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New Paradigms for Familiar Diseases: Lessons Learned on Circulatory Bacterial Signatures in Cardiometabolic Diseases

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ABSTRACT

Despite the strongly accumulating evidence for microbial signatures in metabolic tissues, including the blood, suggesting a novel paradigm for metabolic disease development, the notion of a core blood bacterial signature in health and disease remains a contentious concept. Recent studies clearly demonstrate that under a strict contamination-free environment, methods such as 16 S rRNA gene sequencing, fluorescence in-situ hybridization, transmission electron microscopy, and several more, allied with advanced bioinformatics tools, allow unambiguous detection and quantification of bacteria and bacterial DNA in human tissues. Bacterial load and compositional changes in the blood have been reported for numerous disease states, suggesting that bacteria and their components may partially induce systemic inflammation in cardiometabolic disease. This concept has been so far primarily based on measurements of surrogate parameters. It is now highly desirable to translate the current knowledge into diagnostic, prognostic, and therapeutic approaches.
This review addresses the potential clinical relevance of a blood bacterial signature pertinent to cardiometabolic diseases and outcomes and new avenues for translational approaches. It discusses pitfalls related to research in low bacterial biomass while proposing mitigation strategies for future research and application approaches.

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Introduction

In the light of our long co-evolution with microbes [1], the human microbiome can be seen as an accessory genome including diverse bacteria, viruses, archaea, and fungi, expanding over several bodily niches and extending our functional potential. While most bacteria are commensal, many inhabiting our body are mutualistic, and some are detrimental. They are kept in check by the immune system and the surrounding cell communities contributing to the compositional niche, whose survival depends on the livelihood of the entire "human meta-organism". After a period of what can be considered a prolonged cold war with bacteria, brought on by the advent of the hygiene hypothesis [2] and the golden era of the antibiotics, growing evidence of microbial "ecosystem services" [3], the inextricable link between our health and our microbiome, including efficacy and response to medication such as cardiac drugs and chemotherapeutics, have led to a rekindling of interest in mucosa-associated bacterial microbiomes [4–6].

For the last two decades, this area of research has been propelled forward by several consortial efforts, including MetaHit [7], Human Microbiome Project (HMP) [8], Metacardis [9, 10], and Flemish Gut Flora Project [8, 11], in which the intestinal microbiome and, in a minority of cases (HMP), the oral, dermal and vaginal microbiome have been sequenced in large cohorts to define: a) a "healthy microbiome state", b) factors contributing to microbiome composition (including medication, nutrition, and environment)[11–13], and c) the associations of these microbiomes with disease [7, 14]. This has been further facilitated and expedited by the introduction of whole metagenome shotgun sequencing as well as accessible and scalable analyses methods. These population studies have linked dysbiosis – a state of deviation from a core functional and taxonomic composition- with the pathogenesis of several diseases, including obesity, non-alcoholic fatty liver disease, and cardiovascular disease [15]. While a transient presence of bacteria in the blood has been described after teeth brushing or gingival manipulation [16, 17], their consistent presence has been interpreted as indicative of infection or sepsis. This notion changed only recently with the description of low grade or subclinical inflammation in metabolic disease.

Low-grade inflammation is consensually perceived as a potential trigger for metabolic dysfunction, insulin resistance, and diabetes [18] and is often reflected in subtle increases in systemic inflammatory markers such as C-reactive-Protein (CRP). Increased translocation of lipopolysaccharides (LPS), mainly from the gut to the blood, defined as endotoxemia [19], has also been described as a potential trigger for chronic inflammation in metabolic disease, supported by the onset of insulin resistance in healthy subjects after LPS-transfusion [19, 20]. Therefore, it is surprising that the notion of a core blood bacterial signature in health and its divergence in disease with accompanying systemic response remains contentious. However, converging evidence for microbial signatures in metabolic tissues, including the blood, suggests a novel paradigm for metabolic disease development [19–24]. In this review, we address the potential clinical relevance of a blood bacterial signature pertinent to disease development and outcomes and new avenues for translational approaches. We, moreover, discuss pitfalls related to research in low bacterial biomass while suggesting mitigation strategies for future research and application approaches.

Schrödinger's blood bacteria: 'you don't see me, now you do.'

The concept of bacterial presence in the blood has been reported as early as 1969, where living and metabolically active bacteria [25] were localized in the blood of healthy human subjects. In the meantime, a commendable body of work has combined classic molecular and microbiological techniques to evidence the presence of bacterial cells and sequences in the blood of presumably healthy humans, either as a study cohort or as healthy controls for specific disease states (recently reviewed in [26]). These methods include quantitative polymerase chain reaction (qPCR) [27–35] of 16 S rRNA or targeted bacterial genes [29, 35], fluorescence in-situ hybridization (FISH) and other employment of fluorescent probes [35], transmission electron microscopy [36], dark field microscopy [36], PCR followed by electrospray ionization-mass spectrometry [37] as well as classical bacterial culture [32, 38, 39]. Several of these results have been met with criticism, considering the longstanding dogma of the blood being an innocuous environment and controversy regarding visual confirmation of bacteria deemed by some authors as L-form bacteria and by others as merely membrane vesicles or aggregated proteins [40].

The advent of efficient and scalable technologies, including next-generation sequencing of the 16 S rRNA gene (present in all bacterial cells), whole metagenome sequencing, RNA-sequencing [38, 40, 41], and the ongoing expansion of microbial reference genomes have facilitated the detection of non-culturable or hard-to-culture bacteria across several tissues and diseases. This is especially important considering several blood-borne bacteria can persist in a dormant state [42]. Moreover, the democratization of analytical tools, including the development of microbiome tools with friendly graphic user interfaces [43] and comprehensive documentation [44], have made this research area accessible to a broader scientific community.

