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Gut virome in inflammatory bowel disease and beyond

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ABSTRACT

Objective The gut virome is a dense community of viruses inhabiting the gastrointestinal tract and an integral part of the microbiota. The virome coexists with the other components of the microbiota and with the host in a dynamic equilibrium, serving as a key contributor to the maintenance of intestinal homeostasis and functions. However, this equilibrium can be interrupted in certain pathological states, including inflammatory bowel disease, causing dysbiosis that may participate in disease pathogenesis. Nevertheless, whether virome dysbiosis is a causal or bystander event requires further clarification.

Design This review seeks to summarise the latest advancements in the study of the gut virome, highlighting its cross-talk with the mucosal microenvironment. It explores how cutting-edge technologies may build upon current knowledge to advance research in this field. An overview of virome transplantation in diseased gastrointestinal tracts is provided along with insights into the development of innovative virome-based therapeutics to improve clinical management.

Results Gut virome dysbiosis, primarily driven by the expansion of *Caudovirales*, has been shown to impact intestinal immunity and barrier functions, influencing overall intestinal homeostasis. Although emerging innovative technologies still need further implementation, they display the unprecedented potential to better characterise virome composition and delineate its role in intestinal diseases.

Conclusions The field of gut virome is progressively expanding, thanks to the advancements of sequencing technologies and bioinformatic pipelines. These have contributed to a better understanding of how virome dysbiosis is linked to intestinal disease pathogenesis and how the modulation of virome composition may help the clinical intervention to ameliorate gut disease management.

KEY MESSAGES

1. Gut virome dysbiosis, primarily driven by an expansion in *Caudovirales* bacteriophages, is associated with the onset of gut pathologies including inflammatory bowel disease (IBD), irritable bowel syndrome, colorectal cancer and *Clostridioides difficile* infection.
2. Studies highlight the impact of gut virome on intestinal immunity and barrier functions, and the evidence keeps growing with advances in genomics, metabolomics, metaproteomics and spatial metatranscriptomics.
3. Large-scale virome databases have expanded the catalog of gut viruses and enabled virome profiling, but are biased in input datasets and lack taxonomic or functional annotations.
4. The integration of spatial transcriptomics with viral metagenomics is a promising method to study the spatial distribution of viruses and virus–host cell interactions along the gastrointestinal tract.
5. Interindividual and methodological variations hinder the generalisability of IBD-related virome biomarkers.
6. Phage therapies, though currently restricted to compassionate use, have demonstrated promising therapeutic potential in *in vivo* animal models and preclinical studies.

INTRODUCTION

The intestine is the body's most extended interface where the majority of the exchanges between the host and the external environment occurs; it is also where the stimulation and development of mucosal immunity take place.[1] The human gut is populated by billions of commensal microbes, collectively known as intestinal microbiota,[2] living in a delicate equilibrium that has to be preserved to ensure proper immune responses and mucosal homoeostasis.[1] Imbalances of this state alter mucosal functions and activate a series of responses, mainly immune, aimed at restoring equilibrium. When immune and non-immune reactions fail to self-resolve, chronic inflammatory disorders ensue.[2]

Inflammatory bowel disease (IBD) is a class of chronic inflammatory disorders including Crohn's disease (CD) and ulcerative colitis (UC), affecting an estimated 4.9 million individuals as of 2019.[3] Although these two forms of chronic intestinal inflammation have overlapping symptoms and proinflammatory signals, they differ in the pattern and extent of inflammation and disease location,[3] as well as the composition of the intestinal microbiota.[2]

Intestinal dysbiosis, an altered composition of the gut commensals, has been previously linked to IBD pathogenesis.[2] However, whether dysbiosis causes, directly or indirectly, or is a consequence of IBD is still contentious. Microbes can directly interact with the host's cells, modulating specific immune pathways and activating both immune and non-immune

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Viruses are associated with gastrointestinal diseases and, together with the other gut commensals, with the maintenance of mucosal homoeostasis. However, their aetiological role is still debated.

WHAT THIS STUDY ADDS

⇒ Recent studies have highlighted a possible causal role of virome components of the microbiota in triggering the disease in *in vivo* models, although clinical trials are lacking. Moreover, the latest advances in computational biology may help to better characterise the virome composition and its spatial organisation within a tissue, eventually uncovering novel virome-triggered or virome-mediated disease mechanisms.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ The full understanding of virome structure in the gut and its exact functions may promote advancements in therapeutic strategies, aimed at the reconstitution of the 'good virome' for amelioration, or even cure, of gastrointestinal diseases.

cells that can participate in IBD pathogenesis. At the same time, changes in the intestinal milieu due to inflammation might alter the composition of its inhabitants, causing dysbiosis.[4]

This conflicting evidence is partly attributed to the sheer complexity of the intestinal microbiota, which is composed of trillions of diverse micro-organisms, including bacteria, archaea, protists, fungi and viruses. While the role of bacteria in health and disease is starting to be characterised, that of other entities, such as viruses, has been neglected until recently. Viruses are 10-fold more abundant than prokaryotes in the gut and have attracted interest mainly because of their ability to interact with both bacteria (bacteriophages) and host cells (eukaryotic-targeting viruses), hence influencing intestinal homoeostasis.

In this review, we aim to summarise the latest evidence on the role of gut virome in gastrointestinal diseases, with a particular focus on IBD, and the novel techniques available to investigate it. We also highlight current technical challenges that are holding back the field and outline the therapeutic potential of targeting the virome.

Intestinal virome in IBD and its impact on the host's physiology

For their evolutionary characteristics, viruses can be classified into bacteriophages and eukaryotic-targeting viruses, both of which can be constituted by single-stranded or double-stranded RNA or DNA genomes.[5] This genomic diversity complicates analysis and since multiple conversion steps are necessary to sequence single-stranded DNA and RNA, these viral populations are often understudied.[6] Classically, bacteriophages can directly shape bacterial composition, while eukaryotic viruses can interact with both the human host's cells and other eukaryotic components of the microbiota, such as fungi.[7] Alterations in gut virome structure have been implicated in the onset and severity of IBD.[8 9] As an example, a previous comprehensive review, while discussing the dynamics of the gut virome and methods to find out the candidate mechanisms underlying disease pathogenesis, has also pointed out the virome dysbiosis as related to IBD development.[10] Specifically, metagenomics analysis showed an increase in tailed bacteriophage (*Caudovirales*) and a decrease in the spherical *Microviridae*. However, given that these findings vary considerably by cohorts and methodologies,[10] the full picture of the virome's role in IBD is still unclear.

