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Dark matter in host-microbiome metabolomics: Tackling the unknowns—A review

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GRAPHICAL ABSTRACT

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ABSTRACT

The “dark matter” in metabolomics (unknowns) represents an exciting frontier with significant potential for discovery in relation to biochemistry, yet it also presents one of the largest challenges to overcome. This focussed review takes a close look at the current state-of-the-art and future challenges in tackling the unknowns with specific focus on the human gut microbiome and host-microbe interactions. Metabolomics, like metabolism itself, is a very dynamic discipline, with many workflows and methods under development, both in terms of chemical analysis and post-analysis data processing. Here, we look at developments in the mutli-omic analyses and the use of mass spectrometry to investigate the exchange of metabolites between the host and the microbiome as well as the environment within the microbiome. A case study using HuMiX, a microfluidics-based human-microbial co-culture system that enables the co-culture of human and microbial cells under controlled conditions, is used to highlight opportunities and current limitations. Common definitions, approaches, databases and elucidation techniques from both the environmental and metabolomics fields are covered, with perspectives on how to merge these, as the boundaries blur between the fields. While reflecting on the number of unknowns remaining to be conquered in typical complex samples measured with mass spectrometry (often orders

Abbreviations: (APCI), Atmospheric Pressure Chemical Ionization; (APPI), Atmospheric Pressure Photo-ionization; (CI), Chemical Ionization; (CASE), Computer Assisted Structure Elucidation; (CASMI), Critical Assessment of Small Molecule Identification; (EI), Electron Ionization; (ESI), Electrospray Ionization; (GC), Gas Chromatography; (LGG), *Lactobacillus rhamnosus* GG; (LC), Liquid Chromatography; (MS), Mass Spectrometry; (MSTFA), N-methyl-N-(trimethylsilyl)trifluoroacetamide; (NMR), Nuclear Magnetic Resonance; (RI), Retention Index; (TOF), Time of Flight; (TPs), Transformation Products; (TMS), Trimethylsilylation; (UVCBs), Chemicals of Unknown and Variable Composition, Complex Reaction Products and Biological Materials; (CEC), Contaminants of Emerging Concern; (HR), High Resolution.

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of magnitude above the “knowns”), we provide an outlook on future perspectives and challenges in elucidating the relevant “dark matter”.

1. Introduction

1.1. The human gut microbiome

Complex assemblages of microorganisms populate the human body and these microbiomes are emerging as key players in human health and disease [1]. The largest reservoir of microbial biomass is the gastrointestinal tract. The human gut microbiome is considered a central hub that integrates environmental inputs, such as diet and surroundings, with genetic and immune signals to affect the host's overall physiology, including metabolism [2]. The gut microbiome confers essential metabolic and other functions to human physiology including digestion of food components [3], synthesis of essential vitamins [4], stimulation and regulation of the immune system [5], out-competition of pathogens [6], removal of toxins and carcinogens [7], and support of intestinal function [8]. Many of these functions are interconnected as the gut microbiome contributes to overall human metabolism [9,10] and the microbial metabolites produced play essential roles in immunomodulatory processes [11]. In the context of the human immune system, there is a tight interconnection whereby the immune system may affect the gut microbiome and its metabolic capacity, and *vice versa* [12]. The gut microbiome also interfaces with other body systems via the circulatory, immune, endocrine and nervous systems. Changes to the microbial ecology of the gut may culminate in dysbiosis, a pathological imbalance in the gut microbiota with implied dysfunctions in the complex set of processes governing human health [13].

