

UNIVERSITÀ VITA-SALUTE SAN RAFFAELE

CORSO DI DOTTORATO DI RICERCA IN MEDICINA
MOLECOLARE

Curriculum: Project-Based Fellowship - Experimental and
Clinical Medicine (ECM) 2

Unveiling the microbial impact on
intestinal fibrosis: new insights for the
pathogenesis and treatment of Crohn's
disease-associated complications

Supervisor: Prof. Silvio Danese 

Co-Supervisor: Prof. Gianluca Matteoli

Tesi di Dottorato di Ricerca di: Tommaso Lorenzo Parigi

Matricola: 021564

Ciclo di dottorato: XXXVIII

SSD: 06/D4

Anno Accademico 2024/2025

Declaration

This thesis has been written by myself and has not been used for any previous other application for a degree.

Throughout the text I use both "I" and "we" interchangeably.

The text has been written according to the editing guidelines approved by Università Vita-Salute San Raffaele.

I actively participated in the generation of all the data presented in this thesis, though significant contributions came from the following lab colleagues and external collaborators:

- Luca Massimino PhD and Matteo Riva (both at Ospedale San Raffaele) for bioinformatics analyses
- Sabrina Nicolò PhD and Carmela Errico PhD (both at Ospedale San Raffaele) for *in vivo* and *in vitro* experiments and immunostains.
- Federica Ungaro PhD (Ospedale San Raffaele) for data interpretation, experimental design and mentorship.
- Luca Albarello MD (Ospedale San Raffaele) and Vincenzo Villanacci MD (Spedali Civili, Brescia) for the histological interpretation of human tissues
- Immunostains and R.mucosa stimulation experiments were performed in collaboration and Dr.Bragonzi's lab (Ospedale San Raffaele)
- Niels Vande Casteele PhD (AcelaBio, San Diego, CA) for spatial transcriptomics sequencing
- Silvio Danese MD, PhD (Ospedale San Raffaele) experimental design and supervision

All sources of information are acknowledged by means of reference.

A considerable part of this work, particularly from figures 1 to 22 has been published in July 2025 in the Journal of Crohn's and Colitis and can be accessed at DOI: 10.1093/ecco-jcc/jjaf130 (Massimino L, Parigi TL, et al. Journal of Crohn's and Colitis, 2025 – shared first authorship)

CONSULTAZIONE TESI DI DOTTORATO DI RICERCA

Il/la sottoscritto Tommaso Lorenzo Parigi

Matricola 021564

Nato a Salem, Virginia, USA

il 17 Gennaio 1993

autore della Tesi di Dottorato di Ricerca dal titolo:

Unveiling the microbial impact on intestinal fibrosis: new insights for the pathogenesis and treatment of Crohn's disease-associated complications

AUTORIZZA la Consultazione della Tesi

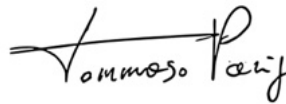
Si rende noto che parti della Tesi sono indisponibili in relazione all'utilizzo di dati tutelati da

segreto industriale

E' fatto divieto di riprodurre, in tutto o in parte, quanto in essa contenuto

Data 01/12/2025

Firma

A handwritten signature in black ink, appearing to read "Tommaso Parigi". The signature is fluid and cursive, with a horizontal line extending from the start of the first name.

Acknowledgments

The work hereby presented is the result of a collaborative effort involving multiple people. I particularly wish to thank: Luca Massimino PhD and Matteo Riva (bioinformatics analyses); Sabrina Nicolò PhD and Carmela Errico PhD (*in vivo* and *in vitro* experiments); Federica Ungaro PhD (mentorship, supervision and data interpretation); Luca Albarello MD, Maurilio Ponzoni MD, Federico Scarfò MD, Andrea Municchi MD, Vincenzo Villanacci MD, Mattia Bugatti PhD, Sara Mino MD (human tissue processing and histological interpretation); Pierpaolo Sileri MD, Andrea Vignali MD, Alice Frontali MD (acquisition of human samples); Silvio Danese MD, PhD (mentorship and supervision); Theresa Pizarro PhD and Fabio Cominelli, MD, PhD (mentorship and supervision);

Funding

The work was funded by the European Union - Next Generation EU - NRRP M6C2 - Investment 2.1 Enhancement and strengthening of biomedical research in the NHS" under the grant agreements #PNRR-MAD-2022-12375729 (CUP master C43C22001270007) to Silvio Danese. and #PNRR-MCNT2-2023-12377779 (CUP master C43C24000410007) to Pierpaolo Sileri. and Federica Ungaro; by the International Organization for the study of IBD (IOIBD) Operating Grant 2024 to Federica Ungaro, by the "Ricerca Finalizzata" program by the Italian Ministry of Health under the grant agreement #RF-2021-12372637 to Silvio Danese. Tommaso Parigi's PhD scholarship was funded by an unrestricted grant by FIRMAD.

Index

• English Abstract.....	5
• Italian Abstract.....	6
• Introduction.....	8
• Results	10
• Discussion.....	38
• Methods.....	42
• References.....	47

English Abstract

Background and aims

Crohn's disease (CD) is a chronic inflammatory condition of the gastrointestinal tract often complicated by the development of fibrotic strictures for which there is no medical treatment or prevention. The biological mechanism that connects long-standing inflammation to tissue scarring and the potential role of intestinal microbiota in modulating this remain insufficiently understood. Here, we sought to clarify these mechanisms using a computational framework and *in vitro* and *in vivo* validation.

Methods

We applied spatial transcriptomics to 13 surgical samples encompassing inflamed and fibrotic regions from CD patients, along with non-affected intestinal tissue. Spatial profiles were combined with single-cell RNA sequencing to reconstruct cellular states and molecular programs. Analyses of ligand–receptor communication and pseudotime trajectories were used to map dynamic interactions and lineage evolution. Principal computational observations were confirmed by immunostaining and tested in a murine model of chronic TNBS-induced colitis. Furthermore, 16S RNA sequencing was used to identify microbiota components associated with disease phenotypes.

Results

Our analyses revealed a progressive remodeling of the intestinal architecture accompanying the transition to chronic inflammation. Fibrotic changes characteristic of Crohn's disease arose within the mesenchymal niche and were linked to activation of PECAM2 pathways mediated through the PECAM1–CD38 axis. At the same time, apolipoprotein-A-related signaling—most notably the APOA1–ABCA route—appeared important for sustaining epithelial and stromal equilibrium; reduced activity in this pathway correlated with fibrogenic progression. In experimental chronic colitis induced by TNBS, pharmacologic suppression of CD38 signaling alleviated inflammatory manifestations and attenuated colonic wall thickening. Finally, we found that *Roseomonas mucosa* was associated with fibrostenotic complications.

Conclusions

Our findings support a key contribution of CD38-mediated pathways in the fibrotic process and indicate that disrupting PECAM2-related signaling could help prevent stricture development in Crohn's disease, offering a possible therapeutic strategy. We also observe that *R. mucosa* may play a role in this transition.

Italian Abstract

Introduzione e obiettivi

Il morbo di Crohn (CD) è una malattia infiammatoria cronica del tratto gastrointestinale spesso complicata da stenosi fibrotiche per cui non esistono terapie. I meccanismi che legano l'infiammazione cronica alla cicatrizzazione tissutale, e il potenziale ruolo del microbiota intestinale, rimangono poco chiari. Abbiamo cercato di chiarire tali meccanismi con un approccio computazionale e validazioni *in vitro* e *in vivo*.

Metodi

Abbiamo eseguito transcriptomica spaziale su 13 campioni chirurgici comprendenti aree infiammate e fibrotiche di pazienti con CD, e tessuto non malato. I profili spaziali sono stati integrati con dati di RNA sequencing a singola cellula per ricostruire gli stati cellulari e i pathway molecolari. L'analisi delle interazioni ligando-recettore e le traiettorie di pseudotempo sono state utilizzate per mappare le interazioni dinamiche e l'evoluzione cellulare. Le osservazioni sono state confermate tramite immunocolorazione e il target identificato (CD38) è stato testato in un modello murino di fibrosi intestinale (TNBS). Abbiamo inoltre ricercato componenti del microbiota associate alla malattia stenotica tramite sequenziamento dell'RNA 16S.

Risultati

Abbiamo osservato un rimodellamento progressivo dell'architettura intestinale nella transizione verso la stenosi, in cui le aree fibrotiche originano dalle cellule mesenchimali legate all'attivazione di PECAM2 mediate dall'asse PECAM1-CD38. Parallelamente, la segnalazione legata all'apolipoproteina A (APOA1) è risultata rilevante per mantenere l'omeostasi epiteliale e stromale e una sua riduzione correla con la progressione fibrogenica. Nel modello murino, l'inibizione di CD38 ha attenuato le manifestazioni infiammatorie e fibrotiche. Infine, *Roseomonas mucosa* è stata associata alle complicanze fibrostenotiche.

Conclusioni

I nostri risultati evidenziano un ruolo centrale di CD38 nel processo fibrotico e suggeriscono che interferire con la segnalazione correlata a PECAM2 potrebbe contribuire a prevenire lo sviluppo delle stenosi nel morbo di Crohn, offrendo una potenziale strategia terapeutica. Inoltre, abbiamo osservato che *R. mucosa* potrebbe avere un ruolo in questa transizione.

Introduction

Crohn's disease (CD) is a chronic inflammatory condition characterized by a complex and heterogeneous etiology, with marked variability in both anatomical distribution and clinical behavior (1). Although CD can involve any portion of the gastrointestinal tract from the oral cavity to the perianal region, it most frequently affects the terminal ileum and/or the colon.

Unlike ulcerative colitis, the other major form of inflammatory bowel disease, CD is defined by transmural inflammation, extending from the mucosa through the full thickness of the bowel wall. This deep tissue involvement, in combination with impaired wound-healing responses, is thought to drive the development of excessive fibrosis and ultimately the formation of intestinal strictures. While the precise histological definition of a stricture remains debated, two features are unanimously recognized: an abnormal accumulation of extracellular matrix—primarily in the lamina propria but also within the muscularis propria—and a marked thickening of the muscular layer due to both hypertrophy and hyperplasia (2). More than half of individuals with CD will develop strictures during their disease course, with a predilection for the terminal ileum (2). Management remains predominantly invasive, relying on surgical resection or endoscopic balloon dilation. Even these interventions are not definitive: endoscopic or surgical recurrence at the anastomosis approaches 70% within the first year, and over a quarter of patients eventually require repeat surgery. Consequently, strategies to prevent or effectively treat fibrostenotic complications represent a major unmet clinical need.

Persistent tissue damage associated with chronic intestinal inflammation, together with defective repair mechanisms, is considered the main driver of fibrostenosis (3). Nevertheless, the cellular and molecular determinants that underlie fibrotic remodeling remain poorly defined, moreover there is no information on whether the intestinal microbiota plays a role in this process which results in a complete lack of targeted anti-fibrotic therapies and preventive strategies.

The gut is a highly structured, multilayered organ comprising diverse cell types and signaling networks; thus, examining single components in isolation provides only a partial view of CD pathogenesis. A more integrated, multi-scale assessment is therefore required. Advances in Next Generation Sequencing (NGS) technologies have introduced powerful tools to dissect biological systems and reveal molecular mechanisms that would otherwise remain inaccessible (4). Bulk and single-cell RNA sequencing have contributed substantially to understanding CD biology and generating new hypotheses (5–8), but neither approach captures the spatial context of gene expression within intact tissue architecture. Spatial transcriptomics (ST) addresses this limitation by combining transcriptional profiling with positional information, without requiring tissue dissociation (9).

In this study, we leveraged ST, applied for the first time to CD across multiple stages of disease, together with advanced computational methods, to map the pathways involved in the progression from inflammation to fibrosis. This multimodal approach enabled us to uncover a key role for PECAM2-related signaling in the transition toward fibrotic remodeling, and we further showed that inhibiting this pathway ameliorated colitis and reduced colon thickening in experimental models. Finally, we identified a particular bacteria, *R. mucosa*, that was associated with strictures and able to induce pro-fibrotic changes in fibroblasts.

Overall, our findings shed light on molecular circuits that govern Crohn's disease progression.

Results

The progression of the disease is accompanied by a rearrangement of the anatomical compartments and changes in their molecular signatures.

Surgical specimens from intestinal resections due to stricturing CD serve as an ideal model of the unfolding sequence of changes from inflammation to fibrosis. The two ends of each specimen are selected by the surgeon to be macroscopically healthy or at least non-involved by fibrosis. Moving toward the center of the segment, the features of the disease, such as the inflammatory infiltrate and the thickening of the submucosa and *muscularis propria* become more evident, reaching their maximum at the point of the stricture (**Figure 1A**).

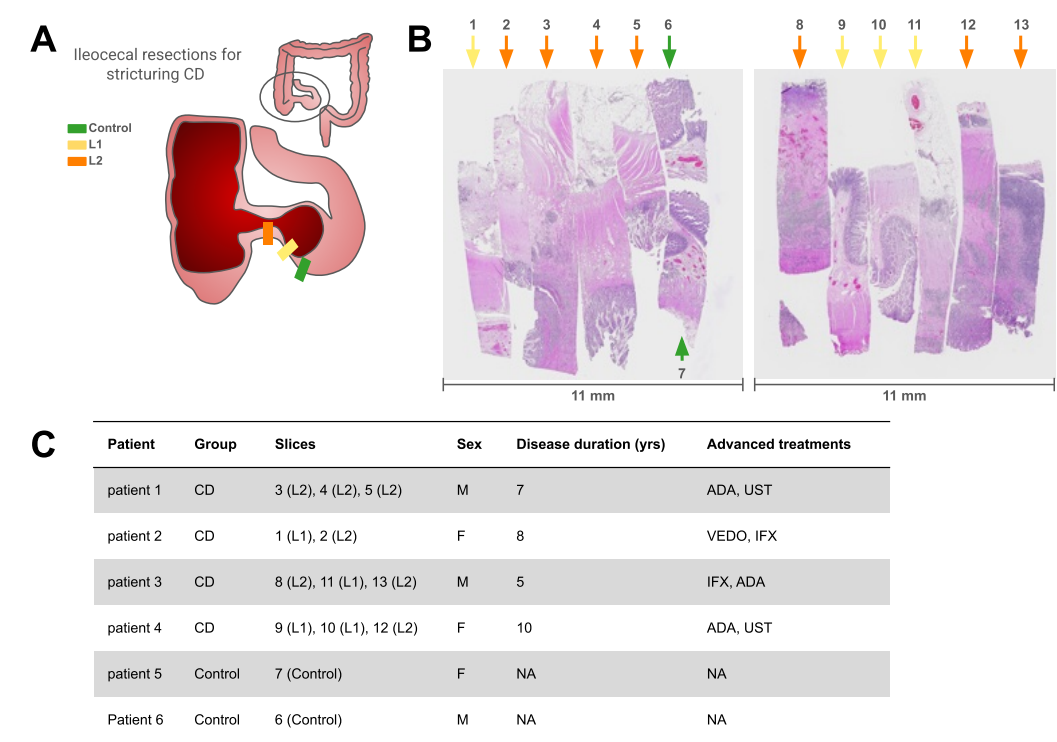


Figure 1. Intestinal tissue selection for spatial transcriptomics sequencing.