Blood bacterial signatures in health and cardiometabolic disease

While the role of systemic inflammation in cardiometabolic disease, partially induced by bacteria, has been widely appreciated, most of the published studies related this observed systemic inflammation to bacterial components and/or selectively analyzed surrogate parameters. This includes measurements of host response patterns (e. g., LPS binding protein (LBP)) or wall components of gram-negative bacteria (LPS) in the circulation to assess bacterial burden indirectly. However, there is scarce but increasing research investigating direct bacterial presence, that is, bacterial cells or bacterial nucleic acids, and its quantitative and qualitative potential to contribute to inflammation and dysfunction of both the immediate microenvironment and the whole system. Several studies have evidenced the presence of bacterial genetic material, whole bacteria, or even bacterial RNA in the blood of healthy subjects [26, 42] (► **Fig. 1a**), and research dissecting the contribution of circulating bacteria or bacterial genetic material to cardiometabolic disease has been accumulating steadily (► **Fig. 1b**). From a clinical perspective, it has been noted early on that bacteremia of unknown origin was more prevalent in subjects with type 2 diabetes (T2D) compared to non-diabetic subjects [45], with higher occurrences of *Staphylococcus aureus* and *Klebsiella* in the blood along

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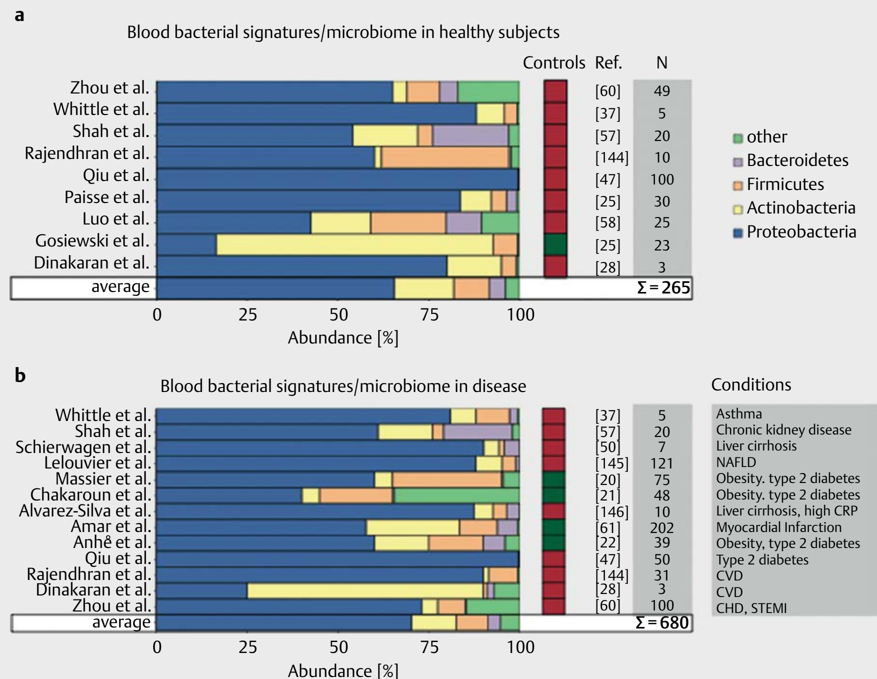

▶ **Fig. 1** **a) Overview of phyla proportions, controls inclusion, and the number of included subjects in selected study cohorts reporting blood bacterial signatures in healthy cohorts and healthy subjects of cohorts with cardiometabolic and cardiovascular disease**. Names of the studies are to the left of the bar. Bar length equals 100 % (total proportions of reported phyla), and each color in the bar represents a specific phylum (color code specified in the legend to the right). Controls: specifies whether experimental and collection controls were included to account for contamination. A red box corresponds to a lack of controls, a green box to the availability of adequate controls. Ref.: refers to the reference number in the bibliography (Paisse and Gosiewski et al. are reviewed in ref [25]) and N the number of healthy subjects in the cohort. An average distribution for all studies is given in the lowest bar, and N on the right refers to the total number of subjects included. **b) Overview of phyla proportions, included controls, and the number of included subjects in selected study cohorts reporting blood bacterial signatures in disease (focusing on the cardiometabolic disease).** Names of the studies are to the left of the bar. Bar length equals 100 % (total proportions of reported phyla), and each color in the bar represents a specific phylum (color code specified in the legend to the right). Controls: specifies whether experimental and collection controls were included to account for contamination. A red box corresponds to a lack of controls, a green box to the availability of adequate controls. Ref.: refers to the reference number in the bibliography and N the number of diseased subjects in the cohort. Conditions: refers to the medical conditions of the cases in the studies. An average distribution for all studies is given in the lowest bar, and N on the right refers to the total number of subjects included. *Abbreviations*: NAFLD: non-alcoholic fatty liver disease, CVD: cardiovascular disease, CHD: chronic heart disease, STEMI: ST-elevation myocardial infarction.

relatively 'harmless infections' such as urinary tract infections, leading to septic shock more often (OR 1.9) [46] and motivating the early application of targeted empiric antibiotic treatment. A hallmark study was published in 2011 by Amar et al., analyzing blood bacterial load by measuring 16 S rRNA copy numbers in more than 3000 subjects followed over nine years. The authors report an increased abundance of Proteobacteria in visceral obesity as well as onset of T2D [30] and cardiovascular disease at follow-up [47]

In 2014, Sato et al. undertook a nested case-control study reporting that prevalent T2D was associated with an increased inflammatory response with higher levels of LBP independently linked to HbA1c and BMI. Higher abundances of *Clostridium coccoides*, *Atopobium* clusters [31, 47], *Sediminibacterium* spp.[48], and higher detection of the 16 S rRNA gene was positively associated with the disease [31]. This is particularly important, as increased bacterial DNA detection after bariatric surgery in subjects with T2D and obesity revealed a blunted response to the intervention with minor improvement in glucose tolerance or inflammation [49]. In contrast, subjects with a high abundance of the genus *Bacteroides* were less likely to develop T2D during a two-year follow-up [48, 49].

Likewise, there is increasing evidence for a link between circulating bacteria in liver fibrosis and steatosis, which is associated with an impaired immune system and glucose metabolism and major cardiovascular events [50]. In 2016, Lelouvier et al. reported increased levels of circulating 16 S rDNA and proportions of Proteobacteria (specifically *Sphingomonas, Bosea,* and *Bradyrhizobiaceae* taxa) in liver fibrosis. This was further substantiated by other studies showing similar results in liver cirrhosis in several blood compartments [51] and the increased detection of bacterial DNA via *in situ* hybridization in particularly severe cases of liver decompensation [52]. Chronic kidney disease (CKD), a well-established cardiovascular risk factor [53], has similarly been linked to changes in blood bacterial signature, as it was associated with a decreased alpha diversity – a hallmark of several non-communicable diseases – and the expansion of bacterial taxa expressing uricase as well as indole- and *p*-cresyl-forming uremic enzymes [54]. These bacte-

