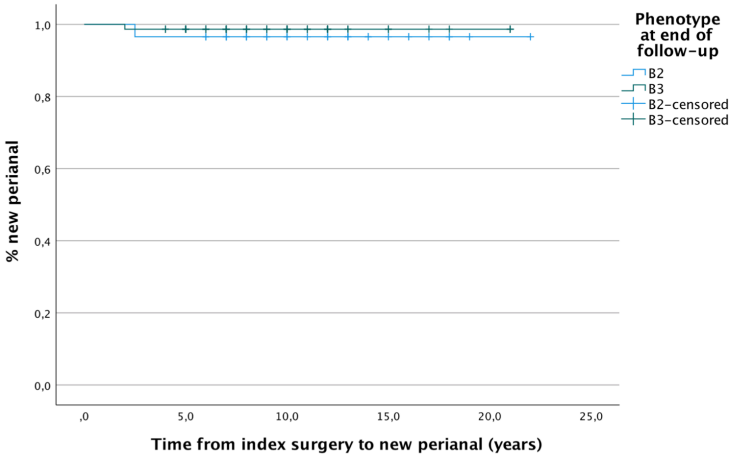

Phenotype at end of follow-up
B2
B3
B2-censored
B3-censored
% change IS
1,0
0,8
0,6
0,4
0,2
0,0
,0
5,0
10,0
15,0
20,0
25,0
Time from index surgery to change IS (years)


Phenotype at end of follow-up
B2
B3
B2-censored
B3-censored
% BT
1,0
0,8
0,6
0,4
0,2
0,0
,00
5,00
10,00
15,00
20,00
25,00
Time from index surgery to BT (years)


Phenotype at end of follow-up
B2
B3
B2-censored
B3-censored
% change BT
1,0
0,8
0,6
0,4
0,2
0,0
,00
5,00
10,00
15,00
20,00
25,00
Time from index surgery to change BT (years)


Phenotype at end of follow-up
B2
B3
B2-censored
B3-censored
% new stricturing
1,0
0,8
0,6
0,4
0,2
0,0
,00
5,00
10,00
15,00
20,00
25,00
Time from index surgery to new stricturing (years)


Phenotype at end of follow-up
B2
B3
B2-censored
B3-censored
% new penetrating
1,0
0,8
0,6
0,4
0,2
0,0
,0
5,0
10,0
15,0
20,0
25,0
Time from index surgery to new penetrating (years)


Phenotype at end of follow-up
B2
B3
B2-censored
B3-censored
% new perianal
1,0
0,8
0,6
0,4
0,2
0,0
,0
5,0
10,0
15,0
20,0
25,0
Time from index surgery to new perianal (years)

Table, Supplemental Digital Content 4. Histopathological scoring per section

	Margin	Inflamed	Most affected
Inflammation, n (%)			
0	10 (10)	0 (0)	0 (0)
1	40 (39)	0 (0)	8 (8)
2	23 (22)	10 (10)	21 (20)
3	30 (29)	93 (90)	74 (72)
Fibrosis, n (%)			
0	18 (18)	4 (4)	1 (1)
1	85 (82)	75 (73)	94 (91)
2	0 (0)	24 (23)	8 (8)
Total score, n (%)			
0	4 (4)	0 (0)	0 (0)
1	13 (13)	0 (0)	0 (0)
2	37 (36)	1 (1)	9 (9)
3	22 (21)	12 (12)	20 (19)
4	27 (26)	66 (64)	66 (64)
5	0 (0)	24 (23)	8 (8)

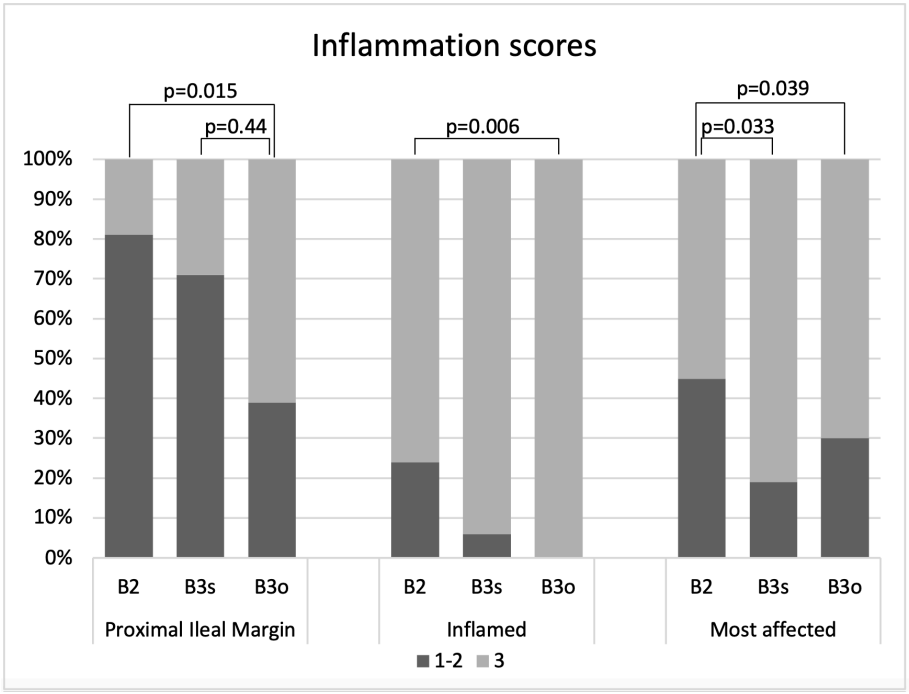
Table, Supplemental Digital Content 5. Histopathological scoring variation according to section location.

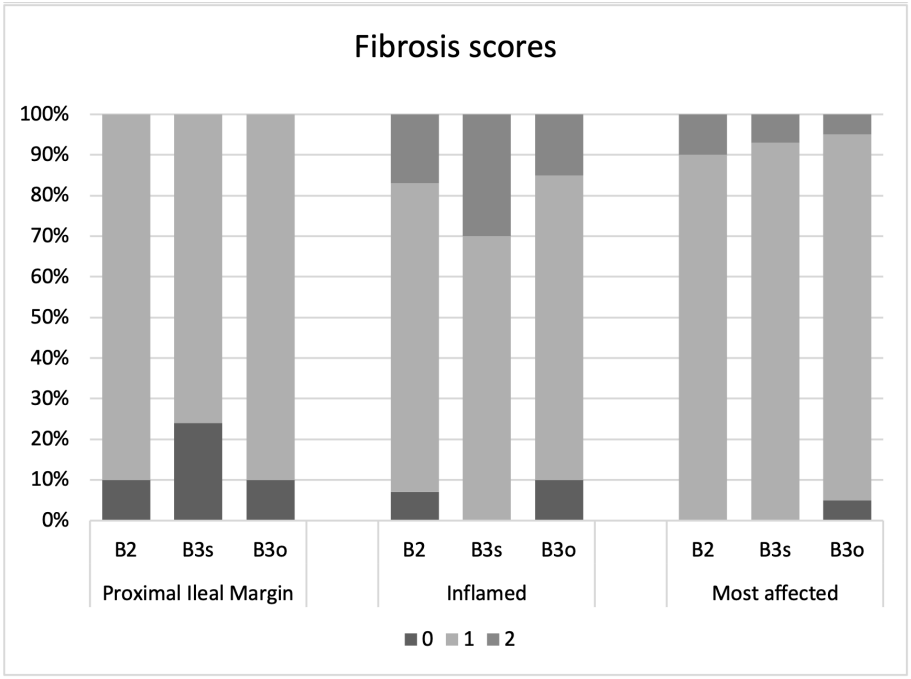
	Increased		Not increased	
Margins vs inflamed, n (%)				
Inflammation	70	(68)	33	(32)
Fibrosis	38	(37)	65	(63)
Total score	76	(74)	27	(26)
Inflamed vs Most affected, n (%)				
Inflammation	3	(3)	100	(97)
Fibrosis	5	(5)	98	(95)
Total score	7	(7)	96	(93)

Table, Supplemental Digital Content 6. Correlation between histopathological inflammation and fibrosis scores.

			Inflammation score		
			Margins	**Inflamed**	**Most affected**
Fibrosis score	**Margin**	Spearman correlation	0.169	0.108	0.042
		p-value	0.088	0.277	0.673
	Inflamed	Spearman correlation	0.010	**0.198**	0.213
		p-value	0.918	**0.045**	0.031
	Most affected	Spearman	-0,034	0.079	0.057
		p-value	0.736	0.428	0.570

Figure, Supplemental Digital Content 7. Graphical representation of histopathological scoring per section and CD phenotype: stricturing (B2) vs penetrating without associated stricture (B3o) vs penetrating with associated stricture (B3s). Caption: A – Inflammation scores; B – Fibrosis scores.





Table, Supplemental Digital Content 8. Presence of selected histological features per (sub)phenotype CD (sub)phenotype.

	B2		B3o		B3s					
	n	(%)	n	(%)	n	(%)	p-value[1]	p-value[2]	p-value[3]	p-value[4]
Margins										
Submucosal inflammation	5	(17)	2	(10)	17	(32)	0.100	-	-	-
Transmural inflammation	5	(17)	11	(59)	14	(26)	**0.013**	**0.018**	>0.999	0.057
MP hyperplasia >25%	12	(41)	9	(45)	18	(33)	0.590	-	-	-
MM hyperplasia >25%	23	(79)	17	(85)	34	(63)	0.099	-	-	-
MM splay >25%	14	(48)	9	(45)	24	(44)	0.944	-	-	-
Muscularization of SM	9	(31)	2	(10)	7	(13)	0.073	-	-	-
Adipose tissue in SM	8	(28)	6	(30)	10	(19)	0.474	-	-	-
Inflamed										
Submucosal inflammation	2	(7)	0	(0)	1	(2)	0.426	-	-	-
Transmural inflammation	25	(86)	20	(100)	50	(93)	0.197	-	-	-
MP hyperplasia >25%	21	(72)	14	(70)	37	(69)	0.934	-	-	-
MM hyperplasia >25%	27	(93)	18	(90)	54	(100)	0.064	-	-	-
MM splay >25%	27	(93)	16	(80)	53	(98)	**0.020**	0.610	0.720	**0.010**
Muscularization of SM	19	(66)	9	(45)	33	(61)	0.328	-	-	-
Adipose tissue in SM	11	(34)	9	(45)	19	(35)	0.742	-	-	-
Most affected										
Submucosal inflammation	8	(28)	5	(25)	9	(17)	0.475	-	-	-
Transmural inflammation	16	(55)	15	(75)	43	(79)	0.058	-	-	-
MP hyperplasia >25%	19	(66)	11	(55)	44	(82)	0.053	-	-	-
MM hyperplasia >25%	28	(97)	19	(95)	52	(96)	>0.999	-	-	-
MM splay >25%	26	(90)	16	(80)	44	(81)	0.602	-	-	-
Muscularization of SM	18	(62)	9	(45)	28	(52)	0.474	-	-	-
Adipose tissue in SM	12	(41)	7	(35)	20	(37)	0.888	-	-	-

[1] p-value among 3 sub-phenotypes, Chi Square test; [2] p-value B2 vs. B3o with Bonferroni correction; [3] p-value B2 vs. B3s with Bonferroni correction; [4] p-value B3o vs. B3s with Bonferroni correction; MP: *muscular propria*; MM: *muscularis mucosae*; SM: submucosa.

Table, Supplemental Digital Content 9. Presence of adipose tissue in submucosa and muscularization of submucosa according to histopathological scoring and section location.

	Adipose tissue in submucosa				Muscularization of submucosa					
	yes		no		yes		no			
	n	(%)	n	(%)	n	(%)	n	(%)	p-value[1]	p-value[2]
Margins										
Inflammation									0.681	0.781
1-2	15	(71)	48	(67)	12	(71)	51	(67)		
3	6	(29)	24	(33)	5	(29)	25	(33)		
Fibrosis									>0.999	0.733
0	4	(17)	14	(18)	4	(22)	14	(17)		
1	20	(83)	65	(82)	14	(78)	71	(84)		
2	0	(0)	0	(0)	0	(0)	0	(0)		
Inflamed										
Inflammation									0.129	0.466
1-2	6	(15)	4	(6)	7	(12)	3	(7)		
3	33	(85)	60	(94)	54	(89)	39	(93)		
Fibrosis									0.426	0.466
0	2	(5)	2	(3)	2	(3)	2	(5)		
1	31	(80)	44	(69)	42	(69)	33	(79)		
2	6	(15)	18	(28)	17	(28)	7	(17)		
Most affected										
Inflammation									0.371	0.863
1-2	9	(23)	20	(31)	16	(22)	13	(45)		
3	30	(77)	44	(69)	58	(78)	16	(55)		
Fibrosis									0.679	0.208
0	0	(0)	1	(2)	0	(0)	1	(2)		
1	37	(95)	57	(89)	49	(89)	45	(94)		
2	2	(5)	6	(9)	6	(11)	2	(4)		

[1] p-value for adipose tissue in submucosa; Chi Square test; [2] p-value for muscularization of submucosa; Chi Square test.

Table, Supplemental Digital Content 10. Progressive disease outcomes per CD (sub)phenotype.

	B2 (n=29)		B3o (n=20)		B3s (n=54)		p-value[1]
Reoperation, n (%)	3	(10)	1	(5)	6	(11)	0.669
Hospitalization, n (%)	10	(34)	4	(20)	16	(30)	0.594
Starting steroids, n (%)	14	(48)	5	(25)	13	(24)	0.061
Starting IS, n (%)	12	(41)	11	(55)	17	(31)	0.186
Changing IS, n (%)	2	(7)	2	(10)	3	(6)	0.870
Starting biologics, n (%)	14	(48	4	(20)	22	(41)	0.134
Changing biologics, n (%)	7	(24)	3	(15)	15	(28)	0.510
New event, n (%)							
Stricturing	10	(34)	2	(10)	11	(20)	0.119
Penetrating	0	(0)	0	(0)	6	(11)	**0.043**
Perianal	1	(3)	1	(5)	0	(0)	0.224
Progressive disease, n (%)	23	(79)	14	(70)	15	(70)	0.650

[1] Chi Square test; IS: immunosuppressive therapy

CHAPTER 5 – Fibrosis-Related Transcriptome Unveils a Distinctive Matrix Remodelling Pattern in Penetrating Ileal Crohn's Disease

5. Fibrosis-Related Transcriptome Unveils a Distinctive Matrix Remodelling Pattern in Penetrating Ileal Crohn's Disease

The third and last objective of this thesis was to investigate and compare the fibrosis-related transcriptome profiles in stricturing and penetrating CD full-thickness ileal specimens, given the histopathologic similarities regarding fibrosis degrees and fibromuscular changes between the two phenotypes found in this thesis' second study, herein presented on Chapter 4. This study focused on RNA extracted from full-thickness sections retrieved from the most affected area (as defined in this thesis' second study) of CD ileal surgical specimens. The study comprised 12 B2 and 24 B3 cases, according to Montreal phenotype classification, with B3 including 12 B3s and 12 B3o (as defined in this thesis' second study). Through Nanostring targeted technology, the expression of 787 genes covering the core pathways and processes involved in fibrosis was obtained and analyzed. Nanostring software and comparative bioinformatics were used for the transcriptome analyses and data interpretation.

This study produced a **core scientific paper** published in a **Q1** Gastroenterology journal, with a 2023 Clarivate/Web of Science Journal Impact Factor of 8.0

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Advance access publication 3 May 2024
Original Article

Fibrosis-related Transcriptome Unveils a Distinctive Remodelling Matrix Pattern in Penetrating Ileal Crohn's Disease

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Abstract

Background and Aims: Stricturing [B2] and penetrating [B3] ileal Crohn's disease have been reported to present similar levels of histopathological transmural fibrosis. This study aimed to compare the fibrosis-related transcriptomic profiles of penetrating and stricturing ileal Crohn's disease.

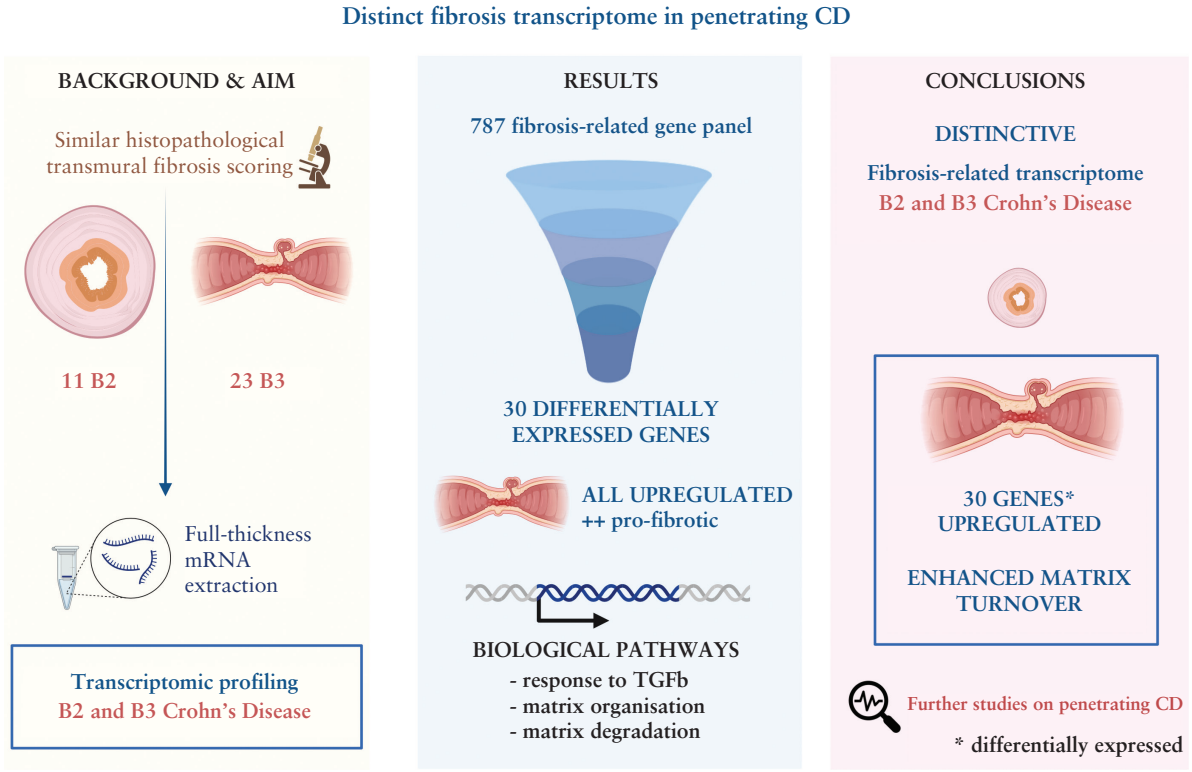
Methods: Using Nanostring technology and comparative bioinformatics, we analysed the expression of 787 fibrosis-related genes in 36 ileal surgical specimens, 12 B2 and 24 B3, the latter including 12 cases with associated stricture[s] [B3s] and 12 without [B3o]. Quality control of extracted RNA was performed according to Nanostring parameters and principal component analysis for the distribution analysis. For the selection of the differentially expressed genes, a *p*-adjusted <0.05 and fold change ≤-1.5 or ≥1.5 were adopted. Quantitative polymerase chain reaction (qPCR) and immunohistochemistry analyses were used to validate selected differentially expressed genes.

Results: We included 34 patients with B2 and B3 phenotypes, balanced for age at diagnosis, age at surgery, gender, Crohn's disease localisation, perianal disease, and therapy. Inflammation and fibrosis histopathological scoring were similar in all cases. B2 and B3 groups showed a very good clustering regarding 30 significantly differentially expressed genes, all being remarkably upregulated in B3. More than half of these genes were involved in Crohn's disease fibrogenesis, and eight differentially expressed genes were so in other organs. The most significantly active biological processes and pathways in penetrating disease were response to TGFβ and matrix organisation and degradation, as validated by immunohistochemistry.

Conclusions: Despite the histopathological similarities in fibrosis between stricturing and penetrating ileal Crohn's disease, their fibrosis-related transcriptomic profiles are distinct. Penetrating disease exhibits a distinctive transcriptomic landscape related to enhanced matrix remodelling.

Key Words: Crohn's disease; fibrosis; transcriptome; TGFβ expression

Graphical Abstract



1. Introduction

Crohn's disease [CD] is a chronic inflammatory disorder of the digestive tract, with transmural inflammation and submucosal fibrosis as disease hallmarks.[1] Intestinal fibrosis is defined by excessive extracellular matrix accumulation [ECM] and mesenchymal cell expansion, usually in response to chronic inflammation, but also in the absence of inflammatory mediators.[2,3] Fibrosis underlies disease complications that usually require surgery, such as strictures, intestinal obstruction, and penetrating events.[2] Moreover, the presence of internal penetrating disease is associated with strictures in over 85% of cases.[2]

Current treatments cannot avoid fibrosis progression nor revert established complications.[4] Understanding the molecular and cellular networks underpinning inflammation and fibrogenesis in CD might contribute to the identification of therapeutic targets to prevent disease progression. Given the transmural nature of CD, such understanding requires full-thickness study of the intestinal wall.[5] With few exceptions,[6–9] research on CD fibrosis has focused on stricturing disease of the terminal ileum.[5] This derives from poor accessibility to human tissue representing fistulizing disease but also from inexistent *in vitro* and scarce *in vivo* models.[10] However, it is essential to understand the biological processes behind fissure/fistula development and fibrosis, including those not related to inflammation.[10,11] Histopathologically, intestinal strictures present inflammatory and fibrotic components,[3,12,13] and the same applies to fistulizing CD.[9–11] In a previous study, our group could not find differences in transmural fibrosis and fibromuscular changes between penetrating and stricturing ileal CD by histopathological analysis.[8] In this setting, our study intended to unveil differences between stricturing and penetrating CD at the molecular level, focusing on the transcriptomic landscape related to fibrosis, and to correlate the transcriptomic findings with histopathological inflammation and fibrosis scorings.

2. Materials and methods

2.1. Case series

Our case series included ileal surgical specimens from CD patients previously characterized by our group.[8] From the original case series [n = 103],[8] 36 cases were selected: 12 with pure stricturing disease [B2, Montreal classification],[14] 12 with penetrating disease and also stricture[s] [B3s], and 12 with penetrating disease without stricture[s] [B3o]. On the basis of the macroscopic pictures and/or description, sections representative of "*the most affected area*" were selected for molecular study by Nanostring nCounter assay by two gastrointestinal pathologists [IG and FC]. The most affected area corresponded to the narrowest caliber area of the ileal stricture for cases only showing strictures or to the most severely inflamed ileal area involved by fistulas, fissures, and/or deep ulcers. Only sections with well-preserved, non-necrotic tissue and sections representative of the whole thickness of the ileal wall were considered.

The histopathological scoring system data were retrieved from our previous study.[8] Inflammation and fibrosis were graded following a specific histopathological scoring developed for transmural alterations in CD, as described by Chiorean MV et al.[15]

2.2. Gene expression profiling by Nanostring nCounter assay

Prior to ribonucleic acid [RNA] extraction, tissue sections were manually macrodissected, using a scalpel, excluding contaminants [e.g. extensive neutrophilic exudate, granulation

tissue, necrosis, debris], to ensure adequate and representative transmural changes due to CD. RNA input levels were increased according to fragmentation status, following NanoString recommendations.

Formalin-fixed and paraffin-embedded [FFPE] total RNA from each sample was isolated at Ipatimup Diagnostics from three independent extractions, each containing three sections of 20 µm thick, using automated extraction—Maxwell RSC 16 equipment with RCS RNA FFPE Kit [Promega, USA]. After isolation, the three extractions were pooled and column concentration was performed using Amicon Ultra centrifugal filters 3K [Merck Millipore, Germany] to increase the final concentration of RNA.

RNA concentrations were quantified by using Qubit 3.0 Fluorimeter [Invitrogen, USA] and fragmentation was evaluated through Agilent 2100 Bioanalyser. A minimum of 400ng of total RNA was used for each sample. To detect any procedural error during the material processing, three separate batches were run on different dates [Supplementary Table 1].

