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Microorganisms as triggers in acute severe ulcerative colitis : the multi-omics ITAC project.

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Abstract

Acute severe ulcerative colitis is a specific ulcerative colitis (UC) flare characterised by systemic inflammation on top of bloody diarrhoea, leading to a 20% risk of colectomy and 1% mortality risk. Little is known about acute severe ulcerative colitis pathophysiology. Microorganisms have been proposed as triggers for acute severe ulcerative colitis because of the similarity, most notably systemic inflammation, between this phenotype and infectious colitis. Moreover, gut microbiota are key players for protection against pathogens and in UC inflammation.

We hypothesised that a dysfunctional gut microbiome, characterised by a lack of diversity and the loss of anti-inflammatory bacterial species, would allow the proliferation of a pathobiont in the colonic lumen eliciting a systemic inflammatory response in hosts with permissive gut mucosal immunity leading to an acute severe ulcerative colitis flare.

The general objective of my study was to identify the microbiome component(s) and the host factors leading to acute severe ulcerative colitis.

We had three specific aims: (i) to compare the gut microbiota of patients with acute severe ulcerative colitis compared to patients with a non-severe ulcerative colitis flare using 16S rRNA gene sequencing of stool samples and rectal biopsies. Patients with acute severe ulcerative colitis displayed significant alterations in their gut microbiota, characterised by reduced alpha-diversity, an increased presence of Proteobacteria, particularly members of the *Escherichia/Shigella* genus, and a reduction in the abundance of Lachnospiraceae and Ruminococcaceae family members; (ii) to identify the cellular subtypes and pathways involved in gut mucosal inflammation in acute severe ulcerative colitis patients compared to non-severe ulcerative colitis patients by single-cell RNA-Seq of rectal biopsies. In severe cases, plasmablasts exhibited a distinct transcriptomic profile with increased IgG production,

and a specific T cell population expressing *IL26* was expanded compared to non-severe cases. Innate immune cells displayed a pro-inflammatory profile. Both T cells and innate immune cells indicated a pro-Th17 mucosal environment; (iii) to determine the host pathways mediating the systemic inflammatory outburst using whole blood RNA-Seq in acute severe ulcerative colitis patients compared to non-severe ulcerative colitis patients. We found no clear distinction between severe and non-severe cases and did not identify any pathways enriched with differentially expressed genes. This observation suggests that in acute severe ulcerative colitis, the systemic inflammation is less likely to be orchestrated by cytokines originating from circulating cells but rather from inflammatory cells located in the colonic mucosa.

This multi-omics study contributes valuable insights into the pivotal cellular and bacterial components involved in the pathogenesis of acute severe ulcerative colitis. These findings have the potential to guide future clinical research, directing efforts toward microbiome modulation, targeted interventions on plasmablasts, or nuanced inhibition of the Th17/IL-23 axis.

Keywords: ulcerative colitis - acute severe ulcerative colitis - microbiota - immune response.

Résumé

La colite aiguë grave est un phénotype spécifique de poussée de rectocolite hémorragique caractérisé par une inflammation systémique associée à une diarrhée sanglante, associé à un risque de colectomie de 20 % et une mortalité de 1 %. On en sait peu sur la physiopathologie de la colite aiguë grave. Les micro-organismes ont été proposés comme déclencheurs de la colite aiguë grave en raison de la similitude entre ce phénotype et les colites infectieuses, en particulier la présence de l'inflammation systémique. De plus, le microbiote intestinal joue un rôle clé dans la protection contre les agents pathogènes et dans la physiopathologie de la rectocolite hémorragique.

Nous avons émis l'hypothèse qu'un microbiote intestinal dysfonctionnel, caractérisé par un appauvrissement de sa diversité et la perte d'espèces bactériennes anti-inflammatoires, permettrait la prolifération d'un pathobionte dans la lumière colique, déclenchant une réponse inflammatoire systémique chez les hôtes dotés d'une immunité muqueuse intestinale permissive, conduisant ainsi à une colite aiguë grave.

L'objectif général de notre étude était d'identifier le ou les composants du microbiote et les facteurs liés à l'hôte responsables de la colite aiguë grave. Nous avions trois objectifs spécifiques : (i) comparer le microbiote intestinal des patients atteints de colite aiguë grave à celui des patients présentant une poussée non sévère de rectocolite hémorragique en utilisant le séquençage du gène de l'ARNr 16S sur des échantillons de selles et des biopsies rectales. Les patients atteints de colite aiguë grave présentaient des altérations significatives de leur microbiote intestinal, caractérisées par une réduction de l'alpha-diversité, une présence accrue de Proteobacteria, en particulier de membres du genre *Escherichia/Shigella*, et une réduction de l'abondance des membres des familles Lachnospiraceae et Ruminococcaceae ; (ii) identifier les sous-types cellulaires et les voies impliquées dans l'inflammation de la

muqueuse intestinale chez les patients atteints de colite aiguë grave par rapport aux patients atteints de poussées de rectocolite hémorragique non sévère en utilisant un séquençage de l'ARN sur cellules uniques provenant de biopsies rectales. Dans les cas sévères, les plasmocytes présentaient un profil transcriptomique distinct avec une production accrue d'IgG, et la présence d'une population spécifique de lymphocytes T exprimant l'*IL26* était augmentée par rapport aux cas non sévères. Les cellules immunitaires innées présentaient un profil pro-inflammatoire. À la fois, les lymphocytes T et les cellules immunitaires innées indiquaient un environnement muqueux pro-Th17 ; (iii) déterminer les voies de l'hôte qui sous-tendent la poussée inflammatoire systémique en utilisant le séquençage d'ARNm sanguin chez les patients atteints de colite aiguë grave par rapport aux patients atteints de poussées non sévères. Nous n'avons observé aucune distinction claire entre les cas graves et non graves et n'avons identifié aucune voie enrichie en gènes différentiellement exprimés. Cette observation suggère qu'en cas de colite aiguë grave, l'inflammation systémique est moins susceptible d'être orchestrée par des cytokines provenant de cellules circulantes mais plutôt provenant des cellules inflammatoires coliques.

Cette étude apporte des informations sur les composants cellulaires et bactériens essentiels impliqués dans la physiopathologie de la colite aiguë grave. Ces résultats ouvrent des perspectives pour la recherche clinique future. Ils indiquent que les efforts pourraient s'orienter vers la modulation du microbiote, des interventions ciblées sur les plasmocytes ou une inhibition nuancée de l'axe Th17/IL-23 pour la gestion des patients atteints de colite aiguë grave.

Mots-clés: rectocolite hémorragique - colite aiguë grave - microbiote - réponse immunitaire.

List of abbreviations

ASUC: Acute severe ulcerative colitis

ASV: Amplicon sequence variant

CI: Confidence Interval

C.diff: Clostridioides difficile

CMV: Cytomegalovirus

CRP: C-reactive protein

ESR: Erythrocyte Sedimentation Rate

F.prau: Faecalibacterium prausnitzii

GO: Gene Ontology

KEGG: Kyoto Encyclopedia of Genes and Genomes

IBD: Inflammatory bowel disease

IL: Interleukin

IQR: Interquartile range

IV: Intravenous

NSAID: non-steroidal anti-inflammatory drugs

NSUC: Non-severe ulcerative colitis

UC: Ulcerative colitis

UCEIS: Ulcerative colitis endoscopic index of severity

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Thesis preface

This manuscript-based thesis consists of six chapters.

Chapter 1 introduces the theoretical framework relevant to the thesis project, including a literature review for which the thesis author is the first author, accepted for publication in *Lancet Gastroenterology and Hepatology* in 2023. It also describes the overall objectives, hypothesis and research questions, detailed in three specific aims, of the thesis project.

Chapters 2-4 are original research chapters, containing manuscripts for which the thesis author is the first author. Chapter 2 contains a manuscript that was published in *Digestive and Liver Disease* in 2023. Chapter 3 and 4 contain manuscripts currently in preparation corresponding to Aims 1 and 2 of the thesis project. The specific contribution of each author of the manuscripts presented in Chapters 2-4 are detailed in the preface of each chapter.

Chapter 5 contains preliminary results for the third aim.

Chapter 6 contains concluding statements and future directions for the research project.

Appendix A lists a selection of other publications to which the thesis author has contributed during the course of the thesis project. Appendix B contains supplementary material for Chapters 3-5.

1. Chapter 1: Introduction

This thesis project primarily stemmed from a medical question. In the gastroenterology unit of Bordeaux University Hospitals, where I held the position of a senior clinical fellow during the conception of this PhD project, we admit patients with severe ulcerative colitis flares on a weekly basis. As a referral centre, we are responsible for selecting the appropriate medical therapies and, at times, determining the necessity of colectomy to prevent life-threatening outcomes. Conversely, in our outpatient clinics, we regularly manage patients with less severe ulcerative colitis flares, where the focus shifts from survival to improving their quality of life in the absence of systemic inflammation. Therefore, when the time came for me to choose a subject for my PhD project, I, in collaboration with my supervisor and clinical head Pr. David Laharie, decided to investigate why certain patients experience such dramatic inflammatory episodes with life-threatening consequences while others do not.

In this chapter, we will first discuss the definition and the therapeutic management of acute severe ulcerative colitis. Next, we will develop the rationale and hypothesis of this PhD project.

1.1. Acute severe ulcerative colitis: definition, current management and future directions

This review has been accepted for publication in *Lancet Gastroenterology and Hepatology* in 2023. The paper is reproduced here in full length.

Specific author's contributions:

P Rivière conceived the outline of the manuscript, drafted parts of the manuscript, integrated and rewrote when necessary contributions from co-authors, contributed to and finalised tables and figures and critically reviewed the manuscript.

Christopher Li Wai Suen drafted specific parts of the manuscript, contributed to tables and figures and critically reviewed the manuscript.

María Chaparro drafted specific parts of the manuscript, contributed to tables and figures and critically reviewed the manuscript.

Peter De Cruz drafted specific parts of the manuscript, contributed to tables and figures and critically reviewed the manuscript.

Antonino Spinelli drafted specific parts of the manuscript, contributed to tables and figures and critically reviewed the manuscript.

David Laharie conceived the outline of the manuscript and critically reviewed the manuscript.

Acute severe ulcerative colitis management: unanswered questions and latest insights.

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1.1.1. Abstract

Acute severe ulcerative colitis (acute severe ulcerative colitis) is a distinctive ulcerative colitis (ulcerative colitis) flare presentation characterised by the presence of systemic inflammation on top of classical bloody diarrhoea, occurring at least once in 25% of ulcerative colitis patients during their disease course. Each episode carries a risk of complications, need for colectomy and mortality. Little is known about acute severe ulcerative colitis pathogenesis although impaired host-microbiota crosstalk involving pathobionts is suspected. Here, we review unanswered questions and results from the latest research on medical - first-line, second-line and potential third-line therapy - and surgical management of acute severe ulcerative colitis. We detail promising options, such as the use of enteral nutrition as an adjunct therapy to steroids, the ability to predict early failure of first or second-line therapies and the emerging role of JAK inhibitors. The optimal framework to personalise therapy based on multi-omics tools is yet to be developed.

1.1.2. Introduction

Acute severe ulcerative colitis is a distinctive ulcerative colitis flare presentation characterised by the presence of systemic inflammation in addition to bloody diarrhoea. Systemic inflammation was identified as early as in 1954 by Truelove and Witts as a major driver of complications and mortality in active ulcerative colitis[1]. Indeed, during their pioneering randomised controlled trial evaluating the efficacy of cortisone in ulcerative colitis, 14/66 (20%) of patients with a severe flare died in the first three months compared to 2/97 (3%) with a moderate flare and 0/47 (0%) with an absence of systemic inflammation[1]. Since their seminal description, Truelove and Witts criteria have been slightly modified to add C-Reactive protein (CRP) to erythrocyte sedimentation rate (ESR) as a marker of systemic inflammation (Table 1)[2]. It should be noted that this score has never been validated in clinical practice. It is noteworthy that the CRP cut-off that originally replaced ESR was defined arbitrarily. More recent data suggest that a CRP ≥ 12 mg/L correlates with an ESR of > 30 mm/hour[3].

Table 1: Definition of acute severe ulcerative colitis.

According to Truelove & Witts criteria adapted by the European Crohn and Colitis Organization[2]

Digestive symptoms	≥ 6 bloody stools/24h
Associated systemic inflammation (one criteria needed)	Pulse > 90 bpm
	Temperature > 37.8°C
	Haemoglobin < 10.5 g/dl
	Erythrocyte sedimentation rate > 30 mm/h
	C-Reactive protein > 30 mg/l

Modified from *Second European evidence-based consensus on the diagnosis and management of ulcerative colitis. Part 1: Definitions and diagnosis. Spinelli 2022 A et al. Journal of Crohn's and Colitis (2012) 6, 965-990.*

It is estimated that 25% of ulcerative colitis patients experience at least one acute severe ulcerative colitis episode during their disease course[4]. Importantly, more than one quarter of acute severe ulcerative colitis occurs within the ulcerative colitis index presentation[5]. Each episode carries a risk of complications, including bowel perforation, haemorrhage, thrombo-embolic events and electrolyte disturbance, and a 13% risk of colectomy[6]. A meta-analysis of population-based studies from the last 20 years found that acute severe ulcerative colitis was associated with a 1% mortality[7], as confirmed by the recent UK study[6]. Most deaths occurred after colectomy, in patients older than 50 years and among those with a prolonged delay between admission and surgery[8,9]. Given the poor prognosis of acute severe ulcerative colitis, the European Crohn's and Colitis Organization (ECCO) recommends urgent admission in a dedicated unit for intravenous (IV) therapy[2]. Truelove et al. had already described venous thromboembolism as a major mortality driver in acute severe ulcerative colitis[1]. Although no randomised controlled trial has been conducted to evaluate this strategy, thromboprophylaxis is recommended for patients with acute severe ulcerative colitis throughout the hospital stay[2]. Extended prophylaxis after hospital discharge, especially in case of colectomy, is not yet validated but may be considered[10]. Even though acute severe ulcerative colitis diagnosis is based on Truelove and Witts criteria, imaging techniques are useful to exclude complications requiring urgent colectomy such as bowel perforation and toxic megacolon[11]. In centres with dedicated expertise, abdominal ultrasound could be used to guide management[12]. Illustrative pathologic, endoscopic and radiologic images of patients with acute severe ulcerative colitis are presented in Figure 1 and short-term outcomes in Figure 2.

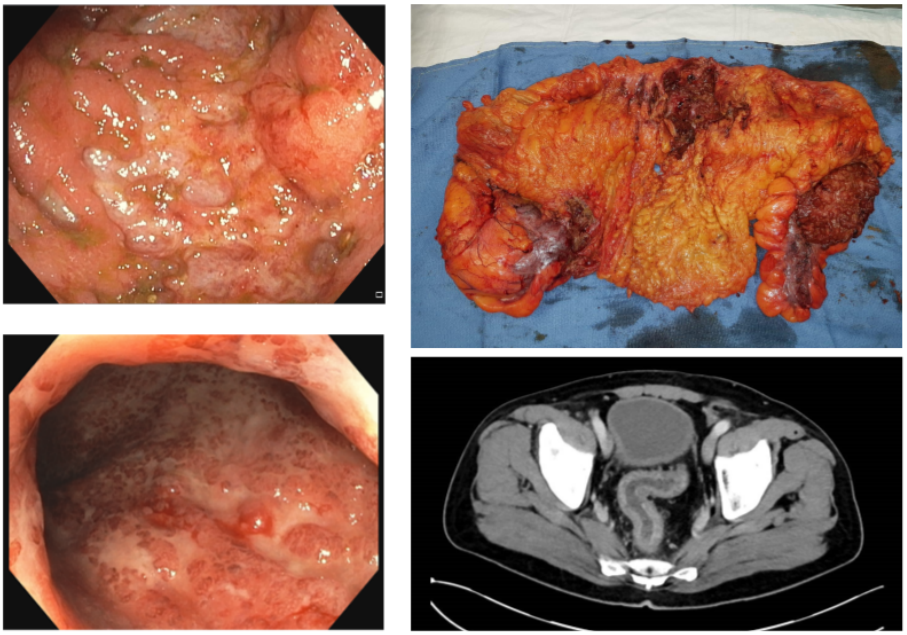

Figure 1: Illustrative features from patients with acute severe ulcerative colitis.
Top left: endoscopic view of deep rectal ulcers. Bottom left: endoscopic view of superficial rectal ulcers. Top right: colectomy specimen with deep ulcers. Bottom right: Computed Tomography-scanner with inflammatory features of the rectum (increased wall thickness, ulcers, fat infiltration). All patients have given their permission to use this data collected during routine care.

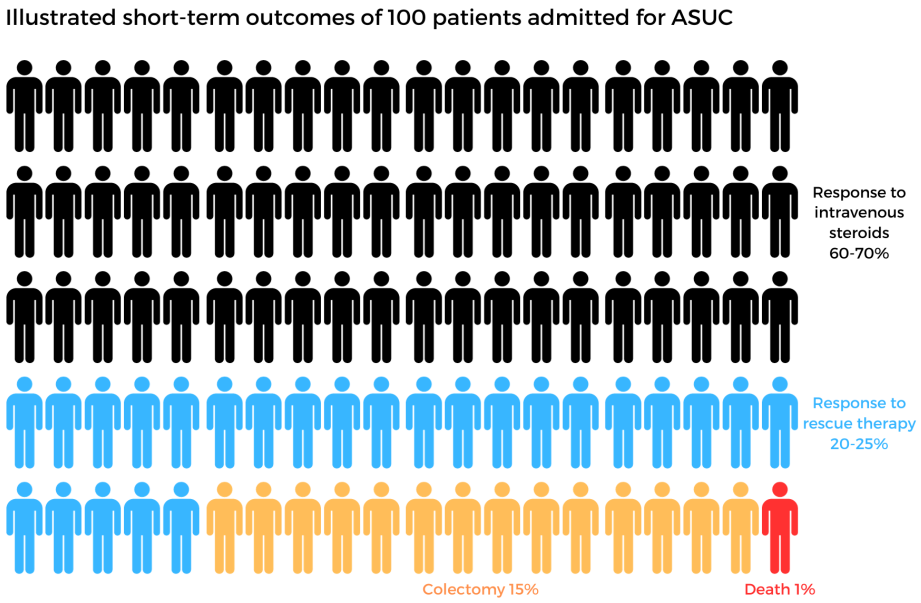

Figure 2: Schematic representation of short-term outcomes of 100 patients admitted with acute severe ulcerative colitis.
Based on[2,4,6,7,35,36]. ASUC: acute severe ulcerative colitis.

Little is known about acute severe ulcerative colitis pathogenesis. Few studies have investigated genetic and microbial determinants of ulcerative colitis severity in relation to risk of colectomy[13,14]. These studies have not distinguished between acute severe ulcerative colitis and medically refractory non-severe ulcerative colitis requiring colectomy. Microorganisms have been proposed as triggers for acute severe ulcerative colitis because of the similarity between this presentation and infectious colitis, especially given the presence of systemic inflammation. Cytomegalovirus (CMV) or *Clostridioides difficile* (CDif) are found in 10-30% of acute severe ulcerative colitis patients[15,16]. However, specific antimicrobial treatment of the CMV or CDif colitis do not modify colectomy rate, suggesting that factors other than these pathogens alone determine the acute colonic inflammation[17,18]. The susceptibility of ulcerative colitis patients to triggering digestive pathogens might be related to the composition of their gut microbiota. A loss of bacterial diversity has been found in the gut of ulcerative colitis patients compared to healthy individuals and correlates with the subsequent risk of flare[19]. The diversity impairment seems to be even more drastic in acute severe ulcerative colitis compared to non-severe ulcerative colitis[20]. Commensal microbes play a crucial role in the host defence against pathogenic agents through direct microbe-microbe competition and the release of intermediary mediators influencing the immune activation threshold of the host[21]. Certain bacterial species, such as CDif, can act as “pathobionts”, i.e. a normal component of the gut microbiome causing disease only when specific genetic and/or environmental conditions are met[22]. Such mechanisms could be involved in acute severe ulcerative colitis pathogenesis and are currently investigated in a multi-omics pilot study (NCT04272307)[23]. For now, no tailored strategy is available for patients with acute severe ulcerative colitis. Management is based on sequential use of medical therapy alongside close monitoring to identify the best timing for colectomy in case of non-response to medical therapy.

Here, we will review unanswered questions and results from the latest research on medical - first-line, second-line and potential third-line therapies - and surgical management of acute severe ulcerative colitis. A summary of medical strategies available is given in Figure 3.

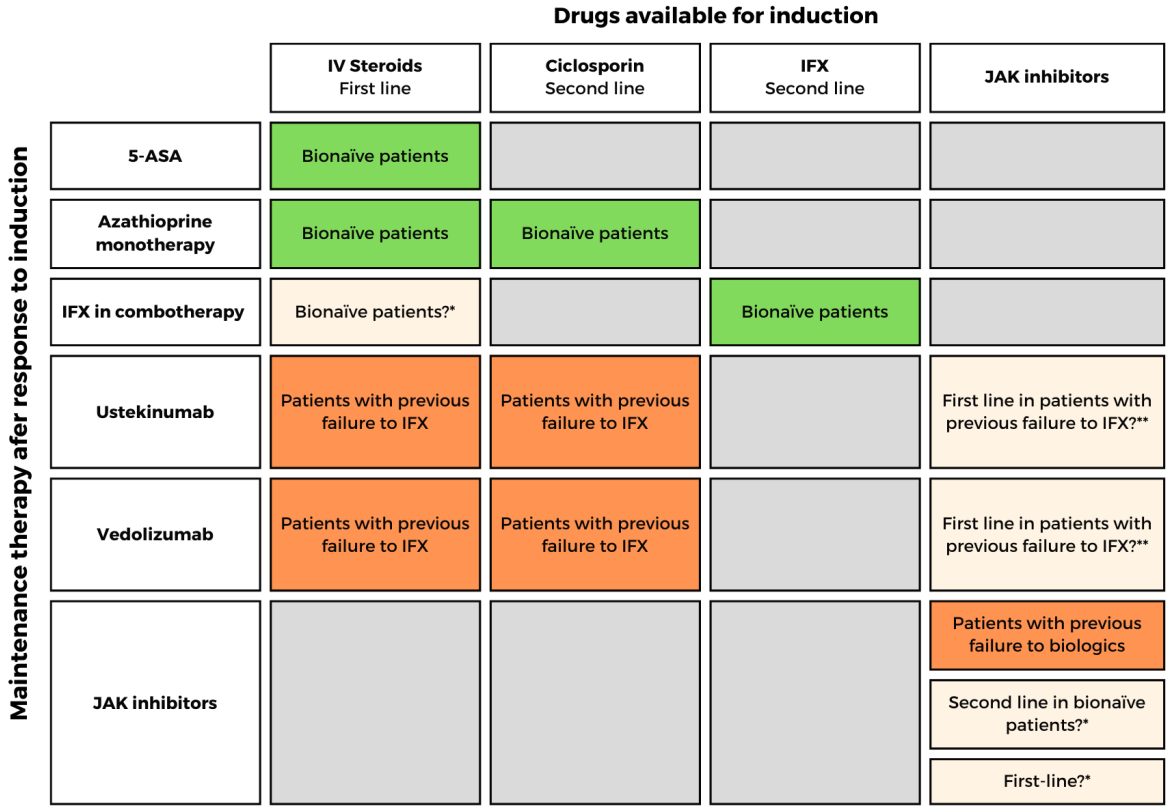

Figure 3: Positioning drugs for the medical management of acute severe ulcerative colitis currently and in the near future.
Top are represented available drugs acting rapidly enough to be used for the treatment of acute severe ulcerative colitis. Left are represented available drugs for maintenance therapy in ulcerative colitis. Cells illustrate the sequential bridging strategies between fast-acting and maintenance drugs depending on patient's profile. Colours represent the level of evidence supporting the data: green corresponds to strategies evaluated in randomised controlled trials, light orange in large retrospective cohorts, dark orange in case series and white in ongoing or forthcoming trials.
Surgery should be considered at any step in case of absence of response to induction therapy before occurrence of complications.
IV: intravenous.*Strategies currently evaluated in clinical trials.**Strategies that should be evaluated in clinical trials in the forthcoming years.

1.1.3. Intravenous steroid therapy as first-line therapy: what's new?

Since the nineteen-fifties, IV steroid therapy has remained the mainstay of management of acute severe ulcerative colitis[1]. It is recommended as first-line therapy for patients with acute severe ulcerative colitis[2] resulting in clinical response within one week in 70% of patients[24]. In the recent UK study, 369/375 (98%) of patients admitted with acute severe ulcerative colitis were treated with IV steroids[6]. Administration of IV steroids in outpatient care to patients with acute severe ulcerative colitis has been used during the COVID-19 pandemic to avoid hospital admission. However, a large proportion of patients subsequently needed hospital admission showing that this strategy may not be safe and effective in acute severe ulcerative colitis patients[6]. Being the first prescribed treatment in acute severe ulcerative colitis, IV steroids remain a vivid area of research. We will here review how to improve prediction of response to IV steroids, which adjunct therapy can be used in combination and which maintenance therapy may be selected in patients responding to IV steroids.

Can we predict response to IV steroids at admission?

Anticipating steroid failure before starting or just after initiation may allow clinicians to use second-line therapies earlier. The Oxford team, the Ho index and the Swedish fulminant colitis index defined criteria of IV steroids failure as early as day 3 of therapy whereas the Seo index was based on parameters at week 2 (Table 2)[25–28]. Admission parameters such as mucosal damage severity, evaluated by the Ulcerative Colitis Endoscopic Index of Severity (UCEIS) or faecal calprotectin, and the level of systemic inflammation by ESR or CRP level have been repeatedly found to be associated with IV steroids failure (Table 2)[29–31]. Recently, the ADMIT-ASC team demonstrated in a retrospective and in a validation cohort that 13/13 (100%) of patients with CRP $\geq$ 100 mg/l, UCEIS $\geq$ 7 and albumin $\leq$ 25 g/l will not respond to IV steroids (Table 2)[5]. Furthermore, more specifically than an overall high UCEIS score, there

is ongoing debate about the prognostic value of deep ulcers in acute severe ulcerative colitis when starting steroids[32–34]. One could hypothesise that in patients with deep ulcers, illustrated by elevated CRP in the blood[23], mucosal damage is such that steroids will not be able to induce remission in contrast to biological therapies with greater healing efficacy[35,36]. These elements may suggest that in patients with high ADMIT-ASC score and/or deep ulcers, first-line therapy could be bypassed. Emerging data suggest that newer therapies, such as tofacitinib, could be used to replace IV steroids as a first-line therapy for patients with acute severe ulcerative colitis. In a recent randomised pilot trial in moderate ulcerative colitis, tofacitinib demonstrated similar efficacy and safety than oral steroids[37]. However, IV steroids having proven to reduce mortality in acute severe ulcerative colitis[1], randomised controlled trials are needed to validate this risk-stratified strategy modifying the treatment paradigm in acute severe ulcerative colitis.

For clinical practice use, the Oxford index is the best-validated predictive score for determining IV steroid failure after three days of treatment[26]. The ADMIT-ASC score that is able to predict steroid failure at admission needs to be confirmed further[5].

Table 2: Scores predicting intravenous steroids failure in acute severe ulcerative colitis.

Scores are listed by chronological order of publication.

Reference	Criteria	Time-point	Score and its predictive value
Seo index[20]	Bloody stools Bowel movements ESR Haemoglobin, albumin	Week 2	60 x bloody stool (0=No, 1=Yes) + 13 x bowel movements/day (0=0-3, 1 = 4, 2 = 5-7, 3=≥ 8) + 0.5 x ESR – 4 x Haemoglobin (g/l) – 15 x albumin (g/l) + 200 Index > 180 = 65% probability of failure
Oxford criteria[21]	Bowel movements CRP	Day 3	> 8 bowel movements or 3-8 bowel movements and CRP > 45mg/l = 85% probability of failure
Fulminant colitis index[22]	Bowel movements CRP	Day 3	Bowel movements/day + (0.14 x CRP) Score ≥ 8.0 = 72% probability of failure
Ho index[23]	Bowel movements Colonic dilatation Albumin	Day 3	Bowel movements/day (0 = < 4, 1 = 4-6, 2 = 7-9, 4 = > 9) + Colonic dilatation (0=No, 4=Yes) + Albumin < 30 g/l (0=No, 1=Yes) Score ≥ 4 = 85% probability of failure
ACE score[26]	CRP, albumin Endoscopic severity	Admission	CRP ≥ 50 mg/l and albumin ≤ 30 g/l and severe endoscopic activity = 78% probability of failure
ADMIT-ASC score[4]	CRP, albumin UCEIS	Admission	CRP ≥ 100 mg/l = 1 point, albumin ≤ 25 g/l, UCEIS ≥ 4 = 1 point, UCEIS ≥ 7 = 2 points Scoring of ≥ 3 = 84% probability of failure

CRP: C-Reactive protein, ESR: Erythrocyte Sedimentation Rate, IV: Intravenous, UCEIS: Ulcerative Colitis Endoscopic Index of Severity.

Can we improve the efficacy of IV steroids?

Diet and artificial nutrition

There has been emerging speculation about the role of dietary intervention in ulcerative colitis. A recently published Indian open-label randomised trial compared IV steroids alongside exclusive enteral nutrition (EEN) to IV steroids alone in 62 patients admitted for acute severe ulcerative colitis[38]. In a per-protocol analysis, 5/27 (19%) of patients failed IV steroids at day 7 in the EEN group compared to 13/30 (43%) in the IV steroids alone group (p=0.04). Notably, 5/32 (16%) of patients did not tolerate EEN and stopped before day 7. Mechanisms of the benefit of EEN remain elusive and may be related to restoration of gut barrier function by increasing nutrients intake to colonocytes and modulation of gut microbiome composition[38]. The low number of patients in this trial did not allow for a robust analysis of their microbiome. Following the growing interest in exclusion diets associated with partial enteral nutrition for Crohn's disease, randomised trials are awaited to evaluate the benefit of exclusive and partial enteral nutrition in acute severe ulcerative colitis.

Antibiotics

As described above, an infectious trigger has been suspected in acute severe ulcerative colitis given the similarity of the presentation with infectious colitis and the known role of the gut microbiota in ulcerative colitis. However, several randomised controlled trials evaluating the benefit of antibiotics associated with IV steroids in acute severe ulcerative colitis were negative[39,40]. Only one open-label randomised study including 28 children with acute severe ulcerative colitis found improved disease activity at day 5 in the group receiving a combination of 4 antibiotics and IV steroids compared to children receiving only IV steroids[41]. Therefore, the American Gastroenterological Association advises against adjunctive antibiotics in acute severe ulcerative colitis[42]. In patients with a gut microbiota characterised by a dramatic loss of diversity, a strategy combining faecal microbiota

transplantation (FMT) and/or enteral nutrition and exclusion diet may be of more value to rebalance host-microbiota crosstalk than antibiotics[43]. It has been previously shown that faecal microbiome composition and mucosal antibacterial profiles at ulcerative colitis diagnosis are associated with mild versus moderate/severe disease course[19]. Restoration of a diverse microbiome could influence positively the mucosa towards repair. To date, no dedicated study beyond case reports has been conducted to evaluate the benefit and the safety of FMT in patients with acute severe ulcerative colitis.

Of note, a recently published international randomised controlled trial found no benefit of oral mesalamine as adjunct therapy for IV steroids in acute severe ulcerative colitis[44]. Another randomised controlled trial using anakinra, an interleukin-1 blocker, in combination with IV steroids in acute severe ulcerative colitis did not demonstrate its efficacy over IV steroids alone[45].

What maintenance treatment should we give to patients responding to IV steroids?

In steroid-responder patients admitted for acute severe ulcerative colitis as index presentation, historical cohorts suggest that the disease course seems to be similar to patients with mild-to-moderate ulcerative colitis beyond the first three months[46]. Consequently, this suggests that maintenance therapy could be selected irrespective of initial disease severity in steroid-responders. The available evidence supporting this approach remains limited so far. A retrospective multicentre French study found that disease relapse after a first acute severe ulcerative colitis episode was lower in patients receiving maintenance therapy with monoclonal antibodies directed against Tumour Necrosis Factor (anti-TNF) as compared to 5-aminosalicylates (5-ASA) or conventional immunosuppressants[47]. Results from the ACTIVE trial comparing azathioprine alone to azathioprine with infliximab in patients with acute severe ulcerative colitis responding to IV steroids are awaited (NCT02425852).

1.1.4. Second-line therapy: which treatment should be selected based on the patient's profile?

In steroid-refractory patients, calcineurin inhibitors and infliximab are employed as rescue therapy[2]. Importantly, none of the other available molecules have been evaluated in clinical trials of acute severe ulcerative colitis and are therefore not recommended as second-line therapy.

Are infliximab and ciclosporin equally effective in acute severe ulcerative colitis?

Infliximab and calcineurin inhibitors, especially ciclosporin, used as second-line medical rescue therapies, are effective in patients with acute severe ulcerative colitis who have failed to respond to IV corticosteroids[2]. Comparison trials suggest that infliximab and ciclosporin are equally effective. CySIF (Ciclosporin With Infliximab in Steroid-refractory Severe Attacks of Ulcerative Colitis), a randomised controlled trial, compared the outcomes of 116 patients enrolled in either the ciclosporin arm (2 mg/kg daily IV ciclosporin for 1 week followed by 4 mg/kg daily oral ciclosporin until day 98) or infliximab arm (5 mg/kg infusions at weeks 0, 2, 6)[35]. No difference was observed between the two arms in immediate outcomes (day 7) or short-term outcomes (day 98). Safety profiles did not differ significantly between the two arms (9/58 (16%) of patients reported adverse events in the ciclosporin arm *vs.* 15/57 (25%) in the infliximab arm).

In a long-term follow-up of the CySIF study (median 5.4 years), there was no significant difference in colectomy-free survival rates at 1 and 5 years, respectively (71% (95% Confidence Interval (CI) 59-83%) and 61% (95%CI 49-74%) in ciclosporin-treated patients, and 69% (CI95% 57-81%) and 65% (CI95% 52-78%) in infliximab-treated patients)[48]. Seventy-seven percent (26/34) of failures in the ciclosporin group occurred within the first

year and infliximab was used as further therapy in these patients; in this context infliximab was well tolerated with approximately half of patients avoiding colectomy at 5 years. Comparable outcomes were observed between infliximab and ciclosporin in the CONSTRulcerative colitisT pragmatic trial (Comparison of Infliximab And Ciclosporin in Steroid-Resistant Ulcerative Colitis Trial)[49]. At 3.5 years of follow-up of 270 patients, no significant difference in quality-adjusted survival, colectomy rates, mortality and adverse events was found between infliximab and ciclosporin. A considerable proportion of patients originally commenced on ciclosporin switched to infliximab upon completion of the study. It remains to be seen whether more intensive infliximab schedules make any difference to outcome when compared with ciclosporin.

Ciclosporin has been the most studied calcineurin inhibitors in acute severe ulcerative colitis. Few data specific to acute severe ulcerative colitis are available for tacrolimus. Data suggest that, similarly to ciclosporin, tacrolimus may have comparable efficacy than infliximab in steroid-refractory acute severe ulcerative colitis patients[50].

Should we use dose-escalated infliximab in acute severe ulcerative colitis?

The optimal infliximab dosing regimen in acute severe ulcerative colitis remains under debate[51]. Standard infliximab induction regimen is 5 mg/kg at week 0, 2 and 6. Doses higher than 5 mg/kg are referred to as “high dose” or “dose-escalated infliximab” whereas infusions administered more frequently than this scheme as an accelerated induction. The last ECCO recommendations update were inconclusive regarding the optimal infliximab dosing regimen in this setting[2]. Despite a lack of evidence, use of dose-escalated infliximab is becoming increasingly common in acute severe ulcerative colitis The rationale behind dose-escalated strategies is to overcome unfavourable infliximab pharmacokinetics which preliminary studies suggest may be responsible for non-response or loss of response to therapy. In particular, a

drug exposure-response relationship may exist in severe colitis as infliximab trough levels at day 14 have been shown to be lower in patients with acute severe ulcerative colitis compared with patients with moderate ulcerative colitis[52]. Furthermore, faecal infliximab loss has been demonstrated to occur in acute severe ulcerative colitis; however, results correlating extent of loss with clinical outcome have been divergent[53,54]. In a retrospective single-centre study, Battat *et al.* found that in 39 patients with acute severe ulcerative colitis, a higher calculated infliximab clearance was associated with increased colectomy rate [55]. However, the formula used to calculate the clearance in this study included only albumin and sex, which may not recapitulate the whole pharmacokinetics of infliximab. Indeed, emerging data suggest that combination of clinical and biological characteristics of individual patients and trough levels measurements in so-called model-informed precision-dosing may perform better to predict infliximab clearance[56].

High-dose induction strategies have not been found to improve short or long-term outcomes in acute severe ulcerative colitis. A meta-analysis of 43 studies concluded that dose intensification was not superior to standard induction; however, the high-dose cohort had higher levels of disease activity thereby confounding the results[51]. A more recent retrospective study found no difference in colectomy rates between standard and high-dose groups at 1, 3 and 24 months despite similar baseline characteristics and disease severity[57].

Accelerated induction involves shortening the dose interval to complete 3-dose induction within 3-4 weeks. While such strategies appear to reduce short-term progression to colectomy, data are conflicting and longer-term colectomy rates appear similar to those following standard 6-week induction[51,57,58]. These data suggest that intensified infliximab regimens in acute severe ulcerative colitis may be postponing colectomies rather than avoiding them altogether. Given the increased risk of post-operative complications with delayed surgery,

dose-escalated infliximab or accelerated induction should be used cautiously, especially in elderly or frail patients[9].

More recent retrospective studies have suggested that colectomy rates following dose-escalated infliximab in patients with more severe disease (higher CRP-to-albumin ratios, persistently elevated CRP, higher Mayo Endoscopic subscore) are comparable to standard induction in lower risk patients[58]. A recent prospective cohort including 38 children with acute severe ulcerative colitis initiating infliximab using a pharmacokinetics dashboard found that increased clearance was associated with colectomy and lack of steroids free-remission at six months[59]. However, it is unknown whether this can be overcome by novel dosing strategies which is currently being evaluated by two prospective randomised controlled trials (NCT02770040 and NCT03937609). In particular, the PREDICT-ulcerative colitis study (NCT02770040) is an ongoing multi-centre randomised controlled trial evaluating whether intensified infliximab 10 mg/kg induction is superior to accelerated 5mg/kg induction (week 0, 1 and 3) in patients with acute severe ulcerative colitis, with a standard induction group as control group.

Can we predict the response to second-line therapy?

Several studies have aimed to identify predictors or factors associated with non-response to infliximab or ciclosporin rescue therapy in acute severe ulcerative colitis in an attempt to help guide management[4,35,58,60–64]. Factors associated with response can be broadly divided into clinical, biochemical/laboratory and endoscopic factors and are displayed in Table 3.

Which factors guide the selection of rescue therapy?

Whilst there is insufficient evidence to indicate which patients are better suited for ciclosporin versus infliximab, some criteria may help physician guidance. Ciclosporin seems better

indicated for patients without associated comorbidities including renal impairment. In thiopurine-naive patients, it is used as a bridging agent for thiopurine maintenance. We will describe below new bridging strategies with ciclosporin (Figure 3). Although ciclosporin is cheaper, clinicians more often prefer infliximab to its supposed easier use. Indeed, ciclosporin that is started by a continuous IV infusion for the first five to seven days, requires trough levels monitoring and a prophylaxis against *Pneumocystis jiroveci*. In patients with extra-intestinal manifestations associated, such as ankylosing spondylitis or psoriasis. infliximab is a better choice given its efficacy to control extraintestinal inflammation in patients with inflammatory bowel disease[65].In contrast, ciclosporin is not being currently recommended for ankylosing spondylitis or psoriasis[66,67] Safety profile of ciclosporin is deemed poorer than infliximab since the retrospective description of the Leuven cohort of patients with acute severe ulcerative colitis treated with ciclosporin in 2004 including 3 deaths from opportunistic infections out of 86 treated patients[68]. However, subsequent publication of cohorts of patients with acute severe ulcerative colitis treated with infliximab found comparable mortality rates. In 2007, Kohn *et al.* reported one death from opportunistic infections among 83 patients[69] and Lees *et al.* one death out of 39 patients[70]. Notably, in the CySIF and the CONSTRulcerative colitisT randomised trials, there was no difference in terms of severe adverse events between the infliximab and the ciclosporin groups[35,49]. With almost twenty years of distance, these studies confirm that, whatever the drug used, mortality in acute severe ulcerative colitis is mostly related to infectious events especially in the postoperative setting. We will discuss below the implications of these findings for patient management (see Third-line and Surgery sections).

Table 3: Factors predicting failure to rescue therapy in acute severe ulcerative colitis.

		Time-point	Drug	Type of studies
Clinical	Age ≥ 50 years[30]	-	Ciclosporin Infliximab	Randomised controlled trial
	Fever, tachycardia[50] Higher number of Truelove & Witts criteria[3]	At day of rescue therapy initiation	Ciclosporin Infliximab	Retrospective
Biological	CRP > 30 mg/l[51,52] Albumin < 30 g/l[52] or < 25 g/l[53]	Admission	Infliximab Ciclosporin	Retrospective
	Increased CRP/albumin ratio[49]	At day of rescue therapy initiation	Infliximab	Retrospective
	Increased CRP/albumin ratio[54]	Day 3 after infliximab infusion	Infliximab	Retrospective
Endoscopic	Severe endoscopic lesions[50]	At admission	Ciclosporin	Retrospective

CRP: C-Reactive protein

1.1.5. How to use new molecules in patients with previous exposure to anti-TNF?

Patients admitted with acute severe ulcerative colitis who have previously failed infliximab or other biologics are becoming more prevalent, necessitating alternative non-anti-TNF biologics and small molecules rescue therapies. New strategies have emerged within the last few years but their effectiveness in acute severe ulcerative colitis is yet to be confirmed (Table 3).

How to use vedolizumab and ustekinumab?

Vedolizumab, a selective humanised immunoglobulin G1 monoclonal antibody to the integrin $\alpha_4\beta_7$, was approved for its use in patients with moderate to severe ulcerative colitis. It has been suggested that the onset of action of vedolizumab may be slow, with increasing response and remission rates demonstrated over the first 10 weeks of treatment[71]. The speed of action of vedolizumab is insufficient for the treatment of acute severe ulcerative colitis. However, it can be considered as maintenance therapy in combination with a fast-acting agent such as IV steroids or calcineurin inhibitor as induction therapy.

Seven studies including 145 patients have been published so far on the effectiveness and safety of vedolizumab in acute severe ulcerative colitis[50]. Most of the studies included a limited number of patients, the majority of which were retrospective and patients included were biologic-failures. In addition, some of the studies included a mixed group of ulcerative colitis patients in terms of disease severity rather than being a pure acute severe ulcerative colitis cohort. In almost all of them, vedolizumab had not been used as monotherapy but in combination with a calcineurin inhibitor as a bridge for the induction of remission. In a systematic review, Gisbert *et al*. found a colectomy-free rate in patients treated with vedolizumab for acute severe ulcerative colitis of 69% (weighted mean; 95%CI=61-76%) and the rate of serious adverse events (or adverse events leading to discontinuation) was only

1.8%, while there was no death attributable to vedolizumab and ciclosporin[50]. These data suggest that a sequential strategy of calcineurin inhibitors followed by vedolizumab may be an effective and low-risk strategy in patients with acute severe ulcerative colitis.

Limited information is available evaluating the effectiveness of ustekinumab, an antagonist of the p40 subunit of interleukin-12 and interleukin-23, in acute severe ulcerative colitis, with only three studies including a total of 13 patients (the largest one included 10 patients)[50]. All studies were retrospective, and all patients had previously failed anti-TNF and vedolizumab. Of note, in all cases sequential treatment was used starting with calcineurin inhibitor as induction (ciclosporin/tacrolimus) followed by ustekinumab maintenance. With this strategy, colectomy was avoided in all patients. In addition, the rate of serious adverse events and mortality rate attributable to the drug were both 0%. Hence, ustekinumab in combination with a calcineurin inhibitor bridge may be effective and safe in acute severe ulcerative colitis. However, the data supporting its use are limited, making it difficult to draw conclusions.

How to use Janus-kinase (JAK) inhibitors?

