Immunological Networks Defining the Heterogeneity of Inflammatory Bowel Diseases

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Abstract

Current practice in IBD is to classify patients based on clinical signs and symptoms and provide treatments accordingly. However, the response of IBD patients to available treatments is highly variable, highlighting clinically significant heterogeneity among patients. Thus, more accurate patient stratification is urgently needed to more effectively target therapeutic interventions to specific patients. Here we review the degree of heterogeneity in IBD, discussing how the microbiota, genetics, and immune system may contribute to the variation among patients. We highlight how molecular heterogeneity may relate to clinical phenotype, but in other situations may be independent of clinical phenotype, encouraging future studies to fill the gaps. Finally, we discuss novel stratification methodologies as a foundation for precision medicine, in particular a novel stratification strategy based on conserved genes across species. All of these dimensions of heterogeneity have potential to provide strategies for patient stratification and move IBD practice towards personalised medicine.

Key Words: IBD; heterogeneity in IBD; personalised medicine; molecular stratification of IBD patients; immunology.
Inflammation outside the gut, so called extraintestinal manifestations (EIMs), contribute to the clinical heterogeneity in IBD patients. Wide heterogeneity in EIMs exists when it comes to both clinical manifestations and underlying pathological mechanisms. It is still unknown why some patients get unifocal and the others multifocal inflammation, and the potential of these observations to assist patient stratification has not yet been exploited.5

Given the heterogeneity of the disease, classification and division of IBD into different entities is a complicated task and will require a better understanding at the cellular and molecular levels. In future, clinically observable phenotypes will likely be less relevant since molecular characterisation will likely provide deeper understanding of the diverse pathogenic mechanisms of IBD and may underpin therapeutic decision making. However, single-cell RNA sequencing [scRNA-seq] data on immune cell populations in both gut and blood confirm that the division into UC and CD is still biologically relevant, as distinguishing features can be observed between these groups.11

1.1. Age at disease onset
Based on age at IBD onset, patients can be defined into very early onset (VEO)-IBD that debuts before the age of 6 years, paediatric IBD that starts before age of 18, adult onset IBD that starts at ages 18 to 60, and elderly onset with debut at over 60 years age. In particular VEO-IBD, which is discussed in more detail below, is often a very different type of disease compared with later onset disease; however, there is also more general heterogeneity among patients based on the age of disease onset. In CD, ileocolonic location dominates in paediatric patients, whereas colonic location is the most common in elderly onset disease.32–34 However, in VEO-IBD, isolated colitis is the most common manifestation.35,36 Also, after 5–10 years diagnosis over half of the paediatric onset patients have stricture or penetrating disease behaviour, whereas the number is only around 30% in elderly onset patients even after 15 years of follow-up.32,34,35

In UC, extensive colitis is more common in childhood compared with adult and elderly onset disease, where left-sided colitis dominates.35,36 Further, EIMs have been reported to be more common in paediatric onset and lower in elderly onset IBD.13–15 Together these differences in clinical manifestations indicate more severe disease with earlier onset.

There are also aetiological differences between different age groups. The genetic component has a greater influence in paediatric onset IBD.18 Positive first-degree relative family history has been reported to be slightly over 10% in the paediatric population

Table 1. Possible and existing clustering strategies with clinical implication in IBD.

| Feature         | Clustering strategies or suggested clustering strategies with clinical implication |
|-----------------|-----------------------------------------------------------------------------------|
| Clinical        | Montreal classification                                                          |
| Genetics        | Increased risk of colectomy, colorectal cancer, and death in PSC-IBD patients compared with non-PSC-IBD patients8 |
| Microbiota      | At least four gene loci have been associated with prognosis in CD36 |
| Immune system   | In UC, extensive colitis is more common in childhood compared to later adulthood; in CD, ileocolonic location dominates in paediatric patients whereas colonic location is the most common in elderly onset disease.12–15 Further, EIMs have been reported to be more common in paediatric onset and lower in elderly onset IBD.13–15 Together these differences in clinical manifestations indicate more severe disease with earlier onset.11

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IBD, inflammatory bowel disease; PSC, primary sclerosing cholangitis; CD, Crohn’s disease; UC, ulcerative colitis; IL, interleukin; ILC, innate lymphoid cell; NK, natural killer; Treg, regulatory T cell; BP, binding protein; TNF, tumour necrosis factor; Ig, Immunoglobulin; TLR, Toll-like receptor; OSM, oncostatin M; NOD2, nucleotide-binding oligomerisation domain-containing protein 2; eQTL, expression quantitative trait loci study.

K. A. Selin et al.
in general and over 20% in VEO-IBD.16,17 In a French population-based registry, the rate of family history with IBD fell with increasing age of disease onset, with 16% and 13% positive family history for paediatric CD and UC patients, respectively, and 7% and 3% for CD and UC patients with elderly-onset disease.16 It is also clear that many environmental exposures are relevant for gut inflammation, for example smoking and polypharmacy, as well as hormonal factors differing between different age groups.19 The microbiota also undergoes changes throughout the lifetime.18 In paediatric patients, an increase in mucosa-associated aerobic and facultative-anaerobic bacteria has been reported in contrast to increase in anaerobic bacteria in adult onset disease, but otherwise microbial differences in relation to IBD disease onset have not been well studied.16-25 Similarly, the immune system is known to undergo many functionally relevant changes through the lifetime, but its significance in IBD is not well defined.16 Thus, IBD heterogeneity in relation to disease onset is an important future research area and may inform optimal management strategies for these different groups which may in future be classified as different diseases.