For Nanostring nCounter assay, we used the nCounter® Fibrosis Consortium Panel comprising 787 genes covering the core pathways and processes involved in fibrosis in several human tissues and organs [Supplementary Table 2], and 11 internal reference genes for data normalization. The data analysis was conducted using ROSALIND®, which utilizes a HyperScale architecture developed by ROSALIND, Inc. in San Diego, CA. As part of the quality control [QC] process, various visual representations, including read distribution percentages, violin plots, identity heatmaps, and sample MDS plots were generated. Normalization, fold change calculations, and p-value determinations were executed following the criteria outlined by Nanostring. ROSALIND® adheres to the nCounter® Advanced Analysis protocol. The calculation of fold changes and p-values follows the fast method described in the nCounter® Advanced Analysis 2.0 User Manual, with p-value adjustment performed using the Benjamini-Hochberg method to estimate false discovery rates [FDR]. We performed a comprehensive QC analysis, with evaluation of the quality of the extracted RNA and the distribution of cases. Principal Component Analysis [PCA] was employed using the prcomp function from the 'stats' R package [version 4.2.2] to assess potential batch effects and identify outlier cases. This analysis aimed to ensure the reliability of the data and to detect any sources of variation, such as batch effects or unusual observations. For the selection of the significantly differentially expressed genes [DEG] we adopted a p-adjusted < 0.05 and a Fold Change ≤ -1.5 or Fold Change ≥ 1.5. Gene clustering for the final heatmap of differentially expressed genes was carried out using the Partitioning Around Medoids [PAM] method.

Enrichment analysis was performed using cluster Profiler R package along with Gene ontology [GO] terms, Kyoto Encyclopedia of Genes and Genomes [KEGG] and Reactome databases.

2.3. Immunohistochemical analysis

Based on the data obtained from Nanostring nCounter assay, TGFβ1 protein expression was analysed by immunohistochemistry [IHC] in 12 selected specimens with representative lesions. The same formalin-fixed, paraffin-embedded [FFPE] block was used for Nanostring nCounter assay, quantitative polymerase chain reaction [qPCR] and IHC analysis. From each selected FFPE tissue block, serial 3-µm tissue sections were prepared. Immunohistochemical staining was performed in an automated Ventana BenchMark ULTRA Staining System, using the OptiView DAB IHC Detection Kit [Roche/Ventana Medical Systems, Tucson, AZ], according to the manufacturers' instructions. TGFβ1 antibody [clone TB21; Invitrogen, Thermo Fisher SCIENTIFIC, USA] was used with 1:2000 dilution, following an antigen retrieval step using pH = 6.0 acidic citrate buffer. For each stained tissue slide, external positive and negative controls were used [breast cancer and normal colon wall specimens, respectively]. TGFβ1 expression was evaluated by two board-certified pathologists with experience in gastrointestinal pathology [IG, FC].

2.4. qPCR analysis

To validate the Nanostring nCounter assay transcriptomics data, RNA from three B2 samples, three B3 samples and one cell line used as positive control [MKN74] were used to perform qPCR analysis. Samples were selected from B3 and B2 groups on the basis of available extracted RNA combined with the values of normalised counts of four selected genes: transforming growth factor β1 [*TGFβ1*], connective tissue growth factor [*CTGF*], the A disintegrin, and metalloprotease 17 [*ADAM17*], and carcinoembryonic antigen cellular adhesion molecule 3 [*CEACAM3*] [Supplementary Table 3].

cDNA was synthesised using 1 µg of RNA from B2 and B3 samples and 200 ng of RNA for MKN74, with SuperScript reverse Transcriptase II [Invitrogen], according to the manufacturer's protocol. The qPCR reaction was performed with KAPA PROBE FAST qPCR Master Mix [2x] kit [Applied Biosystems] and probes for *ADAM17* [Hs.PT.58.1898859, IDT], *TGFβ1* [Hs.PT.58.39813975, IDT], *CTGF* [Hs.PT.58.4521527.g, IDT], *CEACAM3* [Hs.PT.58.2763884, IDT], and 18S [custom assay, IDT] as endogenous control. Expression analysis of 18S was performed in MKN74 cells to assess the RNA quality. Reactions were sequenced on a 7500 Real-Time PCR System [Applied Biosystems].

2.5. Statistics

All statistical analyses were conducted using the IBM SPSS Statistics for Mac, Version 27.0 [IBM, Armonk, NY]. A level of significance at 5% was established. Descriptive data were expressed as absolute [n] and relative frequencies [%] for categorical variables. Continuous variables are summarised as mean with standard deviation [SD] or median with interquartile range [IQR], depending on the statistical distribution. Comparison of categorical variables was tested through chi square test and of continuous variables using paired samples t test or Wilcoxon test, based on statistical distribution.

2.6. Ethical statement

Patients were selected from the population of a previous study from our group,[8] hence the same ethical considerations apply.

3. Results

3.1. Setting the sample for analysis with quality control and batch effects analysis

The quality control [QC] process revealed that the majority of the NanoString quality parameters were met in all the included samples [Supplementary Table 1]. The principal component analysis [PCA] graph showed a normalisation of the distribution of the 36 cases, meaning that this feature should not interfere with the transcriptomic results and that no batch

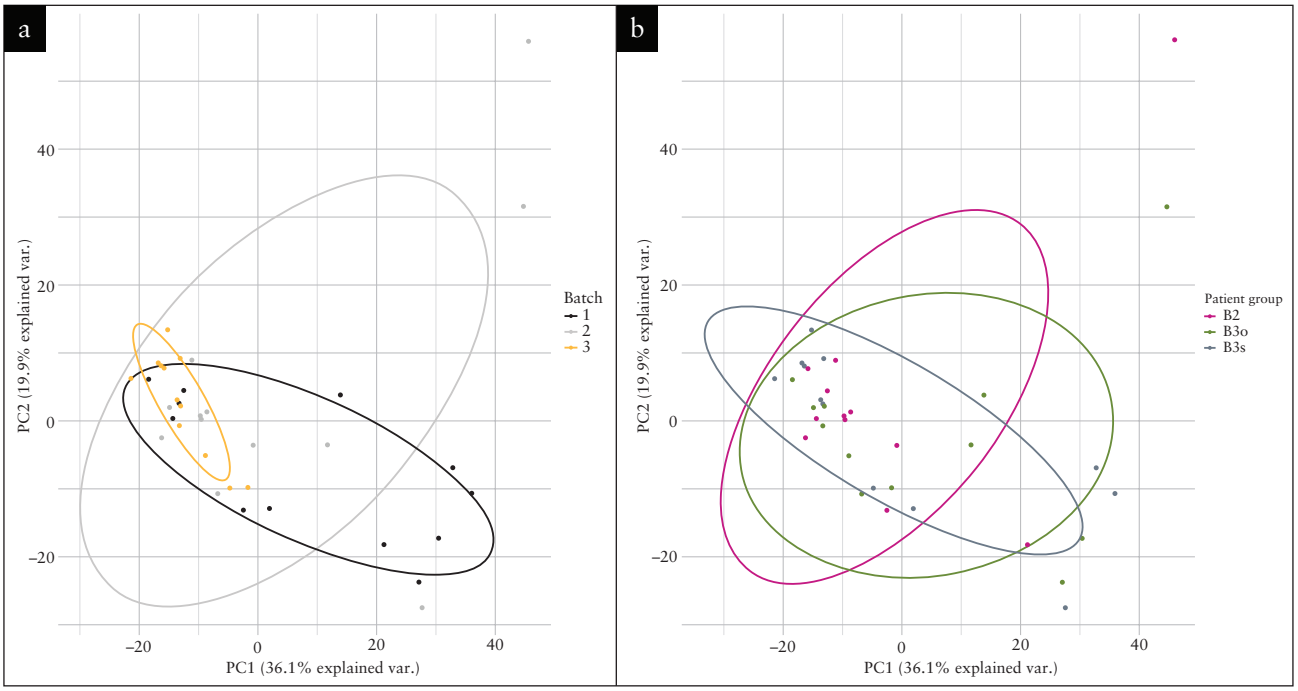


Figure 1 Principal component analysis [PCA] graphs per batch [1a] and per patient group [1b].

effect was noticed [Figure 1a]. However, PCA graph per patient group evidenced two outliers, one from the B2 group and one from the B3o group [Figure 1b], sufficiently distant to be considered outliers. This observation was further validated using the 'pca.outliers' function from the 'mt' R package [version 2.0-1.20], with a confidence level of 0.99. Additionally, upon examining various clinical variables through PCA, no distinct clustering by these features was observed. Thus, no clinical criteria suggest a significant difference. These two cases were excluded from further analyses.

3.2. The study sample is balanced for demographic, clinical, and histopathological features

The study sample comprised 34 patients: 11 B2, 12 B3s, and 11 B3o, who were followed up for a median time of 16.0 [IQR 15.0–17.0], 14.0 [13.8–14.3], and 14.0 [13.0–14.0] years, respectively. The demographic and clinical variables and histopathological scoring are displayed in Table 1. The groups were globally balanced in all variables [p >0.05].

Histopathological scoring regarding fibrosis and inflammation were comparable for all three groups. For the fibrosis score, a median of 1 [min-max: 1-2 for B2 and B3o; 1-1 for B3s] was observed, and for the inflammation score a median of 3 [min-max: 1-3 for the three groups] was noticed. In particular, the amount of fibrosis, as evaluated by haematoxylin and eosin sections and Masson trichrome staining, was similar in B2 and B3 phenotypes [Figure 2], as previously reported.[8] The histopathological scoring resemblances hampered a correlation study with the transcriptomic findings.

3.3. Penetrating disease sub-groups are genetically similar and can be clustered for further analyses [B3]

The analysis of the transcriptome of the 23 penetrating CD patients demonstrated that B3s and B3o patients showed similar transcriptomic profiles, as no differentially expressed genes [DEGs] were found between the two groups [Figure 3]; these two groups were grouped as B3 for further analyses. On the contrary, 46 DEGs effectively clustered the B2 and B3s groups, which were thus distinct groups that should not be grouped [Supplementary Figure 1].

3.4. B3 and B2 groups display distinct transcriptomic fibrosis signatures, with upregulation of all differentially expressed genes in B3 patients.

The fibrosis-related transcriptomic landscape of the 34 cases, grouped by Montreal phenotype [11 B2 and 23 B3], revealed a total of 30 DEGs between the two groups. A very good clustering of the cases was observed, with B3 and B2 patients grouping in different clusters [Figure 4]. A total of 30 DEGs highlighted significant differences between the B3 and B2 phenotypes, with upregulation in B3 patients [Figures 4 and 5].

The volcano plot in Figure 5 displays the distribution of the 30 DEGs, according to the p-value and fold change. The connective tissue growth factor [*CTGF*] gene showed the highest consistency in the B3 group, whereas the phosphatidic acid phosphatase type 2 domain containing 1A phospholipid phosphatase 4 [*PPAPDC1A/PLPP4*] gene appeared as the most differentially expressed between the two groups, followed by the hydrogen voltage-gated channel 1 [*HVCN1*] and the *CEACAM3* genes. The key profibrotic gene *TGFβ* displayed a significant level of fold change, underscoring the significance of this gene in distinguishing the two groups.

3.5. The most differentially expressed pathways between B3 and B2 groups are related to TGFβ response and matrix organisation and degradation

The biological process that included more DEGs and was most significantly activated in the penetrating CD group was the response to TGFβ [p adjusted = 6.01E-08], followed by ECM organisation [p adjusted = 6.61E-08] [Figure 6a and

Table 1 Demographic and clinical variables and histopathological scoring.

Demographical and clinical variables	B2 [*n* = 11]	B3s [*n* = 12]	B3o [*n* = 11]
Gender, *n* [%]			
Female	6 [56]	6 [50]	5 [46]
Male	5 [46]	6 [50]	6 [56]
Age at diagnosis [years], mean ± SD	34 ± 10	28 ± 11	31 ± 10
CD localisation, *n* [%]			
L1, terminal ileum [TI]	7 [64]	6 [50]	4 [36]
L1 + L4, TI + upper digestive tract	1 [9]	1 [8]	1 [9]
L3, ileocolonic	2 [18]	4 [33]	6 [56]
L3 + L4, ileocolonic + upper digestive tract	1 [9]	1 [8]	0 [0]
Perianal disease, *n* [%]	1 [9]	4 [33]	4 [36]
Pre-operative therapy, *n* [%]			
5-aminosalicylic acid	7 [64]	7 [58]	5 [46]
Steroids	9 [82]	10 [83]	6 [56]
Immunosupressives	5 [46]	8 [67]	2 [18]
Anti-tumour necrosis factor alpha	0 [0]	2 [17]	2 [18]
Age at index surgery [years], mean ± SD	37 ± 10	38 ± 12	35 ± 10
Total follow-up [years], median [P25-P75]	16.0 [15-17]	14.0 [13.8-14.3]	14.0 [13.0-14.0]
Histopathological score[a]			
Inflammation scoring [1-3], median [min-max]	3 [1-3]	3 [1-3]	3 [1-3]
Fibrosis scoring [0-2], median [min-max]	1 [1-2]	1 [1-1]	1 [1-2]

B2, stricturing CD; B3s, penetrating CD with stricture[s]; B30, penetrating CD without stricture[s]; CD, Crohn's disease; L, localisation of CD; SD, standard deviation.
[a]Histopathological scoring in: Chiorean MV, Sandrasegaran K, Saxena R, Maglinte DD, Nakeeb A, Johnson CS. Correlation of CT enteroclysis with surgical pathology in Crohn's disease. *Am J Gastroenterol* 2007;**102**:2541–50. doi: 10.1111/j.1572-0241.2007.01537x.

Supplementary Table 4a]. In both, *TGFβ1* is the most differentially expressed gene [*p* adjusted = 0.014623] followed by the collagen type 1, 3, and 4 genes, *COL1A1* [collagen type I α1 chain, *p* adjusted = 0.014839], *COL1A2* [collagen type I α2 chain, *p* adjusted = 0.01791], *COL3A1* [collagen type III α1 chain, *p* adjusted = 0.018271] and *COL4A2* [collagen type IV α2 chain, *p* adjusted = 0.018848] [Supplementary Table 5].

Likewise, the biological pathways that included more DEGs and were most significantly activated in the penetrating CD group were related to collagen degradation [*p* adjusted = 3.21E-10], ECM degradation [*p* adjusted = 2.37E-09] [Figure 6b and Supplementary Table 4b], and ECM organisation [*p* adjusted = 3.30E-08]. These pathways exhibited similar DEGs, namely collagen genes *COL1A1* [*p* adjusted = 0.014839], *COL1A2* [*p* adjusted = 0.01791], *COL6A3* [collagen type VI α3 chain, *p* adjusted = 0.017942], *COL3A1* [*p* adjusted = 0.018271], *COL4A2* [*p* adjusted = 0.018848], *COL5A3* [collagen type V α3 chain, *p* adjusted = 0.032931], and *ADAM17* [*p* adjusted = 0.025322]. ECM organisation pathway also included the *TGFβ1* gene [*p* adjusted = 0.014623] [Supplementary Table 5].

3.6. TGFβ1 pathway activation and protein expression are more frequent in penetrating CD [B3 groups] compared with pure stricturing CD [B2 group]—validation of transcriptomic data by immunohistochemistry analysis

To validate the results obtained by Nanostring nCounter Assay regarding *TGFβ1* pathway enrichment in the penetrating CD group, we performed IHC analysis to evaluate TGFβ1 expression in 12 samples, selected on the basis of available material after RNA extraction. This subset of cases included five B2 specimens, five B3s specimens, and two B3o specimens. The results of IHC analysis and comparison with mRNA expression levels [normalised counts by Nanostring nCounter Assay] are shown in Table 2. The B3 cases showed higher levels of *TGFβ1* mRNA expression and higher TGFβ1 protein expression [57%] compared with B2 cases [20%] [Figure 7a vs 7c]. Statistical analysis was not performed due to the low number of cases in which IHC staining was performed. Positive cases showed strong TGFβ*1* expression [in at least 5% of analysed tissue section] in fibroblasts-like cells and surrounding collagenous matrix [Figure 7b], as described previously.[16,17] Positive areas were observed mostly in the submucosa and subserosa layers.

3.7. The qPCR approach did not validate the current analysis

As mentioned, B2 and B3 samples were selected according to the available extracted RNA and to the normalised counts of the *ADAM17*, *TGFβ1*, *CTGF*, and *CEACAM3* genes, which were higher in the B3 group than in the B2 group [Supplementary Table 3].

The qPCR analysis of the four genes returned undetermined Ct [cycle threshold] values for most samples [Supplementary Table 6a–e and Supplementary Figure 2a–e].

4. Discussion

This study demonstrated that despite the histopathological similarities of the fibromuscular changes between stricturing

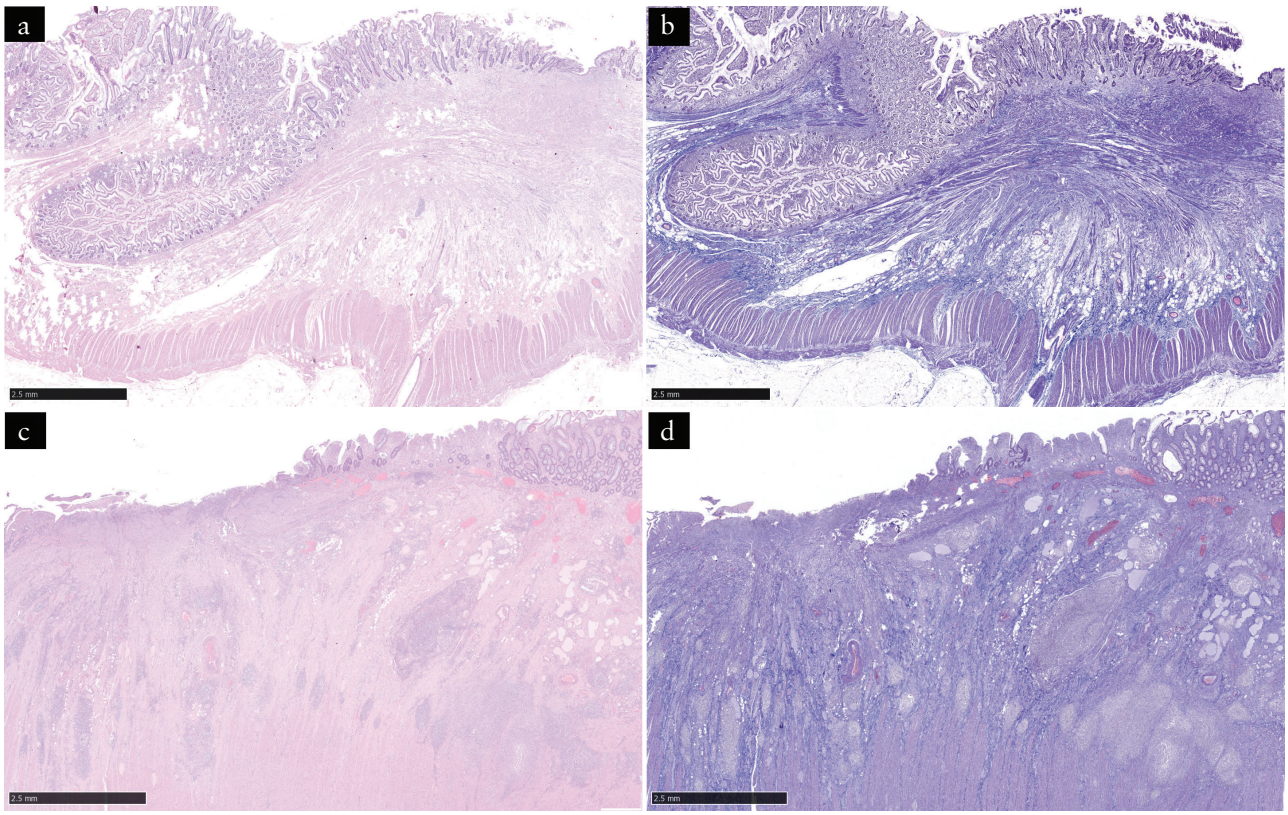


Figure 2 Similar transmural fibrosis score in B2 [a, HE, low magnification; b, Masson trichrome, low magnification] and B3 [c, HE, low magnification; d, Masson trichrome, low magnification] phenotypes were observed. HE, haematoxylin and eosin.

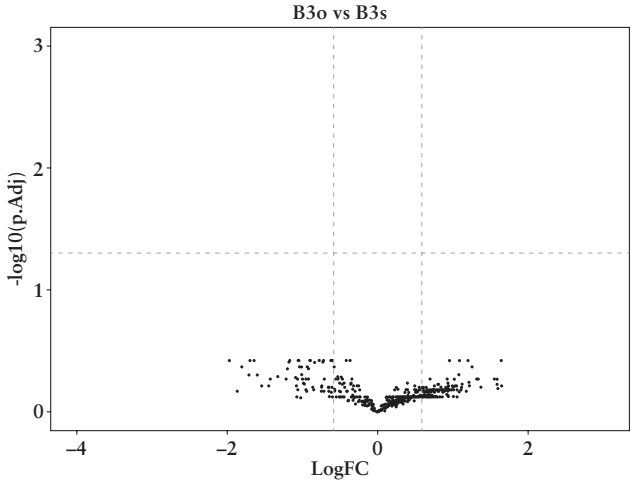


Figure 3 Vulcano plot of differentially expressed genes in patients with [B3s] and without associated stricture [B3o].

[B2] and penetrating [B3] ileal CD,[8] the transcriptomic landscape differed in 30 highly discriminative and significantly DEGs, out of a 787 fibrosis-related gene panel.

Over half of the 30 significant DEGs are known to be involved in CD pathogenesis; eight genes are well established in CD fibrogenesis, namely those encoding the key profibrotic mediator *TGFβ1*,[18–21] its downstream effector *CTGF*,[5,22] and the collagen constituents, *COL1A1*, *COL1A2*, *COL3A1*, *COL4A2*, *COL5A3*, and *COL6A3*.[2,6,23,24] Although their role is not fully understood, further eight DEGs are also involved in CD fibrogenesis, namely *BCL2* [B cell lymphoma 2], *LATS2* [large tumor suppressor kinase 2], *POSTN* [encoding for periostin], *XBP1* [X-box binding protein 1], *CEACAM-3*, *S100A12* [encoding for calgranulin C], *ADAM17*, and *HVCN1*.

BCL2,[25,26] *LATS2*,[27,28] *POSTN*[29], and *XBP1*[30] have been related to profibrotic activity in CD, and *POSTN*[31] and *LATS2*[27] were also associated with increased inflammatory activity. The *CEACAM-3*,[32] *S100A12*,[33] *ADAM17*,[34] and *HVCN1*[35,36] genes are all pro-inflammatory genes in CD. *CEACAM-3* might play a role in the onset of the disease through involvement in bacterial breaking of the intestinal barrier.[37–39] Both *S100A12* and *ADAM17* genes are involved in the early neutrophilic phase of intestinal inflammation and correlate with CD activity.[33,34] This evidence suggests that these genes might have a role in the development of the acute phase of the inflammatory cascade of CD fibrogenesis.

