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Forum

The gut microbiome molecular complex in human health and disease

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The human gut microbiome produces a functional complex of biomolecules, including nucleic acids, (poly) peptides, structural molecules, and metabolites. This impacts human physiology in multiple ways, especially by triggering inflammatory pathways in disease. At present, much remains to be learned about the identity of key effectors and their causal roles.

A relatively small fraction of human chronic diseases can be explained by genetic factors alone (Rappaport and Smith, 2010). Exposure to environmental factors, the exposome, and the resulting gene-environment interactions have been postulated to play an important role in disease initiation and progression (Rappaport and Smith, 2010). The microbiome, i.e., the community of commensal, symbiotic, and pathogenic microorganisms including bacteria, archaea, microeukaryotes, and viruses that share our body space, has emerged as a driver of various diseases along with its complex of distinct yet connected biomolecules (which we refer to here as the "expobiome"). The largest reservoir of microbial biomass is localized along the gastrointestinal tract, and it encodes a genetic repertoire that outnumbers human genes by at least two orders of magnitude (Miyauchi et al., 2022). The gut microbiome must therefore be considered a central hub of exposures, which integrates environmental inputs with genetic and immune signals to affect host physiology.

In health, the gut microbiome confers essential and systemic functionalities including digestion of dietary components, synthesis of vitamins, education and regulation of the immune system, out-competition of pathogens, removal of toxins and carcinogens, and support of intestinal function. Many of these functions are interconnected as the gut microbiome contributes to overall human metabolism, and microbial metabolites play essential roles in immunomodulation. The gut microbiome also interfaces with other body systems via the circulatory, immune, endocrine, and nervous systems.

High-throughput metagenomics has provided essential insights into the gut microbiome's diversity and functional potential. Attributes uncovered include extensive genetic diversity, distinct community types, apparent functional stability, the influence of host genetics on microbial community structure, inter-individual variability and intra-individual stability as well as the effects of extrinsic and intrinsic host factors. Although a lot has been learned, much remains to be discovered about the biology of the gut microbiome especially with respect to its functional interrelationship with human physiology. In this Forum, we discuss recent literature on how gut microbiome-derived molecules can shape innate and adaptive immune responses in the context of health and disease. Using specific disease examples, we offer future directions on uncovering current functional unknowns in the microbiome in an integrative way.

Microbial modulation of immune system and inflammation

Expression of the gut microbiome's functional repertoire culminates in the synthesis of a diverse set of biomolecules which stimulate the host's immune system. The best characterized effectors include lipopolysaccharides (LPS), lipoteichoic acid, polysaccharide A, lipoproteins, peptidoglycans, short-chain fatty acids (SCFAs), secondary bile acids, and fungal glucans. Even when molecules belong to the same class, subtle structural, species- or strain-specific differences may lead to distinct immunogenic properties. Importantly, additional microbial molecules including secondary metabolites, nucleic acids, and (poly)peptides are also immunogenic. An initial, systematic characterization of the gut microbiome-derived biomolecular complex has demonstrated the uniqueness of the extracellular biomolecular fractions (DNA, small and large RNA, (poly)peptides, and metabolites) with respect to their taxonomic and functional affiliations within as well as between distinct individuals (De Saedeleer et al., 2021).

This vast number of chemically diverse microbiome-derived molecules are sensed by epithelial, innate immune and dendritic cells (DCs), which bridge to adaptive immunity. The human immune system is activated through the recognition of microbe-associated molecular patterns (MAMPs) by pattern recognition receptors (PRRs) and other sensing receptors, which can activate pro-inflammatory pathways. PRRs comprise, among others, membrane-associated Toll-like receptors (TLRs) and C-type lectin receptors as well