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rial uremic toxins have been linked to worsening kidney function, endothelial dysfunction, cardiac fibrosis, macrophage activation, and insulin resistance [55]. CKD is also linked to a dysbiosis in the gut, which has been associated with impairment of gut epithelial barrier via depletion of claudin-1, occludin, and zonula occludens-1 proteins in addition to dysfunction of colonic T-regulatory cells [56] as well as further aggravation by direct effects of uremic toxins [56, 57]. Therefore, it is not surprising that CKD is reflected in a decreased bacterial diversity in the blood and associated with a higher abundance of proteobacteria, which were inversely correlated with kidney function [58]. Comprehensive studies on the specific compartmentalization of bacterial DNA in metabolic disease remain rare but promise a more holistic approach. For example, Anhê, Jensen et al. compared the bacterial DNA load and taxonomy in plasma samples, liver, subcutaneous, visceral, and mesenteric adipose tissues, noting a relatively reduced bacterial load in plasma samples compared to other tissues and a preferential presence of two specific genera (*Rodoferax* and *Polaromonas*) in the blood, but did not find distinct differences in the tissues between subjects with and without T2D [23]. Results were supported and expanded by findings from our research group, where 16 S rRNA gene content was quantified and sequenced in 75 patients with obesity and with or without T2D. Bacterial composition in the blood was associated with circulating tumor necrosis factor-α, CRP, LBP, and interleukin-6. Moreover, amounts of *Lactococcus* in the blood correlated negatively with homeostasis model assessment-estimated insulin resistance while *Acinetobacter* and *Tahibacter* were found more often in the blood than in several studied adipose tissues and correlated positively with diabetes status [21]. In another recent study, we evidenced reduced bacterial diversity in the blood of subjects with T2D vs. non-T2D, but a reduced richness after bariatric surgery paralleled with an increased bacterial quantity. The latter was surprisingly negatively associated with leukocyte counts. We also evidenced early shifts in taxa after surgery with a reduction in *Rhizobacter*, *Anoxybacillus*, *Streptococcus*, and *Ureibacillus.* In our cohort, several of these genera were positively correlated with inflammation, fat mass, and body mass index (BMI). Most importantly, 40% of the variance in the blood bacterial composition could be explained by 27 host variables, including medication intake [22]. Similarly, bacterial composition predicted the clinical classification of patients according to metabolic disease robustly. The strength of the last three studies is the extensive experimental and bioinformatic contaminant control, which has since become more common in recent studies [59].

At the other end of the cardiometabolic disease spectrum, infections in the hospital setting as well as outpatient infections very often preceded coronary heart disease (CHD) and stroke in the ARIC study, which analyzed cardiovascular risk in 15792 subjects, showing that in a total of 1312 incident CHD and 727 incident stroke cases, infections were associated with an up to 12.83 OR for CHD (inpatient infections, up to 14 days prior to CHD presentation) [60]. Increased strain can lead to overt manifestations of cardiovascular events in predisposed subjects. This can be exemplified in type 2 myocardial infarction (MI) in infection and anemia, where an imbalance between myocardial oxygen demand and supply becomes apparent. That being said, one can not preclude that systemic inflammation in the realm of infection can further trigger immunological processes in local sites of preexistent vascular lesions in the heart or carotid arteries. In line with a potential mediating effect of blood bacteria and increased bacterial translocation from the gut, Zhou et al. demonstrated higher levels of LPS and D-lactate (bacteria-derived) in the blood of subjects two days post-MI along with an increased alpha diversity in ST-elevation MI (STEMI) and enrichment of gut-derived bacteria. At the same time, chronic CHD presented with lower bacterial diversity in the blood compared to controls. Mediation analysis revealed a mediating role for bacterial translocation in inflammation (CRP and monocytes), left ventricular ejection fraction, and major adverse cardiovascular events. More importantly, in an experimental MI mouse model, the reversal of bacterial translocation via antibiotic treatment reduced serum LPS, alleviated monocytosis, and reduced cardiomyocyte injury post-infarction [61]. More recently, Amar et al. showed that subjects with an acute onset MI displayed a 1.3-fold increase in blood bacterial load compared to control subjects with concurrent metabolic risk factors, which was driven by high LDL cholesterol. In the 99 subjects with MI, several genera known to metabolize cholesterol, such as *Nocardiaceae*, *Aerococcaceae*, *Gordonia*, *Propionibacterium*, *Chryseobacterium*, and *Rhodococcus*, were depleted [62]. In conclusion, most studies found a similar pattern of phyla distribution in the circulation, which was dominated by Proteobacteria (~65%), followed by Actinobacteria (16%), Firmicutes (10%), and Bacteroidetes (4%) (▶**Fig. 1**). With some exceptions, changes in cardiovascular disease (CVD) followed the same trend, showing a relative increase of Proteobacteria by 7% and a 10% reduction in Firmicutes on average (▶**Fig. 2**). As depicted, individual studies show more considerable changes but are not always congruent. No objective conclusion can be drawn on lower taxonomic levels, as reported results vary greatly, but for example, *Pseudomonadaceae* and *Streptococcus* were enriched in CVD in more than two studies (▶**Table 1**).

Translational avenues: are we there yet?

High throughput sequencing of blood-borne bacteria and other microorganisms is relevant as a potential extension of the repertoire of current assessments in health and disease. It has already shown paradigmal potential for the gut microbiome and promises a similar potential for blood-derived disease markers [15] or liquid biopsies [24] in non-communicable and infectious diseases. Moreover, it will be critical for downstream applications, including novel, cost-effective techniques to detect new pathogens in an ever-evolving environment [63, 64]. Exploring functional potential using highly granular whole genome sequencing and genome assembly paired with metabolomics and metaproteomics will further underpin disease mechanisms [65], allowing targeted and possibly personalized treatment options.

First things first: traditional applications for non-classical diagnostics

A seemingly immediate application for the analysis of bacterial sequences in the blood, along with other bodily niches, is the potential to precisely identify bloodstream pathogens at the strain level. This helps account for pathogenicity, transmission potential, and antimicrobial resistance [66] and define their source early on in order to inform targeted interventions in clinical infection management. Considering that the latter is in most cases dictated by as-