The virome can impact the other components of the microbiota, as reported in the study by Norman and colleagues, which described the expansion of *Caudovirales* bacteriophages in IBD compared with controls, coupled with decreased bacterial richness and diversity, two hallmarks of IBD-associated intestinal dysbiosis.[11] Similarly, the dynamics of bacteriophages-bacteria parasitism have been studied in the case of *Faecalibacterium prausnitzii*, a bacterium generally depleted in IBD, whose low abundance in IBD has been associated with a higher prevalence of *F. prausnitzii* phages compared with controls, suggesting enhanced phage-mediated mortality of *F. prausnitzii* in IBD.[12]

Several additional studies and meta-analyses found an increase in *Caudovirales* and a concurrent decrease in overall virome diversity[13 14] in both paediatric ileal CD[15] and very early onset IBD.[16] In addition to bacteriophages such as *Caudovirales*, eukaryotic viruses have also been associated with early stages of intestinal inflammation, suggesting a possible role in the pathogenesis of IBD supported by their ability to interact with the host's cells. In this regard, a higher abundance of the eukaryotic *Orthohepadnaviridae* transcripts was found in early-diagnosed treatment-naive patients with UC, when compared with those with CD and controls. Furthermore, patients with CD showed a higher prevalence of *Hepeviridae* and a lower prevalence of *Virgaviridae*.[17] These findings were based on data derived from mucosal biopsies, highlighting the possibility of a direct interaction of the eukaryotic viruses with the host's immune and non-immune cell functions. Similarly, a study reported a positive association between the eukaryotic *Anelloviridae* prevalence in early-onset patients with IBD along with immunosuppressive treatment.[16] IBD-associated virome dysbiosis is summarised in table 1.

In summary, despite some variability across the studies, virome dysbiosis is associated with IBD pathogenesis. *Caudovirales* are likely to be the most enriched bacteriophages in patients with IBD, which may contribute to bacterial dysbiosis. In parallel, eukaryotic viruses, such as *Hepadnaviridae* and *Hepeviridae*, were found to be associated with early stages of intestinal inflammation, posing the question of whether these can represent a leading cause in IBD pathogenesis as we discuss in the following section.

How the virome may interact with the host's mucosal physiology and health implications

The intestinal mucosa is a multilayered tissue, composed of different cell types that together orchestrate pathways, signal synergistically and interact with each other and with the microbiota to maintain homoeostasis. However, in IBD, this equilibrium is disrupted.[18]

Intestinal viruses were shown to interact with the host's mucosal cells, and in some cases, have been demonstrated to have a detrimental effect on intestinal physiology, where several specialised cells, both immune and non-immune, take part in the homoeostatic regulation. All these cells are essential to protect and defend against external insults.[18]

The first line of defence of the intestine is the epithelium lining the mucosal surface.[19] This is key in maintaining mucosal protection against luminal commensals, and in favouring the exchange of materials with the external environment, which is necessary for the digestion of food, absorbing vitamins, or training our immune system.[19]

Table 1 Viruses involved in IBD.

Virus	Host	Alteration	Potential mechanisms	Clinical significance
Caudovirales	Cyanobacteria, heterotrophic bacteria, archaea	↑ in IBD[10 11 13–16]	Induction of the production of interferon γ through toll-like receptor 9 signalling	Escalating disease severity reduction of Caudovirales diversity, richness and evenness reduced bacteria diversity
Microviridae	Gram-negative bacteria	↓ in IBD[10]	Their loss is probably due to the higher fluid flow or loss of mucosa that do not retain phages	Contribution to bacterial dysbiosis in IBD (expansion of bacterial commensals). Exacerbation of colitis severity in mice
crAss-like phage	*Bacteroidota*	↑ in UC[24]	Impact on bacterial dysbiosis	Exacerbation of colitis severity in mice
Siphoviridae	Bacteria, archaea	↑ in UC[24]	Impact on bacterial dysbiosis	Exacerbation of colitis severity in mice
Podoviridae	Bacteria	↑ in UC[24]	Impact on bacterial dysbiosis	Exacerbation of colitis severity in mice
Faecalibacterium prausnitzii phages	*Faecalibacterium prausnitzii*	↑ in IBD[12]	Enhanced temperate phage-mediated mortality of *F. prausnitzii*	Gut inflammation might increase prophage induction thereby promoting bacterial lysis and aggravating dysbiosis and reinforcing the inflammatory loop
Orthohepadnavirus	Mammals	↑ in UC[4 17]	Disruption of epithelial cells and induction of ulcers	The expansion may be involved in the early cause of UC in a subcohort of UC patients
Hepeviridae	Mammals	↑ in CD[17]	Unknown	The expansion may be involved in the early cause of CD
Virgaviridae	Plant cells	↓ in CD[17]	Unknown	The reduction may be associated with low fibre diet
Enterovirus B	Mammals	↑ in IBD[31]	Unknown	Trigger of proinflammatory response
Picornaviridae	Mammals	↑ in IBD[31]	Unknown	Trigger of proinflammatory response
Anelloviridae	Mammals	↑ in IBD[16]	Unknown	Positive correlation with immunosuppressive treatments Abundance is likely related to reduced immune surveillance

Upwards arrow means increased abundance in disease by comparison with the healthy controls.
Downwards arrow means reduced abundance in disease by comparison with the healthy controls.
CD, Crohn's disease; IBD, inflammatory bowel disease; UC, ulcerative colitis.

The epithelial layer, organised in a very sophisticated manner, prevents excessive contact between immune cells with microorganisms and their antigens, thereby protecting the gut from unwanted immune reactions (figure 1A). This is possible thanks to tight junction proteins connecting a single layer of columnar intestinal epithelial cells (IECs) and other specialised cells such as goblet cells and Paneth cells, which are crucial to the innate immune response.[19] Goblet cells create a thick mucus layer as part of the physical epithelial barrier, preventing direct contact of bacteria with the epithelium.[20] Many commensals live within the mucus layer. However, the mucin protein layer can be disrupted by some parasites, pathogenic bacteria or even dietary emulsifiers,[21] or by several other mechanisms, including aberrations in the immune system and impairment of the epithelial cell-forming barrier.[22] When intestinal commensals reach the epithelial surface or cross it, the immune system is activated, triggering the canonical series of events at the basis of IBD pathogenesis[4]

Viruses contribute to barrier damage in various ways. Phages indirectly fuel the immune response through the release of bacterial products following bacterial lysis or translocation across the epithelium (transcytosis), activating pattern recognition receptors on the IECs or resident immune cells[23] (figure 1B).