as cytosolic nucleotide oligomerization domain (NOD)-like receptors or retinoic acid-inducible gene (RIG)-I-like receptors. In permanent contact with the microbial molecules, gut epithelial and immune cells have evolved to maintain homeostasis through promoting tolerogenic responses to commensal microbiota while retaining the capacity to defend against pathogenic agents. Effector lymphocytes, such as T helper type 1 (T_H1), T helper 17 (T_H17), follicular helper T (T_{FH}), and innate lymphoid cells (ILCs; mainly ILC3s) and B cells producing antibodies, contribute to host defense. Regulatory T (T_{reg}) cells play critical roles in immune tolerance and resolution of inflammation. Under healthy conditions, regulatory and effector T cells balance each other to preserve homeostasis. Furthermore, the gut microbiome tunes host responses to microbial molecules to stabilize its niche via stimulating the production of antimicrobial peptides and regulating mucosal homeostasis (Blander et al., 2017). This feedback is based on host cell recognition of microbial molecules, which triggers recruitment and activation of immune cells in the gut but, also at a systemic level, leads to a healthy balance between anti-inflammatory and inflammatory states (Blander et al., 2017) (Figure 1).

In diseases with inflammatory signatures, the balance between cytotoxic and anti-inflammatory, pro-healing immune activation is dysregulated (Figure 1). Increases in inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8, and interferons), hyper-activation of effector lymphocytes, including T_H1, T_H17, T_{FH}, and ILCs or impaired T_{reg} functions, are common markers of the chronic inflammation contributing to pathological damages in host tissues (Blander et al., 2017). Impacts on the microbiome may contribute to chronic inflammation. These include an unbalanced diet, administration of antibiotics, infections, and erosion of the mucus layer. Indeed, differences in the structure of the gut microbiome between healthy individuals and patients have been described for several chronic diseases including autoimmune, metabolic and neurodegenerative diseases as well as cancer (Duvallet et al., 2017). These alterations reflect a microbial imbalance (dysbiosis), which may be characterized by the loss of beneficial species and the overgrowth of pathobionts (Blander et al., 2017; Duvallet et al., 2017) (Figure 1). However, the consequences of such alterations are poorly understood, especially in relation to the triggering of inflammatory processes.

Microbial dysbiosis also likely leads to differential enrichments in microbiome-derived molecules, as a cause or consequence of dysregulated microbiome-immune system interactions (Figure 1). In this context, functional microbiome differences may delineate healthy versus diseased individuals more clearly than simply taxonomic alterations (Heintz-Buschart and Wilmes, 2018). Although changes in predicted functional potential have been resolved using metagenomics, integrated multi-omic analyses also involving metatranscriptomic, metaproteomic, and (meta-)metabolomic analyses have so far only been applied in a subset of diseases, for example inflammatory bowel disease (IBD) (Jansson et al., 2009) and type 1 diabetes (Heintz-Buschart et al., 2016). In general, the functional implications of changes in the abundances of microbial taxa in disease are largely unknown. This lack of knowledge also precludes an understanding of which functions are essential to human health and how shifts in microbiome structure and function may trigger the onset and progression of disease over a lifetime.

Gut microbial factors affecting distal organs and tissues

Taxonomic alterations are also apparent in many diseases which are typically not directly associated with the gut, for example Parkinson's disease (PD) (Heintz-Buschart et al., 2018; Romano et al., 2021) and rheumatoid arthritis (RA) (Miyauchi et al., 2022; Scher et al., 2013). This reflects how gut microbial dysbiosis may trigger or aggravate disease processes in distal organs and tissues, through the production of bioactive compounds. We use PD and RA as case studies below to illustrate this point (Figure 2).

