Title: **Challenges in IBD Research 2024: Preclinical Human IBD Mechanisms**

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Introduction and Background

To address the unmet medical needs of patients with inflammatory bowel diseases (IBD) and move toward the ultimate goal of cure, preclinical human-relevant research is critical and must center on mechanistic questions relevant to patients with IBD in the three areas of disease interception, remission, and restoration. Gaps previously highlighted in "Challenges in IBD Research 2019" in preclinical human IBD mechanisms included: (1) triggers of immune response, (2) intestinal epithelial homeostasis and wound repair, (3) developmental and age-related pathophysiology, (4) biology of complications, (5) biological determinants of disease location, and (6) new therapies and response to treatment[1].

Over the past five years, significant progress in these areas has been achieved. "Triggers of Immune Response," called for greater research into the host, diet, and microbiome interactions as instigators for dysregulated immune responses associated with IBD. Significant strides were made in microbiome science through advancements in technology and analytic tools. In conjunction with multi-omic analyses of well-curated IBD patient cohorts[2], studies have shed light on how microbial metabolites shape intestinal homeostasis[3] and how microbes influence the efficacy of widely used drugs for IBD[4] among a multitude of other important discoveries. The application of single cell and other 'omic technologies to samples from patient cohorts and experimental model systems have advanced our understanding of the other

previously identified gaps, specifically, the epithelial and mesenchymal cell diversity and function in health and IBD[5–7]. We also have gained insight into factors that shape age-related pathophysiology[8–11], the biologic determinants of disease location and complications[12,13], and drivers of resistance to anti-TNF therapy[14,15].

Although substantial progress has been achieved, discoveries over the last five years have also revealed new gaps in knowledge that form the basis for the identified Challenges in IBD Research 2024 Preclinical Human IBD mechanisms priorities. Along with the other Challenges in IBD Research 2024 Work Groups, we place these gaps in knowledge within the context of disease interception (prevention of progression to the clinical phase), maintenance of remission and restoration of mucosal homeostasis. The challenges identified herein are guided by the Crohn's & Colitis Foundation's mission to cure IBD and improve the quality of life for those affected by these diseases.

The new 2024 gaps encompass five areas of focus for IBD preclinical research. They are as follows. (1) Genetics*, Risk Alleles, and Epigenetics* investigations that leverage current knowledge to understand how these factors contribute to IBD development and how they may be targeted for personalized medicine approaches. (2) *Microbiome* studies that are mechanistically oriented and prioritize the identification and validation of microbially-rooted, remission-inducing and resiliency-affording approaches that restore health to the IBD gut. (3) *Cell States and Interactions* research centered on understanding the interactions and modulation of immune cell responses and gut barrier function. Specifically, it is critical to close knowledge gaps in the contributions of non-immune cells (such as fibroblasts and smooth muscle cells), cell states and interactions (immune and stromal) and other cells and tissues (mesentery, nerves, muscle) in initiating and driving pathobiology so that we can address the differences between pre-disease and remission states in IBD and identify potential points for therapeutic intervention. (4) *Barrier Function* research remains underdeveloped as a target for therapeutic intervention and represents a tremendous

opportunity to improve patient outcomes in interception, restoration, and remission. (5) Pathways to *Complications and Extraintestinal Manifestations*: strictures, fistulas, and extra-intestinal manifestations (EIM)s all cause significant morbidity in patients with IBD. A greater understanding of the mechanisms and pathways driving these will shape future interventions and therapies.

The Challenges in IBD Research 2024 Preclinical Human IBD Mechanisms Work Group also identified two important concepts that touch all the gaps described. First, consideration to diversity in gender, ethnicity, age, and race must be a priority of all studies. There is an overarching need for expansion of research pertaining to patients across populations underrepresented in existing IBD cohorts to facilitate personalized medicine approaches. Second, improvements in preclinical models pertaining to characterization, availability, reliability, and consistency are also needed (vertebrate models, organoids, and multicellular cultures). In response to this, we direct attention to the *Inflammatory Bowel Diseases* Journal's recent call for basic science submissions focused on Translational Preclinical Models of IBD https://academic.oup.com/ibdjournal/pages/basic-science-cfp.

The following sections will describe in detail the five gaps identified by the Challenges in IBD Research 2024 Preclinical Human IBD Mechanisms Work Group and how these gaps pertain to interception, remission, and restoration of IBD when appropriate. Some of these gaps and challenges clearly cross all three domains, such as genetics and the microbiome, while others focus primarily on one of the domains. In some cases, the rationale for the domains is similar and multiple domains are covered together.

Gap 1: Genetics, risk alleles and epigenetics

As pertaining to ***interception, remission, restoration*****,** there is a need to leverage results from genomic studies to identify how individual or compound genetic variants contribute to IBD development and/or treatment response as well as impact on restoration and may ultimately be targeted for personalized

medicine approaches. Similarly, there are large knowledge gaps in understanding the role of epigenetics in driving IBD development and/or treatment response and the impact on restoration.