(A) Schematic representation of the selection of intestinal areas included in the analysis. L1 denotes the intermediate mainly inflammatory state, while L2 the more fibrotic state. (B) Hematoxylin and Eosin staining of the analyzed tissue areas. Numbers on the top of panel B indicate from which patient the samples were collected. (C) Table listing clinical characteristics of patients included in the analysis.

Importantly, the anatomical distribution of lesions reflects both the timing and duration of disease activity. The earliest changes—such as erosions and ulcers—tend to arise at the site where the stricture will eventually form, while areas adjacent to

the original lesion often show more recent inflammation. This premise serves as the rationale for our study. We selected surgical specimens from patients who underwent ileocecal resection for stricturing CD. Experienced gastrointestinal pathologists examined the formalin-fixed, paraffin-embedded (FFPE) tissues to delineate three regions of interest in each sample: a healthy area (referred to as control, confirmed to have a Robarts Histological Index of activity below 3 with no neutrophils in the lamina propria or epithelium), an intermediate segment showing inflammation with minimal fibrotic changes (L1), and the most severely affected region exhibiting pronounced fibrosis and maximal stricture (L2) (**Figure 3A**). After identifying these intestinal regions of interest, the FFPE tissues were reorganized to generate a composite specimen (**Figure 1B**) containing both CD-derived and healthy control intestinal samples (n=13 slices, 11 CD and 2 controls, **Figure 1C**). Spatial transcriptomics was subsequently applied to map the spatial distribution of cellular compartments.

Because the transcriptomics kit includes two separate sequencing areas in the same slide, we anticipated possible batch effect that we evaluated and overcome using Harmony, a computational tool (10) (**Supplementary Figure 2A, 2B**). A quality assessment of read counts was then conducted, confirming that no sequencing bias was detectable across the whole tissue regions and clusters (**Supplementary Figure 2C, 2D**).

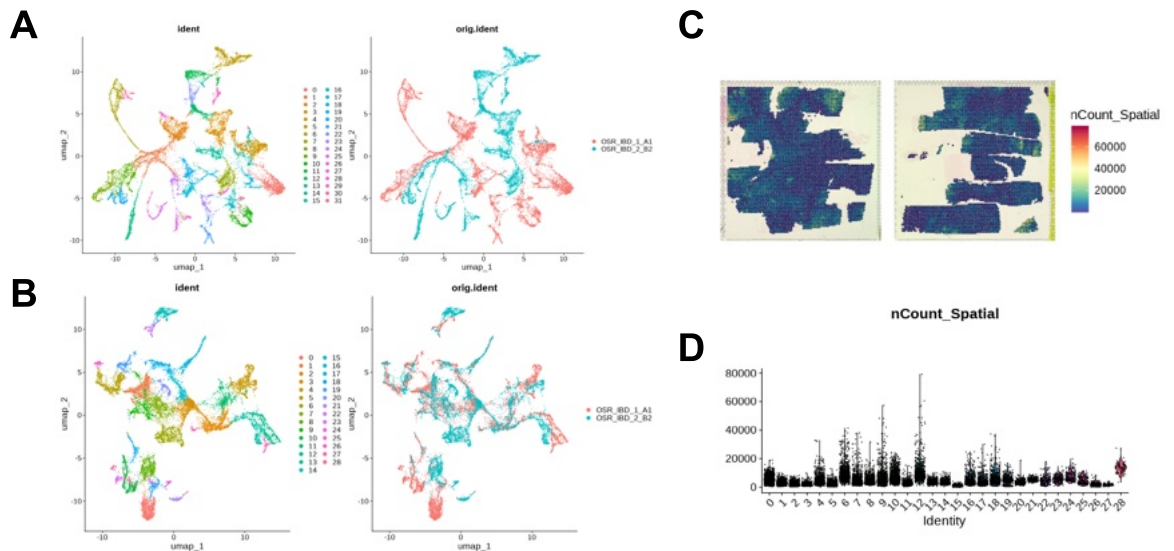


Figure 2. Batch correction and quality check of spatial transcriptomics analysis.

(A, B) Multidimensional scaling of the spatial transcriptomics from 10x Visium analysis by UMAP before (A) and after (B) batch correction. (C) Spatial feature plot showing the number of

normalized counts within the tissues. (D) Violin plots showing the number of normalized counts within Seurat clusters.

To explore the transmural progression of inflammation in CD (11), we applied unsupervised clustering based on spatial distance, tissue morphology, and gene expression profiles, using the Louvain community detection algorithm. This approach identified 21 clusters, each defined by distinct transcriptomic signatures and spatially mapped across disease stages (control, L1, and L2) and tissue layers within each slice (**Figure 3B**). Pathologists then annotated each cluster according to its features (**Figure 3C**), ultimately defining compartments such as epithelial, muscular, stromal, nervous, fibrotic, and inflammatory (including lymphoid aggregates and granulation tissue). Within these compartments, further transcriptomic differences were observed, corresponding to both anatomical location and inflammatory state (e.g., deep versus superficial; inflamed versus non-inflamed, **Figure 3C**).

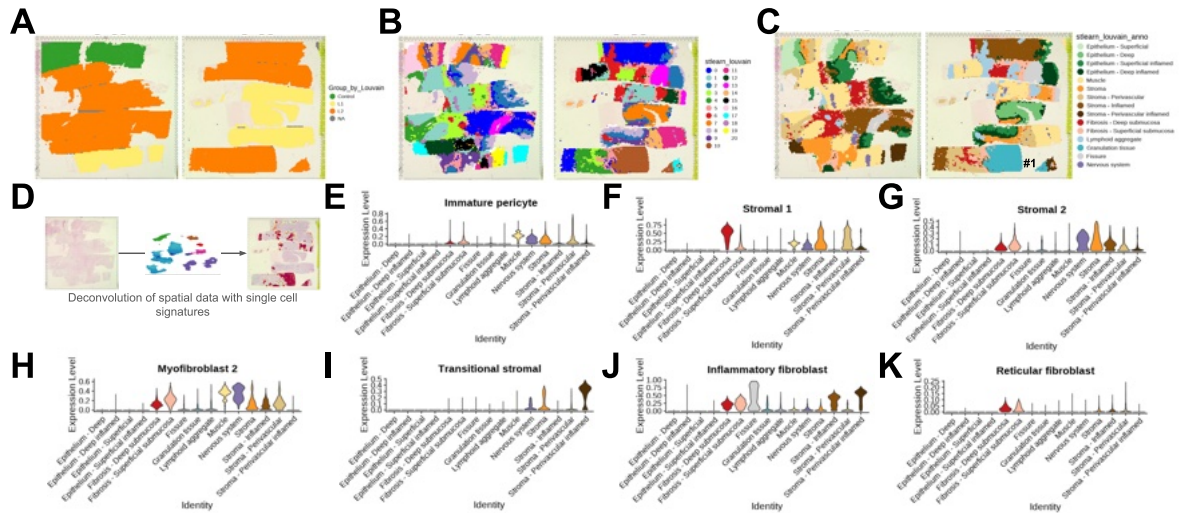


Figure 3. Intestinal mucosa compartments rearrange while inflammation progresses.

(A) 10x Visium Spatial Gene Expression slides with tissue slices from control (green), L1 (yellow), and L2 (orange) areas. (B-C) Unbiased clustering by Louvain algorithm (B) and pathologist's cluster annotation (C) of spatial transcriptomics data in control, L1, and L2 tissue slices. (D) Schematic representation of deconvolution of spatial data with single-cell signatures. (E-K) Violin plots showing gene expression profiles related to the indicated specific cell types of the annotated cell clusters.

We observed progressive disruption of intestinal cytoarchitecture across the different disease stages, reflecting worsening pathology from control to L1 and L2, as expected. The healthy region (control) exhibited a well-organized, intact epithelium with two distinct layers—superficial and deep. In L1 and L2 slices, these layers lost their normal structure and were replaced by inflamed epithelium (**Figure 3C**). Notably, a thin layer of deep inflamed epithelium was also detected in the control, likely reflecting residual inflammation in the healthy margin of resected tissue. Similarly, in healthy tissue, the submucosa—including the stroma and perivascular stroma—was well-organized above the muscular layer and associated with scattered enteric nervous tissue. In contrast, this organization was disrupted in L1 and L2. All stromal compartments became inflamed, fibrotic tissue—distinguishable as deep and superficial—accumulated, surface breaks and fissures appeared, and extensive lymphoid aggregates and granulation tissue developed (**Figure 3C**). These spatial analyses corroborate previous observations of CD-associated rearrangement of intestinal layers (12), highlighting changes in both localization and molecular signatures as inflammation progressed and complications arose.

Cell clustering deconvolution reveals cell phenotype shifts during the transition from healthy mucosa to fibrosis

Next we deconvolved the spatial transcriptomics data with single-cell RNA-seq profiles from the scIBD platform, which integrates well-curated single-cell datasets from both CD patients and healthy controls (13) (**Figure 3D**). This allowed to further characterize the cell compartments whose phenotypes changed as disease progressed (**Figure 4A**).

The analysis revealed that both deep and superficial epithelial layers, enriched for crypts and postmitotic villi respectively, shared similar cell-type-specific signatures, primarily reflecting enterocyte-associated gene expression (**Figure 4B**). However, with increasing inflammation, the superficial epithelium became particularly enriched for Goblet cell-specific features, whereas the deep inflamed epithelium showed reduced Goblet cell signatures and was dominated by genes associated with transit-amplifying (TA) cells, Paneth cells, and Tuft cells (**Figure 4C–3G**). These findings suggest that the deep epithelial layer undergoes the most pronounced phenotypic changes during disease progression.

(A-F) Violin plots showing expression levels of the indicated cell type signature within spatial compartments.

In the fibrotic compartments, we distinguished two types of intestinal fibrosis, each defined by specific molecular markers and cellular compositions. Notably, inflammatory fibroblast signatures were reduced in both superficial and deep fibrotic regions compared with the inflamed stroma, including the perivascular areas (**Figure 3J**). Conversely, reticular fibroblast gene signatures were elevated and predominated in the fibrotic compartments (**Figure 3K**), which also exhibited myofibroblast-associated gene expression (**Figure 3H**). Additionally, superficial submucosal fibrosis displayed a mesoderm-related gene signature (**Figure 5E**). Interestingly, genes specific to stromal 1 and stromal 2 populations were enriched in deep and superficial fibrosis, respectively (**Figure 3H** and **3G**).

As expected, the muscular compartment showed gene signatures associated with mature and immature pericytes (**Figure 5C** and **4D**), stromal cells, myofibroblasts (**Figure 3F–3H**), and smooth muscle cells (**Figure 5F**).

Overall, these findings indicate that fibrosis primarily reflects remodeling of the mesenchymal compartments—both stromal and muscular—without substantial involvement of the epithelial layer.

Fibrosis develops from the mesenchymal compartment

Although several studies have explored the mechanisms of fibrosis (14), relatively little is known about the molecular pathways driving this process and many questions remain on how inflammation contributes or triggers fibrosis development. To better clarify the progression from chronic inflammation to fibrosis, we integrated publicly available single-cell RNA-seq data from a large collaborative dataset (13) with the spatial transcriptional signatures identified in our samples (**Figure 6A**). The UMAP projection illustrated the diversity of cell populations present in the single-cell dataset (**Figure 6B**), including epithelial, lymphoid (CD8, CD4, ILC, plasma cells), myeloid, mesenchymal, endothelial, and neural clusters.

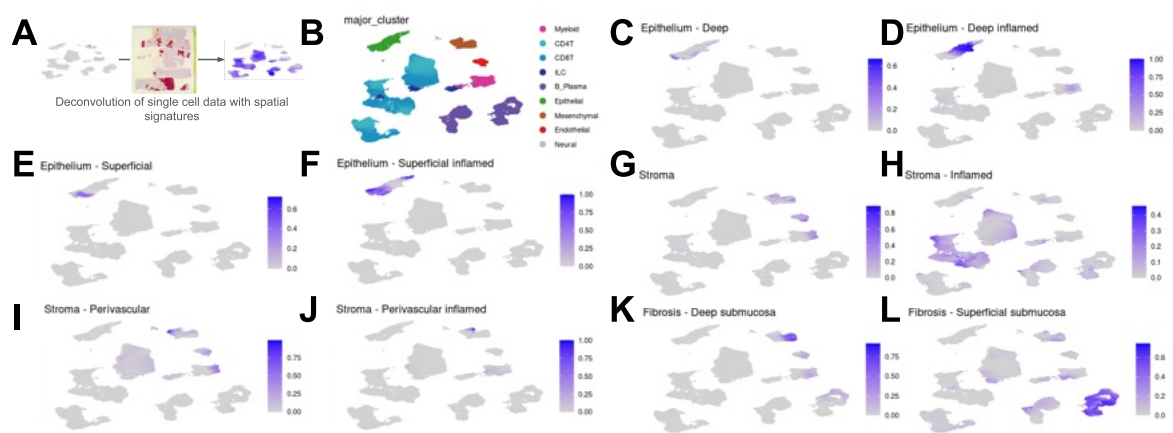
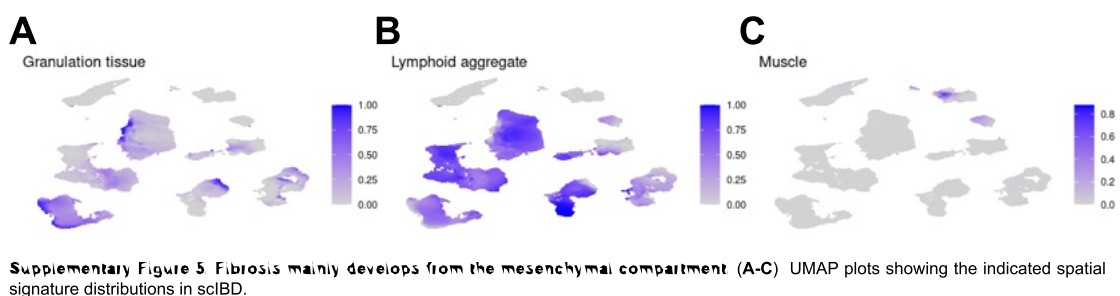


Figure 6. Fibrosis mainly develops from the mesenchymal compartment.