Our study showed that the most important biological processes and pathways in penetrating disease involve response to TGFβ and ECM organisation and degradation. The activation of these processes is supported not only by an accurate molecular profiling platform, but was also validated on the protein level by immunohistochemical analysis of TGFβ1 expression. Response to TGFβ seems pivotal, not only because *TGFβ1* and its effector gene *CTGF* are among the most significant DEGs, but also because *TGFβ1* and its collagen target genes were the most significant DEGs in the most activated pathways. Moreover, the CD profibrotic genes *BCL2*, *LATS2*, *POSTN,* and *XBP1*[25,26,28] and four of the eight profibrotic genes in other organs [Supplementary Table 7], namely *TXNDC5*, *LRRC32*, *CD44*, and *LTB*,[40–47] are all TGFβ mediated. Response to oxygen levels and

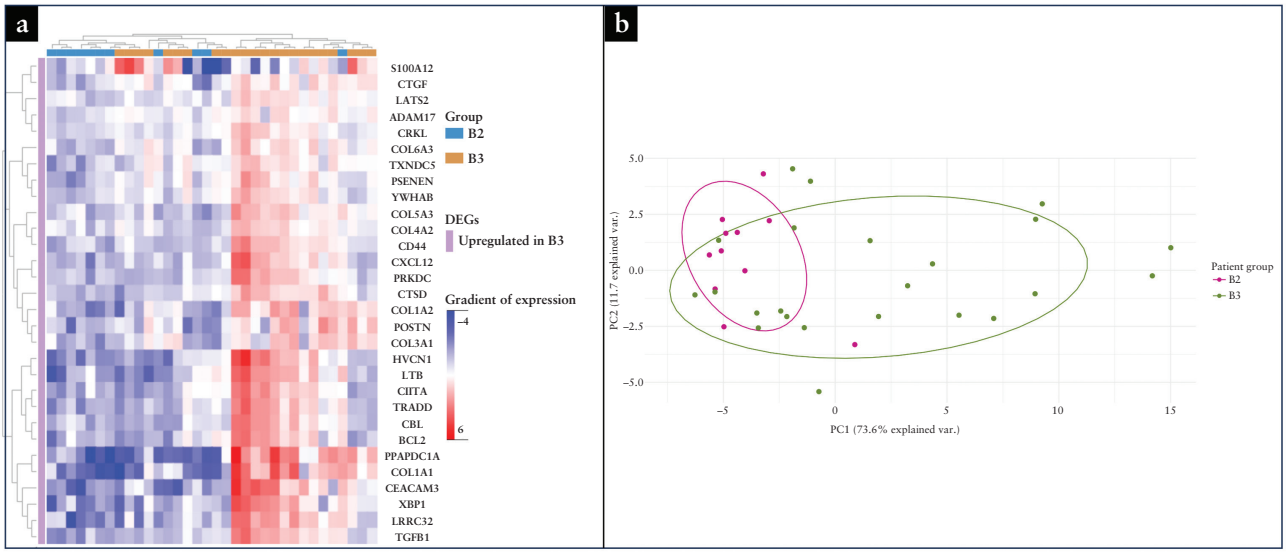


Figure 4 Differentially expressed genes profile. 4a, Heatmap of the transcriptional profile for the 30 differentially expressed genes [DEGs] in penetrating [B3] and stricturing [B2] Crohn's disease; 4b, PCA graph per patient group of DEGs in B2 and B3. PCA, principal component analysis.

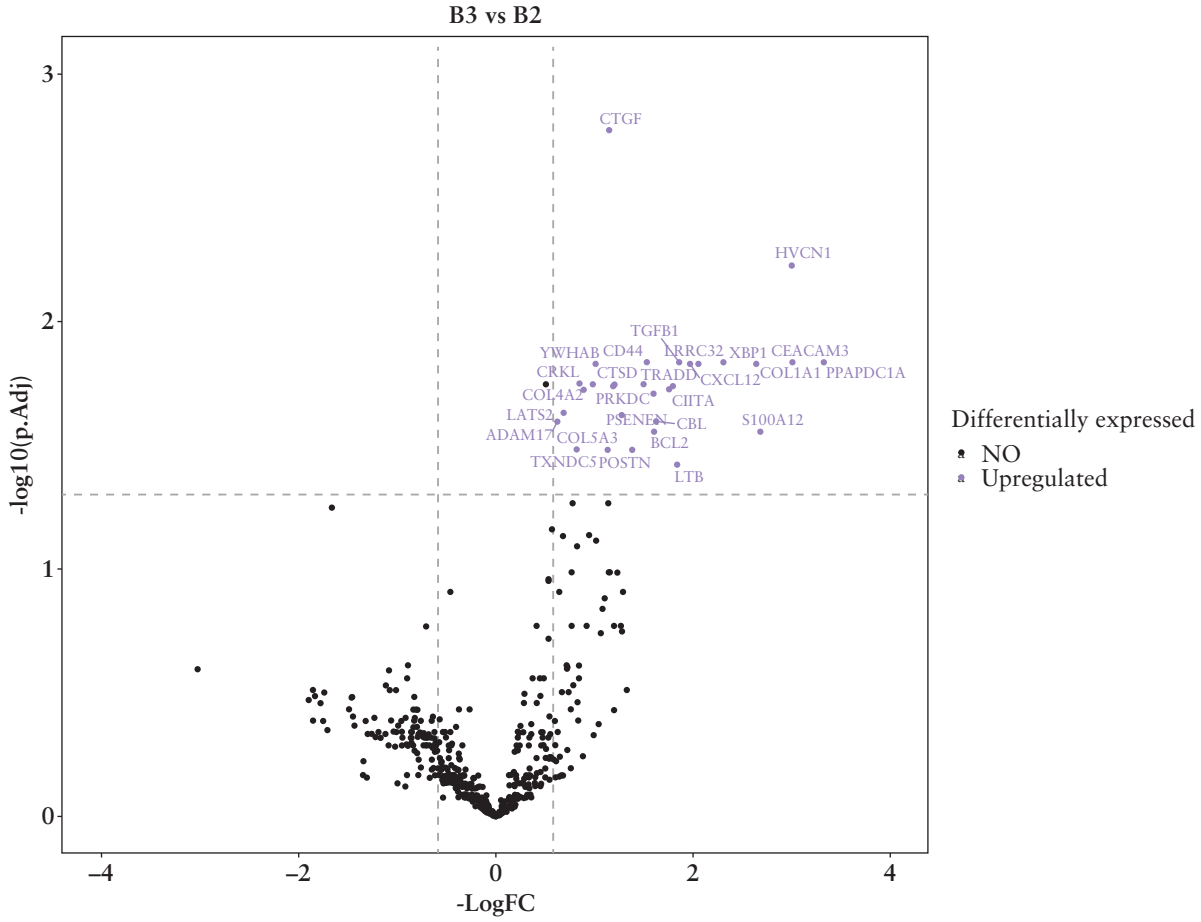


Figure 5 Vulcano plot of 30 differentially expressed genes between penetrating [B3] and stricturing [B2] Crohn's disease groups. FC, fold change.

muscle development were also significantly activated biological processes in the penetrating group. Thickening of the muscle layers is a histopathological characteristic of CD, as a result of the uncontrolled expansion and transdifferentiation of mesenchymal cells, upon exposure to TGFβ1.[48] These cellular processes require rapid energy-generating mechanisms, like the fibroblasts' shifting from oxidative phosphorylation to aerobic glycolysis, which in turn leads to the production of profibrotic metabolites, some by means of TGFβ1 activation.[49,50]

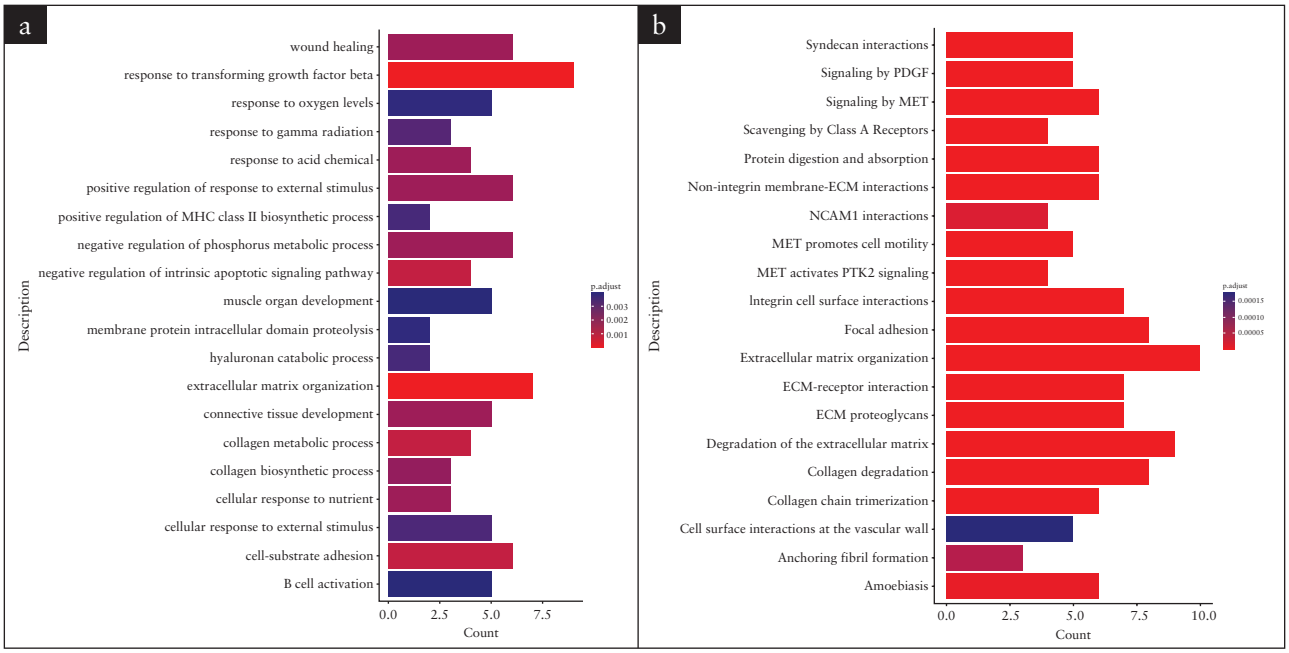


Figure 6 Enrichment analysis for 30 differentially expressed genes [DEGs] between penetrating [B3] and stricturing [B2] Crohn's disease. 6a, gene ontology terms [biological processes, molecular function and cellular component]; 6b, biological pathways [reactome and KEGG databases]. Right vertical red-blue fading bar: adjusted *p*-value [brighter red means more significant]; bar height: number of DEGs involved.

Table 2 TGFβ1 mRNA and protein expression in stricturing [B2] and penetrating [B3] Crohn's disease cases selected for immunohistochemistry analysis.

Case ID	TGFβ1 mRNA expression [normalised counts]	TGFβ1 protein expression
B2 group [*n* = 5]	Median value = 7.98	Positive cases [n = 1/5, 20%]
#1	7.59	Negative
#2	7.61	Negative
#3	7.98	Positive
#4	8.05	Negative
#5	8.14	Negative
B3 group [*n* = 7]	Median value = 10.61	Positive cases [n = 4/7, 57%]
#1	9.12	Negative
#2	9.79	Positive
#3	10.05	Negative
#4	10.61	Positive
#5	11.00	Positive
#6	11.34	Negative
#7	12.73	Positive

The simultaneous and significant activation of ECM and collagen degradation pathways indicate an accelerated ECM turnover in penetrating disease. ECM turnover continues in established intestinal fibrosis and it may even accelerate with increased ECM deposition,[2] as occurred herein. Even though fibrogenesis is accompanied by an imbalance between MMPs and tissue inhibitors of metalloproteinases [TIMPs], it remains unproven that this imbalance drives the excessive ECM deposition in CD fibrosis.[3,51] Given the similar histopathological fibrosis [Figure 2], we did not find a differential expression of MMP or TIMP genes between the two phenotypes.[51] However, *ADAM17* was involved in ECM and collagen degradation pathways, suggesting a role as a matrix protease.

Recent evidence suggested that the pathogenesis of penetrating CD might include a combination of epithelial-mesenchymal transition [EMT] and overexpression of MMPs in response to an epithelial defect mediated by pro-inflammatory cytokines,[10] which herein may be related to *CEACAM-3*, *S100A12*, and *ADAM17* genes. It was also proposed that penetrating and stricturing CD pathophysiology may differ, in part, through different responses to the same key cytokines TNF-α, IL-13, and TGFβ.[52–54] Importantly, the TGFβ induction of EMT and activation of the pro-invasive β6-integrin seem exclusive of penetrating CD.[7,10,53,55] Herein, after the possible break of the epithelial barrier mediated by *CEACAM-3* gene, the TGF-β-driven invasiveness of the

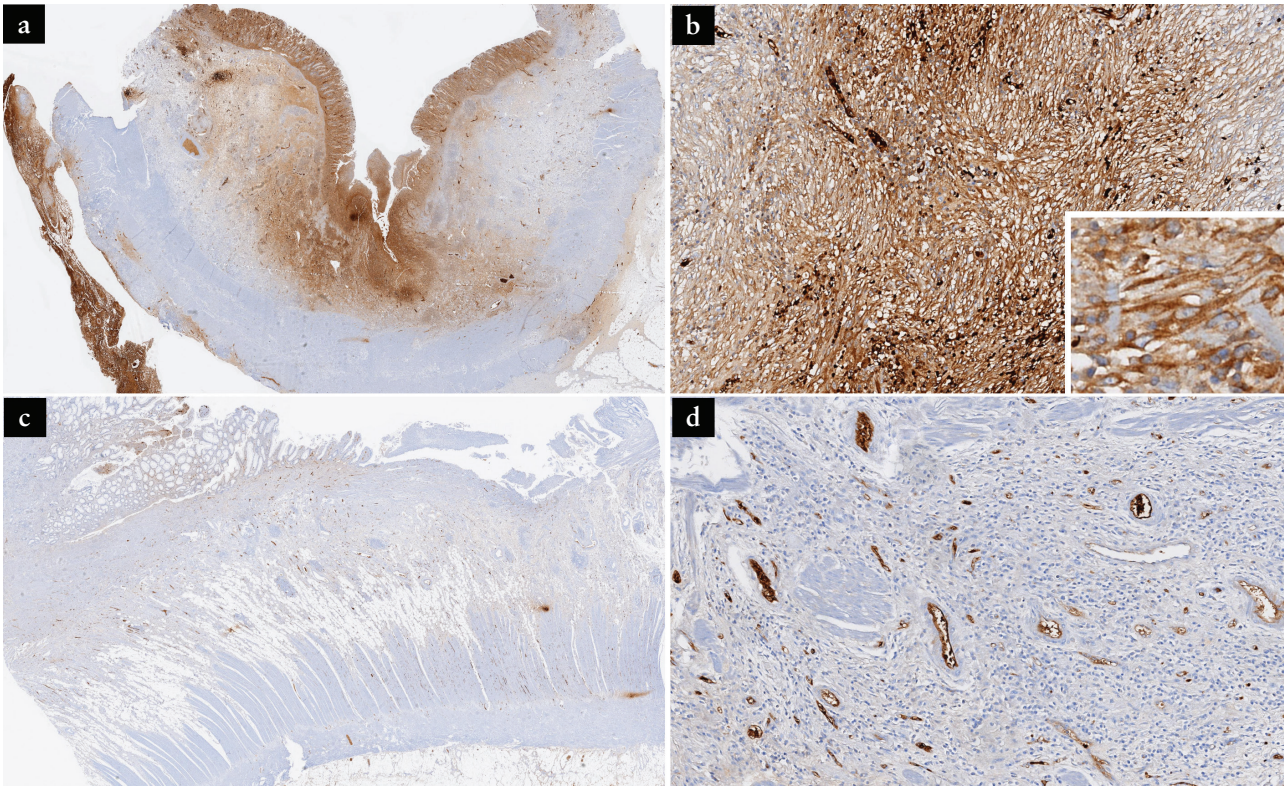


Figure 7 B3 cases showed more frequently expression of TGFβ1 in the submucosa and subserosa layers [7a, TGFβ1 immunostaining, low magnification], compared with B2 cases, with pure structuring phenotype, [7c, TGFβ1 immunostaining, low magnification]. Positive cases showed TGFβ1 expression in fibroblast and surrounding collagenous matrix, [7b, TGFβ1 immunostaining, 100x amplification and inset, 400x amplification]. Negative cases showed expression only in red blood cells [d, TGFβ1 immunostaining, 100x amplification].

intestinal wall along with penetrating inflammation could result in the development of deep ulcers, fissures, and fistulae. *ADAM17* gene involvement in matrix and collagen degradation might further facilitate penetration phenomena, and the concomitant strong profibrotic activity would promote ECM reorganisation. Taking all together, we propose that in penetrating disease, *TGFβ1* is strongly involved not only in profibrotic events but also, and most distinctively, in the promotion of invasive behaviour in a way that the increased ECM turnover becomes incapable of healing or closing the fissures and tracts. Our results are also strengthened by the demonstration of higher levels of TGFβ1 expression in B3 phenotypes as compared with B2 pure stricturing CD. Moreover, we speculate that the increased ECM turnover is specifically related to an ongoing attempt of the tissue to achieve this closure.

This study has some limitations. First, although no batch effect was noticed in the PCA graph, two outlier patients were identified and excluded, reducing the study sample to 34 patients. Second, gene expression analysis of FFPE samples is challenging because the RNA is usually more degraded than RNA from other sources. The impact of this issue was evidenced by the failure of the qPCR approach to validate the results obtained by Nanostring nCounter Assay, which may be due to the poor quality of the material used. In fact, the NanoString platform has been designed and validated as being able to retrieve reliable results from very poor-quality samples or small quantity of FFPE tissues, which cannot be matched with a regular qPCR result,[56] as herein observed. Moreover, previous studies have demonstrated that NanoString data from FFPE samples is highly correlated to data from fresh-frozen samples, if high amounts of RNA are available,[56] as occurred in our study. Third, most of the included cases present grade 1 fibrosis with predominant submucosal fibrosis, which could potentially introduce a bias in the results. However, some cases presented grade 2, transmural, fibrosis. Hence, the full thickness of the intestinal wall is mandatory to adequately assess the fibrosis-related transcriptome of CD.

The strengths of our study rely on the consistency of the transcriptomic findings, where the two CD phenotypes were highly clustered by the 30 DEGs, with upregulation in penetrating disease. Moreover, their involvement in the most active biological pathways adds to the current knowledge on CD fibrogenesis and fistula formation.

5. Conclusion

This study demonstrated, for the first time, that the fibrosis-related transcriptomic profiles of stricturing and penetrating ileal CD are distinct, despite similarity of fibrosis amount in the two phenotypes by histopathological analysis. Even though the study sustains the unmet need of CD antifibrotic therapy, and fibrosis underlies both penetrating and stricturing disease, therapeutic targets may differ in the two phenotypes. Further studies on penetrating disease are needed to unveil the mechanisms underlying this more aggressive phenotype.

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Conflict of Interest

The authors have no competing interests to report.

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Author Contributions

HTS: planned and designed the study, conducted the study, collected and critically interpreted the data, drafted the manuscript, and revised it critically for important intellectual content. MF: collected and critically interpreted the data, drafted the manuscript, and revised it critically for important intellectual content. IG and AMF: planned and designed the study, conducted the study, collected and critically interpreted the data, drafted the manuscript, and revised it critically for important intellectual content. AP and DL: after first revision, collected and critically interpreted the data [qPCR and IHC analysis, respectively], drafted the new contents of the manuscript, and revised the last version of the manuscript. CO, FC, and FM: planned and designed the study, critically interpreted the data, drafted the manuscript, and revised it critically for important intellectual content. All authors are accountable for all aspects of the work. All authors approved the final version of the article, including the authorship list.

Conference presentations: Digital Oral Presentation [DOP 56] at the 19th Congress of ECCO—Inflammatory Bowel Diseases 2024, in Stockholm, Sweden, at the 2024 Annual Meeting of the IBD Portuguese Study Group [GEDII] in Lisbon, Portugal and at the Portuguese Digestive Week in Algarve, Portugal. Also as a poster [Tu1760] at the Digestive Disease Week 2024, in Washington DC, USA.

Data Availability

The transcriptome data from NanoString nCounter® were deposited at GEO [https://www.ncbi.nlm.nih.gov/geo/], and their accession number is GSE259353. Other data underlying this article will be shared on reasonable request to the corresponding author.

Supplementary Data

Supplementary data are available at *ECCO-JCC* online.

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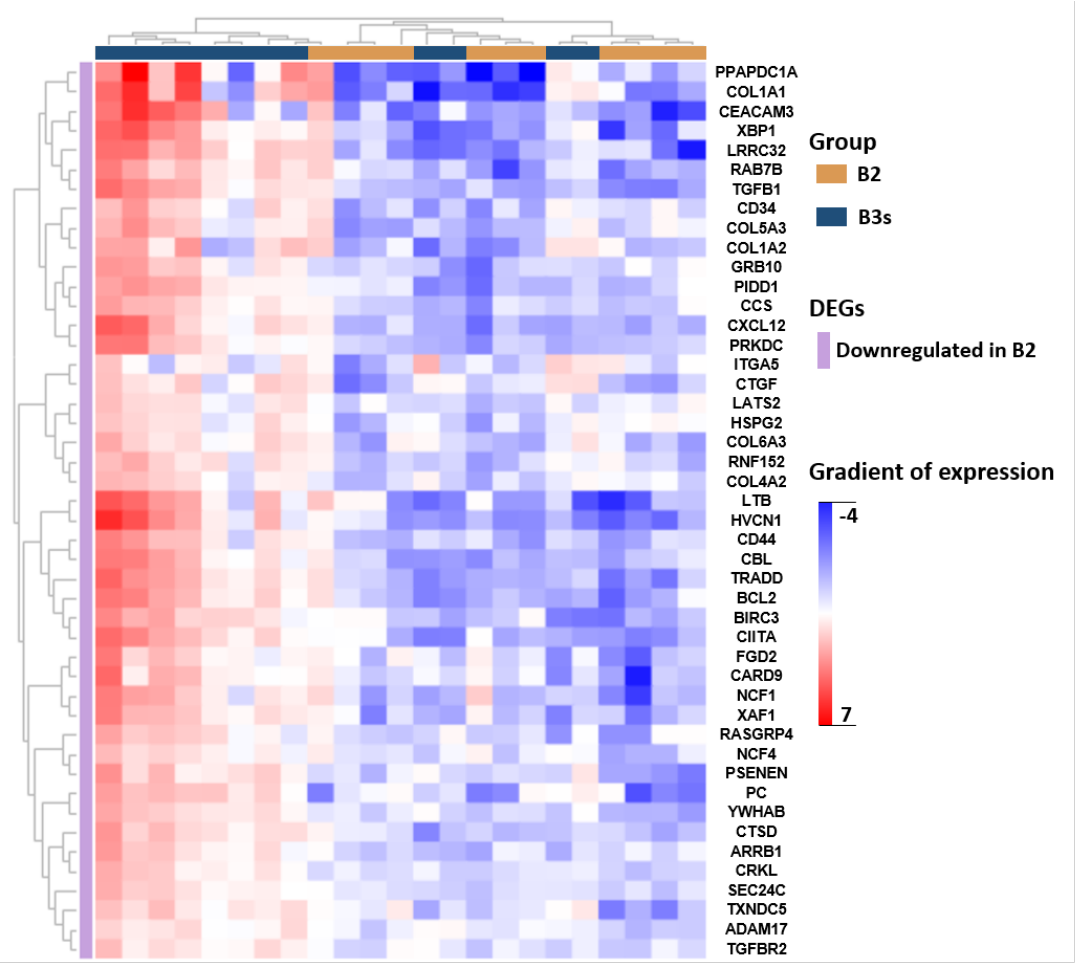
SUPPLEMENTARY MATERIAL

Supplementary Figure 1. Heatmap of the transcriptional profile for the 46 differentially expressed genes (DEGs) in stricturing (B2) and penetrating Crohn's disease with associated stricture (B3s).



Supplementary Table 1. Demographical and quality control per batch.