Genetics in IBD

Over the past two decades, genome-wide association (GWAS) studies have provided key insights into the biological underpinnings of IBD. Over 200 genetic loci have been linked to risk of disease[16–22]. Importantly, many of the identified coding variants converge upon common pathways, act through specific cell types, and affect core mechanisms of mucosal immunity and intestinal epithelial barrier function. These points of convergence have become the focus of rigorous mechanistic studies[23], like the role of the NOD2 signaling pathway (including NOD2/CARD15, ATG16L1, CARD9, and RIPK2) in response to microbes and the IL-17/IL-23 immune axis in development of chronic inflammation. Mechanistic studies around GWAS-identified variants in genes such as IL-23R[16] contributed to the basis for the development of currently used anti-IL23 therapies (e.g., ustekinumab, risankizumab, mirikizumab) and highlight the importance of preclinical research.

While GWAS studies have given clues into the underlying biology of IBD, the variants identified to date do not account for all cases. Moreover, IBD is a complex, most often polygenic disease. While polygenic risk scores provide a measure of disease risk, the combination of variants necessary to trigger disease remains elusive. Many disease-associated variants are in non-coding regions, making mechanistic studies challenging. As a call to action, human-derived cell culture models and innovative approaches such as massively parallel reporter assays (MPRA)[24,25] and CRISPR-based technologies should be employed to conduct large-scale genetic screens, using both single-gene modification and combinatorial approaches, to link the hundreds of disease-associated variants, including those in non-coding regions of the genome, to their biological function within the landscape of IBD. Results from such screens will allow for generation of hypotheses that should then be further tested in preclinical cellular and *in vivo* models of

relevance to IBD. This will allow for eventual identification of therapeutic intervention points for interception and to impact remission and restoration.

Population-based differences in disease-associated risk alleles further complicate the role of genetics in IBD. For example, while variants of NOD2 are associated with disease in patients of European ancestry, they are typically not present in patients of African[26] or South Asian ancestry[27]. The expansion of GWAS studies to populations of non-European ancestry in recent years has uncovered significant differences in disease-associated variants[26] outside of NOD2. Variants that are extremely rare and undetectable in patients of European ancestry could be identified in future studies focusing on different patient populations, giving new biological insight into disease and moving us closer to personalized medicine approaches. As a call to action, investigators should determine genetic-based mechanisms involved through analysis of data stemming from diverse patient populations, and through conducting experiments with these patient-derived samples (e.g., cells, organoids), genetically engineered cells and animals to identify specific mechanisms implicated in driving IBD and/or treatment response, restoration and to identify potential drug targets. Finally, leveraging genetics to understand heterogeneity in disease course and response to treatment remains a significant gap in understanding disease biology. Why do some patients develop fistulizing disease or perianal disease? Why do some patients respond well to treatments while others relapse? A better understanding of how polygenic risk translates into specific disease phenotypes will help stratify patients, improve the use of current therapies, and accelerate the development of novel, personalized diagnostic and therapeutic approaches (see Challenges in IBD 2024 Precision Medicine Work Group Report). As a call to action, researchers should utilize data from precision medicine biomarker studies implicating epigenetic mechanisms (see below) and clinical studies implicating genotypic risk groups more likely to experience medication failure or response to particular therapies to generate and test mechanistic hypotheses in preclinical experimental systems.

Epigenetics in IBD

If genetic variants were the only driver of IBD, one would expect that concordance for identical twins would reach 100 percent. While twins are more likely to both develop IBD, concordance only reaches 50 percent[28], suggesting a strong environmental component to disease (see Challenges in IBD 2024 Environmental Triggers Work Group Report). Gene activity can be adjusted throughout life, with no change to the DNA sequence, by modifying the accessibility of genes. These epigenetic modifications can alter host biology in response to the environment and are emerging as key players in human disease, including IBD. Epigenetic modifications fall into three classes: histone modifications, expression of non-coding RNAs, and DNA methylation (DNAm). Specific changes in DNAm patterns have been identified in the intestinal epithelium of pediatric IBD patients and correlate with disease outcomes[29]. DNAm is largely stable in *ex vivo* patient-derived organoid models[29], enabling the conduct of mechanistic studies on how changes in DNAm affect downstream cellular function. These studies will determine whether epigenetic modifications and genetic risk alleles converge upon common pathways or cellular processes that can be assessed in preclinical models and ultimately therapeutically targeted.