2. Aetiology

2.1. Genetics

IBD develops as a complex interaction between environment, genes, microbiota, and immune system.3,4 However, there are also more than 50 observed forms of IBD-like intestinal inflammation in which rare high-penetrance genetic variants cause a specific phenotype that is often associated with immune defects and VEO-IBD.21-24 For example, mutations in either IL-10 or the IL-10 receptor cause a phenotype of VEO-IBD with severe perianal disease and colitis.21,23 Despite the significant heterogeneity in GWAS hits between different demographic populations,3,4,21,29 Genome-wide association studies (GWAS) have revealed over 260 loci that are associated with the risk of sporadic IBD, some of which are associated with the function of the immune system and have revealed relevant pathological pathways.30,31 There exists significant heterogeneity in GWAS hits between different demographic populations.32 For example, three coding variants of nucleotide-binding oligomerisation domain-containing protein (NOD)2, with odds ratio of 2.13–3.03 for IBD development in the European population, were not present in the East Asian population and there did not significantly contribute to the risk of IBD.32 One limitation in GWAS studies is that common variation does not account for all the heritability in IBD,27 as highlighted also in the previous section. So far, disease risk alleles discovered through GWAS have not been converted into new clinical strategies. This is likely due to the lack of assigned functions to IBD risk variants, many of which are located in non-coding regions.28 Integration of genetic and molecular data offers possibilities for clinically meaningful patient stratification;30 for example, expression quantitative trait loci studies [eQTLs] can link hits on non-coding regions to functional genes.31 Indeed, Mariangola et al. found that transcriptional risk score based on eQTLs data could predict which paediatric CD patients will develop strictureing and/or penetrating disease within 3 years from disease onset, but a genetic risk score based on GWAS could not46 [Table 1]. Moreover, Lee et al. demonstrated that GWAS variants have no or minor impact on disease prognosis in CD but found instead four separate loci associated with prognosis in a within-cases GWAS.3 Thus, genetic analyses should not only be applied to prediction of susceptibility to developing IBD. However, some variants, such as NOD2, IL-6, and Toll-like receptor [TLR]4 have been associated with both susceptibility to CD and phenotypic features such as ileitis without colitis, ileitis, and surgery at disease onset, respectively.34

2.2. Heterogeneity in experimental models of IBD

Analogous to the heterogeneous group of human genetic defects that ultimately cause IBD-like syndromes, there is heterogeneity in the range of experimental mouse models which result in acute and chronic gut inflammation.37 The dextran sodium sulphate [DSS] murine model of colitis is one of the most widely used for its simplicity. DSS induces damage in epithelial cells with the consequent barrier disruption and bacterial translocation. Its initiation is dependent on signalling through NOD-like receptor protein 3 [NLRP3] inflammasome, which activates caspase-1 leading to interleukin [IL]-1β production.38 Further, DSS-induced IL-1β release was shown to require functional lysosomes and reactive oxygen species [ROS] production.39 Although it is considered a T cell-independent model, expression of both Th1 and Th2 as well as Th17-type cytokines has been reported to be dysregulated in the DSS model.40 By contrast, the oxazolone colitis, a model of UC at histological level, is mediated mainly by T helper [Th] 2-type response and is dependent on initiation by natural killer [NK] T cells, giving rise to high IL-13 production.40,41

Alternatively, adaptive transfer of CD4⁺CD45RB⁺ naïve T cells from a wild-type [WT] mouse into a recipient lacking T and B cells [RAG1- or RAG2-deficient mouse] may resemble CD.39,42-43 This model, commonly termed ‘T cell transfer model of colitis’, develops transmural pannicolitis in combination with small bowel inflammation 5–8 weeks after cell transfer.42-43 In this model, inflammation is mainly of Th1 type and colitis development can be prevented by anti-interferon [IFN]-γ or anti-tumour necrosis factor alpha [TNFα] antibodies, or by administration of recombinant interleukin [rIL]-10.43 However, the expression of some Th2- and Th17-type cytokines has also been reported to be dysregulated.44 Further, IL10⁻/⁻ mice develop a spontaneous colitis after weaning that is mainly Th1 mediated, but also with contribution of a Th17 immune response.44-46 Also, cytokines IL-23 and IL-17 may be critical for the development of colitis in IL-10 knockout mice.44-47

Important differences in colonic transcriptomic profiles, possibly having functional relevance, have been described for different murine IBD models.39 For example, in IL10⁻/⁻ mice where spontaneous colitis is accelerated with piroxicam [PAC IL10⁻/⁻ model], the expression of...
genes associated with tissue remodelling were relatively high compared with the T cell transfer model of colitis, whereas those associated with bacterial sensing were lower. Generally, IBD-associated genes were upregulated in PAC IL10 to a higher extent compared with the T cell transfer model of colitis. Furthermore, the penetrance of the disease and mechanism of action might differ between animal facilities even if the same animal background and method are used, which is attributed to microbial variations.

For example, it has been shown that different microbial communities in identical genetic backgrounds induce DSS-induced colitis by distinct mechanisms, namely either neutrophil- or T cell-dependent, thus indicating a high degree of experimental IBD heterogeneity dictated also by environmental factors, which is analogous to human IBD. Although this complicates the interpretation and generalisation of results from animal studies, it provides a great opportunity to study complex interactions between genetics, environmental, and immunological factors as well as heterogeneity in IBD. Moreover, the fact that environmental, particularly microbial, factors have turned out to be so significant in experimental IBD further emphasises the fundamental role of the genotype-environment interface in human IBD.

Animal models are a vital tool facilitating the disentanglement of the multiple pathogenetic elements of a complex diseases such as IBD. Each model facilitates focus on different aspects of IBD pathogenesis which may then be reconstructed into an enhanced understanding of the human disease. However, the potential of animal models to accurately recapitulate specific IBD phenotypes remains limited. The development of new models that reflect the pathogenesis of specific forms of IBD, such as PSC-IBD or fistulising CD, would greatly enhance the personalisation of IBD management.

### 2.3. Microbiota

Altered microbiota [dysbiosis] in gut is an extensively studied hallmark of IBD. The microbial signature in IBD is characterised by instability and more frequent fluctuations compared with healthy controls. Also changes in abundance of species, taxonomic changes, reduced diversity, and functional metabolic changes characterise microbiota in IBD.

In colonic surgical samples, IBD patients had enrichment of Proteobacteria and the Bacillus subgroup of Firmicutes, whereas Bacteroidetes and the Lachnospiraceae subgroup of Firmicutes were depleted compared with non-IBD controls. Interestingly, approximately one-third of CD and one-fourth of UC patients had more pronounced alterations in microbiota, with overall lower proportion of Firmicutes and Bacteroidetes together with manifold increase in relative abundance of Proteobacteria and Actinobacteria compared with non-IBD controls.