In addition, specific virome-derived factors can interfere with barrier integrity and impact intestinal physiology, often through interaction with the other commensals. As an example, Sinha *et al* pooled viral-like particles (VLPs) from three UC patients, mainly enriched in *Microviridae* phages and, to a lesser extent in *crAss-like*, *Siphoviridae* and *Podoviridae* phages, and transplanted them into human microbiota-associated mice. UC VLP transplantation exacerbated colitis severity[24]

Another recent study showed that the gut-virome colonising *Orthohepadnavirus* genus was associated with UC pathogenesis in both paediatric and adult patients.[4 17] In particular, the viral protein Hepatitis B X (HBx) disrupts the epithelial barrier in the intestine by shaping epithelial cell functions toward a dedifferentiated state, eventually leading to alteration of the immune milieu and intestinal inflammation in vivo[4] (figure 1C). As per the literature, the primary site of *Orthohepadnavirus* infection is the liver, where this genus of virus replicates and induces an immune response, leading to chronic inflammation and cancer.[25] However, non-human hepatitis B virus (HBV) lymphotropism and its ability to use lymphoid cells as extrahepatic reservoirs have been reported in lymphoid tissues, including spleen and lymph nodes.[26–28] This supports the concept that the virome is made up of a large plethora of entities not necessarily colonising their preferential tissues but residing on the mucosal surfaces while stimulating tissue immunity, even in the absence of a canonical infection cycle.[7 29] As an example, Massimino *et al* reported HBx positivity in a subcohort of UC patients independently of HBV chronic or acute infections, suggesting that HBx presence in their intestines can be the result of environmental exposure to waters contaminated with non-human infecting *Orthohepadnavirus*.[4] This may represent one of the environmental factors predisposing to UC development.[30]

Similarly, Adiliaghdam *et al* found that the healthy virome directly elicited atypical anti-inflammatory innate immune activity, while viromes isolated from UC and CD, mainly enriched in *Picornaviridae* and *Enterovirus B*, induced inflammation, successfully reverted by non-IBD viromes.[31]

Other studies pointed out the impact of viruses on innate immunity. For instance, filamentous Pf bacteriophages produced from *Pseudomonas aeruginosa* are internalised by dendritic cells (DCs), macrophages and B-cells to induce type-I interferon responses, thereby facilitating infection by related bacteria.[32]

Interestingly, a recent study reported that viral infections with enteric viruses promoted the expansion of some specific immune cell populations in the intestine, such as colonic and small intestinal lamina propria leucocytes, including effector memory T cells, macrophages and plasmacytoid DCs.[33]

Despite these pieces of evidence, the investigation of the virome's impact on intestinal immunity and barrier functions is still in its infancy. Apart from the characterisation of virome dysbiosis in IBD, studies describing virome-induced pathogenic

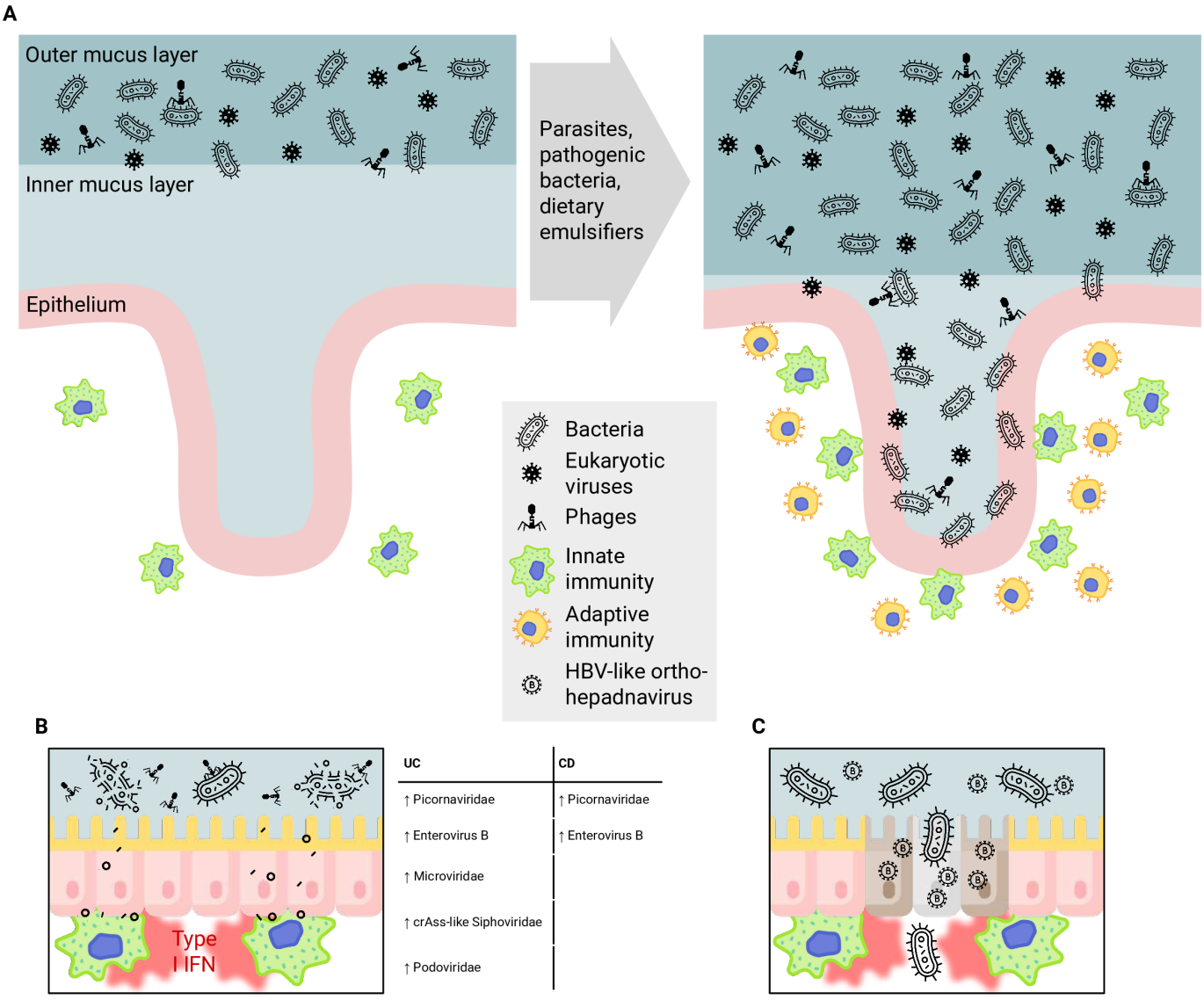

Figure 1 (A) Environmental effects on mucus barrier function. The healthy colon features a layer of mucus that separates the microbiota and its products from the epithelium (left). (B, C) On infection by parasites, pathogenic bacteria or viruses (B), or orthohepadnavirus-derived factors (C) results in enhanced microbial penetration eventually triggering the activation of both innate and adaptive immune systems. HBV, hepatitis B virus.

events in the intestinal mucosa are still limited. However, compelling, these studies need further validation, particularly because interindividual and intercohort variabilities limit the generalisability of their results.