Patient Group	Patient sex	Imaging quality	Binding density	Positive control linearity	Mean of Negative Controls	SD of Negative Controls	Positive Control E	Positive Control A	Housekeeping Genes > 50
					Batch 1				
B2	M	1	0.34	1	4.38	5.21	55	13998	100
B2	M	1	0.32	0.99	6.38	2.07	49	18730	90
B2	F	0.99	0.26	0.99	6.12	8.31	43	14157	80
B2	F	1	0.59	0.99	5.38	4.37	49	13654	100
B3o	F	1	0.22	0.99	4.88	4.7	43	15441	80
B3o	M	1	0.32	0.99	9	10.98	55	18704	80
B3o	F	1	0.32	0.99	5.12	4.45	49	16529	100
B3o	M	1	0.39	0.98	6.38	5.07	31	12955	90
B3s	M	1	0.25	0.99	6.25	5.9	50	18386	80
B3s	F	1	0.36	0.99	10.62	13.08	56	18255	80
B3s	M	1	0.57	0.99	7.5	3.82	43	14585	100
B3s	F	1	0.58	0.99	6.88	6.9	43	15845	100

Batch 2									
B2	F	1	0.14	0.98	1.25	2.12	14	6338	20*
B2	M	1	0.19	0.99	0.88	1.46	7	2523	40*
B2	F	0.98	0.81	0.99	5.38	4.53	46	13671	90
B2	F	1	0.29	0.99	2.88	3.36	43	11055	90
B2	M	1	0.21	0.98	3.5	5.1	34	15837	80
B2	F	1	0.11	0.99	2.5	3.3	43	11539	30*
B2	F	1	0.18	0.99	3.5	4.54	35	11974	80
B3o	F	1	0.25	1	2.12	4.05	35	6338	80
B3o	M	1	0.12	0.99	1.38	2.33	29	8330	40*
B3o	M	1	0.3	0.98	4.62	4	33	9435	80
B3o	M	1	0.32	0.99	4	5.1	36	14182	80
B3s	F	1	0.17	0.99	4.5	5.63	53	13914	20*
Batch 3									
B2	M	1	0.36	0.99	6	3.7	38	12909	100
B3o	F	1	0.3	0.99	6.38	7.39	40	15730	80
B3o	F	1	0.13	0.99	3.38	3.93	26	8262	20*
B3o	M	1	0.11	0.98	2.75	3.96	29	12757	20*
B3o	F	1	0.43	0.99	6	5.73	31	11194	100
B3s	M	1	0.12	0.99	5	5.01	39	14831	10*
B3s	F	1	0.15	0.99	4.12	4.49	35	12373	20*
B3s	F	1	0.22	0.99	4	3.85	37	13926	80
B3s	M	1	0.19	0.99	5.75	6.36	31	11896	80
B3s	M	0.99	0.4	0.98	4.88	4.94	28	10464	100

B3s	M	0.99	0.11	0.99	3.75	2.31	39	14771	20*
B3s	F	1	0.19	0.99	4.88	4.22	41	12240	70

F – Female; M – male; SD – standard deviation; *Low percentage of housekeeping genes above 50 counts.

Supplementary Table 2. Pathways and corresponding number of genes included on the nCounter® Fibrosis Consortium Panel.

Pathways	Number of Genes
Antimicrobial peptides	14
Circadian Clock	10
Gene expression (Transcription)	82
Interleukin-6 family signaling	7
Fc epsilon receptor (FCERI) signaling	24
Intracellular signaling by second messengers	41
Metabolism of lipids	75
Organelle biogenesis and maintenance	15
Metabolism of vitamins and cofactors	19
Post-translational protein modification	64
Muscle contraction	18
NLR signaling pathways	16
Metabolism of carbohydrates	15
Interleukin-10 signaling	28
Signaling by PTK6	9
Complement cascade	7
Hemostasis	91
Interferon Signaling	27
Signaling by Receptor Tyrosine Kinases	94
Metabolism of amino acids and derivatives	11
Signaling by GPCR	94
Diseases of signal transduction	65
Chaperonin-mediated protein folding	6
Signaling by Rho GTPases	19
Neutrophil degranulation	63
Metabolism of RNA	12
Signaling by WNT	25
Regulation of IGF transport and uptake by IGFBPs	23
Developmental Biology	93
Programmed Cell Death	26
Biological oxidations	22
Interleukin-20 family signaling	8
Surfactant metabolism	6
Death Receptor Signaling	14
Transport of small molecules	30
TNFR2 non-canonical NF-kβ pathway	8
Integration of energy metabolism	7
Interleukin-2 family signaling	16
Amyloid fiber formation	7
Interleukin-4 and 13 signaling	59
Cellular responses to external stimuli	58
Extracellular matrix organization	82

Adaptive Immune System	74
eNOS activation and regulation	5
Interleukin-12 family signaling	15
Neuronal System	15
Vesicle-mediated transport	34
Toll-Like Receptors Cascades	46
Unfolded Protein Response (UPR)	12
MAPK family signaling cascades	45
Interleukin-7 signaling	9
Signaling by NOTCH	18
Metabolism of nucleotides	7
Cell-Cell communication	15
TOTAL	**787**

eNOS - Endothelial nitric oxide synthase; GPCR - G protein-coupled receptors; GTPases - family of hydrolase enzymes that bind to the nucleotide guanosine triphosphate (GTP) and hydrolyze it to guanosine diphosphate (GDP); IGF - Insulin-like growth factor; IGFBPs - Insulin-like growth factor binding proteins; MAPK - mitogen-activated protein kinase; NOTCH - highly conserved cell signaling system based on the NOTCH receptor, a single-pass transmembrane receptor protein; NF-κβ - factor nuclear κβ; NLR - Nucleotide-binding domain, leucine rich repeat containing receptor; PTK6 - Protein tyrosine kinase 6; RNA - ribonucleic acid; TNFR2 - Tumor necrosis factor receptor 2; WNT - signal transduction pathways, including proteins that pass signals into a cell through cell surface receptors.

Supplementary Table 3. ADAM17, TGFβ1, CTGF, and CEACAM3 mRNA expression in structuring (B2) and penetrating (B3) Crohn's disease cases selected for qPCR analysis.

Case ID	mRNA expression (normalized counts)			
	TGFβ1	**CTGF**	**ADAM17**	**CEACAM3**
B2 group (n=3)				
#1	7.59	8.10	6.19	1.33
#2	7.61	8.23	5.89	2.85
#3	8.42	7.98	6.10	3.70
Median value	7.61	8.10	6.10	2.85
B3 group (n=3)				
#1*	11.34	9.78	7.46	7.95
#2**	8.87	10.16	7.38	3.86
#3**	9.47	9.74	7.21	4.92
Median value	9.47	9.78	7.38	4.92

Supplementary Table 4a. Biological Processes with differentially expressed genes (DEGs) between penetrating (B3) and stricturing (B2) Crohn's disease, per process.

ID	Description	Gene Ratio	Bg Ratio	*p* adjusted	geneID	Count	ONT
GO:0071559	response to transforming growth factor beta	09/28	258/18800	6.01E-08	LATS2/ADAM17/CRKL/COL4A2/ COL1A2/COL3A1/COL1A1/ LRRC32/TGFB1	9	BP
GO:0030198	extracellular matrix organization	07/28	307/18800	6.61E-05	COL5A3/COL4A2/COL1A2/ POSTN/COL3A1/COL1A1/TGFB1	7	BP
GO:2001243	negative regulation of intrinsic apoptotic signaling pathway	04/28	102/18800	0.001295	CD44/CXCL12/BCL2/XBP1	4	BP
GO:0032963	collagen metabolic process	04/28	101/18800	0.001295	COL1A2/CIITA/COL1A1/TGFB1	4	BP
GO:0031589	cell-substrate adhesion	06/28	364/18800	0.001295	CRKL/COL5A3/CD44/COL3A1/ BCL2/COL1A1	6	BP
GO:0042060	wound healing	06/28	429/18800	0.00206	ADAM17/CD44/COL3A1/COL1A1/ XBP1/TGFB1	6	BP
GO:0061448	connective tissue development	05/28	260/18800	0.00206	CD44/COL3A1/COL1A1/XBP1/ TGFB1	5	BP
GO:0001101	response to acid chemical	04/28	129/18800	0.00206	COL1A2/COL3A1/COL1A1/XBP1	4	BP
GO:0010563	negative regulation of phosphorus metabolic process	06/28	441/18800	0.00206	LATS2/CRKL/YWHAB/PRKDC/ CBL/TGFB1	6	BP
GO:0031670	cellular response to nutrient	03/28	42/18800	0.00206	POSTN/COL1A1/XBP1	3	BP
GO:0032103	positive regulation of response to external stimulus	06/28	442/18800	0.00206	S100A12/ADAM17/CXCL12/PRKD C/TRADD/TGFB1	6	BP
GO:0032964	collagen biosynthetic process	03/28	47/18800	0.002227	CIITA/COL1A1/TGFB1	3	BP
GO:0010332	response to gamma radiation	03/28	56/18800	0.003329	PRKDC/CBL/BCL2	3	BP
GO:0071496	cellular response to external stimulus	05/28	309/18800	0.003478	POSTN/CBL/BCL2/COL1A1/XBP1	5	BP
GO:0030214	hyaluronan catabolic process	02/28	10/18800	0.003565	CD44/TGFB1	2	BP
GO:0045348	positive regulation of MHC class II biosynthetic process	02/28	10/18800	0.003565	CIITA/XBP1	2	BP
GO:0070482	response to oxygen levels	05/28	324/18800	0.003804	ADAM17/CXCL12/CBL/BCL2/ COL1A1	5	BP
GO:0031293	membrane protein intracellular domain proteolysis	02/28	11/18800	0.003858	PSENEN/TGFB1	2	BP
GO:0007517	muscle organ development	05/28	334/18800	0.0039	COL6A3/COL3A1/BCL2/XBP1/ TGFB1	5	BP
GO:0042113	B cell activation	05/28	336/18800	0.003924	ADAM17/PRKDC/BCL2/XBP1/ TGFB1	5	BP

Bg ratio is M/N, where M is the size of the geneset (is the number of genes within that distribution that are annotated (either directly or indirectly) to each term), and N is the size of all of the unique genes in the collection of genesets (is the total number of genes in the background distribution (universe); BP – Biological Process; geneID – gene acronym; Gene ratio is k/n, where k is the size of the overlap of 'a vector of gene id' you input with the specific geneset (the number of genes within that list n, which are annotated to the term and n is the size of the overlap of 'a vector of gene id' you input with all the members of the collection of genesets, is the size of the list of genes of interest; GO – gene ontology; ID – Identification number in GO; ONT – Ontology; *p* adjusted – *p* value adjusted.

Supplementary Table 4b. Biological Pathways with differentially expressed genes (DEGs) penetrating (B3) and stricturing (B2) Crohn's disease, per pathway.

ID	Description	Gene Ratio	Bg Ratio	*p* adjusted	GeneID	Count	ONT
R-HSA-1442490	Collagen degradation	08/26	64/10891	3.21E-10	ADAM17/COL6A3/COL5A3/COL4A2/ CTSD/COL1A2/COL3A1/COL1A1	8	R
R-HSA-1474228	Degradation of the extracellular matrix	09/26	140/10891	2.37E-09	ADAM17/COL6A3/COL5A3/COL4A2/ CD44/CTSD/COL1A2/COL3A1/COL1A1	9	R
R-HSA-3000178	ECM proteoglycans	07/26	76/10891	2.96E-08	COL6A3/COL5A3/COL4A2/COL1A2/ COL3A1/COL1A1/TGFB1	7	R
R-HSA-1474244	Extracellular matrix organization	10/26	300/10891	3.30E-08	ADAM17/COL6A3/COL5A3/COL4A2/ CD44/CTSD/COL1A2/COL3A1/COL1A1/ TGFB1	10	R
R-HSA-216083	Integrin cell surface interactions	07/26	85/10891	3.30E-08	COL6A3/COL5A3/COL4A2/CD44/ COL1A2/COL3A1/COL1A1	7	R
R-HSA-8948216	Collagen chain trimerization	06/26	44/10891	3.30E-08	COL6A3/COL5A3/COL4A2/COL1A2/ COL3A1/COL1A1	6	R
R-HSA-3000170	Syndecan interactions	05/26	27/10891	1.27E-07	COL5A3/COL1A2/COL3A1/COL1A1/ TGFB1	5	R
R-HSA-3000171	Non-integrin membrane-ECM interactions	06/26	59/10891	1.27E-07	COL5A3/COL4A2/COL1A2/COL3A1/ COL1A1/TGFB1	6	R
R-HSA-6806834	Signaling by MET	06/26	79/10891	5.53E-07	CRKL/COL5A3/COL1A2/COL3A1/CBL/ COL1A1	6	R
R-HSA-8875878	MET promotes cell motility	05/26	41/10891	7.51E-07	CRKL/COL5A3/COL1A2/COL3A1/ COL1A1	5	R
hsa04512	ECM-receptor interaction	07/22	85/5894	7.67E-07	COL6A3/COL5A3/COL4A2/CD44/ COL1A2/COL3A1/COL1A1	7	K
R-HSA-3000480	Scavenging by Class A Receptors	04/26	19/10891	1.70E-06	COL4A2/COL1A2/COL3A1/COL1A1	4	R
R-HSA-186797	Signaling by PDGF	05/26	58/10891	3.57E-06	CRKL/COL6A3/COL5A3/COL4A2/ COL3A1	5	R
hsa04974	Protein digestion and absorption	06/22	81/5894	6.18E-06	COL6A3/COL5A3/COL4A2/COL1A2/ COL3A1/COL1A1	6	K
hsa04510	Focal adhesion	08/22	200/5894	6.18E-06	CRKL/COL6A3/COL5A3/COL4A2/ COL1A2/COL3A1/BCL2/COL1A1	8	K
R-HSA-8874081	MET activates PTK2 signaling	04/26	30/10891	1.04E-05	COL5A3/COL1A2/COL3A1/COL1A1	4	R
hsa05146	Amoebiasis	06/22	106/5894	2.30E-05	COL5A3/COL4A2/COL1A2/COL3A1/ COL1A1/TGFB1	6	K
R-HSA-419037	NCAM1 interactions	04/26	42/10891	3.69E-05	COL6A3/COL5A3/COL4A2/COL3A1	4	R
R-HSA-2214320	Anchoring fibril formation	03/26	15/10891	7.01E-05	COL4A2/COL1A2/COL1A1	3	R
R-HSA-202733	Cell surface interactions at the vascular wall	05/26	137/10891	0.000175	CD44/COL1A2/COL1A1/CEACAM3/ TGFB1	5	R

Bg ratio is M/N, where M is the size of the geneset (is the number of genes within that distribution that are annotated (either directly or indirectly) to each term), and N is the size of all of the unique genes in the collection of genesets (is the total number of genes in the background distribution (universe); BP – Biological Process; geneID – gene acronym; Gene ratio is k/n, where k is the size of the overlap of 'a vector of gene id' you input with the specific geneset (the number of genes within that list n, which are annotated to the term and n is the size of the overlap of 'a vector of gene id' you input with all the members of the collection of genesets, is the size of the list of genes of interest; GO – gene ontology; ID – Identification number in GO; K – KEGG; ONT – Ontology; *p* adjusted – *p* value adjusted; R – REACTOME.

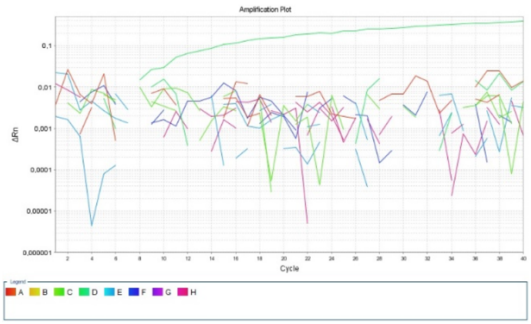
Supplementary Table 5. List of differentially expressed genes (DEGs) between penetrating (B3) and stricturing (B2) Crohn's disease.

Name	Description	Fold Change	Log Fold Change	p value	p adjusted	Average Log2 Expression
CTGF	connective tissue growth factor	2.22	1.15	2.79E-06	0.001685	9.44
HVCN1	hydrogen voltage gated channel 1	8.04	3.01	1.97E-05	0.005941	6.37
XBP1	X-box binding protein 1	4.96	2.31	0.00017	0.014623	11.67
CD44	CD44 molecule (Indian blood group)	2.89	1.53	0.000163	0.014623	10.03
CEACAM3	carcinoembryonic antigen-related cell adhesion molecule 3	8.09	3.02	0.000155	0.014623	3.99
TGFB1	transforming growth factor. beta 1	3.64	1.86	0.000127	0.014623	9.18
PPAPDC1A	phosphatidic acid phosphatase type 2 domain containing 1A	10.08	3.33	8.22E-05	0.014623	5.43
COL1A1	collagen. type I. alpha 1	6.26	2.655	0.000271	0.014839	13.12
YWHAB	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein. beta	2.02	1.02	0.000268	0.014839	8.32
CXCL12	chemokine (C-X-C motif) ligand 12	3.93	1.97	0.000259	0.014839	8.49
LRRC32	leucine rich repeat containing 32	4.16	2.06	0.000223	0.014839	4.72
CRKL	v-crk avian sarcoma virus CT10 oncogene homolog-like	1.81	0.85	0.000355	0.017855	7.45
COL1A2	collagen. type I. alpha 2	2.83	1.50	0.000386	0.01791	9.85
COL6A3	collagen. type VI. alpha 3	1.98	0.99	0.000446	0.017942	10.42
CTSD	cathepsin D	2.31	1.21	0.000439	0.017942	10.65
COL3A1	collagen. type III. alpha 1	2.29	1.19	0.000545	0.018271	12.21
TRADD	TNFRSF1A-associated via death domain	3.48	1.80	0.000538	0.018271	6.85
CIITA	class II. major histocompatibility complex. transactivator	3.38	1.76	0.000625	0.018848	7.50
COL4A2	collagen. type IV. alpha 2	1.86	0.89	0.000602	0.018848	9.41
PRKDC	protein kinase. DNA-activated. catalytic polypeptide	3.03	1.60	0.000681	0.019543	8.92
LATS2	large tumor suppressor kinase 2	1.61	0.69	0.000853	0.023392	8.36
PSENEN	presenilin enhancer gamma secretase subunit	2.43	1.28	0.000911	0.023883	5.79
CBL	Cbl proto-oncogene. E3 ubiquitin protein ligase	3.10	1.63	0.00105	0.025322	7.84
ADAM17	ADAM metallopeptidase domain 17	1.55	0.63	0.001012	0.025322	6.85
BCL2	B-cell CLL/lymphoma 2	3.05	1.61	0.001249	0.027884	8.48
S100A12	S100 calcium binding protein A12	6.45	2.69	0.001205	0.027884	4.18
COL5A3	collagen. type V. alpha 3	2.20	1.13	0.001638	0.032931	8.01
POSTN	periostin. osteoblast specific factor	2.61	1.39	0.001625	0.032931	6.84
TXNDC5	thioredoxin domain containing 5 (endoplasmic reticulum)	1.77	0.82	0.001625	0.032931	10.39
LTB	lymphotoxin beta (TNF superfamily. member 3)	3.58	1.84	0.001953	0.037986	8.49

Supplementary Figures 2a – 2e and Supplementary Tables 6a – 6e. mRNA expression analysis and corresponding Ct values of qPCR.

Supplementary Table 6a

Ct, Ct Mean and Ct SD values of qPCR analysis for *CEACAM3* gene.

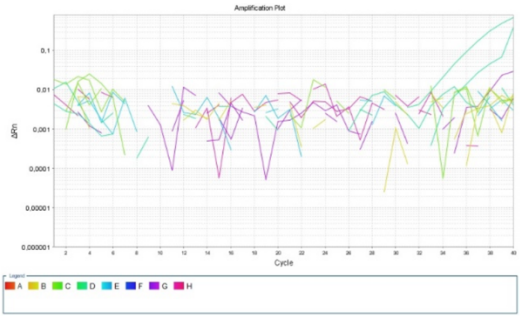


Sample	Group	Gene	Cт	Cт Mean	Cт SD	Gene	Cт	Cт Mean	Cт SD
#1		*CEACAM3*	Undetermined			18S	30,87977	30,06891	0,777899
#1		*CEACAM3*	Undetermined			18S	29,99815	30,06891	0,777899
#1		*CEACAM3*	Undetermined			18S	29,32881	30,06891	0,777899
#3		*CEACAM3*	Undetermined			18S	22,42614	22,38994	0,032006
#3	B3	*CEACAM3*	Undetermined			18S	22,36539	22,38994	0,032006
#3		*CEACAM3*	Undetermined			18S	22,3783	22,38994	0,032006
#2		*CEACAM3*	Undetermined			18S	20,37294	19,97235	0,657358
#2		*CEACAM3*	Undetermined			18S	20,33042	19,97235	0,657358
#2		*CEACAM3*	Undetermined			18S	19,2137	19,97235	0,657358
#1		*CEACAM3*	Undetermined			18S	27,18642	27,47083	0,56304
#1		*CEACAM3*	Undetermined			18S	28,11934	27,47083	0,56304
#1		*CEACAM3*	Undetermined			18S	27,10672	27,47083	0,56304
#3		*CEACAM3*	Undetermined			18S	26,26703	25,96453	0,309233
#3	B2	*CEACAM3*	Undetermined			18S	25,97758	25,96453	0,309233
#3		*CEACAM3*	Undetermined			18S	25,64898	25,96453	0,309233
#2		*CEACAM3*	Undetermined			18S	26,23173	25,90973	0,340937
#2		*CEACAM3*	Undetermined			18S	25,94487	25,90973	0,340937
#2		*CEACAM3*	Undetermined			18S	25,55258	25,90973	0,340937
MKN74						**18S**	**7,93136**	**7,93136**	

Supplementary Figure 2a – mRNA expression analysis for *CEACAM3*.

Supplementary Table 6b

Ct, Ct Mean and Ct SD values of qPCR analysis for *TGFB1* gene.

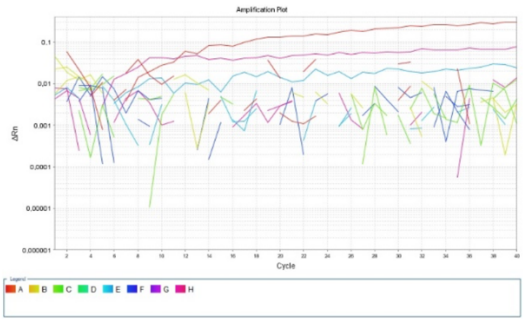


Sample	Group	Gene	Cт	Cт Mean	Cт SD	Gene	Cт	Cт Mean	Cт SD
#1		*TGFB1*	Undetermined			18S	30,87977	30,06891	0,777899
#1		*TGFB1*	Undetermined			18S	29,99815	30,06891	0,777899
#1		*TGFB1*	Undetermined			18S	29,32881	30,06891	0,777899
#3		*TGFB1*	Undetermined	36,56988		18S	22,42614	22,38994	0,032006
#3	B3	*TGFB1*	36,56988	36,56988		18S	22,36539	22,38994	0,032006
#3		*TGFB1*	Undetermined	36,56988		18S	22,3783	22,38994	0,032006
#2		*TGFB1*	31,8101	31,1179	3,625107	18S	20,37294	19,97235	0,657358
#2		*TGFB1*	27,1966	31,1179	3,625107	18S	20,33042	19,97235	0,657358
#2		*TGFB1*	34,347	31,1179	3,625107	18S	19,2137	19,97235	0,657358
#1		*TGFB1*	Undetermined			18S	27,18642	27,47083	0,56304
#1		*TGFB1*	Undetermined			18S	28,11934	27,47083	0,56304
#1		*TGFB1*	Undetermined			18S	27,10672	27,47083	0,56304
#3		*TGFB1*	Undetermined			18S	26,26703	25,96453	0,309233
#3	B2	*TGFB1*	Undetermined			18S	25,97758	25,96453	0,309233
#3		*TGFB1*	Undetermined			18S	25,64898	25,96453	0,309233
#2		*TGFB1*	Undetermined			18S	26,23173	25,90973	0,340937
#2		*TGFB1*	Undetermined			18S	25,94487	25,90973	0,340937
#2		*TGFB1*	Undetermined			18S	25,55258	25,90973	0,340937
MKN74						**18S**	**7,93136**	**7,93136**	

Supplementary Figure 2b – mRNA expression analysis for *TGFB1*.

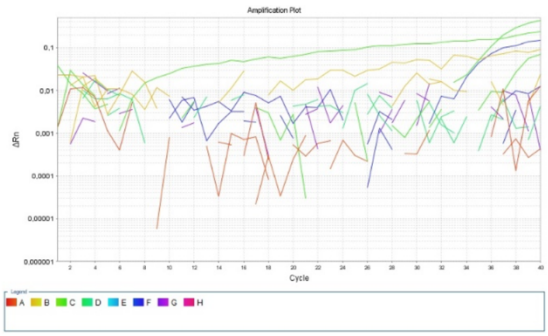
Supplementary Table 6c

Ct, Ct Mean and Ct SD values of qPCR analysis of all samples for *CTGF* gene.