The heterogeneity in microbial signature observed in cross-sectional studies is complemented by longitudinal studies which also reveal instability of the microbiota within individual IBD patients, including states where the microbiota is indistinguishable of those observed in non-IBD controls. However, although the microbiota is highly heterogeneous within one individual at different time points, there are still possible clustering strategies based on microbiota composition.

Generally, CD patients have more pronounced alterations in microbiota than UC patients, and this is most evident in ileal CD, especially after ileo-caecal resection. For example, CD patients show both lower diversity and more pronounced fluctuations and inter-individual variation in microbial composition compared with UC.

Further, CD patients show greater abundance in Proteobacteria and Bacteroidetes and decrease in Clostridia compared with UC patients. In faecal samples from IBD and irritable bowel syndrome patients, as well as healthy controls, Vich Vila et al. could identify 477 different taxa of which 219 were associated with CD and 102 with UC, among which 15 were UC specific, further demonstrating differences between CD and UC patients in microbial composition.

CD patients who respond to anti-TNFα treatment have been shown to have increase in Bifidobacterium, Collinsella, Lachnospira, K. A. Selin et al. 2021.

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**Figure 1.** Comparison of factors that differ between UC and CD. There are remarkable differences between patients with diagnoses UC and CD, but also great heterogeneity within these patient groups. Generally, CD patients have, for example, lower diversity and greater instability in their microbiota compared with UC patients. Also, in both CD and UC, ILCs produce IL-22, IL-17A, IL-17F, and IFN-γ upon stimulation by IL-23, but in CD there is a selective increase in IL-17A, IL-17F, and IFN-γ producing ILCs. Further, in CD, colonic DC show increased expression of IL-12 and IL-16 during inflammation compared with UC patients. Also, CD is characterised by intestinal dominance of Th1-related gene expression, whereas Th2- and Th17-related gene expression dominates in UC. CD, Crohn’s disease; UC, ulcerative colitis; IL, interleukin; T, T helper cell; ILC, innate lymphoid cell; IEL, intestinal epithelial lymphocyte; Mac, macrophage; ENS, enteric nerve system; DC, dendritic cell; RA, retinoic acid; TNF, tumour necrosis factor.
Lachnospiraceae, Roseburia, and Eggerthella taxa and reduction in Phascolarctobacterium compared with anti-TNFα non-responders. Also, higher pre-treatment abundance of Faecalibacterium prausnitzii predicted better response to anti-TNFα treatment in both UC and CD. Furthermore, higher abundance of Faecalibacterium prausnitzii on infliximab treatment cessation predicted sustained remission in CD. Also, in CD, increased Eggerthella, Clostridiales, and Oscillospira have been associated with quiescent clinical disease whereas increase in Enterobacteriaceae, Enterobacteriaceae, and Klebsiella have been found to predict more aggressive disease course. Furthermore, post-surgical CD patients have significantly reduced microbiota diversity compared with patients who have not undergone surgery. Moreover, in CD, post-surgical disease recurrence 1 year after ileocaecal resection is associated with an altered microbial profile. Endoscopic recurrence of CD 6 months after ileal resection can also be predicted by lower abundance of Faecalibacterium prausnitzii at the time of the surgery.

In conclusion, there are some overarching characteristics of the IBD microbiota but much inter- and intra-individual variation exists. Furthermore, the relationship between abundance of various species and the alteration in function is not well characterised.

### 2.4. Environment

Studies on monozygotic twins have shown significant discordance when it comes to both occurrence of IBD and phenotype of developed IBD, indicating the influence of environmental factors in addition to genetic factors. Early life events affecting the risk of IBD through changes in microbiota, especially antibiotic use and mode of feeding, are among the most frequently identified environmental risk factors. There is also evidence implicating urbanisation, air pollution, non-steroidal anti-inflammatory drug use, hypoxia, and diet in the risk of IBD.

Since environmental factors affect individuals in different ways it is reasonable to propose that environmental pressures significantly account for the heterogeneity in IBD patients. As discussed above, the microbiota is associated with heterogeneity in IBD patients and, further, in healthy individuals there is robust evidence that diet is a significant environmental factor.

![Diagram of Immunological Networks Defining the Heterogeneity of Inflammatory Bowel Diseases](image)

**Figure 2.** Examples of heterogeneity between UC, CD and healthy subjects. The first row illustrates how in UC all the DCs have decreased capacity to produce RA (green DCs) compared with healthy individuals (white DCs), whereas DCs from CD patients show heterogeneity in their RA production capacity. The second row illustrates that in early CD the abundance of colonic ILC1s is selectively increased, whereas in early UC the abundance of ILC2s is increased. Later in the disease course, ILC1s and ILC2s are increased compared with healthy individuals in both disease groups. The third row shows an example of microbiota-based clustering, where CD patients responding to anti-TNF treatment have increased abundance of Bifidobacterium, Collinsella, Lachnospira, Lachnospiraceae, Roseburia, and Eggerthella taxa and reduction in Phascolarctobacterium compared with anti-TNFα non-responders, whereas UC patients do not seem to show clustering based on these bacteria and anti-TNF response. The fourth row exemplifies a possible clustering strategy based on CD49b+LAG3- IL-10-producing type 1 Treg cells that show more variable frequencies in UC compared with CD. The fifth row illustrates that UC patients with PSC have higher abundance of colonic ILCs compared with UC patients without PSC. CD, Crohn’s disease; UC, ulcerative colitis; IL, interleukin; TH, T helper cell; ILC, innate lymphoid cell; DC, dendritic cell; RA, retinoic acid; TNF, tumour necrosis factor; PSC, primary sclerosing cholangitis.
affecting gut microbial heterogeneity. Thus, it is likely that diet significantly contributes to heterogeneity also among IBD patients. Further, dietary compounds can have direct effects on the immune system and cause epigenetic modifications. Dietary long chain n-6:n-3 PUFA intake ratio affect the risk of CD. Polymorphism in CYP4F3 has also been shown to modify the effect of n-6:n-3 PUFA intake ratio in the risk of adult UC. Further, the interaction between polymorphism of rs7657746 [IL-21] and potassium intake affects the risk of CD; there is increasing disease risk with increasing potassium intake in individuals with GG genotype, whereas people with GA and AA genotypes have decreasing risk of CD with increasing potassium intake.