Precision medicine biomarker studies have also shown that DNAm of specific genetic loci is stable in peripheral blood mononuclear cells (PBMCs) of IBD patients[30]. Results from such studies should be independently validated and used to generate and subsequently test mechanistic hypotheses in preclinical experimental systems. These studies may be used in the future to help diagnose, stratify, and predict treatment outcomes of patients, moving toward a personalized or precision medicine approach. Ultimately, the identification of the environmental factors causing DNAm changes as well as the pathways and components involved utilizing preclinical model systems will reveal points that can be perturbed to prevent disease in the subset of patients where epigenetics plays an outsized role.

Gap 2. Microbiome

In 2019, the microbiome was contextualized, from the perspective of the Challenges in IBD 2019 Preclinical Work Group, as a trigger of immune responses in IBD[1], our current vision has expanded. The microbiome or microbiota, in the context of IBD, is the constellation of archaea, bacteria, fungi, viruses, protists, and helminths that inhabit the body and shape resistance and susceptibility to IBD disease onset, symptoms, flares, remission, and response to treatment. Microbiome science overall and as it is related to IBD has grown tremendously since the Challenges in IBD Research report was published in May 2019[1,31–34]. Developments in microbiome studies are driven by expansion of well-curated human cohorts with a gut microbiome focus, advancements in microbiome data generation and analysis tools (especially regarding metabolomics) applied to IBD, and innovations in preclinical models[2–4,35–48]. In this gap section, we highlight recent impactful discoveries, existing knowledge gaps, and calls to action regarding IBD and the microbiome and provide ideas and concepts for how the microbiome can be approached using preclinical models, first for IBD interception and then for IBD remission and restoration.

Interception

A knowledge gap persists in the fundamental understanding of how the microbiome influences disease initiation in both Crohn's disease (CD), ulcerative colitis (UC), and microscopic colitis. Intrinsic to this gap is the need for a more mechanistic understanding of the microbial–host interactome to identify individuals at risk for developing IBD. Is one microbe the culprit or rather how does one microbe or microbes act in a consortium to trigger IBD symptom onset. What are the consequences of particular microbial behaviors (biogeography, metabolite production, interactions with other host or microbial cells) for a host with a particular susceptibility under certain environmental conditions? Are instigating microbes bacterial, fungal, viral or all of the above and what are their features, metabolite or structural, that are problematic for some hosts and why? While there has been significant microbiome characterization in

newly diagnosed and established IBD patients while they are symptomatic and in remission with appropriate healthy control populations[2,49,50], building cohorts of individuals at risk for IBD prior to symptom development has been more challenging. To begin to resolve this biological complexity, The Crohn's and Colitis Canada Genetic, Environmental, Microbial (GEM) Project[51] built prospective cohorts studying healthy first-degree relatives of CD patients. A recent study from this consortium analyzed stool microbiome data from these individuals prior to the onset of CD to generate microbiome-based IBD risk scores[52]. This cohort is and will be immensely useful to IBD researchers and patients alike[53–55] and should direct the efforts of basic and translational IBD investigators so that they mechanistically interrogate the microbial-host signaling features and pathways that are inflection points for manifestation of IBD symptoms.

We seek to call investigators to action to identify and validate microbiome bioactive features and their modulators. Our definition of features encompasses specific strains of bacteria, fungi, viruses, microbial pathways, biosynthetic gene clusters, and metabolites that are viable targets for either preventing symptomatic IBD or sustaining health in individuals at risk for IBD. Beyond identification and validation of such features, we also encourage the development of strategies to provision these beneficial features or remediate them. Our working definition of bioactive is holistic and integrated with the other gaps put forward by this group. Bioactivity can be immunoregulatory such as the recently elucidated function of certain bile acid metabolites[56,57] or inulin elicited-microbiome metabolites[58]. However, bioactivity is not restricted to traditional immune cell populations; effects on the epithelial barrier, specific cell populations within in the epithelium, enteric neurons, and stromal cell populations are welcome areas of investigation when and where robust assays and measures are available.

Specific actions we encourage are development of preclinical pipelines to prioritize microbiome bioactive features that precipitate symptomatic IBD and that can be targeted for interception. Prioritization workflows can encompass advancement in mouse models bearing human IBD risk alleles combined with the use of gnotobiotics and human microbial communities (e.g. as in [45,46]) in the context of microbial feature testing, more reductionist methods, e.g., such as organoid-bacterial co-culture systems[59,60], that enable the growth of anaerobic bacteria, or computational pipelines paired with cell-based interrogative screening assays of features.