Sample	Group	Gene	Cт	Cт Mean	Cт SD	Gene	Cт	Cт Mean	Cт SD
#1		*CTGF*	Undetermined			18S	30,87977	30,06891	0,777899
#1		*CTGF*	Undetermined			18S	29,99815	30,06891	0,777899
#1		*CTGF*	Undetermined			18S	29,32881	30,06891	0,777899
#3		*CTGF*	Undetermined			18S	22,42614	22,38994	0,032006
#3	B3	*CTGF*	Undetermined			18S	22,36539	22,38994	0,032006
#3		*CTGF*	Undetermined			18S	22,3783	22,38994	0,032006
#2		*CTGF*	Undetermined			18S	20,37294	19,97235	0,657358
#2		*CTGF*	Undetermined			18S	20,33042	19,97235	0,657358
#2		*CTGF*	Undetermined			18S	19,2137	19,97235	0,657358
#1		*CTGF*	Undetermined			18S	27,18642	27,47083	0,56304
#1		*CTGF*	Undetermined			18S	28,11934	27,47083	0,56304
#1		*CTGF*	Undetermined			18S	27,10672	27,47083	0,56304
#3		*CTGF*	Undetermined			18S	26,26703	25,96453	0,309233
#3	B2	*CTGF*	Undetermined			18S	25,97758	25,96453	0,309233
#3		*CTGF*	Undetermined			18S	25,64898	25,96453	0,309233
#2		*CTGF*	Undetermined			18S	26,23173	25,90973	0,340937
#2		*CTGF*	Undetermined			18S	25,94487	25,90973	0,340937
#2		*CTGF*	Undetermined			18S	25,55258	25,90973	0,340937
MKN74						**18S**	**7,93136**	**7,93136**	

Supplementary Figure 2c – mRNA expression analysis for *CTGF*.

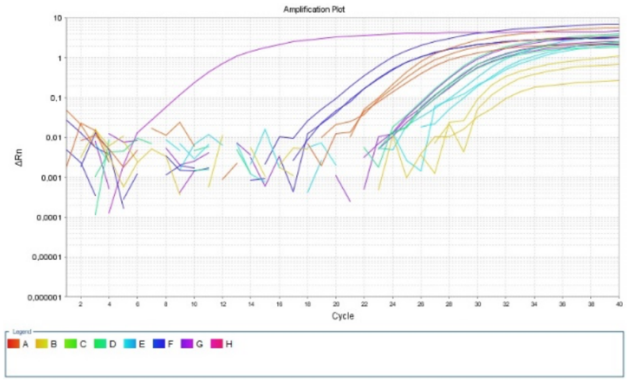


Supplementary Table 6d

Ct, Ct Mean and Ct SD values of qPCR analysis of all samples for *ADAM17* gene.

Sample	Group	Gene	CT	CT Mean	CT SD	Gene	CT	CT Mean	CT SD
#1		*ADAM17*	Undetermined			18S	30,87977	30,06891	0,777899
#1		*ADAM17*	Undetermined			18S	29,99815	30,06891	0,777899
#1		*ADAM17*	Undetermined			18S	29,32881	30,06891	0,777899
#3		*ADAM17*	32,93926	32,93926		18S	22,42614	22,38994	0,032006
#3	B3	*ADAM17*	Undetermined	32,93926		18S	22,36539	22,38994	0,032006
#3		*ADAM17*	Undetermined	32,93926		18S	22,3783	22,38994	0,032006
#2		*ADAM17*	Undetermined	34,61821		18S	20,37294	19,97235	0,657358
#2		*ADAM17*	36,31911	34,61821		18S	20,33042	19,97235	0,657358
#2		*ADAM17*	32,91731	34,61821		18S	19,2137	19,97235	0,657358
#1		*ADAM17*	Undetermined	19,13719		18S	27,18642	27,47083	0,56304
#1		*ADAM17*	Undetermined	19,13719		18S	28,11934	27,47083	0,56304
#1		*ADAM17*	19,13719	19,13719		18S	27,10672	27,47083	0,56304
#3		*ADAM17*	Undetermined			18S	26,26703	25,96453	0,309233
#3	B2	*ADAM17*	Undetermined			18S	25,97758	25,96453	0,309233
#3		*ADAM17*	Undetermined			18S	25,64898	25,96453	0,309233
#2		*ADAM17*	Undetermined			18S	26,23173	25,90973	0,340937
#2		*ADAM17*	Undetermined			18S	25,94487	25,90973	0,340937
#2		*ADAM17*	Undetermined			18S	25,55258	25,90973	0,340937
MKN74						**18S**	**7,93136**	**7,93136**	**18S**

Supplementary Figure 2d – mRNA expression analysis for *ADAM17.*



Supplementary Table 6e

Ct, Ct Mean and Ct SD values of qPCR analysis of all samples, and MKN74, for 18S.

Sample	Group	Gene	CT	CT Mean	CT SD
#1		18S	30,87977	30,06891	0,777899
#1		18S	29,99815	30,06891	0,777899
#1		18S	29,32881	30,06891	0,777899
#3		18S	22,42614	22,38994	0,032006
#3	B3	18S	22,36539	22,38994	0,032006
#3		18S	22,3783	22,38994	0,032006
#2		18S	20,37294	19,97235	0,657358
#2		18S	20,33042	19,97235	0,657358
#2		18S	19,2137	19,97235	0,657358
#1		18S	27,18642	27,47083	0,56304
#1		18S	28,11934	27,47083	0,56304
#1		18S	27,10672	27,47083	0,56304
#3		18S	26,26703	25,96453	0,309233
#3	B2	18S	25,97758	25,96453	0,309233
#3		18S	25,64898	25,96453	0,309233
#2		18S	26,23173	25,90973	0,340937
#2		18S	25,94487	25,90973	0,340937
#2		18S	25,55258	25,90973	0,340937
MKN74		**18S**	**7,93136**	**7,93136**	

Supplementary Figure 2e – mRNA expression analysis for 18S.

Supplementary Table 7. Upregulated differentially expressed genes (DEGs) previously not described in Crohn's disease. Role in non-intestinal organ fibrosis described in literature.

Gene	Protein	Role in fibrosis	Function in fibrogenesis	Pathways	Involvement in organ fibrosis	Human / model	Observations
TXNDC5	Thioredoxin domain-containing protein 5	Pro-fibrotic	Cell protection from oxidative stress, promotion of cell proliferation and migration. inhibition of apoptosis[1]	Pro-inflammatory by inducing VEGF, IL-6, and IL-8.[2] Inhibites expression of IGF-1-binding protein, promoting IGF-1 binding to its receptor, which exhibits tyrosine-kinase activity activating signaling pathways as PI3K-AKT/PKB and RAS/MAPK, regulating cell proliferation, differentiation, survival and apoptosis.[3] Promotes joint inflammation through activation of NF-kβ.[4] Enhances synovial fibroblasts and cartilage destruction through increased expression of MMP-3[5] Transcription is controlled by TGF-β1-induced ATF6-dependent ER stress pathway. Profibrogenic through increased TGF-β signaling activity, including posttranslational stabilization and upregulation of type I TGF-β receptor.[6–10]	Joints (RA)[2,4,5]	Human Model	
					Kidney[7]	Model	
					Liver[6]	Human Model	
					Heart[9]	Human Model	
					Lung[8]	Human Model	
LRRC32	Leucine Rich Repeat Containing 32 protein (LRRC32) / glycoprotein-A	Pro-fibrotic	LRRC32 protein is critical in anchoring the large latent TGF-β complex to the surface of regulatory T cells (Tregs) and platelets.[11]		Systemic sclerosis[12]	Model	
					Liver[13,14]	Human Model	

	repetitions predominant (GARP) / filaggrin				Lung[15]	Human	
CTSD	cathepsin D	Pro-fibrotic	Acts as a lysosomal protease, secreted as pro-enzyme outside the cells. Promotes apoptosis via the mature form of the enzyme inside cells.[16]	Glycosylation of asparagine 233 (N233) determined pro-CTSD secretion. The steroid hormone 20-hydroxyecdysone (20E) promoted CTSD expression. (Macro)autophagy triggered CTSD maturation and localization inside midgut cells to activate CASP3 and promote apoptosis.[16]	Kidney[17–19] Liver[20,21] Heart[22]	Model Human Model Model	
CD44	CD44 molecule	Pro-fibrotic	Transmembrane glycoprotein acting as cell-surface receptor for multiple ECM proteins.[23] One of the multiple β-catenin target genes. Canonical WNT/β-catenin signaling is involved in myofibroblast activation and subsequent organ fibrosis.[24]	The activation of receptor complex CD74/CD44 activates intracellular signal pathways, such as the activation of ERK 1 and 2, PI3K-AKT/PKB signal transduction cascade, NFκβ, and the AMPK pathway.[25] TGF-β-mediated HA/CD44/STAT3 pathway plays a crucial role in the development of atrial fibrosis.[26]	Kidney[27] Liver[28] Heart[23,26,29] Lung[30]	Model Model Model Human Model	β-catenin signaling dysregulation is involved in chronic inflammation, organ fibrosis, and human cancers.[24]
CXCL12	chemokine (C-X-C motif) ligand 12 (also known as SDF-1)	Pro-fibrotic	CXCL12 is the only ligand of CXCR4, the main chemokine receptor on fibrocytes.	The CXCR4/CXCR7-CXCL12 axis shows distinct effects and pathways depending on different (patho)physiological conditions. and the interplay between the three molecules is highly complex.[32]	Joints (RA)[34] Liver[35,36]	Human Model Model	CXCR4/CXCR7-CXCL12 axis have also been

			CXCL12/CXCR4 is involved in several pro-fibrotic mechanisms such as inflammation, immunity, EMT, and angiogenesis.[31] CXCL12 shows higher binding affinity to CXCR7.[32] Marker of mast cell maturation chemokine.[33]		Heart[37,38] Lung[33,39] Retroperitoneal[40] Spleen[41]	Model Model Model Model	described in cancer[31,32] Other gene-related proteins have been associated to inflammation and/or fibrosis in CD (CXL9[42], CXCL10[43], CXCL14[44], CXCL1,2,8[45]).
TRADD	TNFRSF1A-associated via death domain	Pro-fibrotic	Regulates cell proliferation and apoptosis via TNF-α-mediated signaling pathways.[46]	Key signaling intermediate in TNFα-activation of NFκβ.[47]	Skin[46,48] Liver[49] Lung[47]	Model Model Model	
LTB	Lymphotoxin β	Pro-fibrotic	LTα and LTβ are members of the TNFβ superfamily, having chronic inflammation effects.[50]	In the lung. the activation of LTβR on stromal cells by LTα and β expressed by activated lymphocytes during chronic inflammation, triggers downstream non-canonical NF-κβ signalling via activation of NIK.[51]	Liver[52,53] Biliary[54]	Human Model Human	
YWHAB	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase	Pro-fibrotic	Adapter protein that mediates signal transduction	Adapter protein that mediates signal transduction by binding to phosphoserine-containing proteins[55]	Kidney[56]	Human	Kidney interstitial fibrosis in offspring of

	activation protein β						DEHP -exposed women[56]
CBL	calcineurin B-like protein	Anti-fibrotic	Adapter protein that functions as a negative regulator of many signaling pathways that are triggered by activation of cell surface receptors.[57]	Mediates the transfer of ubiquitin from ubiquitin conjugating enzymes (E2) to specific substrates. Recognizes activated receptor tyrosine kinases, including KIT, FLT1, FGFR1, FGFR2, PDGFRA, PDGFRB, CSF1R, EPHA8, and KDR and terminates signaling, by target them for proteasome degradation.[57,58]	Only if mutated: Myelofibrosis[59] Liver[60]	Human Model	Anti-fibrotic role demonstrated in kidney[61,62], skin[63] and lung.[64]
PRKDC	PRKDC/DNA-PKcs	Anti-fibrotic	Promotes autophagy[65]	Acts as a positive regulator of basal and DNA damage-induced autophagy. In autophagy-regulating signaling cascades PRKDC acts upstream of AMPK complex and ULK1 kinase[65]	In knocked-down mice models: Adipose tissue[66] Systemic sclerosis[67,68] Lung[69]	Model Model Model	Also involved in carcinogenesis[70]
CIITA	class II, major histocompatibility complex (MHC), transactivator	Anti-fibrotic	MHC class II transactivator	IFN-γ stimulates CIITA expression. which activates MHC II and represses collagen expression.[71]	None		Anti-fibrotic role demonstrated in dendritic[72] and stellate cells[73,74]
CRKL	Protein kinase containing SH2 and SH3 domains	Unknown	Plays a role in fibroblast transformation by BCR-ABL tyrosine kinase[75]	Activate the RAS and JNK kinases signaling pathways[76]	None		Has ubiquitous expression. including the gut.[76] Roles mostly showed

						on carcinogenesis.
PSENEN	v-crk avian sarcoma virus CT10 oncogene homolog-like /Presenilin enhancer-2	Unknown	The smallest subunit of the γ-secretase complex, an intramembrane protease that cleaves proteins within their transmembrane domains, like NOTCH1, APP, and CDH-1.[77]	Cleaves proteins within their transmembrane domains, like NOTCH1, APP, and CDH-1.[77] Also involved in autophagy-lysosome system in a γ-secretase activity-independent manner.[77]	None	Mutations in this gene (and of other components of γ-secretase) have been described in hidradenitis suppurativa.[78]
PPAPDC 1A /PLPP4	phosphatidic acid phosphatase type 2 domain containing 1A / phospholipid phosphatase 4	Unknown	Promotes proliferation and tumorigenesis and cancer-related inflammation.	Promotes proliferation and tumorigenesis *via* activating influx of intracellular Ca^{2+} in lung adenocarcinoma.[79] Involved in cancer-related inflammation and the infiltration of immune cells.[80]	None	Involved in carcinogenesis[80] and cancer dissemination.[79,81]

AMPK - AMP-activated protein kinase; APP - amyloid precursor protein; ATF6 - activating transcription factor 6α; CASP3 - caspase 3; CBL - calcineurin B-like protein; CD - Crohn's disease; CD74/44 – CD74/44 molecule; CDH-1 – Cadherin-1; CIITA - class II, major histocompatibility complex (MHC) transactivator; CTSD – cathepsin D; CXCL - C-X-C motif ligand; CXCL12 - chemokine (C-X-C motif) ligand 12; CRKL - Protein kinase containing SH2 and SH3 domains; CXCR4 - C-X-C receptor 4; CXCR7 - C-X-C receptor 4; DEHP - Di-(2-ethylhexyl)phthalate; ECM - extracellular matrix; EMT - epithelial-mesenchymal transition; ER - endoplasmic reticulum; ERK - extracellular signal regulated kinase; GARP - glycoprotein-A repetitions predominant; HA – hyaluronan; IFN-γ - Interferon gamma; IL – interleukin; IGF-1 - Insulin-like growth factor 1; JNK - c-Jun NH2-terminal kinase;; LRRC32 - Leucine rich repeat containing 32 protein; LTB - lymphotoxin β; LTBP - latent TGF-β-binding protein; LTβR - lymphotoxin β receptor; MMP3 - matrix metalloproteinase-3; NIK - NF-κβ inducing kinase; NF-κβ - fuclear factor κβ; PI3K-AKT/PKB - phosphatidylinositol 3-kinase/ Protein kinase B; PPAPDC1A/PLPP4 - phosphatidic acid phosphatase type 2 domain containing 1A/1A / phospholipid phosphatase 4; PRKDC/DNA/PKcs - Protein kinase, DNA-activated, catalytic subunit; PSENEN - crk avian sarcoma virus CT10 oncogene homolog-like /Presenilin enhancer-2; RA – rheumatoid arthritis; RAS/MAPK - RAt sarcoma/ mitogen-activated protein kinase; SDF-1 - stromal-derived-factor-1; STAT3 - signal transducers and activators of transcription; TNFα - Tumor necrosis factor α; TRADD - TNFRSF1A associated via death domain; TNFRSF1A – tumor necrosis factor superfamily member 1; TGFβ - transforming growth factor β; TXNDC5 - Thioredoxin domain-containing protein 5; VEGF - vascular endothelial growth factor; WNT - Wingless-Int-1; YWHAB - Tyrosine 3 monooxygenase/tryptophan 5-monooxygenase activation Protein β.

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CHAPTER 6 – General Discussion

6. General Discussion

This thesis aimed to add knowledge regarding inflammatory and fibrosis changes in stricturing and penetrating CD, bearing in mind that most clinical and molecular research on fibrosis in CD has focused on stricturing disease, leaving penetrating disease pathogenesis poorly described. We believe this thesis' research accomplished its global aim, through the achievement of the three specific objectives, summarized as follows.

- Objective 1 - To uncover a liable transmural histopathological scoring system for the study of inflammation and fibrosis both in stricturing and penetrating CD.
- Objective 2 - To grade inflammation and fibrosis in ileal CD resection specimens, according to a liable CD transmural histopathological scoring system in stricturing and penetrating CD.
- Objective 3 - To compare the fibrosis-related transcriptome profiles in stricturing and penetrating CD.

This section summarizes the main conclusions of each objective and discusses the potential translational impact of the produced research in the field of IBD.

6.1. Summary of Main Findings

6.1.1. A Transmural Histopathologic Scoring System for The Study of Inflammation and Fibrosis in Crohn's Disease

The first objective of this thesis was accomplished by means of a systematic review of 29 original CD transmural scoring systems[1] [CHAPTER 3]. Due to high heterogeneity, a meta-analysis was not feasible. Instead, a triple quality appraisal was performed: First, through the assessment of the methodological quality of each study describing an original scoring system, according to the 10-item COSMIN checklist [internal consistency, reliability, measurement error, content validity, structural validity [factor analysis], hypothesis testing, cross-cultural validity, criterion validity, responsiveness to change, and interpretability], with each property being rated on a four-point scale [1 = poor, 2 = fair, 3 = good, 4 = excellent]; Second, through the analysis of the operating or psychometric properties [validity, reliability, responsiveness, and feasibility] of each scoring system, which were graded as unknown [?], moderately assessed [+/-], and well assessed [+]; Third, through a detailed analysis of the variables in each score, regarding the ability to assess the histopathological changes of transmural inflammation and fibrosis in CD.

Two highly comprehensive scores, by Chen et al[2] and by Shaeffer et al,[3] showed good operating properties and high methodological quality of the corresponding studies (75% and 85%, respectively), as well as the lowest risk of bias, given they were the only ones using two independent and blinded pathologists. Importantly, both derive from studies specifically designed to describe the intestinal histopathological changes caused by CD. The upcoming anti-fibrotic agents' clinical trials demand for high-quality, comprehensive

and sensitive scoring systems. Hence, although extensive and cumbersome, we believe that Chen et al[2] and Shaeffer et al[3] systems could suitable for this type of research, although still requiring validation studies. Additionally, three scores emerged as the most widely reproduced, probably due to ease of application in clinical studies. Of these, the scores by Chiorean et al[4] and by Adler et al[5] include both inflammation and fibrosis components and resulted equally from cross-imaging-pathological correlation studies. Their adequate operating properties and high methodological quality of the corresponding studies (80% and 77.5%, respectively) makes them suitable for both clinical research and practice.

It is important to mention that, as of today, still no histopathological scoring system for the evaluation of inflammation and fibrosis changes due to CD has been validated or is widely accepted.[6] In addition to our paper, other systematic-reviews performed on the same topic but restricted to stricturing disease, all concluded for a lack of suitable scoring system.[7–9] This gap, attributed to lack of standardized variables,[10] led to the publication of an international consensus on the key findings that a transmural histopathological scoring system for CD should include, although also limited to small-bowel stricturing disease.[9] It was our belief in the value of standardization of research measures that led us to recommend some selected scoring systems for use in different research settings, according to our results.[1] The importance of setting methodological standards in order to adequately and objectively measure and compare subjects, was also addressed by the candidate in an Editorial published in 2022 (vide APPENDICES, Section III.a.).[11] In this Editorial, the huge effort of the Stenosis Therapy and Anti-fibrosis Research Consortium [STAR] in developing endpoints,[12] and clinical[13], imaging[12,14] and histopathologic[9] standards for the non-invasive assessment of inflammation and fibrosis in stricturing CD

in view of the building up of anti-fibrotic agents' clinical trials for stricturing CD was mentioned. Recently, the same group published a set of consensus statements on definitions, diagnosis and management of ileal CD to be used in clinical practice.[15] Taking all together, yet a research gap in on CD persists: with few exceptions,[16–18] the vast majority of clinical and molecular research on fibrosis in CD has been focused on stricturing disease, mostly of the terminal ileum.[19–22]

Hence, we decided to contribute with scientific knowledge regarding the study of inflammation and fibrosis in CD by studying it in both stricturing and penetrating disease, as accomplished in this thesis' second study. For this purpose, we had to choose one of the histopathological scoring systems we proposed in our systematic review. Due to time-constraints and workload of the research group pathologists, a simple system had to be adopted, e.g. Chiorean's[4] or Adler's[5], which are displayed on Table 1 and 2, respectively.

Score	Grade	Features	Score
Inflammation	Mild	Aphthous ulcers affected surface <50%; Cryptitis <50%; Inflammation limited to mucosa	1
	Moderate	Large, superficial ulcers (0.5-2cm) Ulcerated surface <50%; affected surface 50-100% Cryptitis >50%; crypt abscesses; submucosal inflammation	2
	Severe	Deep[1] ulcers or ulcers >2cm in size; circumferential ulcers; deep[1] fissures; transmural inflammation	3
Fibrosis	None	None or minimal fibrosis limited to submucosa (<25% thickness)	0
	Mild/moderate	Mild stricture (>15mm) with non-dilated lumen Submucosal fibrosis and muscular hyperplasia >25% with preserved layers	1
	Severe	Massive transmural fibrosis; effacement of normal layers; severe stricture	2

Table 1. Chiorean et al[4] transmural histopathological scoring system for CD. [1] – Deep – beyond the submucosa.

Score	Features	Score
Inflammation	No inflammation or distortion	0
	Lamina propria inflammation only	1
	Submucosal foci of inflammation	2
	Foci of transmural inflammation	3
	Significant, dissecting, confluent transmural inflammation	4
Fibrosis	No Fibrosis	0
	Minimal fibrosis in submucosa or subserosa	1
	Increased submucosal fibrosis, septa into muscularis propria	2
	Septa through muscularis propria, increase in subserosal collagen	3
	Significant transmural scar, marked subserosal collagen	4
Muscle	Normal thickness	0
	Increased thickness of muscularis propria layer	1

Table 2. Adler's et al[5] transmural histopathological scoring system for CD.

Although Adler et al[5] scoring system presented a detailed fibrosis component, it did not include the expansion of the muscular layers and the inflammation part seemed vague, as inflammation variables were not defined.

In the end, Chiorean et al[4] scoring system was considered suitable for the histopathological objective study of inflammation and fibrosis in CD ileal surgical specimens, due to the descriptive equilibrium of both components, despite some deficiencies which were addressed on this thesis' second study.

6.1.2. Similar Transmural Fibrosis in Penetrating and Stricturing Crohn's Disease

The second objective of this thesis was addressed through a histopathological study of archived ileal resection surgical specimens[23] [CHAPTER 4]. Herein we innovatively demonstrated that, in advanced CD, fibrosis scores and fibromuscular changes were similar irrespective of phenotype, with the degree of inflammation being the major differentiator of penetrating disease.