New technologies for studying the gut virome in the intestine

There are conflicting findings on overall virome richness in IBD and inconsistent bacteriophage and eukaryotic viral biomarkers.[11 13 16 34] These inconsistencies could be attributed to cohort variation and database-dependent profiling methods, which may introduce biases or inaccurate taxonomic assignments.[35] Clooney *et al*, who adopted a database-independent method for profiling, did not find significant changes in overall virome richness, and only observed significant increases in *Caudovirales* richness in CD compared with controls.[36] However, numerous virome studies included a multiple displacement amplification (MDA) step before sequencing,[11 16 36] which may favour the amplification of small, circular and single-stranded DNA viruses,[37 38] although introducing the undesired amplification bias.[39] To address this, Stockdale *et al* omitted the MDA step and compared results to matched 16S sequencing analyses of the same cohort.[38] They found that interindividual variation was increased using unamplified viromes, causing differences in gut virome richness between IBD and controls to be less pronounced compared with 16S sequencing. By contrast, the unamplified approach showed the opposite, with the healthy gut virome enriched in viruses compared with the IBD viromes. Thus, these findings raise the need to identify and characterise commensal viruses shared in populations. Given the high interindividual variation of viromes, these shared communal viruses likely vary by geographical regions and disease states.

By harnessing the potential of molecular biology, metabolomics and metaproteomics, researchers have employed mass spectrometry to analyse the proteins and metabolites in the microbial environment and their interactions with the host. This comprehensive understanding of biochemical processes has shed light on the underlying mechanisms of certain human diseases.[40] Additionally, it is essential to profile transcriptomics, metatranscriptomics and biological processes through functional enrichment methods.[41] Metatranscriptomics provides valuable insights

into the active micro-organisms present in a specific environment and their ability to encode specific gene products. Since gene expression plays a significant role in phenotypic expression, investigating differences in gene expression between various conditions, including the presence and absence of a disease, can aid in comprehending the molecular mechanisms responsible for human disorders.

Today, metagenomics and metatranscriptomic methods predominantly rely on Illumina sequencing technologies, such as HiSeq or NovaSeq, which offer high throughput and cost-effectiveness.[42] For gene expression analysis, one of the most widely used tools is DESeq2.[14] To classify taxonomic information from microbial reads obtained through sequencing, tools like Kraken2 are crucial.[14] However, in microbiota gene expression analysis, the annotated genes' structural and functional complexity can be overwhelming, necessitating the use of tools like Cd-hit[43] and DeepNOG to reduce structural and functional redundancy in the identified gene set.[44] Such reductions help in lowering computational expenses, making it more feasible to use protein structure prediction tools like AlphaFold, which provides insights into protein structure and reactivity,[45] further aiding in understanding their function.

Recently, single-virus sequencing has emerged as a promising tool for uncovering the diversity and abundance of viruses[46 47] and for the identification of host–phage interactions.[48] Coupled with third-generation long-read sequencing,[49] single-virus sequencing may serve as a powerful framework for the accurate recovery of viral genomes and high-throughput cataloguing of host–phage interactions. Both will facilitate the formation of phage cocktails targeting IBD-related bacteria.

Virome organisation with spatial transcriptomics: an innovative technology for investigating host–microbe interaction

Spatial transcriptomics is a cutting-edge technology that combines traditional gene expression analysis with spatial information.[50] It enables analysing gene expression patterns within tissues while maintaining their spatial context, providing a deeper understanding of how genes are regulated and interact within specific regions of an organ or tissue. In the gut, spatial transcriptomics typically involves RNA sequencing of spatially barcoded tissue sections or laser capture microdissected tissues.[50] In combination with single-cell RNA sequencing, these spatial sequencing approaches allow for the construction of spatial transcriptional maps at almost single-cell resolution.

Lötstedt *et al* presented spatial host-microbiome sequencing, an all-sequencing-based approach that captures tissue histology, polyadenylated RNAs, and bacterial 16S sequences directly from tissues on spatially barcoded glass.[51] Application of this approach, coupled with deep-learning-based data mapping, revealed spatial niches that were impacted by microbial biogeography in mouse gut.[51]

In the human context, Niño *et al* applied *in situ* spatial profiling technologies and single-cell RNA sequencing to oral squamous cell carcinoma and colorectal cancer (CRC) to reveal spatial, cellular and molecular host–microbe interactions.[52] They showed that instead of being randomly distributed, microbiota within a tumour is highly organised in micro niches with immune and epithelial cell functions that promote cancer progression.

Looking to the future, spatial transcriptomics holds significant potential for studying the intestinal virome. By integrating spatial transcriptomics with viral metagenomics/metatranscriptomics, researchers may be able to investigate the spatial distribution of viruses within the intestinal tissue and examine their interactions with the host cells. This approach could help identify specific viral species, characterise their gene expression patterns and explore their potential roles in health and disease. However, efforts are needed to develop, optimise and standardise this approach.

VIROME DATABASES

Several recently published studies have used bulk and/or VLP-enriched metagenomes to catalogue their diversity, identifying thousands to hundreds of thousands of novel viruses, and vastly expanding the catalogue of known human gut viruses.