PD is a progressive neurodegenerative disease whose classical pathological hallmarks are aggregations of the protein α-synuclein (αSyn) in the dopaminergic *substantia nigra* of the central nervous system. αSyn aggregation has also been observed in the peripheral nervous system and has been hypothesized to represent a main source of the disease (Heintz-Buschart et al., 2018). In epidemiological studies, a decreased risk for PD has been shown after complete (but not selective) truncal vagotomy (Heintz-Buschart et al., 2018; Sampson et al., 2016), suggesting that the vagus nerve may be involved in the αSyn pathology, which may ascend from the peripheral to the central nervous system. Inflammation and increased permeability of the colonic mucosal lining have also been shown in PD (Heintz-Buschart et al., 2018; Romano et al., 2021). In general, an inflammatory cellular environment is known to enhance αSyn aggregation, which may promote a positive feedback loop culminating in αSyn propagation and progression of the disease. Such effects are associated with the function of the gut microbiome. Work involving a transgenic αSyn-overexpressing mouse model has demonstrated that gut motility, neuroinflammation, motor symptoms, and αSyn aggregation can be modulated by manipulating the gut microbiome for example by antibiotic treatment (Sampson et al., 2016). Transplanting fecal matter from human PD patients to αSyn-overexpressing mice enhances symptoms in comparison to transplants from healthy human donors (Sampson et al., 2016). Although there is variation in the reported results, many studies, including our own work (Heintz-Buschart et al., 2018), as well as meta-analyses, have consistently reported increased relative abundances in the gut of PD patients of the genera *Akkermansia*, *Bifidobacterium*, *Lactobacillus*, and *Methanobrevibacter* and decreased abundances in *Faecalibacterium* and *Roseburia* (Heintz-Buschart et al., 2018; Romano et al., 2021). Functionally, PD-associated microbiota may affect gut barrier integrity (possibly also the blood-brain barrier), SCFA production and inflammation as well as protein misfolding and aggregation (Heintz-Buschart et al., 2018; Romano et al., 2021; Sampson et al., 2016).

RA is a systemic autoimmune disease mainly characterized by inflammation of the synovial joints. Its etiology remains unknown, but it is considered a multifactorial disease requiring both environmental and genetic factors for onset. The gut microbiome has emerged as a candidate for triggering aberrant systemic immunity in RA (Miyauchi et al., 2022) but also as treatment target as studies in murine models of arthritis have demonstrated that disease

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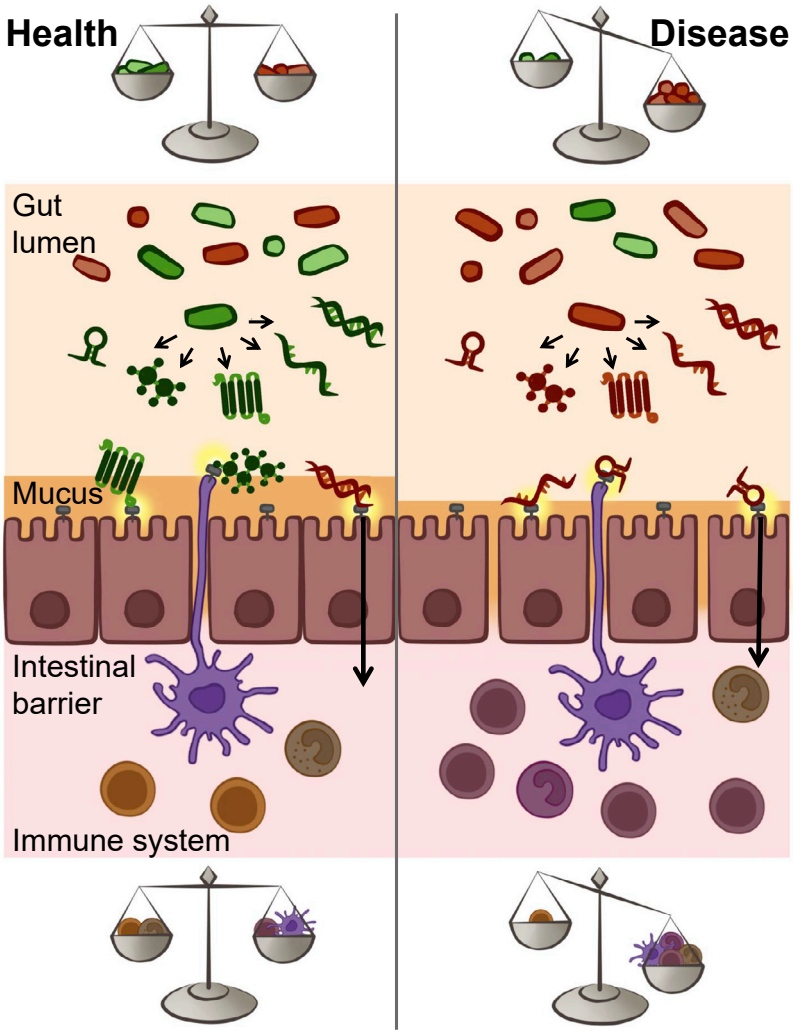