We consider these results to be robust, as an objective score-based and double-blinded histopathological analysis of 3 different sections of each of the 103 ileal surgical specimens (ileal proximal margin, inflamed area and most affected area) was performed. Moreover, both stricturing (B2, n=29) and penetrating (B3, n=74) CD were studied, including mixed stricturing/ penetrating cases which constituted over half of the study sample (B3s, n=54). The adopted histopathologic scoring system was the one by Chiorean et al[4], chosen by the group pathologists considering its adequate operating properties and high (80%) methodological quality of the corresponding study on this thesis' first study,[1] together with its inflammation and fibrosis components and applicability in an acceptable time and workload. However, acknowledging the scoring system limitations, the term "muscular hyperplasia >25%" was applied both for muscularis propria hyperplasia >25% and muscularis mucosae hyperplasia or splay (no cellular hyperplasia) >25%, emphasizing the role of the thickening muscle layers on the reduction of the intestinal lumen.[6] The presence of smooth muscle and, innovatively, of adipose tissue[24] in the submucosa was also assessed, so all the important histopathologic inflammatory and fibromuscular changes of CD were included.

This study secondarily aimed to correlate the inflammation and fibrosis scoring with progressive disease, previously defined[25] as the occurrence of at least one of eight post-operative outcomes, namely reoperation, hospitalization, need for steroid therapy, starting and changing of immunosuppressive or biologic therapy and the occurrence of new events such as new stricture(s), penetrating event(s) or perianal disease. As per definition, progressive disease was observed in 73% of patients. However, the 10-year reoperation rate was around a quarter of that previously reported. New penetrating events occurred only in B3s patients. Postoperative change of biologic therapy correlated with severe inflammation at the proximal ileal margin, which could represent as a "red flag" for non-response to ongoing biologic therapy. No other significant correlation was shown for CD subgroups, histopathologic scores or variables, which somewhat came as a disappointment. One might speculate that the histopathologic variables found in end-stage disease may indeed not present a direct relation with its post-operative course.

In summary, we strongly believe that our findings of a similarity for histopathologic fibrosis scores and fibromuscular changes between stricturing and penetrating CD were innovative. We proposed, and hold, that the term "fibrostenosing disease" as a synonym for stricturing disease should be abandoned. Furthermore, we anticipated that therapeutic targeting of markers of inflammation-dependent and -independent fibrogenesis could prevent progression of both stricturing and penetrating CD.

To achieve the targeting of fibrogenesis markers, an increased comprehension of its pathogenesis is required. Thus, we sought to add knowledge through the genetic study of stricturing and penetrating CD biologic tissue.

6.1.3. Distinctive Fibrosis-Related Transcriptomic Pattern in Penetrating Crohn's Disease

The third objective of this thesis was addressed through a transcriptomic study performed on full-thickness sections retrieved from the most affected area (as previously defined)[23] of archived ileal resection surgical specimens penetrating and stricturing CD[26] [CHAPTER 5].

Herein we innovatively demonstrated that the two CD phenotypes showed a very good clustering due to 30 differentially expressed genes (DEGs), all of which were upregulated in the B3 group, and more than half having been described as involved in CD fibrogenesis. Interestingly, there were no DEGs between B3s and B3o subgroups, which may indicate that, genetically, the penetrating behavior is driver. This is reinforced by the findings of 46 DEGs differentiating the B2 and B3s groups. The most significant DEGs included TGFβ1, for which a central role was disclosed for most of the 30 DEGs, and its effector gene CTGF. Moreover, the most significantly active biologic processes and pathways in the B3 group were related to response to TGFβ and matrix organization and degradation, unveiling a continuous process of tissue healing and destruction distinctive of this aggressive phenotype. The key role of TGFβ1 gene in penetrating CD was demonstrated on the immunohistochemistry validation analyses.

Taken the literature and our data together, we proposed that after the possible break of the epithelial barrier mediated by CEACAM-3 gene, the TGF-β-driven invasiveness of the intestinal wall along with penetrating inflammation could result in the development of deep ulcers, fissures, and fistulae. ADAM17 gene involvement in matrix and collagen degradation might further facilitate penetration phenomena. The simultaneous TGF-β-

derived strong pro-fibrotic activity would promote ECM reorganization. The net result would be an increased ECM turnover incapable of “healing” or “closing” the fissures and fistulous tracts.

This study showed that, despite the histopathological similarities of fibrosis and fibromuscular changes between stricturing and penetrating CD, their fibrosis-related transcriptomic profiles are distinct. Even though fibrosis underlies both phenotypes and, thus, anti-fibrotic therapy is still an unmet need, the distinctive transcriptomes may insinuate different therapeutic targets even (or especially) in end-stage disease.

6.2. Limitations

Despite the contribution of this thesis to the knowledge on histopathology and pathogenesis of stricturing and, specially, penetrating CD, this thesis presents some limitations. Beyond the specific limitations of each study, addressed in the corresponding paper,[1,23,26] this section will focus on a constraint shared by the second and third studies. The most important limitation of this thesis is related to the use of archived formalin-fixed and paraffin-embedded (FFPE) blocks of CD ileal resection surgical specimens at the Pathology Department of São João University Hospital Center in Porto. The limited archive capacity of the Pathology Department (the most ancient archived material used in the second study dated from 1998) together with some cases of material degradation, led to many case exclusions, resulting in a relatively small sample of stricturing CD (29 out of 103 cases) in the second study and to some difficulties in the histopathological analyses, which were overcame as described.[23] Still, the biggest impact of using FFPE archived blocks revealed during the procedures necessary for an adequate RNA extraction, as it is known that RNA is usually more degraded than that from other sources. Even though, in the end, a very good quality RNA was ensured for analysis, as described in the Chapter 4 paper,[26] the processes concerning RNA extraction and preparation were difficult and cumbersome, justifying the absence of thesis' core scientific publications throughout 2022 and 2023. Another consequence of using RNA retrieved from FFPE archived block was the impossibility to validate our results through qPCR, although it could be accomplished by immunohistochemistry.

6.3. Future Research

Despite the added information that this thesis provides in the field of CD knowledge, there are still unmet needs concerning this thesis objectives.

First, even after Gordon et al[9] proposal concerning the items that a histopathological scoring system should include (regardless this consensus' focus on stricturing disease), as of today, still no histopathological scoring system for the evaluation of inflammation and fibrosis changes due to CD was validated or is widely accepted.[6] It was not an objective of the thesis to develop such a system. Yet, a histopathological scoring system for evaluation of inflammation and fibrosis in CD, irrespective of phenotype, is lacking.

Second, our second study results have not been reproduced. Clinical research remains very focused on stricturing disease - a recent example is the paper of Liu Q et al (vide abstract in APPENDICES, section IV),[19] which motivated the candidate to send a Letter to the Editor (vide APPENDICES, section III.b.).[27] Still, we strongly believe there is still room for expanding histopathological investigation of CD to penetrating disease.

Third, multi-omics constitute most of today's basic and clinical research on CD. Even if the main driver for these investigations is the identification of new targets for the treatment of CD (which constitute by itself a crucial research field), progresses in the understanding of the disease physiopathology are still warranted to better comprehend its clinical course and response to therapy. Hence, we believe that multi-omics will keep on leading research in the field of CD for the near and medium future.

To sum up, an increased focusing on penetrating CD by both molecular and clinical researchers is much needed to better understand this more aggressive phenotype.

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CHAPTER 7 – Conclusions

7. Conclusions

This thesis' objectives conclusions may be summarized as follows.

1. Although no validated or widely accepted transmural histopathological scoring system for the evaluation of inflammation and fibrosis changes due to CD exists as of today[1], we do believe a systematic review of the existing systems may indicate the one(s) more suitable(s) for different research settings, in view of achieving objective and comparable data.
2. The highly comprehensive and sensitive histopathological scoring systems by Chen et al[2] and Shaeffer et al[3] deemed suitable for new therapies clinical trials, although requiring validation studies[4].
3. The histopathological scoring systems by Chiorean et al[5] and Adler et al[6], while bearing some deficiencies, were considered suitable for clinical research and practice[4].
4. When using an objective histopathological transmural scoring system, there were no histopathological differences on transmural fibrosis and fibromuscular changes between penetrating and stricturing ileal CD, with inflammation degree being the major differentiator between phenotypes, as it is highest in the penetrating group[7].
5. The fibrosis-related transcriptomic profile of penetrating CD was distinct from stricturing CD, being remarkable for the activation of all the 30 DEGs and of biological pathways and processes concerning response to TGF-β and increased matrix turnover[8].
6. We propose that in penetrating disease, ECM becomes disorganized and degraded due to intense inflammatory infiltrates, combined with protease activity and potent

TGF-β-induced invasiveness leading to fissures and fistulae formation. The concomitant mostly TGF-β-related pro-fibrogenic activity would lead to increased matrix turnover[8].

7. Despite the similarities of fibrosis and fibromuscular changes between stricturing and penetrating CD at the histopathological level, the differences of their fibrosis-related transcriptomic profiles, may unveil different therapeutic targets even in end-stage disease, which may impact anti-fibrotic therapy.

Finally, "Are Crohn's disease phenotypes a myth?'"

With this thesis we can properly answer the question in two not mutually exclusive ways. First, we could not find any evidence contradicting the modern concept of advanced / complicated disease (e.g. with strictures, penetrating events or both) as a clinical entity, as stricturing and penetrating CD share not only the clinical consequences of fibrosis-derived progressive intestinal damage but also the histopathologic characteristics of transmural fibrosis and fibromuscular changes. Moreover, literature report that over 70% of patients with CD will progress to complicated disease within 10 years of diagnosis,[9] with a cumulative risk of surgery due to stricturing or penetrating complications ranging from 40% to 71%.[10–12] Hence, both from a clinical and histopathological perspectives, the traditional phenotype Montreal classification could be considered as outdated or a myth. However, this thesis unveiled a distinctive fibrosis-related transcriptome for penetrating CD as compared to stricturing disease. Specifically, the fibrosis-related transcriptomic profile of penetrating CD was remarkable for the activation of all differentially expressed genes and of biological pathways and processes related to matrix production and degradation, with a key role for TGF-β. Hence, despite the histopathological similarities,

at the genetic and transcriptomic levels, the fibrosis-related events might differ, with a potential impact on therapeutic targets. In this way, the traditional classification of CD in stricturing and penetrating phenotypes may still make sense.

As a closure, there is not a definite and closed answer to this thesis' prime question, as it will depend on the perspective or level considered. Yet, like for virtually any disease, molecular biology precedes and drives physiopathology, which, for CD, will result in histopathological changes and subsequent clinical events. The clarifying of the complete multi-omics behind CD will most probably provide the final answer.

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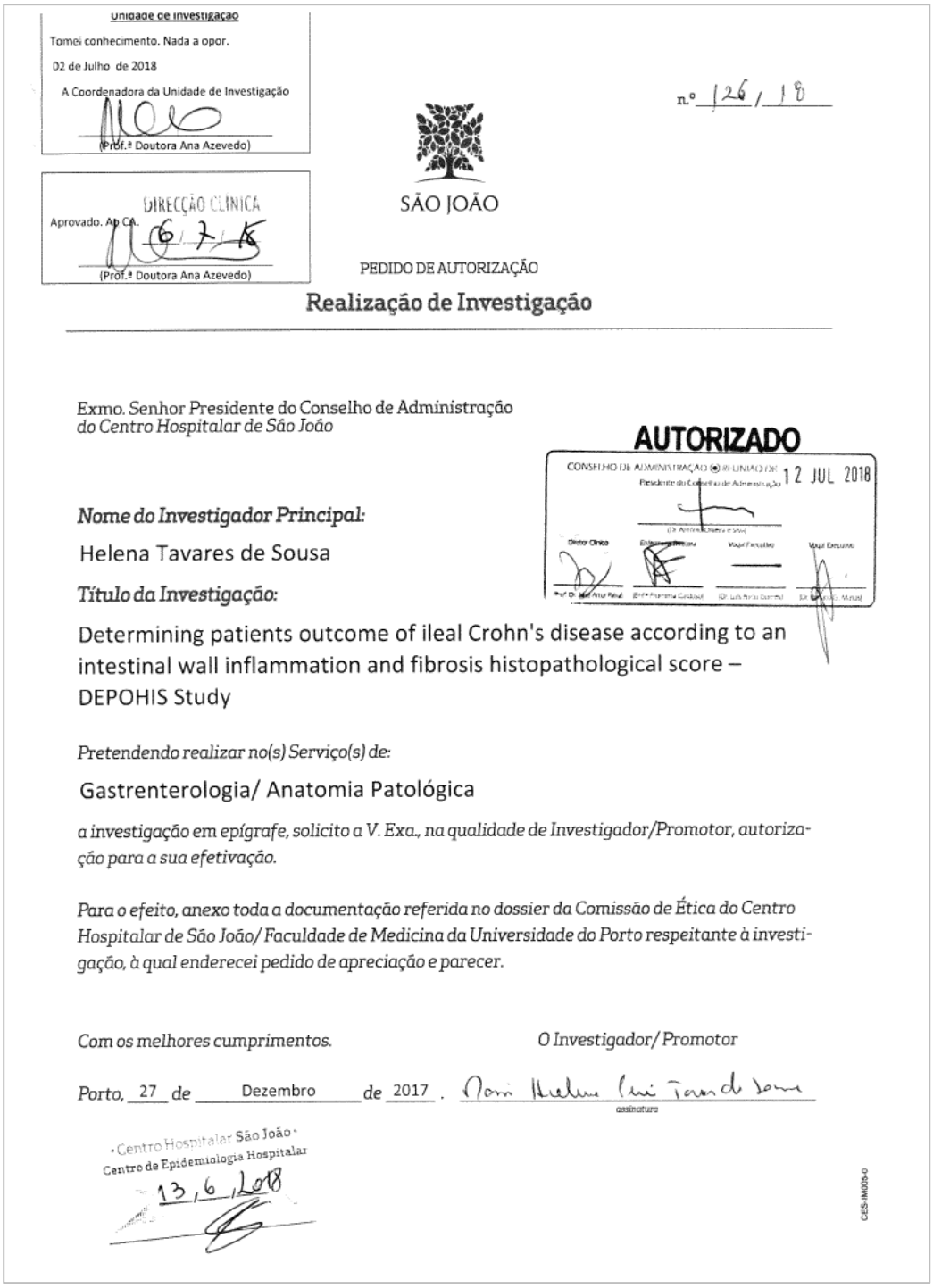
APPENDICES

I. Ethical Permissions

Permission from "Comissão de Ética para a Saúde" from "Centro Hospitalar Universitário de São João"



Unidade de Investigação
Tomei conhecimento. Nada a opor.
02 de Julho de 2018
A Coordenadora da Unidade de Investigação
(Prof.ª Doutora Ana Azevedo)

DIRECÇÃO CLÍNICA
Aprovado. Ap CA. 6/7/18
(Prof.ª Doutora Ana Azevedo)

nº 126/18

SÃO JOÃO

PEDIDO DE AUTORIZAÇÃO

Realização de Investigação

Exmo. Senhor Presidente do Conselho de Administração
do Centro Hospitalar de São João

AUTORIZADO
CONSELHO DE ADMINISTRAÇÃO REUNIÃO DE 12 JUL 2018

Nome do Investigador Principal:

Helena Tavares de Sousa

Título da Investigação:

Determining patients outcome of ileal Crohn's disease according to an intestinal wall inflammation and fibrosis histopathological score – DEPOHIS Study

Pretendendo realizar no(s) Serviço(s) de:

Gastrenterologia/ Anatomia Patológica

a investigação em epígrafe, solicito a V. Exa., na qualidade de Investigador/Promotor, autorização para a sua efetivação.

Para o efeito, anexo toda a documentação referida no dossier da Comissão de Ética do Centro Hospitalar de São João/ Faculdade de Medicina da Universidade do Porto respeitante à investigação, à qual enderecei pedido de apreciação e parecer.

Com os melhores cumprimentos. *O Investigador/ Promotor*

Porto, 27 *de* Dezembro *de* 2017 . Maria Helena Rui Tavares de Sousa
assinatura

Centro Hospitalar São João
Centro de Epidemiologia Hospitalar
13/6/2018

CES-IM005-0

Parecer da Comissão de Ética para a Saúde do
Centro Hospitalar de São João / Faculdade de Medicina da Universidade do Porto

Título do Projeto: Determining patients outcome of ileal Crohn's disease according to an intestinal wall inflammation and fibrosis histopathological score – DEPOHIS Study

Nome da Investigadora Principal: Dra. Helena Tavares de Sousa

Onde decorre o Estudo: No Serviço de Gastrenterologia e Anatomia Patológica do CHSJ. Dispõe de autorização do Prof. Doutor Guilherme Macedo e da Prof.ª Doutora Fátima Carneiro.

Objectivos do Estudo:
Aplicar um score histopatológico para a quantificação e caracterização da inflamação e fibrose da parede intestinal na DC e correlacioná-lo com o resultado pós-operatório a médio prazo.

Concepção e Pertinência do estudo:
Não existe um score histopatológico validado que quantifique a gravidade da fibrose intestinal na DC.
Mediante o estudo de secções de peças de ressecção de íleon terminal em doentes com fenótipo estenosante e penetrante, propomo-nos quantificar e caracterizar a inflamação e fibrose da parede intestinal na DC de acordo com um score histopatológico e correlacioná-lo com o outcome do doente. Este estudo deverá confirmar não apenas que estes dois fenótipos apresentam diferentes cargas e padrões de fibrose, mas também que estas diferenças são determinantes do resultado pós-operatório a médio prazo.

Benefício/risco: Não aplicável

Confidencialidade dos dados:
Doentes são identificados através de um número aleatório.

Respeito pela liberdade e autonomia do sujeito de ensaio: Não aplicável

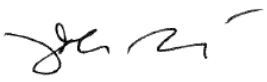
***Curriculum* da investigadora**: Adequado à investigação.

Data previsível da conclusão do estudo: Dezembro de 2019

Conclusão: Proponho um parecer favorável à realização deste projecto de investigação.

Porto, 25 de Maio de 2018

O Relator da CES, Dr. John Preto



Comissão de Ética Centro Hospitalar São João / / Faculdade de Medicina da Universidade do Porto n.º 126 / 18

SÃO JOÃO

U.PORTO FMUP FACULDADE DE MEDICINA UNIVERSIDADE DO PORTO

Questionário para submissão de Investigação

Exmo. Sr. Presidente da Comissão de Ética do Centro Hospitalar de São João/ Faculdade de Medicina da Universidade do Porto,

Pretendendo realizar a investigação infracitada, solicito a V. Exa., na qualidade de Investigador, a sua apreciação e a elaboração do respetivo parecer. Para o efeito, anexo toda a documentação requerida.

IDENTIFICAÇÃO DO ESTUDO

Título da investigação: Determining patients outcome of ileal Crohn's disease according to an intestinal wall inflammation and fibrosis

Nome do investigador: Helena Tavares de Sousa

Endereço eletrónico: helenatsousa@gmail.com **Contacto telefónico:** 962307915

Caracterização da investigação:

☒ Estudo retrospetivo ☐ Estudo observacional ☐ Estudo prospetivo

☐ Inquérito ☐ Outro. Qual? _____

Tipo de investigação:

☐ Com intervenção ☒ Sem intervenção

Formação do investigador em boas práticas clínicas *(GCP)*: ☒ Sim ☐ Não

Promotor *(se aplicável)*: _____

Nome do orientador de dissertação/tese *(se aplicável)*: _____

Endereço eletrónico: _____

Local/locais onde se realiza a investigação: Serviço Gastrenterologia CHSJ/ Serviço Anatomia Patológica CHSJ

Data prevista para início: 1 / 6 / 2018 Data prevista para o término: 31 / 12 / 2019

PROTOCOLO DO ESTUDO

Síntese dos objetivos:

Aplicar um score histopatológico para a quantificação e caracterização da inflamação e fibrose da parede intestinal na DC e correlacioná-lo com o resultado pós-operatório a médio prazo.

Fundamentação ética (ganhos em conhecimento/inovação; ponderação benefícios/riscos):

Não existe um score histopatológico validado que quantifique a gravidade da fibrose intestinal na DC.
Mediante o estudo de secções de peças de ressecção de íleon terminal em doentes com fenótipo estenosante e penetrante, propomo-nos quantificar e caracterizar a inflamação e fibrose da parede intestinal na DC de acordo com um score histopatológico e correlacioná-lo com o outcome do doente. Este estudo deverá confirmar não apenas que estes dois fenótipos apresentam diferentes cargas e padrões de fibrose, mas também que estas diferenças são determinantes do resultado pós-operatório a médio prazo.

CONFIDENCIALIDADE

De que forma é garantida a anonimização dos dados recolhidos de toda a informação?

Doentes são identificados através de um número aleatório

O investigador necessita ter acesso a dados do processo clínico? ☒ Sim ☐ Não

Está previsto o registo de imagem ou som dos participantes? ☐ Sim ☒ Não

Se sim, está prevista a destruição deste registo após o sua utilização? ☐ Sim ☐ Não

CONSENTI MENTO

O estudo implica recrutamento de:

Doentes: ☐ Sim ☒ Não Voluntários saudáveis: ☐ Sim ☒ Não

Menores de 18 anos: ☐ Sim ☒ Não

Outras pessoas sem capacidade do exercício de autonomia: ☐ Sim ☒ Não

A investigação prevê a obtenção de Consentimento Informado: ☐ Sim ☒ Não

Se não, referir qual o fundamento para a isenção:

Para este estudo será analisado material biológico recolhido no passado e armazenado no Serviço de Anatomia Patológica do Centro Hospitalar de São João; este material biológico consiste em lâminas e blocos de parafina com tecido de ileon terminal de doentes operados no passado por doença de Crohn complicada (estenose obstrutiva ou complicação penetrante). Para a caracterização da

Existe informação escrita aos participantes: ☐ Sim ☒ Não

PROPRIEDADE DOS DADOS

A investigação e os seus resultados são propriedade intelectual de:

☒ Investigador ☐ Promotor ☐ Ambos ☐ Serviço onde é realizado

☐ Não aplicável Outro: _____

BENEFÍCIOS, RISCOS E CONTRAPARTIDAS PARA OS PARTICIPANTES

Benefícios previsíveis:

Potencial de optimização do seguimento de doentes com Doenças de Crohn

Riscos/incómodos previsíveis:

Não existem riscos para os participantes

São dadas contrapartidas aos participantes:

- *pela participação* ☐ Sim ☐ Não ☒ Não aplicável
- *pelas deslocações* ☐ Sim ☐ Não ☒ Não aplicável
- *pelas faltas ao emprego* ☐ Sim ☐ Não ☒ Não aplicável
- *por outras perdas e danos* ☐ Sim ☐ Não ☒ Não aplicável

CUSTOS / PLANO FINANCEIRO

Os custos da investigação são suportados por:

☒ Investigador ☐ Promotor ☐ Serviço onde é realizado

☐ Não aplicável Outro: _____

Existe protocolo financeiro? ☐ Sim ☒ Não

LISTA DE DOCUMENTOS ANEXOS

- [x] Pedido de autorização ao Presidente do Conselho de Administração do Centro Hospitalar de São João *(se aplicável)*
- [ ] Pedido de autorização à Diretora da Faculdade de Medicina da Universidade do Porto *(se aplicável)*
- [x] Protocolo do estudo
- [x] Declaração do Diretor de Serviço onde decorre o estudo
  (sendo um estudo na área de enfermagem deve anexar também a concordância da chefia de enfermagem)
- [x] Profissional de ligação
- [ ] Informação dos orientadores
- [ ] Informação ao participante
- [ ] Modelo de consentimento
- [ ] Instrumentos a utilizar *(inquéritos, questionários,escalas, p.ex.)*: _____
- [x] Curriculum Vitae abreviado *(máx. 3 páginas)*
- [ ] Protocolo financeiro
- [ ] Outros:

COMPROMISSO DE HONRA E DECLARAÇÃO DE INTERESSES

Declaro por minha honra que as informações prestadas neste questionário são verdadeiras. Mais declaro que, durante o estudo, serão respeitadas as recomendações constantes da Declaração de Helsínquia (1960 e respetivas emendas), e da Organização Mundial da Saúde, Convenção de Oviedo e das "Boas Práticas Clínicas" (GCP/ICH) no que se refere à experimentação que envolve seres humanos. Aceito, também, a recomendação da CES de que o recrutamento para este estudo se fará junto de doentes que não tenham participado em outro estudo, nos últimos três meses. Comprometo-me a entregar à CES o relatório final da investigação, assim que concluído.