Additional interception approaches we endorse draw from the disciplines of immunology and infectious diseases, specifically vaccinology. Vaccination remains the single most effective strategy to prevent or mitigate the severity of bacterial and viral infections. If the symptoms of IBD or its triggers are rooted in the microbiome (be it bacterial, viral, or fungal), vaccine approaches (either quasi-personalized or bacterial-straining targeting broadly) may be an option, as technology to generate RNA-based vaccines is now within reach of many laboratories. Recently data analyses from the GEM projects highlighted that *Ruminococcus torques* is a key contributor to the IBD microbial risk score they identified in first-degree relatives of CD patients[52]. *R. torques* is frequently observed in microbiome studies of IBD as is another member of its genus *R. gnavus* for which certain strains in particular have robust IBD associations[61,62]. Beyond these ruminococci, adherent and invasive *Escherichia coli* have longstanding and well validated associations with ileal CD[63]. Rather than enumerate strains associated with risk for IBD or potential vaccine antigens, we encourage vaccine development approaches as an interception or prevention strategy of interest and preclinical models provide ample opportunities for testing such vaccine-based approaches. Adjacent to vaccine strategies would be the further optimization of phage-based approaches in preclinical models for IBD interception[64,65]. The opportunity to introduce phages to reverse a pro-inflammatory microbial environment before IBD develops in at-risk individuals is also appealing.

In summary, robust and innovative preclinical systems investigating the microbiome and IBD focused on the topics described above will be pivotal for effective translation of preventive strategies for IBD interception.

Remission and Restoration

While cure is the Crohn's & Colitis Foundation's ultimate goal, achieving remission and restoration is the penultimate step in that path. Over the past five years, advancements in microbiome science as cited above have identified specific microbes and microbial factors associated with mucosal healing and maintenance of barrier function, yet a gulf remains between these handful of studies and effective translation for therapeutic approaches. Additionally, we still need to identify specific microbes and microbial products that can restore healthy host-microbial interactions in IBD. Fecal microbiome transplant and transfer of consortia have shown promise, and these approaches merit further refinement[66,67]. A recent study of the dextran sodium sulfate (DSS) model of colonic injury and inflammation was laudable in its systematic approach for unraveling microbial drivers of the DSS weight loss and histology providing insight for optimizing this accessible and quick model for more reproducible and robust remission and restoration studies[47]. While there are clearly gaps in the knowledge of microbes and features that restore health, there are similar knowledge shortfalls in understanding of host factors that influence microbial localization and behavior than affect effective barrier function. Intelectin-1 influences the localization of *Akkermansia mucinphilia,* a mucus-loving bacteria, with immunomodulatory and barrier modifying effects[68]. At first glance intelectin-1 might be seen as a beneficial host defensive factor but experiments in preclinical models have revealed that its excessive production in IBD results in disadvantageous effects for the epithelial barrier and mucus layers.

When we observe host or microbial features that are differentially abundant in active IBD, it can be difficult to predict whether there is too little or too much of the factor for the host. Preclinical models are pivotal in resolving such Goldilocks zone questions and whether a feature is ultimately beneficial or pathogenic and under what conditions. We encourage preclinical investigators to generate hypotheses from existing and emerging datasets in human cell systems and humanized animal models to identify specific microbes, microbial features, and their host modifiers to restore a healthy host-microbial status. These investigations can be grounded in combining *in silico* approaches with preclinical *in vitro* and *in vivo* models. For example, when one identifies an inflammatory and wound healing program or cell state signature, as was recently done to better understanding stricturing in pediatric Crohn's disease[69], such signatures can be compared with microbial metabolites cell screening data sets to identify shared differentially expressed gene signatures. Such approaches can prioritize molecules for additional testing in primary cell cultures or even relatively simple cell line wound healing assays to winnow the list of bioactives prior to *in vivo* testing in models humanized from a human genetics or microbiome perspective.

We acknowledge that non-bacterial members of the microbiota have often been understudied in IBD in the context of remission and restoration. A recent study sought to rectify that knowledge gap and uncovered that *Debaryomyces hansenii* was enriched in intestinal wounds of mice that were incompletely healed and also in inflamed CD patient biopsies[70]. The investigators went on to show that *D. hansenii* impaired wound healing via a macrophage-type 1 interferon-CCL5 program. Additional studies have profiled the mycobiome of well-phenotyped CD and UC patients with extensive outcome data (e.g., [71]). This recent study is quite useful for the IBD community. However, more attention to these fascinating microbes as regards to their roles in IBD etiopathogenesis is warranted, specifically with a focus on refining understanding of fungi in IBD for improved remission and restoration outcomes in patients via preclinical

investigations that embrace reverse translation methods[72,73]. The human virome is also very important and we anticipate breakthroughs in this area from the NIH-funded initiative on this topic. Regarding research opportunities for preclinical investigators, this Preclinical Work Group prioritizes identification and validation of microbially-rooted, remission-inducing and resiliency-affording approaches that restore healthy to the IBD gut.