In the same way, in individuals with the GG genotype of UC susceptibility locus rs1801274, increasing intake of haem iron decreases the risk of UC, whereas the opposite is true for those with the AA phenotype. There are as yet no ways to cluster patients based on diet and other environmental exposures, but this may become an interesting field of research as incidence of IBD grows globally also in areas where IBD has previously been rare.

2.5. Immune system

Both innate and adaptive immune cells are implicated in the pathogenesis of IBD and balance between distinct immune responses likely lies at the root of much of the heterogeneity between patients. In homeostasis, intestinal dendritic cells [DC] are specialised in producing retinoic acid [RA] during the process of imprinting naïve T lymphocytes in the intestinal mucosa, inducing the homing molecules C-C chemokine receptor type 9 [CCR9] and integrin α4β7, and serves as an adjuvant to generate Foxp3 regulatory T cells [Foxp3 TREG]. It is believed that breakdown of this process might contribute to IBD pathogenesis, which is supported by the observation that IBD patients have lower numbers of RA-producing DCs. Interestingly, Magnusson et al. reported decreased frequencies of RA-producing cells in all 15 UC patients but in only four out of nine CD patients compared with control tissue, suggesting greater heterogeneity in RA-producing cells in CD compared with CD103+DC and CD1c+CD103+DC frequencies in CD. In the same study, there was an overall decrease of CD141+CD103+DC and CD1c+CD103+DC in inflamed mucosa of CD patients compared with non-inflamed mucosa from the same individual, but this was not observed in a subset of CD and in UC patients. Similar results have been reported for CD103+DC in total in CD. These differences are likely to have a functional role because CD103+DC decrease in CD patients after treatment with an anti-TNFα agent. Hart et al. showed that DC from both inflamed UC and CD intestinal tissue express significantly higher levels of Toll-like receptor [TLR]-2 and TLR-4 compared with intestinal DC from healthy controls. Further, they showed that intestinal DC from patients with active CD show significantly higher levels of the maturation/activation marker CD40 compared with both healthy controls and DC from non-inflamed tissue of CD patients, but levels decreased after anti-TNF treatment irrespective of the response. Also, DC from inflamed intestinal tissue of CD patients show significantly higher expression of both IL-6 and IL-12 compared with DC from both healthy controls and inflamed tissue of UC patient [Figure 1], suggesting heterogeneity in DC-produced cytokines.

Platt et al. showed in murine colitis that the dominant population of inflammatory macrophages are distinct from gut-resident macrophages and are derived from blood monocytes in a CCR2-dependent manner. In the study by Magnusson et al., both CD and UC mucosa showed heterogeneity in the extent of accumulation of HLA-DR+ macrophages in the inflamed to relation to the non-inflamed mucosa and similar results have been obtained by Dige et al. for CD, suggesting specific importance of these cells for inflammation in a subset of patients irrespective of the diagnosis. It is likely that these differences have a functional role in IBD, since the proportion of HLA-DR+ macrophages of total lamina propria myeloid cells [LPMC] decreases in CD patients after anti-TNFα treatment. Moreover, the proportion of total colonic lamina propria myeloid cells which were HLA-DR+ macrophages was decreased in inflamed mucosa compared with non-inflamed mucosa in few patients despite the lack of significant difference in the total abundance of these cells.

Innate lymphoid cells [ILCs] have lymphoid origin and mirror functions of T cells. Among the most well-known subtypes are ILC1s, ILC2s, and ILC3s. ILC1s function mainly in type 1 immunity reacting against tumours and intracellular microbes, and ILC2s function in type 2 immunity reacting against large extracellular pathogens and allergens. ILC3s in turn function mainly in type 3 immunity against extracellular microbes such as bacteria and fungi. Thus, ILC1s, ILC2s, and ILC3s mirror functions of Th1, Th2, and Th17 cells, respectively. There are data suggesting that ILCs are involved in intestinal inflammation in IBD by IL-23 driven induction of secretion of cytokines IL-17A, IL-17F, interferon-γ [IFN-γ], and granulocyte-macrophage colony-stimulating factor [GM-CSF]. ILCs are also able to produce IL-22 and IL-26 upon stimulation by IL-23, but numbers of IL-17A-, IL-17F-, and IFN-γ-producing ILCs are selectively increased in the inflamed ileal and colonic tissue of CD patients compared with IL-22- and IL-26-producing cells [Figure 1].

However, one can observe marked heterogeneity between individual CD patients in the relative abundance of these cells, suggesting differential importance between CD patients. Moreover, many more alterations in ILC populations, including the dynamics of the changes along the disease course, have been described in IBD [Figure 2]. Also, ILCs are increased in the colon of patients with PSC-UC compared with both UC patients without PSC and controls [Figure 2]. Thus, it can be speculated that ILCs could contribute to higher risk of colon cancer in patients with PSC-UC since there are studies implicating ILCs in intestinal cancer development. NKp44+ILC3 are decreased in inflamed tissue and correlate with disease severity [Table 1]. In treatment-naïve, relatively newly diagnosed IBD, ILC1s are increased in inflamed gut tissue in CD patients whereas ILC2s are increased in inflamed gut tissue in UC patients, functionally mirroring their adaptive T cell counterparts [Figure 2]. In contrast, in patients with IBD established for at least 1 year before sampling, both ILC1s and ILC2s are increased in inflamed gut tissue in both UC and CD [Figure 2]. Also, there is accumulation of IL-17-producing ILC3s in inflamed tissue in CD but not in UC. Frequencies of ILCs do not differ between IBD patients and controls generally in blood and non-inflamed tissue. However, frequencies of ILC1s and ILC2s are increased among total ILCs in the blood of patients with PSC-UC compared with patients with UC and controls.