(**A**) Schematic representation of single-cell data deconvolution with spatial cell signatures. (**B**) UMAP showing major clusters found in the scIBD dataset. (**C-L**) UMAP plots showing the indicated spatial signature distributions in scIBD.

Next, we confirmed that the clusters associated with inflammation in our spatial transcriptomics dataset (i.e., granulation tissue and lymphoid aggregates) matched the proinflammatory single-cell data (**Figures 7A and 7B**).



Supplementary Figure 5 Fibrosis mainly develops from the mesenchymal compartment (A-C) UMAP plots showing the indicated spatial signature distributions in scIBD.

Figure 7. Fibrosis mainly develops from the mesenchymal compartment.

(A-C) UMAP plots showing the indicated spatial signature distribution in scIBD.

Within the epithelial cluster, integrating the spatial signatures with the single-cell profiles highlighted transcriptomic differences between the deep and superficial epithelial layers. Both compartments exhibited inflammation-associated shifts (**Figure 6C–6F**). As anticipated, the stromal regions aligned with mesenchymal and endothelial clusters and also showed signals within the myeloid cluster (**Figure 6G**), consistent with the presence of resident or circulating innate immune cells that physiologically contribute to stromal surveillance (15). With inflammation, the stromal compartment became enriched in granulation tissue and lymphoid aggregates (**Figure 6H** and **Supplementary Figure 5A, 5B**). The perivascular stroma displayed mesenchymal and endothelial signatures, along with myeloid features, but did not undergo major transcriptional changes in the inflamed state (**Figure 6I, 6J**). These observations indicate that structural stroma acquires an activated inflammatory profile during disease progression, whereas the perivascular stroma—known to regulate immune cell trafficking—remains comparatively stable.

In parallel, fibrosis in the deep submucosa originated predominantly from mesenchymal cells, with only minor contributions from endothelial and myeloid populations (**Figure 6K**). By contrast, superficial submucosal fibrosis showed a strong enrichment for B-cell signatures (**Figure 6L**). The muscular compartment, as expected, clustered within the mesenchymal population (**Figure 7C**).

Collectively, these findings demonstrate that epithelial cells undergo transcriptional alterations in response to inflammation, but do not support the presence of epithelial-to-mesenchymal transition (EMT) at this stage. In contrast, fibrotic tissue arises mainly from mesenchymal sources, with a prominent inflammatory infiltrate in the superficial submucosa.

To further dissect the mesenchymal-to-fibrosis transition, we conducted sub-clustering of the mesenchymal cell population (**Figure 8A**). Fibrosis in the deep submucosa was predominantly associated with a single cluster (**Figure 8B**, cluster #1), whereas superficial fibrosis involved an additional population (**Figure 8C**, clusters #1 and #2). Cluster #1 contained stromal cells—including fetal and non-fetal subsets—cycling stroma, and reticular fibroblasts, while cluster #2 was enriched for transitional stroma and mesoderm (**Figure 8D**). Based on these distinctions, reticular fibroblasts appear to predominantly define deep submucosal fibrosis, whereas mesodermal cells are characteristic of the superficial fibrotic layer.

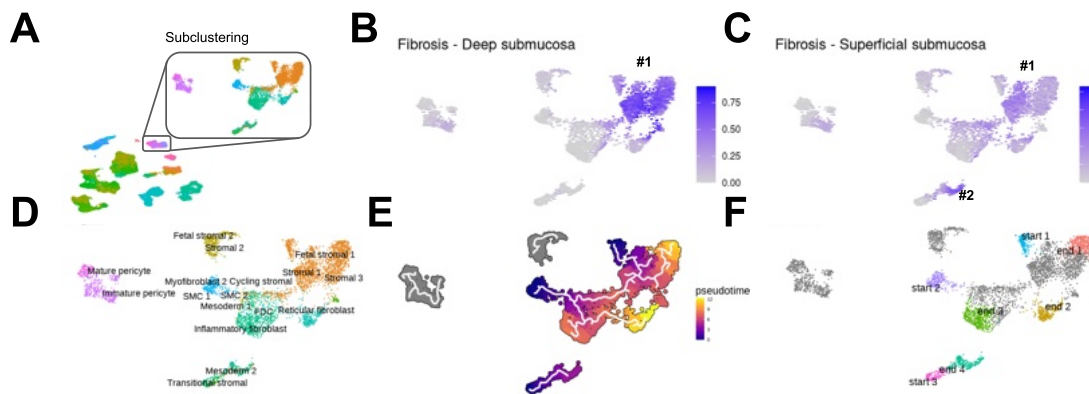


Figure 8. Pseudotime analysis of the mesenchymal compartment subclusters reveals 3 types of cellular transition defining fibrosis.

(A) Schematic representation of mesenchymal compartment subclustering. (B-C) UMAPs showing the deep (B) and superficial (C) submucosal fibrosis spatial signature distributions in scIBD-defined cell types. (D) UMAP showing minor clusters in scIBD mesenchymal subclustering. (E) Pseudotime analysis in the mesenchymal subclustering. (F) Seurat clustering with 200% resolution to identify pseudotime start and end clusters.

To investigate how fibrosis emerges within the mesenchymal compartment, we conducted a pseudotime analysis. In cluster #1, two origins of differentiation (start 1 and start 2) were identified, corresponding to fetal stromal cells and myofibroblasts, respectively, whereas in cluster #2 the transitional stroma represented the most likely starting point (start 3, **Figure 8D–8F**). Along this trajectory, fetal stromal cells in cluster #1 progressed toward differentiated stromal cells (end 1). In parallel, myofibroblasts transitioned into reticular and inflammatory fibroblasts (end 2 and end 3) through an intermediate state defined by cycling stroma. In cluster #2, transitional stroma evolved toward mesoderm (end 4), a process uniquely associated with superficial submucosal fibrosis.

To further dissect these transitions, we carried out pathway enrichment analysis on the pseudotime sub-clusters to identify biological processes (BP) most characteristic of, and activated during, fibrogenesis. Start 1 was associated with BPs linked to the negative regulation of fibroblast growth factor (FGF) signaling and to microvillus organization—both of which were also activated as cells progressed toward the stromal state (**Figure 9A, 9B**, and **Figure 10A**, top panel). At the completion of this trajectory (end 1), pathways related to cell adhesion became prominent (**Figure 9C, 9D**, and **Figure 10A**, bottom panel).

Start 2, corresponding to myofibroblasts, showed activation of pathways involved in muscle contraction and intermediate filament assembly (**Figure 9E, 9F**, and **Figure 10B**, top panel). During the differentiation toward end 2, in addition to cell adhesion pathways (**Figure 4C** and **4D**), biological processes linked to T-cell costimulation and antibacterial responses were induced (**Figure 9G, 9H**, and **Figure 10B**, middle panel). The route toward end 3 was instead dominated by pathways reflecting innate immune activation (**Figure 9I–9L**, and **Figure 10B**, bottom panel). Finally, the transition from start 3 to end 4 was marked by strong upregulation of inflammatory pathways (**Figure 9K** and **9L**, and **Figure 10C**).

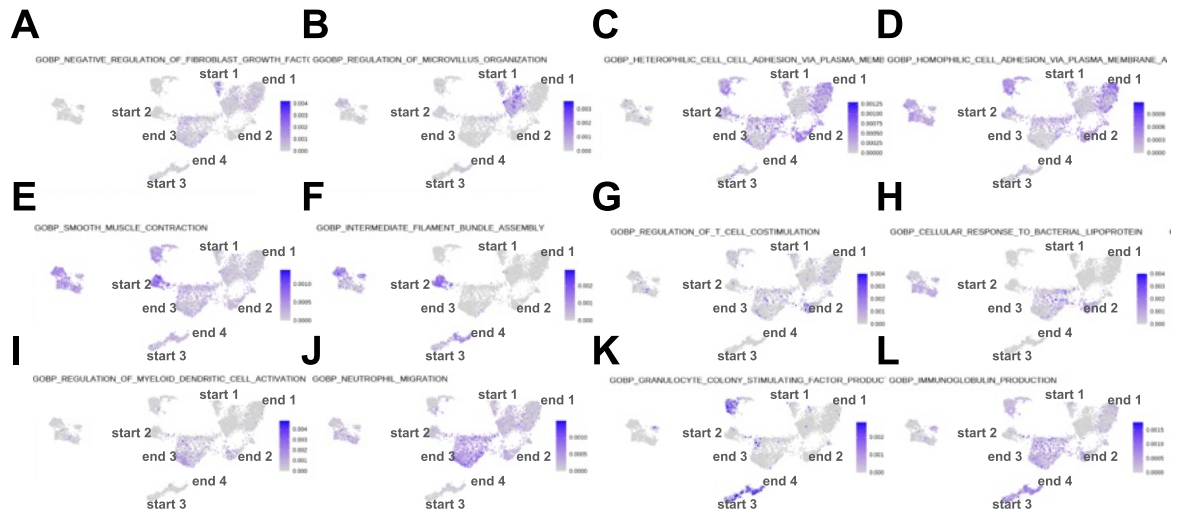


Figure 9. Transition to fibrosis is associated with changes in cell shape and inflammatory response-related gene expression.

(A-L) UMAPs showing the indicated gene ontology biological process (GO BP) enrichment distributions in accordance with the pseudotime subclustering.

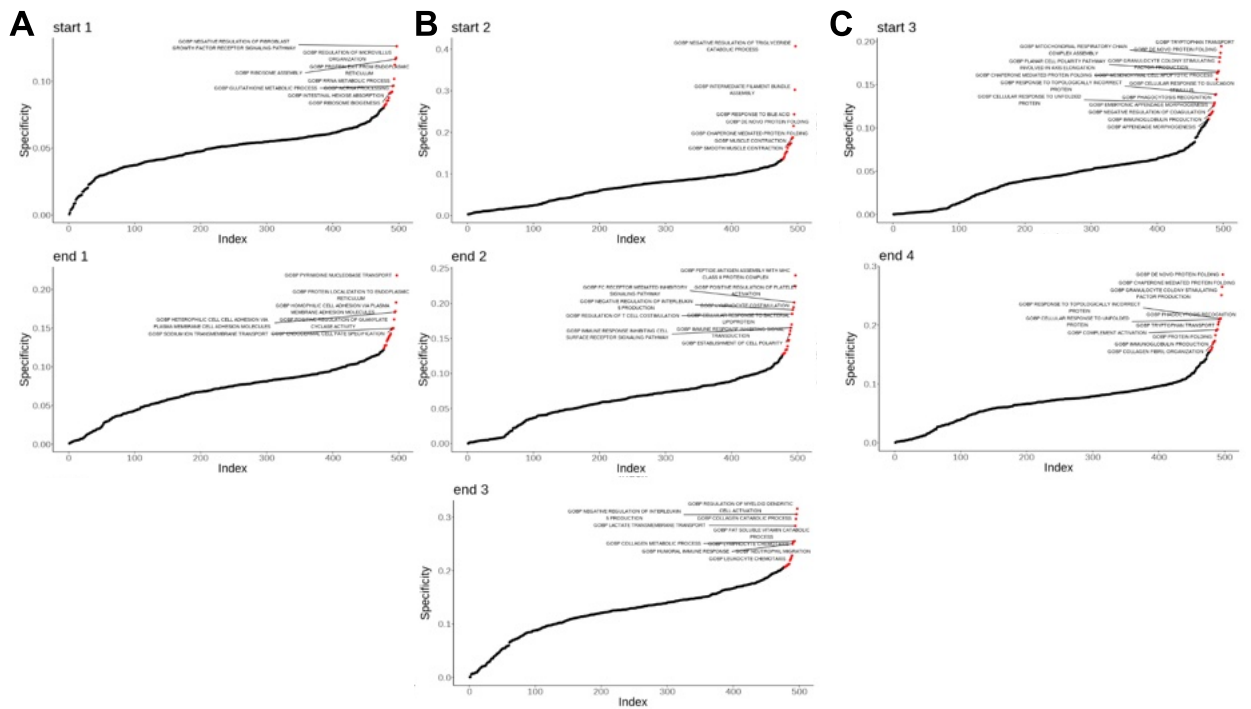


Figure 10. Specificity of biological processes associated with the transition to fibrosis.

(A-C) Plots showing gene ontology biological processes (GO BP) specificity as a function of ranking in pseudotime subclusters.

Taken together, these findings indicate that fibrosis arises from mesenchymal-lineage cells and is composed of stromal populations that include both inflammatory and reticular fibroblasts, with the latter being particularly abundant in deep submucosal fibrosis. In addition, the two fibrotic phenotypes differ not only in their cellular origins but also in the biological processes that become activated. In particular, deep submucosal fibrosis is mainly defined by pathways involved in structural and cytoskeletal remodeling, whereas superficial submucosal fibrosis is driven predominantly by inflammatory programs.

Communication between cells broadens but weakens as inflammation progresses to fibrosis

Signaling exchanges between tissue compartments through ligand–receptor interactions are essential for maintaining normal and pathological tissue function (16). To explore how these communication networks shift across control, L1, and L2 regions, we applied CellChat, a tool that leverages a curated ligand–receptor database to infer, depict, and analyze intercellular signaling patterns(17). In the control samples, the strongest communication occurred among the muscular layer, the stromal compartment, and the perivascular stroma (**Figure 11A**), with an average interaction strength close to 2. Of note, the neural compartment also engaged in signaling—with moderate intensity—with both stromal and muscular regions, underscoring the role of the enteric nervous system in guiding proper mucosal physiology through its interactions with the muscle layer.