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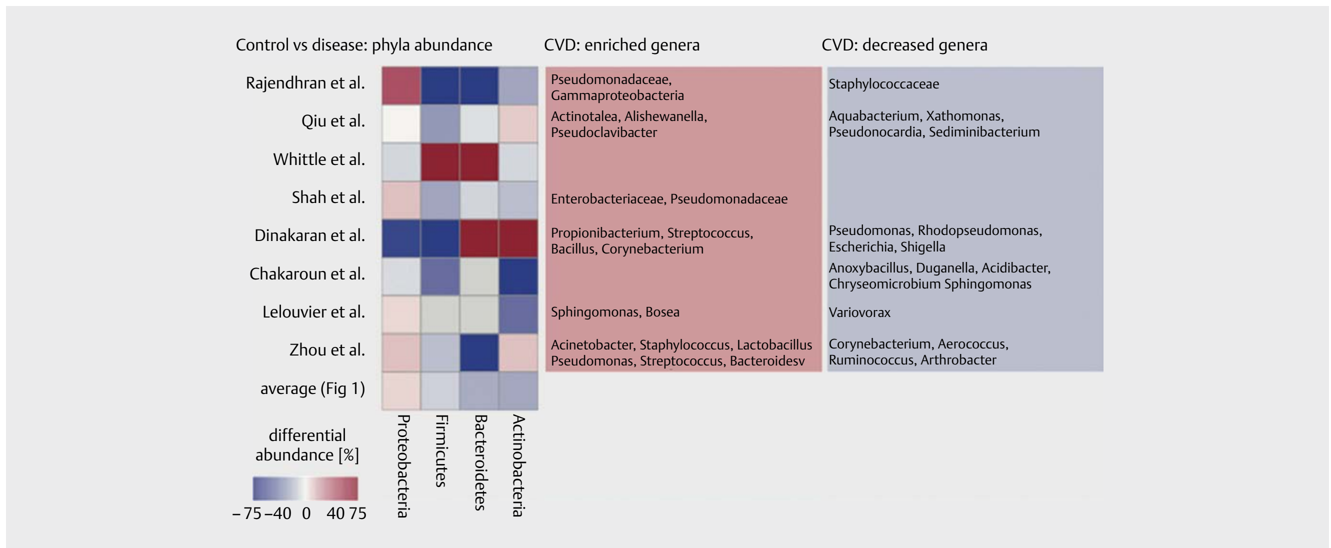

▶ **Fig. 2** Heatmap referring to the differential abundance [increased (in red) or decreased (in blue)] of phyla in studies with disease vs. healthy controls. Studies are given to the left of the heatmap rows; phyla constitute the columns of the heatmap. A look at differentially abundant genera in CVD in selected studies are shown in the red box (for increased genera) and the blue box (for reduced genera) next to the survey referred. Abbreviation: CVD: cardiovascular disease.

▶ **Table 1** Studies showing evidence of blood bacterial signatures in cardiometabolic disease in human

Author	Method	Matrix	Conditions and number	Year	Reference
Rajendhran	16 S V3	Whole blood	133 CVD vs 118 controls, thereof positive for 16 S: 31 vs 10 (not matched)	2013	[153]
Dinakaran	Metagenomic, 16 S quantification	Plasma	80 CVD vs 40 controls for quantification, 3 vs 3 for sequencing	2014	[29]
Lelouvier	16 S V3/V4	Buffy coat	Cohort 1: 11 fibrosis vs 26 controls; Cohort 2: 11 fibrosis vs 60 controls	2016	[154]
Whittle	16 S V4	Plasma	5 atopic asthmatic vs 5 controls	2018	[38]
Schierwagen	16 S	Buffy coat	3 variceal bleeding, 4 refractory ascites	2018	[51]
Zhou	16 S V4	Buffy coat	100 ST-segment elevation myocardial infarction, 50 CHD, 49 controls	2018	[61]
Qiu	16 S V5/V6	Whole blood	50 T2D vs 100 healthy (matched for age, sex)	2019	[48]
Alvarez-Silva	16 S V3/V4	Whole blood and ascitic fluid	33 cirrhosis	2019	[155]
Shah	16 S V3/V4	Buffy coat	20 CKD vs 20 healthy (not matched)	2019	[58]
Amar	16 S V3/V4	EDTA blood	103 high CVD risk, 99 with myocardial infarction	2019	[62]
Anhê	16 S V3/V4	Plasma	20 T2D, obese vs 20 normal glucose tolerance, obese	2020	[23]
Massier	16 S V4/V5, CARD-FISH	EDTA blood	75 with obesity, 42 no T2D vs 33 T2D	2020	[21]
Chakaroun	16 S V4/V5, CARD-FISH	EDTA blood	64 at baseline with obesity, 24 no T2D vs 24 T2D with 12-month follow-up (matched for age, sex, and BMI)	2021	[22]

CARD-FISH: catalyzed reporter deposition – fluorescence in situ hybridization; CVD: cardiovascular disease; CHD: chronic heart disease; CKD: Chronic kidney disease; EDTA: ethylenediaminetetraacetic acid; T2D: type 2 diabetes

sumptions of infection source and the fact that culture-based methods are slow and fail to identify potential pathogens in 40 % of cases [67], this application seems particularly central in subjects with sepsis [68] and high-risk subjects such as immunocompromised patients and those after hematopoietic cell transplantation (HCT) [69] or subjects with liver cirrhosis [52], as well as patients with diabetes. In the latter, the higher risk of primary bloodstream infections may arise from the translocation of organisms from damaged mucosal sites like the oral cavity or the gut. Additionally, it might be helpful in patients who are already treated with antibiotics, and in whome blood cultures usually fail. A promising approach was published by Tamburini et al., who developed a bioinformatic tool, which utilizes the potential of whole-genome sequencing to profile strain variations between metagenomes to match bloodstream pathogens in HCT to a candidate source. They found concomitant dominant gut colonization with known enteric pathogens a few days prior to bloodstream infection becoming clinically apparent. Interestingly, they found cases where typically non-enteric pathogens (e. g., *Pseudomonas aeruginosa* and *Staphylococcus aureus*) were present in the gut microbiota before infection and as-

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certained functional relatedness by showing concordant predicted and clinical antibiotic resistance [70]. While turnaround time in sample preparation and sequencing and the high cost remain hindersome for efficient integration of whole-genome sequencing in clinical care, strain-level identification of pathogens and their reservoirs has the potential to improve infection prevention by reconstructing the trajectories and timing of colonization, the evolution of pathogenicity, and microbial adaptation [71]. This can further enhance management procedures by rapidly identifying resistance and escape mechanisms in real-time [63]. Considering this manuscript was written amid the coronavirus 2 (SARS-CoV-2) pandemic, it would almost be tone-deaf not to stress how metagenomics has been pivotal in helping us develop a holistic ecological understanding of microbial evolution over time and geography. This is particularly true for detecting subclonal mutations (used routinely in cancer research), which have helped delineate the chains of infection and interspecies transmission and the higher frequency of which has been associated with severe acute respiratory distress syndrome in SARS-CoV-2 [72]. The same holds for bacterial infection: bacterial DNA in the bloodstream more effectively identifies subjects at higher risk of death or more severe illness from suspected sepsis [73], regardless of the isolation of viable bacteria via blood culture alone [37]. While immunotherapy is promising but practically nonexistent in cardiometabolic disease [74], the implementation of screening for blood bacterial DNA might be beneficial in critically ill subjects, where adjunctive immunotherapy [75] or treatment with specific toll-like-receptor (TLR) antagonists is an option [76].