From 5742 gut metagenomic assemblies, Benler *et al* identified 3738 putative phage genomes, which were further dereplicated into 1886 genomes (at 95% average nucleotide identity, ANI).[53] However, around 69% of these genomes could not be assigned a viral family approved by the International Committee on the Taxonomy of Viruses, and 71% did not have a predicted host.[53] Similarly, using bulk metagenomes (n=11 810), Nayfach *et al* identified nearly 190 000 viral draft genomes that represent 54 118 viral species genomes (clustered at 95% ANI and 85% alignment fraction (AF)) to form the Metagenomic Gut Virus catalogue.[54] Of these viral genomes, 53% did not cluster with any then-known viral genomes, 43% could not be assigned a family-level taxonomy and 10% did not have a predicted host.[54] In the largest study to date, Camarillo-Guerrero *et al* analysed over 28 000 bulk gut metagenomes and 2898 gut bacterial reference genomes to form the Gut Phage Database, consisting of 142 809 non-redundant phage genomes (95% ANI and 75% AF).[8] Similarly, a majority (71.3%) of these phages could not be linked to a host. The authors further clustered these phage genomes with those in existing databases (90% ANI, 75% AF) to generate 21 012 non-singleton viral clusters with at least one genome from the GDP, 80% of which could not be assigned a viral family.[8] In addition, using 2697 bulk (48%) and VLP-enriched metagenomes (52%), Gregory *et al* created the human Gut Virome Database (GVD) of 33 242 viral species genomes (95% ANI), with <15% assigned a family-level taxonomy and 56% having a host identified.[55] Furthermore, Zhao *et al* applied ultra-deep third-generation and next-generation sequencing to a faecal DNA sample with VLP enrichment (collected from a middle-aged woman) and obtained 1058 novel gut viral genomes, including 13 long ones.[56] These new genomes improved viral profiling, and a combination of 14 of them differentiated patients with CRC from controls with an AUROC of 0.85 and 0.73 in independent cohorts.[56] Several other studies have constructed virome databases from the metagenomes or metatranscriptomes of multiple human body sites[55 57 58] or of all sources—mainly environments[59]—and reported a great amount of previously uncharted genetic material therein. These studies highlight the immense unexplored viral diversity in the human gut and facilitate the characterisation and analysis of gut virome without *de novo* assembly.

However, studying the role of the gut virome in IBD remains challenging. First, there is biased geographical coverage of sampling. Although more prevalent in European and American populations, IBD cases have accumulated most rapidly in Asia and Africa over the past two decades.[60 61] Yet, only one-third of the samples used in database creations were rooted in Asia or Africa.[62] Greater efforts are needed to improve the representativeness of the viral diversity for these populations. Second, documented gut viral populations are biased toward DNA viruses. While a few databases cataloguing RNA viromes are

available,[63–65] only a handful of metatranscriptomes collected from the human gut have been included. By contrast, the DNA virome databases, except GVD, were sourced primarily or exclusively from bulk metagenomes.[55] Given that bulk and VLP-enriched metagenomes likely capture different portions of the viral population,[55] the generation of more VLP-enriched datasets would allow for comprehensive profiling. Furthermore, the assembly of complete viral genomes from shotgun metagenomic sequencing data is challenging due to genomic mosaicism. This may be circumvented by applications of single-virus sequencing paired with long-read sequencing. Given various bioinformatic tools, a robust and standardised analytical workflow, such as the one proposed by Li *et al*, should also be used to improve *in silico* annotations, reduce cross-study heterogeneity and enable meta-analysis.[62] Finally, being genetically distinct from known viruses, most metagenome-derived viral genomes lack taxonomic and functional annotations. Isolation and characterisation of phages are therefore necessary to further characterise the viral 'dark matter' and determine their links to biological functions and diseases.

In summary, existing GVDs have immense viral genomic diversity but are biased towards bulk metagenomic data and have incomplete annotations. Improvements in sampling, extraction of genetic materials, phenotyping and database annotation will be necessary to unravel the complexity of the human gut virome, potentially revealing novel avenues for screening, diagnosing, preventing and treating human diseases including IBD.

The role of the virome in other intestinal pathologies

Clostridioides difficile infection

The main alterations of the gut virome observed in IBD have also been found in the context of *Clostridioides* (formerly *Clostridium*) *difficile* infection. In particular, patients with *C. difficile* infection have a higher abundance of bacteriophage *Caudovirales* compared with healthy controls while featuring reduced overall diversity.[9] At the same time, the success of faecal microbiota transplant, an approved treatment for recurrent *C. difficile* infection, has been associated with the colonisation of donor *Caudovirales* and restoration of microbial diversity.[9] These observations support the importance of phages in the bidirectional regulation of gut homoeostasis.

Colorectal cancer

CRC is the third most commonly diagnosed cancer and the second-leading cause of cancer deaths worldwide. Aetiogenic factors underlying CRC pathogenesis are multiple, both genetics and environmental, and are still partially unknown.[66]

Similarly to IBD, CRC is also associated with microbiota dysbiosis, including that of the virome, whose direct and indirect links with CRC have been proposed only recently.[67] At least one-sixth of the entire global cancer burden has been attributed to viral infections including Epstein-Barr virus (EBV), human papillomavirus (HPV), human Kaposi sarcoma virus, HBV, hepatitis C virus (HCV), human immunodeficiency virus (HIV), human T-cell lymphotropic virus genotype 1 and Merkel Cell Polyomavirus (MCPyV or MCV).[68] Although mainly associated with other malignancies, all these eukaryotic viruses have been associated also with CRC risk and pathogenesis. However, evidence of causality between viral infections and CRC is still lacking.[66]

Community-based viral shotgun NGS techniques have revealed alterations in the colon virome diversity in patients with CRC. Nakatsu *et al* identified *Orthobunyavirus*, *Tunalikevirus*, *Phikzlikevirus* and 19 other viral genera that discriminate patients with CRC from controls, with the evidence that different virome signatures correlate with the cancer stage and that specific virome-associated risk groups had independent prognostic significance.[69]

In another study, while the overall alpha diversity (richness and Shannon diversity) and beta diversity (Bray-Curtis dissimilarity) were similar between healthy subjects and patients with cancer, specific colonic virus communities were associated with CRC and altered the overall bacterial composition of the gut,[70] supporting the predator–prey relationship.[11]

While there have been some successes in predicting CRC from viral alterations, mechanistic links between the altered gut virome and CRC onset and/or progression have yet to be elucidated. Virome–CRC associations could potentially involve host bacteria. Among the aforementioned meta-analyses, two found increased abundances of *F. nucleatum* or *Fusobacterium*-related phages in CRC.[71 72] Given that the phages' potential host *F. nucleatum* is enriched in CRC across different cohorts, these phages may simply 'piggyBack' *F. nucleatum*. Regardless, *Enterobacteriaceae*-targeting phages were also found to be enriched in CRC.[72 73] These phages may kill commensals such as *Escherichia coli*, resulting in dysbiosis and favouring the colonisation of CRC-promoting pathogens.[74] Phages that have highly antigenic outer capsid proteins may also contribute to CRC. The capsid immunoglobulin-like domains allow them to adhere to mucins and form an antibacterial layer.[75] Since cancer cells have altered glycosylation, it is possible that their interactions with these phages are altered and their defence against pathogens weakened.