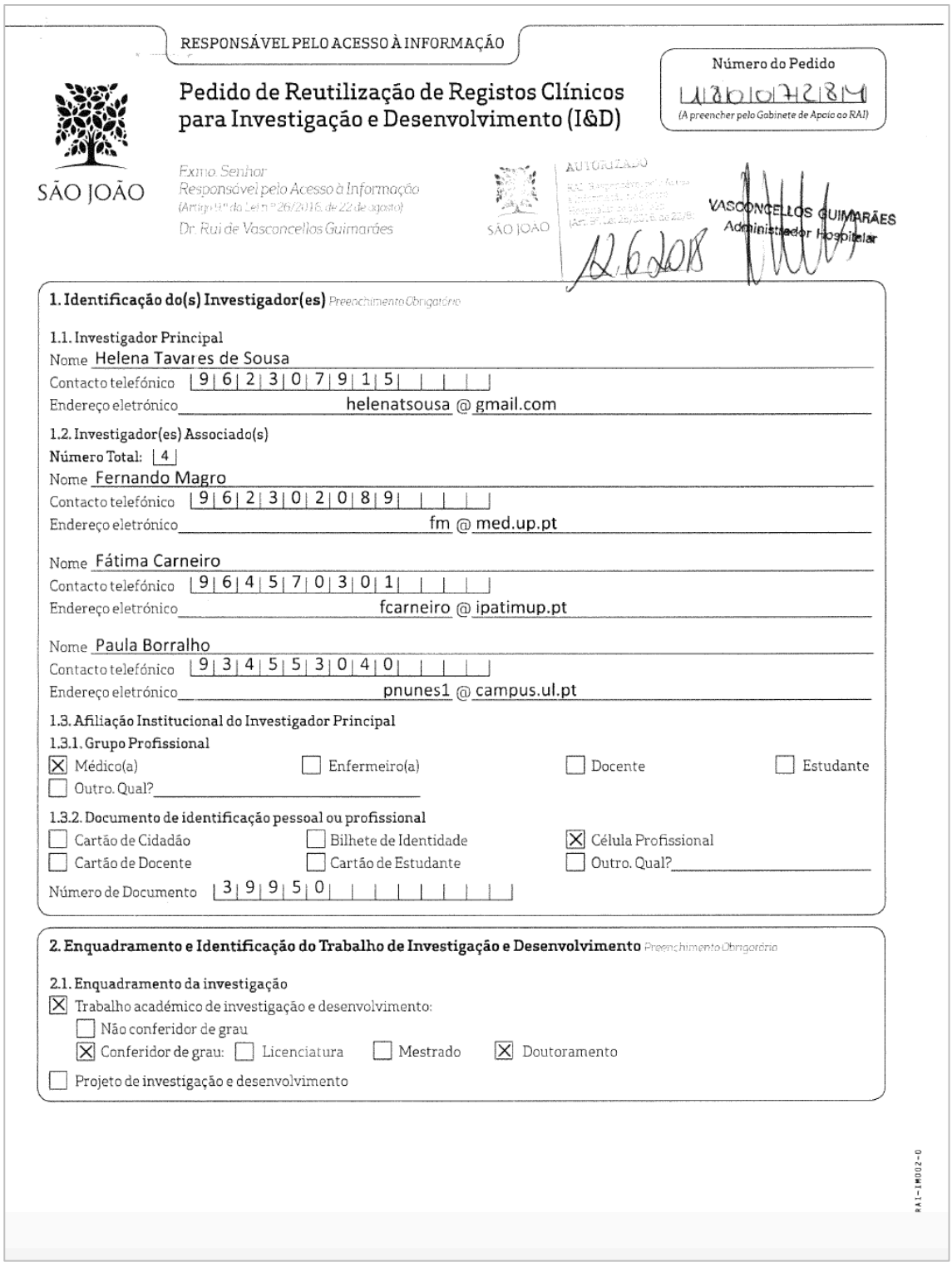
Porto, 27 de Dezembro de 2017

Nome legível: Helena Tavares de Sousa

assinatura

Parecer da Comissão de Ética do Centro Hospitalar de São João/ FMUP Emitido na reunião plenária da CE de _____ / _____ / _____

Permission for Clinical Data Acess and Use for Investigational Purposes from “Centro Hospitalar Universitário de São João”



RESPONSÁVEL PELO ACESSO À INFORMAÇÃO

SÃO JOÃO

Número do Pedido
1 1 3 6 1 0 7 2 8 4
(A preencher pelo Gabinete de Apoio ao RAI)

Pedido de Reutilização de Registos Clínicos para Investigação e Desenvolvimento (I&D)

Exmo. Senhor
Responsável pelo Acesso à Informação
(Artigo 9.º da Lei n.º 26/2016, de 22 de agosto)
Dr. Rui de Vasconcellos Guimarães

SÃO JOÃO

AUTORIZADO
VASCONCELLOS GUIMARÃES
Administrador Hospitalar
12.6.2018

1. Identificação do(s) Investigador(es) Preenchimento Obrigatório

1.1. Investigador Principal
Nome Helena Tavares de Sousa
Contacto telefónico 962307915
Endereço eletrónico helenatsousa @ gmail.com

1.2. Investigador(es) Associado(s)
Número Total: 4
Nome Fernando Magro
Contacto telefónico 962302089
Endereço eletrónico fm @ med.up.pt

Nome Fátima Carneiro
Contacto telefónico 964570301
Endereço eletrónico fcarneiro @ ipatimup.pt

Nome Paula Borralho
Contacto telefónico 934553040
Endereço eletrónico pnunes1 @ campus.ul.pt

1.3. Afiliação Institucional do Investigador Principal
1.3.1. Grupo Profissional
☒ Médico(a) ☐ Enfermeiro(a) ☐ Docente ☐ Estudante
☐ Outro. Qual?

1.3.2. Documento de identificação pessoal ou profissional
☐ Cartão de Cidadão ☐ Bilhete de Identidade ☒ Célula Profissional
☐ Cartão de Docente ☐ Cartão de Estudante ☐ Outro. Qual?
Número de Documento 39950

2. Enquadramento e Identificação do Trabalho de Investigação e Desenvolvimento Preenchimento Obrigatório

2.1. Enquadramento da investigação
☒ Trabalho académico de investigação e desenvolvimento:
☐ Não conferidor de grau
☒ Conferidor de grau: ☐ Licenciatura ☐ Mestrado ☒ Doutoramento
☐ Projeto de investigação e desenvolvimento

RAI-IM002-0

2.2. Entidade(s) que tutela(m) a investigação

☒ Centro Hospitalar de São João

Serviço: Gastrenterologia e Anatomia Patológica

☐ Universidade do Porto

Faculdade / Instituto: _____

☐ Outra Instituição. *Qual?* _____

Há alguma parceria entre instituições?

☐ Não ☐ Sim. Qual(is)? _____

2.3. Orientador *Se Aplicável*

Contacto telefónico 962302089

Endereço eletrónico fm @ med.up.pt

2.4. Título provisório

Determining patients' outcome of ileal Crohn's disease according to an intestinal wall inflammation and fibrosis histopathological score – DEPOHIS Study

Deverá posteriormente indicar o título definitivo para emissão do Certificado de Reutilização pelo RAI – DAta REuse Certificate for Research – DARE através dos contactos disponíveis no fim deste formulário.

2.5. Acesso requerido

☐ Ficheiro

Descrição do património informacional a que pretende ter acesso, identificando a informação a obter, i.e. nome, morada, diagnóstico, idade, códigos dos distritos, entre outros.

☐ Consulta de processos clínicos em ambiente papel:

☐ Bloco ☐ Consulta Externa ☐ Hospital de Dia ☐ Internamento ☐ MCDT ☐ Urgência

Deverá anexar ficheiro(s) contendo a identificação do pretendido, i.e. números de processos, episódios, números de utente, entre outros.

Anexar ficheiro no ato de envio

☒ Consulta de registos clínicos eletrónicos

Especificar os Sistemas de Informação:

Diários Clínicos de Consulta Externa, Internamento e Hospital de Dia. Relatórios Operatórios. Relatórios

Data previsível de fim de utilização das credenciais de acesso 2020-06-30

☐ Outro Acesso. Qual?

2.3. Pareceres e Autorizações

☐ Autorização da Hierarquia

☐ Protocolo Científico Aprovado [1]

☐ Parecer da Comissão de Ética para a Saúde (CES) [1]

☐ Parecer do Centro de Epidemiologia Hospitalar [1]

Deverá anexar ficheiro(s) contendo cópia dos documentos referentes às opções selecionadas.

Anexar ficheiro no ato de envio

3. Observações *Preenchimento Facultativo*

Este trabalho será integrado em Tese de Doutoramento, embora o Programa Doutoral propriamente dit

4º Investigador Associado: Guilherme Macedo, Tm: 963972028, email: guilhermemacedo59@gmail.com

4. Aceitação dos Termos e Condições da Reutilização

Cumulativamente com as obrigações decorrentes da lei já citada *(n.º 2 e 3 do artigo 21 e o n.º 1 e 2 do artigo 12, ambos da Lei n.º 26/2016, de 22 de agosto)* ao submeter o presente pedido concordo e fico ainda vinculado aos seguintes termos e condições:

- Comprometo-me a manter confidencial toda a informação à qual vou ter acesso;
- Não vou elaborar registos, susceptíveis de identificar ou tornar identificável a identidade das pessoas a quem os mesmos dizem respeito;
- Não vou elaborar, nem ficar na posse, de cópias de bases de dados utilizadas na recolha de informação;
- Comprometo-me a obter junto da Comissão Nacional de Proteção de Dados (CNPD) as necessárias autorizações, para eventuais bases de dados que venha a conceber e utilizar no âmbito da presente investigação;
- Comprometo-me a devolver ao Centro Hospitalar de São João, na pessoa do seu Diretor Clínico, as bases de dados e o resultado da investigação;
- Comprometo-me a ocultar os elementos de identificação da(s) pessoa(s) a quem os registos digam respeito, em futuras e eventuais publicações de resultados;
- Comprometo-me a consultar os processos clínicos nas instalações que me forem indicadas para o efeito;
- Comprometo-me a obter os necessários pareceres, quer da Comissão de Ética do Hospital, quer do Centro de Epidemiologia Hospitalar, sempre que necessário;
- Comprometo-me a citar as fontes sempre que publicitar o trabalho de investigação independentemente de requerer a Certidão de Reutilização (DAta REuse Certificate for Research – DARE);
- Tomei conhecimento, que a violação de qualquer dos compromissos aqui assumidos, resultará no apuramento de responsabilidades disciplinares, civis e penais e ainda, à impossibilidade futura de aceder a informação de saúde para fins de investigação.

5. Decisão do investigador sobre requerer a DAta REuse Certificate for Research – DARE *Preenchimento Obrigatório*

☒ Pretendo desde já requerer a Certidão de Reutilização (DARE) cujo sentido, valor e significado consultei em *http://portal-chsj.min-saude.pt/pages/710*.

☐ Não pretendo requerer a Certidão de Reutilização (DARE) cujo sentido, valor e significado consultei em *http://portal-chsj.min-saude.pt/pages/710*.

6. Assinatura

Nota 1: Se o presente pedido for submetido eletronicamente ou faz assinatura digital qualificada; ou posteriormente vem ao Centro Hospitalar de São João exibir o seu documento de identificação pessoal, ou no âmbito do seu espaço de liberdade e como manifestação expressa do seu consentimento envia cópia do referido documento, neste caso, concluído o processo ser-lhe-á devolvida ou eliminada a cópia do documento de identificação pessoal, conforme as indicações que dê.
Nota 2: Se o presente pedido for entregue presencialmente, assina e exibe o documento de identificação a quem recebe o pedido.

Data 2018-06-10

Maria Helena Pereira Tomaz de Sousa
Investigador Principal

Em caso de dúvida no preenchimento contacte através dos endereços eletrónicos
rai.reutilizacao.id@chsj.min-saude.pt **ou** ruiguimaraes@chsj.min-saude.pt
ou pelos números de telemóvel 962 204 194 **ou** 918 880 299

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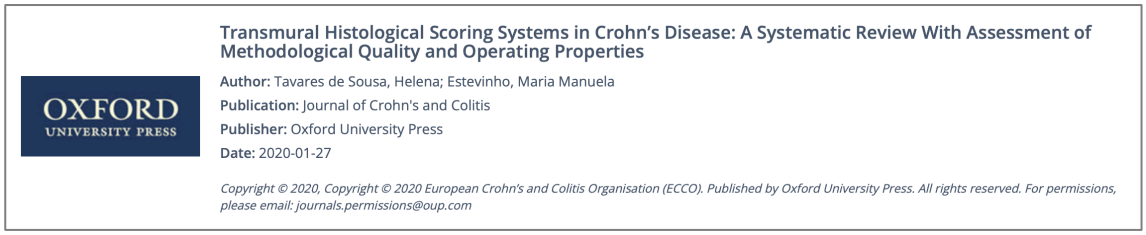

Transmural Histological Scoring Systems in Crohn's Disease: A Systematic Review With Assessment of Methodological Quality and Operating Properties

Author: Tavares de Sousa, Helena; Estevinho, Maria Manuela

Publication: Journal of Crohn's and Colitis

Publisher: Oxford University Press

Date: 2020-01-27

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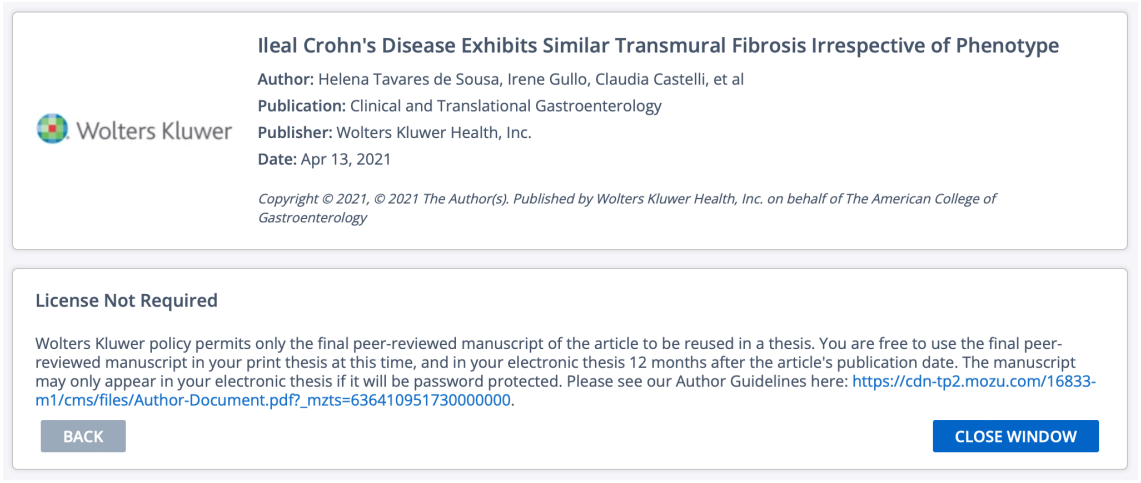

Ileal Crohn's Disease Exhibits Similar Transmural Fibrosis Irrespective of Phenotype

Author: Helena Tavares de Sousa, Irene Gullo, Claudia Castelli, et al

Publication: Clinical and Translational Gastroenterology

Publisher: Wolters Kluwer Health, Inc.

Date: Apr 13, 2021

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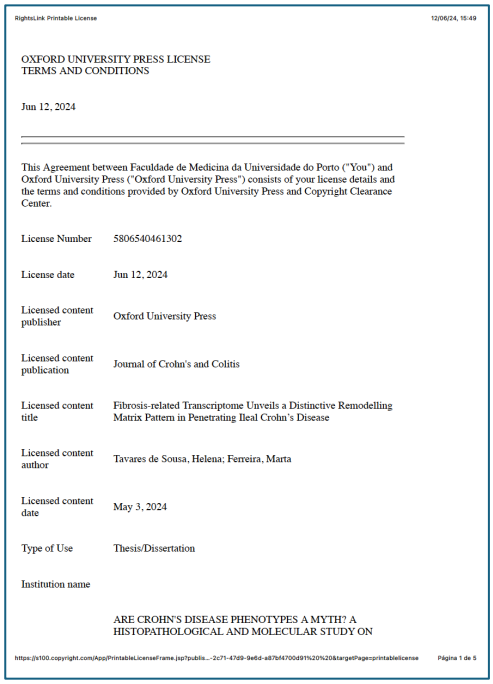
Fibrosis-related Transcriptome Unveils a Distinctive Remodelling Matrix Pattern in Penetrating Ileal Crohn's Disease

Author: Tavares de Sousa, Helena; Ferreira, Marta

Publication: Journal of Crohn's and Colitis

Publisher: Oxford University Press

Date: 2024-05-03



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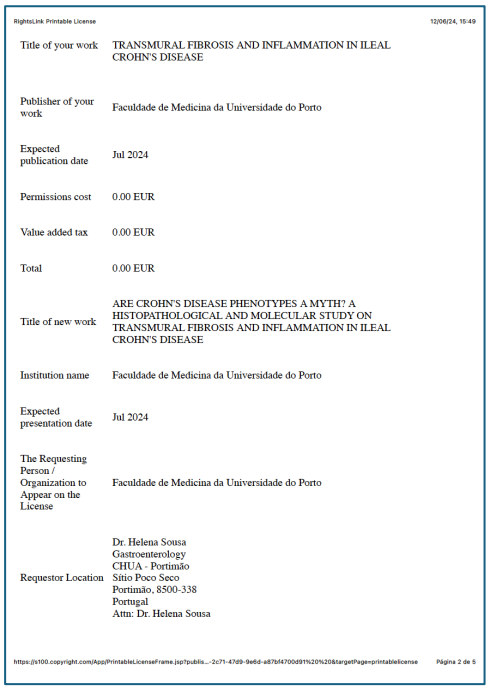

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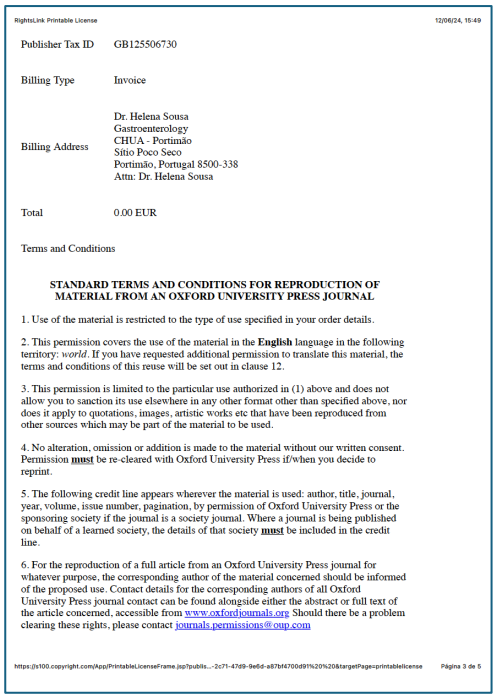

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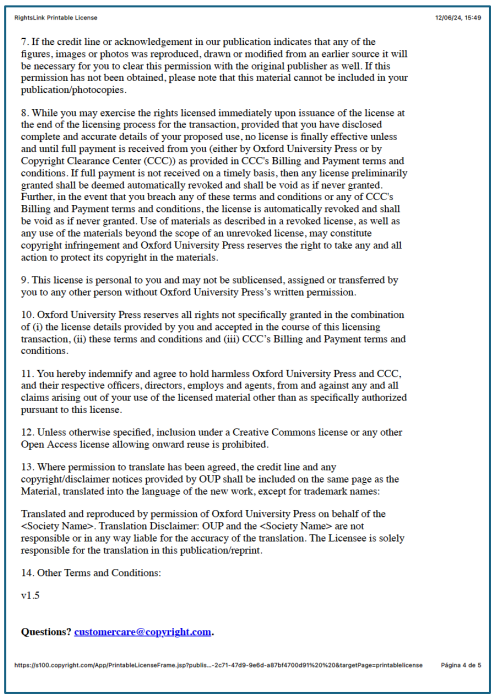

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EDITORIAL | Open Access

Understanding progression of strictures in ileal Crohn's disease—The importance of setting methodological standards

Helena Tavares de Sousa, Fátima Carneiro

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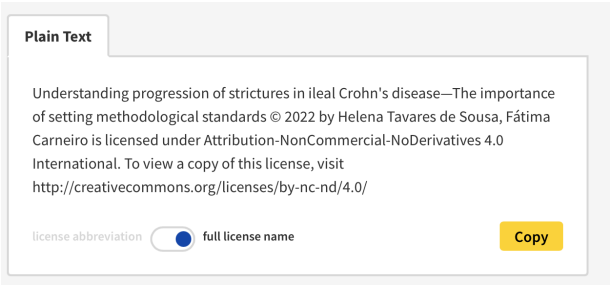

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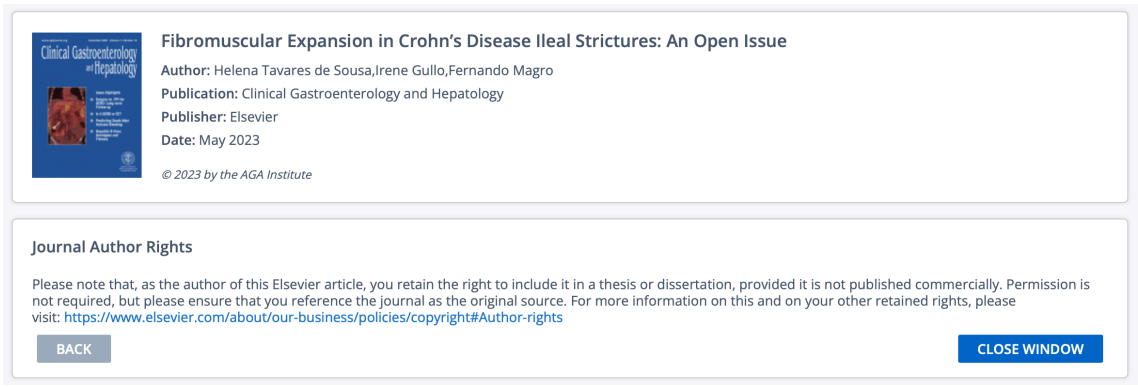

Fibromuscular Expansion in Crohn's Disease Ileal Strictures: An Open Issue

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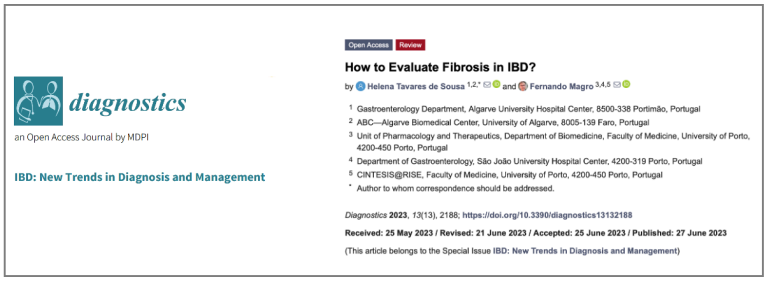

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IBD: New Trends in Diagnosis and Management

Open Access | Review

How to Evaluate Fibrosis in IBD?

by Helena Tavares de Sousa [1,2,*] and Fernando Magro [3,4,5]

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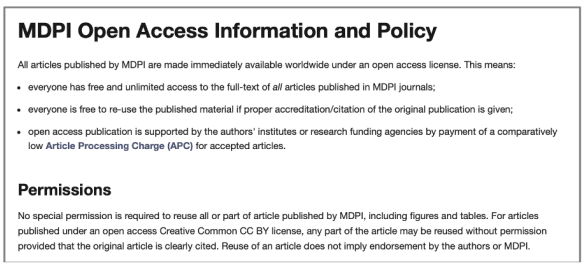

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III. Non-Core Papers a. and b.

a. Tavares de Sousa H, Carneiro F. **Understanding progression of strictures in ileal Crohn's disease - The importance of setting methodological standards**. United European Gastroenterol J. 2022 Nov;10(9):915-916. doi: 10.1002/ueg2.12327. Epub 2022 Oct 17. PMID: 36251489; PMCID: PMC9731658

Editorial.[1]

Journal Impact Factor (2022): 6.866[2] (Q1 Gastroenterology)[3]

[1] **Concerning the paper** by El Ouali S, Baker ME, Lyu Ret al. Validation of stricture length, duration and obstructive symptoms as predictors for intervention in ileal stricturing Crohn's disease. United European Gastroenterol J. 2022 Nov;10(9):958-972. doi: 10.1002/ueg2.12314. Epub 2022 Dec 7. PMID: 36479841; PMCID: PMC9731662 – **vide abstract in CHAPTER 8 - APPENDICES, section IV.**

[2] Journal Citation Reports™ from Clarivate/Web of Science, 2022

[3] From https://www.scimagojr.com/journalrank.php?category=2715&year=2022

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EDITORIAL

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Understanding progression of strictures in ileal Crohn's disease—The importance of setting methodological standards

Crohn's disease (CD) is a chronic inflammatory bowel disease most frequently involving the terminal ileum and right colon. Given its transmural nature, CD can lead to progressive bowel damage and complications (e.g., strictures, fistulas and abscesses).[1] Over 10% of patients present strictures at diagnosis,[2] with 15% to over 20% of the remainders developing them through the next 10 and 20 years, respectively.[3] Importantly, strictures coexist in over 85% of penetrating CD.[4] Population-based studies showed a 10-year cumulative risk of surgery (due to stricturing and/or penetrating complications) between 40% and 71%.[5] After intestinal resection, anastomotic recurrence is the rule, leading to re-stricturing and need of intervention, either through endoscopic balloon dilation (EBD) or surgery.[6] Despite the high rates of stricturing disease, a gap remains in the understanding of the risk factors and progression rate for stricture development, which are crucial for patient risk stratification and selection of those benefiting the most from intervention. Noteworthy, prior studies on stricturing CD presented significant methodological caveats: (1) lack of a standard definition of stricture, (2) mixed populations (stricturing disease with and without associated fistulae, ileal and colonic strictures, anastomotic and primary strictures), (3) variable, non-validated outcomes (subjective obstructive symptoms, different success criteria for therapeutic interventions), and (4) different imaging modalities and protocols, overall contributing for heterogeneous results.