Gap3: Cell states and interactions

The maintenance of mucosal homeostasis requires a delicate balance of cell states and cellular interactions of the different components within the intestine including immune, stromal, epithelial, and muscle cells as well as the enteric nervous system (ENS). Of these, how the dysregulation of the immune and epithelial components contribute to IBD onset, progression, and remission have been the most studied. However, data suggest that other cell types such as stromal cells (fibroblasts, endothelial cells and smooth muscle cells) are critical to the intricate balance between tolerance and inflammation[6,74,75]. Yet, there is limited knowledge about which cell types are important, how they communicate with one another, where they are located, and how they are remodeled during disease. There is also a lack of understanding of how adjacent tissues, such as the mesentery, contribute to IBD. Finally, it is almost entirely unknown whether cell states return to pre-IBD baseline during remission, or alternatively a new baseline is reached after experiencing inflammation. Are there IBD related epigenetic alterations to these cells? Do these cells return to their pre-inflammatory niches with restoration of the cell-cell communication to homeostatic baseline? Additionally, is there a retained "memory" of the inflammatory state beyond epigenetic regulations and rather through a gut-brain axis as suggested by Koren et al., where areas of the brain store and retrieve memory of immune responses[76]? These key questions are further divided below into those that affect IBD interception, remission, and restoration to identify gaps in our understanding and approaches to address them.

Interception

Several recent studies have used systems biology approaches to understand cell types, their abundances, and interactions in a combination of healthy and IBD tissues from UC and CD patients[6,7,14,74,75,77–86]. To further improve our understanding of key drivers of IBD onset, there is a need to apply similar approaches to cohorts beyond those that have already developed IBD, to subjects at risk of developing the disease, as in the GEM project mentioned in Gap 2. This will ensure that states and cellular interactions that predict the onset of IBD can be identified with the goal of modulating these interactions to prevent disease initiation. Furthermore, it is important that these new cohorts are diverse to determine whether mechanisms are conserved between diverse patient populations. Alternatively, can unique signatures be identified that correlate with different ethnicities?

The suspension multi-omic data should be correlated with and complemented by special techniques both at the transcriptional and/or at the protein level. Commercialization of these techniques (Visium by 10X genomics[87], GeoMx®[88] and CosMx™ by Nanostring Technologies[89], MERFISH[90], and imaging mass cytometry) has enhanced our understanding of tissue architecture, cellular locations, and cell-cell crosstalk. With the improvement in their resolution and analysis pipelines, it is important to apply these techniques to IBD to identify cellular neighborhoods and interactions that drive IBD onset.

Additionally, the ENS plays important roles in maintaining mucosal homeostasis that could potentially be critical to IBD onset. For example, studies have suggested that certain disease states, such as infectious colitis, drive “neurogenic inflammation” through altering neuropeptides released from neurons and cytokines from pericytes[91]. Additional studies have suggested that regulatory T cells (Tregs) are found near and regulated by enteric neurons that themselves receive signals from the microbiome, suggesting a microbiome-ENS-immune circuit[92]. Whether these interactions are perturbed in people at risk of IBD is unknown and should be investigated further. It is also known that stress impacts the intestinal system,

where IBD flares can be linked to increased stress. An elegant study has recently suggested that the ENS mediates intestinal inflammation during episodes of chronic stress where elevated levels of glucocorticoids promote the generation of inflammatory glia that recruit TNF-producing monocytes to the intestine and additionally cause dysmotility[93].

Finally, the mesentery, or the mesenteric fat, has been linked to IBD[94–97], to stricturing CD in particular[98]. The mesentery contains numerous immune and non-immune cell populations (endothelial cells and fibroblasts) that potentially could contribute to IBD pathogenesis and should be investigated further.

Remission

To improve our understanding of how remission can be achieved and maintained, we need to identify drivers of therapeutic responses and treatment failures. Little is known about the contribution of non-immune cells, cell states and interactions, and other tissues (mesentery) in initiating and driving progression, loss of response, and post-surgical recurrence in patients with IBD. Key questions remain unanswered such as are there particular "cellular signatures" before the initiation of therapy that could predict individuals' responses to treatment and are there signatures in the intestine or other tissue including blood that correlate with remission? As such, there is a need for a deeper understanding of disease states, those that lead to remission and those that predict response failure, to facilitate the identification of new pathways to maintain remission. Some of the recent scRNAseq datasets have begun to do just that and have identified signatures that correlate with TNF failure for example[75]. Much more work is needed to identify similar signatures for other biologic and small molecule-based therapies and those that correlate with therapeutic responses and disease remission. This can be accomplished by using multi-omic datasets directly from patients on various medications where the responses to therapies are known. Moreover, there is a need to establish more sophisticated patient-derived organoid co-cultured models where epithelial cells are cultured with other intestinal components such as immune cells and or

fibroblasts to resemble the *in vivo* system where drug responses can be optimized to establish drivers of therapeutic responses. Both the organoid and patient sample multi-omic datasets should be assessed for signatures that could then lead to hypothesis generation that can be evaluated in preclinical models.