In the adaptive immune system, regulatory T cells [Treg], including both Foxp3+ TREG, and IL10+Foxp3+ T regulatory type 1 [Tr1] cells, are key regulators of intestinal homeostasis, and dysfunction of these
cells may be a key feature of IBD pathogenesis. Increased numbers of colonic and ileal Foxp3+ Treg, have been shown in both active and inactive CD and in active, but not in inactive, UC but marked heterogeneity is observed between patients. Holmén et al. reported increase of Treg with increasing disease activity in UC, and same has also been reported for CD. Interestingly, Reikvam et al. showed that paediatric ileal CD patients have even greater accumulation of Treg in ileal mucosa compared with adult ileal CD patients, which the authors suggested may contribute to the relative rarity of isolated ileal enteritis in paediatric CD patients. Treg from IBD patients show approximately 60% decreased ability to suppress autologous T cell proliferation. One explanation could be increase in circulating RORγt+IL17a+Foxp3+ Treg cells among IBD patients compared with healthy controls. Also, Treg in inflamed colonic lamina propria have increased apoptosis rate compared with Treg in non-inflamed tissue. This may have a functional role in the pathogenesis of IBD, since the apoptosis rate decreases after treatment with an anti-TNFα agents together with decreasing disease activity. Further, Foxp3 negative IL-10-producing Tr1 cells can be identified by the expression of the CD49b and lymphocyte-activation gene 3 (LAG-3) surface markers, which are decreased in CD and UC patients compared with controls.

Interestingly, UC patients show more variable frequencies of these CD49b+/LAG-3− IL-10-producing Tr1 compared with CD patients. Figure 2, suggesting that frequencies of CD49b+/LAG-3− IL-10-producing Tr1 cells might allow stratification of UC but not CD patients. Furthermore, the same cell type can show great heterogeneity even within an individual. For example, IL-10-producing Foxp3+ CD4+ T cells are transcriptionally heterogeneous and can display both regulatory and pro-inflammatory activity.

In contrast to the regulatory activity of Treg, Th1, Th2, and Th17 lymphocytes have a pro-inflammatory phenotype. The expression of Th17- and Th2-related genes is shown to be increased in UC compared with CD, whereas the expression of Th1-related genes is increased in CD. However, one can observe wide heterogeneity in the expression of specific cytokines also within patient groups. For example, expression of the Th2 cytokine IL-13 shows dichotomy among UC patients, and expression levels are associated with mucosal microbiota composition as well as clinical characteristics including sex, age at disease onset, steroid/immunosuppressive/anti-TNF-α drug use, and presence of extensive colitis [Butera, 2020]. Also, there is heterogeneity between patients with both CD and UC in the intestinal expression of Th17 signature genes IL-22, IL-17A, IL-17F, and IL-26. The Th17-secreted cytokines are increased in the inflamed tissue of IBD patients, which correlates with infiltration of activated Th17 into the intestinal tissue. IL-17 acts as a paracrine signal to increase the production of additional pro-inflammatory cytokines, chemokines, and inflammatory mediators that eventually contribute to tissue damage. In intestinal lamina propria, CD14+CD163 low myeloid cells induce Th17 cell differentiation and IL-17 production in a way that is dependent on IL-1β, transforming growth factor [TGF]β, IL-6, and IL-23.

Interestingly, the role of IL-23 in the pathogenesis of IBD seems to be heterogeneous and may depend on the genetic and/or environmental background, since the primary risk variant, as well as two secondary risk variants of IL-23 receptor (IL23R) gene which are significantly associated with the risk of IBD in European populations, are not significantly associated with the risk in East Asian populations. This is particularly remarkable since IL23R risk variants have the second largest effect size on the risk of IBD after NOD2 variants, in population with European ancestry. Further, risk variants in IL23R contribute to greater risk of developing CD versus UC (odds ratio [OR] = 1.4) though it is significantly associated with the risk of both diagnoses, suggesting variation in the importance of this risk variant based on disease phenotype. Blocking antibodies against IL-23 and IL-12 [ustekinumab] has been shown to be effective in treating CD and UC and is now licensed for the treatment of IBD, including in Europe, the USA, Canada, and Japan. Relatively good response to ustekinumab has been shown in CD patients resistant to anti-TNF therapy, highlighting CD heterogeneity in response to different biologic therapies and need for patient stratification. Initially, both preclinical and human studies indicated the importance of IL-12 and IL-23 in CD but not in UC. However, evidence of the clinical efficiency of ustekinumab in UC has recently led to the approval of this drug for UC, demonstrating that cytokine profiling of groups of patients does not directly translate into effective therapeutic strategies—although individual cytokine profiling may be more clinically informative.

IL-22 is part of the IL-10 superfamily and an important cytokine in the pathogenesis of IBD. It has heterogeneous functions and is involved in epithelial regeneration and healing as well as pro-inflammatory responses in the gut. IL-22 is elevated in IBD patients and levels correlate positively with disease activity in both CD and UC. However, IL-22 gene expression is highly heterogeneous in both inflamed and uninfamed intestinal tissue of IBD patients, suggesting a possibility for patient stratification. Not only IL22 itself but regulators of IL22 display heterogeneity in IBD patients. IL-22 activity can be controlled by IL-22-binding protein.
[IL-22BP] which blocks IL-22 receptor [IL-22R] activation.\textsuperscript{123,124} Interestingly, IL-22BP expression is increased in intestinal tissue from CD and UC patients compared with healthy controls, yet with wide heterogeneity,\textsuperscript{122} suggesting decreased IL-22 signalling regardless of IL-22 serum levels. Interestingly again, IL-22BP produced by CD4 R T cells, but not DC, varies in association to response to anti-TNF treatment\textsuperscript{125} [Table 1], and therefore, frequencies of IL-22BP- T cells might be a criterion for patient stratification.