Tofacitinib is an oral small molecule, non-selective blocker of JAK-STAT pathways, that regulates signalling of multiple immune mediators, which has been approved for the treatment of moderate-to-severe ulcerative colitis[72]. Despite being an oral drug, tofacitinib is promptly absorbed and has a rapid mechanism of action, showing a benefit over placebo after three days of treatment as shown by the post-hoc analysis of the phase 3 trials[73]. In addition, the rapid plasma clearance has the theoretical benefit of minimising risks in case emergency colectomy is required or if another rescue therapy is initiated. Tofacitinib therefore represents an attractive therapeutic option in acute severe ulcerative colitis.

A total of 15 studies including 143 patients treated with tofacitinib for acute severe ulcerative colitis have been published so far - most of them retrospective and with a limited number of patients in each (<10)[50]. The largest study performed so far was published by Uzzan *et al*[74].

Authors included 55 patients: 49 previously exposed to infliximab and 19 to ciclosporin. Rates of colectomy-free survival at three and six months were 78.9% (CI95% 69-91%) and 73.6% (CI95% 62-87%) respectively, and rate of steroid-free clinical remission at week 14 was 32.7%. In the above mentioned review published by Gisbert et al., including 14 studies with 134 patients, the proportion of patients able to avoid colectomy with tofacitinib treatment for acute severe ulcerative colitis was 77% (weighted mean; 95%CI=70-85%)[50].

The optimal tofacitinib dosing regimen for acute severe ulcerative colitis is unknown. Whilst it is suggested that a higher dose of drug may be needed, further investigation is required to establish whether such an approach is warranted. Most of the series published thus far employed standard tofacitinib dosing (10 mg twice a day) for the induction, whereas a few studies used higher doses (10 mg three times a day) based on the short half-life (approximately 3.2 hours) of tofacitinib and the reported efficacy of 30 mg daily in a phase 2 trial[72]. In the study performed by Berinstein *et al*. tofacitinib was protective against colectomy at 90 days only in patients treated with tofacitinib three times a day; while the standard dose of 10 mg twice a day did not demonstrate this benefit compared to matched controls[75]. However, the number of patients were small in each group, reducing the statistical power to detect differences. Gisbert *et al*. analysed the studies prescribing the standard dose (10 mg bid) and the colectomy-free rate was 73%, similar to that of the entire cohort[50]. Therefore, the benefit of higher doses of tofacitinib in acute severe ulcerative colitis remains to be demonstrated.

Regarding safety, the overall rate of serious adverse events was about 3%[50], with no death attributable to tofacitinib treatment. The effectiveness and mainly the safety of tofacitinib in combination with other biologics or calcineurin inhibitors is unknown. Nevertheless, given its rapid onset of action, tofacitinib monotherapy may be reasonable for this indication.

In summary, tofacitinib seems to be an effective and safe therapeutic option at least in biologic-experienced hospitalised patients with acute severe ulcerative colitis. An ongoing clinical trial is currently evaluating whether tofacitinib could be an option (compared to ciclosporin) as second-line therapy in steroid-refractory patients with acute severe ulcerative colitis (NCT05112263). The position of tofacitinib in the acute severe ulcerative colitis therapeutic strategy remains to be elucidated. An Indian trial has evaluated the benefit of tofacitinib as adjunct therapy to steroids in patients with acute severe ulcerative colitis. Only interim results have been presented yet as an abstract and did not show any difference between the two groups[76]. A case of a patient with acute severe ulcerative colitis treated successfully by a combination of tofacitinib and ciclosporin has also been recently published[77]. However, there is a real safety concern of these non-conventional strategies, regarding the mortality risk in acute severe ulcerative colitis associated with delayed surgery and multiple lines of immunosuppressants[8,68,78]. Therefore, newer treatment strategies should be used only in expert centres after discussion in multidisciplinary meetings with gastroenterologists and colorectal surgeons.

The role of newer more selective JAK inhibitors in this setting is yet to be demonstrated. To date, there is no data in acute severe ulcerative colitis with filgotinib. A recent case-series was published gathering data from six patients with acute severe ulcerative colitis treated with upadacitinib and followed for 16 weeks. All patients responded to upadacitinib. Only one patient required a colectomy at week 15[79].

1.1.6. Third-line therapy: in which cases is it reasonable?

Although surgery is a life-saving procedure, it may decrease the patient's quality of life in the long-term and is often associated with psychological morbidity. For these reasons, among several highly selected patients with steroid-refractory acute severe ulcerative colitis in whom

one rescue therapy (mainly infliximab or calcineurin inhibitors) have failed, a salvage therapy could be considered to avoid colectomy. In fact, deferral of urgent surgery to the elective setting could also be a successful outcome of such sequential treatment. However, a sequential strategy could pose risks because of higher levels of immunosuppression with another drug, and delays in surgery, which has been associated with increased postoperative morbidity and mortality especially in elderly patients[8,9].

Several studies with a limited number of patients have evaluated the effectiveness of infliximab after ciclosporin failure or vice versa. In the above-mentioned review, authors identified 23 studies including 340 patients. Sequential therapy avoided colectomy in 53% (CI95% 47-58%) of patients with acute severe ulcerative colitis[50]. In the same review, the proportion of adverse events was 26% and mortality rate was 0.88% (three deaths). The order of drug administration did not seem to influence either the efficacy or safety of the strategy.

Salvage therapy with infliximab after failure of ciclosporin (or vice versa) may be associated with an acceptable risk of complications in selected younger patients without comorbidities treated in specialised centres[2]. Following non-response to second-line medical therapy, assessed within one week, the decision to either proceed to alternative rescue therapy or surgery should be made promptly and jointly with the colorectal surgeon. Importantly, the response to the third-line drug must be closely monitored and patients without an adequate response within the first few days should proceed to emergency colectomy.

1.1.7. Surgery: what to discuss with the colorectal surgeon?

Despite all risk mitigation strategies, colectomy in acute severe ulcerative colitis patients is still associated with an increased risk of complications. Timing of surgery is crucial in this setting: prolongation of medical treatments, especially steroids, delays surgery, and considerably increases the risk of perioperative morbidity and mortality[9,80]. Mortality in acute

severe ulcerative colitis seems to be more related to opportunistic infections or sepsis after surgery than directly to bowel perforation[68–70,81]. Postoperative morbidity, especially infections, are strongly associated with the length of medical therapy before surgery[78]. A joint assessment of acute severe ulcerative colitis patients starting from admission and throughout the course of inpatient stay by an expert team of gastroenterologists and colorectal surgeons, helps establish the best time to stop medical rescue therapies and proceed to surgery.

Even though improvements in therapeutic algorithms have led to a decline in colectomy rates over time[82], the risk of colectomy for acute severe ulcerative colitis patients remains high. According to the most recent reports, nearly 16-40% of acute severe ulcerative colitis patients require surgical intervention[4,6]. The development of highly standardised therapeutic algorithms - combined with predictive scores[5,26–28,31,58] – has enabled a decrease in emergent procedures, which have been associated with higher mortality, and has also improved surgical planning and postoperative outcomes[9,80].

In cases of semi-elective surgery, acute severe ulcerative colitis patients have the option to receive the gold standard surgical treatment that is offered to medically refractory chronically active moderate-to-severe ulcerative colitis, which involves a staged procedure including an initial subtotal colectomy with ileostomy, followed by ileal pouch-anal anastomosis (IPAA) construction with or without protective ileostomy (three-stage and modified two-stage restorative proctocolectomy)[2]. In the two-stage procedure, a proctocolectomy with ileostomy is the first step. While a two-stage procedure has been found to have comparable outcomes than three-stage in medically refractory ulcerative colitis, such an approach involving dissection of the rectum is considered too risky in acute severe ulcerative colitis patients who are often too weak to recover from such extensive surgery[2]. Instead of a traditional three-stage strategy, the surgical team may opt for a modified two-stage procedure, which avoids the

stoma in the IPAA construction step, once the patient has completely recovered following colectomy[2].

In the emergent setting of acute severe ulcerative colitis, even in the presence of complications such as toxic megacolon, minimally invasive surgery is the preferred approach in most referral centres whenever feasible, providing shorter postoperative recovery and length of hospitalisation compared with open surgery[2]. Recently, the single access laparoscopic approach has been introduced as a valid alternative to standard multiport surgery, with preliminary advantages also observed in acute severe ulcerative colitis patients[83]. An emerging evolution of the classical laparoscopic approach is robotic colectomy, with preliminary evidence suggesting its feasibility and safety in acute severe ulcerative colitis patients, in non-emergent settings[84].

One of the most critical issues after colectomy for acute severe ulcerative colitis is the management of the rectal stump, which may lead to severe postoperative morbidity in cases of blow-out[2]. Surgical management includes the creation of an intraperitoneal, subcutaneous, or mucosal fistula. Although low quality evidence suggests the superiority of subcutaneous fistula placement, this approach is usually avoided in favour of intraperitoneal placement, due to suboptimal compliance, risk of abdominal wall infection and hernia[2].

A systematic review estimated that at least one in three patients experience early or late complications from colectomy for ulcerative colitis. In the long-term, the mean incidence rate of pouchitis was 29%, faecal incontinence was 21% and small bowel obstruction was 17%. The mean rate of severe problems leading to permanent ileostomy was 5%[85]. Newer surgical techniques have improved outcomes after colectomy: rates of early infections and late pouch failure decreased after 2010[85] and laparoscopic colectomy and IPAA has been shown to be able to preserve female fertility[86]. Enhanced recovery after surgery protocols, including among others early feeding and mobilisation, anaemia optimisation and nutritional support,

have been widely adopted in colorectal surgery but evidence is lacking in the specific context of inflammatory bowel disease[87]. Anaemia and malnutrition are key elements of acute severe ulcerative colitis but prehabilitation may be difficult to manage in this emergent setting. Future studies should focus on the benefit of perioperative care in acute severe ulcerative colitis especially nutritional support beyond its therapeutic use as described above and anaemia optimisation, i.e. iron supplementation or whole blood transfusion.

Emerging data show that one in four inflammatory bowel disease patients experience post-traumatic stress related to their disease, mainly after negative hospitalisation experiences. The need for surgery during hospitalisation may be associated to post-traumatic stress severity[88]. Poor communication and information exchange are listed by patients as major drivers of trauma[89]. Patients admitted with acute severe ulcerative colitis are at high risk of post-traumatic stress given the severity of symptoms, the threat of colectomy and the mortality risk perceived by patients.

1.1.8. What to do in specific situations?

Elderly patients

Older age has been associated with a poorer response to medical therapy[35] and a higher mortality after colectomy in acute severe ulcerative colitis, especially in cases of delayed surgery[8,9]. In patients older than 50 years, the therapeutic strategy should be discussed early in a multidisciplinary forum to define the best timing for surgery in the event of non-response to medical therapy. Third-line medical therapy, and possibly even second-line therapy in frail patients, should not be attempted without a rigorous benefit-risk balance assessment.

Pregnant women

Few data exist on acute severe ulcerative colitis during pregnancy. Usual drugs for acute severe ulcerative colitis are not contraindicated in pregnancy, including IV steroids and

infliximab, except for JAK inhibitors[90]. Due to the potential adverse events, ciclosporin is not the preferred option during pregnancy[90]. A recent multicentre case series reported good response to medical therapy in 19/20 patients. However, a high rate of adverse foetal outcomes was observed: 2 spontaneous abortions, 6 premature births and 4 low birth weight infants. Only one patient required colectomy during pregnancy without consequences for the foetus[91]. Colectomy remains a risky procedure during pregnancy as reported recently in an ECCO case series. In this series of 44 patients operated for inflammatory bowel disease during pregnancy, 10 underwent colectomy for acute severe ulcerative colitis, of whom 3 experienced foetal loss (week 18, week 16 and week 10 of gestation)[92]. Pregnant women with acute severe ulcerative colitis should be managed in expert centres involving surgeons in a multidisciplinary discussion early on to avoid emergent colectomy which is associated with poorer outcomes[9,80]. Given the increased thrombo-embolic risk in pregnancy, prophylaxis should be even more rigorous in this setting[90].

1.1.9. Conclusion

In the coming years, therapeutic strategies in acute severe ulcerative colitis may evolve to include nutritional support and innovative bridging strategies. Given the growing evidence on the role of diet in ulcerative colitis, development of controlled trials evaluating exclusion diets including exclusive or partial enteral nutrition, in conjunction with medical therapy should be a priority for the scientific community. In parallel, one could envision that fast-acting small molecules such as JAK inhibitors will change the historical paradigm of steroids as first-line therapy in acute severe ulcerative colitis. Trials comparing JAK inhibitors to IV steroids or as add-on therapy are awaited in the coming years. Better positioning of maintenance therapy with safer tolerance profiles albeit with slower mechanisms of action, such as vedolizumab or ustekinumab, represent attractive options in acute severe ulcerative colitis patients responding

to induction therapy. Despite increasing therapies becoming available, surgery will always be required for some refractory patients especially if elderly or frail. However, colectomy does not mean the end of the inflammatory disease[85]. Ongoing research should focus on prevention and management of complications of pouch surgery such as pouchitis.

Finally, in the medium-term, with the advent of multi-omics tools, one may be able to predict drug response on admission based on individualised immunological and/or microbiota profiles of patients. A retrospective pilot study on micro-RNA in colonic mucosa has shown good performance in predicting response to IV steroids, ciclosporin or infliximab in patients with acute severe ulcerative colitis using a deep learning algorithm[93]. In an ideal world, inflammatory bowel disease centres would grow colonic-derived organoids for each patient as a personalised testing hub for tailored therapeutic strategies.

In an era of increasing numbers of biological therapies and small molecules, acute severe ulcerative colitis remains an event of major importance for patients with ulcerative colitis that represents one of the last clinical situations in inflammatory bowel disease associated with a real risk of mortality[6,7]. The future remains bright as clinicians, surgeons and basic scientists work together to nullify this risk.

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1.2. Understanding the pathogenesis of ulcerative colitis: the microorganism hypothesis

1.2.1. Background

Little is known so far about pathogenesis of acute severe ulcerative colitis. No genetic variants related to colectomy risk or disease extent in ulcerative colitis have been found in genome-wide association study[1]. The theory that microorganisms may serve as triggers has been proposed to explain this inflammatory outburst, drawing from the clinical similarities observed between infectious colitis and acute severe ulcerative colitis, such as the presence of fever and elevated C-Reactive protein (CRP) levels. Digestive infectious agents are found more frequently in inflammatory bowel disease (IBD) patients experiencing a disease flare than in patients with inactive disease or healthy controls[2–4]. Cytomegalovirus (CMV), a virus from the *Herpesviridæ* family, and *Clostridioides difficile* (*C.diff*) infections are considered to be the most suspicious enteropathogens in this setting. Depending on the diagnostic tools, CMV infections have been found retrospectively in 6% to 30% of acute severe ulcerative colitis patients[5,6]. Conflicting data exist about whether CMV could be an innocent bystander in inflamed tissue or could worsen the disease. A high load of CMV copies in the colonic mucosa is associated with failure of medical therapy[7]. However, treatment of the CMV colitis does not modify the colectomy rate[8]. *C.diff* is an anaerobic Gram-positive spore-forming bacterium, pathogenic for the colonic mucosa when toxins are produced[9]. Toxigenic *C.diff* is present in 6% to 20% of IBD patients[2,10] and is related to higher surgery and mortality rates even with appropriate antibiotic therapy[11–14]. The hypothesis proposing microorganisms as triggers in acute severe ulcerative colitis is supported by the established role of the microbiota in this condition. Encouraging results have emerged from randomised controlled trials involving faecal microbiota transplantation, showing promise in inducing both clinical and

endoscopic remission among patients with ulcerative colitis[15]. Alterations in the composition and functions of the gut microbiota have been found in ulcerative colitis patients during flares[16]. Compared to healthy controls, the gut microbiota in ulcerative colitis patients is characterised by a decrease in bacterial diversity and richness, an expansion of specific bacteria that activate inflammatory response in the gut mucosa, and a decreased abundance of short-chain fatty acids-producing bacteria, which are important for maintaining the health of the intestinal epithelium[16–18]. In a prospective cohort of newly diagnosed ulcerative colitis patients, baseline microbiota composition was associated with the number of flares during follow-up[19]. Diet and antibiotics influence the microbiota composition and activity in IBD[20]. Commensal microbes play a crucial role in the host defence against pathogenic agents, notably through the release of intermediary mediators influencing the immune activation threshold[19,21]. For example, presence of *Fæcalibacterium*, an anti-inflammatory commensal bacterium well studied in Crohn's disease[22], negatively influences the production of IL-17 by the colonic mucosa in response to *Staphylococcus aureus* infection[21]. The production of small intermediary molecules by the microbiota is associated with microbiota alterations observed in IBD patients. During flares, the bacterial metabolic activity, observed in blood and stool, is reduced[23,24] and is correlated with inflammation levels of the colonic mucosa[25].

The relationship between the host and the microbiota is based upon reciprocal control. From the host's perspective, an impairment of the host defence mechanisms against pathogens has been repeatedly highlighted in IBD. In genome-wide association studies, from more than 160 genetic variants found to be associated with IBD, many are involved in sensing and elimination of microbe through epithelial barrier function, autophagy, interaction with the gut microbiome, pathways of response to molecules of bacterial origin and involvement of the tumour necrosis factor superfamily[1,26–28]. Analysis of the expression profile of gut mucosa in ulcerative colitis confirms these data and shows increased expression of genes involved in

control of bacterial proliferation and epithelial barrier function[29,30]. Several pathways of gut defence are altered in ulcerative colitis. The mucus production is impaired, allowing bacteria to penetrate close to the epithelium[31,32]. Neutrophil infiltration in the mucosa is seen early in ulcerative colitis and is associated with a higher epithelial permeability[33]. Dendritic cells in the gut mucosa, the antigen presenting cells, display an increased expression of Toll-like receptors, signifying a more activated state, in ulcerative colitis patients than in healthy subjects[34,35]. Regarding the adaptive immune system, plasma cells are more abundant in the colonic mucosa of ulcerative colitis patients and express a different pattern of defensins, potent antimicrobial peptides[36]. An insufficient suppressor function by the regulator T lymphocytes has also been found, possibly participating in the loss of mucosal tolerance[37].

Single-cell RNA sequencing (scRNASeq) has recently contributed to the better understanding of the cellular and molecular changes present in the intestinal mucosa of patients with ulcerative colitis. This technique has advanced the study of complex diseases by offering a high-resolution perspective on the cellular and molecular alterations within afflicted tissues, without being constrained by rigid pre-existing hierarchies or reliant on predetermined markers[38]. Notably, scRNASeq allows identification of disease-specific cell types and characterization of disease-associated gene expression patterns at the single-cell level[39]. A seminal paper, published by Smillie *et al.* in 2019, provided the first atlas of the cellular landscape of colonic mucosa in ulcerative colitis. The study identified networks of inflammatory cells, including CD8+IL-17+ T cells and inflammatory monocytes, characteristic of patients with ulcerative colitis. These inflammatory cells expressed genes with established risk alleles for ulcerative colitis[40]. The role of CD8+ T cells was further supported by a subsequent paper by Corridoni et *al.*. They observed expanded populations of effector and post-effector terminally differentiated CD8+ T cells in the colonic mucosa of patients with ulcerative colitis. The effector T cells could incite tissue damage and produce

tumor necrosis factor (TNF), whereas the post-effector cells could acquire innate signatures and transition to regulatory functions, potentially mitigating excessive inflammation and being deleterious in the context of chronic inflammation[41]. However, these papers included samples from patients with ulcerative colitis at diverse severity grades. They did not provide specific insights into the mechanisms of acute severe ulcerative colitis.

1.2.2. Hypothesis

We hypothesised that a dysfunctional gut microbiome, characterised by a lack of diversity and the loss of anti-inflammatory bacterial species, would allow the proliferation of a pathobiont in the colonic lumen. The rise of this pathobiont would trigger a systemic inflammatory response in hosts with permissive gut mucosal immunity leading to an acute severe ulcerative colitis flare.

1.2.3. General objective and specific aims

The general objective of this project was to identify the microbiota and host factors leading to an acute severe ulcerative colitis flare. To attain this objective, we had three specific aims:

1) Aim 1: To confirm a reduced microbiome diversity and identify candidate pathobionts among the gut microbiota of acute severe ulcerative colitis patients using 16S rRNA sequencing in stool and rectal biopsy samples.

2) Aim 2: To identify the cellular subtypes and pathways involved in the gut mucosal inflammation in acute severe ulcerative colitis patients using scRNAseq in rectal biopsy samples.

3) Aim 3: To determine the host pathways mediating the systemic inflammatory outburst in the blood of acute severe ulcerative colitis patients using whole blood RNA sequencing.

To investigate potential triggers in acute severe ulcerative colitis, an ideal approach would have been to prospectively track a cohort of newly diagnosed ulcerative colitis patients, collecting biological samples at diagnosis, and subsequently analysing these samples if an acute severe ulcerative colitis episode were to occur, in order to identify potential triggers. However, the inherent unpredictability of acute severe ulcerative colitis poses a significant logistical challenge to this approach. To have a reasonable chance of capturing the triggering events, patients would need to be sampled at very frequent intervals, which is impractical and cost-ineffective. Moreover, as mentioned above, more than 25% of acute severe ulcerative colitis episodes correspond to index presentation. Therefore, we opted to conduct a cohort study involving two groups, each comprising 20 participants: one group with acute severe ulcerative colitis and the other with non-severe ulcerative colitis flares. This study, funded by Bordeaux University Hospitals and the French IBD patients‘ association (AFA Crohn-RCH), was named “ITAC” or “mIcroorganisms as Triggers in Acute severe ulcerative Colitis”. The size of the study population was decided based on feasibility and available funding. The uniqueness of this thesis work lies in its comprehensive approach, which combines extensive clinical phenotyping with multi-omics techniques, including microbiome analysis, scRNASeq and whole blood RNASeq.

1.2.4. References

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2. Chapter 2: Investigating the association between systemic and local inflammation in ulcerative colitis

2.1. Preface

Before diving into multi-omics analysis, our first research question was to determine if systemic inflammation, which is the cornerstone of the seminal definition of ASUC, was the sign of a more severe local disease, *i.e.* more advanced colonic mucosal damage. To that end, we analyse the clinical data from the ITAC study and data from a retrospective cohort of patients undergoing subtotal colectomy for acute severe ulcerative colitis.

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Specific author's contributions:

- P. Rivière: conceived and wrote the ITAC study protocol, obtained the funding and ethics approval, led the clinical research study team, collected the data on the electronic database, performed the statistical analysis, interpreted the data and wrote the manuscript.

- A. Le Chevillier: participated in the conception of the retrospective study, collected the data and reviewed the imaging from the retrospective cohort.

- A. Rullier and M. Marty: participated in the conception of the retrospective study and reviewed the histology slides of the retrospective cohort.

- E. Schurr: participated in discussion on the research question about systemic and local inflammation.

- B. Lapuyade: participated in the conception of the retrospective study and reviewed the imaging of the retrospective cohort.

- B. Célérier and B. Fernandez: participated in the conception of the retrospective study and obtained the colectomy specimen.

- T. Bessissow, X. Treton, M. Uzzan, F. Poullenot, A. Berger and F. Zerbib: were active investigators in the ITAC study.

- D. Laharie: conceived the retrospective study and participated in the conception of the ITAC study, interpreted the data, participated in the discussion on the research question about systemic and local inflammation.

All authors critically revised the manuscript and approved the final version.

Deep ulcers are associated with increased C-Reactive protein in active ulcerative colitis.

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2.2. Abstract

Background: Increased C-Reactive protein (CRP) is used to diagnose and predict response to treatment in acute severe ulcerative colitis (UC). As deep ulcers are considered to be a negative prognosis marker in UC, our aim was to investigate the connection between CRP elevation and deep ulcers in UC.

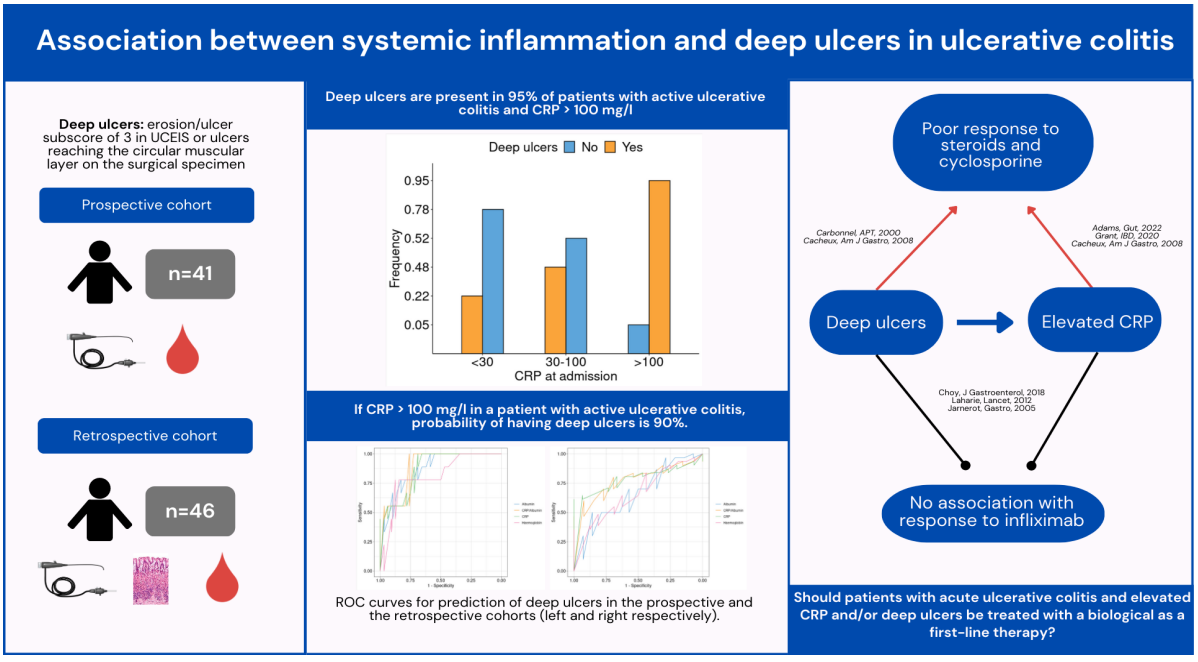
Methods: Patients with active UC were enrolled in a multi-center prospective cohort; deep ulcers were defined by an erosion/ulcer subscore of 3 in the Ulcerative Colitis Endoscopic

Index of Severity. An additional retrospective cohort of consecutive patients undergoing colectomy for active UC from 2012 to 2019 was analysed; deep ulcers were defined by ulcers reaching the circular muscular layer on the surgical specimen.

Results: Forty-one (9 (22%) with deep ulcers) patients were included in the prospective cohort: 4/5 (80%) patients with CRP > 100 mg/L, 2/10 (20%) patients with CRP between 30 and 100 mg/L and 3/26 (12%) patients with CRP < 30 mg/L had deep ulcers (p=0.006). In the retrospective cohort [46 patients (31 (67%) with deep ulcers)], 14/14 (100%) patients with CRP > 100 mg/L, 11/17 (65%) patients with CRP between 30 and 100 mg/l and 6/15 (40%) patients with CRP < 30 mg/L had deep ulcers (p=0.001). Positive predictive value of CRP > 100 mg/l for presence of deep ulcers was 80% and 100% in both cohorts, respectively.

Conclusions: CRP elevation is a robust surrogate marker for presence of deep ulcers in UC. Elevated CRP or presence of deep ulcers could influence the choice of medical therapy in acute severe UC.

Graphical abstract



2.3. Introduction

Ulcerative colitis (UC) is a chronic disease characterised by acute episodes of bloody diarrhoea with varying degrees of severity. The most feared event, acute severe UC, can lead to life-threatening and systemic complications[1]. The Truelove and Witts criteria are used for rapid identification of those patients with acute severe UC requiring hospital admission and intensive treatment[2]. Historically, systemic inflammation was evaluated by the erythrocyte sedimentation rate (ESR)[3]. The European Crohn's and Colitis (ECCO) guidelines suggested replacing ESR by C-Reactive protein (CRP). Compared to what is described in Crohn's disease, CRP elevation has been shown to be preferentially associated with severe clinical activity in UC[4]. Next to biomarkers, a flexible sigmoidoscopy is recommended to assess disease severity in UC[2]. In a retrospective series of patients admitted for an acute severe UC episode, presence of deep ulcers of the colorectal mucosa was associated with treatment failure and colectomy[5–7]. However, these results may also reflect a circular argument if the decision of colectomy was based on endoscopic findings[8]. Moreover, most research on severe lesions in acute UC was conducted before the implementation of biological therapies in routine clinical practice, dramatic improvement of endoscopy and wide use of the Ulcerative Colitis Endoscopic Index of Severity (UCEIS)[9]. Overall, the relationship between systemic inflammation and severity of colorectal lesions has not been thoroughly investigated in acute UC. These factors point out that deep ulcers may be an overlooked topic in acute UC.

The objective of the present study was to describe in-depth the relationship between systemic inflammation, measured by CRP elevation and low albumin and haemoglobin levels, and deep ulcers in acute UC.

2.4. Methods

Study population: ITAC cohort

Consecutive adult patients admitted for acute severe UC from May 2020 to May 2021 in Bordeaux University Hospitals (Bordeaux, France), Beaujon University Hospitals (Paris, France) and McGill University Health Centre (Montréal, Canada) were included prospectively in an observational prospective cohort. Acute severe UC was defined according to the Truelove and Witts criteria: six or more bloody daily stools and at least one of the following: fever (temperature > 38.5°C), tachycardia (> 90/min), anaemia (haemoglobin less than 10.5 g/dL) or CRP > 30 mg/L[2]. In parallel, a control group of patients with non-severe active UC was enrolled, defined as disease activity symptoms corresponding to a partial Mayo score ≥ 4 with a rectal bleeding subscore ≥ 1 without Truelove and Witts criteria. Patients with features of Crohn's disease (perianal lesions, ileal lesions or suggestive endoscopic lesions) were excluded. The cohort was observational only and therapeutic management was not standardised.

Study population: retrospective cohort

An additional retrospective cohort was constituted by consecutive patients > 15 years old who underwent colectomy for active UC in a single tertiary care centre (Bordeaux University Hospitals) from January 2012 to February 2019. Patients were excluded in case of colectomy for dysplasia or cancer, segmental colectomy, Crohn's disease diagnosed on the surgical specimen. Patients without an endoscopy within three months before surgery were removed from the analysis. Enrollment date corresponded to the date of colectomy. For acute severe UC patients and non-severe active UC patients treated by corticosteroids, a staged colectomy was performed as recommended by ECCO guidelines. At time of colectomy, rectum and

distal sigmoid were left in place and anchored to the abdominal wall and a terminal ileostomy was created[10].

Assessment of deep ulcers

In the prospective cohort, endoscopic activity was evaluated by flexible sigmoidoscopy or total colonoscopy at enrollment. Deep ulcers were defined as an erosion/ulcer subscore of 3 in the UCEIS score corresponding to presence of deep ulcerations[9].

In the retrospective cohort, deep ulcers were assessed by examination of the surgical specimen and the last endoscopic examination before colectomy that was scored based on reports, pictures or videos when available. Two IBD-specialised pathologists (AR and MM) blinded from the original pathology report and patient outcomes reviewed the slides for presence and grading of ulcers on each surgical specimen. In case of disagreement, a third revision was performed by one of them (AR). An average number of ten histology blocks from the whole colectomy specimen were done and slides were examined following hematoxylin-eosin staining. Deep ulcers were defined as at least one ulcer reaching the circular muscle layer in the worst lesions of the retrospective specimen. In case of subtotal colectomy, patients without deep ulcers on surgical specimens but having deep endoscopic ulcers located in the rectum or the lower sigmoid were assigned to the deep ulcers group.

Biomarkers

In the prospective cohort, haemoglobin, CRP and albumin were measured on the same day as endoscopy. In the retrospective cohort, albumin (last measurement before albumin infusion or parenteral nutrition initiation), CRP and haemoglobin (last measurement before blood transfusion) were retrieved from electronic health records. In case of multiple measurements of one of the biomarkers, the latest taken before colectomy was retained.

Objectives

The primary objective of this study was to compare the proportion of patients with deep ulcers according to prespecified CRP thresholds. Patients were divided into three groups according to their CRP serum levels as described below. Two thresholds of CRP were selected: 30 mg/L that corresponds to the cut-off defining acute severe UC in the ECCO guidelines[2] and 100 mg/L recently shown to be associated with non-response to steroids in acute severe UC[11].

Secondary objectives were to *i)* compare the proportion of deep ulcers according to albumin levels in patients with active UC; *ii)* compare the proportion of deep ulcers according to CRP/albumin ratio in patients with active UC; *iii)* compare the proportion of deep ulcers according to haemoglobin levels in patients with active UC; *iv)* investigate the correlation between CRP, albumin and haemoglobin in patients with active UC.

Albumin was considered as low if < 35 g/L[12]. haemoglobin was considered as low if < 10.5 g/dL[2]. Based on literature review, a cut-off of 0.32 was selected for CRP/albumin ratio predicting avoidance of colectomy at 12 months in a retrospective multicentric cohort of acute severe UC patients[13].

Statistical analyses

Continuous data and categorical variables were expressed as median (interquartile range [IQR]) and frequencies, respectively, and compared using a Student's t-test and a chi-square test, respectively. Receiver operating characteristic (ROC) curves were plotted using sensitivity against (1-Specificity). Correlation of continuous variables was evaluated using the Spearman method. Two-sided statistical tests were used for all analyses. A p-value <0.05 was considered as significant. For controlling false discovery rate related to multiple testing, we report p-values for the primary objective of the study only. Statistical analyses were performed using R version 3.5.1 (R Development Core Team, Vienna, Austria).

Ethical considerations

This study was conducted in accord with the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans (2014), as well as in respect of the requirements set out in the applicable standard operating procedures of the participating centres. The study was approved by the *Comité de Protection des Personnes Ouest IV - Nantes* (Reference 69/19-2, 19.09.19.61935).

2.5. Results

Study population: prospective cohort

After exclusion of two patients with features of Crohn's disease, 41 patients [median (IQR) age 42 years (34, 57)] were included in the prospective cohort: 19 (46%) patients having an acute severe UC and 22 (54%) a non-severe active UC. Median (IQR) disease duration at enrollment was 5.3 (1.7, 11.3) years. Twenty-one (51%) patients had a pancolitis, 15 (37%) a left-sided colitis and 5 (12%) a proctitis. Twenty-seven (66%) had never been exposed to biologics at admission. In the whole cohort, median (IQR) CRP, albumin and haemoglobin levels at inclusion were 12 (4, 58) mg/L, 33.8 (26.4, 38.9) g/L and 12.9 (10.4, 13.7) g/dL respectively. They were 59 (38, 95) mg/L, 26.2 (23.4, 30.8) g/L and 10.3 (9.2, 11.6) g/dL in patients having acute severe UC and 4 (3, 8) mg/L ($p<0.01$), 38.6 (35.1, 41.0) g/L ($p<0.01$) and 13.6 (13.1, 14.6) g/dL ($p<0.01$) in those with non-severe active UC, respectively.

At enrollment, all patients were evaluated by endoscopy: median UCEIS score was 5 (4, 6). Nine (22%) patients displayed deep ulcers, all included in the acute severe UC group.

Detailed characteristics of the patients at inclusion are displayed in Table S1.

Study population: retrospective cohort

Among the 50 patients who underwent colectomy for active UC during the study period, 46 [median (IQR) age 39 years (23, 57)] were analysed after removing four patients without an endoscopic assessment performed within the three months before colectomy. Median (IQR) disease duration at enrollment was 1.9 (0.3, 6.0) years. Thirty-seven (82%) patients had pancolitis and 8 (18%) left-sided colitis. Twenty-two (48%) patients had never been exposed to biologics at admission. Five (11%) patients were operated for non-severe refractory UC and 41 (89%) for acute severe UC. A staged colectomy with temporary ileostomy was performed in 44 (94%) patients. Median (IQR) CRP, albumin and haemoglobin levels at baseline were 58 (15, 120) mg/L, 28.6 (24.9, 31.9) g/L and 10.1 (8.9, 11.4) g/dL respectively.

The endoscopic assessment was performed 7 (4, 12) days before colectomy with a median UCEIS score of 6 (5, 7). Twenty-three (50%) patients had deep ulcers on the surgical specimen. From the 23 (50%) patients without deep ulcers on the surgical specimen, 8 had deep ulcers located in the rectum and/or the sigmoid at endoscopy and were also considered having deep ulcers. Overall, 31 (67%) patients had deep ulcers in the retrospective cohort.

Characteristics of patients enrolled in the two cohorts are described in Table 1.

Table 1: Characteristics of the patients enrolled in the two cohorts.

Variable	Prospective cohort n=41	Retrospective cohort n=46
Age in years, median (IQR)	42 (34, 57)	39 (23, 57)
Body mass index, kg/m2, median (IQR)	24.5 (21.8, 28.8)	21.4 (19.2, 23.8)
Disease duration, years, median (IQR)	5.3 (1.7, 11.3)	1.9 (0.3, 6.0)
Disease extent, n (%)		
- Pancolitis	21 (51)	37 (82)
- Left-sided colitis	15 (37)	8 (18)
- Proctitis	5 (12)	0 (0)
Presence of Truelove-Witts criteria, n (%)	19 (46)	41 (89)
Lichtiger score at enrolment, median (IQR)	11 (7, 13)	11 (10, 14)
Never exposed to biological therapy, n (%)	27 (66)	22 (48)
Steroids for current flare, n (%)	16 (39)	33 (72)
C- Reactive protein, mg/L, median (IQR)	11.7 (4.0, 57.7)	57.7 (14.7, 120.8)
Hæmoglobin, g/dL, median (IQR)	12.9 (10.4, 13.7)	10.1 (8.9, 11.5)
Albumin, g/L, median (IQR)	33.8 (26.4, 38.9)	28.6 (24.9, 31.9)
Surgery performed for current flare, n (%)	6 (15)	46 (100)

Association between CRP and deep ulcers

In the prospective cohort, patients' characteristics at enrollment were similar between patients with CRP > 100 mg/L, CRP 30-100 mg/L or CRP < 30 mg/L especially for previous exposure to biologics (p = 0.11) or disease duration (p = 0.40) (Table 2). Four out of five (80%) patients with CRP > 100 mg/l, 2/10 (20%) patients with CRP between 30 and 100 mg/l and 3/26 (12%) patients with CRP < 30 mg/l had deep ulcers (p = 0.006) (Figure 1A). In the retrospective cohort, 14/14 (100%) patients with CRP > 100 mg/l, 11/17 (65%) of patients with CRP between 30 and 100 mg/l and 6/15 (40%) of patients with CRP < 30 mg/l had deep ulcers (p=0.001) (Figure 1B).

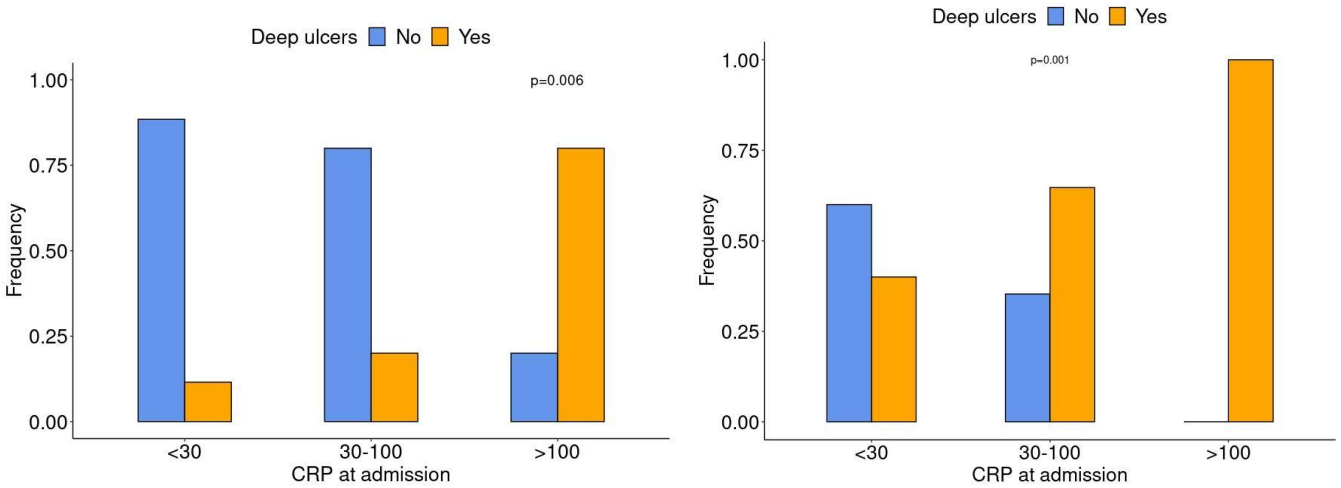


Figure 1: Association between deep ulcers and CRP levels.

A. Frequency of deep ulcers in the prospective cohort according to C-Reactive protein (CRP) levels. B. Frequency of deep ulcers in the retrospective cohort according to CRP levels.

Table 2: Characteristics of the patients in the prospective cohort according to the CRP levels.

Variable	CRP < 30 mg/l, n = 26	30 ≤ CRP ≤ 100 mg/l, n = 10	CRP > 100 mg/l, n = 5	p-value
UCEIS ulcer = 3, n (%)	3 (12)	2 (20)	4 (80)	< 0.01
Body mass index, kg/m2, median (IQR)	24.8 (21.6, 29.0)	23.1 (22.3, 26.0)	23.8 (21.8, 24.6)	0.80
Disease duration, years, median (IQR)	5.3 (1.3, 8?1)	7.9 (4.0, 16.9)	1.7 (1.4, 17.3)	0.40
Disease extent, n (%)				0.50
- Pancolitis	10 (38)	3 (30)	2 (40)	
- Left-sided colitis	11 (42)	7 (70)	3 (60)	
- Proctitis	5 (19)	0 (0)	0 (0)	
Lichtiger score at enrolment, median (IQR)	8.5 (6.0, 11.8)	13.0 (13.0, 14.0)	13.0 (12.0, 13.0)	< 0.01
Never exposed to biological therapy, n (%)	20 (77)	4 (40)	3 (60)	0.11
Hæmoglobin, g/dL, median (IQR)	13.6 (12.5, 14.6)	11.1 (9.3, 11.7)	10.2 (9.2, 12.9)	< 0.01
Albumin, g/L, median (IQR)	36.2 (33.9, 41.0)	27.9 (25.6, 31.8)	21.9 (19.9, 23.9)	< 0.01
Surgery performed for current flare, n (%)	1 (4)	3 (30)	2 (40)	0.03

Association between albumin levels and deep ulcers

In the prospective cohort, 9/22 (40%) of patients with albumin < 35 g/L and 0/16 (0%) patients with albumin ≥ 35 g/L had deep ulcers (Figure 2A). In the retrospective cohort, 29/40 (73%) of patients with albumin < 35 g/L and 1/4 (25%) patients with albumin ≥ 35 g/L had deep ulcers (Figure 2B).

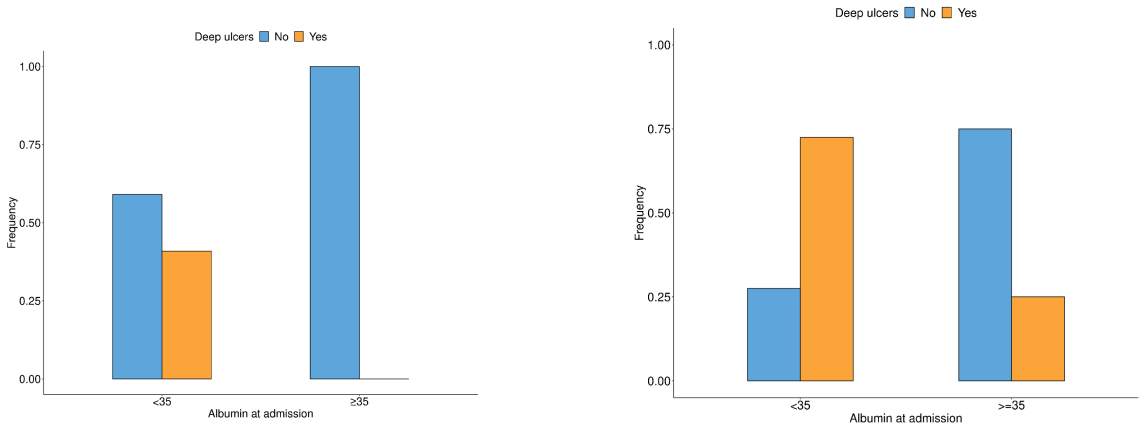


Figure 2: Association between deep ulcers and albumin levels.
A. Frequency of deep ulcers in the prospective cohort according to albumin levels. B. Frequency of deep ulcers in the retrospective cohort according to albumin levels.