The advent of high-throughput sequencing and its application to the complex microbiota of the human gut has provided essential new insights into the structural diversity and functional potential of the gut microbiome. Essential attributes of the human gut microbiome uncovered through these studies include extensive genetic diversity [14,15], distinct community types [16,17], apparent functional stability despite variation in community structure [18], the influence of host genetics in shaping community structure [19], inter-individual variability [20] and apparent intra-individual stability [21,22], and the overall importance of extrinsic and intrinsic host factors in shaping community composition [23]. Furthermore, through the use of sequencing-based methods, largely applied in case-control study designs, dysbiosis has been implicated in the aetiology of numerous idiopathic conditions including irritable bowel syndrome [24], inflammatory bowel disease [25], liver cirrhosis [26], type 1 diabetes [27,28], type 2 diabetes [29], obesity [30,31], cardiovascular disease [32], colorectal cancer [33,34], rheumatoid arthritis [35] and most recently Parkinson's disease [36–38].

Metagenomic analysis involving random shotgun sequencing of community genomic DNA has revealed the genetic potential of the gut microbiota, especially in relation to metabolic transformations and disease [39]. Additionally, metatranscriptomic and metaproteomic analyses have identified which genes are expressed by the microbiota under specific conditions [40]. However, as community-wide metabolism reflects the actual, cumulative phenotypes of the different populations which comprise the microbiome, (meta-)metabolomics is likely the most sensitive indicator for disease-linked processes. This in turn makes it well suited for identifying discriminant features based on which mechanistic hypotheses can be formulated linking, for example, dysbiotic microbiota to disease pathogenesis. Therefore, differences in microbial community structure reflective of dysbiosis have been linked to changes in microbial metabolism in the gut, e.g. alteration of microbial phosphatidylcholine metabolism in the context of atherosclerosis [32] or increased biosynthesis of branched chain amino acids in the context of insulin resistance [41]. Metabolic activity within the gut microbiome also impacts drug metabolism and efficacy [42,43]. A detailed knowledge of gut microbiome-mediated metabolic transformations is therefore essential to understand how the gut microbiome impacts human phenotypes, especially in relation to the panoply of diseases now associated with changes in the gut microbiome. In this context, metabolomic analyses of gut microbiome small molecule extracts have allowed the identification of disease-specific signatures (recently reviewed in Ref. [44]).

1.2. Metabolites and the microbiome

Given the pronounced metabolic activity of the gut microbiome, which includes multiple unique catabolic and anabolic reactions not catalyzed by human cells, microbial metabolism has to be considered as an integral part of human physiology. In general terms, the major known metabolite classes produced and transformed by gut microbiota with known effects on human physiology include organic acids (lactate, succinate, formate, etc.), short chain fatty acids (acetate, propionate, butyrate, etc.), lipids (ceramides, lysophosphatidylcholines, phosphatidylcholines, etc.), branched-chain fatty acids (valerate, isobutyrate, isovalerate, etc.), branched-chain amino acids (leucine, isoleucine, valine), vitamins (biotin, folate, niacin, etc.), bile acids (deoxycholic acid, lithocholic acid, etc.), and neurotransmitters (GABA, serotonin, etc.). Apart from these specific metabolite classes, the gut microbiome catalyzes a broad spectrum of different biotransformations (Fig. 1). Importantly, apart from the known microbiome-driven metabolic reactions, microbiome-derived metabolomic datasets are characterized by a significant fraction (>90%) of as yet uncharacterized metabolite features that do not have any match in public databases [45]. Many of these “unknowns” are highly likely to represent “missing links” in microbial metabolism and human-microbe molecular interactions [46]. The systematic study of the microbiome-conferred metabolome therefore requires extensive future study, not least because a detailed understanding of the functional microbiome represents an essential prerequisite for future rational interventions leveraging the gut microbiome to alter host phenotype. In this context, the unknowns represent an important focus for investigation.

1.3. Mass spectrometry: from metabolites to the environment

Mass spectrometry (MS) is often the analytical method of choice for discovery-based untargeted metabolomics analyses. Although a lot of excellent metabolomics is performed with nuclear magnetic resonance (NMR) techniques, it is not the focus of this article. The post-analysis identification workflows of MS-based approaches depend highly on the analytical set-up used, with many different databases and software tools now available. Separation techniques,

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