In animal models, IL-22 attenuates colitis in a Th2-mediated chronic colitis model [TCR\textsuperscript{R} KI mice].\textsuperscript{131} Moreover, anti-IL-22 antibodies significantly delay recovery from the DSS model of colitis.\textsuperscript{125} Also, a ligand for aryl hydrocarbon receptor is protective against TNBS colitis and the protective effect is partially blocked by IL-22 antibodies.\textsuperscript{125} Further, transfer of IL-22-deficient CD45RB\textsuperscript{lo} CD4\textsuperscript{+} T cells into IL-22-/- Rag1-/- mice causes more aggressive inflammation compared with transfer of wild-type CD45RB\textsuperscript{hi} CD4\textsuperscript{+} T cells.\textsuperscript{127} Kamanaka \textit{et al.} demonstrated that transfer of Foxp3-depleted CD45RB\textsuperscript{lo} CD4\textsuperscript{+} T cells into Rag1-/- mice induces a Th17-type intestinal inflammation compared with Th1-type inflammation caused by transfer of CD45RB\textsuperscript{hi} CD4\textsuperscript{+} T cells.\textsuperscript{128} Interestingly, transfer of IL-22 KO Foxp3-CD45RB\textsuperscript{lo} CD4\textsuperscript{+} T cells caused reduced inflammation compared with transfer of WT Foxp3-CD45RB\textsuperscript{hi} CD4\textsuperscript{+} T cells, demonstrating a pathological role of IL-22 in this model of intestinal inflammation in contrast to the CD45RB\textsuperscript{hi} CD4\textsuperscript{+} T cell transfer model, as well as heterogeneous functions of IL-22 in IBD.\textsuperscript{129} In addition, in anti-CD40 antibody-induced acute innate colitis models in Rag1-/- mice, IL-23R-dependent IL-22 production promoted inflammation, demonstrating that IL-22 can have pathogenic effects also in innate models of colitis.\textsuperscript{125} Thus, results from studies with different animal models demonstrate high functional heterogeneity of IL-22 depending, for example, on cytokine environment.\textsuperscript{130} The mean serum IL-22 is significantly higher in the CD45RB\textsuperscript{hi} CD4\textsuperscript{+} T cell population, with wide heterogeneity,\textsuperscript{122} suggesting decreased IL-22 signalling regardless of IL-22 serum levels.

### 3. Transcriptional stratification strategies

Stratification strategies of IBD patients based on transcriptional profiles have been recently developed. Kugathasan \textit{et al.} showed that at disease onset, paediatric CD patients who later developed strictureing disease behaviour had upregulation of extracellular matrix accumulation-associated genes in ileum, whereas patients who later developed penetrating disease had upregulation of genes associated with acute microbial immune responses\textsuperscript{133} [Table 1]. Biasi \textit{et al.} validated a transcriptional signature based on 17 genes in whole-blood samples from IBD patients which was predictive of aggressive disease course.\textsuperscript{134} The clustering was based on an unbiased patient stratification according to transcriptional profile in circulating CD8\textsuperscript{+} T-cells, which was earlier shown to predict disease course in IBD.\textsuperscript{132,135} Interestingly, both the stratification strategy in whole blood and that in CD8\textsuperscript{+} T cells were independent of the diagnosis UC or CD.\textsuperscript{132,135} However, an attempt at validation of patient stratification based on transcriptional profile in CD8\textsuperscript{+} T cells has recently reported negative results,\textsuperscript{136} highlighting the importance of validating molecular predictors of such clinical importance in independent cohorts. Of note however, in this validation cohort the CD8\textsuperscript{+} T cell profile was associated with the age of the patient, indicating that this insight might shed light on the clinical heterogeneity between childhood- and adult-onset IBD.

#### 3.1. Heterogeneous responses to anti-TNF treatment

Heterogeneous responses to different treatment options among IBD patients have led to active search for predictive biomarkers.\textsuperscript{137} Stevens \textit{et al.} performed in 2018 a literature search and found 92 articles on predictive biomarkers of response to therapies, mostly to anti-TNF agents, but no study was evaluated to have overall low risk of bias.\textsuperscript{138} However, high pre-treatment levels of oncostatin M [OSM] have been shown to predict poor response to anti-TNF treatment.\textsuperscript{139} Further, OSM was shown to have pathological significance in driving anti-TNF-resistant colitis in a murine model, and blocking OSM could attenuate colitis.\textsuperscript{140} Thus, OSM is not only a biomarker but also a potential treatment target with pathological significance.\textsuperscript{139} Further, high OSM expression is associated with a generally different transcriptional profile,\textsuperscript{141} possibly revealing that different pathological mechanisms are relevant in anti-TNF responders and non-responders. On the other hand, OSM expression was significantly correlated with inflammation severity and macroscopic lesions, suggesting that anti-TNF resistance might reflect an escalated state of inflammation.\textsuperscript{137} However, baseline clinical Mayo scores [consisting of categories stool frequency, rectal bleeding, endoscopic findings, and physician’s global assessment, where each is scored between 0–3] were not different between anti-TNF responders and non-responders, although their OSM expression levels were significantly different.\textsuperscript{137}

Developments in scRNA-seq technologies have provided novel ways for patient stratification based on cellular composition. In that way, Martin \textit{et al.} found that a subset of ileal CD patients has distinct cellular profile in the inflamed ileum, with accumulation of IgG plasma cells, inflammatory mononuclear phagocytes, activated T cells, and stromal cells.\textsuperscript{138} Further, they demonstrated that presence of the cellular profile is associated with altered cytokine signalling compared with other ileal CD patients, as well as non-response to anti-TNF therapy.\textsuperscript{139} Correspondingly, Smillie \textit{et al.} demonstrated a cellular profile in UC with increase in inflammatory fibroblasts that are enriched in gene expression associated with colitis, fibrosis, and cancer, and inflammatory monocytes and DC2 subsets that predicted non-response to anti-TNF treatment.\textsuperscript{140,141} Using gene expression profiling to predict disease course has great clinical potential, but the extent to which these profiles identify more aggressive inflammation in a way that is not captured by traditional endoscopic scores such as Mayo, and to what extent these profiles identify patients with immunological signatures that are specifically resistant to particular drugs, has not yet been resolved.