Bacteria in the blood: liquid biopsies of the new era?

As liver cirrhosis has been associated with a circulatory bacterial signature, it is not surprising that hepatocellular carcinoma (HCC) displays a specific alteration of the bacterial sequences found in the blood of diseased subjects. Specifically, the blood of subjects with HCC had a lower bacterial diversity. Moreover, significant differences in the relative abundance of *Pseudomonas*, *Streptococcus*, *Bifidobacterium*, *Staphylococcus*, *Acinetobacter*, *Klebsiella*, and *Trabulsiella* were noted. The latter four bacteria were heavily enriched in HCC, and *Staphylococcus* had the most significant association with HCC (p = 4.0e-08), showing a 4.3-fold increase compared to controls. The authors then identified a marker-based model depending on five bacterial genera, distinguishing HCC from controls (AUC of 0.879, accuracy 81.6 %). Validation in a subgroup of the cohort confirmed that the model accurately differentiated HCC with an AUC of 0.875 and an accuracy of 79.8 % [77]. Substantial contributions of local tissue and gut microbiome have been demonstrated for several cancer types [78–85], which further motivates the search for microorganism-derived molecules to diagnose noncommunicable diseases such as cancers. In a recent publication, Poore et al. reassessed whole-genome and whole-transcriptome sequencing studies in The Cancer Genome Atlas accounting for over 30 types of cancer from 104,814 treatment-naive subjects, detecting cancer-specific microbial signatures (viral and bacterial) in the tissues and blood, and benchmarking signatures from microbial DNA from the plasma against cell-free tumor DNA. They, moreover, used deep metagenomic sequencing on plasma samples from 100 tumor patients vs. 69 healthy subjects and showed that cell-free microbial profiles were discriminatory between healthy and diseased subjects and between different types and stages of cancers. This was only possible for specific cancer types (colon, stomach adenocarcinomas and renal clear cell carcinoma), and the microbial signatures failed to differentiate intermediate cancer stages, suggesting that the microbial community structure might not be associated closely with cancer stages. They further trained a microbial source tracking algorithm and showed that the gut microbiome was most relevant for the bacterial signature in cancers of the gastrointestinal tract, further indicating that *Fusobacterium* spp. was overabundant in GI- vs. non-GI-tumors. That being said, the authors did not replicate the bacterial signatures found in HCC in other studies and relied on tissues more than blood samples, which were very often used as negative controls and did not generally show a differential microbial signature [24]. While bacterial signatures have been associated with cardiometabolic disease and cancer, this has been done in heterogeneous populations, and studies differed extensively in how they processed samples and what analytical tools were used, making it impossible to benchmark a 'meta-population blood bacterial signature' for cancer or cardiovascular disease. Currently, 'good old' circulatory tumor markers and imaging studies will need to suffice. It is desirable to standardize studies on blood bacterial signatures in several diseases and pair them with traditional disease assessment to apply them in the clinical context of prevention in the future.

Blood bacterial signatures derived therapeutic avenues

Targeting bacterial signatures in disease requires an understanding of how these signatures come about in the first place, whether they are a mirror of a common immunological denominator contributing both to metabolic health and increased susceptibility towards bacterial translocation, or whether translocated bacteria (alive or dead) are at the base of immunological cascades contributing to metabolic disease. Moreover, even if bacteria in the blood do not make up an ecological niche, bacterial functionality related to host-bacterial co-metabolism such as cholesterol degradation might still reflect the severity of cardiometabolic diseases. In this sense, consolidating a new therapeutic strategy might not directly require targeting bacteria in the blood but other upstream mechanisms associated with an increased bacterial translocation. Downstream strategies may ensue over time but are currently inaccessible due to the lack of informative mechanisms linking circulating microbial signatures to disease.

Examples for upstream strategies could be targeted at correcting altered gut microbiota via pro-, pre-, syn- or antibiotics, an impaired gut barrier, or the modulation of the crosstalk between the host and the microbiome by harnessing bacterial metabolites but also by targeting the bacterial metabolism of xenobiotics.

Elimination strategies such as via antibiotics

When it comes to antibiotics, studies in humans have been contradictory, showing that children treated with antibiotics are more likely to develop obesity and diabetes in a dose-dependent manner [86–88], while in mice at least, antibiotics treatment-induced improved gut barrier, insulin sensitivity and weight loss [89]. Furthermore, gram-positive bacteria-targeted antibiotic treatment of

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mice with systemic lupus erythematosus reduced bacterial growth in mesenteric lymph nodes and the liver [90].

From a clinical perspective, antibiotic treatment not seldom leads to a selection of pathogens such as *C. difficile*. Considering this, the lack of possibility to target only one specific strain/taxon or bacterial function, the increase of multiresistant pathogens, and lack of informative mechanistic studies, we believe the use of antibiotics to treat metabolic disease is currently questionable. Other strategies such as bacteriophage implementation, growth inhibition via bacteriostatic non-antibiotics, or even blood UV-Irradiation [91] might be alternatives but necessitate very nuanced testing for safety and efficacy. Moreover, these rather experimental approaches are hard to justify in metabolic disease, where pharmacotherapies with clinical evidence for hard outcomes are available.

Supplementation strategies via replenishing missing bugs

Fecal microbiota transplants (FMTs) FMTs has proven to be a valuable tool for the discovery of next-generation microbiota targeted therapeutics and has now been successfully implemented in the effective treatment of recurrent *C. difficile* either via colonic or duodenal infusion or in the form of oral frozen capsules containing fecal material from healthy donors [92, 93]. FMT, on the other hand, appears to only transiently improve insulin sensitivity and insulin secretion without ameliorating obesogenic phenotypes due to the failure of donor's microbiota to colonize the gut of the recipient's long-term [94, 95]. Interestingly, the application of FMT in newly diagnosed type 1 diabetes stabilized residual Beta-cell function mainly seen after autologous as compared to healthy donor FMT [96]. There are no studies proving a reduced translocation of bacterial fragments through the intestinal barrier after FMT. Alas, a case of bacteremia with a multiresistant bacteria with subsequent death has been recently described [97], encouraging the search for more targeted and safe strategies than FMTs in metabolic disease.