Association between CRP/albumin ratio and deep ulcers

In the prospective cohort, 9/20 (40%) of patients with CRP/albumin ratio > 0.32 and 0/18 (0%) patients with CRP/albumin ratio ≤ 0.32 had deep ulcers. In the retrospective cohort, 25/34 (74%) of patients with CRP/albumin ratio > 0.32 and 5/9 (55%) patients with CRP/albumin ratio ≤ 0.32 had deep ulcers.

Association between haemoglobin and deep ulcers

In the prospective cohort, 7/11 (63%) of patients with haemoglobin < 10.5 g/dL and 2/30 (6%) patients with haemoglobin ≥ 10.5 g/dL had deep ulcers. In the retrospective cohort, 20/26 (77%) of patients with haemoglobin < 10.5 g/dL and 11/21 (52%) patients with haemoglobin ≥ 10.5 g/dL had deep ulcers.

ROC curves for CRP, albumin, CRP/albumin ratio and haemoglobin to predict presence of deep ulcers in the two cohorts are displayed in Figure 3. Sensitivity, specificity, positive and negative predictive values for each parameter regarding presence of deep ulcers are presented in Table 3. Table 4 displays area under the curve (AUC) for each parameter in the two cohorts.

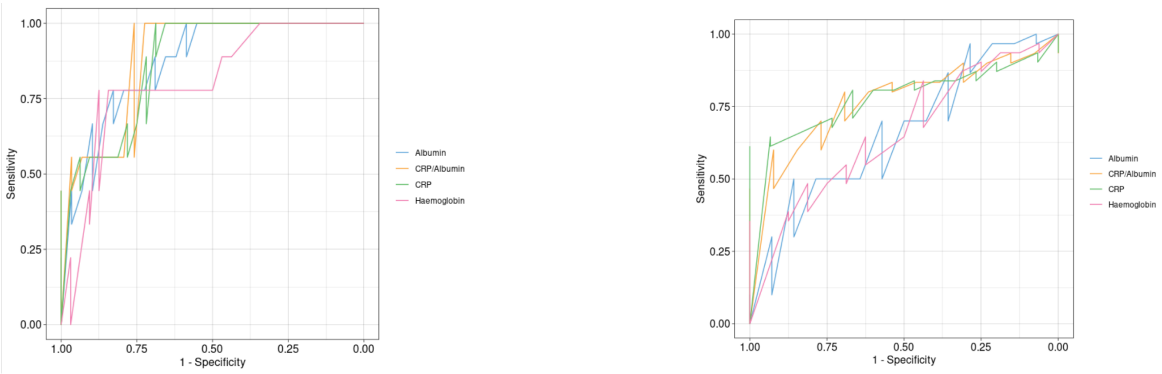


Figure 3: Performances of biological parameters to predict the presence of deep ulcers. Left. ROC curves for CRP, albumin, CRP/albumin ratio and haemoglobin to predict the presence of deep ulcers in the prospective cohort. Right. ROC curves for CRP, albumin, CRP/albumin ratio and haemoglobin to predict the presence of deep ulcers in the retrospective cohort.

Correlation between CRP levels and biological and endoscopic scores

In the prospective cohort, CRP levels were correlated with albumin levels (Spearman coefficient -0.65, $p < 0.001$), haemoglobin levels (Spearman coefficient -0.62, $p < 0.001$) and total UCEIS score (Spearman coefficient 0.57, $p < 0.001$).

In the retrospective cohort, CRP levels were correlated with albumin levels (Spearman coefficient -0.35, $p = 0.02$). No correlation was observed between CRP and haemoglobin levels (Spearman coefficient -0.20, $p = 0.17$) and CRP and total UCEIS score (Spearman coefficient -0.07, $p = 0.70$).Table 4: Area under the curve for receiver operating characteristic curves of CRP, albumin, CRP/albumin ratio and haemoglobin for the presence of deep ulcers in the two cohorts.

Table 3: Performance of biological parameters to predict the presence of deep ulcers in the two cohorts.

Results are presented as percentages (95% Confidence Interval).

	Prospective cohort (n=41)	Retrospective cohort (n=46)
CRP > 100 mg/L		
Sensitivity	44 (14-79)	45 (27-64)
Specificity	97 (84-100)	100 (78-100)
Positive predictive value	80 (28-99)	100 (77-100)
Negative predictive value	57 (32-100)	47 (29-65)
Albumin < 35 g/l		
Sensitivity	100 (66-100)	97 (83-100)
Specificity	55 (36-74),	21 (5-51)
Positive predictive value	41 (21-64)	72 (56-85)
Negative predictive value	100 (79-100)	75 (19-99)
CRP/albumin ratio ≤ 0.32		
Sensitivity	100 (66-100)	83 (65-94)
Specificity	62 (42-79)	31 (9-61),
Positive predictive value	45 (23-68)	74 (56-87)
Negative predictive value	100 (81-100)	44 (14-79)
haemoglobin < 10.5 g/dl		
Sensitivity	78 (40-97)	65 (45-81)
Specificity	88 (71-96)	62 (35-85)
Positive predictive value	64 (31-89)	77 (56-91)
Negative predictive value	25 (7-87)	48 (26-70)

Table 4: Area under the curve for receiver operating characteristic curves of biological parameters for the presence of deep ulcers in the two cohorts.

	Prospective cohort (n=41)	Retrospective cohort (n=46)
CRP	0.87	0.79
Albumin	0.87	0.66
CRP/albumin ratio	0.89	0.77
haemoglobin	0.81	0.68

2.6. Discussion

Evaluation of disease severity is the prime concern in acute UC to prevent morbidity and mortality related to acute severe UC complications[2]. Biomarkers such as CRP and albumin have received the most attention in the last few years. Using data from an international prospective cohort of well-phenotyped patients with active UC, we found a robust association between CRP elevation, low levels of albumin and haemoglobin and presence of deep ulcers. We confirmed these findings in a retrospective analysis of colectomy specimens of patients with active UC. Positive predictive value for presence of deep ulcers in patients with CRP > 100 mg/L reached 100% in the retrospective cohort.

The association between clinical severity and CRP elevation in UC has long been known[4]. Presence of systemic inflammation, measured initially by ESR and next by CRP level, is part of the modified Truelove and Witts criteria used to define acute severe UC[2]. To our knowledge, our study is one of the first to investigate thoroughly the link between CRP elevation and endoscopic severity in UC. We found that CRP elevation is a good surrogate marker for presence of deep ulcers. Most patients with CRP > 100 mg/L present with deep ulcers in the colorectal mucosa. Potential explanatory factors involve an increase production of inflammatory cytokines by monocytes in the context of a greater damage to mucosal

barrier in patients with deep ulcers related to increased systemic transfer of luminal content such as bacterial peptides[14].

In acute severe UC, early identification of steroids non-responders is needed to avoid exposure to multiple immunosuppressants and delayed surgery[15]. The relevance of several biomarkers, such as CRP or albumin and endoscopic findings have been investigated in that context. Buckell *et al.* in 1980 showed in a retrospective cohort of 40 patients with active severe UC that colonic dilatation and perforation were mostly seen in patients with deep ulcers[16]. Retrospective studies from the pre-biologics era showed that patients with acute severe UC and deep ulcers were less prone to respond to steroids compared to patients without such lesions[5–7]. Two recent retrospective studies found that CRP, albumin, and endoscopy at admission were robust predictors of non-response to steroids in acute severe UC[11,17]. However, in those studies, the total UCEIS and the Mayo score were used without discriminating between patients with severe or non-severe endoscopic lesions. Here, we demonstrate that elevated CRP and decreased albumin are strong predictors for the presence of deep ulcers in acute UC both in a prospective and in a retrospective retrospective cohort. This is the plausible missing link between deep ulcers and steroid non-response. Evidence from clinical trials shows that the ability of steroids to induce endoscopic healing of UC lesions is limited[18,19]. Our data suggest that patients with high CRP display deeper ulcers which steroids may not be able to act upon.

Available predictive indices in acute severe UC focus on response to steroids[11,17]. Few data exist on predictive factors of response to cyclosporine. In a retrospective study of 135 patients with steroid refractory-acute severe UC treated with cyclosporine, Cacheux *et al.* found that CRP > 45 mg/l and presence of deep ulcers at admission were associated with colectomy[20]. The picture is thought to be different with biological therapies because infliximab has been shown to be a potent mucosal healing agent in UC.[19] In pivotal randomised trials testing

infliximab in acute severe UC, elevated CRP and presence of deep ulcers at therapy initiation were not predictive of response to therapy[21,22]. Similarly to what is observed with steroids, post-hoc analysis of the CYSIF trial comparing infliximab to cyclosporine in steroid refractory-acute severe UC found that cyclosporine was less prone to heal endoscopic lesions[23]. Consistent with our findings, pretreatment levels of CRP and albumin were not predictive of response to infliximab in a retrospective analysis of 54 patients treated for acute severe UC[13].

We acknowledge several limitations of our study. The relatively limited sample size hampered our ability to investigate the predictive value of biomarkers and endoscopic features for response to therapy. Thus, we restricted ourselves to descriptive objectives in a cross-sectional view of systemic inflammation and endoscopic severity which is currently not well depicted in the literature. In the retrospective cohort, endoscopic reports were retrospectively revised looking for deep ulcers. All included patients underwent colectomy, suggesting a selection bias of the most severe cases and implying a limited sample size. However, this ensured homogeneity of the study population and high reliability of lesions assessment in the whole colon on the retrospective specimen. Colonoscopy is not recommended during acute severe UC flare[24]. Staged colectomy was performed as recommended by ECCO guidelines[15] making histologic assessment of rectal and distal sigmoid ulcers impossible for the majority of patients. That is the reason why we combined the retrospective specimen evaluation to sigmoidoscopy findings. Conversely, we cannot exclude that patients in the prospective cohort without deep ulcers at flexible sigmoidoscopy and high CRP displayed deep ulcers in the transverse or right colon.

In conclusion, using both prospective and retrospective data, we showed that systemic inflammation measured by CRP elevation and hypoalbuminemia is strongly associated with deep ulcers in patients with active UC, especially in case of an acute severe episode. These

biochemical and endoscopic severity features may be predictive of non-response to steroids and cyclosporine in the context of acute severe UC. Prospective trials must evaluate whether patients with acute severe UC and CRP > 100 mg/L at admission and/or deep ulcers should be treated with a biologic as a first-line therapy.

2.7. References

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3. Chapter 3: Differences in microbiome diversity and composition between acute severe ulcerative colitis and non-severe ulcerative colitis

3.1. Preface

The aim of this thesis project is to identify the determinants of acute severe ulcerative colitis which is characterised by systemic inflammation compared to non-severe ulcerative colitis. Clinically, one can observe resemblances between infectious colitis and acute severe ulcerative colitis, specifically fever and C-Reactive protein elevation. In our gastroenterology unit during the years 2017-2018, when we were designing this PhD project, we encountered several cases of patients admitted with infectious colitis concomitant to an episode of acute severe ulcerative colitis. A specific patient, who had not been previously diagnosed with ulcerative colitis, developed severe colitis, and their stool culture tested positive for *Campylobacter jejuni*. He was admitted due to antibiotic non-response, and we observed a clear case of acute severe ulcerative colitis, which necessitated the use of ciclosporin. The challenge we faced in this case was determining whether the underlying cause of the flare was the infection or whether the pathogen was merely an 'innocent by-stander'.

Thus, our second research question was to determine the role of potential pathogens and/or pathobionts and their association with microbiota composition in acute severe ulcerative colitis. We aimed at *i)* evaluating the prevalence of known pathogens in patients with acute severe ulcerative colitis and *ii)* investigating whether systemic inflammation seen in acute severe ulcerative colitis was associated with impaired bacterial composition of the gut microbiome associated with pathobionts. We used stool and biopsy samples collected during the ITAC study to compare microbiota diversity and composition at enrollment and at three months using 16S sequencing between patients with acute severe ulcerative colitis and

non-severe ulcerative colitis. I performed the microbial DNA extraction and the bioinformatics analysis.

This work corresponds to a manuscript currently in preparation.

Specific author's contributions:

P Rivière conceived the study, wrote the clinical research protocol, coordinated the centres, designed and tested the microbial DNA extraction and PCR protocol, performed the microbial DNA extraction for all samples, analysed the data, drafted and corrected the manuscript.

R Enaud provided theoretical support for the analysis of the data including discussion about analysis methods and sharing of code.

T Bessissow was involved in the study's design and contributed to patient recruitment.

X Treton was involved in the study's design and contributed to patient recruitment.

M Uzzan was involved in the study's design and contributed to patient recruitment.

F Poullenot was involved in the study's design and contributed to patient recruitment.

F Zerbib was involved in the study's design and contributed to patient recruitment.

K Dewar provided theoretical and practical support to establish the extraction and PCR protocol.

E Schurr conceived the study, provided theoretical and practical support to establish the extraction protocol and for the analysis of the data and critically revised the manuscript.

D Laharie conceived the study, contributed to study coordination and patient recruitment and critically revised the manuscript.

Acute severe ulcerative colitis is associated with impaired diversity and expansion of pathobionts of the gut microbiota.

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3.2. Abstract

Background: Acute severe ulcerative colitis, characterised by bloody diarrhoea and systemic inflammation, poses a significant lethal risk, yet its pathophysiology remains poorly understood. The role of microorganisms as triggers for this inflammatory condition has been proposed. We aimed to compare the presence of known pathogens, microbiota diversity and composition in patients with acute severe ulcerative colitis to those with non-severe ulcerative colitis.

Methods: We conducted a prospective observational study at three referral centres. Patients meeting Truelove and Witts criteria for acute severe ulcerative colitis and those with non-severe ulcerative colitis (partial Mayo score of 4 or more with a rectal bleeding subscore of 1 or more, but without meeting Truelove severity criteria) were enrolled. We screened for known enteric pathogens and analysed microbiota using 16S ribosomal RNA sequencing on stool and rectal biopsy samples at enrollment and at three months.

Results: Our cohort did not exhibit evidence of overt infectious colitis at enrollment. Patients with acute severe ulcerative colitis displayed significant alterations in their gut microbiota, characterised by i) reduced alpha-diversity, ii) an increased presence of Proteobacteria, particularly members of the Escherichia/Shigella genus, and iii) a reduction in the abundance of Lachnospiraceae and Ruminococcaceae family members. These alterations were reflected in the microbial dysbiosis index and were correlated with systemic inflammation, as indicated by CRP levels.

Conclusion: The microbiota in patients with acute severe colitis is further compromised compared to patients with non-severe ulcerative colitis. These findings suggest the potential for therapeutic modulation of the microbiota as a strategy for managing acute severe ulcerative colitis.

3.3. Introduction

Ulcerative colitis, one of the two entities of Inflammatory Bowel Diseases (IBD) together with Crohn's disease, is characterised by chronic inflammation of the colonic mucosa and progresses through alternating flare and remission periods[1]. Acute severe ulcerative colitis, defined as bloody diarrhoea (≥6/24h) associated with signs of systemic inflammation, occurs in 25% of patients during the disease course[2]. Acute severe ulcerative colitis can manifest as the first flare of ulcerative colitis or develop after several years of stability under medical treatment[3]. Patients with acute severe ulcerative colitis are at risk of life-threatening complications associated with systemic inflammation[2]. Urgent colectomy is the only recourse for the approximately 20% of patients who do not respond to medical therapy, a measure crucial for preventing fatal outcomes[4]. Few data exist so far about the pathophysiology of acute severe ulcerative colitis. The explanation of microorganisms as triggers has been evoked to explain this inflammatory outburst.

Digestive infectious agents are found more frequently in IBD patients experiencing a disease flare than in patients with inactive disease or healthy controls[5]. Cytomegalovirus (CMV), a virus from the *Herpesviridæ* family, and *Clostridioides difficile* (*C.Diff*), an anaerobic Gram-positive spore-forming bacteria, infections are considered to be the most suspicious enteropathogens in this setting. Depending on the diagnostic tools, a CMV or a *C.Diff* infection are found in 10-30% of ASUC patients[6–9]. Conflicting data exist about whether CMV and *C.diff* are innocent bystanders in inflamed tissue or contribute to worsening of the disease. Treatment of CMV colitis does not modify the colectomy rate[10,11]. *C.diff* is related to higher surgery and mortality rates even with appropriate antibiotic therapy[12,13]. The hypothesis of microorganisms as triggers in acute severe ulcerative colitis is supported by the role of gut microbiota in ulcerative colitis. Faecal microbiota transplantation has shown promising results in inducing clinical and endoscopic remission in randomised controlled trials in ulcerative

colitis[14]. Changes in the microbiota composition compared to healthy subjects have been observed in patients with ulcerative colitis, mostly characterised by a decrease in butyrate-producing species, known to have anti-inflammatory properties on the colonic mucosa[15]. Commensal microbes play a crucial role in the host defence against pathogenic agents, through direct microbe-microbe competition and the release of intermediary mediators influencing the immune activation threshold of the host[16,17]. Certain bacterial species, such as *C.Diff*, can act as "pathobionts", i.e. a normal component of the gut microbiome causing disease only when specific genetic and/or environmental conditions are met. Diet and antibiotics influence the microbiota composition and activity in IBD[18].

We hypothesised that a dysfunctional gut microbiome, characterised by a lack of diversity and the loss of anti-inflammatory bacterial species, would allow the proliferation of a pathobiont in the colonic lumen associated with a systemic inflammatory response.

3.4. Materials and Methods

This was a prospective physiopathological and prognostic observational pilot study conducted in three IBD referral centres, Bordeaux University Hospital (France), Beaujon Hospital (Paris, France) and McGill University Health Centre (Montréal, Canada).

Study population

From May 2020 to May 2021, consecutive willing adult patients diagnosed with ulcerative colitis according to usual criteria[4] were enrolled if they corresponded to the following criteria:

- acute severe ulcerative colitis group: patients admitted with acute severe ulcerative colitis defined according to Truelove criteria, i.e. ≥6 bloody daily stools with one or more of the

following criteria: temperature >37.8°C, pulse >90 beats/min, haemoglobin <10.5g/dl or C Reactive-Protein >30 mg/l[2,4]. Patients with acute severe ulcerative colitis had to be enrolled within three days of admission.

- non-severe ulcerative colitis group: patients seen in outpatients clinic with disease activity symptoms, corresponding to a partial Mayo score of 4 or more with a rectal bleeding subscore of 1 or more, without Truelove severity criteria[19], whatever previous or on-going medical therapy.

We excluded patients with perianal lesions, ileal lesions or endoscopic lesions suggestive of Crohn's disease acute severe colitis.

Medical therapy and surgical indications were managed according to routine practice and current guidelines in both groups.

Procedures at enrollment

At baseline, a standard clinical evaluation with general state assessment and abdominal examination was performed. The Lichtiger score[20] and the Mayo score[21] were calculated. We retrieved use of antibiotics (class and indication if used), non-steroidal anti-inflammatory drugs (NSAID) and vaccines (name if used) in the past three months. We performed faecal calprotectin measurement on stool and C-Reactive protein (CRP) and albumin measurement in blood as disease activity biomarkers.

We screened for known enteric pathogens by standard diagnostic tests. We measured *Cytomegalovirus* (CMV) in the blood by quantitative PCR. We collected stool samples to perform a stool culture and *Clostridioides difficile* (*C.diff*) detection by immunochemical detection of Glutamate dehydrogenase and toxins A and B gene amplification in case of positivity, as recommended by the Infectious Disease Society of America guidelines[22]. We

performed a gene amplification for enteropathogenic viruses (rotavirus, norovirus, sapovirus, astrovirus, adenovirus) and ova and parasite tests. We collected one stool sample, which was frozen immediately at -80°C for microbiome analysis.

As routine clinical practice, patients undergoing flexible sigmoidoscopy had biopsies collected from the rectum, with a five mm forceps biopsy. We placed two biopsies in formol for histological examination, CMV detection by inclusions in standard Hemalun and Eosin coloration and immunohistochemistry with antibodies directed against CMV. We performed an aspiration of five ml of intestinal fluid through the endoscope for standard culture and *C.diff* detection. We collected two additional biopsies for mucosal microbiome analysis which were placed in RNA later and stored at -80°C until analysis. We used the Mayo score and Ulcerative Colitis Endoscopic Index of Severity (UCEIS) for endoscopic severity assessment.

Dietary habits questionnaires and scores

At enrollment, patients completed a Food Frequency Questionnaire concerning their dietary habits over the preceding seven days, based on a provided list of foods. Patients were asked what they consumed during the study period and specify the frequency. Following the seminal study published by Levine *et al.*[23], we calculated a 'pro-inflammatory diet' score by summing the frequency of consumption of 'pro-inflammatory' foods (including white bread, industrial food and sauces, fried food, red meat, and dairy), as well as an 'anti-inflammatory' score using a similar approach, considering foods such as fruits, vegetables, rice, and home-cooked meals.

Procedures at three month

Patients in both groups were seen again at the outpatients' clinic or the infusion unit at three months from enrollment. We calculated the partial Mayo score for clinical evaluation. Response to medical therapy at three months was defined as a partial Mayo score ≤2 without

bleeding (blood subscore < 1)[21]. We collected plasma for CRP and albumin measurement and stool samples for faecal calprotectin measurement and bacterial microbiome analysis. Patients underwent a flexible sigmoidoscopy. We collected two biopsies for routine histological examination and two additional biopsies were stored at -80°C in RNA later for mucosal microbiome analysis.

Ethical aspects

This research was carried out in accordance with law no. 2012-300 implemented on 5th March 2012, pertaining to research involving the human subjects, as well as in agreement with Good Clinical Practice guidelines (International Conference on Harmonisation, version 4 [9th November, 2016 and 24th November, 2006) and the Declaration of Helsinki. This study was approved by the Committee for the Protection of Persons Ouest IV – Nantes (ref 69/19-2, 19.09.19.61935) for French centres and by the Research Ethics Board of the McGill University Health Centre (ref 2020-5968).

Microbial DNA extraction

We extracted DNA from stool and biopsies using the QIAamp Powerfecal Pro DNA Kit and the QIAcube system following the manufacturer's instructions. Briefly, 50-100 mg of stool or one biopsy were placed in PowerBead Pro Tubes in a biosafety cabinet, incubated 10 minutes with Solution CD1 at 70°C, then lyzed using the PowerLyzer 24 Homogenizer and centrifuged. The next steps of extraction were automated in the QIAcube and performed in batches of 12. Two replicates were processed for each sample. A randomization list was established to ensure proper distribution of phenotypes and replicates in batches. A stool sample from a healthy donor was used as a positive control. A negative control (where the entire protocol was executed without any actual starting material) was included for each

Powerfaecal Pro Kit. Quality of the extraction was verified using migration in 1% agarose gel for one sample per QIAcube batch, the positive and the negative controls.

Amplification of the 16S ribosomal RNA gene and sequencing

The 16S ribosomal RNA (rRNA) library preparation and sequencing was performed using the Illumina MiSeq platform at the McGill Genome Center (Montréal). Primers targeting the V3-V4 (Primers: 341F, 805R) region of the 16S rRNA gene were used in the first step and a unique barcode and Illumina adapters were added to each library in the second step. The MiSeq250 platform was used for 2 x 250 nucleotides (nt) paired-end sequencing of the resulting PCR products.

Reads preprocessing and assignation

After sequencing, using DADA2 version 1.26.0, reads were truncated at 240 nt or at first instance of a quality score $\leq 2c$[24]. After truncation, reads with higher than two expected errors were discarded. Next, the Dada algorithm was used to remove sequencing errors using the error model computed from the data. Reverse and forward reads were assembled in pairs if the overlap region measured at least 12 nt without mismatch. Counts of each unique sequence across samples were summarised in an amplicon sequence variant (ASV) table. Finally, after identification from pooled sequences and by consensus across samples, chimeras were removed. Assignation was performed using the Ribosomal Database Project Naive Bayesian Classifier algorithm[25], with kmer size 8 and 100 bootstrap replicates and the Silva database version 138.1[26]. Exact matching against the Silva species database was used to assign genus-species binomials to the input sequence.

Amplicon sequence variant preprocessing

Preprocessing and analysis of ASV was performed using Phyloseq version 1.42.0 separately for stool and biopsy samples[27]. Sequences from outside the bacterial kingdom, as well as phyla including only one taxon and sequences found in fewer than ten samples, were excluded. Using microDecon[28] and the negative controls as references, reads attributed to kit contamination were eliminated. Replicates were pooled by averaging the ASV counts from both replicates. After filtering, we obtained 122 samples with a median number of reads of 9,714 (minimum 1,306 and maximum 65,454 reads) corresponding to 399 taxa.

Microbiota alpha- and beta-diversity and dysbiosis index evaluation

Microbiota alpha-diversity was expressed by observed richness, Shannon index and Simpson index. These indices assess the heterogeneity within a sample's community by considering both the diversity of species and their relative abundances. The microbial dysbiosis index, an overall summary statistic proposed to summarise the microbiota changes observed in IBD[29], was calculated for each sample. The formula is log of [total abundance in organisms increased in IBD] over [total abundance of organisms decreased in IBD]. Diversity and microbial dysbiosis indices were compared between acute severe ulcerative colitis and non-severe ulcerative colitis patients using a Wilcoxon rank sum test. Correlations between indices and biomarkers were calculated using the Pearson method.

Between sample beta-diversity differences (measured using Bray Curtis dissimilarity) were tested using a permutational multivariate ANOVA (Permanova) from the “vegan” package with 10,000 permutations, while accounting for individual identity as a covariate[30]. The Bray-Curtis dissimilarity ranges between 0 and 1, where 0 indicates that two samples have zero dissimilarity, meaning they share the exact same number of each type of species, while 1

indicates that they have complete dissimilarity, meaning they share none of the same type of species. This reflected how similar was the composition of the microbiota between the samples included in the analysis.

Differential bacterial abundance between groups

Two complementary methods were used to identify differentially abundant taxa between the groups: the linear discriminant analysis (LDA) effect size (LefSe) from microbiomeMarker package[31] and the Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC)[32] The combination of these two methods is recommended to ensure results are robust to methodological variations and lower the false positive rate[33].

General statistical considerations

Descriptive analysis was performed overall and by severity group. The analyses were performed on available data without imputation of missing data. All estimates were performed with a type I error rate of 5%. Qualitative variables were described by numbers and percentage and quantitative variables by median, range, and interquartile range (IQR) and compared using a chi-square test and a Student's t-test, respectively. All statistical analyses were performed using R (version 3.5.1).

3.5. Results

Study population at enrollment

Forty-one patients (23 (56%) female, median (IQR) age 42 (34 – 57) years were included, 19 (46%) in the acute severe ulcerative colitis group and 22 (54%) in the non-severe ulcerative colitis group. No difference was observed between the two groups in terms of gender, age, disease duration, previous exposure to biological therapies and duration of symptoms of current flare before enrollment. Severity features were more pronounced in the acute severe ulcerative colitis group: the median (IQR) Lichtiger clinical score was higher (13.0 (12.5 – 14.0) *versus* 7.0 (5.3 – 10.8), $p < 0.01$, respectively); the median (IQR) UCEIS score was higher (6.0 (5.0 – 7.0) *versus* 4.0 (4.0 – 5.0), $p < 0.01$, respectively); median (IQR) CRP levels were higher (59 (38 – 95) mg/dl *versus* 4 (3 -8) mg/dl, $p < 0.01$, respectively), median (IQR) haemoglobin and albumin levels were lower (10.3 (9.2 – 11.7) g/dl *versus* 13.7 (13.1 – 14.6) g/dl, $p < 0.01$ and 26 (23 – 31) g/l *versus* 39 (35 – 41) g/l, $p < 0.01$, respectively). Characteristics of the two groups are displayed in Table 1.

Table 1: General characteristics of the study patients at enrollment.

Variable	Acute severe ulcerative colitis, n = 19	Non-severe ulcerative colitis, n = 22	p-value
Female gender, n (%)	12 (63)	11 (50)	0.40
Age, median (IQR)	42 (35 - 55)	43 (33 - 56)	> 0.90
Body mass index, kg/m2, median (IQR)	23.4 (21.9 - 26.3)	24.8 (21.6 - 29.1)	0.40
Smoking status, n (%)			> 0.90
- Currently smoking	1 (5)	1 (5)	
- Former smoker	7 (37)	8 (36)	
- Never	11 (58)	13 (59)	
History of appendectomy, n (%)	0 (0)	0 (0)	> 0.90
Disease duration, years, median (IQR)	4.2 (1.7 - 15.0)	5.5 (1.9 - 9.8)	> 0.90
First flare, n (%)	2 (11)	4 (18)	0.70
Never exposed to biological therapy, n (%)	9 (47)	5 (23)	0.10
Disease extent, n (%)			0.09
- Pancolitis	8 (42)	7 (32)	
- Left-sided colitis	11 (58)	10 (45)	
- Proctitis	0 (0)	5 (23)	
Symptoms duration in weeks, median (IQR)	8.4 (2.8 – 18.3)	4.3 (2.0 – 10.2)	0.40
Lichtiger score at enrollment, median (IQR)	13.0 (12.5 - 14.0)	7.0 (5.3 - 10.8)	< 0.01
C-Reactive protein (mg/l), median (IQR)	59.0 (37.8 - 94.9)	4.2 (2.9 - 8.3)	< 0.01
Haemoglobin, g/dL, median (IQR)	10.3 (9.2 – 11.7)	13.7 (13.1 – 14.6)	< 0.01
Albumin, g/l, median (IQR)	26.2 (23.4 - 30.8)	38.6 (35.1 - 41.0)	< 0.01
Faecal calprotectin, mg/kg, median (IQR)	618 (100 – 1,490)	489 (169 – 1,273)	> 0.90
UCEIS score, median (IQR)	6.0 (5.0 - 7.0)	4.0 (4.0 - 5.0)	< 0.01

IQR: interquartile range; UCEIS: Ulcerative Colitis Endoscopic Index of Severity.

Screening for known clinical microbiota disruptors at enrollment

In the acute severe ulcerative colitis group, 7/19 (37%) patients had received antibiotics in the past three months compared to 1/22 (5%) in the non-severe ulcerative colitis group ($p = 0.02$). Antibiotics were administered for dental infection in the one patient of the non-severe ulcerative colitis group, for pneumonia in 1/7 patients and for diarrhoea in 6/7 patients in the acute severe ulcerative colitis group. A positive blood PCR for CMV was found in 2/19 (12%) and 2/22 (13%) patients in the acute severe ulcerative colitis and the non-severe ulcerative colitis group respectively, at 150 and 456 copies/ml in the acute severe ulcerative colitis group and 200 and 1564 copies/ml in the non-severe ulcerative colitis group. None of the patients showed histological signs of CMV colitis in their rectal biopsies. Stool culture was positive in 1/19 (6%) patients in the acute severe ulcerative colitis group and 1/22 (6%) patients in the non-severe ulcerative colitis group. Identified bacteria were *Aeromonas veronii* and *Pseudomonas aeruginosa*, respectively. Ova and parasite stool tests were positive in 2/22 (17%) patients of the non-severe ulcerative colitis group, one for *Blastocytis hominis* and one for *Dientamoeba fragilis* and none of the acute severe ulcerative colitis group. No *C.diff* or viral infections were diagnosed. No difference was observed between the two groups regarding dietary habits. Comparison of the two groups regarding known microbiota disruptors is displayed in Table 2.

Table 2: Description of known microbiota disruptors in the two groups.

Variable	Acute severe ulcerative colitis, n = 19	Non-severe ulcerative colitis, n = 22	p-value
Antibiotics intake in the past three months, n (%)			0.02
- None	12 (63)	21 (95)	
- Ongoing	4 (21)	0 (0)	
- Stopped	3 (16)	1 (5)	
Vaccine intake in the past three months, n (%)	0 (0)	2 (9)	0.50
Non-steroidal anti-inflammatory drug intake in the past three months, n (%)	1 (5)	3 (14)	0.60
Positive blood CMV PCR, n (%)	2 (12%)	2 (13%)	> 0.90
Positive stool culture, n (%)	1 (6)	1 (6)	> 0.90
Positive ova and parasite test, n (%)	0 (0)	2 (17)	0.20
Positive viruses multiplex PCR, n (%)	0 (0)	0 (0)	> 0.90
Positive *C.diff* screening, n (%)	0 (0)	0 (0)	> 0.90
Anti-inflammatory diet score, median (IQR)	47.5 (33.8 - 52.8)	54.0 (46.5 - 63.0)	0.14
Pro-inflammatory diet score, median (IQR)	57.0 (53.5 - 82.8)	59.5 (50.0 - 77.3)	0.90

C.diff: Clostridioides difficile; CMV: Cytomegalovirus; IQR: interquartile range.

Study population at three months

Among the 41 included patients, 29 (70%) were re-evaluated at three months, 5 (12%) ended the study earlier because they underwent subtotal colectomy (all from the acute severe ulcerative colitis group) and 7 (18%) ended the study earlier for other reasons (details are

presented in Supplementary Figure 1). Overall, 13/19 (68%) patients in the acute severe ulcerative colitis group and 16/22 (72%) patients in the non-severe ulcerative colitis group were assessed at three months. Characteristics of the two groups at three months are displayed in Supplementary Table 1. No difference was observed between the two groups regarding clinical, biological or endoscopic features. Considering patients who underwent subtotal colectomy within three months as non-responders, therapy response rates were 6/18 (33%) in the acute severe ulcerative colitis group *versus* 11/16 (69%) in the non-severe ulcerative colitis group (p = 0.09). At enrollment, differences in severity characteristics were observed between patients who would respond to therapy at three months and those who would not, *i.e.* a higher Lichtiger score (13 (13-14) *versus* 10 (7-12), respectively, p = 0.01), a higher CRP (42 (16-77) *versus* 9 (4-19), respectively, p = 0.03) and a lower median (IQR) UCEIS score (4.0 (4.0 – 5.0) *versus* 5.5 (5.0 – 7.0), respectively, p = 0.01). Comparison of the responders and the non-responders for enrollment characteristics is displayed in Supplementary Table 2.

Microbiota diversity at enrollment

At enrollment, 399 taxa were present in the 66 available samples (35 biopsies and 31 stool samples). Alpha-diversity measured by the observed richness, the Shannon index and the Simpson index was significantly lower in the acute severe ulcerative colitis group compared to the non-severe ulcerative colitis group (Figure 1A). Alpha-diversity indices were significantly inversely correlated with CRP levels irrespective of the severity groups: patients with higher CRP had lower microbiome diversity (Figure 1B). We observed a correlation between lower diversity and low haemoglobin and albumin levels for two out of three indices each (Supplementary Figure 2).

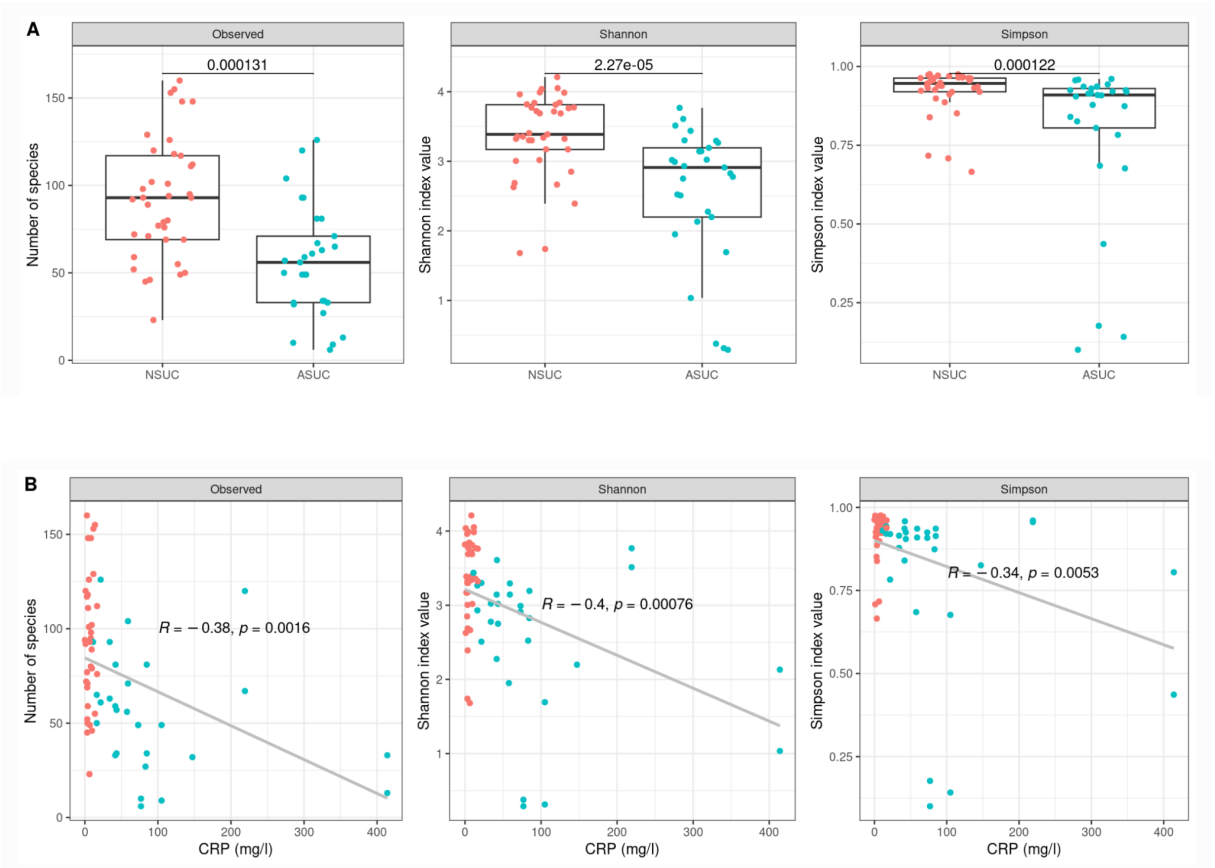


Figure 1: Alpha-diversity of the gut microbiota is reduced in the severe group and correlates with systemic inflammation.

Panel A: Box-plot representing the alpha-diversity at enrollment in the two groups (n=66 samples) using three different indices displayed as Y-axis. The box in the plot represents the middle 50% of the data, with the median line dividing the box into two parts. The whiskers extend from the box to indicate the variability outside the upper and lower quartiles. P-values were calculated using a Wilcoxon test to compare the two severity groups. ASUC: acute severe ulcerative colitis; NSUC: non-severe ulcerative colitis.

Panel B: Scatter-plot representing the alpha-diversity of the gut microbiota at enrollment according to plasmatic C-Reactive protein levels (X-axis) on the same day in the two groups (n=66 samples) using three different indices displayed as Y-axis. Correlation was calculated using Pearson coefficients and p-value.

Left subpanel: Observed richness, corresponding to the number of different species observed by sample. Middle subpanel: Shannon index takes into account the number of species and their abundance. Right subpanel: Simpson index takes into account the number of species and their abundance and is more sensitive to their abundance than the Shannon index.

Each dot represents one sample. Red and blue dots correspond to non-severe and acute severe colitis groups respectively.

The microbial dysbiosis index was significantly higher in the acute severe ulcerative colitis group, the total abundance of taxa known to be decreased in IBD[29] was lower in the acute severe ulcerative colitis group and the total abundance of taxa known to be increased in IBD[29] was higher in the acute severe ulcerative colitis group (Figure 2A). The microbial dysbiosis index was significantly correlated with the CRP levels at enrollment, with a stronger association observed with the total abundance of taxa known to be increased in IBD (Figure 2B). We observed an inverse correlation between the microbial dysbiosis index and haemoglobin, without significant correlation with the relative abundances of increased or decreased taxa (Supplementary Figure 3A), and between the microbial dysbiosis index and albumin with a significant correlation with the relative abundance of increased taxa only (Supplementary Figure 3B).

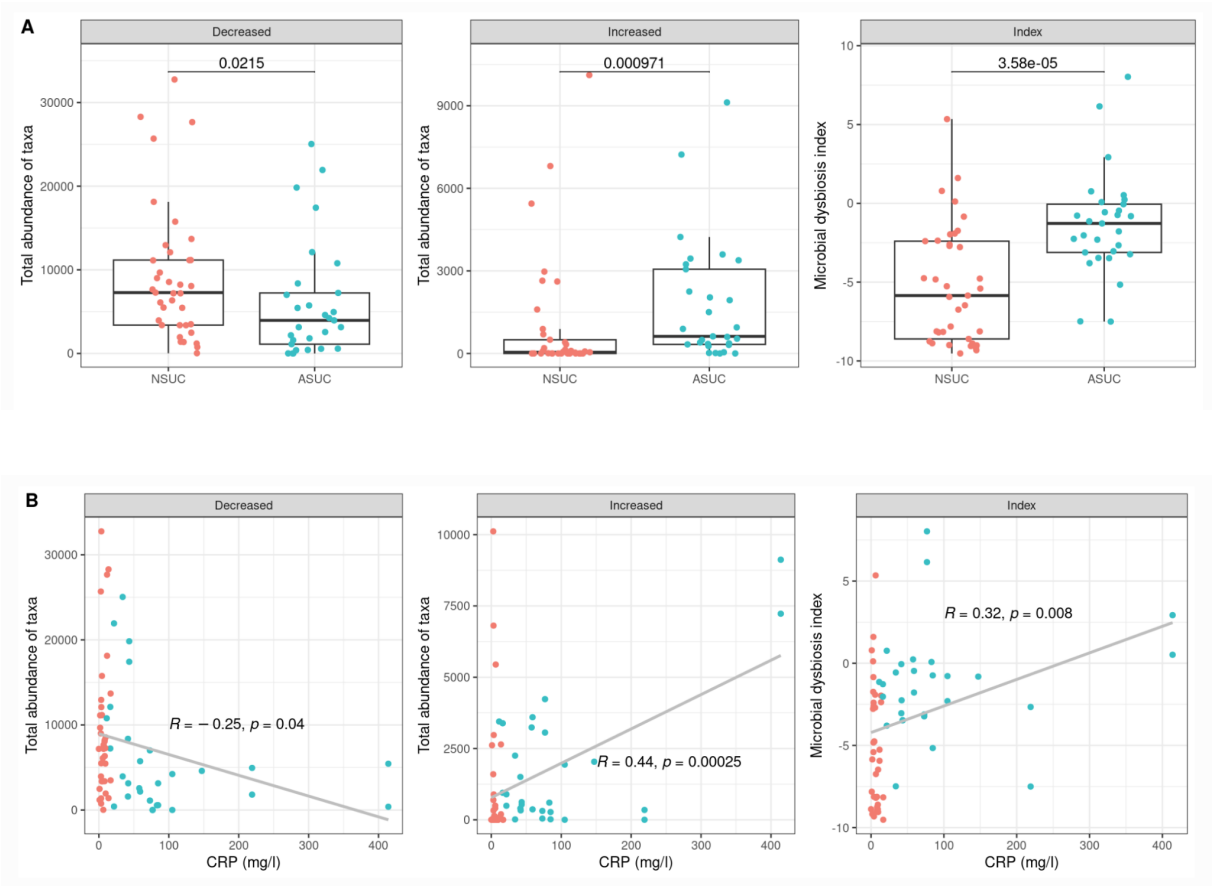


Figure 2: The microbial dysbiosis index is increased in the severe group and correlates with systemic inflammation.

Panel A: Box-plot representing the microbial dysbiosis index at enrollment in the two groups (n=66 samples). The box in the plot represents the middle 50% of the data, with the median line dividing the box into two parts. The whiskers extend from the box to indicate the variability outside the upper and lower quartiles. P-values were calculated using a Wilcoxon test to compare the two severity groups. ASUC: acute severe ulcerative colitis; NSUC: non-severe ulcerative colitis.

Panel B: Scatter-plot representing the microbial dysbiosis index (Y-axis) of the gut microbiota at enrollment according to plasmatic C-Reactive protein levels (X-axis) on the same day in the two groups (n=66 samples). Correlation was calculated using Pearson coefficients and p-value.

Left subpanel: Total abundance of taxa known to be decreased in IBD in Y-axis, as described by Gevers et al[29]. Middle subpanel: Total abundance of taxa known to be increased in IBD in Y-axis, as described by Gevers et al[29]. Right subpanel: Overall index corresponding to the log of [total abundance in organisms increased in IBD] over [total abundance of organisms decreased in IBD] in Y-axis, as described by Gevers et al[29].