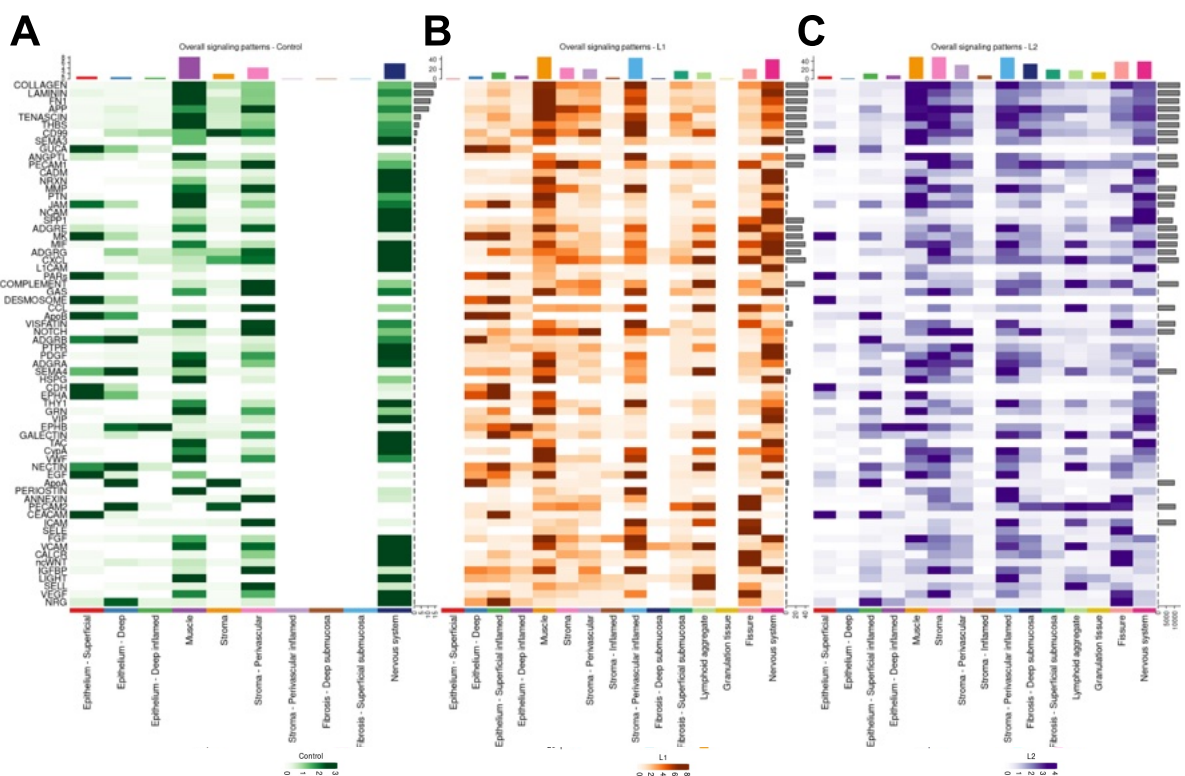


Figure 11. Estimation of cell-cell communication within inflamed and fibrotic tissues using CellChat.

Heatmap showing the summary of the signaling pathways that contribute to outgoing or incoming communication. The color bar represents the relative signaling strength of a signaling pathway across cell types. The bars indicate the sum of the signaling strength of each cell type or pathway.

In contrast, the pattern of intercellular communication shifted considerably in the L1 and L2 regions. In L1, interactions among muscle, stroma, perivascular stroma, and the nervous system increased both in frequency and magnitude, with the inflamed perivascular stroma showing the strongest connectivity (interaction strength between 4 and 6) (**Figure 11B**). Notably, lymphoid aggregates started to participate in signal exchange at this stage, although their activity remained lower than that of the other compartments. As anticipated, granulation tissue—typical of chronic inflammation—showed limited signaling activity. Fibrotic areas, especially those closer to the mucosal surface, communicated primarily with the inflamed perivascular stroma.

Overall, mean interaction strength during L1 was approximately 4, higher than in control tissues, indicating that chronic inflammation amplifies cellular cross-talk, particularly through inflamed perivascular stroma. This aligns with the elevated angiogenic activity characteristic of inflammation, which orchestrates immune cell trafficking within the mucosa(18).

Progressing to L2, nearly all compartments engaged in mutual communication (**Figure 11C**), with the notable exception of the epithelium, which remained relatively disconnected. Similarly, lymphoid aggregates and granulation tissue maintained low interaction levels compared with other stromal components. Whereas deep submucosal fibrosis showed minimal signaling activity in L1, in L2 it became more engaged, interacting broadly with surrounding compartments—more so than the superficial fibrotic regions—and especially with other mesenchymal structures. The inflamed perivascular stroma continued to represent the most active signaling hub in L2. Despite the overall rise in the number of interactions in L2, the average interaction strength decreased to around 2, suggesting that cell–cell communication peaked during L1 and became more diffuse but individually weaker in L2.

Ligand-receptor analysis reveals the main actors in driving inflammation to fibrosis

To identify the most relevant pathways among the large number of interactions observed by CellChat(19) analysis, we calculated the number of ligand-receptor signaling patterns describing the co-contribution of sent and received different pathways, for control, L1, and L2 stages (**Figure 12D-12F**). The number of patterns

was chosen to minimize the pathways featuring compartment-specific contributions and maintain sufficient cophenetic and silhouette scores.

To trace the molecular progression of the disease, we began our analysis at the L2 stage, focusing specifically on pathways most strongly associated with superficial and deep fibrosis, and then worked backward toward the control stage to identify the compartments potentially initiating these processes. In L2, both superficial and deep fibrotic regions were recipients of signaling pattern 4 (black arrow), with PECAM1 and PECAM2 as the predominant contributors. These signals were primarily sent (pattern 1) by the superficial fibrosis and, to a lesser degree, by deep fibrosis, perivascular stroma, inflamed stroma, and granulation tissue (**Figure 13A**).

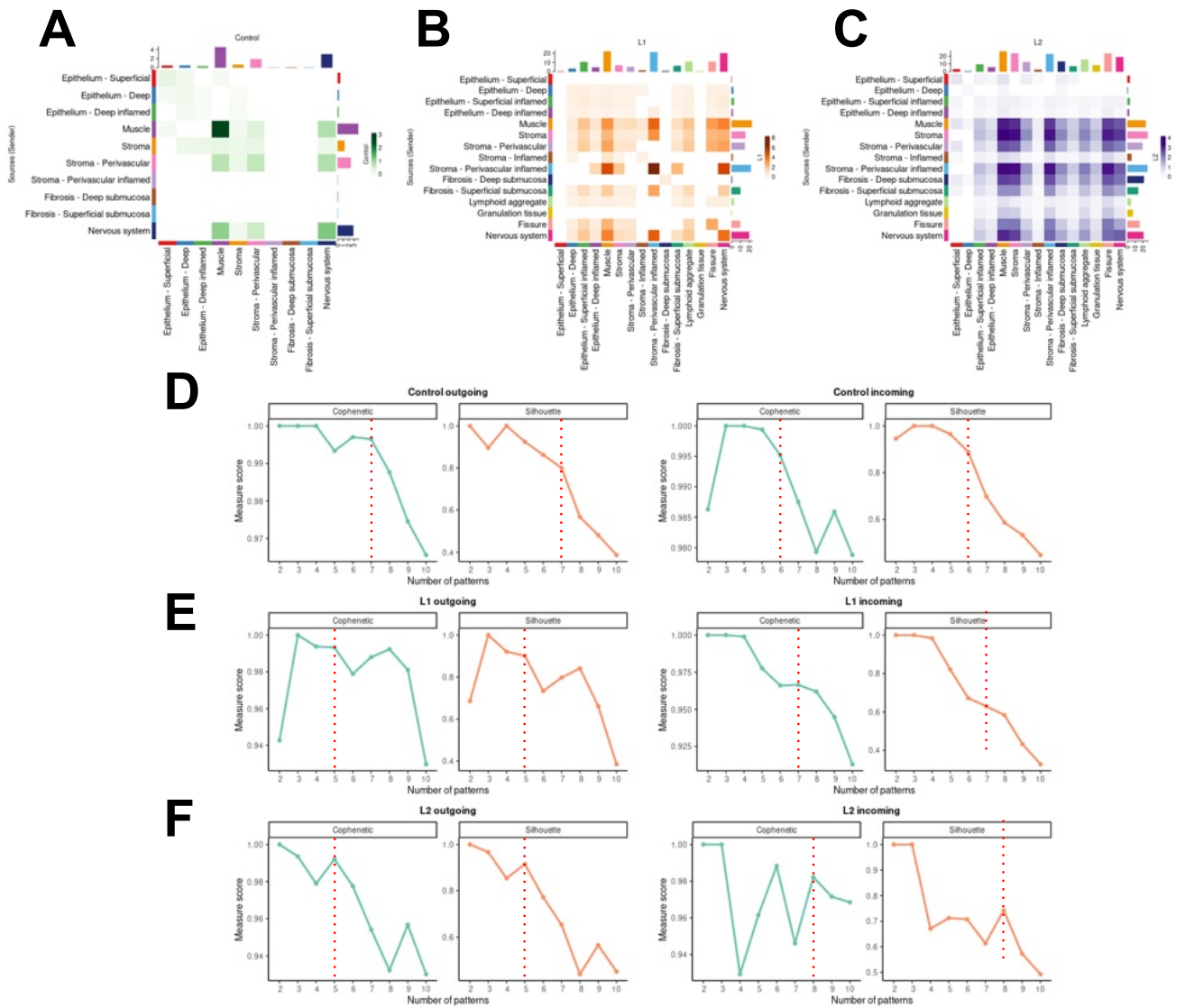


Figure 12. Intercellular communication broadens but weakens as inflammation progresses to fibrosis.

(A-C) Heatmaps showing the interaction numbers (top and left axis) and the relative strengths between the indicated spatial compartments in control (A), inflamed or L1 (B) and fibrotic or L2 (C). (D-F) Trend charts showing the number of co-contribution patterns as a function of Cophenetic and Silhouette scores for outgoing (left panel) and incoming signaling pathways in control (D), inflamed or L1 (E) and fibrotic or L2 (F)

We then examined which signals connected the deep epithelium and stroma in the control. In the control heatmap, the deep epithelium sends ApoA (pattern 3, light blue arrow), which is received by the stroma (pattern 4, Figure 13C). This epithelial-stromal crosstalk diminished as the disease progressed, with ApoA signaling maintaining strong activity within the epithelium but no longer effectively engaging the stroma (**Figure 13A and 13B**).

From these observations, we concluded that under physiological conditions, the deep epithelium and stroma communicate through ApoA and PECAM2 signaling, while the perivascular stroma maintains itself via PECAM1 (**Figure 12A**). With the onset of inflammation, epithelial-stromal crosstalk diminishes, and the epithelium increasingly supports itself through ApoA. Meanwhile, the stroma, perivascular stroma, and fibrotic tissue send PECAM2-mediated signals to themselves and to lymphoid aggregates (**Figure 12B**). By the L2 stage, when chronic inflammation is fully established, fibrosis becomes self-sustaining through PECAM1 and PECAM2 signaling, also contributed by granulation tissue and stroma (**Figure 12C**). Importantly, PECAM1-mediated signaling continues to support mesenchymal compartments throughout all disease stages.

Spatial Transcriptomics reveals fibrosis as induced by CD38-mediated signals

Ligand-receptor analysis allowed us to construct a molecular map of CD, pinpointing and assigning significance to signaling pathways within specific compartments. Using this approach, we aimed to chart the molecular progression of the disease from control to L2 by spatially mapping the ligand-receptor pairs that contributed to overall signaling.

ApoA signaling comprised the APOA1-ABCA1 and APOA1-(CUBN+AMN) pairs. In control tissues, APOA1 and ABCA1 expression was confirmed at the epithelial and stromal levels, respectively, supporting the relevance of APOA1-ABCA1-mediated crosstalk between these compartments under healthy conditions (**Figure 13A**). In L1 and L2 samples, the APOA1-ABCA1 distribution persisted but was weaker and, particularly in the epithelial compartment, associated with APOA1-(CUBN+AMN) signaling (**Figure 13B, 13C**). Notably, CUBN was absent in control tissues, indicating

that APOA1-(CUBN+AMN)-mediated signaling was not active at that stage. This pattern was also confirmed in single-cell data from CD samples, where APOA1+ and ABCA1+ cells were abundant in epithelial and stromal clusters (**Figure 14A–14C**), whereas CUBN+ and AMN+ cells were primarily localized to the epithelium (**Figure 14D, 14E**).

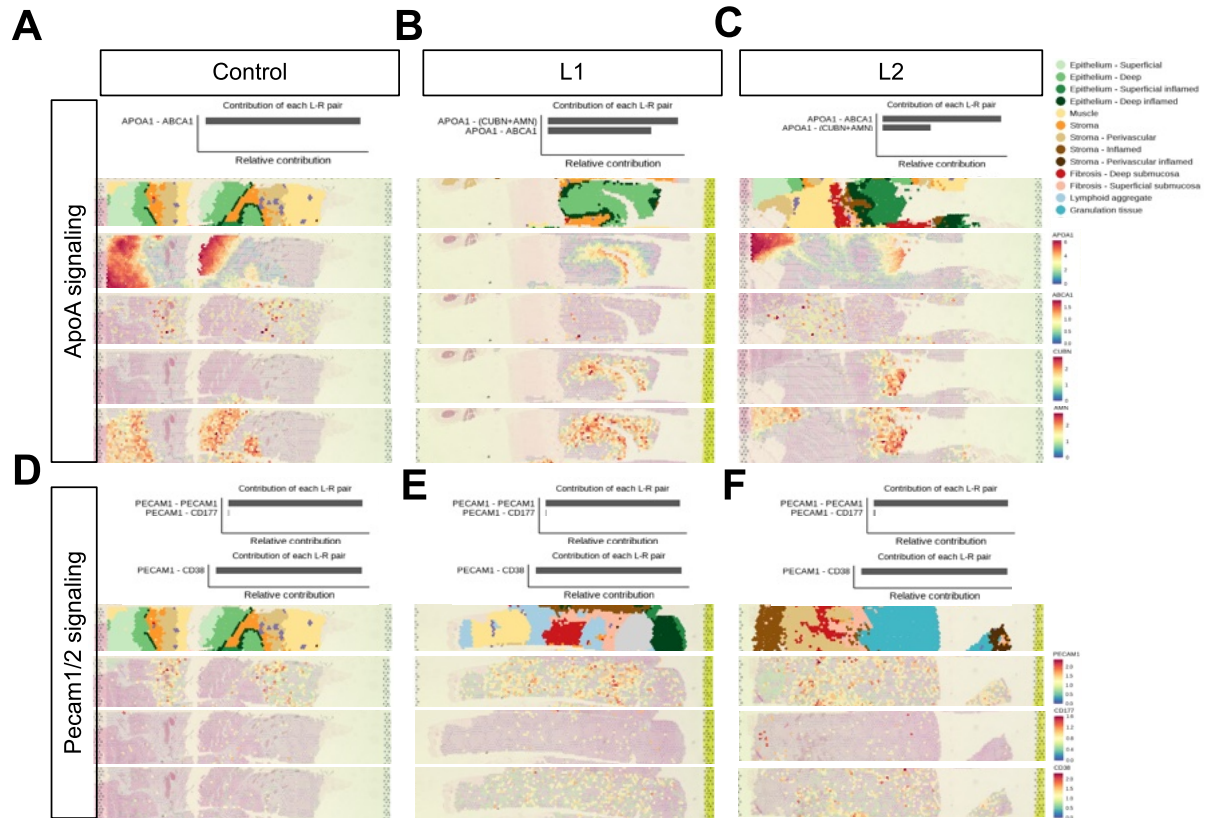


Figure 14 Ligand-receptor pair identification reveals PECAM1-CD38 interaction as the main contributor to fibrosis.