Supplementation of single bacteria to alleviate metabolic disease

Given the medical risk of FMTs and their short-lived success, there has been increasing focus on the therapeutical use of single taxa or consortia. *Akkermansia muciniphila* has received much attention, proving to alleviate obesity, insulin resistance, and increased gut permeability in mice [98]. Supplementation with pasteurized rather than live *A. muciniphila* mildly improved insulin sensitivity [99] (phase 1–2 Study) and improved gut barrier function via the thermostable outer-membrane protein Amuc_1100's interaction with TLR2 in the host [100]. Similarly, evidence of improved insulin sensitivity being associated with *Eubacterium hallii* (*Anaerobutyricum*) [101] after FMT and in mice [102] has led to a pilot study to investigate the effects of direct supplementation of *E. hallii* in humans, which implicated the supplementation in improving peripheral insulin sensitivity [103]. Similarly, *Faecalibacterium prausnitzii* is relatively increased in metabolic health [14, 104], which led to further studies to delineate potential beneficial effects on metabolism. While a microbial anti-inflammatory molecule derived from *F. prausnitzii* reduced inflammation in mice models of colitis via nuclear factor-kappaB inhibition [105], there is, to date, no direct evidence for an anti-hyperglycemic effect. Several bacteria could be considered in the quest for precision medicine in metabolic disease [106] but currently do not embed the notion of bacterial translocation as a clearly defined outcome.

Bacterial metabolites and postbiotics

Several bacterial metabolites have been shown to contribute to metabolic regulation in the host. Secondary bile acids, produced after hydrolysis of primary bile acids by the bacteria in the gut, seem to increase body expenditure and tissue-specific glucose [107–109] uptake as well as pancreas secretion of insulin [110]. But these effects have not been associated with bacterial translocation and are hard to track intracellularly considering the target farnesoid X receptor can be both activated and inhibited in different tissues with similar effects on the metabolism, suggesting further exploration of tissue-specific receptor pathways [109, 111]. On the other hand, short-chain-fatty acids are well-known microbial metabolites with pleiotropic effects on the host produced by bacterial fermentation of fibers [112]. Importantly, butyrate, observed to be ubiquity reduced in T2D (along with a decrease in butyrogenic groups [14, 113]), can directly reduce colonic inflammation by regulating interleukin-18 secretion in the epithelium and immunosuppressive T-cells [114] and hence maintain intestinal homeostasis and integrity via inhibition of histone deacetylase activity [114]. Several other metabolites have been clearly and robustly associated with metabolic diseases such as imidazole propionate in diabetes [115], trimethylamine N-oxide and tyrosine derived metabolites with cardiometabolic disease [116, 117] but also tryptophan-derived microbial metabolites, which improve inflammation and the gut barrier function [118, 119] as well as microbially produced polyphenols such as urolithin A, which have been shown to reduce increased gut permeability [120] and improve mitochondrial health in humans [121], motivating the identification and application of postbiotics (bacteria-derived non-living components) or other therapeutic strategies targeting deleterious bacterial metabolites.

In summary, there is a plethora of microbially based next-generation therapeutics which need to be further tested in humans in the specific condition of disease, where their actions could indeed be scrutinized. In which case, it would be helpful to analyze whether these treatments modify bacterial translocation and bacterial blood signatures while changing the course or severity of the metabolic disease. This will further substantiate the contribution of these signatures to disease and place them in a more strategic context for diagnostic or therapeutic implementation.

Important considerations: Contamination, biases in assessment and mitigation strategies

While the technological and analytical developments in the microbiome field can be seen as overall monumental for the scientific landscape, inspiring curiosity, leading the way into personalized medicine of both communicable and non-communicable diseases [121, 122], and helping relinquish established dogmas, low bacterial biomass samples such as the blood require careful considerations of pitfalls and measures to mitigate for several biases [121–123]. Specifically, these samples are highly prone to contamination from background environmental DNA and to technical biases, not

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the least of them being over-amplification during PCR [122–124]. The lack of standard operating procedures, benchmarked techniques, and best practices both on the experimental and analytical sides of research in working with low biomass samples has led to sensationalized science and wide controversy [125]. Hence, with increasing bacterial signature mining in low biomass samples, it is vital to unify experimental and analytical procedures aiming to overcome limiting biases, which should be fully understood and accounted for during study design, data interpretation but more importantly, during the development of diagnostic and therapeutic tools based on these results.

Technical aspects

Routinely, microbiome studies will usually consist of the following procedures before any analytical pipeline can be implemented: sample collection, DNA extraction, and sequencing library preparation (possibly with or without bacterial enrichment techniques such as culture [39, 126, 127] or bacterial DNA enrichment), high-throughput sequencing technologies [128] such as amplicon-based sequencing (e. g., 16 S rRNA gene), shotgun metagenomic sequencing [129], as well as RNA sequencing-based approaches [38]. Because procedural controls, which take contamination of samples during collection and cross-contamination during processing and sequencing into account, are rarely implemented, studies in low microbial biomass samples relying on sensitive metagenomic techniques highly confound and inflate the diagnostic implications for microbial contribution to disease [130]. Therefore, it is pivotal to identify the sources of contamination during sampling and laboratory procedures. These are 1) personal (doctors, nurses, doctor assistants, and technicians) and their direct 'personal cloud' [131], 2) the immediate environment of sampling, storage, and processing, and 3) laboratory reagents and equipment.

Accordingly, they can be mitigated: 1) contamination from a sampling/processing person could be reduced by wearing sterile masks, gloves, surgical headdress, surgical robes (for single use) and relying on automation where possible, which can be later better accounted for by correction for batch effects [132]. 2) Environmental contaminants are hard to control and account for, primarily because the sampling usually occurs in a different place from where the samples are stored and processed, and the environmental signatures of these locations can very well depend on the subjects working there [133]. Because these contaminants are most likely to be found in the air and on surfaces, it might be worthwhile to sample these environments during subject inclusion and processing times. 3) low biomass samples are most amenable for contamination during processing with lab reagents and equipment, which might still contain bacterial DNA even when they are 'bacteria free' with even the most promising kits for DNA extraction containing clear DNA signatures [124] and can overwhelm genuine bacterial signatures from the sampled tissue [134]. More interestingly and maybe less intuitively, low input DNA concentration can highly bias population levels because it results in overamplification of the template DNA, increases duplication rates, and favors AT-rich sequences [135], which does not, for example, occur in negative controls, leading hence to the overrepresentation of a particular species or even a contaminant and confounding population-level analyses. This requires a multi-pronged approach whereby a) reagents and equipment are subjected to UV radiation, b) controls (extraction blanks) from the sampling and laboratory environments, equipment and reagents are carried through the entire procedures as the actual samples, samples are randomized, and standard operating procedures are consistently followed during lab work with the inclusion of no-template amplification (possibly by the same subjects for a specific project) and c) bioinformatics approaches are included to assess contamination [136, 137], track its source [138, 139], account for it, and remove it [139]. Similarly, reviewers, editors, and readers should be versed when looking at studies analyzing the impact of bacterial signatures in disease. For the interested experimenter and reader, we wholeheartedly recommend reviewing the RIDE-criteria and the recent publication by Eisenhofer et al. [130, 140].