Each dot represents one sample. Red and blue dots correspond to non-severe and acute severe colitis groups respectively.

Microbiota composition at enrollment

Composition of the communities was significantly different between the two groups (Figure 3, PERMANOVA p = 0.01).

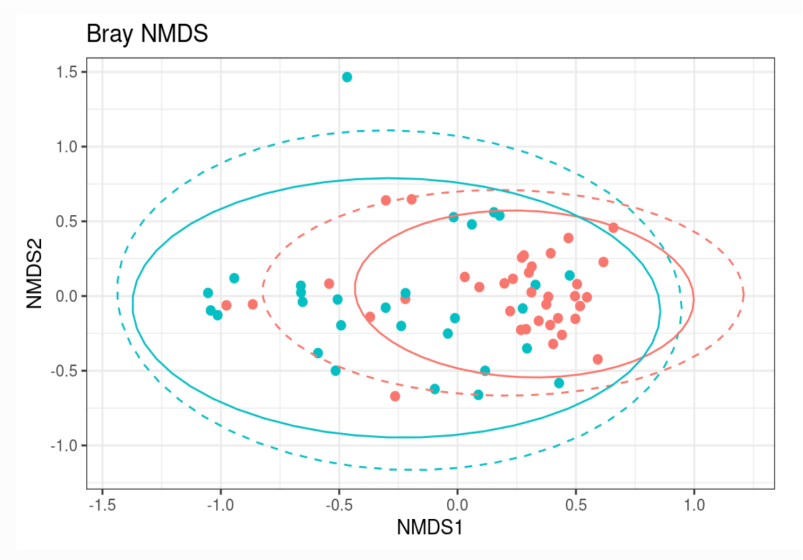


Figure 3: Non-metric multidimensional scaling representation of the microbiota composition at enrollment.

Two-dimensional graph displaying the similarities and differences between microbial communities based on their Bray-Curtis dissimilarity. Each dot on the plot represents a sample, and the distance between the points represents the degree of similarity or dissimilarity between the communities. The closer the dots are to each other, the more similar the microbial communities are in terms of their composition. Red and blue dots correspond to non-severe and acute severe colitis groups respectively. The ellipses show the standard deviation of the data points in the plot. ASUC: acute severe ulcerative colitis; NMDS: Non-metric multidimensional scaling; NSUC: non-severe ulcerative colitis.

At the phylum level, Proteobacteria were increased in the acute severe ulcerative colitis group (ANCOMBC and LEFSe $p < 0.01$, ANCOMBC log fold change 2.08, Figure 4, Supplementary Figure 4). At the genus level, five were significantly increased in the acute severe ulcerative colitis group – *Escherichia/Shigella* (Proteobacteria, Enterobacteriaceae family), *Parvimonas* (Firmicutes, Peptostreptococcales family), *Actinomyces* (Actinobacteria, Actinomycetaceae family), *Lactobacillus* (Firmicutes, Lactobacillaceae family) and *Enterococcus* (Firmicutes, Enterococcaceae family) – and 11 were significantly decreased in the acute severe ulcerative colitis group, all except two from the Firmicutes phylum –

Subdoligranulum and *Incertae* (Firmicutes, Ruminococcaceae family), *Agathobacter, Coprococcus* and *Dorea* (Firmicutes, Lachnospiraceae family), *Paraprevotella* (Bacteroidota, Prevotellaceae family), *Butyricicoccus* (Firmicutes, Butyricoccaceae family), *Intestinimonas* and *Oscillospira* (Firmicutes, Oscillospiraceae family), *Erysipelatoclostridium* (Firmicutes, Erysipelatoclostridiaceae family), *Victivallis* (Verrucomicrobiota, Victivallaceae family). Addition of the microbial dysbiosis index on the beta-diversity representation showed a gradient along the non-metric multidimensional scaling axis (Figure 5).

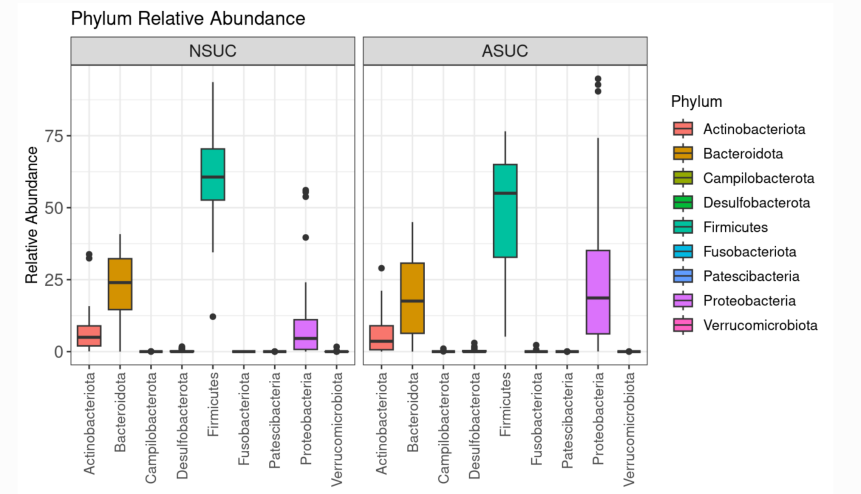


Figure 4: Proteobacteria are increased in the severe group at enrollment.

Box-plot showing the abundance of each phylum in the two groups at enrollment (n=66 samples). The box in the plot represents the middle 50% of the data, with the median line dividing the box into two parts. The whiskers extend from the box to indicate the variability outside the upper and lower quartiles. Each phylum is represented by a color. ASUC: acute severe ulcerative colitis; NSUC: non-severe ulcerative colitis. The only significant difference between the two groups was the abundance of Proteobacteria (in purple).

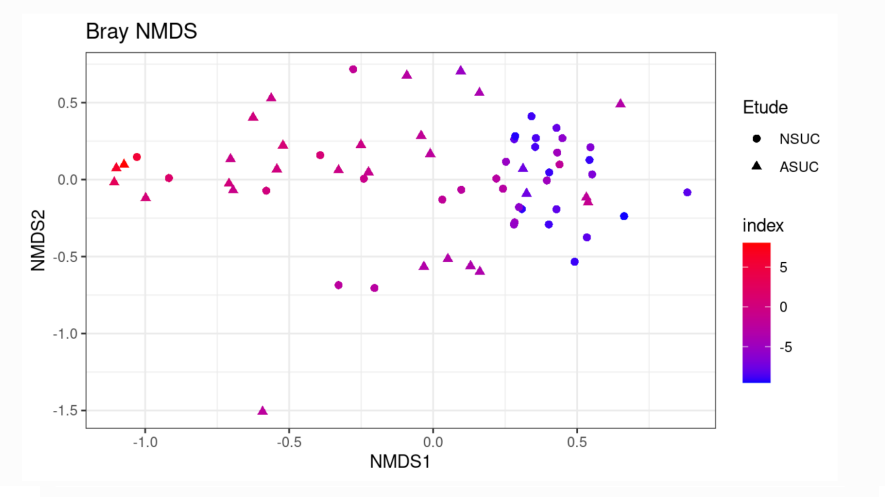


Figure 5: The microbial dysbiosis index accurately recapitulates the dissimilarity between the samples.

Two-dimensional graph displaying the similarities and differences between microbial communities based on their Bray-Curtis dissimilarity. Each dot on the plot represents a sample, and the distance between the points represents the degree of similarity or dissimilarity between the communities. The closer the dots are to each other, the more similar the microbial communities are in terms of their composition. Each sample is coloured according to the microbial dysbiosis index value corresponding to the log of [total abundance in organisms increased in IBD] over [total abundance of organisms decreased in IBD] in Y-axis, as described by Gevers et al[29]. ASUC: acute severe ulcerative colitis; NMDS: Non-metric multidimensional scaling; NSUC: non-severe ulcerative colitis.

Microbiota diversity at three months

At three months, 385 taxa were present in the 35 available samples (20 biopsies and 15 stool samples). Alpha-diversity measured by the observed richness and the Shannon index were not different between the two groups. Only the Simpson index was significantly lower in the acute severe ulcerative colitis group compared to the non-severe ulcerative colitis group (Figure 6A, p = 0.03). No difference regarding alpha-diversity at three months was observed between patients with response to therapy at three months and those who did not respond (Figure 6B). Concerning sampling time-point, alpha-diversity measured by the Shannon and the Simpson index was higher at three months compared to enrollment but not using observed richness (p = 0.03, p = 0.03 and p = 0.06 respectively, Figure 6C, Supplementary Figure 5A).

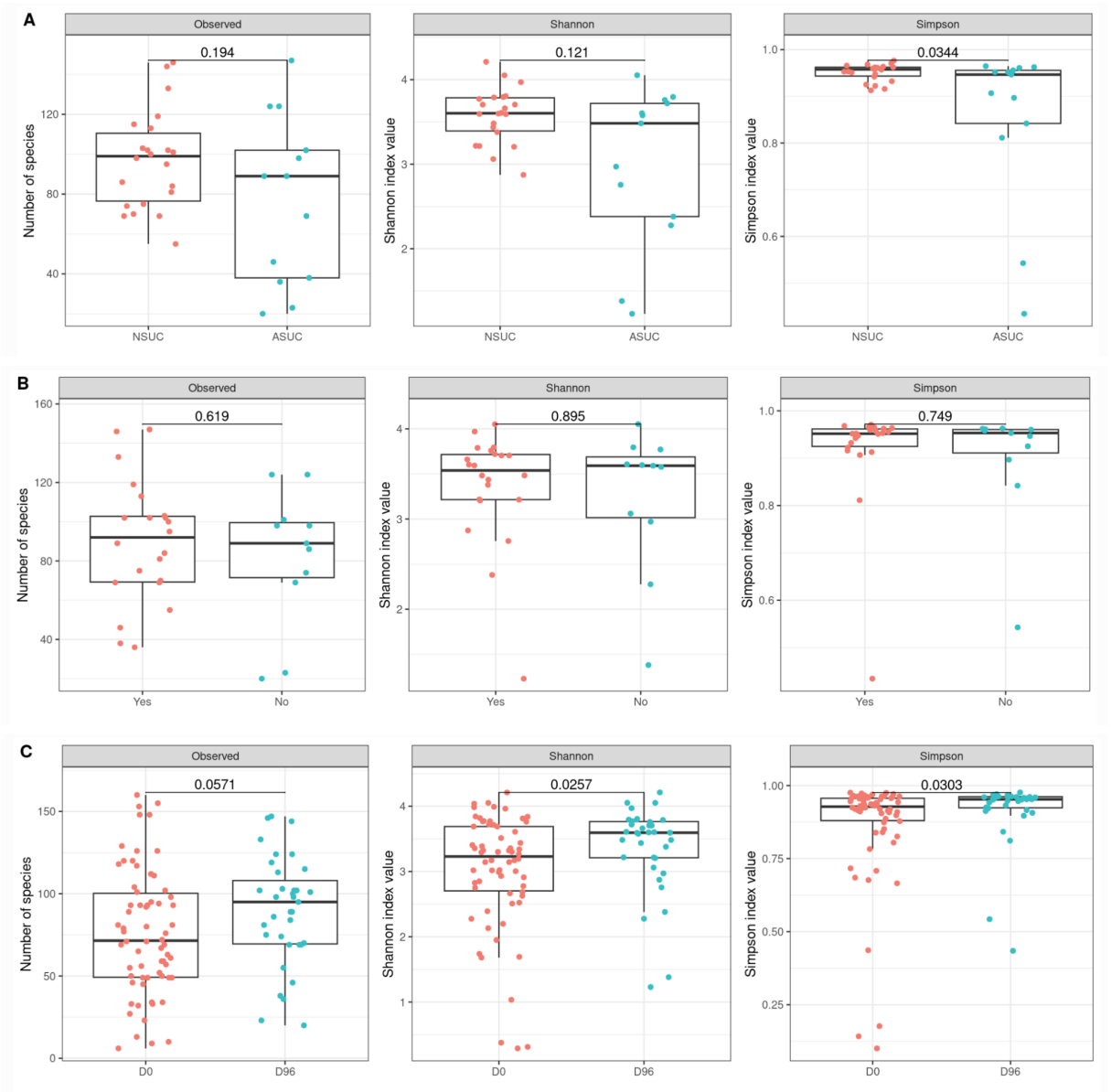


Figure 6: Alpha-diversity is partially restored after three months.

Panel A: Box-plot representing the alpha-diversity at three months in the two groups (n=35) using three different indices displayed as Y-axis. Red and blue dots correspond to non-severe and acute severe colitis groups respectively. Only the Simpson index shows a significant difference between the two severity groups.

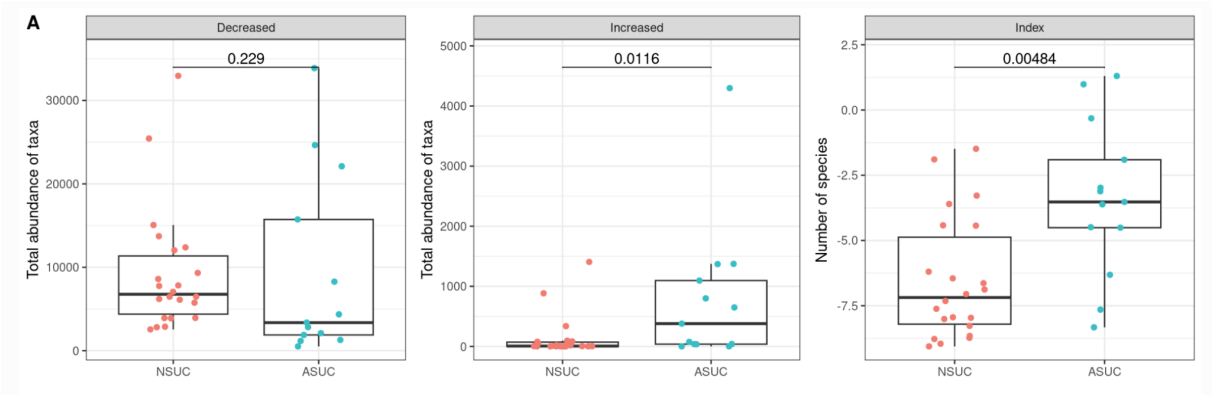
Panel B: Box-plot representing the alpha-diversity at three months according to response to therapy at three months (n=35). Red and blue dots correspond to responders and non-responders respectively. Alpha-diversity at three months is not associated with response to therapy at three months.

Panel C: Box-plot representing the alpha-diversity of the gut microbiota at enrollment and at three months in all patients (n=101). Red and blue dots correspond to enrollment (D0) and three months (D96) respectively. Alpha-diversity is increased at three months compared to enrollment according to the Shannon and Simpson indices.

The box in the plot represents the middle 50% of the data, with the median line dividing the box into two parts. The whiskers extend from the box to indicate the variability outside the upper and lower quartiles. Each dot represents one sample. P-values were calculated using a Wilcoxon test to compare the groups. ASUC: acute severe ulcerative colitis; NSUC: non-severe ulcerative colitis.

Left subpanel: Observed richness, corresponding to the number of different species observed by sample. Middle subpanel: Shannon index takes into account the number of species and their abundance. Right subpanel: Simpson index takes into account the number of species and their abundance and is more sensitive to their abundance than the Shannon index.

At three months, the microbial dysbiosis index remained significantly higher in the acute severe ulcerative colitis group ($p < 0.01$), the relative abundance of taxa known to be decreased in IBD was not different in the acute severe ulcerative colitis group while the relative abundance of taxa known to be increased in IBD was higher in the acute severe ulcerative colitis group (Figure 7A). Concerning sampling time-points, the microbial dysbiosis index was significantly lower at three months compared to enrollment ($p = 0.03$), without significant difference when looking specifically to the relative abundance of taxa known to be decreased in IBD and to the relative abundance of taxa known to be increased in IBD ($p = 0.37$ and $p = 0.05$, respectively, Figure 7B, Supplementary Figure 5B).



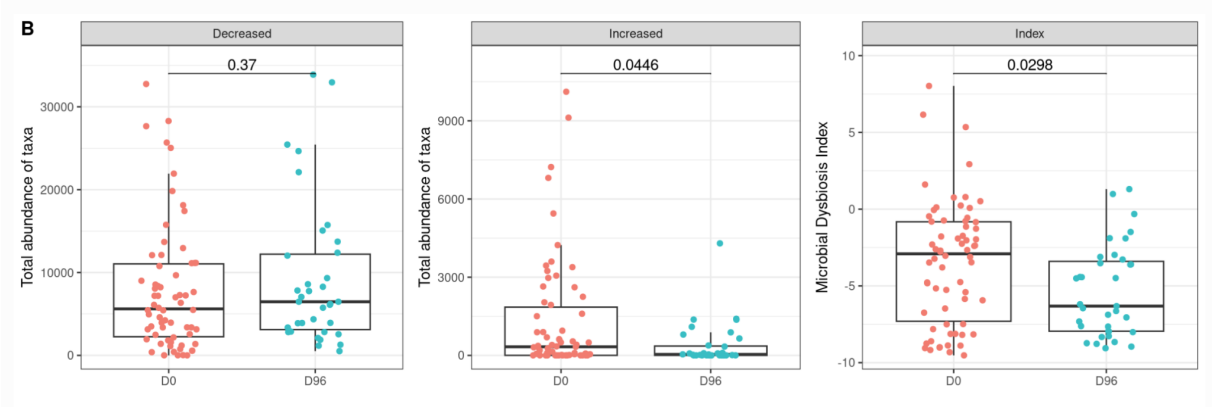


Figure 7: The microbial dysbiosis index remains increased in the severe group after three months.

Panel A: Box-plot representing the microbial dysbiosis index at three months in the two groups (n=35 samples). Red and blue dots correspond to non-severe and acute severe colitis groups respectively. ASUC: acute severe ulcerative colitis; NSUC: non-severe ulcerative colitis.

Panel B: Box-plot representing the microbial dysbiosis index at enrollment and at three months in all patients (n=101). Red and blue dots correspond to enrollment (D0) and three months (D96) respectively.

The box in the plot represents the middle 50% of the data, with the median line dividing the box into two parts. The whiskers extend from the box to indicate the variability outside the upper and lower quartiles. P-values were calculated using a Wilcoxon test to compare the two severity groups. Each dot represents one sample.

Left subpanel: Total abundance of taxa known to be decreased in IBD in Y-axis, as described by Gevers et al[29]. Middle subpanel: Total abundance of taxa known to be increased in IBD in Y-axis, as described by Gevers et al[29]. Right subpanel: Overall index corresponding to the log of [total abundance in organisms increased in IBD] over [total abundance of organisms decreased in IBD] in Y-axis, as described by Gevers et al[29].

Microbiota composition at three months

At three months, composition of the communities was significantly different between the two groups (PERMANOVA p = 0.01). No significant difference was observed at the phylum level between the two groups (Supplementary Figure 6). At the genus level, one taxon was significantly decreased in the acute severe ulcerative colitis group *Phascolarctobacterium* from the Firmicutes phylum, Negativicutes family (ANCOMBC p < 0.01 and LEFSe p = 0.04, ANCOMBC log fold change – 1.35). Concerning sampling time-point, composition of the communities were significantly different comparing enrollment and three months

(PERMANOVA p = 0.02). No significant difference was observed at the phylum level between the two time-points (Supplementary Figure 7). At the genus level, one taxon was significantly increased at three months *Blautia* from the Firmicutes phylum, Clostridium family (ANCOMBC p = 0.03 and LEFSe p < 0.01, ANCOMBC log fold change 1.19).

Microbiota diversity and composition at enrollment according to response to therapy at three months

We analysed the 66 samples taken at enrollment to identify microbiome features predictive of response to therapy at three months. Alpha-diversity at enrollment measured by the observed richness, the Shannon index and the Simpson index and the microbial dysbiosis index were not different between patients who would respond at three months and patients who would not (Figure 8, Supplementary Figure 8). Composition of the communities at enrollment was significantly different between responders and non-responders at three months (PERMANOVA p = 0.02). At the phylum level, no difference was observed (Supplementary Figure 9). At the genus level, one taxon was significantly increased at enrollment in the group of responders at three months – *Faecalibacterium* from the Firmicutes phylum and Clostridia family (ANCOMBC p < 0.01 and LEFSe p = 0.02, ANCOMBC log fold change 0.53) and one taxon was significantly decreased at enrollment in the group of responders at three months – *Catenibacterium* from the Firmicutes phylum and Bacilli family (ANCOMBC p < 0.01 and LEFSe p = 0.04, ANCOMBC log fold change – 0.79).

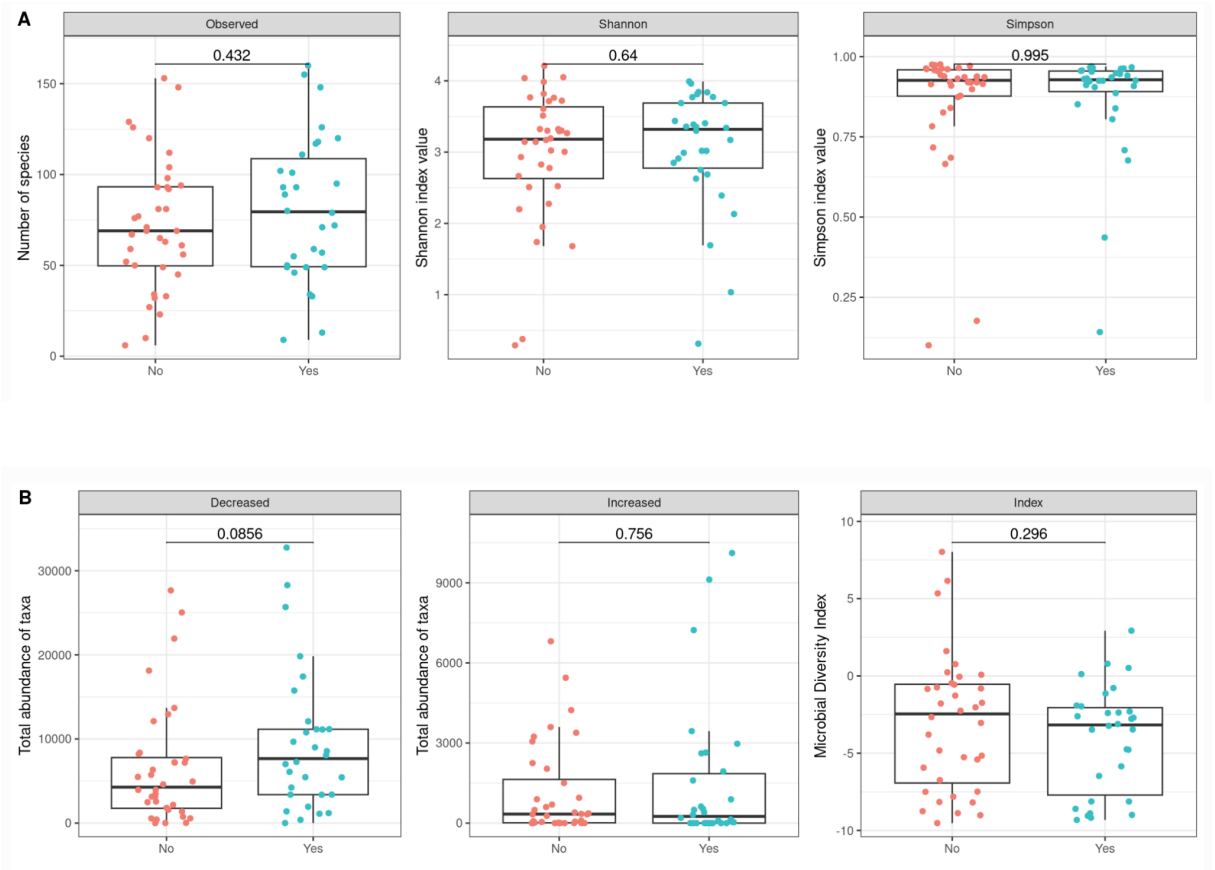


Figure 8: Alpha-diversity at enrollment does not predict response to therapy.

Panel A: Box-plot representing the alpha-diversity of the gut microbiota at enrollment according to response to therapy at three months (n=66). Red and blue dots correspond to non-responders and responders at three months respectively. Left subpanel: Observed richness, corresponding to the number of different species observed by sample. Middle subpanel: Shannon index takes into account the number of species and their abundance. Right subpanel: Simpson index takes into account the number of species and their abundance and is more sensitive to their abundance than the Shannon index.

Panel B: Box-plot representing the microbial dysbiosis index at enrollment according to response to therapy at three months (n=66). Red and blue dots correspond to non-responders and responders at three months respectively. Left subpanel: Total abundance of taxa known to be decreased in IBD in Y-axis, as described by Gevers et al[29]. Middle subpanel: Total abundance of taxa known to be increased in IBD in Y-axis, as described by Gevers et al[29]. Right subpanel: Overall index corresponding to the log of [total abundance in organisms increased in IBD] over [total abundance of organisms decreased in IBD] in Y-axis, as described by Gevers et al[29].

The box in the plot represents the middle 50% of the data, with the median line dividing the box into two parts. The whiskers extend from the box to indicate the variability outside the upper and lower quartiles. P-values were calculated using a Wilcoxon test to compare the two severity groups. Each dot represents one sample.

3.6. Discussion

The objective of our study was to compare known pathogens, microbiota disruptors, microbiota diversity and composition in patients with acute severe ulcerative colitis with those in patients with non-severe ulcerative colitis.

For this, we first identified candidate pathobionts in the gut microbiota of patients with acute severe ulcerative colitis. We used a well-phenotyped cohort of 41 ulcerative colitis patients, divided into acute severe and non-severe groups. We analysed clinical and biological features, as well as microbiota characteristics from stool and rectal biopsies at enrollment and at three months. At enrollment, we found no evidence of overt infectious colitis in our cohort or significant differences regarding diet intake between the two groups. In acute severe ulcerative colitis, a higher proportion of patients received antibiotics within three months before enrollment. However, the indication for antibiotic treatment was diarrhoea for all but one case, precluding the role of antibiotics as a flare trigger. In patients with acute severe ulcerative colitis, we observed substantial alterations in the gut microbiota, including *i)* reduced alpha-diversity, *ii)* increased abundance of Proteobacteria, specifically *Escherichia/Shigella* genus members and *iii)* a decrease in Lachnospiraceae and Ruminococcaceae family members. These changes were reflected in the microbial dysbiosis index and correlated with systemic inflammation, as measured by CRP levels.

The reduction in gut microbial diversity is a well-documented characteristic of IBD[34,35]. We demonstrated a further reduced diversity in patients with acute severe ulcerative colitis, aligning with recent reports[36,37]. Notably, our investigation revealed a consistent linear inverse correlation between systemic inflammation, a hallmark of acute severe ulcerative colitis, and microbiome diversity. However, it is important to acknowledge that our study design does not allow for definitive conclusions regarding whether reduced microbial diversity is a cause or a consequence of systemic inflammation. Local inflammation alters the microbiome diversity,

as exemplified by the antimicrobial properties of faecal calprotectin, a widely used biomarker of colonic inflammation in IBD[38]. An increased antimicrobial activity of the colonic mucosa has been identified in IBD[39]. Furthermore, factors such as leukocyte infiltration in the lamina propria and epithelial cell loss related to ulcerations can contribute to modifications of the gut microbiome. This can result in increased availability of oxygen and iron, primarily derived from heme degradation, and high levels of oxidative stress, selecting microbial taxa able to adapt to these conditions[40].

The inflammatory environment may favour bacteria originating from the oral microbiota, readily adaptable to high levels of oxygen[37,41]. In line with this hypothesis, we observed an increase in members of the oral microbiota in the acute severe ulcerative colitis group, such as *Parvimonas*, *Actinomyces*, *Lactobacillus* and *Enterococcus*. We also demonstrated an increase in *Escherichia/Shigella* in the acute severe ulcerative colitis group. This genus contains several *Shigella* species and *Escherichia coli*. All included patients had a stool culture with negative specific detection of *Shigella*. Thus, the increase in the genus may be driven by an expansion of *E. coli* in patients with acute severe ulcerative colitis. Several strains of *E. coli* are increased in patients with ulcerative colitis compared to healthy controls and their presence is associated with increased levels of faecal calprotectin[40,42,43]. Proliferation of *E. coli* bacteria could be favoured by their ability to thrive among high concentrations of iron and reactive oxygen species associated with colonic inflammation[40]. However, more than only a consequence of colonic inflammation, a pathogenic role of *E. coli* in ulcerative colitis is suspected. In a randomised controlled trial evaluating the benefit of *E. coli* Nissle as add-on treatment to conventional therapies in ulcerative colitis, a worsening effect was observed in the treated group[42]. Adherent-invasive E. coli isolated from patients with IBD are associated with increased expression of *TNF* and *IL-17 in vitro*[43]. Testing different E. coli strains in a gnotobiotic mice model of chemically induced colitis, Kittana *et al.* demonstrated

strain-specific induction of cytokines and severity of inflammation. Specifically, two strains of *E. coli* were associated with high levels of IL-6 production and severe phenotype[44]. In humans, IL-6 blood levels are associated with ulcerative colitis activity[45]. Moreover, IL-6 is the main driver of CRP production, the hallmark of acute severe ulcerative colitis[46]. In acute severe ulcerative colitis, specific strains of *E. coli* favoured by the local conditions might exacerbate further colonic and systemic inflammation.

The microbiota changes we observed in patients with acute severe ulcerative colitis were also characterised by a decrease in taxa from the Firmicutes phylum, and more specifically from the Lachnospiraceae and Ruminococcaceae families. As described in previous reports[37,41], the abundance of these butyrate-producing bacteria is known to be decreased in patients with active IBD. Their reduction could have a detrimental effect because butyrate plays a crucial role in promoting barrier function of the epithelial cells and inhibiting various inflammatory pathways, including NF-kB and IL-8[47]. We also observed that a lack of *Faecalibacterium prausnitzii (F.prau)* at enrollment was associated with non-response to therapy at three months. Our study was not designed and powered to identify predictors of response to therapy. However, it is noteworthy that *F.prau*, the most replicated finding in the study of the microbiota of IBD[15,39,48], emerged as a response predictor in our small cohort. On top of butyrate, *F.prau* produces metabolites that are able to inhibit NF-kB activation and IL-8 production and to promote IL-10 production[48].

Once more, it remains challenging to ascertain whether the reduction in these bacteria signifies a primary event in ulcerative colitis or, alternatively, a consequence of the inflammatory process. Clues can be obtained by studying scenarios in which inflammation and alterations of the microbiota do not coexist, such as in non-inflamed sections of the colon in ulcerative colitis patients or unaffected relatives of those patients. Investigating the microbiota diversity at different sites of inflamed colon, Sepehri *et al.* found that diversity

was reduced in inflammatory lesions but increased in non-inflamed lesions compared to healthy controls[34]. This suggested the existence of a recruitment phase of pathobionts in the non-inflamed tissue, increasing diversity, leading to the onset of inflammation and finally, a reduction of diversity associated with inflammation. However, this study was performed more than 15 years ago precluding to address the question of the identities of the increased taxa driving the increased diversity in the non-inflamed tissue. In an elegant study investigating the gut microbiota of pairs of twins discordant for ulcerative colitis, Lepage *et al.* found that Lachnospiraceae and Ruminococcaceae were increased in unaffected twins compared to twins with ulcerative colitis but also to healthy controls[35]. They posited that this may constitute a compensatory mechanism protecting these unaffected twins, carrying genetic susceptibility to gut inflammation, against the onset of ulcerative colitis, through the anti-inflammatory properties of these bacteria. On the mucosal side, Verstockt *et al.* recently demonstrated that first-degree relatives of IBD patients displayed an inflammatory state characterised by up-regulation of pro-inflammatory cytokines, including IL-6 and IL-8, and pathways involved in myeloid migration[49]. They did not analyse the microbiota of these patients; however, their results align with the hypothesis proposed by Lepage *et al*. The genetic susceptibility to IBD may induce an inflammatory state, which, in turn, could be counterbalanced by a 'hyper-healthy' microbiota. In acute severe ulcerative colitis, an opposite mechanism may occur: a microbiota lacking anti-inflammatory taxa may fail to compensate for colonic inflammation, which would be triggered or exacerbated by the expansion of pathobionts.

The main limitation of our study is the relative modest sample size. This could explain the absence of known pathogens, i.e. *C.diff* or CMV, found in our cohort despite extensive microbiological testing. We combined stool and biopsy samples to increase the power of our analysis. However, a sensitivity analysis of each sample type separately retrieved the same findings (data not shown). A higher proportion of patients in the acute severe ulcerative colitis

group had been exposed to antibiotics within three months before enrollment. This may have impacted the diversity of the gut microbiome. However, the reduced diversity in the acute severe ulcerative colitis group remained after exclusion of patients exposed to antibiotics (Supplementary Figure 10). The cross-sectional design of our study does not allow us to draw conclusions about causality. To study the potential triggers in acute severe ulcerative colitis, it would have been ideal to follow a cohort of newly diagnosed ulcerative colitis patients, collect biological samples and go back to these samples if an acute severe ulcerative colitis would occur to identify a possible trigger. However, this type of study is hardly feasible due to the unpredictability of the event. Patients would need to be sampled very often to capture the triggering modification(s).

In conclusion, we identified *E. coli* and members of the oral microbiota as putative pathobionts in acute severe ulcerative colitis, proliferating among a less diverse gut microbiota lacking bacteria from the Lachnospiraceae and Ruminococcaceae families able to counterbalance their pro-inflammatory effects. These findings argue in favour of therapeutic modulation of the microbiota to treat acute severe ulcerative colitis. Trials investigating the benefit of antibiotics in this indication have yielded conflicting results[50–52]. This could be attributed to the broad spectrum of antibiotics employed, which not only eliminate pathobionts but also affect the butyrate-producing bacteria. Modulation of the microbiome by diet, and specifically by enteral nutrition, recently emerged as an adjunct therapy to intravenous steroids in acute severe ulcerative colitis, with the publication of a positive open-label randomised trial in 2021[53]. Although clinical trials are difficult to envision in this situation with life-threatening complications, there is a need for research efforts to be directed towards the modulation of the microbiome in patients with acute severe ulcerative colitis.

3.7. References

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4. Chapter 4: Single-cell RNASeq of acute severe ulcerative colitis lesions unveils a pathogenic loop involving the adaptive and innate immune systems focused on the IL-23 axis.

4.1. Preface

Due to the complex relationship between the host and gut microbiota, following our investigation of the microbiota, we proceeded to examine the immune characteristics of the colonic mucosa in cases of acute severe ulcerative colitis. Our objectives were *i)* to assess discrepancies in the mucosal inflammatory profiles of acute severe ulcerative colitis patients and patients with non-severe ulcerative colitis and *ii)* to identify the specific cellular subtypes and signalling pathways characterising acute severe ulcerative colitis. We used rectal biopsy samples collected at enrollment on a subset of patients enrolled in the ITAC study. I analysed the transcriptomic profiles of the samples by single-cell RNASeq technology. I performed the digestion of the biopsies, the preparation of the cDNA libraries and the bioinformatics analysis. I analysed four patients with acute severe ulcerative colitis and five with non-severe ulcerative colitis. The sample size was determined by the available funding and the substantial costs associated with single-cell RNASeq technology.

This work corresponds to a manuscript currently in preparation.

Specific author's contributions:

P Rivière conceived the study, wrote the clinical research protocol, coordinated the sample logistics, designed and tested the digestion protocol, performed the digestion and single cell suspension preparation for all samples, prepared the libraries for all samples except two, analysed the data, drafted and corrected the manuscript.

M Dallmann-Sauer provided theoretical and practical support for the analysis of the data including discussion about analysis methods and sharing of code.

T Bessissow was involved in the study's design and contributed to patient recruitment.

X Treton was involved in the study's design and contributed to patient recruitment.

M Uzzan was involved in the study's design and contributed to patient recruitment.

F Poullenot was involved in the study's design and contributed to patient recruitment.

F Zerbib was involved in the study's design and contributed to patient recruitment.

M Saleh was involved in the study's design and made contributions to establishing the digestion protocol.

J Giraud made contributions to establishing the digestion protocol, helped with the samples management and trained PR for the preparation of libraries.

VM Fava provided theoretical and practical support for the analysis of the data including discussion about analysis methods and sharing of code.

D Laharie conceived the study, contributed to study coordination and patient recruitment and critically revised the manuscript.

E Schurr conceived the study, provided theoretical and practical support for the analysis of the data and critically revised the manuscript.

Mucosal immune response in acute severe ulcerative colitis is characterised by a pathogenic loop involving the adaptive and innate immune systems and focused on the IL-23 axis.

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4.2. Abstract

Background: Acute severe ulcerative colitis represents a distinct phenotype of ulcerative colitis flare, featuring systemic inflammation alongside classical bloody diarrhoea. Recent advances in single-cell RNA sequencing (scRNASeq) have elucidated mucosal immune mechanisms in ulcerative colitis pathogenesis. We applied scRNASeq to assess cellular composition and gene expression in the colonic lamina propria of acute severe ulcerative colitis patients.

Methods: We obtained rectal biopsies from nine patients (four with non-severe and five with severe ulcerative colitis) and performed scRNASeq using the 10x Genomics platform. Bioinformatics analysis was conducted with Seurat v3.

Results: We generated 31,480 high-quality single-cell RNASeq profiles for immune cells and 1,307 for stromal cells. In severe cases, plasmablasts exhibited a distinct transcriptomic profile with increased IgG production, and a specific T cell population expressing IL26 was expanded compared to non-severe cases. Innate immune cells displayed a pro-inflammatory profile. Both T cells and innate immune cells indicated a pro-Th17 mucosal environment.

Conclusion: Colonic mucosa in acute severe ulcerative colitis patients demonstrates heightened inflammation, involving the adaptive and innate immune systems, with a central focus on IgG production and the Th17/IL-23 pathway. These findings suggest potential avenues for further research into drugs targeting the IL-23 pathway for managing patients with acute severe ulcerative colitis.

4.3. Introduction

Acute severe ulcerative colitis is a specific phenotype of ulcerative colitis flare characterised by systemic inflammation on top of the classical bloody diarrhoea observed in mild to moderate ulcerative colitis[1]. This severe phenotype deserves specific management as it has been shown to be associated with life-threatening complications dominated by thrombo-embolic events and septic shock[2]. In recent years, there has been a steady mortality rate of 1% among patients admitted with acute severe ulcerative colitis, even within specialised referral centres[3]. Three drugs are approved for the medical treatment of patients with acute severe ulcerative colitis, intravenous steroids, a monoclonal antibody targeting the *Tumor Necrosis Factor*, infliximab, and ciclosporin[1]. Nevertheless, around 15% of patients do not respond to medical therapy and require a colectomy with potential debilitating complications[3]. To address this pressing medical gap, a deeper understanding of the pathogenesis of acute severe ulcerative colitis is required.

Ulcerative colitis is characterised by a mucosal immune dysregulation and altered gut microbiota associated with genetic and environmental factors[4]. In the last five years, the use of novel techniques such as single-cell RNA sequencing (scRNASeq) has shed light on the mucosal immune mechanisms involved in the pathogenesis of ulcerative colitis. Specifically, in 2019, Smillie *et al.* published the first single-cell atlas of the colonic mucosa of patients with ulcerative colitis. They observed alterations in the cellular composition of the colonic mucosa, including an increase in the number of activated T cells, changes in intercellular communication, with decreased interactions between T and B cells and increased interactions between Th17 cells and inflammatory monocytes, and confirmed the upregulation of the IL-23/Th17 pathway[5].

Our aim was to leverage scRNASeq in the context of acute severe ulcerative colitis to assess the cellular composition and gene expression of immune and stromal cells from the colonic lamina propria.

4.4. Materials and Methods

Study population

Nine patients participating in the ITAC study[6], an observational cohort of patients with acute severe ulcerative colitis and non-severe ulcerative colitis conducted in three inflammatory bowel disease referral centres (Bordeaux University Hospital and Beaujon Hospital in France, McGill University Health Center in Montréal, Canada) were selected at enrollment for the single-cell RNASeq substudy. Patients were offered enrollment based upon the feasibility of immediate processing of biopsies samples for single-cell RNASeq.

Severity of the ulcerative colitis was defined as follows:

- acute severe ulcerative colitis group: patients admitted with acute severe ulcerative colitis defined according to Truelove criteria, i.e. ≥6 bloody daily stools with one or more of the following criteria: temperature >37.8°C, pulse >90 beats/min, haemoglobin <10.5g/dl or C Reactive-Protein >30 mg/l[1,2]. Patients with acute severe ulcerative colitis had to be enrolled within three days of admission.

- non-severe ulcerative colitis group: patients seen in outpatients clinic with disease activity symptoms, corresponding to a partial Mayo score of 4 or more with a rectal bleeding subscore of 1 or more, without Truelove severity criteria[7], whatever previous or on-going medical therapy.

We excluded patients with perianal lesions, ileal lesions or endoscopic lesions suggestive of Crohn's disease acute severe colitis.

In July 2020, four patients (two acute severe ulcerative colitis and two non-severe ulcerative colitis) were enrolled in this substudy. Between February 2021 and November 2021, an additional five patients (two acute severe ulcerative colitis and three non-severe ulcerative colitis) were incorporated into the substudy cohort leading to a total of nine patients (four acute severe ulcerative colitis and five non-severe ulcerative colitis). The clinical characteristics of the nine patients are described in Supplementary table 1.

Sampling and biopsies processing

The digestion protocol was established following several iterations of experimentation, guided by previously published protocols[5,8], with the aim of optimising cell viability yield. During flexible sigmoidoscopy at enrollment, four rectal biopsies were taken using standard disposable biopsy forceps. Upon collection, the biopsies were promptly placed in ice-cold RPMI1640 and transported to the laboratory on the same day for subsequent processing. Only two patients were sampled simultaneously, whereas the remaining samples were individually processed on distinct days.

First, to separate the epithelial layer, the biopsies were placed in five millilitres of dissociation medium (comprising HBSS Ca/Mg-free, EDTA at 5 mM concentration and HEPES at 10 mM concentration) and subjected to agitation using the gentleMACS system. The program *37m_LDK_1* was employed for 15 minutes, followed by the *m_intestine_1* program. Subsequently, the solution was passed through a 70 μm cell strainer, with the filtrate (primarily consisting of epithelial cells) being discarded. Five millilitres of enzymatic digestion mix (comprising HBSS Ca/Mg+, dispase at 0.25 U/ml concentration, liberase at 100 μg/ml concentration and DNAse at 100 μg/ml concentration) were introduced to the residual material on the filter, which corresponded to the lamina propria. This mixture was then subjected to agitation using the gentleMACS system, utilising the *37_h_TDK_2* program.

Following this, the cells were filtered through a 40 μm cell strainer, washed, and centrifuged in complete RPMI for 8 minutes at 400g.

Subsequently, red blood cells were lysed by adding a two millilitres RBC lysis buffer, and the cells were incubated five minutes at 4°C before being washed in PBS/0.04% BSA. Cell counting was performed using a hemocytometer with viability staining conducted using eosine. A suspension of 20,000 cells was prepared in PBS/0.04% BSA, resulting in a total volume of 52.4 μL.

Generation of Single Cell 3' Gene Expression libraries, sequencing and assignation

The single-cell suspension was processed in the 10x Genomics Chromium Single Cell Controller according to manufacturer's instructions and the Chromium Next GEM Single Cell 3' Reagent Kits v3.1. In brief, immediately after preparing the single cell suspension, 16,000 cells were loaded for the GEM Generation and Barcoding. The next day, after post-GEM reverse transcription cleanup, the cDNA was amplified with 12 PCR cycles. Subsequently, 3' gene expression libraries were prepared with 12 PCR cycles, one to four samples per batch depending on the time elapsed between the samples collection.

Barcoded reverse transcription libraries were then sequenced in one single batch on an Illumina NovaSeq at 30,000 reads/cell. Cell Ranger v7 was employed to generate the gene expression matrix for each sample utilising the hg38 human reference genome for alignment. The median (IQR) number of reads per sample was 314,589,034 (271,718,499-330,705,366) and the median (IQR) estimated number of cells per sample was 10,639 (9,706-12,572) with a median (IQR) number of genes per cell per sample at 1,580 (1,088-1,707). Quality metrics after alignment by sample are displayed in Supplementary Table 2.