In addition to shaping the bacterial community, bacteriophages have also been shown to transfer directly into colonic epithelial cells, promoting tumour growth and invasiveness in CRC. For example, bacteriophages from the order *Caudovirales* have been observed to directly interact with human cells, cross epithelial barriers, and produce proinflammatory responses.[76–78]

An altered virome could also affect the risk of CRC independently of bacteria. Zuo *et al* reported that patients with CRC had elevated pathways for fatty acid biosynthesis and depleted production of chemicals in their gut virome that inhibit CRC cell proliferation (L-methionine) or maintain homoeostasis (acetate).[73] These findings suggest that an altered virome may also have a direct role in oncogenesis and/or tumour progression, but more mechanistic studies are needed to establish its validity.

However, the complex relationships between the gut virome, the host and gut bacteria, as well as the intricate interplay between the metabolic and immunological pathways, are not yet fully understood. Novel insights into these areas are crucial for developing accurate diagnostics and efficacious precision therapeutics.[79 80]

CRC-associated virome dysbiosis is summarised in table 2.

Irritable bowel syndrome

Irritable bowel syndrome (IBS) is a spectrum of gastrointestinal disorders characterised by chronic abdominal pain, bloating and altered bowel habits, further subdivided into constipation-prevalent (IBS-C), diarrhoea-prevalent (IBS-D) and mixed (IBS-M). Despite the high prevalence, the pathogenesis of IBS remains elusive the absence of macroscopic alterations has suggested that less evident mechanisms, such as dysbiosis, could be key.

The concept of the human 'virotype'[29] or that the virome regulates transcription in asymptomatic hosts depending on host genetics is particularly intriguing in the setting of IBS. However,

Table 2 Viruses involved in IBS.

Virus	Host	Alteration	Potential mechanisms	Clinical significance
Microviridae, Myoviridae, Podoviridae	Bacteria, archaea	↑ in IBS-D[84]	Unknown	Contribution to IBS bacterial dysbiosis
Microviridae, Myoviridae	Bacteria, archaea	↑ in IBS-C[84]	Unknown	Contribution to IBS bacterial dysbiosis
Lactobacillus virus LBR4 8	*Lactobacillus brevis*	↑ in IBS-C[85]	Reduction of the beneficial *L. brevis* activity	Contribution to IBS bacterial dysbiosis

Upwards arrow means increased abundance in disease by comparison with the healthy controls.
Downwards arrow means reduced abundance in disease by comparison with the healthy controls.
IBS, irritable bowel syndrome; IBS-C, IBS-constipation; IBS-D, IBS-diarrhoea.

despite the elegance of the hypothesis, the evidence remains limited.

Data on gut virome in IBS are very heterogeneous, possibly reflecting the broad spectrum of IBS itself. Overall, most studies point to a reduced alpha diversity of both bacterial-targeting[81] and eukaryotic-targeting viruses,[82] two observations that are broadly consistent with what is known of the bacterial microbiome in IBS. Interestingly, unlike the bacterial microbiome, the virome seems to be temporally stable and independent of IBS symptom flare.[83 84]

No clear viral pathogenic family or species has been identified, although the comparison between IBS subtypes and healthy controls has identified phages, such as the *Microviridae*, *Myoviridae* and *Podoviridae* families, increased in IBS-D, and other *Microviridae* and *Myoviridae* species elevated in IBS-C, as compared with healthy controls[84] (table 3). Predictably, some differentially abundant viruses in IBS and controls have been found to co-vary or inversely vary with bacteria and metabolites, which, in turn, have been associated with positive or negative effects. In fact, most of the supposed clinical implications of these differences are mediated by the impact of viruses on the bacterial microbiome. For example, *Lactobacillus* virus LBR4 8, a phage that infects *Lactobacillus brevis*, commonly considered a probiotic, was increased in IBS-C patients, possibly interfering with *L. brevis* activity.[85]

THERAPEUTIC POTENTIALS OF VIROME: A SUMMARY OF THE ONGOING CLINICAL TRIALS

Given the role of gut virome in modulating the bacteriome, host immunity and consequently gut pathologies, there is a growing research interest in virome and phage-based therapies. Although clinical trials involving these treatments are in their infancy, preclinical studies on their viability are growing at a rapid pace.

Table 3 Viruses involved in CRC.

Virus	Host	Alteration	Potential mechanisms	Clinical significance
Autographiviridae	Bacteria	↑ in CRC[72]	Unknown	Enriched in patients with CRC
Siphoviridae	Bacteria, Archaea	↑ in CRC[73]	Impact on bacterial dysbiosis	Enriched in patients with CRC
Gratiaviridae	Bacteria	↑ in CRC[72]	Unknown	Enriched in patients with CRC
Drexlerviridae	Bacteria	↑ in CRC[73]	Unknown	Enriched in patients with CRC
Inoviridae	Bacteria	↑ in CRC[73]	Unknown	Enriched in patients with CRC
Herelleviridae	Bacteria	↓ in CRC[73]	Typically infecting members of the *Firmicutes* phylum	Therapeutic potential for gastrointestinal infection
Podoviridae	Bacteria	↑ in CRC[73]	Impact on bacterial dysbiosis	Enriched in patients with CRC
Myoviridae	Bacteria, Archaea	↑ in CRC[73]	Unknown	Enriched in patients with CRC
Fusobacterium nucleatum phage	*Fusobacterium nucleatum*	↑ in CRC[71]	FadA promotes cancer cell proliferation through the Wnt signalling	Potential CRC biomarker
Parvimonas micra phage	*Parvimonas micra*	↑ in CRC[71]	Unknown	Potential CRC biomarker
Peptacetobacter hiranonis phage	*Peptacetobacter hiranonis*	↑ in CRC[71]	Unknown	Potential CRC biomarker
Orthobunyavirus	Mammals	↑ in CRC[69]	Unknown	Enriched in patients with CRC
Tunalikevirus	Gram-negative bacteria	↑ in CRC[69]	Capability of infecting commensals or lysing enteropathogenic strains of *Escherichia coli*	Enriched in patients with CRC
Phikzlikevirus	Bacteria	↑ in CRC[69]	Unknown	Enriched in patients with CRC
Inovirus	Bacteria	↑ in CRC[69]	Unknown	Enriched in patients with CRC
L5likevirus	Bacteria	↑ in CRC[69]	Unknown	Enriched in patients with CRC
Betabaculovirus	Arthropods	↑ in CRC[69]	Unknown	Enriched in patients with CRC
Sp6likevirus	Bacteria	↑ in CRC[69]	Unknown	Enriched in patients with CRC
Enterobacteria phage	Bacteria	↑ in CRC[74]	Impact on bacterial dysbiosis	Enriched in patients with CRC
Epstein-Barr virus	Mammals	↑ in CRC[66]	Induction of intestinal damage and inflammation	A possible risk factor for CRC
Human papilloma virus	Mammals	↑ in CRC[66]	E5, E6 and E7 oncoproteins increase cellular alteration	A possible risk factor for CRC
Hepatitis B virus (HBV)	Mammals	↑ in CRC[66]	HBx interferes with p53 with it likely inducing malignant transformation	Chronic HBV infection is a risk factor for CRC
Merkel Cell Polyomavirus	Mammals	↑ in CRC[66]	T-antigen-mediated inactivation of p53 and pRB	A possible risk factor for CRC