Scientific aspects

While this area of research has immense untapped potential, it is vital to avoid methodological pitfalls, which would lead to an inevitable waste of time, money and highly tarnish the credibility of the research. Moreover, it would be important to address limitations in the design and interpretation of current research. For example, while comparing studies, it is essential to understand the effect of the matrix from which the bacterial signature is derived: is it plasma, whole blood, or peripheral blood mononuclear cells? The distribution of microbial DNA between compartments of the blood is not clear. This information is important because the matrix can highly influence technical procedures such as PCR efficiency. This has overarching implications pertaining to the source and the physiological role of the bacteria/bacterial sequences. Similarly, studies, which controlled and corrected for contamination, did so very differently, making the comparison between them non-conclusive. It will therefore be important to establish an optimal method to control for contaminants that goes beyond complete subtraction of flagged taxa. Specifically, a prevalent risk with stringent decontamination is that real signals reflecting commensal, tissue-specific microbial communities and concomitant predictive microbial profiles may be discarded. Poore et al. showed, for example, that a stringent filtering approach might be desirable with the possible downside, if scalable/universal, to preclude biologically relevant and informative results [24].

It is also safe to say that most works in this area currently fail to address causality and rely on correlation. While correlational observations are an important steppingstone and have often been the beginnings of great stories, they remain exactly that: the beginning. Hence, it is important to address outstanding questions to inform future research and applications. Some of these questions pertain to a) whether sequences are indeed cell-free bacterial DNA or come from bacteria that are either dead, alive, or dormant, b) whether bacteria in the blood occupy a real ecological niche, c) the source of the bacterial DNA or bacteria for which examining primary tissue specimens along matched gut epithelia and sampled orifices will be pivotal, and d) the tissue selectivity of bacteria, which could potentially be informative.

While microbial DNA has the potential of inherent pathogenicity because it activates TLR9 in its unmethylated form resulting in inflammatory cascades [141, 142], the critical question to answer is whether microbial DNA in itself is a pathogenic finding, whether

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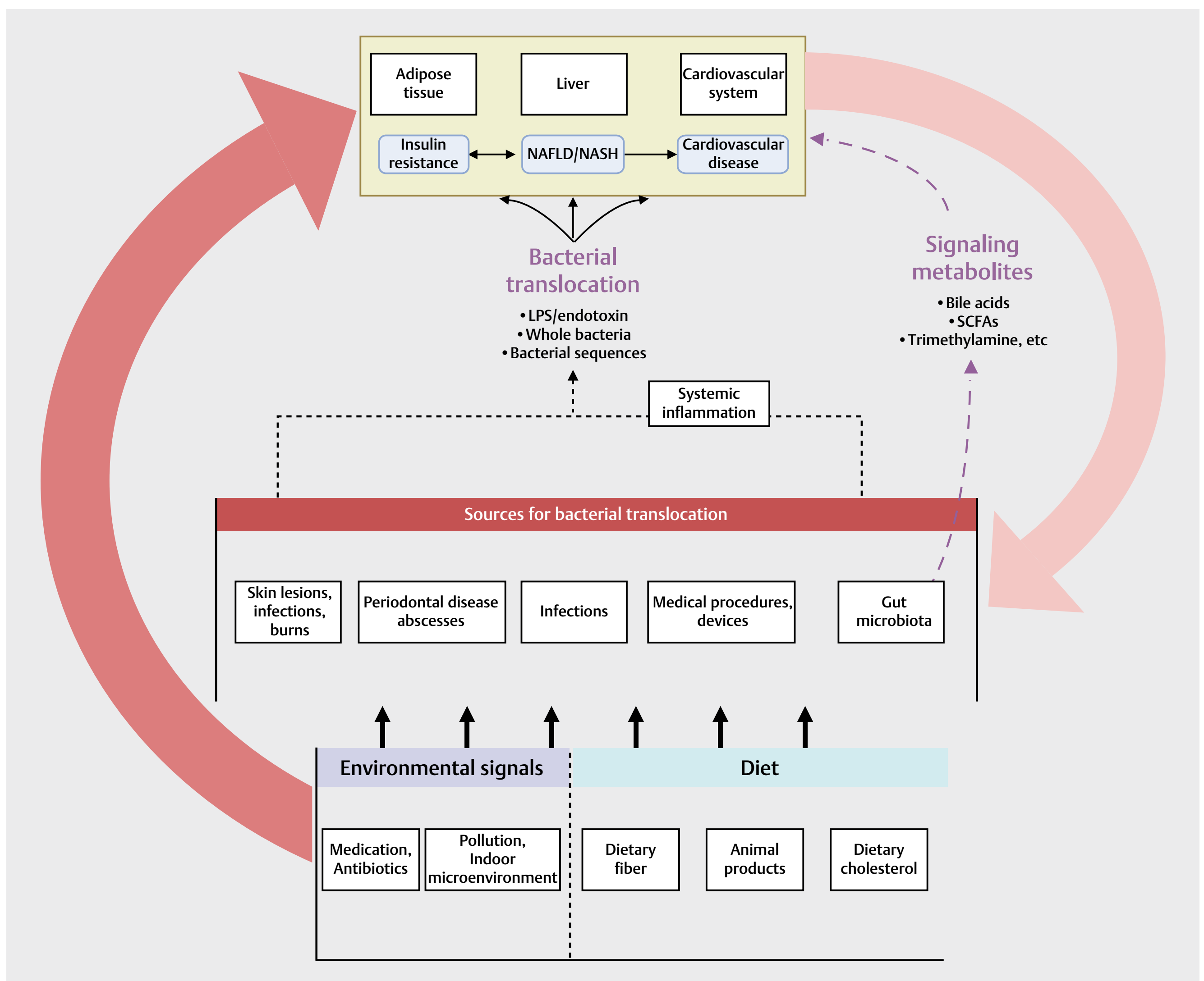
it is an epiphenomenon that reflects overall or specific disease burden or whether both metabolic disease and bacterial signatures are epiphenomena of lurking immunological conditions or active infections (▶ **Fig. 3**), which we yet have to be identified because our tests lack sensitivity.