Pre-processing of scRNAseq data by sample

All subsequent analyses were conducted using R version 4.1.0 with the Seurat package version 3.2.3[9]. A schematic of the analysis workflow is displayed in Supplementary Figure 1. First, the gene expression matrices were loaded in R including only cells with at least 300 genes. The median (IQR) number of cells by sample was 9,119 (8,490-10,301). Next, the raw counts of each sample were log transformed to stabilise the variance of the data and normalised by dividing each gene's expression value by the total counts in the cell and multiplied by 10,000. The 2,000 most variable genes were identified. A first round of clustering was performed by sample using the graph-based clustering function of Seurat. To verify the accuracy of the clustering by identifying cell types, the expression of canonical markers was calculated by cell and by cluster. The list of canonical markers used at this step is displayed in Supplementary Table 3. Further, a score was calculated for each cell and cluster by adding the counts of a list of canonical markers to confirm the cell and cluster broad identities (*i.e.* epithelial, immune or stromal). For epithelial cells, the genes used for the module score calculation were *EPCAM, KRT8* and *KRT18*, for immune cells, *CD52, CD2, IL7R, CD3D, CD3G, CD3E, CD79A, CD79B, CD14, FCGR3A, CD68, CD83, CSF1R, FCER1G, CD27, GZMB, IL32, BANK1* and for stromal cells, *COL1A1, COL1A2, COL6A1, COL6A2, VWF, PLVAP, CDH5, S100B*. Due to the heterogeneity of cell types within the rectal mucosa, low-quality cells were discarded within each cluster based on their gene counts, using criteria of falling below 1.5 standard deviations or exceeding 2 standard deviations in comparison to the gene counts of other cells within the same cluster. Dying cells and erythrocytes were filtered by discarding cells with a percentage of mitochondrial genes higher than 20% or expression of *HBB* gene higher than 0.5. The median (IQR) number of erythrocytes by sample was 8 (7-54). As an initial phase of doublet elimination, cells were scrutinised for co-expression of two deviant canonical markers, such as the concurrent

presence of *CD3D* or *TRAC* alongside *CD79A* and *MS4A1*, designating them as putative doublets composed of T and B cells. The details of manual doublet characterization are displayed in Supplementary Table 4. The median (IQR) number of doublet manually identified by sample was 126 (85-148). Next, doublets were identified and removed using DoubletFinder[10], which computes a metric for each cell reflecting the likelihood of that cell being a pair of cells present in the rest of sample, with a homotypic doublet proportion estimate of 7.6% and decontX[11], which is a Bayesian method to estimate the level of ambient mRNA in each cell, using a contamination cut-off of 50%. Summaries of quality metrics and filtering by sample are displayed in Supplementary Table 5.

Integration of the nine samples

To integrate the nine samples, ribosomal and mitochondrial genes were excluded from the gene expression matrices and counts were transformed using the SCTransform function in Seurat, which combines a variance stabilisation and a regularised negative binomial regression to account for sources of unwanted variability such as technical covariates. Integration was then performed using robust principal component analysis to identify pairs of cells from the nine gene expression matrices that are likely to correspond to the same biological entities, called anchors in Seurat. The module scores, as previously mentioned, which corresponds to epithelial, immune and stromal cells gene signatures were computed for each cluster. Subsequently, cells were allocated to one of the three compartments based on the highest score attained in the calculation. All subsequent analyses were performed analysing all samples by compartment. In each of the immune and stromal compartments, the integrated data was split by sample before repeating preprocessing using SCTransform. Integration was reiterated specifically for immune cells. Specific integration was not performed for stromal cells due to the restriction imposed by the Seurat package's integration function, which

necessitates a minimum of 200 cells per sample—a condition that was not met for the stromal cell compartment.

Successive iterations of clustering, data cleansing, and integration

Following integration by compartment, cells were clustered using the Seurat graph-based Louvain algorithm grouping cells sharing similar gene expression patterns into clusters, with 30 dimensions of reduction and a resolution of 0.5. Cells were then plotted using the Uniform Manifold Approximation and Projection (UMAP) method. UMAP is an algorithm employed for dimensionality reduction, particularly useful for visualising high-dimensional data in a lower-dimensional space. It arranges similar data points (such as cells in our case) closer to each other and distant points farther apart on the lower-dimensional plot, preserving their high-dimensional similarities. In the resulting plot, cells with similar gene expression profiles are positioned closer to each other. Clusters were visually inspected on the UMAP and cells projecting beyond the boundaries of their assigned cluster were manually removed from the dataset. Following this, the dataset was again split by sample before repeating preprocessing using SCTransform and integration as described above. These steps were repeated until all outlier cells had been successfully eliminated. Clusters of immune cells were assigned to broad immune cell types based on canonical marker expression (Supplementary Table 3) and separated in three groups: B cells, T cells, myeloid cells, later divided into neutrophils and myeloid cells, and mast cells. Successive iterations of clustering, data cleansing, and integration - if the condition of 200 cells by sample was met - were repeated for each broad cell type separately. When all samples except one had more than 200 cells, the sample was excluded and integration was performed. A summary of these iterative filtering steps is displayed in Supplementary Table 6.

Annotation, comparison of proportions, differential gene expression and pathway analysis at the cluster level

The likely identity of clusters among high-quality B cells, T cells, myeloid cells, neutrophils, mast cells and stromal cells was determined by assessing the expression of canonical markers and contrasting their gene expression profiles with those of other clusters within the same overarching cell type. To achieve this, the FindMarkers function from Seurat was used to compare the gene expression profiles of cells in one cluster to the remaining cells within the same cell type, for example neutrophils cluster 0 *versus* the rest of neutrophils. Genes detected in at least 25% of cells in either population were subjected to the hurdle model-based analysis of single-cell transcriptomics (MAST) test to determine significance of differential expression.

The proportion of clusters within a cell type was compared between patients with acute severe ulcerative colitis and those with non-severe ulcerative colitis using a Wilcoxon test.

Within each cluster, the gene expression profile of patients with acute severe ulcerative colitis was contrasted with patients with non-severe ulcerative colitis using the FindMarkers function from Seurat. Genes detected in at least 10% of cells in either group were subjected to the Wilcoxon Rank Sum test to determine significance of differential expression. Genes exhibiting a minimum log2 fold change of 0.1 and an adjusted p-value below 0.05 between the two groups were deemed differentially expressed. Pathway analysis was performed using the clusterProfiler package[12] to identify overrepresented biological processes among differentially expressed genes between acute severe ulcerative colitis and non-severe ulcerative colitis patients based on the Gene Ontology (GO)[13], the Kyoto Encyclopedia of Genes and Genomes (KEGG)[14] and the Reactome[15] resources. This analysis aimed to identify terms that are significantly enriched in the differentially expressed gene set compared to the non-differentially expressed genes.

General statistical considerations

The analyses were performed on available data without imputation of missing data. All estimates were performed with a type I error rate of 5%. Qualitative variables were described by numbers and percentage and quantitative variables by median, range, and interquartile range and compared using a chi-square test and a Student's t-test, respectively. All statistical analyses were performed using R (version 3.5.1).

4.5. Results

Atlas of the immune and stromal cells from severe and non-severe patients

After processing rectal biopsies from four patients with acute severe ulcerative colitis and five patients with non-severe ulcerative colitis and integration as described previously, we successfully generated 31,480 high-quality single-cell RNAseq profiles of immune cells and 1307 of stromal cells (Figure 1A-B). Clinical and biological characteristics of patients are displayed in Supplementary Table 1. Among immune cells, 13,047 pertained to patients with acute severe ulcerative colitis (n = 3) and 18,433 to patients with non-severe ulcerative colitis (n = 5) (Figure 1C). We excluded one sample from a patient diagnosed with acute severe ulcerative colitis due to a potential technical issue encountered during the library preparation process, which resulted in the inclusion of only 90 immune cells in the analysis. Using canonical markers (Supplementary Table 3), we identified 13,025 profiles of B cells, 9,699 T cells, 4,671 myeloid cells, 3,262 neutrophils and 823 mast cells (Figure 1D). The distribution of cell subtypes exhibited significant heterogeneity across the patient cohort (Supplementary Figure 1).

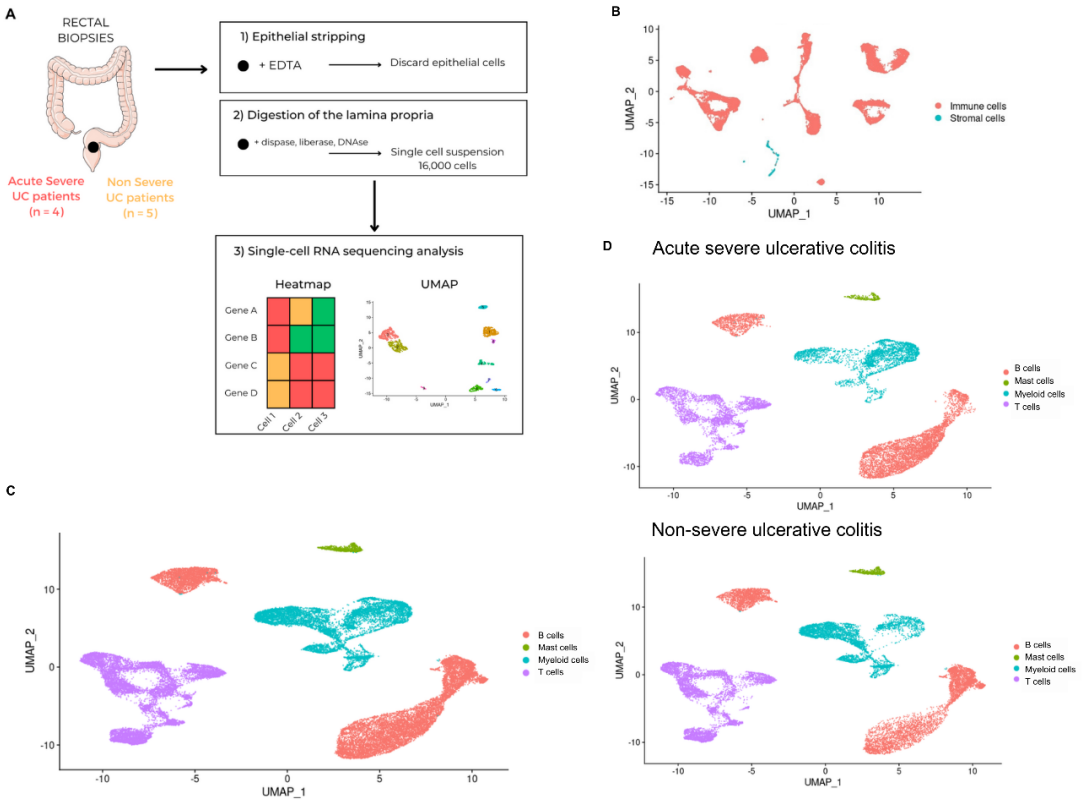


Figure 1: visualisation of the 32,787 immune and stromal cells with a high quality transcriptomic profile.

Panel A: Rectal biopsies collected in four patients with acute severe ulcerative colitis and five patients with non-severe ulcerative colitis (four biopsies by patient) were digested using a cocktail of enzymes and processed for scRNASeq in the 10x Genomics platform. Sequencing data was processed using CellRanger and Seurat to obtain gene expression profile by cell (detailed workflow in Supplementary Figure 1). UC: ulcerative colitis.

Panel B: Uniform Manifold Approximation (UMAP) visualisation of the 32,787 immune and stromal cells retained after quality filtering. In the UMAP plot, each dot represents a cell. Cells with similar gene expression profiles are positioned closer to each other. Stromal and immune cells were annotated using module gene scores adding the counts of a list of canonical markers; for immune cells, *CD52, CD2, IL7R, CD3D, CD3G, CD3E, CD79A, CD79B, CD14, FCGR3A, CD68, CD83, CSF1R, FCER1G, CD27, GZMB, IL32, BANK1* and for stromal cells, *COL1A1, COL1A2, COL6A1, COL6A2, VWF, PLVAP, CDH5, S100B.*

Panel C: UMAP visualisation of the 31,480 immune cells. Cells were clustered using the Seurat algorithm. Clusters of immune cells were annotated using module gene scores. The list of canonical markers used is displayed in Supplementary Table 3.

Panel D: UMAP visualisation of the 31,480 immune cells according to the severity group. Top, cells from patients with acute severe ulcerative colitis (n=13,047); bottom, cells from patients with non-severe ulcerative colitis (n=18,433).

Severity of the colitis is characterised by Ig class switching among B cells

Among the B cells, 5,916 originated from patients with acute severe ulcerative colitis (n=3 patients) and 7,109 from patients with non-severe ulcerative colitis (n=4 patients). Six clusters of B cells were identified. Cluster annotation was performed by examining the differential expression of genes in each cluster compared to the remaining cells. Clusters expressing genes corresponding to immunoglobulins (*IGH*) were classified as plasmablasts. Clusters exhibiting higher expression of *CD83* and *MS4A1* (CD20) genes without expression of immunoglobulin genes were classified as activated B cells. Based on this classification, we identified four clusters of plasmablasts, with two of them showing a higher expression of immunoglobulin G genes (*IGHG*) and classified as IgG+ plasmablasts, and the other two displaying a higher expression of immunoglobulin A genes (*IGHA*) and classified as IgA+ plasmablasts. In addition, three clusters were identified as activated B cells (Figure 2A-C).

Comparing the proportion of B cell clusters in each of the severity group, IgG+ plasma cells cluster 1, was more frequent in patients with acute severe ulcerative colitis whereas IgA+ plasma cells cluster 1, demonstrated a relatively higher prevalence in patients with non-severe ulcerative colitis without showing a significant difference (Figure 2D, p=0.057 for comparison of IgG+ plasmablast cluster 1 ratio in severe patients compared to non-patients using a Wilcoxon test).

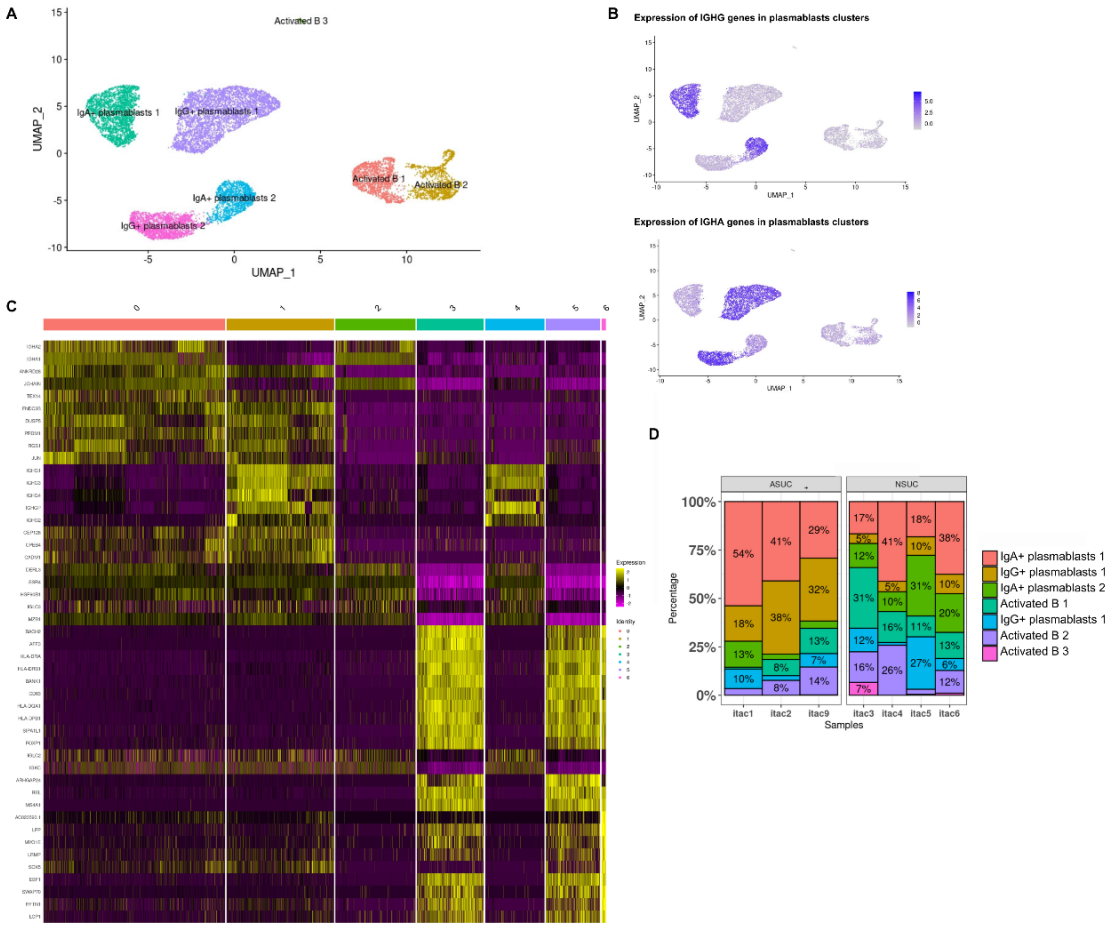


Figure 2: Clusters of plasmablasts and activated B cells.

Panel A: UMAP visualisation of the 13,025 B cells. Cells were clustered using the Seurat algorithm. Clusters of B cells were annotated using the expression of immunoglobulin genes (for IgG, *IGHG1, IGHG2, IGHG3, IGHG4*; for IgA, *IGHA1, IGHA2*) and canonical markers for B cells (*CD83* and *MS4A1*).

Panel B: visualisation of the expression of IgG genes on top (*IGHG1, IGHG2, IGHG3, IGHG4*) and IgA genes (*IGHA1, IGHA2*), bottom, by colouring individual cells on the UMAP plot according to the level of expression of each gene in each cell (yellow - high expression, purple - low expression).

Panel C: Heatmap of the genes differentially expressed in each cluster of B cells compared to the remaining clusters. The rows of the heatmap represent the 10 genes with the highest log fold change for each cluster, and the columns represent the cells, ordered by clusters. The colours represent the level of expression.

Panel D: Distribution of the B cells clusters in each sample, grouped by severity phenotype (ASUC: acute severe ulcerative colitis, NSUC: non-severe ulcerative colitis). Each column represents a patient. coloured bars represent the percentage of cells (Y-axis) within each patient originating from each cluster among all B cells in this patient.

We calculated the combined counts of IgG heavy chain genes (*IGHG1, IGHG2, IGHG3, IGHG4*) and IgA heavy chain genes (*IGHA1, IGHA2*) in the four plasmablast clusters to quantify IgG and IgA production. The cumulative expression of *IGHG* genes by plasmablasts was higher in patients with acute severe ulcerative colitis compared to non-severe ulcerative colitis although no difference was observed for IGHA genes (Figure 3A).

We investigated the differentially expressed genes within each cluster comparing cells from patients with acute severe ulcerative colitis and patients with non-severe ulcerative colitis. IgA+ plasmablasts clusters exhibited 1211 differentially expressed genes between the two groups, 96 being lower expressed and 776 higher expressed in IgA+ plasmablasts cluster 1, and 7 lower expressed and 332 higher expressed in IgA+ plasmablasts cluster 2 (Figure 3B). Notably, the *IGHG4* gene was higher expressed in patients with acute severe ulcerative colitis in the IgA+ plasmablasts cluster. IgG+ plasmablasts clusters exhibited 1198 differentially expressed genes between the two groups, 71 being lower expressed and 697 higher expressed in IgG+ plasmablasts cluster 1, and 15 lower expressed and 415 higher expressed in IgG+ plasmablasts cluster 2 (Figure 3C). Differentially expressed genes were tested for enrichment in specific terms and pathways using GO terms, KEGG and Reactome. Clusters of IgA+ and IgG+ plasma cells from patients with acute severe ulcerative colitis differentially expressed genes related to inflammatory response compared to patients with non-severe ulcerative colitis, including *TNF* and NF-κB pathways for IgA+ clusters and IL-17 pathway for IgG+ clusters (Figures 3D).

Clusters of activated B cells exhibited 634 differentially expressed genes between the two groups, 337 being lower expressed and 54 higher expressed in activated B cells cluster 1, and 136 lower expressed and 107 higher expressed in activated B cells cluster 2. Pathway analysis showed an enrichment in terms related to inflammatory response, inflammatory bowel disease and interferon-gamma signalling among others (Supplementary Figure 3).

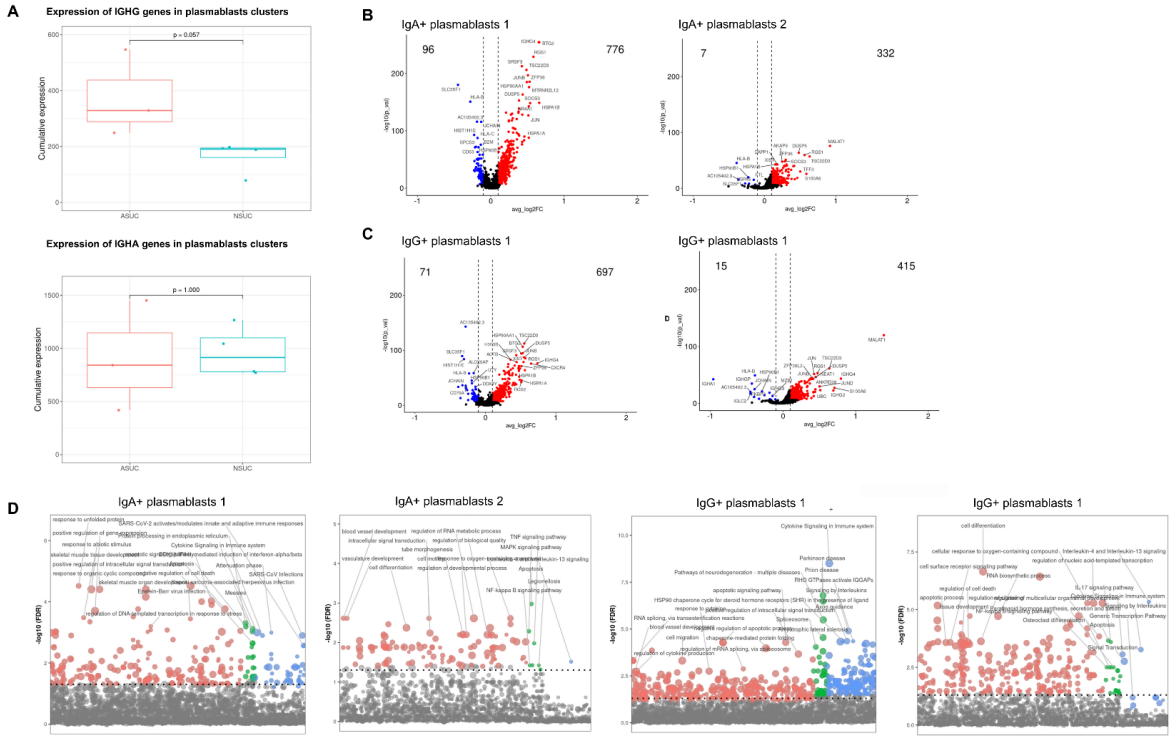


Figure 3: Differential expression profiles of the plasmablasts of patients with acute severe ulcerative colitis.

Panel A: Cumulative expression of IgG genes on top (*IGHG1, IGHG2, IGHG3, IGHG4*) and IgA genes (*IGHA1, IGHA2*), bottom, in the four plasmablasts clusters, compared between the two severity groups. The average expression of the listed genes in the clusters of plasmablasts was calculated for each patient and P-value was calculated using the Wilcoxon test to compare the two severity groups. The box in the plot represents the middle 50% of the data, with the median line dividing the box into two parts. The whiskers extend from the box to indicate the variability outside the upper and lower quartiles. Each dot represents one patient.

Panel B: Volcano plots of genes that are differentially expressed in IgA+ plasmablasts cluster 1 (left) and IgA+ plasmablasts cluster 2 (right) from acute severe ulcerative colitis patients relative to cells from non-severe patients in the same cluster.

Panel C: Volcano plots of genes that are differentially expressed in IgG+ plasmablasts cluster 1 (left) and IgG+ plasmablasts cluster 2 (right) from acute severe ulcerative colitis patients relative to cells from non-severe patients in the same cluster.

The effect size in acute severe ulcerative colitis as average log Fold change (X-axis) is plotted against the statistical significance of expression differences (y axis). The total number of significantly higher (red dots) or lower expressed (blue dots) genes by severe patients is indicated in the top corners of the plot. Effect size and adjusted p-values were computed using FindMarkers and Wilcoxon tests in Seurat. Genes with a minimum log2 fold change of 0.1 and an adjusted p-value below 0.05 between the two severity groups were considered as exhibiting differential expression.

Panel D: Manhattan plot of pathways enriched in differentially expressed genes in clusters of plasmablasts of patients with acute severe ulcerative colitis relative to non-severe patients

using GO (red dots), Reactome (blue dots) and KEGG (red dots) databases. The x-axis represents the pathways and the y-axis represents the statistical significance of the enrichment. Each pathway is represented by a point on the plot, and the height of the point indicates the significance of the enrichment. The size of the point indicates the count of the enriched genes within the pathway. The names of pathways enriched in differentially expressed genes are displayed.

A subset of T cells expressing IL26 is expanded in patients with acute severe ulcerative colitis

Among the 9,699 T cells, 3,770 belonged to patients with acute severe ulcerative colitis (n=3 patients) and 5,929 to patients with non-severe ulcerative colitis (n=4 patients). Twelve clusters were identified and annotated by examining differentially expressed genes in each cluster compared to all other cells and using recently published markers[16] (Figure 4A-B). Briefly, we identified three clusters of naïve T cells expressing *TCF7*, *LEF1, CCR7, SELL*; two clusters of effector CD8+ T cells expressing CD8A and the granzyme K gene *GZMK;* two clusters of regulatory T cells expressing *FOXP3* and *IL2RA*; one cluster of IL-26+ T cells expressing *IL26, RORA, KLRB1* and *CXCR6;* one cluster of NK cells expressing *KLRC1, NKG7* and *TYROBP;* one cluster of intraepithelial lymphocytes expressing *GZMA*, *NKG7, ITGAE* and *CD8A*, one cluster of mucosa associated T cells expressing *CCL20, KLRB1* and *IL7R* and one cluster of cycling cells expressing *MKI67*.

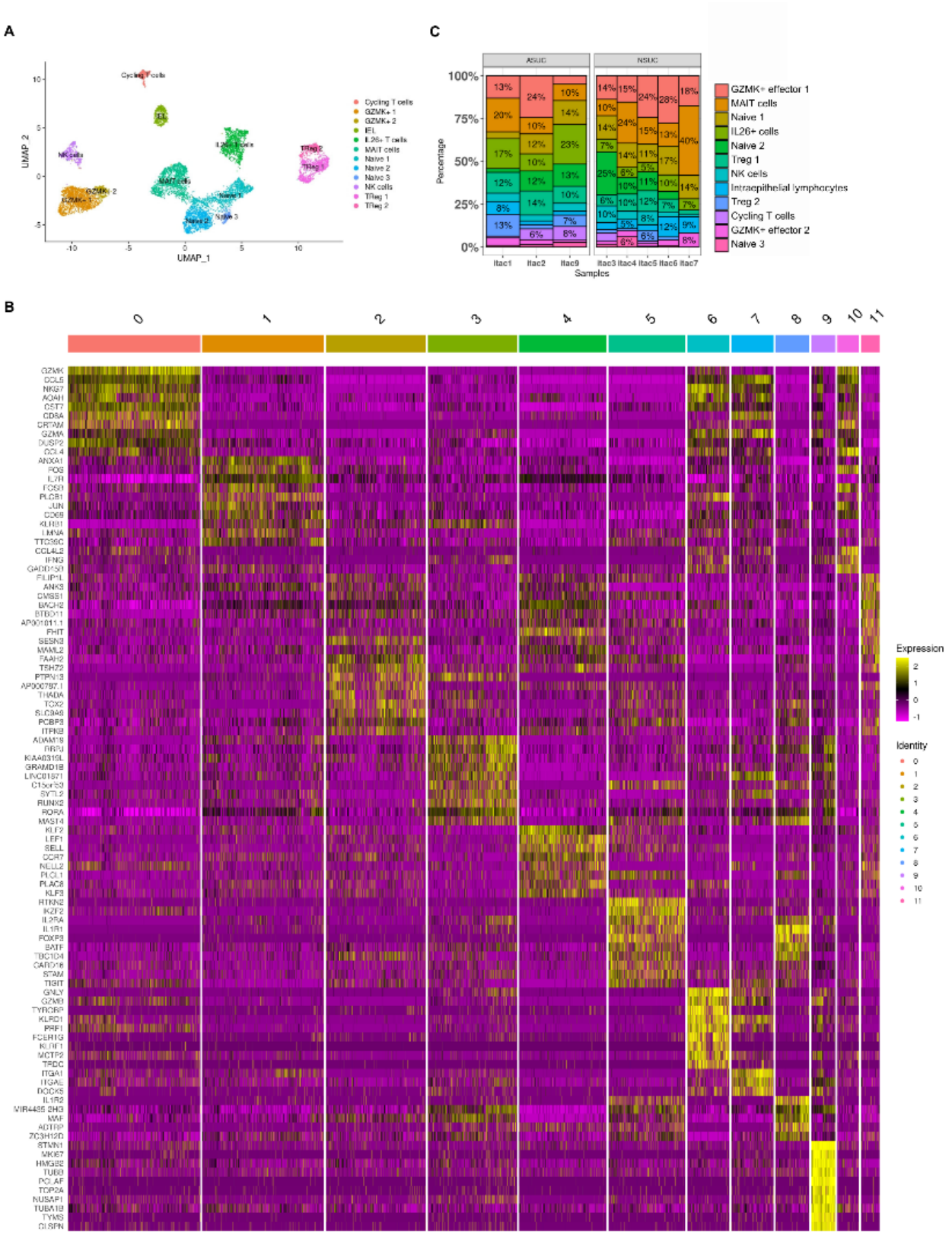
Comparing the proportion of T cell clusters in each of the severity groups, the IL-26+ cluster was higher represented in patients with acute severe ulcerative colitis compared to non-severe ulcerative colitis patients (18% *versus* 7%, respectively, Wilcoxon test p = 0.04, Figure 4C).

Figure 4: Clusters of T cells (next page).

Panel A: UMAP visualisation of the 9,699 T cells. Cells were clustered using the Seurat algorithm. Clusters of T cells were annotated using the expression of canonical markers and published markers for T cells[16] among them *TCF7*, *LEF1, CCR7, SELL* for naïve T cells; CD8A, *GZMK* for effector CD8+ T cells*; FOXP3*, *IL2RA* for regulatory T cells; *IL26, RORA, KLRB1, CXCR6* for IL-26+ T cells*; KLRC1, NKG7*, *TYROBP* for NK cells; *GZMA*, *NKG7, ITGAE*, *CD8A* for intraepithelial lymphocytes; *CCL20, KLRB1* and *IL7R* for mucosa associated T cells (MAIT) and *MKI67* for cycling cells.

Panel B: Heatmap of the genes differentially expressed in each cluster of T cells compared to the remaining clusters. The rows of the heatmap represent the 10 genes with the highest log fold change for each cluster, and the columns represent the cells, ordered by clusters. The colours represent the level of expression.

Panel C: Distribution of the T cells clusters in each sample, grouped by severity phenotype (ASUC: acute severe ulcerative colitis, NSUC: non-severe ulcerative colitis). Each column represents a patient. coloured bars represent the percentage of cells (Y-axis) within each patient originating from each cluster among all T cells in this patient.


A
UMAP 2
UMAP_1
Cycling T cells
GZMK+ 1
GZMK+ 2
IEL
IL26+ T cells
MAIT cells
Naive 1
Naive 2
Naive 3
NK cells
TReg 1
TReg 2
C
ASUC
NSUC
Percentage
Samples
itac1
itac2
itac3
itac4
itac5
itac6
itac7
GZMK+ effector 1
MAIT cells
Naive 1
IL26+ cells
Naive 2
Treg 1
NK cells
Intraepithelial lymphocytes
Treg 2
Cycling T cells
GZMK+ effector 2
Naive 3
B
Expression
Identity

We investigated the differentially expressed genes within each cluster comparing cells from patients with acute severe ulcerative colitis and patients with non-severe ulcerative colitis. Focusing on the cluster of IL-26+ T cells, we found around 300 differentially expressed genes in acute severe ulcerative colitis compared to non-severe ulcerative colitis patients (Figure 5A). Well-known immune genes were among the down-regulated genes, *e.g. CD27, ITGA4* or up-regulated genes, *e.g IL12RB, IL18R1, IL1B, CCL20, ITGB1*. Finally, we found that pathways related to response to inflammatory and immune pathways, and specifically the IL-17 signalling pathway, were enriched for genes down- and up-regulated in the cluster of IL-26+ T cells in acute severe ulcerative colitis patients compared to IL-26+ T cells from patients with non-severe ulcerative colitis (Figure 5B). Notably, in the cluster of cycling cells, we observed a higher expression of markers of IL-26+ cells in acute severe ulcerative colitis patients, such as *IL26* and *RORA* (Figure 5C-D). Similar results were observed in clusters of naive T cells, mucosa associated T cells, regulatory T cells and intraepithelial lymphocytes with the pathways associated with IL-17 signalling, response to bacterium and inflammatory response being enriched for genes down- and up-regulated in acute severe ulcerative colitis patients (Figure 5C-D, Supplementary Figure 4).

Figure 5: Differential expression profiles of T cells of patients with acute severe ulcerative colitis (next page).

Panel A: Volcano plots of genes that are differentially expressed in IL-26+ T cell cluster from acute severe ulcerative colitis patients relative to cells from non-severe patients in the same cluster.

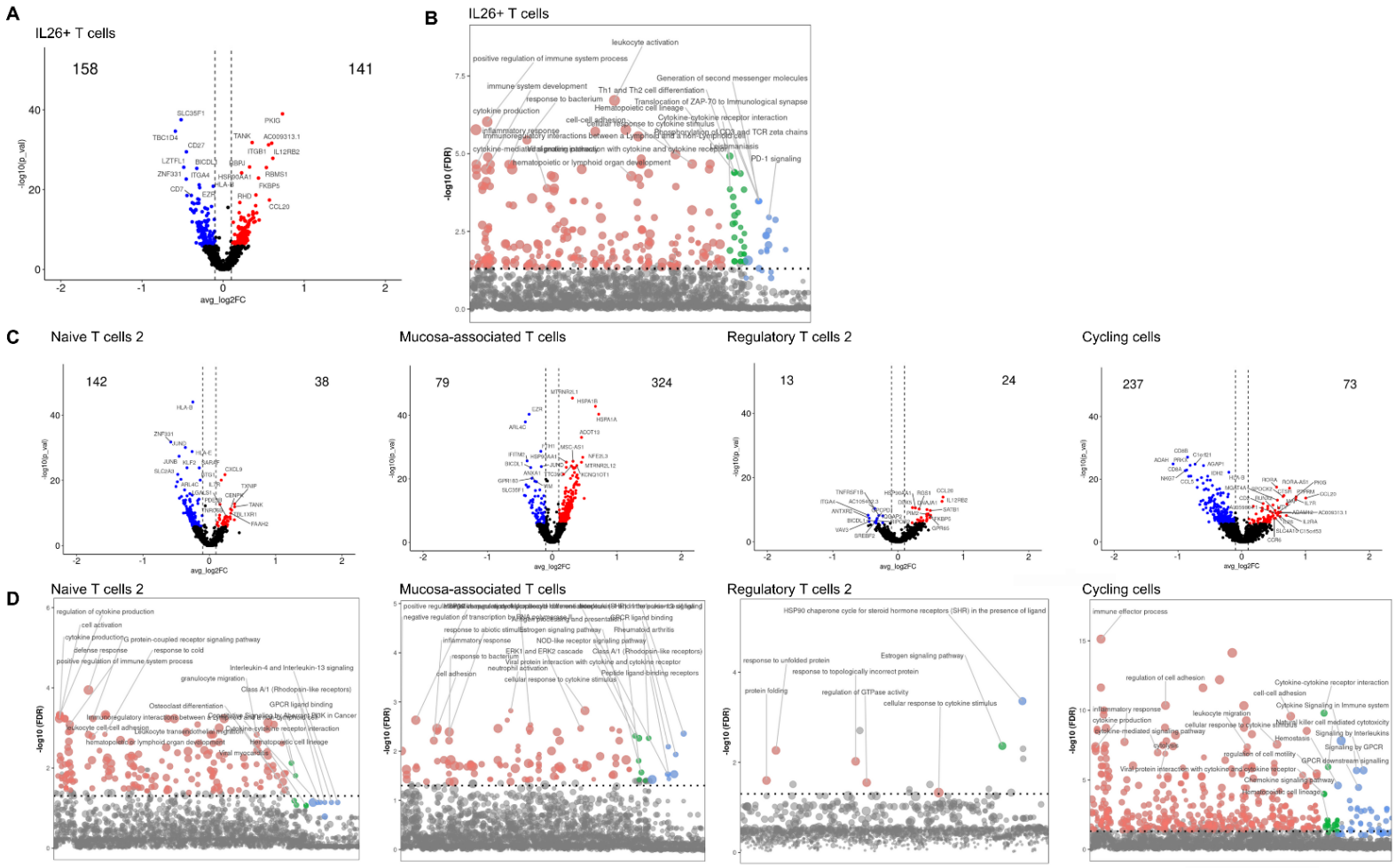
The effect size in acute severe ulcerative colitis as average log Fold change (X-axis) is plotted against the statistical significance of expression differences (y axis). The total number of significantly higher (red dots) or lower expressed (blue dots) genes by severe patients is indicated in the top corners of the plot. Effect size and adjusted p-values were computed using FindMarkers and Wilcoxon tests in Seurat. Genes with a minimum log2 fold change of 0.1 and an adjusted p-value below 0.05 between the two severity groups were considered as exhibiting differential expression.

Panel B: Manhattan plot of pathways enriched in differentially expressed genes in the IL26+ T cells cluster of patients with acute severe ulcerative colitis relative to non-severe patients using GO (red dots), Reactome (blue dots) and KEGG (red dots) databases. The x-axis represents the pathways and the y-axis represents the statistical significance of the

enrichment. Each pathway is represented by a point on the plot, and the height of the point indicates the significance of the enrichment. The size of the point indicates the count of the enriched genes within the pathway. The names of pathways enriched in differentially expressed genes are displayed.

Panel C: Volcano plots of genes that are differentially expressed in T cells clusters from acute severe ulcerative colitis patients relative to cells from non-severe patients in the same cluster, from left to right, naïve T cells 2, mucosa-associated T cells, regulatory T cells 2 and cycling cells.

Panel D: Manhattan plot of pathways enriched in differentially expressed genes in T cells clusters from acute severe ulcerative colitis patients relative to cells from non-severe patients in the same cluster, from left to right, naïve T cells 2, mucosa-associated T cells, regulatory T cells 2 and cycling cells.


A
IL26+ T cells
158
141
-log10(p_val)
avg_log2FC
B
IL26+ T cells
-log10 (FDR)
leukocyte activation
positive regulation of immune system process
Generation of second messenger molecules
immune system development
Th1 and Th2 cell differentiation
response to bacterium
Translocation of ZAP-70 to Immunological synapse
cytokine production
Hematopoietic cell lineage
cell-cell adhesion
Cytokine-cytokine receptor interaction
Cellular response to cytokine stimulus
inflammatory response
Phosphorylation of CD3 and TCR zeta chains
Leishmaniasis
hematopoietic or lymphoid organ development
PD-1 signaling
C
Naive T cells 2
142
38
Mucosa-associated T cells
79
324
Regulatory T cells 2
13
24
Cycling cells
237
73
D
Naive T cells 2
regulation of cytokine production
cell activation
cytokine production
G protein-coupled receptor signaling pathway
defense response
response to cold
positive regulation of immune system process
Interleukin-4 and Interleukin-13 signaling
granulocyte migration
Class A/1 (Rhodopsin-like receptors)
Osteoclast differentiation
GPCR ligand binding
Cytokine-cytokine receptor interaction
leukocyte cell-cell adhesion
hematopoietic cell lineage
Viral myocarditis
Mucosa-associated T cells
response to abiotic stimulus
Estrogen signaling pathway
Rheumatoid arthritis
inflammatory response
NOD-like receptor signaling pathway
response to bacterium
ERK1 and ERK2 cascade
Class A/1 (Rhodopsin-like receptors)
cell adhesion
Peptide ligand-binding receptors
cellular response to cytokine stimulus
Regulatory T cells 2
HSP90 chaperone cycle for steroid hormone receptors (SHR) in the presence of ligand
response to unfolded protein
Estrogen signaling pathway
response to topologically incorrect protein
protein folding
regulation of GTPase activity
cellular response to cytokine stimulus
Cycling cells
immune effector process
regulation of cell adhesion
Cytokine-cytokine receptor interaction
cell-cell adhesion
Cytokine Signaling in Immune system
inflammatory response
leukocyte migration
cytokine production
cellular response to cytokine stimulus
Natural killer cell mediated cytotoxicity
Signaling by Interleukins
Hemostasis
Signaling by GPCR
regulation of cell motility
GPCR downstream signaling
Chemokine signaling pathway
Hematopoietic cell lineage

Innate immune cells from acute severe ulcerative colitis patients differentially express genes related to inflammation

We generated 8,756 single-cell RNASeq profiles of innate immune cells, *i.e.* monocytes and macrophages, neutrophils and mast cells: 4,671 myeloid cells [2,581 from patients with acute severe ulcerative colitis (n=2 patients) and 2,090 from patients with non-severe ulcerative colitis (n=4 patients)]; 3,262 myeloid cells [505 from patients with acute severe ulcerative colitis (n=3 patients) and 2,757 from patients with non-severe ulcerative colitis (n=4 patients)]; 823 mast cells [275 from patients with acute severe ulcerative colitis (n=3 patients) and 548 from patients with non-severe ulcerative colitis (n=4 patients)].

Myeloid cells were classified into six clusters and annotated using previously published markers: one cluster consisted of inflammatory monocytes expressing *CD14, S100A8, S100A9* and *IL1B*[5]; another cluster was composed of macrophages expressing *CD163, CD14* and *MMP12*[17]; a distinct cluster represented M1-macrophages expressing *CCR7*, *MMP12, IDO1* and *LAMP3*[17]; a unique cluster of stressed macrophages was identified in only one patient with acute severe ulcerative colitis, characterised by the expression of genes for heat shock proteins and two clusters of dendritic cells expressing *CD2* and *CD1C*[18] (Figure 6A, Supplementary Figure 5).

Four clusters of neutrophils were identified. The first cluster, labelled 'inflammatory neutrophils 1,' exhibited the expression of inflammatory markers such as *IL1B, IFITM2* and *OSM*; the second cluster, labelled 'inflammatory neutrophils 2,' showed expression of and *S100A8* and *S100A9*, which are also associated with inflammation. The third cluster was characterised as 'migrating neutrophils' due to the expression of chemotaxis markers like *PIK3R5.* The fourth cluster was identified as a 'regulatory neutrophils' based on the expression of macrophage markers, including *APOE* and *CD163* (Figure 6B, Supplementary Figure 6).

Three clusters of mast cells were identified differing by their expression level of the tryptase genes, *TPSAB1,* the alpha subunit of tryptase and *TPSB2*, the beta subunit of tryptase. Mast cells cluster 1 expressed high levels of *TPSAB1*, whereas cluster 2 expressed low levels of *TPSAB1* and cluster 3 high levels of *TPSB2* (Figure 6C, Supplementary Figure 7).

No difference was observed in the distribution of the clusters between the two groups of patients (Figure 6D-F).