Upwards arrow means increased abundance in disease by comparison with the healthy controls.
Downwards arrow means reduced abundance in disease by comparison with the healthy controls.
CRC, colorectal cancer; FadA, fusobacterial adhesin; p53, Tumor protein P53; pRB, retinoblastoma protein; Wnt, Wingless/Integrated.

Faecal virome transplants

By contrast to traditional faecal microbiota transplantation (FMT), faecal virome transplantation (FVT) involves the transplantation of only gut viruses from healthy donors into diseased patients. Most FVT studies were conducted via *in vitro* mouse models of diseases without clear biomarkers, such as obesity[86] and antibiotic-mediated dysbiosis.[87] FVT significantly altered overall bacteriome compositions in terms of *Firmicutes–Bacteroidetes* ratios, diversity,[86 87] and individual bacterial abundances, although the latter accounted for only a small proportion of the bacteriome.[88]

Clinical trials of FVT in IBD are limited. In one study by Ott *et al*, FVT preparations were sterile filtered and transplanted into five patients with *C. difficile* infection,[89] including three that failed FMT and/or antibiotics and one that could not receive FMT due to infectious risk. All five patients recovered from *C. difficile* infection after FVT and remained symptom-free for at least 6 months. Virome analysis, performed only on one patient, revealed that the patient's phageome had changed significantly to resemble that of the donor.[89] Unfortunately, given the nature of the study focused on the efficacy of faecal filtrates rather than the virome specifically, and the limited sample size, no causal links between the virome and patient recovery could be established, nor specific beneficial phages identified.

FVT is advantageous over FMT since it decreases the risk of transferring unknown pathogens or bacteria with undesirable functionalities. Nevertheless, despite the immediate positive outcomes of FVT, its long-term efficacy and effects on the bacteriome are unclear. These studies did not identify the particular taxa, transplanted or displaced, contributing to the improvement in obesity and IBD symptoms. Thus, while studies have demonstrated the virome's therapeutic potential, progress in developing virome-based therapies can only be made if there is a better understanding of the taxa and mechanisms by which viruses affect host metabolism, and, in turn, contribute to both diseased and healthy gut states.[24 88]

Safety concerns regarding FVT have also been raised, including the potential transfer of eukaryotic viruses and prophage-encoded virulence factors. Thus, similar to FMT, FVT must develop donor assessment criteria and genetic screens to remove potential viral hazards of these sorts. In addition, healthy 'super donors' may be identified to provide qualified transplants. With advancements in phage isolation and characterisation, phage cocktails may also be created *ad hoc* to substitute whole-virome transplants (figure 2). Overall, despite promising case series, studies on FVT are poorly generalisable because of the small sample size and phageome interindividual variability, and thus further research is needed.

Phage therapy

The therapeutic use of bacteriophages has been primarily investigated as an alternative to antibiotics for multidrug-resistant bacteria. In the context of gut diseases, 'cocktails' of bacteriophages known as phage therapy could be beneficial for conditions associated with certain bacteria colonisation or infection such as IBD with Adherent-invasive *E. coli*, *Klebsiella pneumoniae*, *C. difficile*[90 91] and CRC with *Fusobacterium nucleatum*.[92]

Compared with faecal transplants or antibiotics, phage therapies are advantageous in that they allow them to target specific commensals, including drug-resistant ones, limiting unintended alterations in gut microbiota without transferring live bacteria.

In murine models of IBD artificially colonised with IBD-related pathobionts, several phage cocktails have been tested. Results have been mainly positive in terms of target bacteria eradication, though clinical effects on disease activity beyond the resolution of the infection are difficult to infer. In addition to the animal model limitations, most studies on phage therapy in IBD targeted a single bacteria strain, an approach far too reductionist considering the microbial complexity of IBD.[93]

Besides IBD, phage therapies have also shown promising results for treating CRC. In a study by Dong *et al*, immunogenic M13 phages were engineered to target *F. nucleatum* (Fn) and were assembled with antibacterial silver nanoparticles.[79] The Fn-specific phages were administered in CRC mice models, in which the phages not only cleared Fn from the gut but also infiltrated CRC tissues via Fn-targeting. Given that M13 phages are immunogenic, phage entry into CRC tissues also facilitated leucocyte activation, undoing the tumour's immunosuppressive characteristics. The treatment delayed CRC tumourigenesis and extended the mice's survival time. However, phage immunotherapy alone was insufficient in removing tumours and all mice died after 23 days. Interestingly, M13 phages have also been engineered to target tumourous antigens, including epidermal growth factor (EGF) and carcinoembryonic antigen.[94]

Although preclinical animal model studies on phage therapies have shown promising results, there are several safety and regulatory concerns to address before extensive clinical applications (figure 2). Multiple early-phase clinical trials have reported that oral administration of phages is well tolerated in humans,[95–97] but these phages were administered in the context of a healthy gut. During inflammation, bacteriophage-induced lysis of pathogens may release pathogen-associated molecular patterns in the gut, potentially worsening inflammatory responses.[19 31] For example, phage therapy worsened the symptoms of one patient suffering from a urinary tract infection, as phage-induced lysis of pathogenic *P. aeruginosa* may have released endotoxins into the patient's gut.[98] Moreover, phage virions may directly interact with host leucocytes and stimulate cytokine production, whose consequences are not fully understood in the context of gut inflammation and dysbiosis.[24 99] Though phage-induced adverse events are rare, further studies are needed to identify the conditions by which they occur. Lastly, regulations for phage therapies have yet to be fully established.[100] As a result, most applications of phage therapies are constrained to compassionate use.