Furthermore, work is required to understand how similar or different the intestinal landscape of remission is to healthy homeostasis. Is "memory" of inflammation retained in the cells of the intestine through epigenetic regulations, so that with certain triggers they are more likely to revert to the inflammatory states? Can these modifications be altered to bring the intestine closer to the "healthy" state?

Finally, it is critical to determine whether there are particular cellular defects in treatment refractory patients. For example, do these patients have an epithelial defect that leads to intestinal barrier loss and can this be identified through simple testing in the clinical setting? Please see the barrier dysfunction section (below) for more details.

Restoration

There is also an incomplete understanding of the mechanisms that affect cell populations causing dysregulation and hampering gut restoration. Longitudinal, multi-omic datasets including suspension and spatial data of subjects in various stages of IBD and healthy controls should be used to determine whether restoration, or return to pre-inflammatory "healthy" state, is possible and if there are signatures during onset and remission that can predict full restoration. These datasets would be instrumental in identifying mechanisms affecting key tolerogenic populations such as Tregs and innate lymphoid cells (ILCs) that play a critical role in maintaining homeostasis and could be harnessed to restore balance.

Gap 4: Barrier function

Decades of preclinical and patient data link intestinal barrier loss to IBD[99]. However, all available therapies target the immune system, either directly or indirectly. Despite the proliferation of effective new

therapies in recent years, there are tremendous opportunities to improve patient outcomes, and the epithelial barrier is an obvious target that has been under-explored[100]. Nearly 40 years ago, the discovery that increased intestinal permeability, i.e., barrier loss, is present in a subset of healthy first-degree relatives of CD patients[101] led many to speculate that increased permeability might be a risk factor for IBD development. However, this was only recently validated, when it was shown that, among healthy CD relatives, increased intestinal permeability was associated with a 3-fold greater risk of developing IBD[102]. Consistent with this, preclinical studies using mouse IBD models have shown that even partial barrier preservation delays disease[103–105]. Together, these clinical and preclinical data suggest that interception, i.e., prevention of IBD progression to a clinically evident phase, may be possible in at-risk individuals with increased intestinal permeability.

In patients with established CD, multiple studies demonstrate that increased permeability during clinical remission is a marker of relapse within the next year[106–108]. Similarly, preclinical data show that barrier restoration at early time points slows progression and reduces severity of clinically evident disease[103–105,109,110]. These data suggest that barrier loss may be a marker of relapse and that barrier restoration may sustain clinical remission. If effective in patients, barrier restoring therapies might also reduce use of biologics and other immune-targeted agents in terms of dose and frequency to lessen associated toxicities.

Clinical and preclinical studies demonstrate that many disease-reducing therapeutic interventions also restore barrier function. In most instances, including the infliximab-induced reductions of intestinal permeability in CD patients[111], effects on inflammatory disease, mucosal repair, and epithelial tight junctions are inextricable. Thus, the contributions of barrier restoration to these therapeutic responses have been impossible to determine. Moreover, nearly all studies have relied on probes that cannot

distinguish mucosal injury-associated barrier loss from that caused by increased tight junction leak pathway permeability[112]. Further, of the two routes across the tight junction (the leak and pore pathways), each can be differentially activated by immune signaling, have unique permeability profiles, and are regulated by distinct cellular processes[113]. Nevertheless, permeability probes that detect pore pathway flux have only been used rarely[112–114]. Commonly used probes, including 4kD FITC-dextran, lactulose, mannitol, sucralose, fluorescein, and ^{51}Cr-EDTA, can detect leak and unrestricted (damage) pathway barrier loss but do not distinguish between these. Moreover, none of these assess pore pathway channels, such as those created by claudin-2[114].

Despite progress in understanding molecular mechanisms of mucosal barrier loss [99,115–124], drugs that target the epithelium have not been developed. One factor related to this has been the absence of even rudimentary characterization of barrier loss, e.g., tight junction-dependent vs. epithelial damage-induced, in patients and preclinical animal models. This information is essential because the mechanisms are distinct. Therefore, an agent that promotes mucosal healing may not impact tight junction permeability while, conversely, a tight junction-targeted therapy would likely be ineffective in the setting of severe mucosal damage, where de-epithelialized areas lack intercellular junctions. Tools that discriminate between these and other processes that increase intestinal permeability are, therefore, desperately needed. Ideal probes would be i) nonmetabolizable by mammals or bacteria; ii) able to efficiently cross the pore, leak, or unrestricted pathways (a pore pathway probe would, of course, also cross via the other two routes); iii) freely-filtered at the glomerulus (to allow analysis in urine); iv) well-tolerated; and v) easily analyzed (without complex assays or interference by endogenous materials). No probes that fit this description are available[112]. If they were, it would be possible to determine pathways, i.e., pore, leak, or unrestricted, responsible for observed changes in barrier function and accurately define and target molecular mechanisms of barrier loss.