In moving towards mechanisms and granular interrogation of causality, it will also be crucial to understand whether the entire bacteria, bacterial cell components, or metabolites are most important for the postulated effects of the identified microorganisms. A question that lends itself to further exploration concomitantly is whether the observed taxa are alive or not. A drawback of culture-independent sequencing methods is their inability to differentiate living from dead bacteria. This is particularly important because DNA, including that from contaminants, can persist in the environment. More importantly, while DNA sequencing and functional annotation can predict bacterial metabolism, only viable bacteria can undertake active metabolic processes. Therefore, methods that do not relay viability information will always overestimate the importance of bacterial metabolism in the host metabolic response, as long as they are not coupled with information on transcription of metabolic modules or direct measurement of metabolites.

Hence, the application of methods that interrogate active metabolism and delineate viability is warranted. Techniques exploring viability can characterize one or several viability aspects, such as the presence of an intact membrane, the replication of genetic material (via RNA detection), and the detection of metabolism or energy. The main challenge being, whether these methods are compatible with next-generation sequencing and whether they are applicable to low-biomass samples.



▶ **Fig. 3** Bacterial **translocation** can be derived from several **sources** such as the skin, the oral cavity, the gut, clinical infection sources, medical procedures including dwelling catheters, and contamination/infection from a hospital stay. The **translocation** can include endotoxins, whole bacteria, or bacterial sequences leading to systemic and local tissue inflammation and culminating in local tissue dysfunction such as insulin resistance in adipose tissue, liver dysfunction, or cardiovascular disease. Similarly, signaling metabolites from the gut can contribute to cardiometabolic disease and specific tissue dysfunctions, which in turn can aggravate bacterial translocation by increasing susceptibility to colonization and infection and increased 'leakiness of the gut.'

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Microbial culture caters to both these needs and is the gold standard to prove viability because it displays the ability of bacterial cells to divide and metabolize nutrients. On the other hand, several bacterial strains are painstakingly hard to culture or require immense optimization to determine optimal culturing conditions. Others are viable but non-culturable because they are either damaged or maintaining the structural and metabolic properties observed or postulated in their environment of origin is not possible [143]. The application of propidium iodine, propidium monoazide (PMA), and ethidium monoazide – all dyes that can bind to DNA in membrane compromised cells and extracellular DNA- enable the labeling of dead bacteria and can be coupled with several other techniques, including epifluorescence microscopy, flow cytometry, but also qPCR, digital PCR and, metagenomics among several other downstream techniques [144]. PMA seems to be more selective and less cytotoxic than other methods, and several of its selectivity drawbacks are known (stains viable cells for some species while failing to stain dead cells from other species). More importantly, it can be used for low-biomass samples, assists in the removal of contaminant DNA (environmental or introduced by the reagents including PCR reagents) [124], has the potential to enrich for rare microbial community members [124, 145], and allows for qPCR quantification to distinguish the viable from the total fraction of microbial cells in several settings [146] (reviewed in [144]). Unfortunately, the samples are required to be in an aqueous solution, and ethylenediaminetetraacetic acid (EDTA, found in specific blood collection tubes) can affect dye permeability through membranes [147], limiting the application of this method to other human low biomass samples such as the blood. Other methods such as RNA analyses (e. g., metatranscriptomics) enable quantification and identification of potentially 'active bacterial community members' and can delineate taxa as well as acute responses to stimuli relatively early on because RNA has a very short half-life [148]. Unfortunately, RNA yield is highly dependent on the matrix it derives from and the preservation method employed until RNA extraction is undertaken [148, 149]. Moreover, significant RNA losses have been described during sample preparation, which is particularly problematic in low-biomass samples, where yield is low to begin with [150, 151]. Similarly, metaproteomics can also be applied for the detection of active bacteria. Coupled with metagenomics, it can enable the identification of active metabolic pathways of bacterial communities and connect them to substrate availability and environmental conditions. A drawback is that only proteins with exact matches in the search databases can be identified. Another drawback is the potential persistence of proteins in specific environments.

Various techniques are available and allow their application in conjunction with several other methods to detect viability, phylogeny, and metabolic activity, lending themselves well to studies assessing the contribution of bacterial cells to overall host health. Several bottlenecks remain, most prominently the necessity for extensive optimization in a setting where human tissue is a matrix, the low throughput of these methods paralleled with the high-cost and the need for careful result interpretation, making this a challenging task but an excellent opportunity for collaboration between groups across the scientific and medical spectrum.

Conclusions

Physicians are familiar with cases where viruses induce tumors (such as. Kaposi-sarcoma) or associate with metabolic disease (such as T1D) and are clinically versed in bacteria-induced rheumatic complications (such as heart rheumatism and Reiter syndrome). More importantly, comprehensive contamination-aware analytical approaches employed in large-scale studies have established a robust link between bacterial translocation to human body compartments such as the blood or adipose tissue and cardiometabolic diseases. This prior knowledge and emerging and converging evidence make it difficult to categorically deny the presence of bacteria and bacterial DNA in the blood and the potential of this genetic material (whether alive or dead) to act as an immunostimulatory factor thus triggering or influencing the course of non-communicable cardiometabolic diseases.

A word of caution is still warranted: while the existence of a tissue bacterial signature for the blood seems likely, considering the presence of comparable taxa in independent studies even in health [26], we believe the presence of a stable, non-pathogenic, highly complex microbial community such as the one we see in the gut rather unlikely, because it is neither consistently detectable [152], nor is it highly prevalent [21–23, 125], nor logical based on ecological principles.

Considering recent advances in analytical and bioinformatics tools coupled with observations from clinical studies within intercontinental research consortia, the emerging area of tissue microbiome research is still in its infancy but promises novel prognostic, diagnostic, and therapeutic strategies with paradigm-shifting potential for health care and disease prevention and treatment. To be able to move into translational avenues, our efforts will need to move beyond pure associations towards functional approaches to understand causative chains better, i. e., define causes and consequences in the highly complex interplay between blood bacterial signatures and metabolic diseases.

Conflict of Interest

The authors declare that they have no competing interests.

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