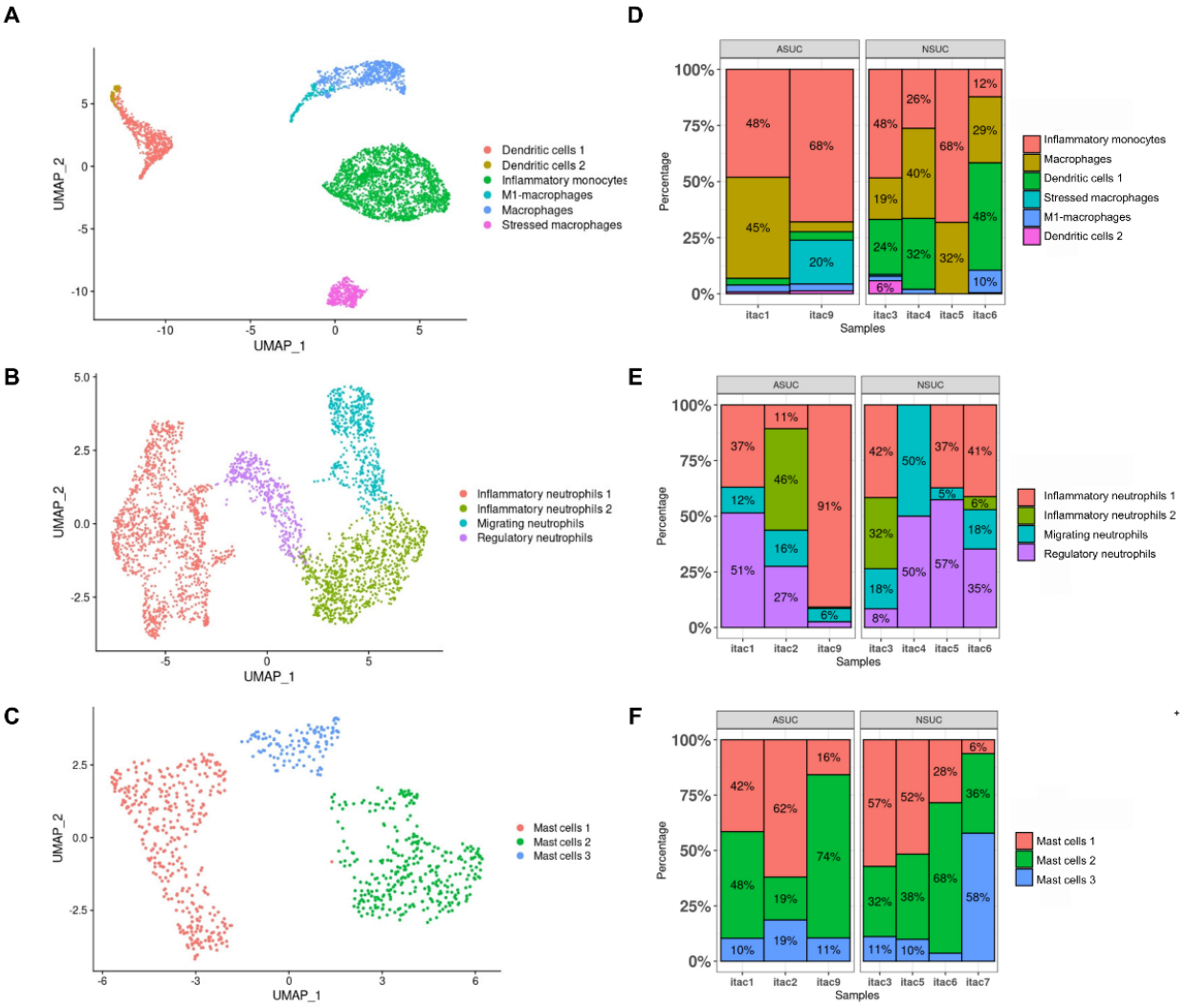


Figure 6: Clusters of innate immune cells.

Panel A: UMAP visualisation of the 4,671 myeloid cells. Cells were clustered using the Seurat algorithm. Clusters were annotated using the expression of canonical markers and published markers[5,17,18] among them *IL1B, S100A8* for inflammatory monocytes; *MMP12, CD163* for macrophages*; CD2, CD1C* for dendritic cells.

Panel B: UMAP visualisation of the 3,262 neutrophils. Cells were clustered using the Seurat algorithm.

Panel C: UMAP visualisation of the 823 mast cells. Cells were clustered using the Seurat algorithm.

Panel D: Distribution of the myeloid cells clusters in each sample, grouped by severity phenotype (ASUC: acute severe ulcerative colitis, NSUC: non-severe ulcerative colitis). Each column represents a patient. coloured bars represent the percentage of cells (Y-axis) within each patient originating from each cluster among all myeloid cells in this patient.

Panel E: Distribution of the neutrophils clusters in each sample, grouped by severity phenotype (ASUC: acute severe ulcerative colitis, NSUC: non-severe ulcerative colitis). Each column represents a patient. coloured bars represent the percentage of cells (Y-axis) within each patient originating from each cluster among all neutrophils in this patient.

Panel F: Distribution of the mast cells clusters in each sample, grouped by severity phenotype (ASUC: acute severe ulcerative colitis, NSUC: non-severe ulcerative colitis). Each column represents a patient. coloured bars represent the percentage of cells (Y-axis) within each patient originating from each cluster among all mast cells in this patient.

We observed 1,376 differentially expressed genes between patients with acute severe ulcerative colitis and those with non-severe ulcerative colitis in the inflammatory monocytes cluster, 1,530 in the macrophages cluster and 244 in the inflammatory neutrophils cluster. Notably, we identified a higher expression of *IL1B, CXCL9, CXCL10* and *CXCL11* in the inflammatory monocytes cluster and dendritic cells cluster 1 and *IL1B, CXCL9, CXCL10* and *IL17RA* in the macrophages cluster, and *IL1B* and *CXCL9* in the cluster of inflammatory neutrophils 1 (Figure 7A, Supplementary Figure 8). Among the remaining neutrophils clusters, expression of *IL1R1, IL1R2* and *IL18R1* were higher in patients with acute severe ulcerative colitis (Supplementary Figure 8). Regarding pathways enriched with differentially expressed genes in clusters of myeloid cells and neutrophils, we found an overrepresentation of genes related to inflammatory response and response to microorganisms, and notably the Jak-stat and the IL6 pathway in macrophages (Figure 7B).

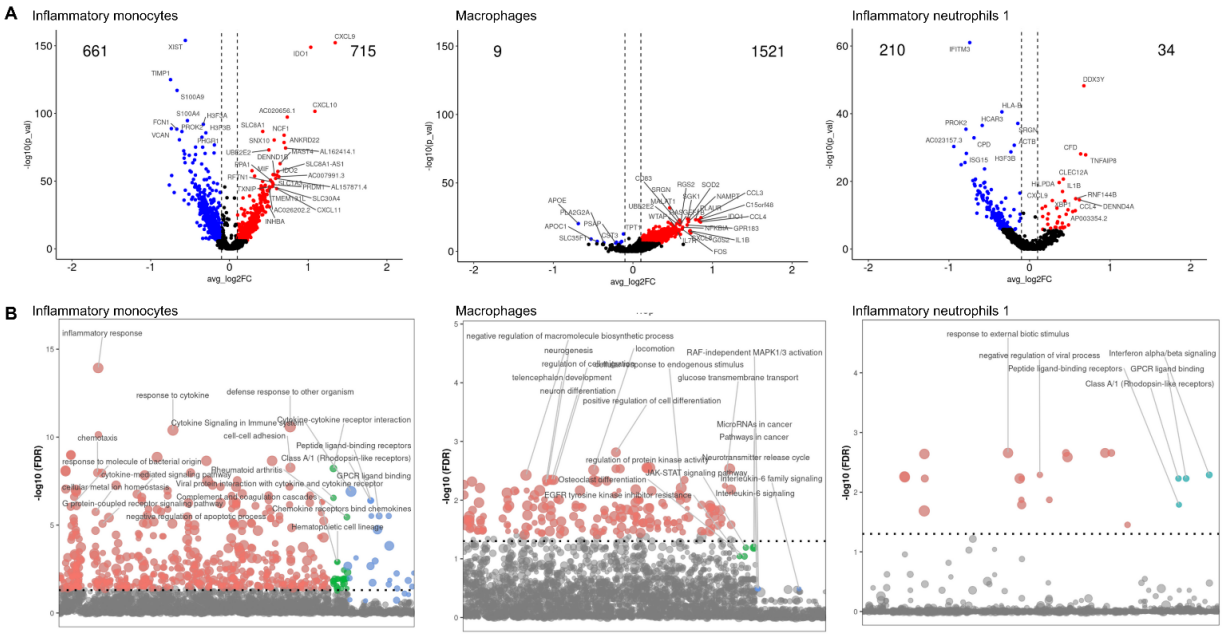


Figure 7: Innate immune cells from patients with acute severe ulcerative colitis exhibit a higher expression of inflammatory genes.

Panel A: Volcano plots of genes that are differentially expressed in innate immune cells clusters from acute severe ulcerative colitis patients relative to cells from non-severe patients in the same cluster. From left to right, inflammatory monocytes, macrophages and inflammatory neutrophils 1.

The effect size in acute severe ulcerative colitis as average log Fold change (X-axis) is plotted against the statistical significance of expression differences (y axis). The total number of significantly higher (red dots) or lower expressed (blue dots) genes by severe patients is indicated in the top corners of the plot. Effect size and adjusted p-values were computed using FindMarkers and Wilcoxon tests in Seurat. Genes with a minimum log2 fold change of 0.1 and an adjusted p-value below 0.05 between the two severity groups were considered as exhibiting differential expression.

Panel B: Manhattan plot of pathways enriched in differentially expressed genes in innate immune cells cluster of patients with acute severe ulcerative colitis relative to non-severe patients using GO (red dots), Reactome (blue dots) and KEGG (red dots) databases. The x-axis represents the pathways and the y-axis represents the statistical significance of the enrichment. Each pathway is represented by a point on the plot, and the height of the point indicates the significance of the enrichment. The size of the point indicates the count of the enriched genes within the pathway. The names of pathways enriched in differentially expressed genes are displayed. From left to right, inflammatory monocytes, macrophages and inflammatory neutrophils 1.

Stromal cells content is heterogeneous among patients in each group

We generated 1,307 single-cell RNASeq profiles for stromal cells, 449 from patients with acute severe ulcerative colitis (n = 3 patients) and 858 from patients with non-severe ulcerative colitis (n = 5 patients). They were classified into 10 clusters annotated according to published markers[5,8,19–21]: three clusters of endothelial cells expressing *PECAM, PLVP, IFI27* and *COL1A1*; one cluster of fibroblasts expressing *ADAMDEC1, DCN* and *CXCL14*; three clusters of myofibroblasts expressing *RSG5, ACTA2, MYH11* and *NOTCH3*; one cluster of subepithelial cells expressing *PDGFRA* and *CXCL14*; one cluster of enteric glia expressing *NRXN1* and one cluster of mixed epithelial cells expressing *EPCAM* and *KRT8* (Figure 8A-B).

Significant interindividual variability in cluster proportions was observed among patients, regardless of their group classification. Notably, the majority of cells in the myofibroblasts 1, myofibroblasts 2, and subepithelial cell clusters were derived from a single patient (Figure 8C). Furthermore, patients with acute severe ulcerative colitis exhibited a lower proportion of fibroblasts and myofibroblasts compared to those with non-severe ulcerative colitis, although this difference was not statistically significant (Figure 8D).

Figure 8: Clusters of stromal cells (next page).

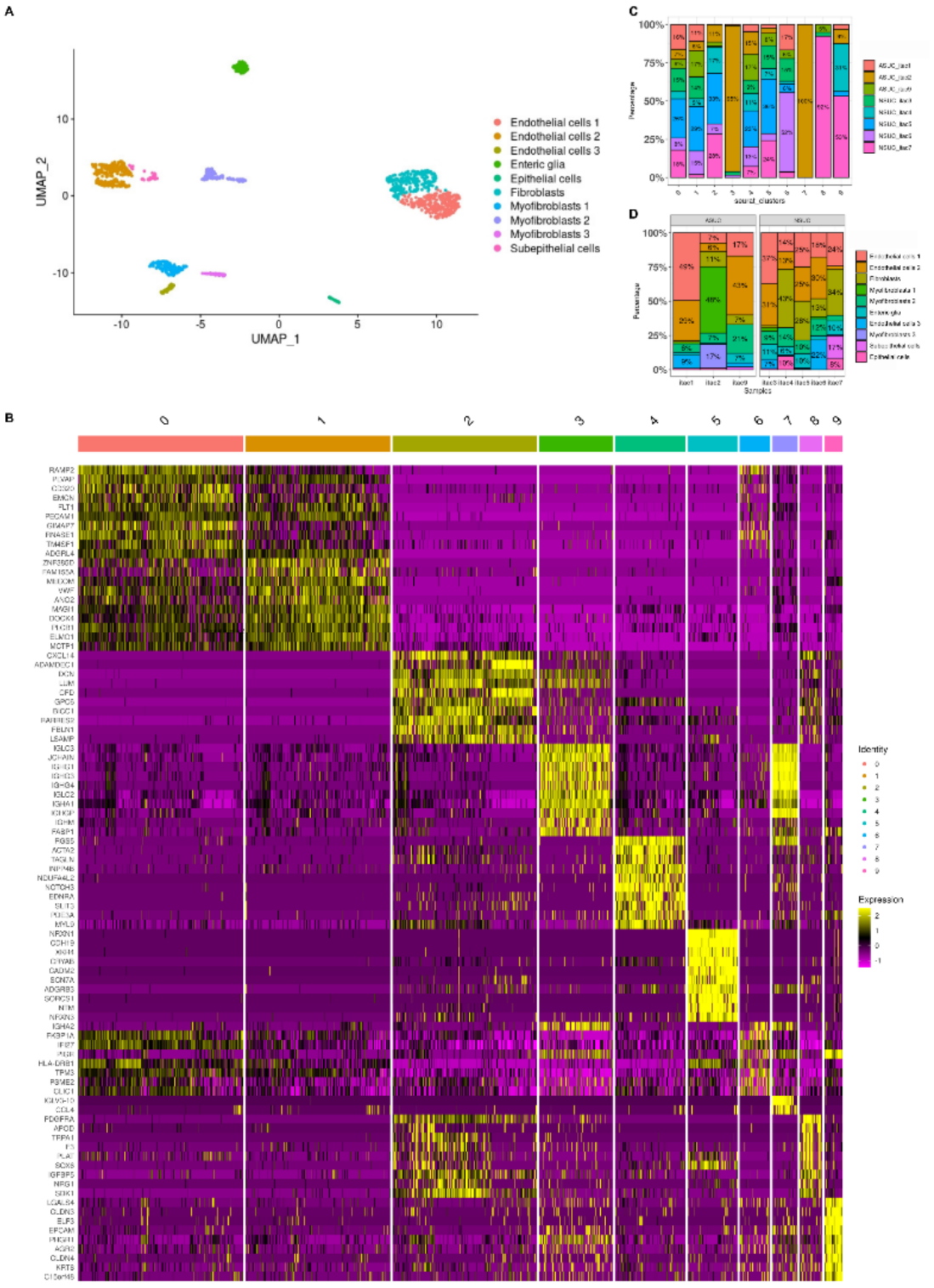
Panel A: UMAP visualisation of the 1,307 stromal cells. Cells were clustered using the Seurat algorithm. Clusters were annotated using the expression of canonical markers and published markers for stromal cells[5,8,19–21] among them *PECAM, PLVP, IFI27* and *COL1A1* for endothelial cells; *ADAMDEC1, DCN* and *CXCL14* for fibroblasts; *RSG5, ACTA2, MYH11* and *NOTCH3* for myofibroblasts; *PDGFRA* and *CXCL14* for subepithelial cells; *NRXN1* for enteric glia and *EPCAM* and *KRT8* for epithelial cells.

Panel B: Heatmap of the genes differentially expressed in each cluster of stromal cells compared to the remaining clusters. The rows of the heatmap represent the 10 genes with the highest log fold change for each cluster, and the columns represent the cells, ordered by clusters. The colours represent the level of expression.

Panel D: Origin of the cells in each cluster. Each column represents a cluster. coloured bars represent the percentage of cells (Y-axis) within each cluster originating from each patient.

Panel D: Distribution of the stromal cells clusters in each sample, grouped by severity phenotype (ASUC: acute severe ulcerative colitis, NSUC: non-severe ulcerative colitis). Each column

represents a patient. coloured bars represent the percentage of cells (Y-axis) within each patient originating from each cluster among all stromal cells in this patient.



4.6. Discussion

In this scRNAseq study, our primary objective was to identify the immunological characteristics within the colonic mucosa of patients experiencing acute severe ulcerative colitis. We reveal in severe cases a distinct transcriptomic profile of plasmablasts, which tend to produce more IgG, and an expansion of a specific T cells population expressing *IL26* compared to non-severe patients. On top of these changes in adaptive cells, we also observed a pro-inflammatory profile of innate immune cells.

In recent years, the increased presence of IgG-producing plasmablasts in the colonic mucosa of patients with ulcerative colitis has been well established[22]. These IgG are directed against bacteria found in the gut microbiota and they have the ability to form immune complexes with these bacteria[23]. Genetic studies have shown that variants in Ig receptors increase susceptibility to ulcerative colitis by lowering the activation threshold of innate immune cells by Ig immune complexes in the colonic mucosa[24]. The activation of innate immune cells by IgG-bacteria complexes results in an excessive production of pro-inflammatory cytokines, including TNF and IL1B, known to be associated with colonic inflammation[23]. We found that IgG-producing plasmablasts are increased in the colonic mucosa of patients with acute severe ulcerative colitis. This finding may be associated with changes in the gut microbiota. In related work (Rivière *et al.* in preparation) we observed a reduced microbial diversity and an elevation in so-called "pro-inflammatory" bacteria in patients with acute severe ulcerative colitis. Our data revealed an elevation in the expression of inflammatory cytokines such as the *IL1B* gene and chemokines involved in the recruitment of T cells, such as *CXCL9, CXCL10* and *CXCL11* by colonic neutrophils and *IL1B*, *CXCL10* and *CXCL11* by inflammatory monocytes of patients with acute severe ulcerative colitis. This upregulation may be attributable to the increased IgG content and the hyperactivation of FCy receptors on neutrophils due to IgG-bacteria complexes in the lamina

propria. Similarly, we found decreased expression of the polymeric immunoglobulin receptor PIGR in dendritic cells among patients with acute severe ulcerative colitis. The activation of PIGR by the microbiota modulates the production of IgA which, reciprocally, influences the composition of the microbiota[25,26]. Among dendritic cells, we also found an increased expression of lysozyme and *S100A4* in patients with acute severe ulcerative colitis. A specific subset of S100A4-lysozyme-producing dendritic cells has been associated with mucosal immunity in mice and humans by bacterial pathogen uptake and regulation of IgA production[27,28]. Our data suggested that in acute severe ulcerative colitis, there are complex interactions between B cells, innate immune cells and the gut microbiota associated with a disrupted regulation of the balance between IgG and IgA and the increased production of pro-inflammatory cytokines by innate immune cells. The cytokine genes upregulated in the neutrophil and monocyte clusters such *IL1B* are characteristic of a pro-Th17 mucosal environment[29].

Th17 cells play a pivotal role in the immune response underlying the pathogenesis of ulcerative colitis. This is evident in the significant therapeutic impact of monoclonal antibodies targeting the IL-23 pathway for the treatment of ulcerative colitis[30]. We observed an increase in the population of IL-26-producing T cells in patients with acute severe ulcerative colitis. This particular subset of T cells has recently been described as increased in the colonic mucosa of patients with ulcerative colitis compared to healthy subjects by Corridoni *et al.*[16]. However, the specific role of this cell subset remains elusive. Notably, IL-26-producing T cells have been shown to correspond to infiltrating pro-inflammatory IL-17-producing T cells in the inflamed colonic mucosa of IBD patients[29]. Production of IL-26 requires IL-23 signalling and IL-26 has direct antimicrobial effect[31]. In the study by Corridoni *et al.*, the expression levels of *IL26* in the colon were correlated with the severity of the disease measured by endoscopy. Elevated levels were specifically detected in patients with substantial colonic ulcers (Ulcerative Colitis Endoscopic Index of Severity at least 4)[16]. Conversely, a protective effect of IL-26 was observed in a mouse model of acute

chemically-induced colitis using dextran-sodium sulfate through the down-regulation of activation and proliferation of lymphocytes. In cases of chronic inflammation, the immunoregulatory properties of IL-26 might have detrimental effects and increase mucosal damage. IL-17 signalling is involved in mucosal barrier repair after damage[32]. In humans, monoclonal antibodies targeting IL-17 worsen the IBD phenotype[33]. This implies that the cytokines of the IL-23/IL-17/IL-26 axis need to be finely regulated to promote intestinal barrier homeostasis. As mentioned earlier, we observed an upregulation of pro-inflammatory cytokines associated with the Th17 cells within innate immune cells clusters. On top of promoting the differentiation of naive T cells into Th17 cells, these molecules primarily function as chemokines, capable of recruiting additional innate immune cells. For instance, CCL4 and CXCL9 are involved in the recruitment of neutrophils[34], while CXCL10 and CXCL11 are associated with the recruitment of inflammatory monocytes[35]. There is a reciprocal cross-talk between innate immune and Th17 cells that might amplify the local accumulation of innate immune and Th17 cells in IBD[36]. Inflammatory monocytes and neutrophils are key players in the disruption of the epithelial barrier and mucosal healing[37]. Recently, we described how patients with acute severe ulcerative colitis exhibit a more severe mucosal damage[6]. This could be related to the pathogenic loop between innate immune cells and immunoregulatory IL-26-producing T cells discussed here.

Our study is one of the first to investigate in-depth the mucosal immunological profiles of well-phenotyped patients with acute severe ulcerative colitis. In previous studies, no distinction was made between patients with mild or moderate ulcerative colitis and patients with severe colitis, including systemic inflammation. The main limitation of our study is the relatively modest sample size. This restricted our ability to conduct a comprehensive investigation of innate immune and stromal cells due to the shortage of available cells. Additionally, it posed challenges in distinguishing whether variations in cell subtypes were linked to individual differences or the severity of the phenotype. We limited our investigation to a single study method, scRNAseq. Our

work could be improved by adding other characterization techniques to validate our results, such as flow cytometry or the assessment of B and T cells repertoire.

In conclusion, we reveal that the colonic mucosa of patients with acute severe ulcerative colitis exhibits heightened inflammatory characteristics, involving both the adaptive and innate immune system, with a central focus on IgG/IgA balance and the Th17/IL-23 pathway. The growing body of literature regarding the efficacy of Jak inhibitors, which suppress the Th17 response[38], in cases of acute severe ulcerative colitis, along with the emergence of potent monoclonal antibodies directed against IL-23 provides hope for the medical treatment of acute severe ulcerative colitis. Nevertheless, further research is needed to gain a deeper understanding of the potential dual effects of the IL-23/IL-17 axis and prevent disease worsening in severe patients exposed to life-threatening complications.

4.7. References

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5. Chapter 5: Characterization of blood transcriptome in patients with acute severe ulcerative colitis.

5.1. Preface

As previously emphasised throughout this thesis, systemic inflammation stands as a hallmark of acute severe ulcerative colitis[1]. In clinical practice, it is primarily quantified through the elevation of plasmatic C-Reactive protein (CRP) levels. To gain deeper insights into the underlying mechanisms of this systemic inflammation, we embarked on an investigation of the blood transcriptome in patients participating in the ITAC study. In this section, I will provide a concise overview of our preliminary findings from the blood transcriptome analysis. It's worth noting that these analyses are still ongoing.

5.2. Context and methods

We hypothesised that genes associated with inflammatory response, particularly those related to pathogen response, would be upregulated in the blood of patients with acute severe ulcerative colitis compared to non-severe cases. This interest emerged after the initiation of the clinical study. Initially, we had not planned to collect whole blood for RNA sequencing (RNASeq) using specialised tubes designed to preserve RNA molecules such as PAXgene tubes. Instead, we collected whole blood in EDTA tubes at enrollment, stored at -80°C, with the intention of conducting genetic analyses in the future once an adequate number of patients were enrolled. However, to address the inflammatory response hypothesis, we decided to use the frozen whole blood in EDTA tubes for RNASeq. We had obtained whole blood samples from 18 patients within the acute severe ulcerative colitis and 19 within the non-severe ulcerative colitis group. In collaboration with the McGill Genome Center, the blood was thawed, transferred to PAXgenes

tubes and RNA was obtained, following the manufacturer's standard RNA extraction protocol[2]. The extracted RNA showed a median [Interquartile Range (IQR)] RNA Integrity Number (RIN) of 5.0 (4.4-5.5), indicating its suitability for library preparation using NEBNext Ultra II Directional RNA kits. Sequencing was carried out on the Illumina NovaSeq 6000 s4 v1.5 platform, generating 100-base pair paired-end reads. After library preparation, the median (IQR) cDNA yield was 50.9 (14.1-90.3) nM, and we obtained a median (IQR) of 68 (55-76) million reads per library.

I conducted the analysis of the FASTQ files using code developed by Monica Dallmann-Sauer and Willian Correa-Macedo from Professor Schurr's team. The raw FASTQ files underwent quality checks with FastQC (v0.11.8)[3]. Subsequently, the sequences were aligned to the human reference genome GRCh38.p13 (ENSEMBL v99)[4] with STAR (v2.7.3a)[5]. The resulting aligned BAM files were used for expression quantification with Salmon version 1.5.1[6]. At this stage, one sample was excluded due to sequencing failure. Text files containing estimated counts and transcript per million values for each subject were employed to create a gene-level expression matrix in R, using tximport (v1.12.3)[7] and biomaRt (2.40.5)[8]. We obtained counts for 60,664 transcripts, which were subsequently filtered and normalised using edgeR.

We examined the sample counts through dimensionality reduction plots to assess potential influences of biological and technical variables. Notably, haemoglobin levels were suspected to affect gene expression (Supplementary Figure 1). No other covariates demonstrated a significant impact on gene expression. To identify differential gene expression, we employed limma[9] and voom (v3.40.6)[10]. The false discovery rate was controlled at a 5% level using the Benjamini-Hochberg procedure. It's worth noting that no adjustment was made for haemoglobin, as haemoglobin is inherently defined as part of the acute severe ulcerative colitis phenotype[1]. Pathway analysis was performed using the clusterProfiler package[11] to identify overrepresented biological processes among differentially expressed genes (adjusted p-value < 0.05) between acute severe ulcerative

colitis and non-severe ulcerative colitis patients based on the Gene Ontology, the Kyoto Encyclopedia of Genes and Genomes and the Reactome resources.

5.3. Results

We analysed whole blood RNASeq data from 18 patients with acute severe ulcerative colitis and 18 patients with non-severe ulcerative colitis. The multidimensional scaling plot in Figure 1 showed no distinct separation of the two severity groups based on gene expression profiles.

In our analysis, we identified 430 differentially expressed genes in patients with acute severe ulcerative colitis compared to those with non-severe ulcerative colitis, with 192 genes being down-regulated and 240 genes being up-regulated (Figure 2). Down-regulated genes included *CXCR5, MADCAM1-AS, IL24* and *IL21R-AS1*, while up-regulated genes included *SLC12A5-AS1, BANF1* and *CLEC12A,* genes shown to be associated with ulcerative colitis. No term/pathway enriched for differentially expressed genes was identified.

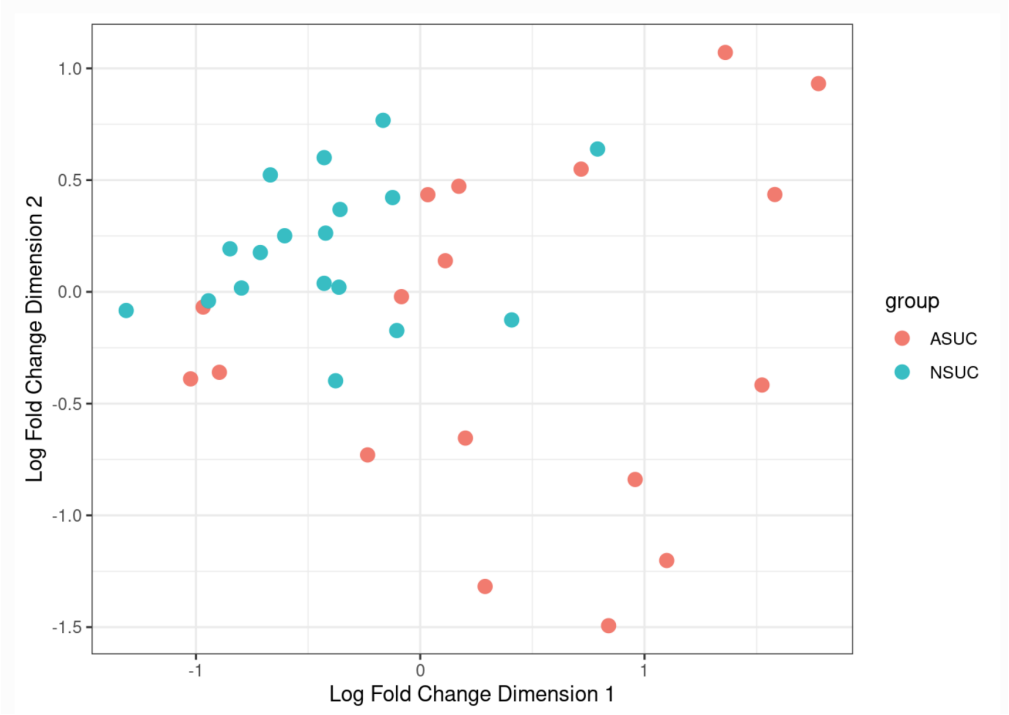

Figure 1: Multidimensional scaling plot showing the gene expression profiles in the two groups.
Two-dimensional scatterplot that shows the distances between samples based on the log2 fold changes between them. Each sample (dot) is coloured according to the severity group. Distances between samples on the plot approximate the typical log2 fold changes for each transcript between the samples. ASUC: acute severe ulcerative colitis; NSUC: non-severe ulcerative colitis.

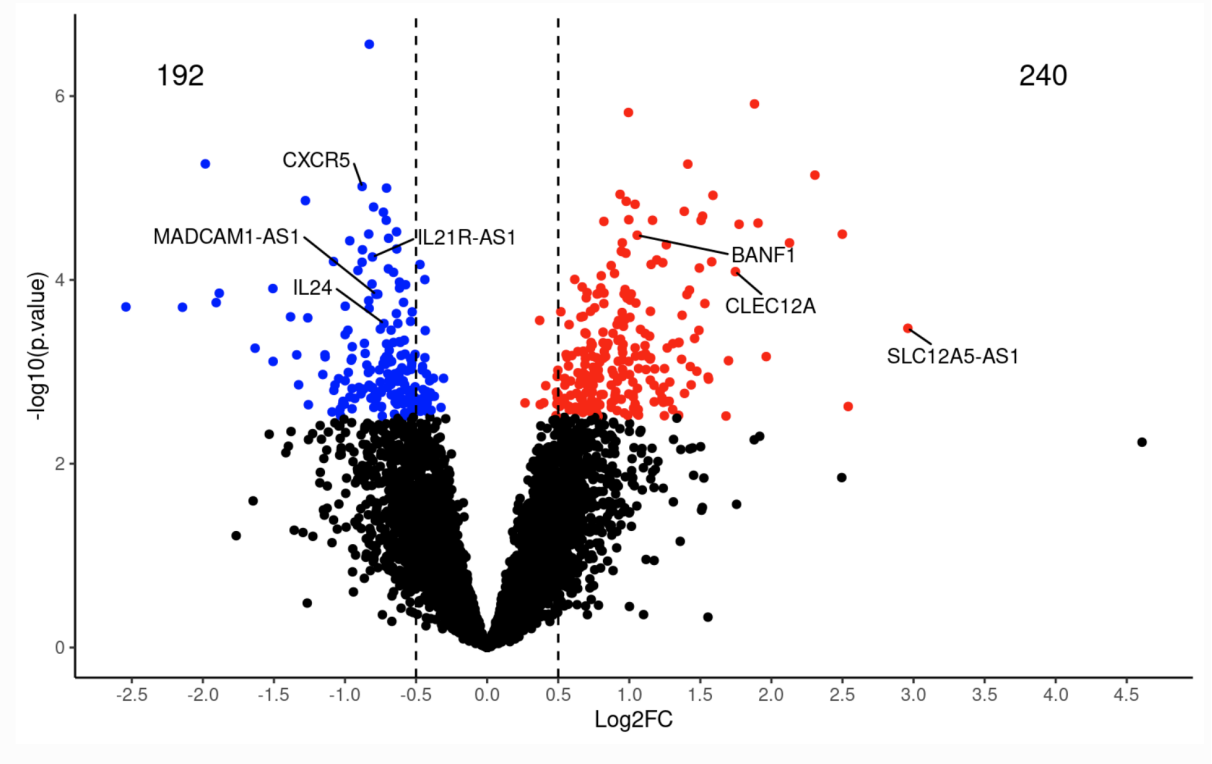

Figure 2: Differential expression of genes.
Volcano plot of genes that are differentially expressed in 18 whole blood of acute severe ulcerative colitis patients relative to 18 non-severe patients. The effect size in acute severe ulcerative colitis as average log Fold change (X-axis) is plotted against the statistical significance of expression differences (y axis). The total number of significantly higher (red dots) or lower expressed (blue dots) genes by severe patients is indicated in the top corners of the plot. Effect size and adjusted p-values were computed using limma-voom. The names of differentially expressed genes of specific interest in ulcerative colitis are provided.

5.4. Discussion

In this whole blood RNASeq investigation of 36 patients with ulcerative colitis, we observed no clear distinction between severe and non-severe cases in the dimensionality reduction plot and no pathway enriched with differentially expressed genes could be identified. Despite our initial clinical intuition that patients with acute severe ulcerative colitis would exhibit significant differences in blood transcriptome, associated with systemic inflammation, our RNASeq data did not support this expectation, aligning with earlier findings[12,13]. Notably, few studies have explored the whole blood transcriptome in ulcerative colitis patients. Planell *et al.* conducted an extensive analysis of whole blood RNASeq in a large cohort of patients with ulcerative colitis compared to healthy subjects,

revealing minimal transcriptional changes in blood opposite to the dramatic changes observed in colonic mucosa[12]. Similarly, Mitsialis *et al.* conducted a comprehensive CyTOF study on blood samples from patients with ulcerative colitis, including severe ones with elevated CRP levels, but found no characteristics able to distinguish cases from healthy controls[13].

Among the 430 differentially expressed genes between the two groups, we identified only seven genes related to immune response and previously implicated in inflammatory bowel disease pathogenesis. Three of them are related to gut barrier function, one to antibacterial defence and three to lymphocyte trafficking and Th17 differentiation.

Starting with genes associated with barrier function, *SLC12A5-AS1* was threefold more highly expressed in the blood of patients with acute severe ulcerative colitis. This long non-coding RNA is known to be up-regulated in the colonic mucosa of patients with ulcerative colitis[14]. It may play a role in up-regulating *MMP-9* expression, a matrix metallopeptidase involved in the degradation of extracellular matrix proteins, via NF-kB signalling[15,16]. In ulcerative colitis, excess amounts of MMP-9 are produced by neutrophils, leading to higher blood levels, and disruption of tight junctions in the colonic mucosa[17,18]. *BANF1*, another one of the up-regulated genes in our data, has been also shown to be involved in the maintenance of gut barrier function through the NF-kB pathway[19], and the pathogenesis of psoriasis, a skin immune mediated disease associated with inflammatory bowel disease[20]. On the other hand, we observed a down-regulation of *IL24*, a member of the IL-10 family with immunosuppressive activity, suggested to protect the integrity of inflamed mucosa of patients with ulcerative colitis[21]. The synthesis of IL-24 is modulated by a spectrum of stimuli, including reactive oxygen species, lipopolysaccharides, and a repertoire of cytokines. Among these cytokines, a nuanced regulation is observed. Specifically, IL-1B functions as an enhancer while TNF exerts an inhibitory influence[22]. In a mouse model of colitis, IL-24 played a beneficial role for mucosal remodelling after the acute phase of inflammation, partly via

induction of MMP-9 production[22]. These three differentially expressed genes suggest an impairment in the regulation of epithelial repair in patients with acute severe ulcerative colitis. This aligns with the results we described in Chapter 2 showing that acute severe ulcerative colitis is often characterised by the presence of deep ulcers in the colonic mucosa.

The last higher expressed gene of interest that we identified was *CLEC12A*. *CLEC12A* is involved in antibacterial autophagy, a defence mechanism of epithelial cells to restrict microbial replication in the gut, and regulated by ATG16L1, a susceptibility gene for Crohn's disease[23,24]. In a mouse model of colitis, *Clec12a* deficiency was associated with worsening of phenotype by failure to control expansion of specific members of the gut microbiota[25]. Although our study design does not allow us to draw conclusions about causality, we can hypothesise that the higher expression of *CLEC12A* is a response to the expansion of pathobionts in the gut lumen described in Chapter 3.

Among the down-regulated genes, we identified *MADCAM1-AS*, an antisense RNA for the *MADCAM1* gene. *MADCAM1* is the receptor of the α4B7 integrin, the target of vedolizumab, a monoclonal antibody used in the treatment of ulcerative colitis[26]. Expression of α4B7 by circulating regulatory T cells is increased in patients with ulcerative colitis leading to the accumulation of these cells in the colonic mucosa[27]. On the ligand side, *MADCAM1* expression by endothelial cells is up-regulated in the colonic mucosa of patients with ulcerative colitis[28]. The lower expression of *MADCAM1-AS* we observed aligns with the described upregulation of *MADCAM1*. Consistent with this differential expression of a gene implicated in lymphocyte trafficking, we also observed a decrease in *CXCR5* in patients with acute severe ulcerative colitis. CXCR5 is involved in the interactions between T and B cells and a subset of circulating T cells lacking CXCR5 is associated with severity in ulcerative colitis[29]. Moreover, in the study by Long *et al.* these CXCR5- T cells were identified as IL-21 producers and their presence was associated with higher serum levels of

IL-21[29]. Consistent with this finding, we observed that *IL21R-AS1*, an antisense RNA for *IL21R*, was also lower expressed in our acute severe ulcerative colitis group. Genetic variants in the IL-21 region are associated with ulcerative colitis[30]. The expression of *IL21R* by CD4+ T lymphocytes in the lamina propria of the colonic mucosa has been shown to be increased in ulcerative colitis, triggering the production of pro-inflammatory cytokines and differentiation into Th17 cells[31]. CXCR5- T cells can also promote B cell differentiation and antibody production[32]. These results suggest that the genes with lower expression we observed are related to circulating subsets of immunosuppressive T cells in patients with acute severe ulcerative colitis. This aligns with the central role of the IL-23/Th17 axis among immune cells in the colonic lamina propria described in Chapter 4.

Our study has several limitations such as the relatively modest sample size. The quality of our cDNA library preparation may have been hampered by the fact that we used frozen EDTA blood. We also observed an effect of haemoglobin on whole blood RNASeq, which could not be accounted for as a covariate in our differential expression model as it is part of the acute severe ulcerative colitis definition. We did not identify a higher expression of genes associated with inflammatory response in the blood of patients with acute severe ulcerative colitis despite systemic inflammation being the hallmark of this phenotype. This suggests that, in acute severe ulcerative colitis, the production of CRP by the hepatocytes is not likely mediated by cytokines from circulating cells but rather originates from the colonic mucosa. In line with this hypothesis, we observed a strong enrichment of pathways involved in inflammatory response in our single-cell RNAseq data of gut lamina propria cells, as described in Chapter 4. The expression of *IL1B* was up-regulated in several clusters of neutrophils, myeloid cells and T cells in patients with acute severe ulcerative colitis. In our clinical practice, we have observed that in patients with acute severe ulcerative colitis, the resolution of systemic inflammation occurs rapidly within a few hours after colectomy. This

observation suggests that the removal of the colon effectively suppresses the source of systemic inflammation. To confirm this hypothesis, it would be informative to measure the serum and colonic levels of CRP-inducing cytokines, i.e. IL-6, TNF and IL-1B[33], in patients with acute severe ulcerative colitis, and to compare our blood RNASeq results with colonic bulk RNASeq investigation on top of our single-cell RNASEq data.

5.5. Outlook

In conclusion, these preliminary results on whole blood RNASeq suggest that the inflammatory outburst observed in acute severe ulcerative colitis is primarily related to a severe colonic inflammation without a preeminent role of circulating cells. In-depth investigation of few inflammatory bowel disease-related genes mainly shows impaired systemic compensatory mechanisms, including epithelial barrier repair, containment of pathobionts and aberrant immunosuppressive T cells. We will pursue the present experiments by focusing on venues not yet explored. For example, the present libraries are not well suited for the study of non-coding RNAs and this is a major shortcoming. Likewise, we have not performed an isoform analysis of the expression data. It is possible that by focusing on genes, *i.e.* collection of isoforms, we missed important disease protective or predisposing transcripts. Finally, we have not yet done a gene set enrichment analysis (GSEA). GSEA is ideally suited to track the cumulative effect of expression differences on biologically important signature pathways. These findings open up opportunities for the development of new therapeutic targets in acute severe ulcerative colitis, such as the correction of the IL-24 deficiency to enhance mucosal barrier remodelling.

5.6. References

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6. Concluding remarks and future directions.

6.1. Rationale and objectives of the thesis project

Acute severe ulcerative colitis represents a unique and clinically challenging presentation of ulcerative colitis, characterised by the concurrent presence of systemic inflammation and bloody diarrhoea[1]. Each acute severe ulcerative colitis episode carries a substantial risk of complications, including bowel perforation, massive haemorrhage, thrombo-embolic events, and toxic megacolon, with a 13% likelihood of colectomy[2]. However, the pathogenesis of acute severe ulcerative colitis remains an area of limited understanding.

A hypothesis has been proposed suggesting that microorganisms might serve as triggers of the pronounced inflammatory response seen in acute severe ulcerative colitis. This hypothesis draws on the clinical resemblances observed between infectious colitis and acute severe ulcerative colitis, including shared features such as fever and elevated C-Reactive Protein (CRP) levels. Support for this hypothesis is derived from the established role of the microbiota in the pathogenesis of acute severe ulcerative colitis[3,4]. Furthermore, the relationship between the host and the microbiota is characterised by mutual control. Notably, previous studies have underlined the host's compromised defence mechanisms against pathogens in the context of inflammatory bowel disease[5,6].

We postulated that a malfunctioning gut microbiome, characterised by diminished diversity and the depletion of anti-inflammatory bacterial species, might provide an environment conducive to the proliferation of a pathobiont within the colonic lumen. The expansion of this pathobiont could, in turn, trigger a systemic inflammatory response in individuals with permissive gut mucosal immunity, leading to the onset of an acute severe ulcerative colitis flare.

The principal aim of this project was to elucidate the role of the microbiota and host factors in driving acute severe ulcerative colitis flares. To address this objective, we formulated three specific aims:

1) Aim 1: To confirm a reduced microbiome diversity and identify candidate pathobionts among the gut microbiota of acute severe ulcerative colitis patients using 16S rRNA sequencing in stool and rectal biopsy samples.

2) Aim 2: To identify the cellular subtypes and pathways involved in the gut mucosal inflammation in acute severe ulcerative colitis patients using scRNAseq in rectal biopsy samples.

3) Aim 3: To determine the host pathways mediating the systemic inflammatory outburst in the blood of acute severe ulcerative colitis patients using whole blood RNA sequencing.

Our investigation encompassed a cohort study involving two distinct groups, each consisting of around 20 participants: one group with acute severe ulcerative colitis and the other with non-severe ulcerative colitis flares. The novelty of this research lies in its comprehensive approach, which melds extensive clinical phenotyping with a multi-omics strategy, integrating microbiome analysis, scRNASeq, and whole blood RNASeq.

6.2. Summary of the findings

In Chapter 2 of this thesis, our objective was to refine our understanding of the phenotype of acute severe ulcerative colitis by exploring the link between colonic and systemic inflammation, as indicated by elevated CRP levels. Utilising data from our international prospective cohort of extensively characterised patients with active ulcerative colitis, we identified a robust association between elevated CRP levels and the presence of deep ulcers, which signify severe colonic inflammation. These findings were subsequently validated in a retrospective analysis of colectomy specimens from patients with active ulcerative colitis. Notably, in the retrospective cohort, a CRP level exceeding 100 mg/L displayed a 100% positive predictive value for the presence of deep ulcers.

Chapter 3 of this thesis was dedicated to exploring the connection between acute severe ulcerative colitis and known pathogens, microbiota disruptors, microbiota diversity and composition. To accomplish this, we conducted an extensive analysis of clinical and biological attributes of the study cohort, as well as microbiota characteristics derived from stool and rectal biopsies collected at the time of enrollment and at a three-month follow-up mark. At enrollment, no evidence of overt infectious colitis was detected in our cohort, and no significant disparities in dietary intake were noted between the two severity groups. In the acute severe ulcerative colitis group, a higher proportion of patients had received antibiotics within the three months preceding enrollment. However, it is noteworthy that antibiotics were primarily prescribed for diarrhoea corresponding to the symptoms of the flare, except for one instance, making it unlikely that antibiotics were the primary triggers for the flare. In patients with acute severe ulcerative colitis compared to patients with non-severe ulcerative colitis, we observed substantial alterations in the gut microbiota, including reduced alpha-diversity, an increased abundance of Proteobacteria (particularly members of the *Escherichia/Shigella* genus) and members of the oral microbiome, and a reduction in members of the Lachnospiraceae and Ruminococcaceae families. These changes were reflected in the microbial dysbiosis index[7] and exhibited correlations with systemic inflammation, as indicated by CRP levels.