Figure 1. Microbiome-derived molecules triggering inflammatory processes
Microbial dysbiosis culminates in the differential enrichment in different microbiome-derived molecules which may trigger inflammatory processes.

activity is reduced under germ-free or specific-pathogen-free conditions, and following antibiotic treatment (Scher et al., 2013). Furthermore, introducing immunostimulatory segmented filamentous bacteria into such models leads to local and systemic enrichments in pro-inflammatory immune cells (T_H17 and T_{FH}), which drive autoimmune arthritis (Scher et al., 2013). The RA-associated gut microbiome is characterized by enrichments in taxa that correlate with disease markers including *Prevotella copri* and *Collinsella* spp., while others are found at lower abundance including *Bacteroides* spp., *Haemophilus* spp., and *Faecalibacterium* spp. (Miyauchi et al., 2022; Scher et al., 2013). Functionally, RA-associated microbial community structures are linked to adaptation to the pro-inflammatory environment, molecular mimicry of RA-associated antigens, and alterations of gut permeability.

The two disease examples highlight how the gut microbiome and its functional repertoire are linked to diseases with thus far underappreciated connections to the gut. Future studies are required to be more integrative to understand how common or distinct shifts in the holobiont culminate in human disease.

Common signatures

Although taxonomic signatures in the gut microbiome have been described for different chronic diseases, coherent and reproducible disease-specific signatures are limited (Duvallet et al., 2017), except in certain diseases, e.g., colorectal cancer, IBD, and PD. A cross-disease meta-analysis of case-control microbiome datasets has suggested that certain taxonomic alterations reflect a shared response to disease (Duvallet et al., 2017). Condition-specific signatures qualified by enrichments in either opportunistic pathogens or commensals appear less consistent across studies and diseases, likely due to different confounders (Duvallet et al., 2017). Inter-individual strain-level variation likely plays an important role in the context of the functional diversity and redundancy observable among gut microbiota (Heintz-Buschart and Wilmes, 2018). In other words, distinct species and strains may play distinct roles across different diseases. Assessment of the microbiome functional repertoire is therefore indispensable to reveal the commonalities and specificities. The differential expression of microbial functions has been reported for chronic inflammatory diseases including IBD (Jansson et al., 2009) and type 1 diabetes (Heintz-Buschart et al., 2016), with functional -omic profiles exhibiting enrichments in microbial molecules and pathways linked to inflammation. In the absence of consistent, disease-specific taxonomic differences, the pool of microbiome-derived molecules is expected to better discriminate between healthy and diseased individuals.

Apart from shared genetic risk alleles for IBD, RA, and PD, common microbiome-linked mechanisms may influence disease initiation and progression as well as response to treatment. Examples of common hallmarks include enrichments in bacteria such as *Akkermansia* and *Collinsella* which are linked to mucus erosion in a fiber poor context (e.g., *Akkermansia* spp. in the case of PD) and systemic inflammation (*Collinsella* spp. in RA). *Akkermansia muciniphila* is often considered a beneficial species and even a potential probiotic (Romano et al., 2021), yet it is more abundant in patients with PD than in healthy controls (Heintz-Buschart et al., 2018) and is elevated in a transgenic rat model of inflammatory arthritis (Blander et al., 2017). Apart from triggering inflammatory pathways through exposure to a higher load of microbial ligands in the case of a