#### 3.2. Molecular taxonomy of IBD

Molecular profiling will likely be central in future clinical decision making.\textsuperscript{142} For example, in patients with multifocal inflammation, finding the driving factors of inflammation with specific EIMs could allow treatment of many inflammatory manifestations simultaneously.\textsuperscript{4} However, in biomarker discovery there is the well-acknowledged problem that inter-individual variability that is not relevant for the disease itself, e.g., differences in environment and lifestyle, accounts for most of the observable data variability.\textsuperscript{14} For example, Lloyd-Price \textit{et al.} followed longitudinally gut microbial constitution in UC and CD patients and controls and showed that inter-individual variability explained most of the variation between samples, surpassing even the explaining effect of the diagnosis.\textsuperscript{12} Thus, to support the field of personalised medicine, new methodologies are required to distinguish data variability that is relevant for the disease itself from random data noise.
Here, as an example of one approach to this problem, we will describe a novel classification of UC patients into two molecularly distinct groups, UC1 and UC2, which differed in immune-mediated transcriptional signatures but had similar partial Mayo score [clinical Mayo score excluding endoscopic findings]. The classification was obtained by unsupervised human patient stratification in respect to evolutionarily conserved transcriptomic profile between mouse and human colitis, which is a totally new approach to study heterogeneity in IBD. Selecting genes that were differentially expressed also during the mouse colitis made it possible to create a clustering based on genes with a true biological significance during inflammation. Also, the fact that they are evolutionarily conserved suggests that they are fundamental to the inflammatory process.

Importantly, it showed up that less than 10% of patients in cluster UC1 were responsive to infliximab [anti-TNF] therapy, whereas on average 70% of patients in cluster UC2 were responsive [Figure 3]. Furthermore, response to vedolizumab was an average 60% and 13% in clusters UC2 and UC1, respectively. Since infliximab and vedolizumab have fundamentally different mechanisms of action, it is unclear how the same clustering can predict response to both drugs. However, this unbiased clustering suggests that responders and non-responders for these medications have biologically different disease types. One could speculate that UC1 patients are generally less responsive to all kinds of treatments or that they would need much higher doses compared with UC2 patients. For example, UC1 could patients leak more drug through a damaged gut barrier. Indeed, it has been demonstrated that infliximab primary non-responders have higher faecal concentration of infliximab during the first day after infusion compared with responders, although the same weight-adjusted dose is given. Also, there are data suggesting that the Fc part of infliximab is needed to obtain its therapeutic effect in colitis, and the Fc part is also present in vedolizumab. Further, it is possible that the UC1-UC2 stratification relates to a downstream immunological pathway common to both drugs despite their differing mechanisms of action. Whether immune function differences between UC1 and UC2 clusters are stable in a given individual or dynamic during the disease course, and how environmental factors among others affect the classification, is still to be discovered.

Among the most differentially expressed genes [DEGs] between clusters UC1 and UC2, higher expression of TNF, S100 calcium-binding protein A8 [S100A8], IL-11, triggering receptor expressed on myeloid cells 1 [TREM-1], IL8, BCL2-related protein A1 [BCL2A1] and cytochrome P450 2b11 [CYP2B11], and lower expression of NADPH oxidase 1 [NOX1], nuclear receptor subfamily 3 group c member 2 [NR3C2], and prostatic androgen-repressed message-1 [PARM-1], defined UC1 from UC2 [Figure 3, Table 2]. Totally there were 56 DEGs that defined the UC1/UC2 clustering [Table 2]. Overall, results were partly in agreement with reports in which biased stratification based on response to therapy was made.

The cluster UC1 is characterised by both genes previously linked to IBD pathogenesis and genes without previous known significance in IBD [Figure 4]. Fasically, the genes expressed at higher level in UC1 cover many of the functions previously identified to be involved with IBD pathogenesis, namely pro-inflammatory cytokine signalling, granulocyte maturation and degranulation, molecules effecting immune amplification, epithelial cell regeneration and gut barrier function, immune cell chemotaxis, regulatory effects of RA, cellular metabolic processes, and apoptosis. This may imply that the UC1 cluster comprehensively captures multiple dimensions of IBD pathogenesis which lead to unresponsiveness to biologic drugs infliximab and vedolizumab [Figure 2]. In contrast, the UC2 cluster is characterised by upregulation of genes that have few specific implications in gut immune functions [Table 2].

It may be speculated that patients in cluster UC1 have more severe colitis, rather than a distinct inflammatory phenotype. Also, in a mouse model of colitis with adoptive transfer of CD4+CD45RB6 T cells, intestinal TREM-1 expression correlates [albeit weakly] with the severity of the inflammation. An earlier study has associated high baseline C-reactive protein and low baseline albumin levels, which are markers of acute severe colitis, with poor response to infliximab in the long term.

Severe disease also predicts poor response for vedolizumab. However, Sjöberg et al. have shown that 50% and 54% of patients receiving infliximab as a rescue treatment for hospitalisation-requiring flare of UC had steroid-free clinical remission at 3 and 12 months, respectively, which are remarkably higher response rates compared with those seen in cluster UC1 patients in our study, supporting that the UC1 and UC2 clustering is more accurate in predicting response to infliximab than clinically observable disease activity. Corresponding results have been reported by Jarnerot et al. Further, the UC1-UC2 stratification appears to provide a greater value for patient stratification than partial Mayo score, which was same between patients in clusters UC1 and UC2. However, among paediatric UC1 patients, total clinical Mayo and Paediatric Ulcerative Colitis Activity Index [PUCAI] scores were higher compared with UC2 patients, although no significant difference existed between UC1 and UC2 for histological score or calprotectin.

One could also speculate that the UC1 cluster represents an earlier state of a flare, that is UC1 and UC2 may represent different phases of the disease and as such may be observed in the same patient at different time points. Indeed, in the DSS mouse colitis model, genes associated with inflammatory responses and neutrophil degranulation were upregulated during the acute phase of DSS colitis but not in later phases. It may be that molecular changes towards recovery occur before clinically observable signs, explaining why UC1 and UC2 patients could not generally be distinguished with partial Mayo score. In that case, it could be also that UC1 patients have a prolonged acute phase of the inflammation and are unable to initiate the recovery phase, whereas UC2 patients may have already activated mechanisms of healing and are thus primed to respond positively to drug treatments. Hence, UC1 and UC2 classification could provide knowledge on optimal drug dose and optimal time point for the start of a biological therapy. Moreover, if UC1 and UC2 represent different phases of the patients’ progression from inflammation to healing, this would provide potential molecular targets to directly enhance the recovery and regeneration process or enhance response to biologic therapies.