(A-C) Bar graphs showing ligand-receptor pair relative contribution to ApoA signaling (top panel) and spatial expression pattern of the contributing genes in representative images of control (A), inflamed or L1 (B), and fibrotic or L2 (C) spatial transcriptomics slides. (D-F) Bar graphs showing ligand-receptor pair relative contribution to PECAM 1 and PECAM2 signalings (top panel) and spatial expression pattern of the contributing genes in representative images of control (A), inflamed or L1 (B), and fibrotic or L2 (C) spatial transcriptomics slides.

PECAM1 signaling involved PECAM1-PECAM1 and PECAM1-CD177 interactions, with the PECAM1-PECAM1 pair serving as the predominant contributor across all disease stages within the stroma (**Figure 13D–13F**). PECAM2 signaling primarily consisted of PECAM1-CD38 interactions. CD38 expression was nearly absent in control tissues but increased during inflammation, appearing in lymphoid aggregates (L1),

granulation tissue (L2), and within the stromal and fibrotic compartments (**Figure 13D–13F**). CD38 was highly expressed in hematopoietic cells (**Figure 14F**) and also detected in the mesenchymal compartment, particularly during the myofibroblast-to-reticular fibroblast transition, and to a lesser degree in the fetal stroma-to-stroma, myofibroblast-to-inflammatory fibroblast, and transitional stroma-to-mesoderm transitions (**Figure 15A, 15B**). Importantly, CD38 expression was also validated in mesenchymal cells within fibrotic regions in an independent cohort of CD patients (**Figure 15C**).

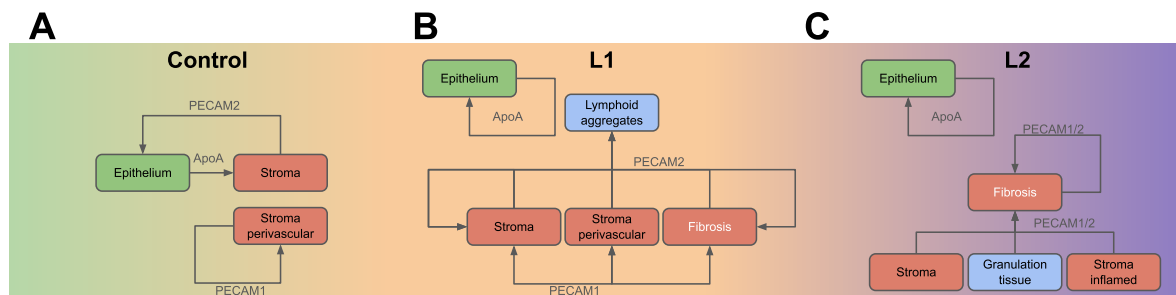


Figure 15 Fibrosis is mediated by PECAM2 signaling pathways.

(**A–C**) In physiological conditions (control, **A**) the deep epithelium and the stroma interact via the ApoA and PECAM2 signaling, while the perivascular stroma sustains itself via the PECAM1. Upon inflammation (L1), the epithelium-stroma crosstalk decreases, and the epithelium starts fostering itself via ApoA. The stroma, along with the perivascular stroma and the fibrosis, sends PECAM2-mediated signals to themselves and the lymphoid aggregates (**B**). In chronic inflammation and fibrosis (L2), fibrosis is self-sustained by PECAM1 and 2 signals, also sent by the granulation tissue and the stroma (**C**). Notably, PECAM1-mediated signals continue sustaining the mesenchymal compartments across all disease stages.

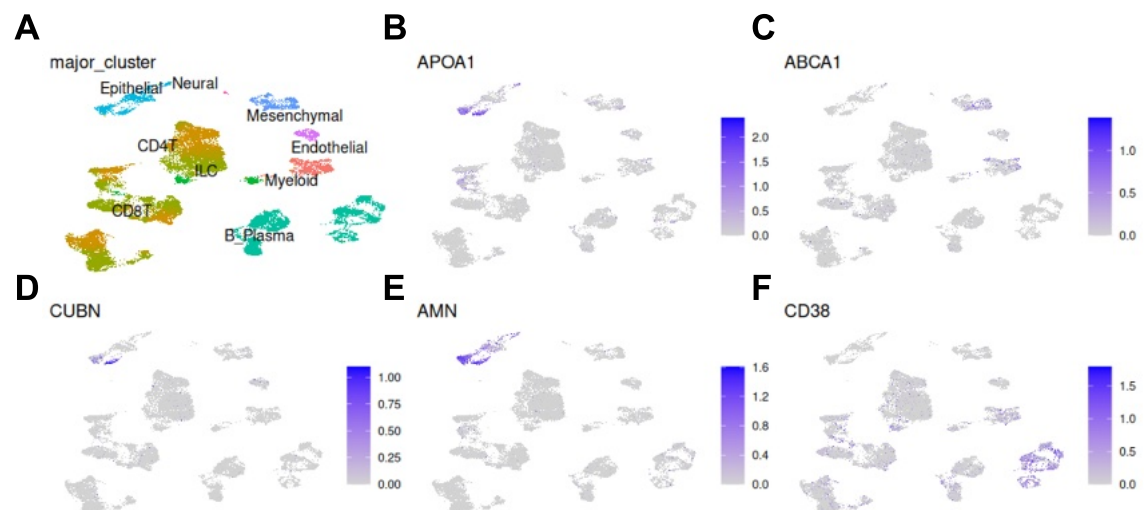


Figure 16 ApoA and PECAM1 signaling distribution in scIBD.

(A) UMAP showing major clusters found in scIBD dataset. (B-F) UMAP plots showing the indicated molecules distribution in scIBD.

To further validate the clinical relevance of our ST data, we correlated CD38 and APOA1 expression profiles with fibrosis development in patients using co-varying neighborhood analysis (CNA) (20). This analysis confirmed the well-established positive association between fibrosis progression and disease duration, after adjusting for age (21) (**Figure 16A, 16B, and Figure 15D**). Notably, the two main factors showed opposite correlations with fibrosis: APOA1 exhibited a negative correlation, whereas CD38 was positively correlated. Moreover, these two molecules were inversely correlated with each other (**Figure 16C–16E**). Supporting our observations further, data from the IBD TaMMA platform (7) demonstrated that CD38 is upregulated and APOA1 downregulated in a larger cohort of surgical CD ilea compared with controls (total samples analyzed $n=1643$, **Figure 17E, 17F**).

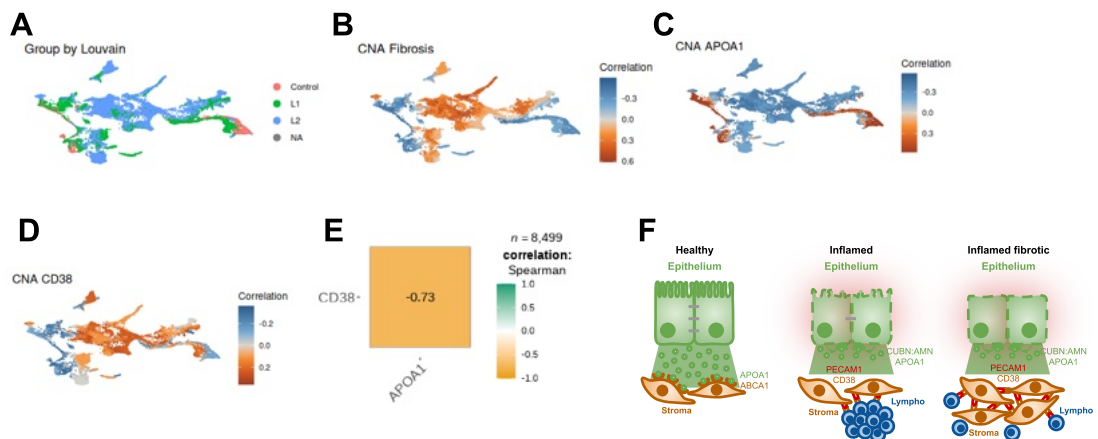


Figure 17 CD38- and ApoA1-mediated signaling contribute to fibrosis.

(A) UMAP showing group distribution within spatial multidimensional scaling. (B-D) UMAPs showing fibrosis (B), APOA1 (C), and CD38 (D) expression profile correlation according to Co-varying Neighborhood Analysis (CNA) in spatial multidimensional scaling. (E) Single-cell gene co-occurrence analysis by Spearman's correlation. (F) Schematic representation of ApoA and PECAM2-controlled fibrosis development.

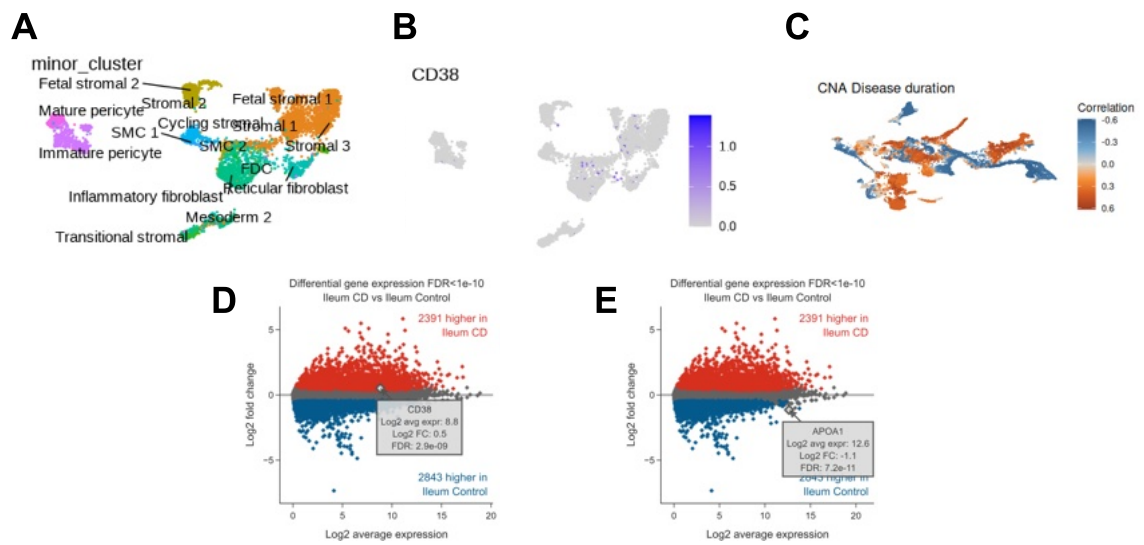


Figure 18 CD38 expression pattern correlates with fibrosis transition.

(**A**) UMAP showing minor clusters found in scIBD dataset. (**B**) UMAP showing CD38 distribution in scIBD mesenchymal subclustering. (**C**) UMAP showing disease duration correlation according to Co-varying Neighborhood Analysis (CNA) in spatial multidimensional scaling. (**D, E**) MA plots showing CD38 (**D**) and APOA1 (**E**) expression statistics in CD ileum (n=1382) versus control (n=261) extrapolated from the IBD TaMMA ⁷. False Discovery Rate (FDR) < 1e-10.

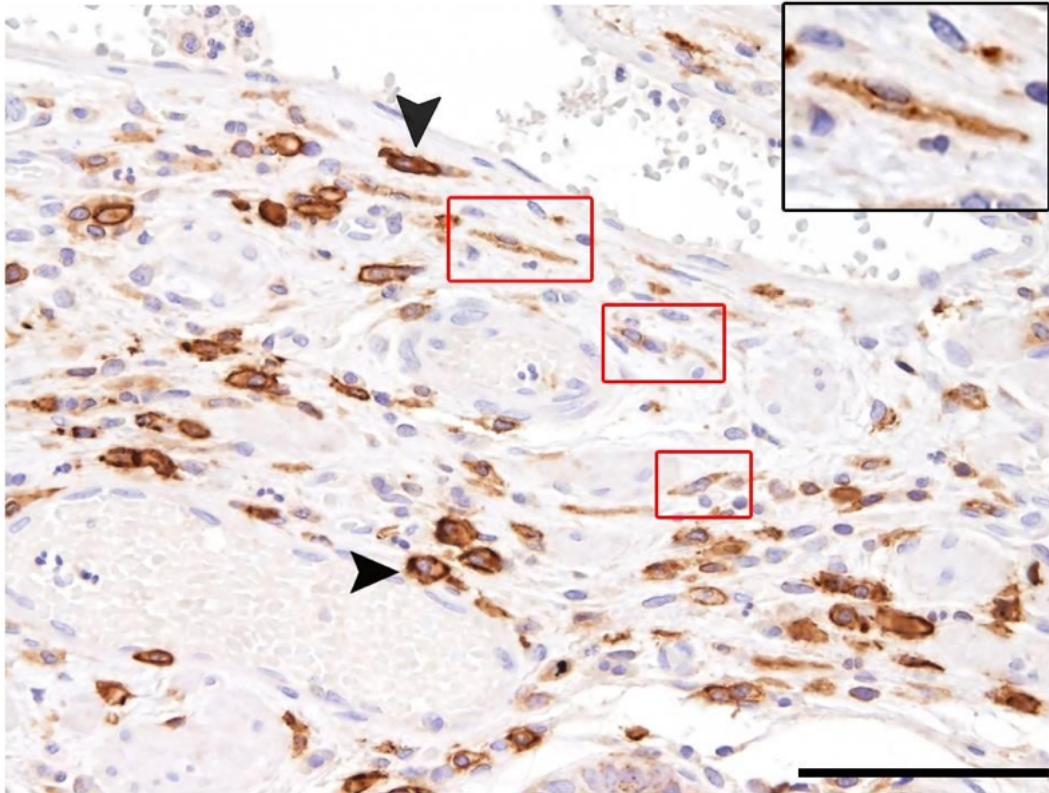


Figure 19 CD38 expression in mesenchymal cells of patients who underwent ileocecal resection due to stricturing Crohn's disease.

Representative image of fibrotic area from CD patients (n=20) showing CD38 expression in the mesenchymal compartment (red and black squares). Black arrows indicate plasma cells positive to CD38. Scale bar 100µm

CD38 inhibition prevents fibroblasts activation *in vitro* and chronic colitis *in vivo*

To investigate the potential clinical translation of our computational results into a therapeutic intervention, we tested the inhibition of CD38 using a small molecule (78c) *in vitro* and *in vivo*.

The stimulation of primary fibroblasts isolated from the colonic mucosa of healthy patients with the profibrotic cytokine TGF- β induced the expected activation response, whereas co-treatment with the selective CD38 inhibitor 78c markedly blunted this effect. Immunofluorescence analysis of vimentin, a marker of fibroblast activation and cytoskeletal remodeling, showed that cells exposed to TGF- β plus 78c maintained a morphology and signal intensity comparable to baseline controls rather than adopting the enhanced vimentin expression pattern seen with TGF- β alone

(Figure 20). These findings support the proposed role of CD38 in promoting fibroblast activation and indicate that its pharmacologic inhibition can attenuate profibrotic transformation *in vitro*.