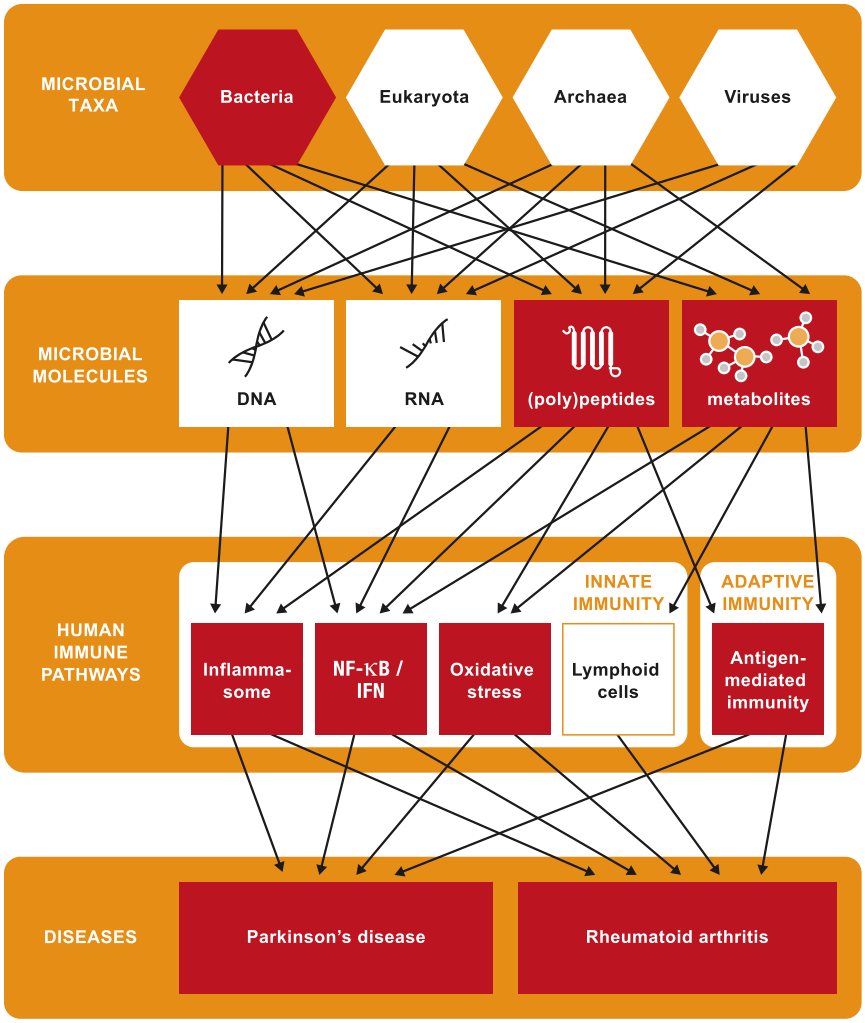

Figure 2. Microbiome-derived biomolecules triggering different immune pathways
Distinct biomolecules trigger different immune pathways dysregulated in human diseases. Based on the published literature, established links are indicated by arrows. Two diseases in which distal organs and tissues are affected, namely Parkinson's disease and rheumatoid arthritis, are highlighted. Nodes highlighted in red are impacted by therapeutic fasting. For information on the corresponding Expobiome Map, please see Aho et al. (2022).

disrupted intestinal barrier, microbial biomolecules may be involved in pathogenesis through affecting protein misfolding in PD (Heintz-Buschart et al., 2018), and/or mimicking human antigens in RA and other autoimmune diseases (Miyauchi et al., 2022). The delineation of common and disease-specific microbiome-linked disease pathways in chronic diseases is therefore essential.