The earlier described stratification strategy by Biasci D et al. which is based on transcriptional profiles in whole blood does not include any genes present among the most differentially expressed genes between UC1 and UC2, demonstrating entirely distinct biological backgrounds of these two stratification strategies. Of note, OSM was not found among differentially expressed genes between UC1 and UC2, though both OSM expression levels and UC1 and UC2 stratification predict response to anti-TNF therapy. This might indicate that OSM is not biologically related to UC1 and UC2 stratification profiles and predicts anti-TNF response based on different biological grounds. However, IL-1β, IL-6, and IL-11 are upregulated in both UC1 patients and patients with high OSM, suggesting that these profiles could be partly overlapping. Smillie et al. demonstrated that
Table 2. All UC1 and UC2 genes. This table summarises all the genes in UC1 and UC2 clustering together with possible implications in colitis.

| Cluster                  | Gene              | Possible functional implications in colitis                                                                 |
|--------------------------|-------------------|-----------------------------------------------------------------------------------------------------------|
| Reduced in UC1 relative to UC2 | PARM1             | Expression increased by inflammatory cytokines, but no found functions in colitis                        |
|                          | NR3C2             | Reciptor activated by cortisol and aldosterone, but no found functions in colitis                         |
|                          | NOX1              | Oxidative stress                                                                                        |
|                          | MAP2K2            | A member of mitogen-activated protein kinase family that has many pro-inflammatory effects in colitis1    |
| Increased in UC1 relative to UC2 | COL15A1          | Collagen synthesis2                                                                                     |
|                          | COL12A1           |                                                                                                          |
|                          | COL4A1            |                                                                                                          |
|                          | COL4A2            |                                                                                                          |
|                          | COL7A1            |                                                                                                          |
|                          | MMP3              | ECM breakdown2                                                                                           |
|                          | MMP1              |                                                                                                          |
|                          | WNT3A             | WNT gene family,2 important in intestinal epithelial regeneration and probably in wound healing in colitis,2 linked to many inflammatory pathways3 and might be possible activators of expression of metalloproteases4 | Collagen synthesis2 |
|                          | WNT2              |                                                                                                          |
|                          | WISP1             |                                                                                                          |
|                          | IL6               | Cytokine and cell-to-cell signalling2                                                                   |
|                          | IL24              |                                                                                                          |
|                          | IL11              |                                                                                                          |
|                          | IL1B              |                                                                                                          |
|                          | IL33              |                                                                                                          |
|                          | IL1R2             |                                                                                                          |
|                          | IL1RN             |                                                                                                          |
|                          | IL7R              |                                                                                                          |
|                          | IL18RAP           |                                                                                                          |
|                          | IL13RA2           |                                                                                                          |
|                          | TNF               |                                                                                                          |
|                          | TNFRSF11B         |                                                                                                          |
|                          | CSF2RB            |                                                                                                          |
|                          | CSF3              |                                                                                                          |
|                          | CSF3R             |                                                                                                          |
|                          | TGFBI             |                                                                                                          |
|                          | SOCS3             |                                                                                                          |
|                          | TREM1             |                                                                                                          |
|                          | CXCR2             |                                                                                                          |
|                          | ICAM1             |                                                                                                          |
|                          | ITGA5             |                                                                                                          |
|                          | CXCR1             |                                                                                                          |
|                          | CXCL8             |                                                                                                          |
|                          | CXCL6             |                                                                                                          |
|                          | CCR1              |                                                                                                          |
|                          | ICAM3             |                                                                                                          |
|                          | TLR1              | Innate immunity, cell migration and neutrophil degranulation2                                           |
|                          | TLR2              |                                                                                                          |
|                          | CD14              |                                                                                                          |
|                          | S100A4            |                                                                                                          |
|                          | S100A9            |                                                                                                          |
|                          | S100A8            |                                                                                                          |
|                          | S100A12           |                                                                                                          |
|                          | PLAU              |                                                                                                          |
|                          | C5AR1             |                                                                                                          |
|                          | SELL              |                                                                                                          |
|                          | SELE              |                                                                                                          |
|                          | FGR               |                                                                                                          |
|                          | BCL2A1            |                                                                                                          |
|                          | PTGS2             |                                                                                                          |
|                          | CYP26B1           |                                                                                                          |
|                          | FAM49A            |                                                                                                          |

UC, ulcerative colitis; ECM, extracellular matrix.

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Immunological Networks Defining the Heterogeneity of Inflammatory Bowel Diseases

1970

inflammation-associated fibroblasts, inflammatory monocytes, and DC2 subsets are not only predictive of non-response to anti-TNF, but also that fibroblasts express OSM receptor whereas monocytes and DC2 subsets express OSM, thus likely being linked to OSM-mediated TNF resistance. Interestingly, they also demonstrated that UC1 genes IL-11, IL13RA2, TNFRSF11B, and CXCL6 are mostly expressed by inflammation-associated fibroblasts, TREM-1 by inflammatory monocytes and DC2s, and UC1 gene PTGS2 by all of these cell types, suggesting that these key cell types might be involved in both OSM signalling and the pathogenesis in the UC1 cluster. Further characterisation of the UC1 and UC2 clusters might provide additional clues on how immunity underlines clinical disease heterogeneity.

4. Concluding remarks

Patient stratification, including prediction of disease course, complications, and treatment responses, are currently the main goals of IBD research. Thus, clustering strategies like UC1 and UC2 are needed to provide the immunological foundation for understanding the observed heterogeneity of IBD. This review has mostly focused on immune functions as a base for heterogeneity in IBD, but many other intestinal cell types and pathways also have their role in observed heterogeneity. For example, the epithelial layer has a key function in intestinal homeostasis and antimicrobial defence, integrating with other intestinal cell types and luminal content. Indeed, integration of data of, for example, genetics, epigenetics, microbiology, immunology, and metabolism in combination with clinical data, so called -omics technology, will now facilitate the search for non-invasive and even more precise biomarkers and enhance understanding of the aetopathogenesis of IBD.

approaches, similar to those employed to detect UC1 and UC2 clusters, provide numerous possibilities to find new approaches for precision medicine. Although there are still few clinically usable clustering strategies, technological developments continue apace, and are likely to revolutionise future clinical management of IBD.

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Author Contributions

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