Patient, animal, and *in vitro* data all confirm that barrier function is dynamic[125–128]. Yet, normal variation, such as the interaction between diurnal variation and alcohol-induced barrier loss reported in mice[128], has not been considered in human studies or characterized in terms of distinct pathways. The availability of improved probes would, therefore, allow determination of how the specific time of day or other recurrent events, such as phase of the menstrual cycle in women, affect properties of all pathways that define the epithelial barrier. If used to assess at-risk individuals or disease status and therapeutic response in patients, it would be reasonable to ask whether repeated measures improve sensitivity or specificity? If so, how many measures? How often? Use could, for example, be modeled after repeated measurements of any clinical parameter, from blood pressure to fecal calprotectin. Integration of longitudinal permeability studies of healthy, at-risk relatives and IBD patients with parallel analyses of tissue or blood RNA and protein expression, immune markers, microbiome composition, stool metabolomics, other parameters, and clinical outcome may both provide insight into events and pathways associated with initial IBD development, remission failure, CD recurrence after surgery, and pouchitis in UC.

Association studies, such as the longitudinal analyses proposed above, would greatly inform other essential studies, including determination of mechanisms that drive barrier loss, such as ER stress, hypoxia, and physiological or pathophysiological stimuli[129–131] and developing approaches to restore epithelial function. Such data could ultimately enable personalized approaches that determine which patients are likely to benefit from therapies that reduce permeability of tight junction pore[109] or leak[105] pathways, limit mucosal damage[34,110,132–136], or augment repair[117,137,138].

Overall, there is a critical need to build on and translate existing data to develop a deeper understanding of epithelial dysfunction in IBD. Beyond general descriptions of changes present in preclinical models, this must include detailed analysis of preclinical animal models and patient materials, alongside studies of

mechanism-based interventions in preclinical *in vitro* and *in vivo* models and, ultimately, patients. Such efforts in the near term will make it possible to achieve the long-term goal of developing new therapies and treatment algorithms that improve outcomes in IBD patients and, potentially, prevent disease development in at-risk populations.

Gap 5. **IBD Complications (focus on fibrosis and fistulas) and Extraintestinal Manifestations**

IBD Complications:

There is a need for a deeper understanding of the pathways and mechanisms involved in driving stricturing and penetrating CD and pathogenic remodeling.

Remission

More than half of CD patients will present with intestinal complications over the course of their disease, including the formation of strictures (fibrostenotic disease) and fistulae (penetrating disease). Fibrostenosis, characterized by bowel wall thickening, luminal narrowing and prestenotic dilation, is a frequent complication in patients with CD occurring in 30-50% of CD patients within 10 years of disease onset[139]. Despite the advances in the treatment of CD, current therapies do little to prevent or reverse stricture formation[140]. The prevailing view of pathogenesis of fibrostenotic CD is that chronic inflammation and prolonged mucosal injury drives an aberrant repair response, resulting in the accumulation of intestinal myofibroblasts within the bowel wall and increased extracellular matrix (ECM) deposition and organ dysfunction. While aspects of this model may hold true, it does not account for the role of intestinal smooth muscle expansion and newly discovered stromal cell subsets in tissue remodeling. Histological analyses of strictures reveal significant expansion of the smooth muscle near regions with significant ECM deposition[141]. The expansion of smooth muscle is reminiscent of tissue remodeling in vascular and airway diseases. Efforts to assess the impact of conserved mechanisms within smooth muscle that regulate growth and phenotype (i.e., contractile vs. synthetic) should be prioritized to identify new targets to address its expansion in fibrostenotic CD.

Advances in single-cell characterization have demonstrated that the cells of the intestinal stromal compartment demonstrate substantial heterogeneity[6,14]. Furthermore, these cell subsets exhibit significant transcriptomic changes in response to inflammation[6,14] and in patients with fibrostenotic disease[13]. As a call to action, there is a need to characterize these stromal cell subsets and understand their respective roles in health and disease to identify new targets to dampen tissue remodeling processes in IBD.

In addition to the extracellular matrix (ECM)-producing properties of stromal cells, continued efforts are required to understand their role in inflammation. Intestinal myofibroblasts can be activated by a variety of inflammatory mediators and express several innate immune receptors, including toll-like receptors[142] and the NOD-like receptors NOD1[142], NOD2[142], and NLRP3[143]. *NOD2* variants are associated with increased risk of developing fibrostenotic CD[144], however our understanding of its role in stromal cells is very limited. Recently, *NOD2* risk alleles were shown to contribute to aberrant CD14+-derived fibrocyte (circulating fibroblast-like cells) function, wherein accumulation of risk alleles was associated with increased expression of an activated fibroblast gene signature in the context of muramyl dipeptide stimulation[145]. These findings highlight the need for continued study into the complex interplay between immune signaling pathways and fibrogenesis in stromal cells.