In Chapter 4, we used single-cell RNA sequencing (scRNASeq) technology to study the immune features of the colonic mucosa in patients with acute severe ulcerative colitis compared to patients with non-severe ulcerative colitis. Our analysis unveiled a distinctive transcriptomic profile of plasmablasts in severe cases, characterised by enhanced IgG production, as well as an expansion of a specific T cell population expressing *IL26*, recently identified as pro-inflammatory IL17-producing T cells expanded in the colonic mucosa of patients with ulcerative colitis[8]. In addition to these changes in adaptive immune cells, we also noted a pro-inflammatory profile among innate immune cells characteristic of a pro-Th17 mucosal environment. In summary, our

scRNAseq findings underscore that the colonic mucosa in acute severe ulcerative colitis patients exhibits elevated inflammatory characteristics, encompassing both the adaptive and innate immune systems, with a central focus on IgG production and the Th17/IL-23 pathway.

Chapter 5 aimed to deepen our understanding of the underlying mechanisms of systemic inflammation. We focused on an investigation of the blood transcriptome in patients participating in the study. Our working hypothesis was that genes associated with inflammatory responses, particularly those related to pathogen responses, would exhibit higher expression in the blood of patients with acute severe ulcerative colitis compared to non-severe cases. However, we found no clear distinction between severe and non-severe cases in the dimensionality reduction plot. Moreover, we did not identify any pathways enriched for differentially expressed genes. This observation suggests that in acute severe ulcerative colitis, the systemic inflammation, characterised by the hepatic production of CRP is less likely to be orchestrated by cytokines originating from circulating cells. Rather, it seems to stem from cytokines produced within the colonic mucosal milieu.

6.3. Drawing connections between the findings

We acknowledge that our study is limited by the absence of a healthy control group. While our research allow us to draw conclusions regarding the distinctive characteristics of acute severe ulcerative colitis in comparison to non-severe patients, it would have been valuable to discern whether the abnormalities observed in acute severe patients represents a unique profile or if there exists a gradient of immunological and gut microbiota alterations from normal to severe. However, in the context of available literature, the combined findings from each chapter contribute significantly to our understanding of the pathogenesis of acute severe ulcerative colitis. Here, we provide a brief overview of the key connections that can be drawn between the results of our study.

Notably, the various specificities observed in patients with acute severe ulcerative colitis, when compared to those with non-severe ulcerative colitis, across clinical, gut microbiota, colonic scRNASeq, and whole blood RNASeq levels, all point toward heightened inflammatory factors at the colonic level. These factors potentially play a crucial role in exacerbating mucosal damage and driving systemic inflammation.

In particular, regarding the interplay between the host and the gut microbiota, the expansion of pathobionts may be initiated by the inflamed state of the colonic mucosa[9]. This expansion can intensify colonic inflammation by stimulating the increased production of pro-Th17 cytokines and IL-6 by immune cells in the mucosa[10,11]. Furthermore, it influences the shift in the production of antibodies by plasmablasts, steering it away from IgA towards IgG[12].

Similarly, the reduction in butyrate-producing bacteria may result from the antimicrobial activity associated with the inflamed mucosa[13]. This reduction further contributes to the inflammatory state by compromising its anti-inflammatory function, particularly in terms of stimulating the production of IL-10[14].

By enhancing the pro-inflammatory components of the colonic immune system, the deeper alterations observed in the gut microbiota of patients with acute severe ulcerative colitis, in comparison to patients with non-severe ulcerative colitis, could potentially play a significant role in the observed differences in terms of clinical severity.

On the host side of the equation, the increased production of IgG, forming immune complexes with pathobionts, might heighten the activation of myeloid cells, particularly in individuals carrying risk variants on Ig receptors[5,15]. This hyper-activated state of myeloid cells and neutrophils can enhance the recruitment of T cells through the expression of *CXCL9, CXCL10,* and *CXCL11*[16]. It also contributes to the differentiation of T cells into Th17 cells through the expression of *IL1B*[17] and

influences the IgA/IgG balance of plasmablasts[6,18]. These pro-inflammatory cytokines that we observed in excess in the transcriptome of colonic myeloid cells and T cells of patients acute severe ulcerative colitis compared to those of with non-severe ulcerative colitis could potentially play a central role in driving the systemic inflammation characteristic of acute severe ulcerative colitis.

A circulating subset of immunosuppressive T cells lacking *CXCR5* expression has the capacity to further promote the differentiation of T cells into Th17 cells and the production of IgG by plasmablasts[19]. The results of our study's whole blood bulk RNASeq suggest the presence of such a subset in patients with acute severe ulcerative colitis compared to those with non-severe ulcerative colitis. This may perpetuate the above-mentioned pathogenic loop involving T cells, innate immune cells, and B cells. As a consequence, these activated immune cells exacerbate mucosal damage, which is not effectively compensated for by systemic mechanisms, as demonstrated by the dysregulation of genes associated with barrier function and the reduction in *IL24* expression in the blood transcriptome. These findings offer potential explanations for the hyper-inflammatory state clinically observed in acute severe ulcerative colitis.

Finally, the hallmark of acute severe ulcerative colitis, systemic inflammation measured by elevation of CRP levels, might be driven mainly by the hyper-inflammatory state of the colonic mucosa rather than by circulating cells. Increased expression of *IL1B* and *IL6* by innate immune cells and T cells from the colonic mucosa, associated with severe mucosal damage, could provide an explanation for the observed association between deep ulcers and elevated CRP levels.

6.4. Future directions

Following the hypothetical connections between the players of colonic inflammation - innate and adaptive immune cells- expansion of pathobionts and reduction of butyrate-producing bacteria, lack

of systemic compensatory mechanisms, an integrative analysis of our multi-omics data will be highly informative.

First, in the scRNASeq dataset, we will use receptor-ligand pairs to infer cell-cell interactions (CellChat)[20]. This technique quantifies the interaction intensity between two cell subsets by counting the unique receptors and ligands that link them, leading to the creation of adjacency matrices that encapsulate all cell-cell interactions in the dataset. Next, cell-cell interaction networks can be plotted using significant interactions. This will allow us to test our hypothesis of cross-talk between B and T cells, B and innate immune cells and T and innate immune cells described above.

Next, in the microbiome dataset, we will test for compositional correlations between abundances of pathobionts and remaining bacteria to identify correlated taxa. This will help us elucidate the dynamics of microbiota changes in acute severe ulcerative colitis.

Finally, we plan to integrate the microbiome and transcriptome data using a machine-learning framework called Lasso penalised regression. This regression model can be used to identify specific interactions between expression of individual host genes and gut microbial taxa by forcing the coefficients of the non-informative features of the model to zero, giving interpretable results only for the taxa significantly associated with the abundance of a transcript[21]. This will be done both for colonic scRNASeq and whole blood bulk RNASeq.

This multi-omics integration will contribute valuable insights into the pivotal cellular and bacterial components involved in the pathogenesis of acute severe ulcerative colitis. These findings will help to guide future clinical research, directing efforts toward microbiome modulation, targeted interventions on plasmablasts, or nuanced inhibition of the Th17/IL-23 axis. To treating clinicians, it may provide valuable information for addressing the concerns of patients who seek to understand why they are experiencing acute severe ulcerative colitis, ultimately assisting them in better coping with this distressing personal experience.

6.5. References

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Appendices

A. Other publications

The contribution of the thesis author to other published manuscripts during the thesis period is listed below.

Rivière P, Vermeire S, Irles-Depe M, Van Assche G, Rutgeerts P, Denost Q, Wolthuis A, D'Hoore A, Laharie D, Ferrante M. Rates of Post-operative Recurrence of Crohn's Disease and Effects of Immunosuppressive and Biologic Therapies. **Clin Gastroenterol Hepatol. 2020** Apr;19(4):713-720.e1.

Rivière P, D'Haens G, Peyrin-Biroulet L, Baert F, Lambrecht G, Pariente B, Bossuyt P, Buisson A, Oldenburg B, Vermeire S, Laharie D. Location but Not Severity of Endoscopic Lesions Influences Endoscopic Remission Rates in Crohn's Disease: A Post Hoc Analysis of TAILORIX. **Am J Gastroenterol. 2021** Jan 1;116(1):134-141.

Rivière P, Pekow J, Hammoudi N, Wils P, De Cruz P, Wang CP, Mañosa M, Ollech J, Allez M, Nachury M, Kamm MA, Ahanori M, Ferrante M, Buisson A, Singh S, Laharie D, Diouf M, Fumery M. Comparison of the risk of Crohn's disease postoperative recurrence between modified Rutgeerts score i2a and i2b categories: an individual patient data meta-analysis. **J Crohns Colitis. 2022** Sep 18:jjac137. doi: 10.1093/ecco-jcc/jjac137. Epub ahead of print. PMID: 36124813.

Rivière P, Bislenghi G, Vermeire S, Domènech E, Peyrin-Biroulet L, Laharie D, D'Hoore A, Ferrante M. Postoperative Crohn's Disease Recurrence: Time to Adapt Endoscopic Recurrence Scores to the Leading Surgical Techniques. **Clin Gastroenterol Hepatol. 2022** Jun;20(6):1201-1204. doi: 10.1016/j.cgh.2022.02.025. Epub 2022 Feb 17. PMID: 35183769.

Torres J, Chaparro M, Julsgaard M, Katsanos K, Zelinkova Z, Agrawal M, Ardizzone S, Campmans-Kuijpers M, Dragoni G, Ferrante M, Fiorino G, Flanagan E, Gomes CF, Hart A, Hedin CR, Juillerat P, Mulders A, Myrelid P, O'Toole A, **Rivière P,** Scharl M, Selinger CP, Sonnenberg E, Toruner M, Wieringa J, Van der Woude CJ. European Crohn's and Colitis Guidelines on Sexuality, Fertility, Pregnancy, and Lactation. **J Crohns Colitis. 2023** Jan 27;17(1):1-27. doi: 10.1093/ecco-jcc/jjac115. PMID: 36005814.

Rivière P, Kanters C, Pellet G, Ni A, Hupé M, Aboulhamid N, Poullenot F, Bitton A, Zerbib F, Lakatos PL, Afif W, Laharie D, Bessissow T. Comparative Effectiveness of Ustekinumab and Anti-TNF Agent as First-Line Biological Therapy in Luminal Crohn's Disease: A Retrospective Study From 2 Referral centres. **Inflamm Bowel Dis. 2023** Jun 1;29(6):923-931. doi: 10.1093/ibd/izac167. PMID: 35917111.

Rivière P, Bislenghi G, Hammoudi N, Verstockt B, Brown S, Oliveira-Cunha M, Bemelman W, Pellino G, Kotze PG; 8 th Scientific Workshop of the European Crohn's and Colitis Organisation; Ferrante M, Panis Y. Results of the Eighth Scientific Workshop of ECCO: Pathophysiology and risk factors of postoperative Crohn's disease recurrence after an ileocolic resection. **J Crohns Colitis. 2023** Apr 18:jjad054. doi: 10.1093/ecco-jcc/jjad054. Epub ahead of print. PMID: 37070326.

B. Supplementary material for each chapter

a. Chapter 3

Supplementary Tables

Supplementary Table 1: Characteristics of the study patients at three months.

Variable	Acute severe ulcerative colitis, n = 13	Non-severe ulcerative colitis, n = 16	p-value
Response to therapy, n (%)	6 (46)	11 (69)	0.20
Partial Mayo score, median (IQR)	2.0 (0.0 – 8.0)	0.0 (0.0 – 2.5)	0.09
C-Reactive protein (mg/l), median (IQR)	7.2 (1.7 - 39.6)	1.9 (0.7 - 3.9)	0.14
Albumin, g/l, median (IQR)	38.6 (36.4 - 40.7)	38.3 (37.4 - 42.3)	0.70
haemoglobin, g/dL, median (IQR)	12.2 (11.2 - 13.3)	13.5 (13.0 - 13.9)	0.07
Faecal calprotectin, mg/kg, median (IQR)	24.0 (16.0 - 32.0)	172.5 (10.3 - 429.0)	0.50
UCEIS score, median (IQR)	6.0 (5.0 - 7.0)	4.0 (4.0 - 5.0)	< 0.01

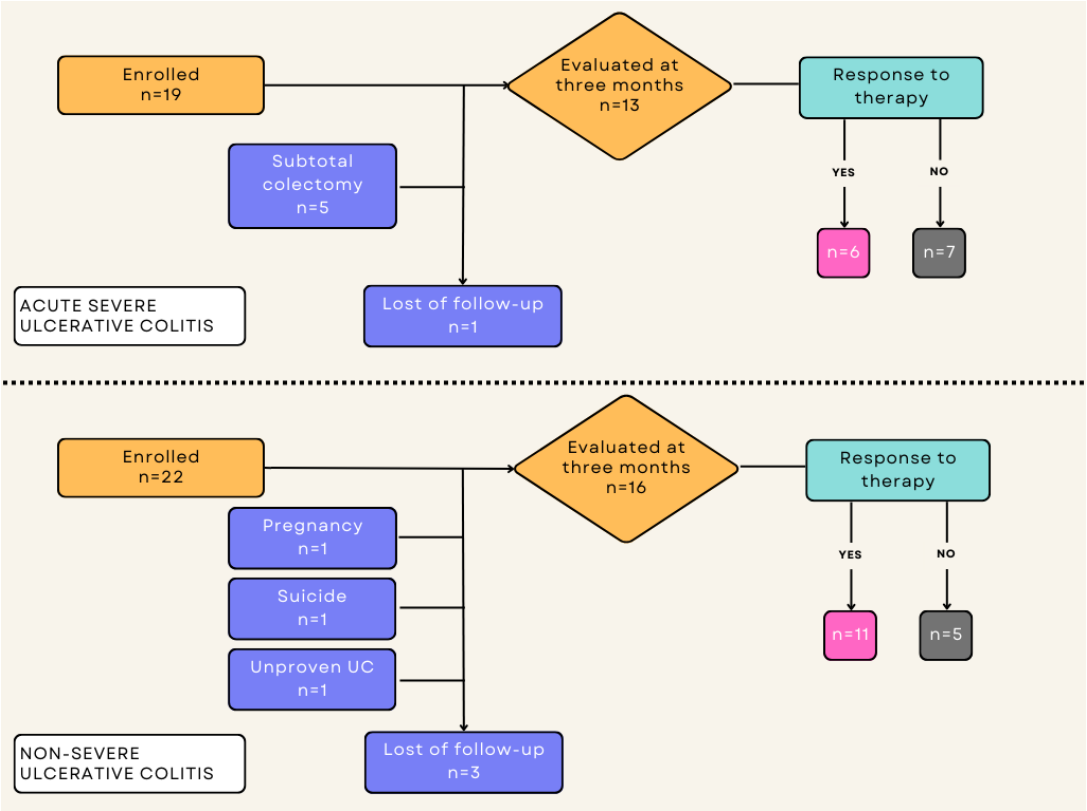
IQR: interquartile range; UCEIS: Ulcerative Colitis Endoscopic Index of Severity.

Supplementary Table 2: Characteristics of the study patients at enrollment according to response to therapy at three months.

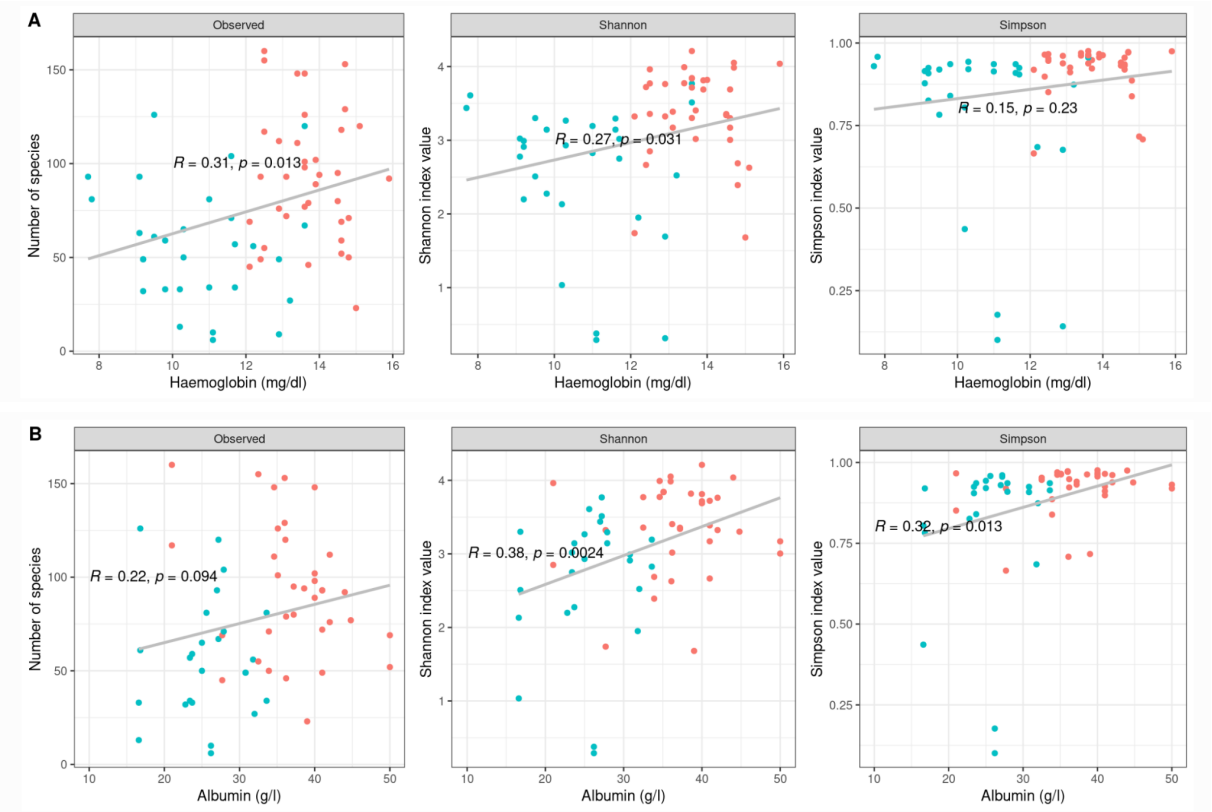
Variable	Non-responders, n = 17	Responders, n = 17	p-value
Severity group, n (%)			0.09
- acute severe ulcerative colitis	12 (71)	6 (35)	
- non-severe ulcerative colitis	5 (29)	11 (65)	
Female gender, n (%)	10 (59)	9 (53%)	0.70
Age, median (IQR)	40.0 (31.0 - 49.0)	49.0 (37.0 - 58.0)	0.30
Body mass index, kg/m2, median (IQR)	22.3 (21.1 - 27.2)	24.6 (22.5 - 27.0)	0.30
Disease duration, years, median (IQR)	3.4 (1.8 - 7.9)	7.0 (3.5 - 14.8)	0.40
First flare, n (%)	1 (5.9%)	3 (18%)	0.60
Never exposed to biological therapy, n (%)	7 (41%)	5 (29%)	0.50
Disease extent, n (%)			0.40
- Pancolitis	8 (47%)	12 (71%)	
- Left-sided colitis	7 (41%)	4 (24%)	
- Proctitis	2 (12%)	1 (5.9%)	
Symptoms duration in weeks, median (IQR)	4.1 (1.7 – 16.6)	6.7 (2.6 - 11.6)	0.70
Lichtiger score at enrollment, median (IQR)	13.0 (13.0 - 14.0)	10.0 (7.0 - 12.0)	0.01
C-Reactive protein at enrollment (mg/l), median (IQR)	41.6 (16.3 - 76.8)	9.3 (4.0 - 19.0)	0.03
haemoglobin at enrollment, g/dL, median (IQR)	11.1 (9.5 - 12.9)	13.1 (11.7 - 13.9)	0.11
Albumin at enrollment , g/l, median (IQR)	27.5 (24.7 - 34.2)	34.1 (28.9 - 36.2)	0.30
UCEIS score, median (IQR)	6.0 (5.0 - 7.0)	4.0 (4.0 - 5.0)	< 0.01

IQR: interquartile range; UCEIS: Ulcerative Colitis Endoscopic Index of Severity. Patients undergoing subtotal colectomy before three months were included in the non-responders groups.

Supplementary Figures



Supplementary Figure 1 : Patients flowchart from enrollment to evaluation at three months.



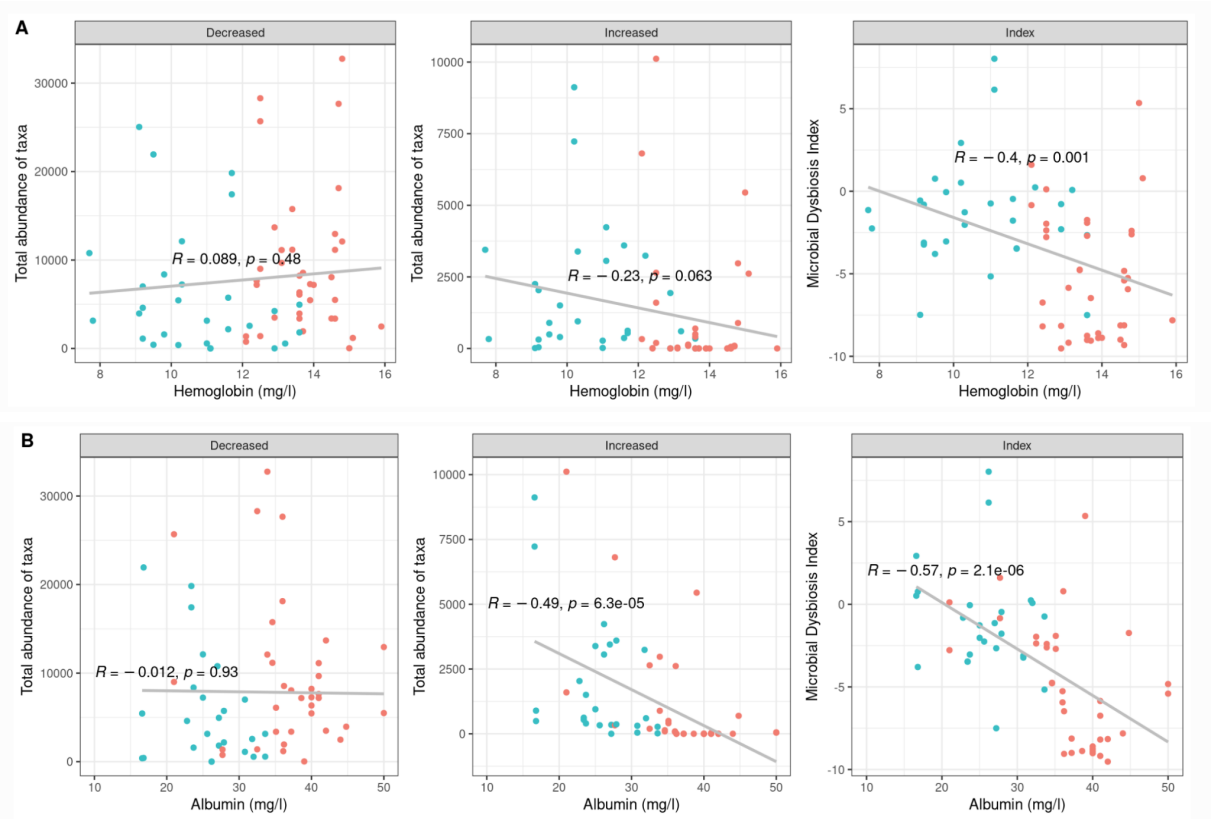
Supplementary Figure 2: Correlation between haemoglobin and albumin levels and alpha-diversity of the gut microbiota at enrollment.

Panel A: Scatter-plot representing the alpha-diversity of the gut microbiota at enrollment according to haemoglobin levels (X-axis) on the same day in the two groups (n=66 samples) using three different indices displayed as Y-axis. Correlation was calculated using Pearson coefficients and p-value.

Panel A: Scatter-plot representing the alpha-diversity of the gut microbiota at enrollment according to albumin levels (X-axis) on the same day in the two groups (n=66 samples) using three different indices displayed as Y-axis. Correlation was calculated using Pearson coefficients and p-value.

Left subpanel: Observed richness, corresponding to the number of different species observed by sample. Middle subpanel: Shannon index takes into account the number of species and their abundance. Right subpanel: Simpson index takes into account the number of species and their abundance and is more sensitive to their abundance than the Shannon index.

Each dot represents one sample. Red and blue dots correspond to non-severe and acute severe colitis groups respectively.



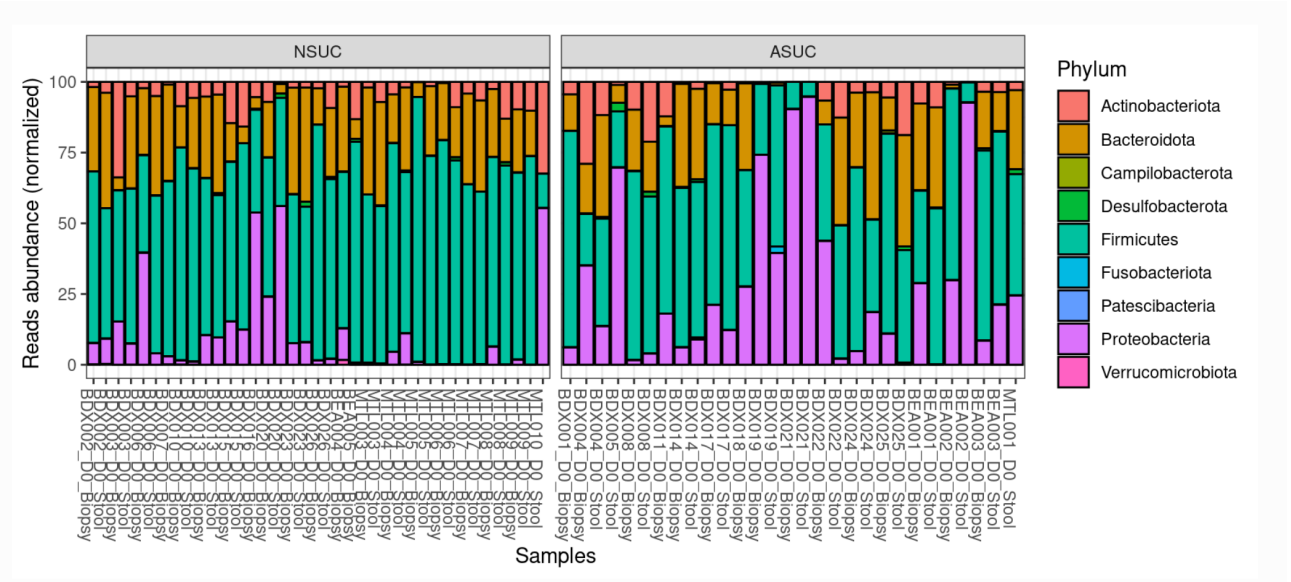
Supplementary Figure 3: Correlation between haemoglobin and albumin levels and the microbial dysbiosis index at enrollment.

Panel A: Scatter-plot representing the microbial dysbiosis index (Y-axis) of the gut microbiota at enrollment according to haemoglobin levels (X-axis) on the same day in the two groups (n=66 samples). Correlation was calculated using Pearson coefficients and p-value.

Panel B: Scatter-plot representing the microbial dysbiosis index (Y-axis) of the gut microbiota at enrollment according to albumin levels (X-axis) on the same day in the two groups (n=66 samples). Correlation was calculated using Pearson coefficients and p-value.

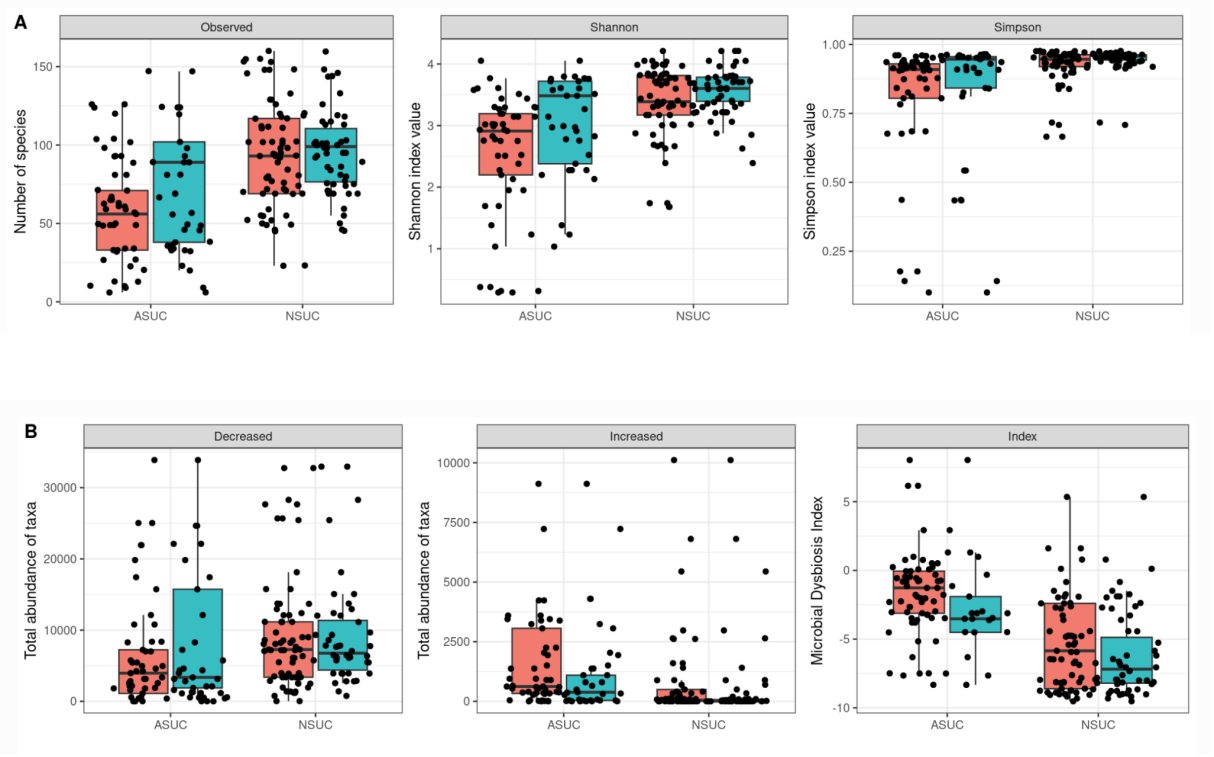
Left subpanel: Total abundance of taxa known to be decreased in IBD in Y-axis, as described by Gevers et al[29]. Middle subpanel: Total abundance of taxa known to be increased in IBD in Y-axis, as described by Gevers et al[29]. Right subpanel: Overall index corresponding to the log of [total abundance in organisms increased in IBD] over [total abundance of organisms decreased in IBD] in Y-axis, as described by Gevers et al[29].

Each dot represents one sample. Red and blue dots correspond to non-severe and acute severe colitis groups respectively.



Supplementary Figure 4: Abundance of each phylum by samples at enrollment.

Bar plot showing the normalised abundance of each phylum (Y-axis, in %) by sample (X-axis) in the two groups at enrollment (n=66 samples). Each phylum is represented by a color. ASUC: acute severe ulcerative colitis; NSUC: non-severe ulcerative colitis.

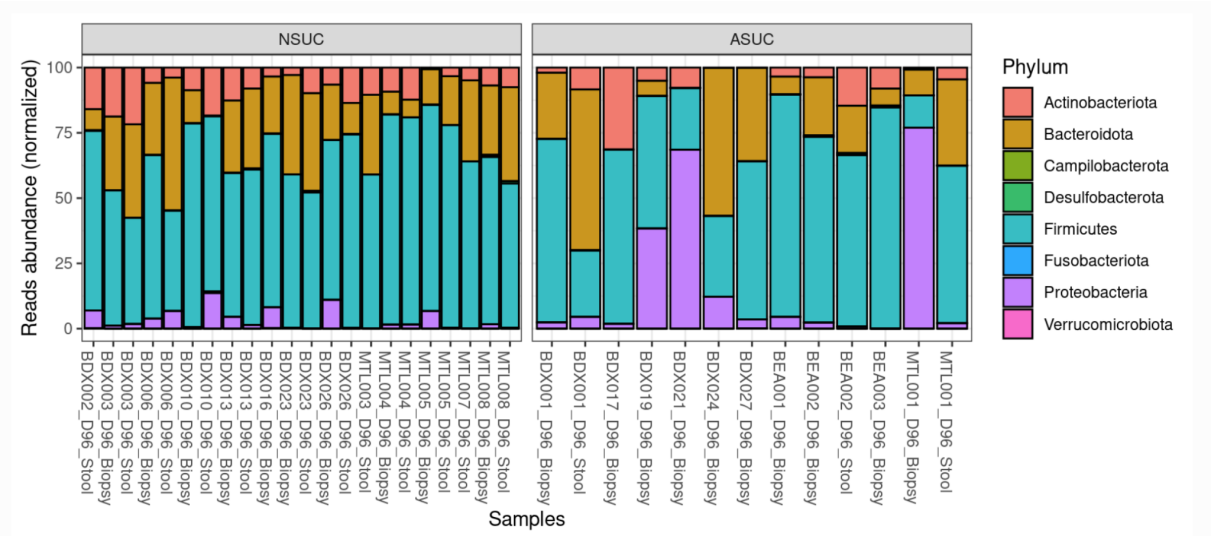


Supplementary Figure 5: Evolution of the alpha-diversity and the microbial dysbiosis index after three months in each group.

Panel A: Box-plot representing the alpha-diversity of the gut microbiota at enrollment and at three months in all patients (n=101). Left subpanel: Observed richness, corresponding to the number of different species observed by sample. Middle subpanel: Shannon index takes into account the number of species and their abundance. Right subpanel: Simpson index takes into account the number of species and their abundance and is more sensitive to their abundance than the Shannon index.

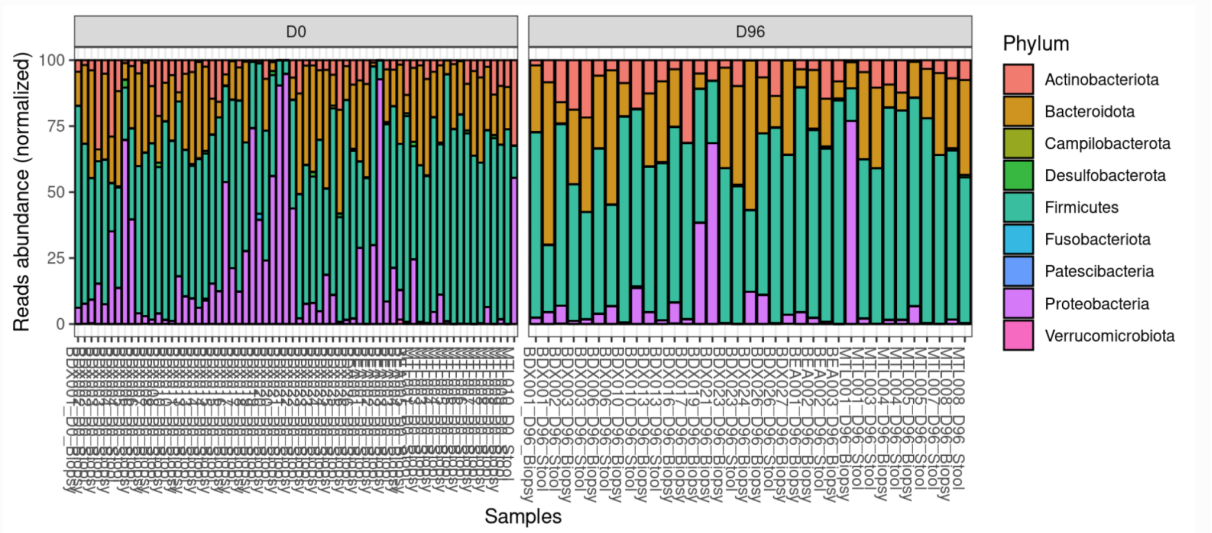
Panel B: Box-plot representing the microbial dysbiosis index at enrollment and at three months in all patients (n=101). Left subpanel: Total abundance of taxa known to be decreased in IBD in Y-axis, as described by Gevers et al[29]. Middle subpanel: Total abundance of taxa known to be increased in IBD in Y-axis, as described by Gevers et al[29]. Right subpanel: Overall index corresponding to the log of [total abundance in organisms increased in IBD] over [total abundance of organisms decreased in IBD] in Y-axis, as described by Gevers et al[29].

The box in the plot represents the middle 50% of the data, with the median line dividing the box into two parts. The whiskers extend from the box to indicate the variability outside the upper and lower quartiles. Red and blue boxes correspond to enrollment (D0) and three months (D96) respectively. Each dot represents one sample. P-values were calculated using a Wilcoxon test to compare the groups. ASUC: acute severe ulcerative colitis; NSUC: non-severe ulcerative colitis.



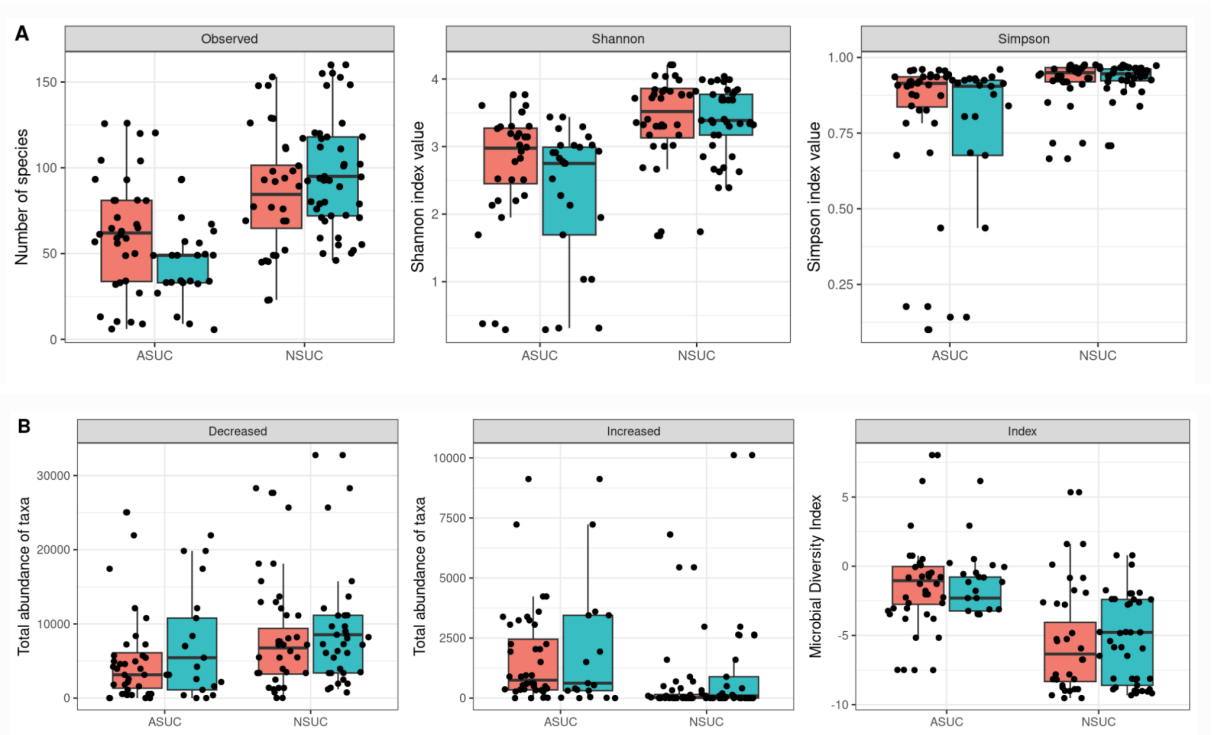
Supplementary Figure 6: Abundance of each phylum by samples at three months.

Bar plot showing the normalized abundance of each phylum (Y-axis, in %) by sample (X-axis) in the two groups at three months (n=35 samples). Each phylum is represented by a color. ASUC: acute severe ulcerative colitis; NSUC: non-severe ulcerative colitis.



Supplementary Figure 7: Abundance of each phylum by samples according to sampling time-point.

Bar plot showing the normalized abundance of each phylum (Y-axis, in %) by sample (X-axis) in the two groups at enrollment (D0) and at three months (D96) (n=101 samples). Each phylum is represented by a color.

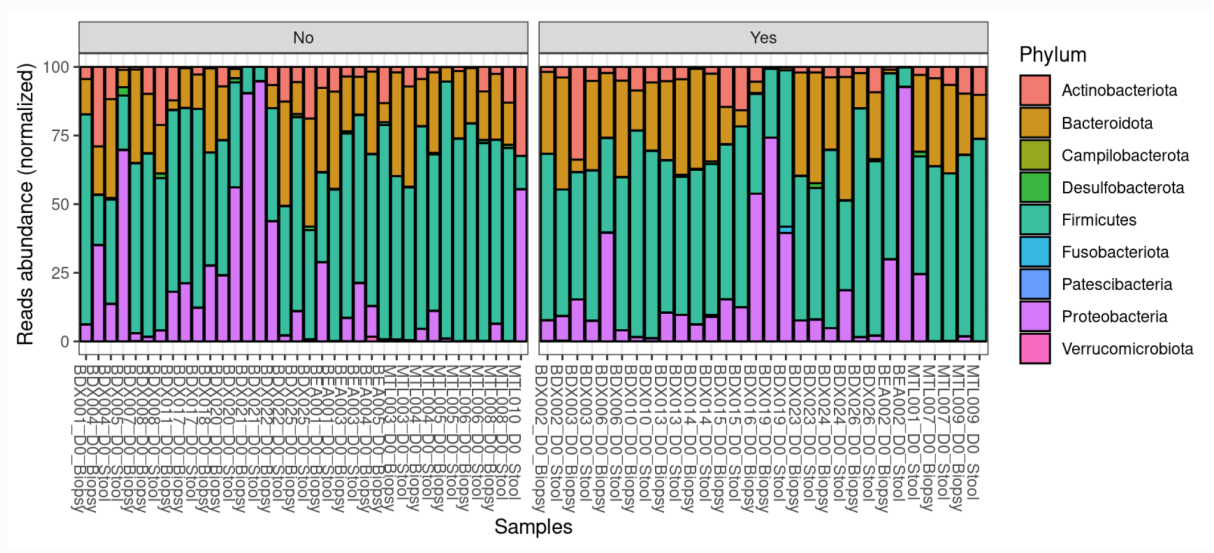


Supplementary Figure 8: Alpha-diversity and microbial dysbiosis index at enrollment according to response to therapy at three months by group.

Panel A: Box-plot representing the alpha-diversity of the gut microbiota at enrollment according to response to therapy at three months (n=66). Left subpanel: Observed richness, corresponding to the number of different species observed by sample. Middle subpanel: Shannon index takes into account the number of species and their abundance. Right subpanel: Simpson index takes into account the number of species and their abundance and is more sensitive to their abundance than the Shannon index.

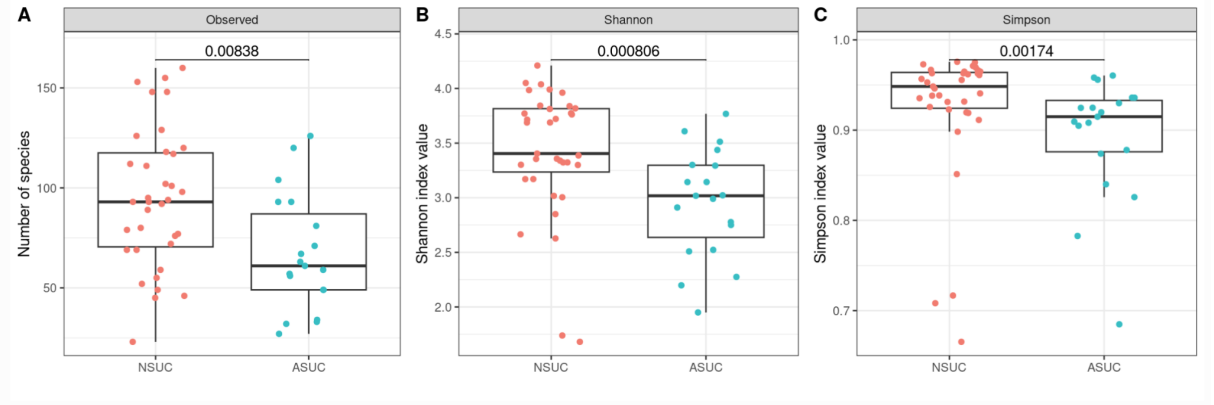
Panel B: Box-plot representing the microbial dysbiosis index at enrollment according to response to therapy at three months (n=66). Left subpanel: Total abundance of taxa known to be decreased in IBD in Y-axis, as described by Gevers et al[29]. Middle subpanel: Total abundance of taxa known to be increased in IBD in Y-axis, as described by Gevers et al[29]. Right subpanel: Overall index corresponding to the log of [total abundance in organisms increased in IBD] over [total abundance of organisms decreased in IBD] in Y-axis, as described by Gevers et al[29].