The study by El Ouali et al.[7] aimed to address many of these gaps in research. The authors included only patients with a strictly-defined "non-penetrating terminal ileum (TI) stricturing CD"—as per the CrOhN's disease anti-fibrotic STRICTure therapies [CONSTRICT] group criteria,[8] treated according to current standard of care. Additionally, they implemented centrally-read (blinded to outcome measures) and state-of-art protocolised-MRE, and rated obstructive symptoms through an own-devised 7-point index. The study included a derivation-cohort from which a predictor model was built and a subsequent validation-cohort that validated the model and its predictors. With this stringent methodology, El Ouali et al.[7] could establish the rate of progression to, the risk factors for, and the need of intervention (EBD or surgery) in "pure" TI stricturing CD. Intervention rates at 12, 24 and 48 months were 26%, 35% and 45%, respectively. Importantly, shorter duration (HR 0.97 [0.95–0.995], $p = 0.016$) and increased length (HR 1.04 [1.01–1.07], $p = 0.007$) of the stricture, together with higher obstructive index (HR 1.44 [1.13–1.85], $p = 0.004$) were validated as predictors of subsequent intervention. On univariate analyses, an anastomotic stricture associated with EBD (HR 8.10 [1.02–64.16]; $p = 0.047$) while decision for surgery associated strongly with restricted diffusion on MRE (HR 10.62 [1.24–91.13]; $p = 0.03$), followed by past smoking (HR 3.75 [1.31–10.77]; $p = 0.01$), nausea/vomiting (HR 2.62 [1.03–6.67]; $p = 0.04$), obstructive index (HR 1.41 [1.07–1.87]; $p = 0.02$) and stricture length (HR 1.04 [1.01–1.07]; $p = 0.003$).

This work raises important issues to clinical practice. First, almost half of the patients required intervention (surgery or EBD) at 48 months of follow up. This might seem to challenge data from previous studies where anti-TNFα treatment prevented surgery in over half of patients with symptomatic stricturing disease at 40–48 months[9,10] Yet, only 26%[10] or 29%[9] of these patients maintained successful response to anti-TNFα (with no add-on medical or endoscopic therapy) at 40 and 48 months, respectively, even if only early disease (median disease duration 2.9 years [0.6–8.6]), biologic-naïve, non-operated patients were included.[10] These data, together with the fact that in this cohort, biologic use did not impact the need for intervention, reinforce the notion that these agents do not alter the natural history of the disease since fibrosis and inflammation always coexist[11] and the first cannot be reversed with current biologic therapy.[4] Second, although 40% of the included patients were asymptomatic at baseline, almost half of them developed obstructive symptoms after 48 months. Clinicians must be aware of the disconnect between symptoms and progression of disease and recognise the impact of the herein identified predictors of intervention. Third, despite some limitations (single-center, observational, retrospective study, including a limited ($n = 86$) number of patients), this work has the potential to have an impact both in clinical practice and in clinical trials. Remarkably, the article provides an accessible online risk calculator for predicting intervention. Future studies should reproduce these findings prospectively and in non-quaternary centers, providing a real-life model validation.

Finally, El Ouali et al.'s study[7] confirms the importance of setting standards for defining study populations and imaging methodology when addressing strictures or their development in CD. This article

can be integrated in the enormous effort that has been put by the Stenosis Therapy and Anti-fibrosis Research Consortium [STAR] for several years, in developing endpoints[8] and standardized methodology for clinical,[12] radiologic[13] and histopathologic scoring systems,[14] in order to build up the much needed clinical trials with antifibrotic agents.[15]

CONFLICT OF INTEREST

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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DATA AVAILABILITY STATEMENT

The data mentioned in this text can be found in the corresponding reference.

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Letter to the Editor.[4]

Journal Impact Factor (2022): 11.382[5] *(Q1 Gastroenterology)*[6]

[4] **In response to the paper** by Liu Q, Zhang X, Ko HM et al. Constrictive and Hypertrophic Strictures in Ileal Crohn's Disease. Clin Gastroenterol Hepatol. 2022 Jun;20(6):e1292-e1304. doi: 10.1016/j.cgh.2021.08.012. Epub 2021 Aug 14. PMID: 34400338 **– vide abstract in CHAPTER 8 - APPENDICES, section IV.**

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[6] From https://www.scimagojr.com/journalrank.php?category=2715&year=2022

active vibration pattern from a simple wearable electronic technology may condition the patient to avoid the right lateral decubitus position, thus significantly alleviating nocturnal reflux symptoms and improving quality of sleep. Of note, on subgroup analysis, treatment success was achieved in patients on proton pump inhibitor therapy and without hiatal hernia. The authors should be commended not only for confirming the link between sleep position and GERD but also for presenting a simple and effective technology that may benefit a large patient population.

We would like to highlight the relationship between sleep position and reflux in patients undergoing esophagectomy and gastric conduit reconstruction for cancer. The pathophysiology of intrathoracic stomach includes disruption of the abdominothoracic pressure gradient, vagal denervation, delayed gastric emptying, and mixed acid and biliary reflux. Up to 60%–80% of patients complain of reflux symptoms after esophagectomy, and progression to severe reflux esophagitis and even Barrett's esophagus is frequently reported.[7] In a large proportion of patients who are cancer-free after curative esophagectomy, troublesome nocturnal regurgitation and choking can severely impair tolerance to daily activity and reduce the likelihood of returning to work. It has been shown that severe reflux occurs in 27% of esophagectomy patients, and these individuals are more likely to suffer from poor sleep quality (odds ratio, 4.9; 95% confidence interval, 1.9–12.4).[8] Proton pump inhibitors are recommended to control heartburn, but volume regurgitation and recurrent aspiration pneumonia remain a major issue. Elevation of the head of the bed using multiple pillows, a wedge pillow, or an ergonomic adjustable bed is generally advised, but it is often uncomfortable because patients tend to slide down, and the overall sleep quality is compromised by refractory regurgitation, morning tiredness, and reduced daytime energy. In a recent survey of 177 patients followed between 1 and 6 years after hybrid Ivor Lewis esophagectomy at our Institution (unpublished data), 88 (48.58%) reported to sleep preferably in the right-lateral position, 57 (32.20%) in the semirecumbent supine position, and 32 (18.07%) either in the right-lateral or semirecumbent position. Interestingly, only 9 individuals (5.08%) were able to sleep in the left-lateral position because this decubitus was commonly associated with volume regurgitation, especially in the postprandial phase. Most patients were using multiple pillows or a wedge, and only a few used an ergonomic adjustable bed. Despite continuous proton pump inhibitor therapy, the median GERD–health-related QOL score was 11 (interquartile range, 6), and about 50% of these patients complained of insomnia and nocturnal regurgitation. At last follow-up endoscopy, the overall prevalence of erosive esophagitis, intestinal metaplasia, and *Helicobacter pylori* infection was 20%, 6.2%, and 7.9%, respectively. Interestingly, sleeping on the right side reduced the prevalence of insomnia and regurgitation ($P < .05$), although the prevalence of endoscopic findings was similar in both groups.

A physiological mechanism accounting for the observed reflux relief in the right lateral decubitus after esophagectomy remains elusive. Besides recommending proton pump inhibitor and a light meal at least 3 hours before going to bed, we suggest testing these patients to sleep on the right side and wear the electronic device described by Schuitenmaker et al.[1] This simple adjunctive treatment may contribute to prevent the "nightmare" of restless sleeping and to improve overall QOL.

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Conflicts of interest
The authors disclose no conflicts.

Most current article
https://doi.org/10.1016/j.cgh.2022.04.030

Fibromuscular Expansion in Crohn's Disease Ileal Strictures: An Open Issue

Dear Editor:

We read with great interest the study by Liu et al[1] published in June's issue of *Clinical Gastroenterology and Hepatology*. Recent pathologic consensus defined small bowel strictures in Crohn's disease (CD) as a combination of decreased luminal diameter and increased thickness of all layers of the intestinal wall, including expansion of the muscularis mucosae (MM) and inner muscularis propria (MP), muscularization of the submucosa, and fibrosis of the submucosa and intestinal wall.[2] Herein Liu et al[1] describe 2 not mutually exclusive types of ileal strictures in CD: the traditional hypertrophic strictures, featuring a normal or expanded external circumference, a concentrically thickened and rigid wall with increased fibrous and smooth muscle content, and high inflammation scores; and a new subset of

constrictive strictures presenting a reduced external circumference, a lean, pliable wall with no increased fibrosis or smooth muscle content and low inflammation scores. Interestingly, all (n = 6) hypertrophic strictures were single, whereas 2/3 (12 of 18) of the constrictive ones were multiple (2–4 per specimen). Although fat wrapping was a universal feature, its extent inversely correlated with decreased intestinal circumference in the constrictive subtype ($P = .003$), indicating a potential role of fat wrapping in their pathogenesis. It was recently demonstrated that TGFβ1-activated smooth muscle cells from the MP outer layer secreted an extracellular matrix in contact with adjacent adipose tissue. This matrix contained increased amounts of fibronectin, which interacted with $\alpha5\beta1$-integrin on the preadipocyte surface, inducing its migration out of the mesenteric fat and de novo formation of creeping fat.[3] No proinflammatory cytokine could promote this migration, contrasting with the established correlation between fat wrapping and intestinal inflammation. However, $\alpha5\beta1$-integrin is crucially involved in mechanotransduction, the key mechanism of inflammation-independent fibrogenesis,[4] which could then be involved in the increased fat wrapping of constrictive strictures.

Notwithstanding the standardization of fibromuscular expansion as an essential feature of CD strictures,[2] Liu et al's[1] work stresses that this is an open issue. It was shown that the most prominent muscular changes occur in the MM, both in the degree of expansion (>17-fold), increase of smooth muscle content (>11-fold), and severity of architectural disarray, when compared with the small expansion and architecture preservation of the MP layers, suggesting a luminally polarized fibromuscular remodeling.[5] Conversely, others demonstrated that the thickening of the intestinal wall was mostly caused by smooth muscle hypertrophy in the MP (>2-fold) and hyperplasia in the MM (2- to 3-fold), followed by minor expansion of the submucosa.[6] Recently, penetrating fibrosis, defined as continuing fibrosis through all intestinal layers with extension into the mesenteric fat, was found in 95% (36 of 38) of ileal CD resection specimens and 88% (59 of 67) of stricturing or penetrating cases, further expanding the range of fibromuscular changes in CD strictures.[7] Through histopathologic assessment of 103 ileal resection specimens, our group demonstrated similar degrees of fibrosis between penetrating and stricturing ileal CD, with the major differentiator being severity of inflammation. When comparing pure strictures (B2; n = 29) with those with associated fistulae (B3; n = 54), there were no differences regarding expansion of MM or MP or submucosal muscularization. Absence of inflammation was found only at the proximal ileal surgical margin (10%), and inflammation limited to the mucosa in only 8% of most affected areas. We did not perform morphometric analyses, so we cannot confirm if the 29 B2 specimens comprised any constrictive stricture as defined by Liu et al.[1] Still, the low rates of isolated mucosal inflammation may indicate that if any, they were rare in our series.[8] The likely predominance of hypertrophic strictures is consistent with the median disease duration of 10 years (interquartile range, 7–12) of B2 patients, because Liu et al's[1] patients with hypertrophic strictures had longer disease duration than those with severely constrictive ones (mean ± SD: 13.7 ± 5.0 years vs 6.3 ± 6.2 years).

We think the work by Liu et al[1] represents a proof of concept of a new pathologic type of CD stricture, with opposing histopathologic features to traditional hypertrophic strictures. Although the most striking feature of constrictive strictures is its thin, flexible wall with almost no increase of smooth muscle and fibrosis, its low inflammation degree is also remarkable and its association to increased extent of fat wrapping may suggest a role of a specific noninflammatory matrix similar to that described by Mao et al.[3] Still, given the established relationship between inflammation and CD strictures, the existence of local proinflammatory, nonhypertrophic, fat-derived mediators cannot be ruled out. Future studies should reproduce the authors' pathologic findings and elucidate the physiopathology underlying the different strictures types.

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Conflicts of interest
These authors disclose the following: Helena Tavares de Sousa received a fee for presenting from Takeda, AbbVie, Janssen, Pfizer, and Biogen. Fernando Magro received a fee for presenting from AbbVie, Ferring, Falk Pharma, Hospira, Pharmakern, MSD, Shering, Lab. Vitoria, Vifor, OmPharma, Janssen, Takeda, and Pfizer. The remaining author discloses no conflicts.

Most current article

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Distinct Relapse Patterns Between HBeAg-Negative Patients Stopping Tenofovir and Entecavir

Dear Editor:

We appreciate the large study (RETRACT-B) by Choi et al[1] on the different relapse patterns after discontinuing entecavir (ETV) and tenofovir (TDF) in chronic hepatitis B. The results have confirmed earlier observations from both East and West that off-TDF relapses occur much earlier and tend to be more severe.[2-6] However, several points deserve clarification and further discussion.

First, there was no clear information regarding onset timing, such as median time (range) to virologic relapse and clinical relapse (CR), and no comparisons of these between off-TDF and ETV patients. A recent study has shown that 43% of off-TDF versus 1% of off-ETV hepatitis flares occur within 3 months and 74% of off-TDF versus 13% of off-ETV hepatitis flares occur within 6 months (both $P < .001$).[6] Such early relapses need more attention for proper management.

Second, the 1- and 2-year cumulative CR incidences were much lower in both off-TDF and ETV patients in the RETRACT-B study than those in our off-Nuc cohort (CGMH off-Nuc cohort) and another study in patients without cirrhosis who were monitored by Asian-Pacific stopping rules.[4,6] The frequency and interval of follow-up after Nuc cessation may be factors for these differences and may need to be considered.

Third, the authors compared the peak alanine aminotransferase levels at CR between off-TDF and ETV patients. However, the incidences of off-Nuc hepatitis flare (alanine aminotransferase >5 × upper limit of normal), hepatic decompensation, and hepatitis flare–related mortality, which are real safety concerns, were not compared between patients stopping TDF and ETV. In CGMH off-Nuc cohort, the overall incidence of off-TDF hepatitis flare was higher than off-ETV flare, and off-TDF was the predictor for hepatic decompensation.[6] Fourth, off-therapy virologic relapse, CR, and retreatment were found to be factors for hepatitis B surface antigen (HBsAg) loss; in particular, sustained response and no retreatment were documented as strong factors for HBsAg loss.[7] These factors were not included in the Cox regression analysis. In addition, the duration of consolidation therapy was >1 year in Asian guidelines, whereas it was >3 years in European guidelines. This may also influence the rate of HBsAg loss, especially in the comparison between HBsAg loss in White and Asian patients.

Last, the regression model in the present study is different from that in the earlier RETRACT-B study,[8] although the sample size of the current study was smaller by only 144 patients, and the aforementioned off-therapy events were similar among these 2 reports.

In conclusion, besides the confirmatory findings, it is anticipated that this large database can provide answers to some of the previously mentioned questions with further analyses.

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Conflicts of interest
The authors disclose no conflicts.

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Most current article

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IV. Abstracts of Papers that Justified Non-Core Papers a. and b.

El Ouali S, Baker ME, Lyu Ret al. Validation of stricture length, duration and obstructive symptoms as predictors for intervention in ileal stricturing Crohn's disease. United European Gastroenterol J. 2022 Nov;10(9):958-972. doi: 10.1002/ueg2.12314. Epub 2022 Dec 7. PMID: 36479841; PMCID: PMC9731662

Clinical Gastroenterology and Hepatology 2022;20:e1292–e1304

Constrictive and Hypertrophic Strictures in Ileal Crohn's Disease

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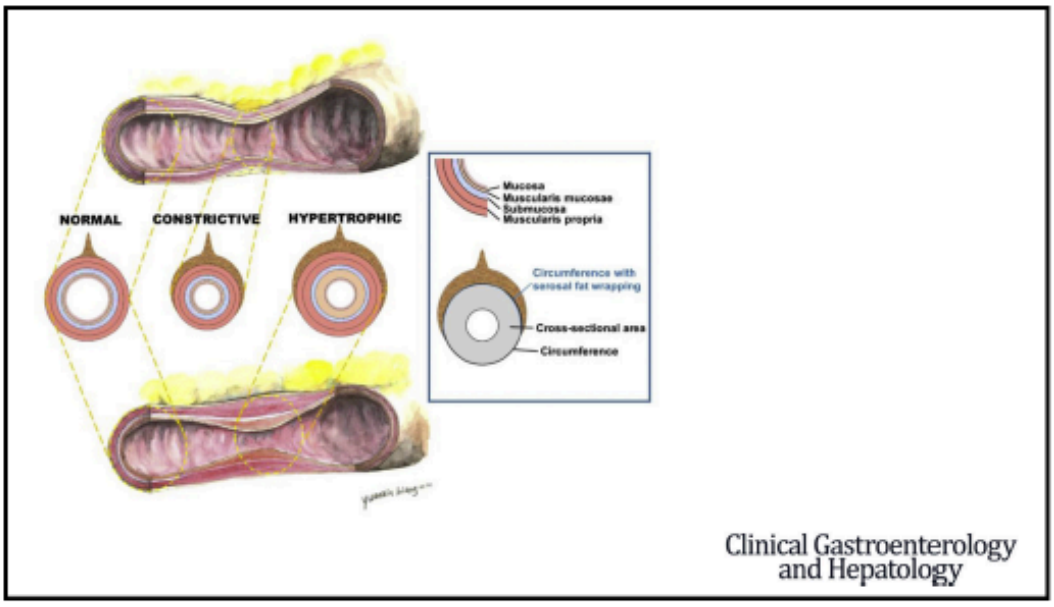

BACKGROUND & AIMS: Strictures in Crohn's disease (CD) are classically attributed to fibromuscular hypertrophy of the intestinal wall. We have identified and characterized CD-related ileal strictures that result instead from mural constriction (ie, reduced external circumference).

METHODS: Twenty-four strictures and internal controls from 17 adults with obstructive CD were analyzed by cross-sectional morphometry.

RESULTS: The stricture-to-control circumference ratios (CRs) ranged from 0.53 to 1.7. Six strictures with CR ≥1.0, designated hypertrophic, had concentrically thickened walls, mean 3-fold increases in cross-sectional area and stainable fibromucular tissue, and high transmural inflammation scores. In contrast, 18 strictures with CR <1.0, designated constrictive, had thin, pliant walls, cross-sectional areas and stainable fibromuscular tissue comparable with control values, and low transmural inflammation scores. Eight mildly constrictive strictures also showed mild fibromuscular mural expansion that fell short of statistical significance. Twelve of 18 constrictive strictures (67%) occurred multiply (2–4 strictures per specimen) in contrast with hypertrophic strictures, all of which occurred singly ($P = .01$). Constriction correlated quantitatively with circumferential serosal fat wrapping ($P = .003$) and was associated with myenteric lymphocytic plexitis ($P = .02$). Disease duration was shortest among subjects with constrictive strictures and correlated with increasing circumference (CR ≤0.8, 6.3 ± 6.2 years; CR >0.8, 8.7 ± 6.4 years; and CR ≥1.00, 13.7 ± 5.0 years, respectively; $P = .03$).

CONCLUSIONS: Constrictive ileal strictures in CD differ pathologically and clinically from hypertrophic strictures, featuring little or no fibromuscular mural expansion, frequent multiplicity, and earlier onset. Mesenteric fat wrapping and myenteric plexitis may contribute to their pathogenesis. Pathologic manifestations of constriction and hypertrophy can coexist, suggesting that stricture heterogeneity may be shaped in part by the dynamics of constrictive and hypertrophic processes.

Keywords: Contrictive; Crohn's Disease; Strictures.

Liu Q, Zhang X, Ko HM et al. Constrictive and Hypertrophic Strictures in Ileal Crohn's Disease. *Clin Gastroenterol Hepatol*. 2022 Jun;20(6):e1292-e1304. doi: 10.1016/j.cgh.2021.08.012. Epub 2021 Aug 14. PMID: 34400338

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ORIGINAL ARTICLE

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Validation of stricture length, duration and obstructive symptoms as predictors for intervention in ileal stricturing Crohn's disease

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Abstract

Background: Risk factors for intervention in terminal ileal (TI) stricturing Crohn's disease (CD) are poorly defined. Novel and rigorous definitions for TI strictures recently became available.

Objective: We aimed to describe the rates of symptoms or need for endoscopic balloon dilation (EBD) or surgery as well as risk factors of progression in a well-defined stricturing CD cohort.

Methods: Consecutive adult patients with non-penetrating stricturing TI CD, as defined by centrally-read magnetic resonance enterography CONSTRICT criteria, were separated into a derivation and validation cohort. Clinical and imaging characteristics were collected following prespecified scoring conventions. Primary outcome was a composite endpoint of EBD or surgery ("intervention"). Multivariable analysis was performed.

Results: Eighty-six patients (48.8% female, median age 36 years) met selection criteria, 17.4% had prior EBD, 59.3% previously received biologics and 58.1% of strictures were anastomotic. Median follow-up was 63.4 [95% CI: 57, 68.9] months. In the derivation cohort, at 12 and 48 months, 26% and 45% of patients had intervention, respectively. Multivariable analysis showed obstructive symptoms (Hazard ratio [HR] 1.444; 95% CI 1.126–1.852), stricture duration (HR 0.974; 95% CI, 0.954–0.995) and length (HR 1.039; 95% CI, 1.011–1.069) predicted intervention. The concordance index for split-sample validation was 0.74 and 0.67, respectively. Biologics were not associated with intervention. An online risk calculator was constructed.

Conclusion: In patients with TI stricturing CD, 26% and 45% required intervention at 1 and 4 years. Obstructive symptoms, stricture duration and length were independent and validated predictors of the need for intervention. These findings are important for clinical practice and aid in the design of future trials for CD strictures.

KEYWORDS
Crohn's disease, dilation, intervention, stricture, surgery