Functional unknowns

Substantial differences exist between predicted/annotated functional potentials based on metagenomic data and actual microbial phenotypes in the gut (Heintz-Buschart and Wilmes, 2018). Although microbial proteins are well-known antigens, the immunogenic capacity of commensals and pathobionts is largely unexplored. Between 40% and 70% of microbiome-borne genes encode proteins of unknown function (depending on the prediction method) and such proteins constitute half of the proteins that are identifiable in proteomics data from fecal protein extracts (Heintz-Buschart and Wilmes, 2018). Similarly, a substantial fraction of gut microbiome-derived small molecules (>90%) does not have any match in public databases (De Saedeleer et al., 2021). Furthermore, RNA transcripts are known to reflect microbial activity and affect antibody responses but microbiome-derived extracellular small and large RNA in the gut remain largely unresolved with respect to their identity and effects on the host side (De Saedeleer et al., 2021). Finally, although it is generally assumed that free DNA, as released from dead bacterial cells, is degraded within seconds to minutes in the gastrointestinal tract, work involving animal models has highlighted the immunomodulatory properties of gut DNA (Hall et al., 2008). In a recent systematic characterization of the gut microbial biomolecular complex, the fraction of extracellular nucleic acid sequences with no functional homology to known sequences was as high as a quarter whereas this was slightly less with respect to their taxonomic affiliations (De Saedeleer et al., 2021). In summary, the gut microbiome harbors a wealth of diverse immunogenic molecules, which have yet to be fully characterized.

Integrated multi-omics

Although we have learned much about the structural and functional characteristics of the gut microbiome through metagenomics, the functional traits conferred by the gut microbiota are much less understood (Heintz-Buschart and Wilmes, 2018). Based on large-scale metagenomic surveys, community structures appear more variable between individuals than the corresponding functional potentials (Heintz-Buschart and Wilmes, 2018). This apparent conservation in functional complement is however not evident when assessing actual functional gene expression (resolved by metatranscriptomics and metaproteomics), which is much more variable (Heintz-Buschart and Wilmes, 2018). Parts of this variation may be attributable to inter-individual and temporal differences in microbial loads affecting host-microbiome interactions as well as other factors such as differences in diet. The variation in microbiome-based functional complements needs to be better understood in relation to its short- and longer-term impacts on host physiology, including in the context of disease.

As many of the gut microbiome-based interactions with the human host involve extracellular or vesicle-borne molecules (De Saedeleer et al., 2021), integrated multi-omic analyses of intracellular biomolecules may result in an incomplete

picture of the functional interlinking between active microbiota and host physiology. To address this challenge, we have recently expanded our biomolecular extraction workflow for integrated multi-omic analyses to allow isolation of concomitant extracellular (including vesicle-associated) DNA (ex-DNA), small and large RNA (ex-sRNA and ex-lRNA), (poly)peptides, and metabolites (De Saedeleer et al., 2021). The relative proportions and absolute amounts of extracellular biomolecules in the respective fractions are distinct from the intracellular compartment, and different from previously reported numbers for gut isolates (e.g., *Escherichia coli*). Taxonomic and functional assignments of the extracellular biomolecules demonstrate the uniqueness of the different fractions and, importantly, metagenomic analysis of the intracellular DNA did not allow inferences regarding the composition of the extracellular complements. The taxonomic affiliations showed higher variability between fractions and samples from distinct individuals than the corresponding functional representations, whereby individual-level variation is the largest factor explaining the observed variation.

Concerning host-microbiome interactions, the ex-DNA along with the ex-lRNA were enriched in genes from known opportunist pathobionts, e.g., some members of the genus *Staphylococcus*, which affect IL-8 via the recognition of CpG motifs by TLR9. The ex-lRNA was further enriched in sequences derived from specific bacterial taxa including *Faecalibacterium* spp., a genus known for its anti-inflammatory properties and which is often depleted in dysbiosis. Interestingly, up to 5% of the ex-lRNA fraction from certain individuals was derived from the hydrogenotrophic archaeon *Methanobrevibacter smithii*. Archaeal RNA has been found to be immunogenic (Vierbuchen et al., 2017), highlighting that microbiome constituents other than bacteria, including archaea but also microeukaryotes, may play essential immunomodulatory functions. The (poly)peptide and small-molecule fractions were enriched in molecules known to be exported or secreted. However, many unknowns exist that require further study, not least in relation to their immunogenic properties. Taken together, this recent work highlights the need to systematically resolve the individual fractions, as the emergent properties with respect to host-microbe interactions cannot be understood if focusing solely on one biomolecular dimension. Quantitative integrated multi-omics have yet to be applied comprehensively to the collection of gut microbiome-derived molecules in relation to human health and disease.