The box in the plot represents the middle 50% of the data, with the median line dividing the box into two parts. The whiskers extend from the box to indicate the variability outside the upper and lower quartiles. Red and blue boxes correspond to non-responders and responders at three months respectively. Each dot represents one sample. P-values were calculated using a Wilcoxon test to compare the groups. ASUC: acute severe ulcerative colitis; NSUC: non-severe ulcerative colitis.



Supplementary Figure 9: Abundance of each phylum by samples at enrollment according to response to therapy at three months.

Bar plot showing the normalised abundance of each phylum (Y-axis, in %) by sample (X-axis) at enrollment according to response to therapy (Yes) or non-response (No) (n=66 samples). Each phylum is represented by a colour.



Supplementary Figure 10: Alpha-diversity of the gut microbiota at enrollment in the two groups in patients not exposed to antibiotics.

Box-plot representing the alpha-diversity of the gut microbiota at enrollment excluding patients exposed to antibiotics in the past three months (n=54). Panel A: Observed richness, corresponding to the number of different species observed by sample. Panel B: Shannon index takes into account the number of species and their abundance. Panel C: Simpson index takes into account the number of species and their abundance and is more sensitive to their abundance than the Shannon index.

The box in the plot represents the middle 50% of the data, with the median line dividing the box into two parts. The whiskers extend from the box to indicate the variability outside the upper and lower quartiles. Red and blue dots correspond to non-severe and severe patients respectively. Each dot represents one sample. P-values were calculated using a Wilcoxon test to compare the groups. ASUC: acute severe ulcerative colitis; NSUC: non-severe ulcerative colitis.

b. Chapter 4

Supplementary Tables

Supplementary Table 1: Characteristics of the nine study patients at enrollment.

Patient	Group	Sex	Age	Symptoms duration (weeks)	Current treatment	CRP (mg/l)	UCEIS ulcer subscore	Presence of pathogen
BDX-011	ASUC	Male	72	3	ustekinumab	83	3	No
BDX-012	ASUC	Female	68	3	mesalamine + steroids	138	3	No
BDX-013	NSUC	Female	26	2	golimumab	13.8	1	Blood CMV PCR:1,500 copies/ml; CMV IHC on rectal biopsies : negative Stool test : *Blastocytis hominis*
BDX-015	NSUC	Male	57	4	mesalamine + azathioprine	5.6	1	No
MTL-009	NSUC	Male	37	8	mesalamine	9.3	2	No
MTL-010	NSUC	Male	60	1	mesalamine	6.3	1	No
MTL-011	NSUC	Male	37	5	mesalamine + steroids	0.2	1	No
BDX-029	ASUC	Female	34	6	None	53	1	Blood CMV PCR:1,500 copies/ml
BDX-030	ASUC	Male	30	16	tofacitinib	177	3	Stool test : *Dientamoeba fragilis*

ASUC: Acute severe ulcerative colitis; CMV: Cytomegalovirus; CRP: C-Reactive protein; IHC: Immunohistochemistry; NSUC: non-severe ulcerative colitis; UCEIS: Ulcerative Colitis Endoscopic Index of Severity.

Supplementary Table 2: Quality metrics after alignment with CellRanger for the nine single-cell RNASeq samples.

Patient	Group	Number of reads	Estimated number of cells	Median UMI counts/cell	Median genes/cell
BDX-011	ASUC	314 589 034	10 241	6 086	1 580
BDX-012	ASUC	330 705 366	15 327	3 639	1 475
BDX-013	NSUC	321 197 777	11 416	3 758	1 088
BDX-015	NSUC	292 669 536	12 572	4 172	1 692
MTL-009	NSUC	263 138 421	12 628	3 000	555
MTL-010	NSUC	414 921 762	9 706	6 052	2 142
MTL-011	NSUC	369 989 830	10 639	11 286	3 261
BDX-029	ASUC	271 718 499	2 321	649	395
BDX-030	ASUC	248 265 877	9 032	4 206	1 707

ASUC: Acute severe ulcerative colitis; NSUC: Non-severe ulcerative colitis.

Supplementary Table 3: Canonical markers used to annotate clusters by sample during the first filtering step of analysis and the immune broad cell types step.

Cell Type	Markers
T cells	CD3D
	CD3G
	CD2
	TRAC
B cells	MS4A1
	CD79A
	CD19
Neutrophils	FCGR3B
Erythrocytes	HBB
Cell-cycle	MKI67
Mast cells	TPSAB1
	TPSB2
Endocrine cells	PYY
	CHGA
	CHGB
	GCG
Epithelial cells	EPCAM
	KRT8
	KRT18
Stromal cells	COL1A1
	COL1A2
	VWF
	CDH5
Myeloid cells	CD14
	CD68
	LAMP3

Supplementary Table 4: Markers used to identify co-expression of deviant canonical markers designating putative doublets.

Markers for cell type 1	Markers for cell type 2	Putative doublet
CD3D, TRAC	CD79A, MS4A1	T cell and B cell
LAMP3	CD79A, MS4A1	Dendritic cell and B cell
FCGR3B	MARCO	Macrophage and neutrophil
HBB	CD68, CD68,CD3D, CD79A,FCGR3B,TPSAB1,EPCAM,VWF	Erythrocyte and any other cell type

Supplementary Table 5: Summary of pre-processing filters for the nine single-cell RNASeq samples.

Patient	Group	Estimated number of cells	Number of doublet (manual curation)	Number of erythrocytes	DoubletFinder filter	decontX contamination level – median (IQR)	decontX doublet filter (n)	Number of cells after filtering (n)	Ratio of cells after filtering (%)
BDX-011	ASUC	7 785	126	7	243	0.012 (0.006-0.086)	173	3 696	47.5
BDX-012	ASUC	14 330	789	337	584	0.130 (0.018-0.319)	3 887	7 483	52.2
BDX-013	NSUC	10 301	121	8	461	0.036 (0.015-0.079)	147	7 499	72.8
BDX-015	NSUC	12 162	148	8	564	0.031 (0.010-0.073)	136	8 619	70.9
MTL-009	NSUC	9 119	251	17	412	0.020 (0.008-0.112)	843	6 156	67.5
MTL-010	NSUC	9 054	144	77	425	0.016 (0.005-0.059)	190	6 499	71.8
MTL-011	NSUC	10 273	38	54	225	0.003 (0.001-0.073)	319	6 811	66.3
BDX-029	ASUC	2 207	12	7	42	0.430 (0.388-0.465)	188	1 426	64.6
BDX-030	ASUC	8 490	85	5	448	0.024 (0.011-0.062)	224	6 797	80.1

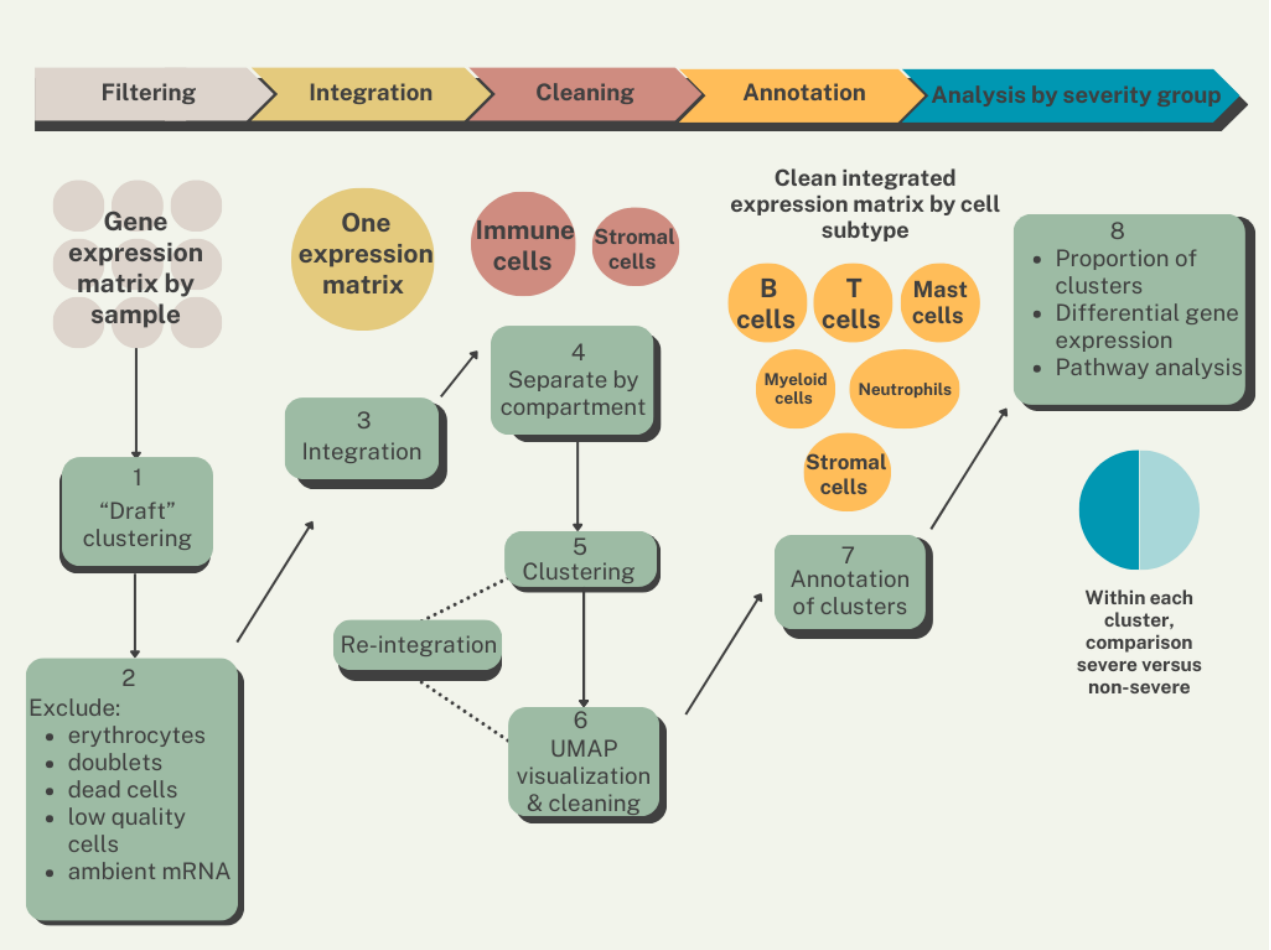
ASUC: Acute severe ulcerative colitis; IQR: Interquartile range; NSUC: Non-severe ulcerative colitis.

Supplementary Table 6: Summary of successive iterations of clustering, UMAP cleansing and integration.

Cell type	Number of cells before UMAP cleansing	Number of cleansing iterations	Excluded samples for insufficient number of cells	Final number of samples from acute severe ulcerative colitis group	Final number of samples from non-severe ulcerative colitis group	Number of cells after UMAP cleansing	Ratio of cells after UMAP cleansing (%)
Immune cells	35 026	1	BDX-029	3	5	34754	99,22
B cells	13 313	3	MTL-011	3	4	13 025	97,84
T cells	11 798	6	None	3	5	9 699	82,21
Mast cells	927	3	MTL-011	3	4	823	88,78
All myeloid cells	8 657	1	MTL-011	3	4	8274	95,58
Myeloid cells	4 962	5	BDX-012	2	4	4671	94,14
Neutrophils	3 450	3	None	3	4	3 262	94,55
Stromal cells	1 662	3	None	4	5	1307	78,64

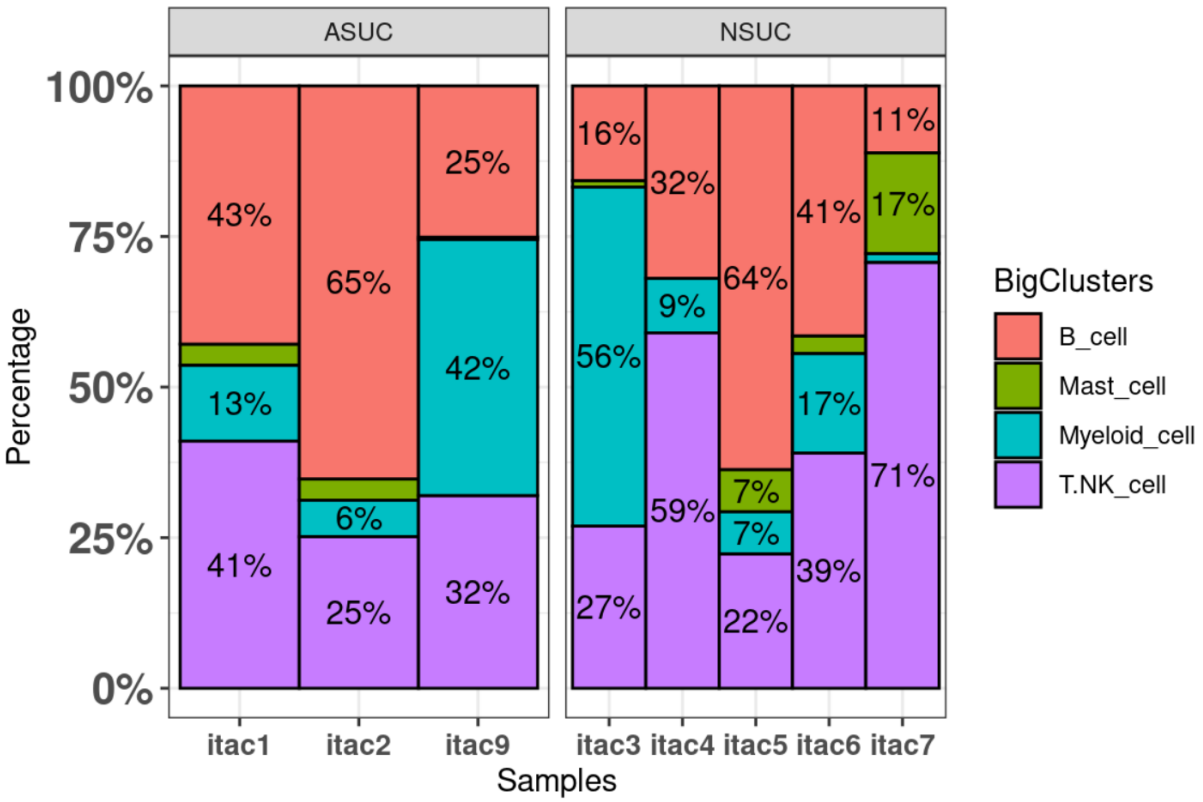
UMAP: Uniform Manifold Approximation Projection

Supplementary Figures



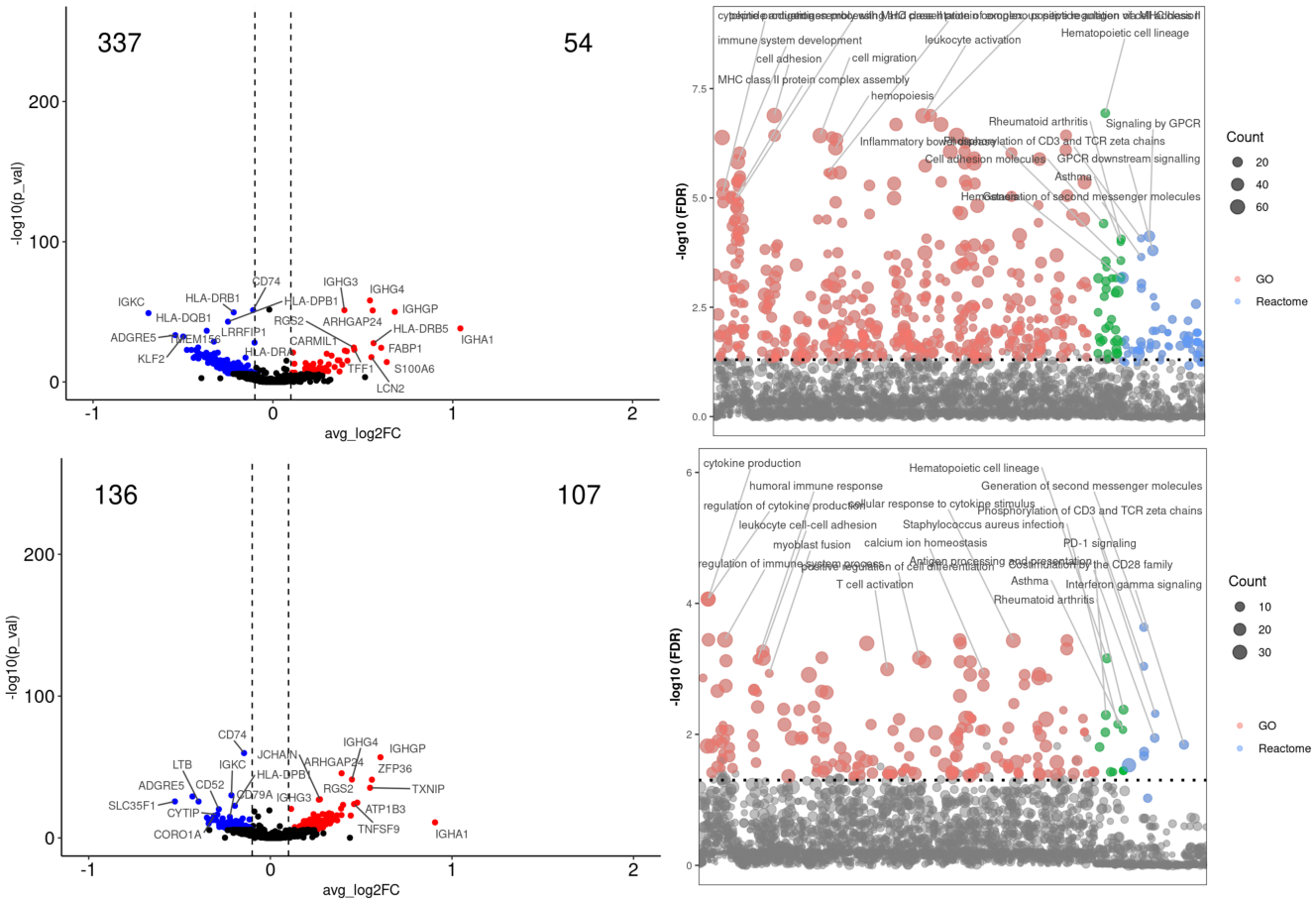
Supplementary Figure 1: Workflow analysis of the scRNASeq data.

Analysis was conducted using the Seurat Package in R, as described in Methods. Briefly, gene expression matrices by sample were filtered to exclude erythrocytes (based on *HBB* gene expression), doublets (manual curation and DoubletFinder), dead cells (mitochondrial genes > 20%) and low-quality cells (number of expressed genes compared to cells from similar cell type) and ambient mRNA (using decontX). The nine samples were then integrated into one matrix. Immune and stromal cells were separated using gene module scores. Within each compartment, and within each cell subtype, cells were clustered, visualized using Uniform Manifold Approximation (UMAP) and outliers cells on the UMAP were excluded. After several rounds of cleaning and re-integration, clean matrices were obtained for each cell subtype. Clusters were annotated using canonical markers and differential gene expression analysis compared to the remaining cells in the same cell subtype. Finally, the proportion of each cluster was compared between severe and non-severe patients and the gene expression within each cluster was compared between cells from severe and cells from non-severe patients. Pathways enriched for differentially expressed genes were studied using clusterProfiler.



Supplementary Figure 2: Distribution of immune cell subtypes among the patients.

Distribution of the immune cells clusters in each sample, grouped by severity phenotype (ASUC: acute severe ulcerative colitis, NSUC: non-severe ulcerative colitis). Each column represents a patient. Myeloid cells and neutrophils have been combined in blue. Red bars correspond to B cells, green to mast cells and purple to T cells.

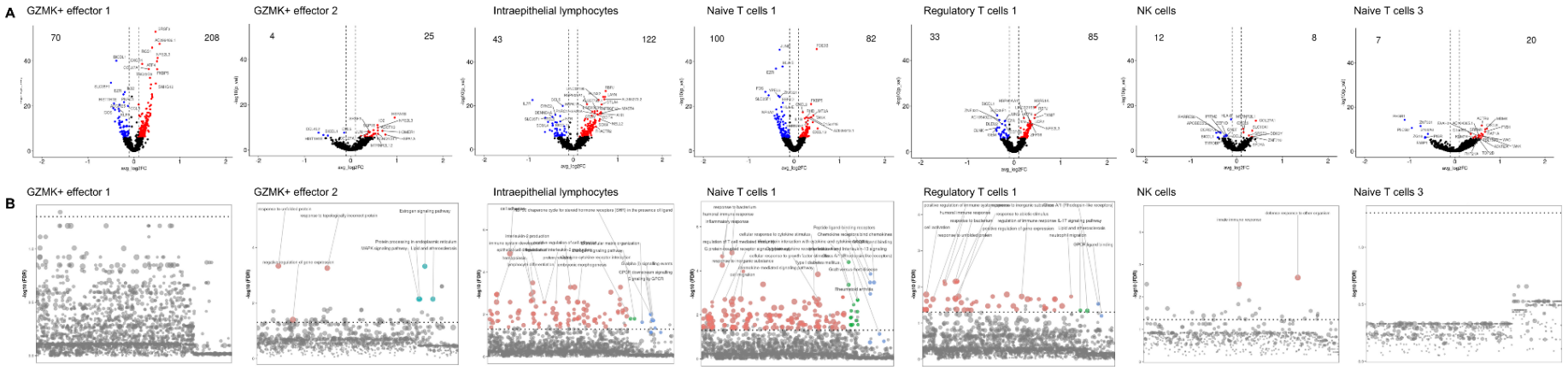


Supplementary Figure 3: Differential expression profile and pathway analysis for activated B cells.

Left: Volcano plots of genes that are differentially expressed in activated B cell cluster 1 (top) and activated B cell cluster 2 (bottom) from acute severe ulcerative colitis patients relative to cells from non-severe patients in the same cluster.

The effect size in acute severe ulcerative colitis as average log Fold change (X-axis) is plotted against the statistical significance of expression differences (y axis). The total number of significantly higher (red dots) or lower expressed (blue dots) genes by severe patients is indicated in the top corners of the plot. Effect size and adjusted p-values were computed using FindMarkers and Wilcoxon tests in Seurat. Genes with a minimum log2 fold change of 0.1 and an adjusted p-value below 0.05 between the two severity groups were considered as exhibiting differential expression.

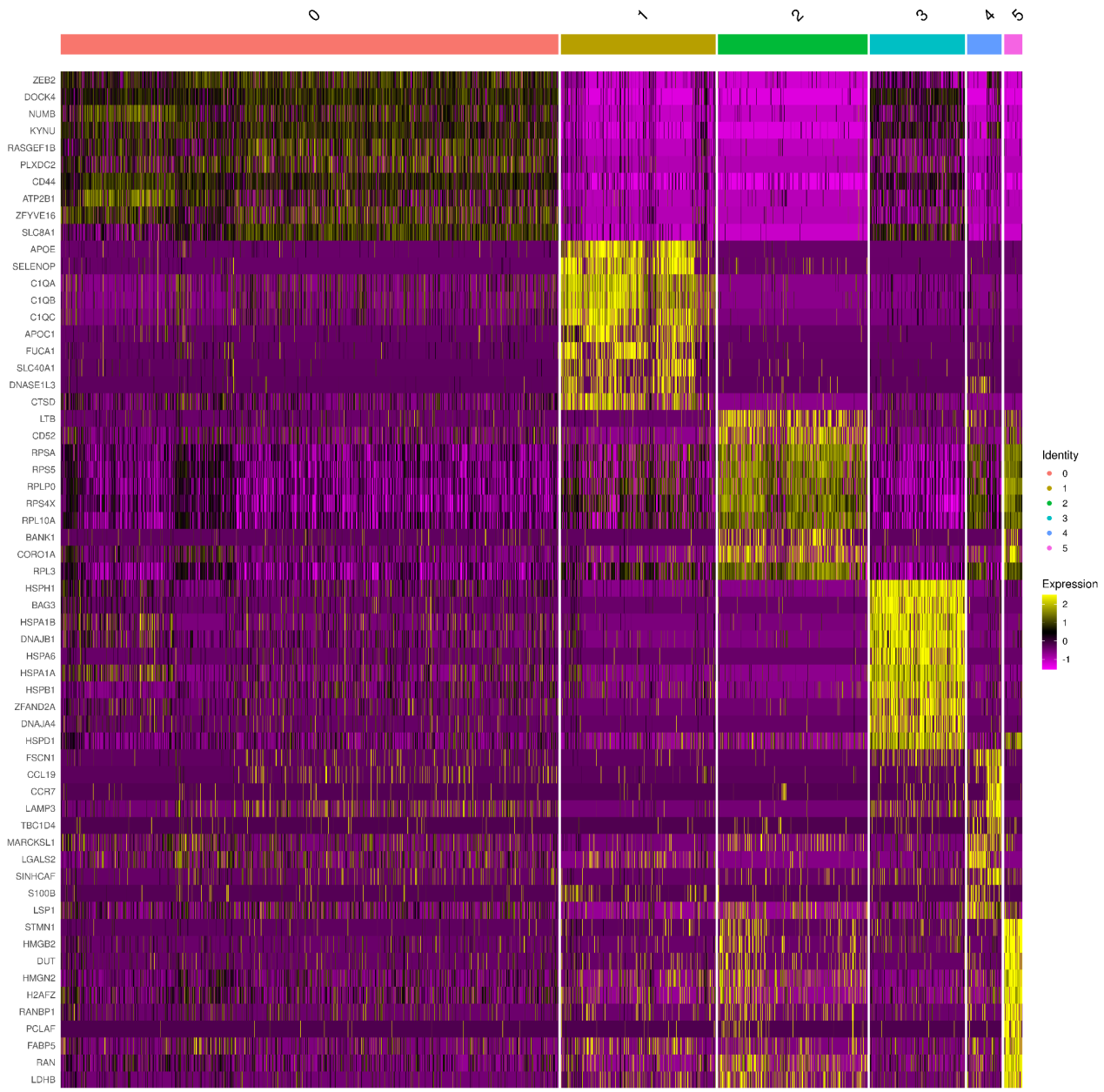
Right: Manhattan plot of pathways enriched in differentially expressed genes in clusters of activated B cell cluster 1 (top) and activated B cell cluster 2 (bottom) of patients with acute severe ulcerative colitis relative to non-severe patients using GO (red dots), Reactome (blue dots) and KEGG (red dots) databases. The x-axis represents the pathways and the y-axis represents the statistical significance of the enrichment. Each pathway is represented by a point on the plot, and the height of the point indicates the significance of the enrichment. The size of the point indicates the count of the enriched genes within the pathway. The names of pathways enriched in differentially expressed genes are displayed.



Supplementary Figure 4: Differential expression profiles of T cells of patients with acute severe ulcerative colitis.

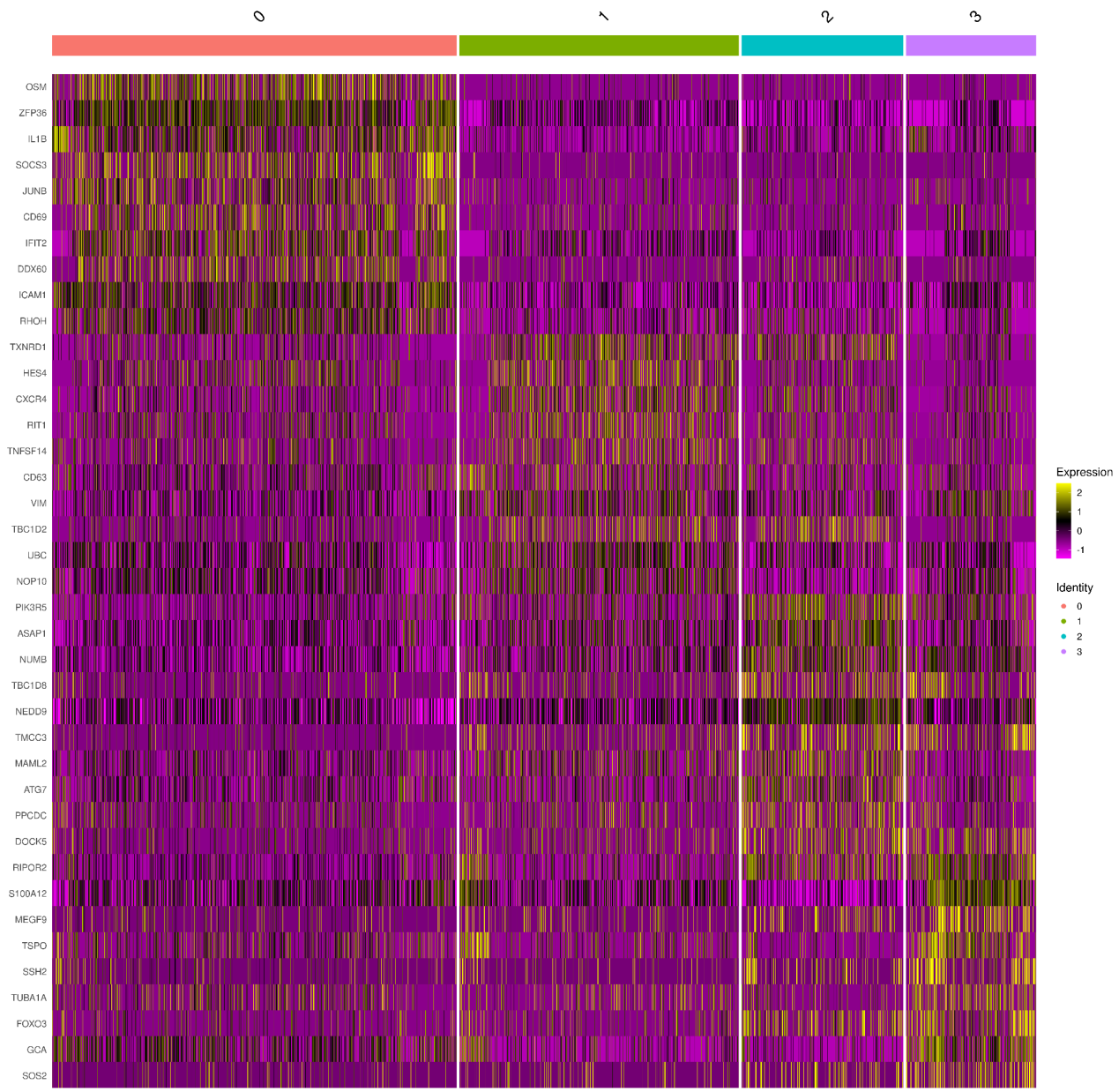
Panel A: Volcano plots of genes that are differentially expressed in T cells clusters from acute severe ulcerative colitis patients relative to cells from non-severe patients in the same cluster. The effect size in acute severe ulcerative colitis as average log Fold change (X-axis) is plotted against the statistical significance of expression differences (y axis). The total number of significantly higher (red dots) or lower expressed (blue dots) genes by severe patients is indicated in the top corners of the plot. Effect size and adjusted p-values were computed using FindMarkers and Wilcoxon tests in Seurat. Genes with a minimum log2 fold change of 0.1 and an adjusted p-value below 0.05 between the two severity groups were considered as exhibiting differential expression.

Panel B: Manhattan plot of pathways enriched in differentially expressed genes in the T cells clusters of patients with acute severe ulcerative colitis relative to non-severe patients using GO (red dots), Reactome (blue dots) and KEGG (red dots) databases. The x-axis represents the pathways and the y-axis represents the statistical significance of the enrichment. Each pathway is represented by a point on the plot, and the height of the point indicates the significance of the enrichment. The size of the point indicates the count of the enriched genes within the pathway. The names of pathways enriched in differentially expressed genes are displayed.



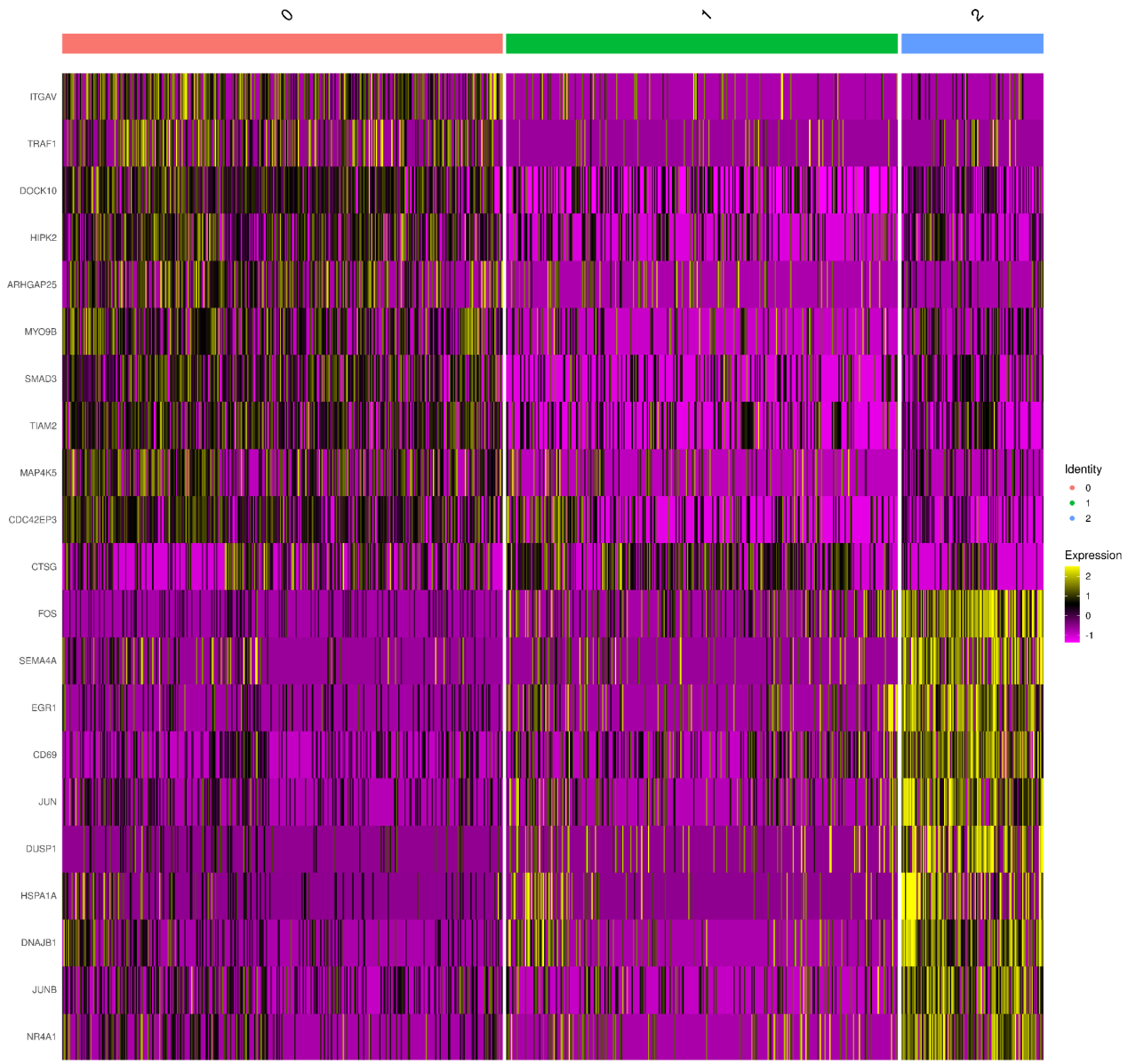
Supplementary Figure 5: Marker genes for myeloid cells clusters.

Heatmap of the genes differentially expressed in each cluster of myeloid cells compared to the remaining clusters. The rows of the heatmap represent the 10 genes with the highest log fold change for each cluster, and the columns represent the cells, ordered by clusters. The colours represent the level of expression. Annotation of the clusters: 0 - Inflammatory monocytes, 1 - Macrophages, 2 - Dendritic cells 1, 3 - Stressed macrophages, 4 - M1-macrophages, 5 - Dendritic cells 2.



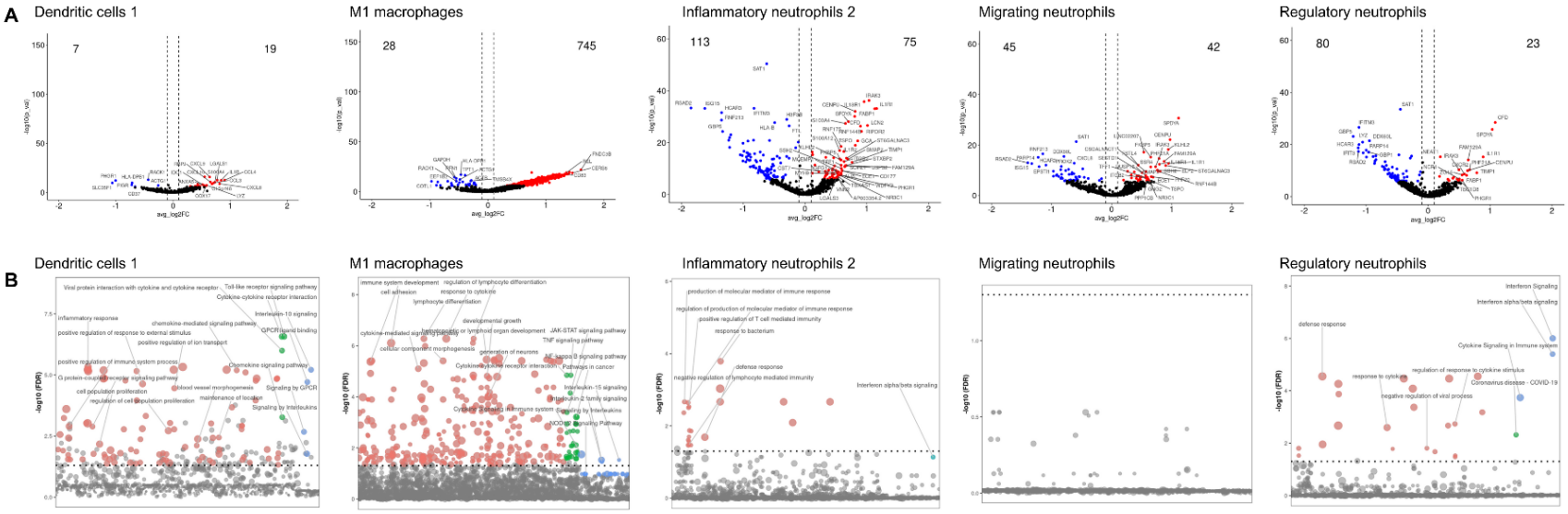
Supplementary Figure 6: Marker genes for neutrophils clusters.

Heatmap of the genes differentially expressed in each cluster of neutrophils compared to the remaining clusters. The rows of the heatmap represent the 10 genes with the highest log fold change for each cluster, and the columns represent the cells, ordered by clusters. The colours represent the level of expression. Annotation of the clusters: 0 - Inflammatory neutrophils 1, 1 - Inflammatory neutrophils 2, 2 - Migrating neutrophils, 3 - Regulatory neutrophils.



Supplementary Figure 7: Marker genes for mast cells clusters.

Heatmap of the genes differentially expressed in each cluster of mast cells compared to the remaining clusters. The rows of the heatmap represent the 10 genes with the highest log fold change for each cluster, and the columns represent the cells, ordered by clusters. The colours represent the level of expression. 0 - Mast cells 1, 1 - Mast cells 2, 2 - Mast cells 3.

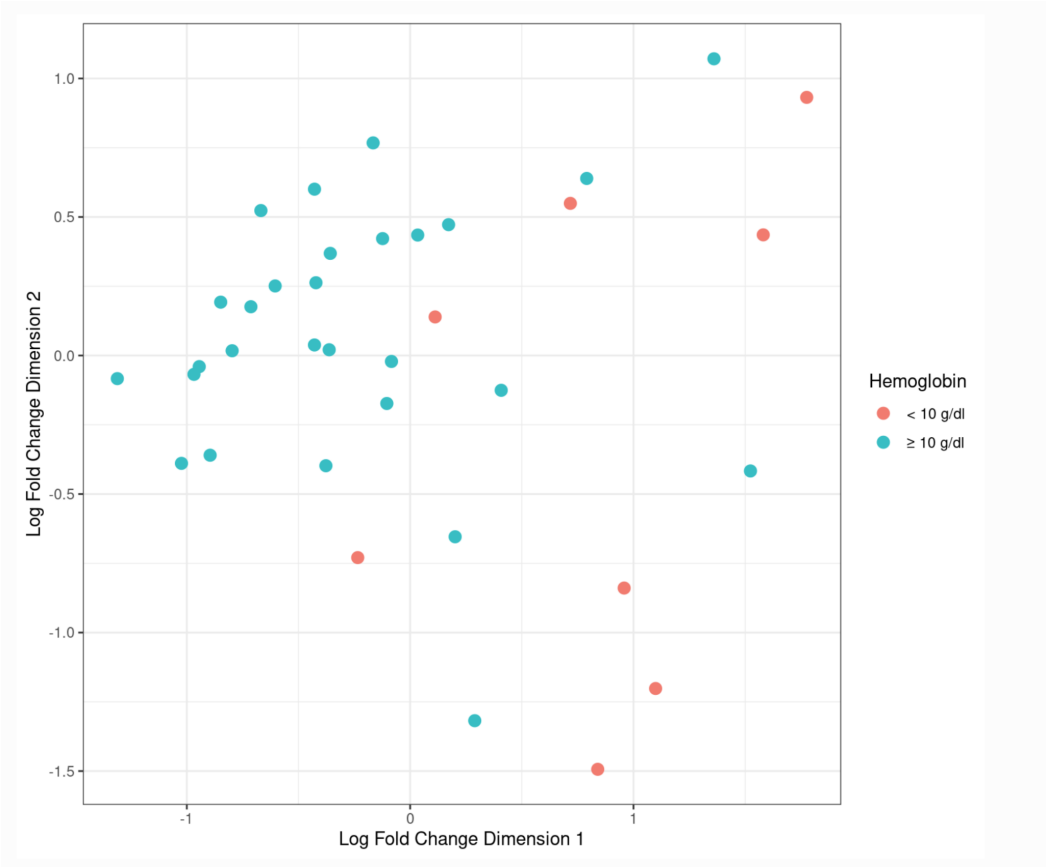


Supplementary Figure 8: Differential expression profiles of innate immune cells of patients with acute severe ulcerative colitis.

Panel A: Volcano plots of genes that are differentially expressed in myeloid cells and neutrophils clusters from acute severe ulcerative colitis patients relative to cells from non-severe patients in the same cluster. The effect size in acute severe ulcerative colitis as average log Fold change (X-axis) is plotted against the statistical significance of expression differences (y axis). The total number of significantly higher (red dots) or lower expressed (blue dots) genes by severe patients is indicated in the top corners of the plot. Effect size and adjusted p-values were computed using FindMarkers and Wilcoxon tests in Seurat. Genes with a minimum log2 fold change of 0.1 and an adjusted p-value below 0.05 between the two severity groups were considered as exhibiting differential expression.

Panel B: Manhattan plot of pathways enriched in differentially expressed genes in myeloid cells and neutrophils clusters of patients with acute severe ulcerative colitis relative to non-severe patients using GO (red dots), Reactome (blue dots) and KEGG (red dots) databases. The x-axis represents the pathways and the y-axis represents the statistical significance of the enrichment. Each pathway is represented by a point on the plot, and the height of the point indicates the significance of the enrichment. The size of the point indicates the count of the enriched genes within the pathway. The names of pathways enriched in differentially expressed genes are displayed

c. Chapter 5



Supplementary Figure 1: Multidimensional scaling plot coloured by haemoglobin levels. Two-dimensional scatterplot that shows the distances between samples based on the log2 fold changes between them. Each sample (dot) is coloured according to the haemoglobin level. Distances between samples on the plot approximate the typical log2 fold changes for each transcript between the samples. Patients with lower haemoglobin seem to have different gene expression profiles than the patients with higher haemoglobin.