CONCLUDING REMARKS

Progress in sequencing technologies and GVDs have advanced our understanding of the gut virome and particularly its role and therapeutic potential in IBD, IBS, *C. difficile* infection and CRC. However, variations in sequencing and analytical methods, incompleteness of database annotations and substantial interindividual and intercohort differences limit the interpretation and generalisation of the findings. Preliminary preclinical and clinical studies have demonstrated the potential for phage therapy in the treatment of IBD and CRC, but further research is certainly needed.

To make valuable advancements in the field, experts should agree on some guidelines and delineate a consensus to conduct virome-based, disease-oriented research, thereby standardised protocols will generate reproducible data and build robust knowledge. For example, key opinion leaders, including clinicians, virologists and biologists, could collaborate to express a consensus where sequencing methods and their analysis are defined as the gold standard for virome-related studies. They

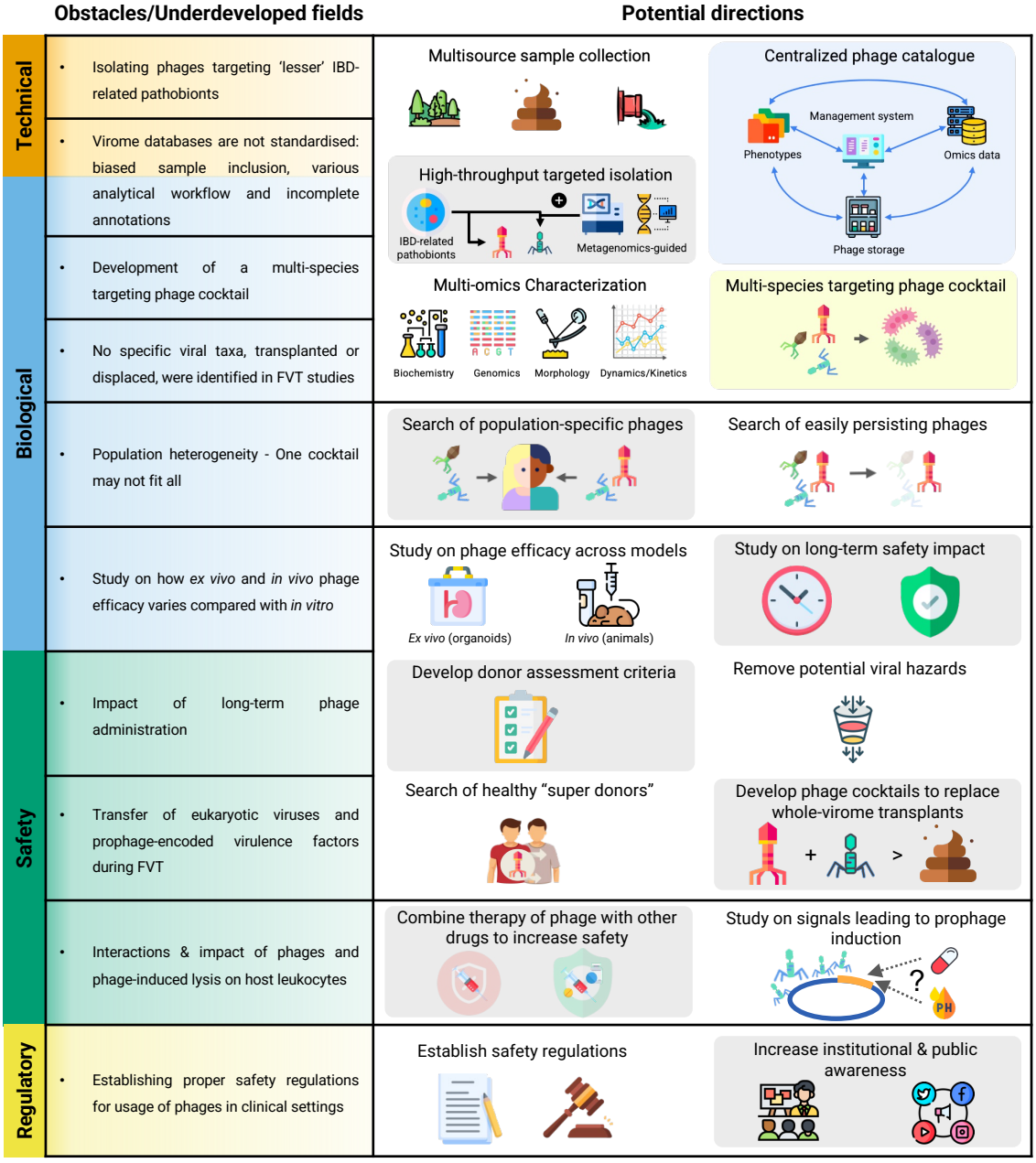

Figure 2 Challenges and potential future directions in establishing phage therapies as a widely accepted approach for treating IBD-related pathobionts. Obstacles and underdeveloped fields are summarised into technical, biological, safety and regulatory categories. Future directions to explore are organised according to their potential to tackle the challenges. FVT, faecal virome transplantation; IBD, inflammatory bowel disease.

should contribute to compiling complete database annotations to make a straightforward interpretation of analysis results. Moreover, large sample-sized cohorts are highly desirable to overcome the intraindividual and interindividual variability, this being a limitation for all microbiota-related research. Patient stratification based on geographical origin, sex, clinical characteristics and daily habits has to be considered a must when a virome-based study is designed since all these traits represent some of the main causes of the high variability and study inconsistency.

Regarding the clinical applications of virome therapy, side effects, although rare, have been registered as discussed in this review. To avoid immune reaction during the virome transplantation, a possibility would be to identify the specific bacteriophage defined as detrimental and to inactivate it before the administration in affected individuals. This could ameliorate tolerance and avoid side effects.

Conclusively, despite the extensive research required, the field is rapidly advancing and shows promising therapeutic potential by improving our understanding of the role of gut virome in IBD and other gastrointestinal diseases. Nevertheless, joint efforts among clinicians, microbiologists, bioinformaticians and regulatory bodies are urgently needed to overcome virome research-related limitations and make important advances in the field, hopefully in the short term.

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