Linking observation to mechanism

The ability to probe the innerworkings of the gut microbiome allows the identification of microbial traits associated with disease. So far, causal relationships have been mostly established using gnotobiotic animal models. However, such approaches are only informative if homology exists with human traits. In the context of immune system stimulation, significant differences exist for example in the complement of PRRs between mice and humans. Therefore, studies aimed at unraveling causal relationships and mechanisms should be conducted using interventions in humans, representative human *in vitro* models and/or humanized animal models (Heintz-Buschart and Wilmes, 2018). Microbiome-modulating interventions, which reduce inflammatory signatures, such as therapeutic fasting (Longo et al., 2021) (see Figure 2 for a representation of the impacts of fasting in the context of PD and RA), offer efficacious and generalizable prevention and treatment modalities for chronic diseases. They represent ideal test cases for identifying common and condition-specific features by tracking the microbiome-derived molecular complex during the intervention using integrated multi-omic analyses coupled with deep immune profiling. Any associations between shifts in the gut microbiome and related physiological impacts on the host require validation in representative experimental models such as human organ-on-chip models. Ultimately, combined approaches will allow us to map the microbiota-derived disease-modulating effector molecules (Figure 2), which will ultimately allow the development of new prevention and treatment modalities.

The complexity of human-microbiome interactions leads to different combinations of molecules, which may trigger or contribute to human disease processes (Figure 1). Based on various publicly available data, we have developed an interactive online tool in the form of the Expobiome Map (https://expobiome.lcsb.uni.lu). This formalization of existing knowledge (Figure 2; see also accompanying SnapShot: Aho et al., 2022) allows inferences to be made on likely links between microbial taxa, their biomolecules, and disease processes via the triggering of human immune pathways. More specifically, gaps in knowledge concerning such linkages allow the formulation of specific hypotheses, which may subsequently be tested *in silico*, *in vitro*, and *in vivo* to result in novel mechanistic insights.

Conclusion

The bioactive attributes of the gut microbiome including the immunogenic potential of commensals and pathobionts remain largely unexplored. Many of these will reflect emergent properties resulting from inter-microorganism and host-microbe interactions. The systematic characterization of the microbiome-derived molecular complex is now possible using advanced integrated multi-omic analyses and appropriate model systems. The unraveling of this complex will ultimately lead to mechanistic understanding of host-microbe interactions as well as new diagnostic, prognostic, preventive, and therapeutic avenues for human diseases.

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DECLARATION OF INTERESTS

P.W. is a member of the Science Advisory Committee of the Predictive Phenomics Initiative at Pacific Northwest National Laboratory (USA), the advisory board of the Swiss National Science Foundation's National Center of Competence in Research Microbiomes (Switzerland), and the scientific steering committee for a clinical trial by 4D Pharma. P.W. is listed as an inventor on patent applications PCT/EP2020/081855, PCT/EP2020/081832, PCT/EP2019/081424, U.S. patent #11,261,414, PCT/EP2016/062024, PCT/EP2013/065718, PCT/EP2013/056607, PCT/EP2013/052134, and PCT/EP2012/065178. V.T.E.A. is an inventor on patents FI127671B, EP3149205B1, and US10139408B2 (issued) as well as US20190137493A1, US20210109098A1, and EP3789501A1 (pending), which are assigned to NeuroBiome Ltd.

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