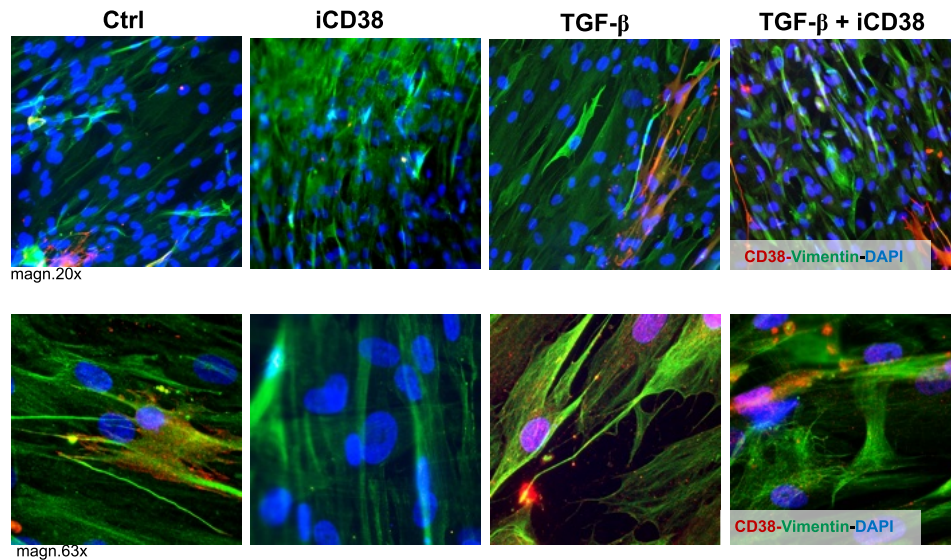


Figure 20. *In vitro* effects of CD38 inhibition on fibroblasts

Representative immunofluorescence co-staining of CD38 (red), Vimentin (green), and DAPI (blue) on healthy human colonic fibroblasts stimulated for 72 hours with 10 ng/mL TGF- β and or 40 μ M iCD38 (CD38 inhibitor). Top panels 20x magnification; bottom panels 63x magnification.

We then progressed to *in vivo* validation using the trinitrobenzene sulfonic acid (TNBS)-induced chronic colitis model, a well-established system for studying chronic intestinal inflammation and fibrosis. Notably, mice receiving the CD38 inhibitor together with TNBS failed to develop chronic inflammation or colonic wall thickening, in contrast to the control group (**Figure 21A-B**). These results substantiate the pro-inflammatory and pro-fibrotic contribution of CD38-mediated signaling in the gut and support the feasibility of targeting CD38. Furthermore, no appreciable signs of drug-related toxicity were detected.

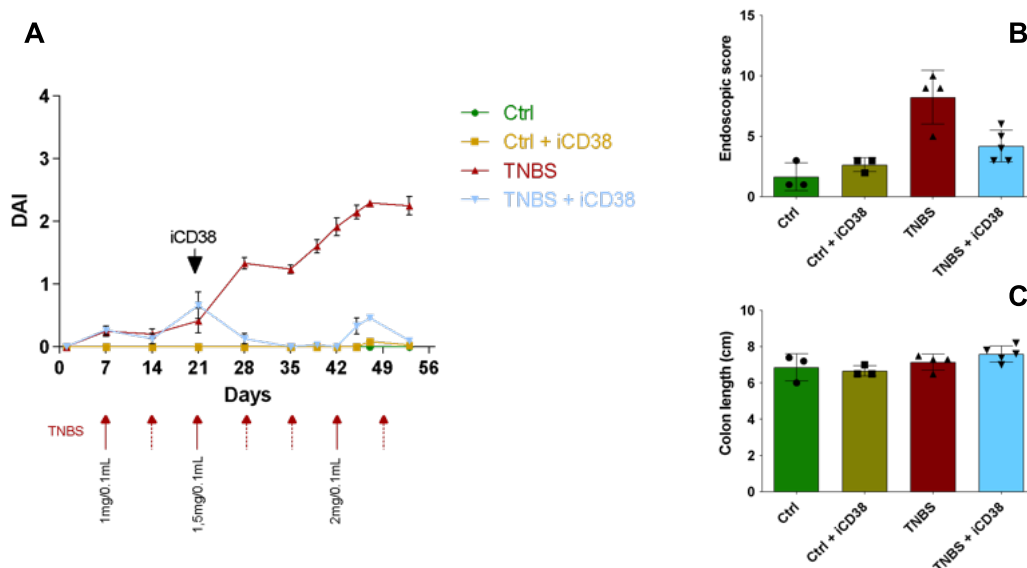


Figure 21. CD38 inhibition prevents chronic inflammation and fibrosis in vivo

(A) Disease Activity Index (DAI) in C57/BL6 mice treated with TNBS with or without a small molecule inhibitor of CD38 (iCD38) or vehicle control with or without the inhibitor (iCD38). The graph shows colitis development after TNBS administration which is instead prevented by the concomitant blockade of CD38 (B) Differences in total endoscopic score between the same groups. Endoscopic score comprises the following 4 parameters: thickening of the colon, granularity of the mucosa, changes in the vascular pattern, presence of fibrin. (C) Colon length did not vary significantly in the experimental conditions.

Overall, our results propose a new mechanisms by which Crohn's disease evolves from a primarily inflammatory disease to a fibrotic one. We propose that APOA1-ABCA1 interaction with the stroma maintains homeostasis in the healthy tissue, exerting anti-inflammatory effects(22) on the stroma. However, during chronic inflammation, when the epithelium is compromised, APOA1-(CUBN+AMN) switches on, supporting the epithelium's survival and diverting ApoA signaling from the stroma. Moreover, during inflammation, immune cells, including the CD38⁺ cells, migrate to the site of injury, and the PECAM1-CD38 interaction increases in both the stromal compartment and the inflammatory sites, contributing to the transformation of the mesenchymal compartment in fibrosis. This function is consistent with CD38's well-established role in inflammation, including in IBD pathogenesis and fibrosis(23–26).

Finally, all disease stages are supported by PECAM1 signaling to ensure the correct activity of the stromal compartment, particularly of the endothelial cells, and in mediating leukocyte trafficking within a tissue, a process known to be central in

regulating intestinal inflammation(27). Thus, PECAM1 signaling is sustained during inflammation to support tissue maintenance, while simultaneously promoting fibrosis.

The possible contribution of intestinal microbiota to the development of fibrotic strictures

The human intestine contains more than 10^{13} microorganisms—including bacteria, viruses, fungi, and archaea—collectively referred to as the microbiota. This complex ecosystem is increasingly recognized as a key regulator of intestinal physiology and disease. Patients with Crohn’s disease (CD) exhibit marked shifts in both the absolute and relative abundance of microbial species.

To explore whether specific bacteria were associated with stricturing CD, we conducted an unbiased meta-transcriptomic analysis on fibroblasts isolated from intestinal biopsies of patients with fibrotic CD and from healthy controls. *Roseomonas mucosa* was increasingly found in fibrotic samples, compared to non-fibrotic controls (**Figure 22**).

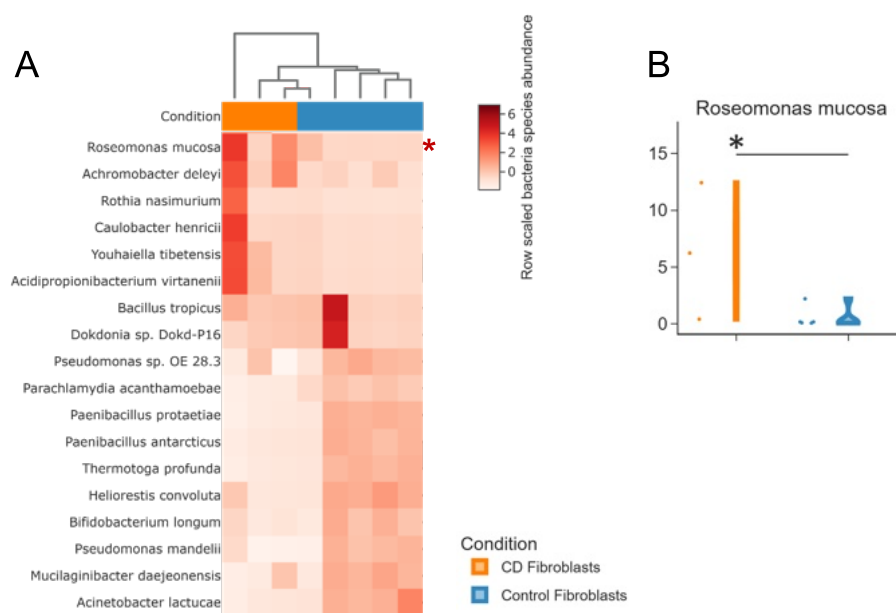


Figure 22. Metatranscriptomics analysis of stricture derived fibroblasts fibroblasts

(A) Heatmap showing scaled abundance of bacterial species across fibroblast samples, with hierarchical clustering revealing distinct microbial signatures. *Roseomonas mucosa* is significantly enriched in CD fibroblasts (red asterisk). (B) Violin plot comparing *Roseomonas mucosa* abundance between CD and control groups, with statistical significance indicated.

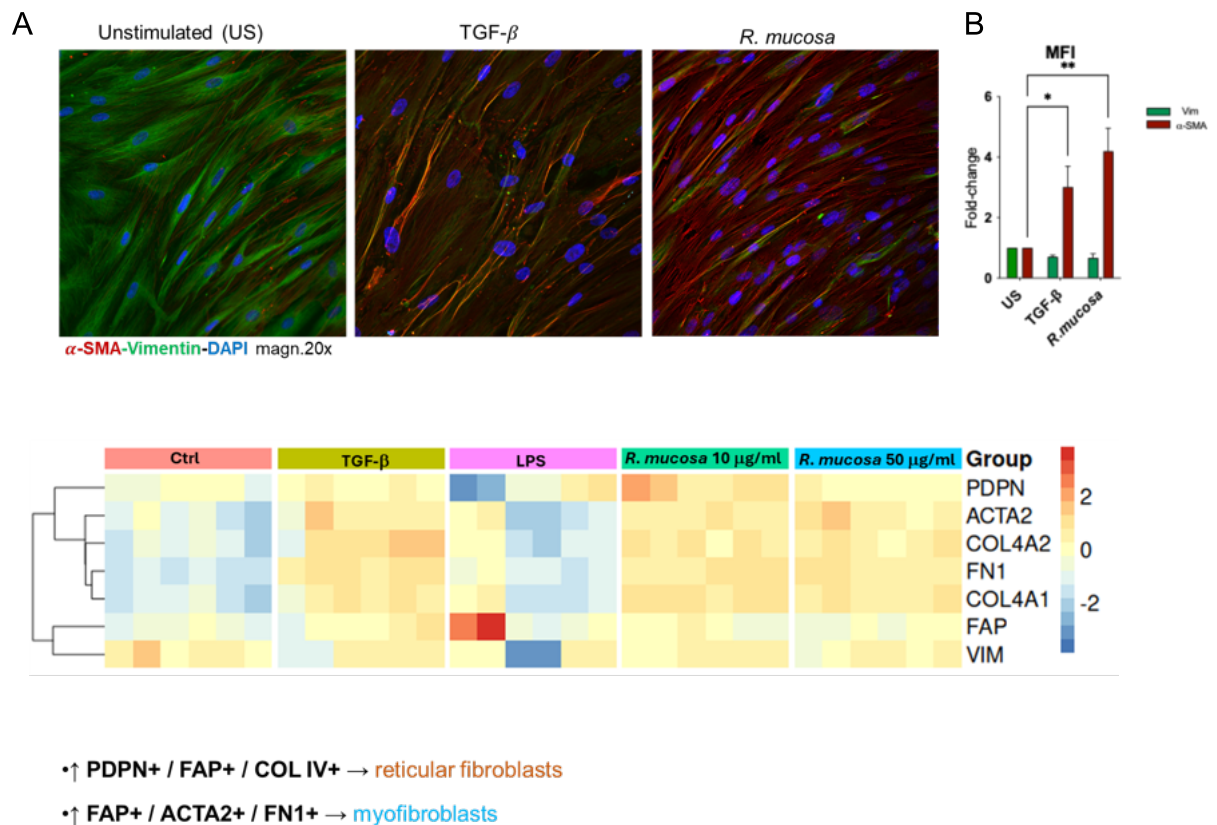


Figure 23. In vitro fibroblasts stimulation with *Roseomonas mucosa*.

Stimulation with *R. mucosa* induces profibrotic changes in human intestinal fibroblasts. (A) Immunofluorescence; (B) Mean Fold Increase of smooth muscle actin (α -SMA) and vimentin (vim) in fibroblasts unstimulated (US), stimulated with TGF- β and with lysates of *R. mucosa*. (C)

To assess whether *R. mucosa* influences fibroblast phenotypes, we stimulated primary human intestinal fibroblasts with *R. mucosa* lysates (10 μ g/mL), comparing the response with that induced by the well-established profibrotic factor TGF- β and with unstimulated controls. Fibroblasts exposed to *R. mucosa* showed a significant increase in smooth muscle actin (SMA) expression—a marker of extracellular matrix production—similar to the effect of TGF- β (**Figure 23**).

Furthermore, stimulation with *R. mucosa* led to upregulation of key profibrotic genes, including ACTA2, FN1, COL4A1/2, and FAP, as well as PDPN, a stromal marker linked to reticular fibroblast expansion in fibrotic CD lesions. Taken together, these results indicate that *R. mucosa* acts as a profibrotic stimulus and promotes activation of PDPN⁺ fibroblasts, supporting a microbial contribution to fibrotic progression in Crohn's disease.

Discussion

This study represents the first spatially resolved characterization of the transition from inflammation to fibrosis in the intestinal tissue of patients with CD, revealing a previously unappreciated mechanism that may regulate fibrogenesis and exploring the potential involvement of the bacterium *R. mucosa* in this process.

By analyzing full-thickness surgical samples from CD patients and healthy controls, we confirmed the profound cytoarchitectural rearrangements, disruption of compartmental structure(12), and the critical contribution of specific epithelial and stromal subsets to fibrotic evolution(28, 29). The spatial granularity of our approach further enabled the identification of two distinct fibrotic niches: a superficial region enriched in inflammatory fibroblasts and a deeper, reticular-fibroblast-dominated cluster uniquely associated with strictures and absent from other inflammatory environments such as fissures or inflamed stroma. Pseudotime reconstruction supported the notion that reticular fibroblasts arise from activated myofibroblasts, constituting the terminal differentiation stage of these cells in fibrotic lesions.