Significantly less is known about the pathogenesis of penetrating disease. Internal and perianal fistulae occur in 30-50% of CD patients. These abnormal passages from the intestine to other regions are often lined with mesenchymal-like cells, in place of a typical epithelial layer[146]. These cells, termed 'transitional cells", are thought to arise from the induction of epithelial-to-mesenchymal transition (EMT), in the context of aberrant epithelial repair processes[146]. The inflammatory milieu of fistulae consists of TGF-β1/TGF-β2[146], TNF[147,148] and IL-13[149] all of which can induce genes related to EMT. In

addition to inflammation, succinate, a microbe-derived metabolite, may contribute to the induction of EMT and fistulae formation[150]. Tissue succinate levels and SUCNR1 (succinate receptor) were elevated in patients with penetrating disease, compared to samples isolated from fibrostenotic disease and non-IBD controls. Furthermore, the authors demonstrated that succinate could trigger EMT through activation of the Wnt pathway[150]. However, it is still unclear how succinate and tissue inflammation contribute to localized EMT and fistula formation. Expanding our understanding of the pathogenesis of fistulae has been significantly hindered by a lack of experimental models and the challenges associated with obtaining samples for primary cell isolation. *In vitro* models can be used to study the mechanism(s) involved in the induction of EMT, however this is likely one step in the disease process. To address this gap, models that incorporate primary cells (e.g., IECs, stromal cells, immune cells) in co-culture (e.g., intestinal organoids; gut-chip) along with conditions that mimic the inflammatory and microbial milieu of CD-associated fistulae are urgently needed. In this regard, an important advancement was the development of a personalized intestinal fibrosis model using human intestinal organoids derived from induced pluripotent stem cells that contains mesenchymal and epithelial cells[151]. Additional improvements are required to optimize the system for use in therapeutic discovery and furthering our understanding of cell-cell interactions in the context of fibrosis.

Extraintestinal Manifestations:

CD and UC are systemic diseases and can affect organ systems beyond the gastrointestinal tract. These extraintestinal manifestations (EIMs) of IBD can significantly impact patient health related quality-of-life and may carry significant morbidity. Commonly affected organs include the skin, joints, and eyes, as well as the liver, lung, and pancreas. While some EIMs improve with the treatment of intestinal inflammation (erythema nodosum [EN], peripheral arthritis, episcleritis, and oral ulcers), some persist or progress independently (e.g., ankylosing spondylitis [AS], primary sclerosing cholangitis (PSC), and uveitis)[152].

A panel of experts recently defined EIMs as "an inflammatory pathology in a patient with IBD that is located outside the gut and for which the pathogenesis is either dependent on extension/translocation of immune responses from the intestine, or is an independent inflammatory event perpetuated by IBD or that shares a common environmental or genetic predisposition with IBD."[153] Several important knowledge gaps remain in the mechanisms of EIMs that could lead to improved IBD patient remission. It is of particular interest to define the mechanisms that drive EIMs that progress despite resolution of intestinal inflammation. Novel discoveries may come from the use of advanced 'omic analyses and single cell resolution spatial technologies. Other guidance may come from deeper interrogation of existing knowledge. For example, IBD genetic risk factors overlap with AS, uveitis, PSC, pyoderma gangrenosum and EN. Emerging evidence links the microbiome and microbial factors with AS, EN and PSC[154]. Some EIMs may respond to TNF-α or JAK inhibition, but not other therapies suggesting ties to these inflammatory pathways. PSC progression on the other hand is not halted by current anti-inflammatory therapies suggesting alternative mechanisms are at play. A few models for EN, spondylarthopathy and PSC have been described[154–157] and more models of EIMs are needed. Finally, further discovery is needed into more recently described IBD EIMs including fatty liver disease and its complications [158,159]. As a call to action, efforts should focus on generating novel or improved preclinical *in vitro* and *in vivo* experimental models that will facilitate testing hypotheses based on analysis of data stemming from expanded cohorts with the future goal of identifying potential drug targets that, when modulated, would eliminate the extraintestinal manifestation.

Conclusions/Summary Statement

The depth, and breadth of preclinical IBD research discovery has rapidly accelerated in the last decade. In parallel, robust systems and resources for clinical-translational validation of preclinical findings continue to develop and are increasingly accessible. This includes the Crohn's & Colitis Foundation's Study of a

Prospective Adult Research Cohort with IBD (SPARC-IBD), a curated database of nearly 7,000 well-characterized (phenotyped) adult patients, with integrated clinical and self-reported outcome data as well as serial biospecimens (tissue, blood, and stool)[160]. Investigators are encouraged to use these resources, to investigate deeply, to collaborate broadly, to work across the gap areas, and integrate/apply discoveries from other inflammatory and neoplastic disease states to IBD. Together, these strategies will propel advancements in the field to advance novel, effective, and targeted interventions for prevention and treatment of IBD.

Summary Figure to be added in final manuscript

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