These findings align with established pathological features of CD. Inflammatory signals activate inflammatory fibroblasts, driving cytokine production(29, 30) and shaping immune responses. Consistently, we observed that the superficial submucosa was heavily infiltrated by B cells and enriched in biological programs related to inflammation. In contrast, reticular fibroblasts—characterized by robust ECM deposition—became predominant in the deep submucosa fibrosis associated with advanced disease(31, 32).

Importantly, we did not find evidence supporting epithelial-to-mesenchymal transition (EMT). Even under inflammatory conditions, epithelial cells failed to exhibit mesenchymal transcriptional signatures. Although EMT has been historically proposed to contribute to fibrosis and is often inferred from mesenchymal marker expression(33), our data do not support such a phenomenon. Instead, our findings agree with recent single-cell studies showing that EMT-like programs are restricted to mesenchymal, not epithelial, compartments(8, 29, 34, 35). We acknowledge that ST may detect isolated EMT-related markers in epithelial regions, but we did not observe transcriptional reprogramming indicative of EMT.

Tracking the molecular progression from control to inflamed to fibrotic states revealed two major shifts in ligand–receptor activity: (1) signaling intensity peaked

in the inflamed L1 stage, when all compartments established extensive aberrant communication, likely reflecting attempts to counterbalance or amplify inflammatory stimuli; and (2) in L2 fibrosis, overall signaling strength diminished while the number of interactions increased, likely reflecting compartmental distortion, loss of organized cross-talk, and the accumulation of transcriptionally inert ECM.

A key insight from our study is that stromal cells orchestrate mesenchymal signaling even before fibrosis arises, primarily through CD38- and ApoA-mediated pathways. These two molecular axes appear central to the progression from inflammation to fibrostenosis. We suggest that APOA1-ABCA1 signaling underlies epithelial-to-stromal immune surveillance essential for homeostasis. APOA1, a well-known anti-inflammatory molecule, marks a distinct epithelial subset in the intestine(36). Its interaction with ABCA1 promotes anti-inflammatory STAT3 activation(22) and modulates stromal metabolism, influencing immune cell function(37–39). This supports a model in which, under physiological conditions, epithelial cells maintain stromal quiescence via APOA1/ABCA1-mediated surveillance(40). During inflammation, however, APOA1 shifts its interaction toward the CUBN/AMN complex—critical for epithelial survival(41, 42)—likely reflecting a compensatory mechanism when epithelial-stromal communication collapses.

Most notably, we identified CD38 as a key driver of chronic inflammation and fibrogenesis. CD38 is a widely expressed, multifunctional membrane bound molecule with both signaling and enzymatic activity. As an enzyme, CD38 degrades nicotinamide adenine dinucleotide (NAD), which is vital for several redox reactions and the functioning of sirtuins, as well as other pathways. Sirtuins are in turn essential for mitochondrial integrity, cellular aging and, central to this work—modulation of inflammation and fibrosis through the stabilization of transcription factors that enhance cytokine signaling, including TGF β /Smad signaling in fibroblasts (25, 26). CD38 role in modulating fibrosis via its NAD⁺-dependent activation of TGF- β pathways has been implicated in fibrosis across multiple organs, including in immune-mediated diseases such as systemic sclerosis, reinforcing the plausibility of a role also in CD (25, 26). We propose that CD38 promotes intestinal fibrosis by directing the reorganization of the mesenchymal compartment, consistent with its expression at transitional states between myofibroblasts, inflammatory fibroblasts, and reticular fibroblasts. Our *in vitro* and - even more so - *in vivo* findings corroborate this model: pharmacological CD38 inhibition attenuated fibroblast activation and prevented inflammation and colon wall thickening, two hallmarks of fibrotic remodeling.

Furthermore, ApoA signaling emerged as a candidate negative regulator of fibrosis, given its inverse correlation with CD38 expression and fibrotic burden in patients, and its progressively diminished influence on the stroma during disease progression although further research is needed to validate this observation.

Collectively, our work delineates—for the first time—the molecular and cellular events underpinning the progression of CD toward fibrotic complications, highlighting specific signaling pathways as central drivers of tissue transformation. While the cohort size was limited, integration with published single-cell datasets and the reproducibility of key observations across analytical approaches strengthen the robustness of our conclusions. Nonetheless, we acknowledge that additional pathways likely contribute, given the substantial heterogeneity of CD among patients(7).

Finally, Our identification of *Roseomonas mucosa* in fibroblasts from fibrotic CD samples, but not in controls, suggests a potential microbial contribution to the profibrotic process. Functional assays further support this, as exposure of primary human intestinal fibroblasts to *R. mucosa* lysates induced markers of extracellular matrix deposition, including SMA, and upregulated profibrotic genes such as ACTA2, FN1, COL4A1/2, FAP, and PDPN. These data indicate that *R. mucosa* can directly modulate fibroblast activation, promoting the expansion of PDPN+ profibrotic fibroblasts. While causality and *in vivo* relevance require further investigation, our observation highlight a previously unrecognized role for the microbiota in contributing in fibrosis development in CD, suggesting that specific microbial signals may act as upstream triggers of mesenchymal activation and tissue remodeling.

In summary, our study provides a revised conceptual framework for intestinal fibrosis in CD, positioning the mesenchymal compartment at the core of disease progression in the setting of inflammation-driven breakdown of homeostatic mechanisms. Importantly, our findings identify CD38 as a pivotal mediator, and a potential therapeutic target, to prevent fibrostenotic complications in CD, and *R. mucosa* as a possible microbial contributor to this process.

Methods

Collection, selection and processing of human samples for spatial transcriptomics

Samples from surgical intestinal resections of CD strictures provide an ideal model of the fibrotic process unfolding. The two ends of the specimen are usually macroscopically non-fibrotic while, progressing toward the center of the segment, the thickening increases reaching its maximum at the point of the stenosis. In addition to this, variable degrees of inflammation can be present throughout the segment. Thus, macroscopically healthy margins are usually present at the resection site, and the affected tract is fully removed. Throughout its length, the surgical specimen displays the transition from the proximal healthy margin to the affected part (usually also inflamed), reaching the stricturing point and reverting to the healthy distal margin. Based on this rationale, we selected surgical leftovers from patients undergoing ileocecal resection due to stricturing CD. The FFPE tissues were inspected by the pathologist to identify the three regions of interest per each sample representing the healthy region, an intermediate tract displaying inflammation with initial fibrotic changes, and the maximally affected areas with marked fibrosis and maximal strictures. After the intestinal regions of interest were identified, the original FFPE blocks were melted. The selected tissue slices were re-assembled in two new paraffin blocks to optimize the sequencing surface. The correct orientation in the re-assembled paraffin blocks was confirmed by examining hematoxylin and eosin (H&E)-stained slides adjacent to those cut for sequencing. Tissues were thus reassembled to create a specimen composite with a diameter of approximately 11 mm with CD-derived intestinal samples encompassing different areas of interest or “stages” of the process. This approach was required to obtain tissue slices accommodating into the 10x Visium Spatial Gene Expression slide from as many patients as possible and

comprising different disease stages.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on a set of human tissue samples affected by Crohn's disease obtained from the archive of the Department of Pathology (ASST Spedali Civili di Brescia, Brescia, Italy). Four micron-thick tissue sections were obtained from formalin-fixed, paraffin-embedded (FFPE) blocks. For immunohistochemical staining, endogenous peroxidase was blocked by incubation with methanol and hydrogen peroxide 0.03% for 20 min during rehydration. Immunostaining was performed using anti-CD38 (clone SPC32, 1:200, Novocastra, code nr. NCL-L-CD38-290). After pre-treatment with microwave in EDTA buffer at pH 8.00, the reaction was revealed using Novolink Polymer (Leica Microsystems) followed by diaminobenzidine (DAB, Dako, Glostrup, Denmark). Finally, the slides were counterstained with Meyer's Hematoxylin.

Spatial transcriptomics

Spatial transcriptomics sequencing was performed by AcelaBio (San Diego, CA). Raw reads were aligned to the human reference genome (GRCh38) and linked to the H&E images with Space Ranger (10x Genomics). The resulting gene expression matrices were quality-checked, normalized, preprocessed, multidimensional scaled, and clustered with Seurat(43). Data harmonization was performed with Harmony(10). Digital segmentation of the different slices was performed with SPATA2(44). Stlearn was used for spatially-resolved multidimensional scaling and clustering(45). Deconvolution of spatial data with single-cell signatures and deconvolution of single-cell data with spatial signatures were performed with Seurat(43). Pseudotime analysis was performed with Monocle 3(46). Pathway analysis was performed with GSdensity(47). CellChat was used to analyze ligand-receptor and cell-cell communication(19).

TNBS model of chronic colitis and intestinal fibrosis

Female C57BL/6 mice were housed under specific-pathogen-free (SPF) conditions in our animal facility and used at 8 weeks of age. All procedures adhered to the guidelines outlined in the Principle of Laboratory Animal Care (directive 86/609/EEC) and were approved by the Italian Ministry of Health. Experiments were conducted in accordance with institutional regulations and national and international laws governing animal research, with additional approval from the ethics committee of the

IRCCS Ospedale San Raffaele. In line with animal welfare recommendations (directive 86/609/EEC) and based on our previous experience with similar models, we determined that two independent experiments with $n=5$ mice per group would provide adequate statistical power while keeping animal use to a minimum. Randomization was performed for group allocation, and both the assignment of animals to treatments and the evaluation of clinical parameters were conducted in a blinded manner. The latter was performed with an independent lab operator removing cage card number before the study investigator assessed parameters without knowing the group.

Seven-week-old C57BL/6N mice were allocated into four experimental groups ($n=5$): group I received 45% ethanol (EtOH) as a control; group II received the vehicle (45% EtOH) together with the CD38 inhibitor; group III was administered trinitrobenzene sulfonic acid (TNBS; Sigma-Aldrich, Italy) dissolved in 45% EtOH; and group IV received both TNBS and the CD38 inhibitor (iCD38; EMD Millipore Corp., USA). Chronic TNBS colitis was induced by weekly intrarectal administration of TNBS in 45% EtOH at escalating doses ranging from 1 mg/0.1 mL to 2 mg/0.1 mL, while control groups received 45% EtOH alone. Mice in groups III and IV also received the CD38 inhibitor intraperitoneally (15 mg/kg) three times per week, beginning in the fourth week of TNBS treatment.

Clinical disease progression was monitored through assessment of stool consistency, body weight, and rectal bleeding, and a disease activity index (DAI) was calculated for each mouse. After the 7-week TNBS treatment period and an additional 2-week follow-up phase, animals were euthanized by CO₂ inhalation. Prior to sacrifice, all mice underwent endoscopic evaluation to quantify inflammation and mucosal injury, as previously described (48).

Microbiome Profiling of Fibroblast from Patients with Stricturing Crohn's disease

Human intestinal biopsy specimens from patients with fibrostenotic CD ($n=3$) and controls ($n=5$) were obtained immediately after tissue collection and transferred into ice-cold sterile PBS. Tissue was minced into approximately 0.5-1 mm fragments and enzymatically dissociated using collagenase IV (1–2 mg/mL) and dispase (1 U/mL) in DMEM for 45–60 min at 37 °C with gentle agitation. The resulting suspension was filtered through a 70 μ m cell strainer, washed with PBS containing 2% FBS, and centrifuged at 400 g for 5 min to obtain a single-cell pellet. Fibroblasts were enriched by magnetic bead-based selection using a negative-selection strategy to remove

epithelial, endothelial, hematopoietic, and erythroid cells. Briefly, the single-cell suspension was incubated with a cocktail of biotin-conjugated antibodies targeting CD45, CD31, EpCAM, and CD235a, followed by anti-biotin magnetic microbeads (Miltenyi Biotec, Germany). The labelled suspension was passed through an LS column placed in a magnetic field; labelled non-fibroblast lineages were retained, whereas unlabeled fibroblasts were collected as the flow-through fraction. Cells were washed, counted, and immediately pelleted for RNA extraction. Total RNA was extracted from freshly isolated fibroblasts using a silica-column purification kit compatible with low-input samples, following the manufacturer's instructions. On-column DNase I digestion was performed to remove genomic DNA. RNA quantity was measured fluorometrically, and integrity was assessed using an Agilent Bioanalyzer. Samples with adequate RNA yield and acceptable integrity were carried forward for metatranscriptomic library preparation. Because both host and microbial transcripts were of interest, ribosomal RNA depletion rather than poly(A) selection was applied. Dual human-bacterial rRNA depletion was performed using a commercially available depletion kit (NEBNext® rRNA Depletion Kit). Depleted RNA was converted to cDNA and processed into strand-specific libraries using an Illumina-compatible library preparation protocol including fragmentation, end-repair, adapter ligation, and limited-cycle PCR amplification. Final libraries were quantified by qPCR and quality-checked for size distribution. Indexed libraries were pooled equimolarly and sequenced on an Illumina platform (NovaSeq) with paired-end 150 bp reads. High sequencing depth was targeted to compensate for the predominance of host RNA, aiming for ≥ 50 million paired reads per sample. Negative controls (extraction blanks and reagent controls) were sequenced concurrently to detect potential contamination. Raw reads were processed by removing adapter sequences and low-quality bases using a standard trimming workflow. rRNA reads that escaped depletion were filtered using sortMeRNA when applicable. Host-derived reads were identified by alignment to the human reference genome (GRCh38) using HISAT2 or STAR. Host-mapped reads were quantified at the gene level (featureCounts) and analyzed for gene expression patterns using established workflows (DESeq2, edgeR).

Non-host reads (unmapped) were retained for microbial analysis. Taxonomic assignment of microbial reads was performed using Kraken2 or Centrifuge with a comprehensive reference database. Microbial metabolic pathway profiling was performed using HUMAnN3, normalizing read counts according to recommended procedures. Differential microbial features were evaluated using compositional data-

appropriate statistical methods. Taxa or gene families present in negative controls were identified and removed from downstream analysis to mitigate low-biomass contamination artifacts.

Differential abundance between fibrotic CD and control fibroblasts was assessed using the Wilcoxon rank-sum test with false discovery rate correction, considering adjusted p-values < 0.05 as significant. Heatmaps with hierarchical clustering (Bray–Curtis dissimilarity) and violin plots were generated in R to visualize microbial community structure and highlight species such as *Roseomonas mucosa*, which showed significant enrichment in fibroblasts isolated from strictures.

Bibliography

1. J. Torres, S. Mehandru, J.-F. Colombel, L. Peyrin-Biroulet, Crohn's disease. *Lancet* 389, 1741–1755 (2017).
2. G. R. Lichtenstein, E. V. Loftus, K. L. Isaacs, M. D. Regueiro, L. B. Gerson, B. E. Sands, ACG clinical guideline: management of crohn's disease in adults. *Am. J. Gastroenterol.* 113, 481–517 (2018).
3. F. Rieder, S. Kessler, M. Sans, C. Fiocchi, Animal models of intestinal fibrosis: new tools for the understanding of pathogenesis and therapy of human disease. *Am. J. Physiol. Gastrointest. Liver Physiol.* 303, G786-801 (2012).
4. J. S. Mandlik, A. S. Patil, S. Singh, Next-Generation Sequencing (NGS): Platforms and Applications. *J. Pharm. Bioallied Sci.* 16, S41–S45 (2024).
5. N. S. Seyed Tabib, M. Madgwick, P. Sudhakar, B. Verstockt, T. Korcsmaros, S. Vermeire, Big data in IBD: big progress for clinical practice. *Gut* 69, 1520–1532 (2020).
6. D. Corridoni, T. Chapman, A. Antanaviciute, J. Satsangi, A. Simmons, Inflammatory Bowel Disease Through the Lens of Single-cell RNA-seq Technologies. *Inflamm. Bowel Dis.* 26, 1658–1668 (2020).
7. L. Massimino, L. A. Lamparelli, Y. Houshyar, S. D'Alessio, L. Peyrin-Biroulet, S. Vetrano, S. Danese, F. Ungaro, The Inflammatory Bowel Disease Transcriptome and Metatranscriptome Meta-Analysis (IBD TaMMA) framework. *Nat. Comput. Sci.* 1, 511–515 (2021).
8. B.-J. Ke, S. Abdurahiman, F. Biscu, G. Zanella, G. Dragoni, S. Santhosh, V. De Simone, A. Zouzaf, L. van Baarle, M. Stakenborg, V. Bosáková, Y. Van Rymentant, E. Verhulst, S. Verstockt, E. Klein, G. Bislenghi, A. M. Wolthuis, J. Frič, C. Breynaert, A. D'Hoore, G. Matteoli, Intercellular interaction between FAP+ fibroblasts and CD150+ inflammatory monocytes mediates fibro-stenosis in Crohn's disease. *J. Clin. Invest.* (2024).
9. C. Zhang, L. Wang, Q. Shi, Computational modeling for deciphering tissue microenvironment heterogeneity from spatially resolved transcriptomics. *Comput. Struct. Biotechnol. J.* 23, 2109–2115 (2024).
10. I. Korsunsky, N. Millard, J. Fan, K. Slowikowski, F. Zhang, K. Wei, Y. Baglaenko, M. Brenner, P.-R. Loh, S. Raychaudhuri, Fast, sensitive and accurate integration of single-cell data with Harmony. *Nat. Methods* 16, 1289–1296 (2019).

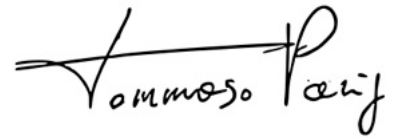
11. M. Dolinger, J. Torres, S. Vermeire, Crohn's disease. *Lancet* 403, 1177–1191 (2024).
12. C. Li, J. F. Kuemmerle, Mechanisms that mediate the development of fibrosis in patients with Crohn's disease. *Inflamm. Bowel Dis.* 20, 1250–1258 (2014).
13. H. Nie, P. Lin, Y. Zhang, Y. Wan, J. Li, C. Yin, L. Zhang, Single-cell meta-analysis of inflammatory bowel disease with scIBD. *Nat. Comput. Sci.* 3, 522–531 (2023).
14. S. D'Alessio, F. Ungaro, D. Noviello, S. Lovisa, L. Peyrin-Biroulet, S. Danese, Revisiting fibrosis in inflammatory bowel disease: the gut thickens. *Nat. Rev. Gastroenterol. Hepatol.* 19, 169–184 (2022).
15. R. Roozendaal, R. E. Mebius, Stromal cell-immune cell interactions. *Annu. Rev. Immunol.* 29, 23–43 (2011).
16. P. O. Valls, A. Esposito, Signalling dynamics, cell decisions, and homeostatic control in health and disease. *Curr. Opin. Cell Biol.* 75, 102066 (2022).
17. S. Jin, C. F. Guerrero-Juarez, L. Zhang, I. Chang, R. Ramos, C.-H. Kuan, P. Myung, M. V. Plikus, Q. Nie, Inference and analysis of cell-cell communication using CellChat. *Nat. Commun.* 12, 1088 (2021).
18. J.-H. Jeong, U. Ojha, Y. M. Lee, Pathological angiogenesis and inflammation in tissues. *Arch. Pharm. Res.* 44, 1–15 (2021).
19. S. Jin, M. V. Plikus, Q. Nie, CellChat for systematic analysis of cell-cell communication from single-cell and spatially resolved transcriptomics. *BioRxiv* (2023).
20. Y. A. Reshef, L. Rumker, J. B. Kang, A. Nathan, I. Korsunsky, S. Asgari, M. B. Murray, D. B. Moody, S. Raychaudhuri, Co-varying neighborhood analysis identifies cell populations associated with phenotypes of interest from single-cell transcriptomics. *Nat. Biotechnol.* 40, 355–363 (2022).
21. B. Pariente, J. Cosnes, S. Danese, W. J. Sandborn, M. Lewin, J. G. Fletcher, Y. Chowers, G. D'Haens, B. G. Feagan, T. Hibi, D. W. Hommes, E. J. Irvine, M. A. Kamm, E. V. Loftus, E. Louis, P. Michetti, P. Munkholm, T. Oresland, J. Panés, L. Peyrin-Biroulet, M. Lémann, Development of the Crohn's disease digestive damage score, the Lémann score. *Inflamm. Bowel Dis.* 17, 1415–1422 (2011).
22. C. Tang, Y. Liu, P. S. Kessler, A. M. Vaughan, J. F. Oram, The macrophage cholesterol exporter ABCA1 functions as an anti-inflammatory receptor. *J. Biol.*

- Chem.* 284, 32336–32343 (2009).
23. M. Schneider, V. Schumacher, T. Lischke, K. Lücke, C. Meyer-Schwesinger, J. Velden, F. Koch-Nolte, H.-W. Mittrücker, CD38 is expressed on inflammatory cells of the intestine and promotes intestinal inflammation. *PLoS ONE* 10, e0126007 (2015).
 24. Z. L. Piedra-Quintero, Z. Wilson, P. Nava, M. Guerau-de-Arellano, CD38: an immunomodulatory molecule in inflammation and autoimmunity. *Front. Immunol.* 11, 597959 (2020).
 25. B. Shi, W. Wang, B. Korman, L. Kai, Q. Wang, J. Wei, S. Bale, R. G. Marangoni, S. Bhattacharyya, S. Miller, D. Xu, M. Akbarpour, P. Cheresch, D. Proccissi, D. Gursel, J. M. Espindola-Netto, C. C. S. Chini, G. C. de Oliveira, J. E. Gudjonsson, E. N. Chini, J. Varga, Targeting CD38-dependent NAD⁺ metabolism to mitigate multiple organ fibrosis. *iScience* 24, 101902 (2021).
 26. B. Shi, A. Amin, P. Dalvi, W. Wang, N. Lukacs, L. Kai, P. Cheresch, T. R. Peclat, C. C. Chini, E. N. Chini, W. van Schooten, J. Varga, Heavy-chain antibody targeting of CD38 NAD⁺ hydrolase ectoenzyme to prevent fibrosis in multiple organs. *Sci. Rep.* 13, 22085 (2023).
 27. A. Woodfin, M.-B. Voisin, S. Nourshargh, PECAM-1: a multi-functional molecule in inflammation and vascular biology. *Arterioscler. Thromb. Vasc. Biol.* 27, 2514–2523 (2007).
 28. L. Kong, V. Pokatayev, A. Lefkovith, G. T. Carter, E. A. Creasey, C. Krishna, S. Subramanian, B. Kochar, O. Ashenberg, H. Lau, A. N. Ananthakrishnan, D. B. Graham, J. Deguine, R. J. Xavier, The landscape of immune dysregulation in Crohn's disease revealed through single-cell transcriptomic profiling in the ileum and colon. *Immunity* 56, 444-458.e5 (2023).
 29. J. Li, A. J. Simmons, C. V. Hawkins, S. Chiron, M. A. Ramirez-Solano, N. Tasneem, H. Kaur, Y. Xu, F. Revetta, P. N. Vega, S. Bao, C. Cui, R. N. Tyree, L. W. Raber, A. N. Conner, J. M. Pilat, J. Jacobse, K. M. McNamara, M. M. Allaman, G. A. Raffa, Q. Liu, Identification and multimodal characterization of a specialized epithelial cell type associated with Crohn's disease. *Nat. Commun.* 15, 7204 (2024).
 30. K. Wei, H. N. Nguyen, M. B. Brenner, Fibroblast pathology in inflammatory diseases. *J. Clin. Invest.* 131 (2021).
 31. M. F. Griffin, H. E. desJardins-Park, S. Mascharak, M. R. Borrelli, M. T. Longaker,

- Understanding the impact of fibroblast heterogeneity on skin fibrosis. *Dis. Model. Mech.* 13 (2020).
32. R. N. Gomes, F. Manuel, D. S. Nascimento, The bright side of fibroblasts: molecular signature and regenerative cues in major organs. *npj Regen. Med.* 6, 43 (2021).
 33. H. Jiang, J. Shen, Z. Ran, Epithelial-mesenchymal transition in Crohn's disease. *Mucosal Immunol.* 11, 294–303 (2018).
 34. Y. Zhang, J. Wang, H. Sun, Z. Xun, Z. He, Y. Zhao, J. Qi, S. Sun, Q. Yang, Y. Gu, L. Zhang, C. Zhou, Y. Ye, N. Wu, D. Zou, B. Su, TWIST1+FAP+ fibroblasts in the pathogenesis of intestinal fibrosis in Crohn's disease. *J. Clin. Invest.* 134 (2024).
 35. P. K. Mukherjee, Q. T. Nguyen, J. Li, S. Zhao, S. M. Christensen, G. A. West, J. Chandra, I. O. Gordon, S. Lin, J. Wang, R. Mao, D. Czarnecki, C. Rayan, I. Goren, S. Banerjee, P. Kotak, T. Plesec, S. Lal, T. Fabre, S. Asano, F. Rieder, Strictureing Crohn's Disease Single-Cell RNA Sequencing Reveals Fibroblast Heterogeneity and Intercellular Interactions. *Gastroenterology* 165, 1180–1196 (2023).
 36. J. Li, A. J. Simmons, S. Chiron, M. A. Ramirez-Solano, N. Tasneem, H. Kaur, Y. Xu, F. Revetta, P. N. Vega, S. Bao, C. Cui, R. N. Tyree, L. W. Raber, A. N. Conner, D. B. Beaulieu, R. L. Dalal, S. N. Horst, B. S. Pabla, Y. Huo, B. A. Landman, Q. Liu, A specialized epithelial cell type regulating mucosal immunity and driving human crohn's disease. *BioRxiv* (2023).
 37. G.-J. Zhao, K. Yin, Y.-C. Fu, C.-K. Tang, The interaction of ApoA-I and ABCA1 triggers signal transduction pathways to mediate efflux of cellular lipids. *Mol. Med.* 18, 149–158 (2012).
 38. Y. Zhu, X. Li, L. Wang, X. Hong, J. Yang, Metabolic reprogramming and crosstalk of cancer-related fibroblasts and immune cells in the tumor microenvironment. *Front Endocrinol (Lausanne)* 13, 988295 (2022).
 39. A. Salminen, K. Kaarniranta, A. Kauppinen, Tissue fibroblasts are versatile immune regulators: An evaluation of their impact on the aging process. *Ageing Res. Rev.* 97, 102296 (2024).
 40. D. Rosenblum, S. Naik, Epithelial-immune crosstalk in health and disease. *Curr. Opin. Genet. Dev.* 74, 101910 (2022).
 41. Y. Ge, M. Zadeh, M. Mohamadzadeh, Vitamin B12 regulates the transcriptional,

- metabolic, and epigenetic programming in human ileal epithelial cells. *Nutrients* 14 (2022).
42. R. Kozyraki, J. Fyfe, M. Kristiansen, C. Gerdes, C. Jacobsen, S. Cui, E. I. Christensen, M. Aminoff, A. de la Chapelle, R. Krahe, P. J. Verroust, S. K. Moestrup, The intrinsic factor-vitamin B12 receptor, cubilin, is a high-affinity apolipoprotein A-I receptor facilitating endocytosis of high-density lipoprotein. *Nat. Med.* 5, 656–661 (1999).
 43. R. Satija, J. A. Farrell, D. Gennert, A. F. Schier, A. Regev, Spatial reconstruction of single-cell gene expression data. *Nat. Biotechnol.* 33, 495–502 (2015).
 44. J. Kueckelhaus, S. Frerich, J. Kada-Benotmane, C. Koupourtidou, J. Ninkovic, M. Dichgans, J. Beck, O. Schnell, D. H. Heiland, Inferring histology-associated gene expression gradients in spatial transcriptomic studies. *Nat. Commun.* 15, 7280 (2024).
 45. D. Pham, X. Tan, B. Balderson, J. Xu, L. F. Grice, S. Yoon, E. F. Willis, M. Tran, P. Y. Lam, A. Raghubar, P. Kalita-de Croft, S. Lakhani, J. Vukovic, M. J. Ruitenber, Q. H. Nguyen, Robust mapping of spatiotemporal trajectories and cell-cell interactions in healthy and diseased tissues. *Nat. Commun.* 14, 7739 (2023).
 46. J. Cao, M. Spielmann, X. Qiu, X. Huang, D. M. Ibrahim, A. J. Hill, F. Zhang, S. Mundlos, L. Christiansen, F. J. Steemers, C. Trapnell, J. Shendure, The single-cell transcriptional landscape of mammalian organogenesis. *Nature* 566, 496–502 (2019).
 47. Q. Liang, Y. Huang, S. He, K. Chen, Pathway centric analysis for single-cell RNA-seq and spatial transcriptomics data with GSDensity. *Nat. Commun.* 14, 8416 (2023).
 48. L. Massimino, O. Palmieri, A. Facoetti, D. Fuggetta, S. Spanò, L. A. Lamparelli, S. D'Alessio, S. Cagliani, F. Furfaro, F. D'Amico, A. Zilli, G. Fiorino, T. L. Parigi, D. Noviello, A. Latiano, F. Bossa, T. Latiano, A. Pirola, L. Mologni, R. G. Piazza, F. Ungaro, Gut virome-colonising Orthohepadnavirus genus is associated with ulcerative colitis pathogenesis and induces intestinal inflammation in vivo. *Gut* 72, 1838–1847 (2023).

Tommaso Lorenzo Parigi, MD

A handwritten signature in black ink that reads "Tommaso Parigi". The signature is written in a cursive style with a horizontal line crossing through the middle of the first name.