

Evaluating Causality of Gut Microbiota in Obesity and Diabetes in Humans

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ABSTRACT The pathophysiology of obesity and obesity-related diseases such as type 2 diabetes mellitus (T2DM) is complex and driven by many factors. One of the most recently identified factors in development of these metabolic pathologies is the gut microbiota. The introduction of affordable, high-throughput sequencing technologies has substantially expanded our understanding of the role of the gut microbiome in modulation of host metabolism and (cardio)metabolic disease development. Nevertheless, evidence for a role of the gut microbiome as a causal, driving factor in disease development mainly originates from studies in mouse models: data showing causality in humans are scarce. In this review, we will discuss the quality of evidence supporting a causal role for the gut microbiome in the development of obesity and diabetes, in particular T2DM, in humans. Considering overlap in potential mechanisms, the role of the gut microbiome in type 1 diabetes mellitus will also be addressed. We will elaborate on factors that drive microbiome composition in humans and discuss how alterations in microbial composition or microbial metabolite production contribute to disease development. Challenging aspects in determining causality in humans will be postulated together with strategies that might hold potential to overcome these challenges. Furthermore, we will discuss means to modify gut microbiome composition in humans to help establish causality and discuss systems biology approaches that might hold the key to unravelling the role of the gut microbiome in obesity and T2DM. (*Endocrine Reviews* 39: 133 – 153, 2018)

The global rise in prevalence of obesity presents an unprecedented challenge to public health and economies of today's world. Obesity has been associated with a plethora of metabolic disturbances including dyslipidemia and insulin resistance; both are considered major risk factors for development of cardiovascular disease (CVD), nonalcoholic fatty liver disease, and several forms of cancer. Obesity therefore is considered one of the greatest public health threats of the 21st century (1). Factors that strongly contribute to the obesity epidemic include decreased physical activity and increased (high-caloric) food intake. However, if the pathogenesis of obesity would have been this simple, Hippocrates' prescription for treatment of obesity: "eat only once a day and take no baths and sleep on a hard bed and walk naked as long as possible" would have been a successful prescription (2). Unfortunately, treatment (and prevention) of obesity and obesity-related complications have been

proven to be more complex. Despite extensive efforts in the field, successful strategies to tackle this pathology are still limited. The need to mechanistically unravel development of obesity and obesity-related disease is therefore high and crucial for development of novel, effective treatment strategies.

The rise in prevalence of obesity coincides with the prevalence of type 2 diabetes mellitus (T2DM), which is a leading cause of CVD in almost all high-income countries (3). It has been estimated that by the year 2040, a staggering 642 million people will suffer from this disease worldwide (3). Numerous researchers have dedicated their careers to unraveling pathophysiological pathways that underlie the development of T2DM in obesity. In 2009, DeFronzo introduced a then-new paradigm in diabetes research: the ominous octet (4). This paradigm describes that in addition to muscle, liver, and β -cells [triumvirate (5)], adipocytes, the gastrointestinal tract, α -cells, kidney,

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ESSENTIAL POINTS:

- Interest in and identification of the role of the gut microbiome in modulation of host metabolism has grown exponentially since the introduction of affordable, high-throughput sequencing technologies
- A complex interplay between ethnicity, host genetics, dietary habits and (history of) medication use plays an important role in shaping the microbial community
- Bacterial metabolites and bacteria-derived components might be important modifiers of human metabolism
- An intriguing interplay between genetics, altered gut microbiome/metabolites, and immunity might play a role in the development of T1DM
- Early intervention in patients who are at high risk to develop obesity and/or T2DM might benefit from gut microbial modulation in a personalized approach, such as microbiota-based dietary strategies or personalized FMT
- A systems biology approach with combined input of different omics data sets will accelerate our understanding of the contributions of the microbiome to human health and metabolic disease
- Prospective and intervention studies in large human cohorts combined with dedicated mechanistic studies in model systems are required to understand if and how gut microbiota affects metabolic disease development

and brain all play important roles in the development of T2DM. The dogma also describes the complexity of development of T2DM: numerous determinants drive disease development; however, the hierarchy of these driving factors remains largely unknown. Additionally, determinants other than those described in the “ominous octet” might play a role in the development of T2DM. In the past decade, the gut microbiome has been identified as a novel, potentially driving, factor in the pathophysiology of T2DM.

In addition to T2DM, the incidence of type 1 diabetes mellitus (T1DM) is rapidly increasing worldwide as well (6, 7). Genetic predisposition or children being born from genetically susceptible mothers cannot simply explain this phenomenon (8). The disproportionate increase in T1DM incidence has therefore largely been attributed to environmental influences such as early enterovirus infection (9). In addition, the clinical onset of T1DM is usually preceded by years of enhanced systemic inflammation and augmented autoimmunity that associate with shifts in gut microbial composition (10). The gut microbiome has therefore been put forward as driving force in pathogenesis of T1DM.

Interest in and identification of the role of the gut microbiome in modulation of host metabolism has

grown exponentially since the introduction of affordable, high-throughput sequencing technologies. These technologies allowed for compositional as well as functional analysis of intestinal microbiota in humans and mouse models. Murine models have provided crucial insight in determinants of gut microbiome composition and the role of the gut microbiome in health and disease. Although studies performed in murine models support the hypothesis that the gut microbiome might play a causal role in development of obesity and diabetes, data showing causality in humans are still scarce.

In this review, we aim to provide insight in (the quality of) evidence that is supportive of a causal role for the gut microbiome in obesity and diabetes development in humans. We will elaborate on factors that drive microbiome composition in humans and discuss possible mechanisms through which the gut microbiome and microbial metabolites affect host metabolism. Challenging aspects in determining causality in humans will be postulated together with strategies that might hold potential to indeed assess a driving role for gut microbiota in metabolic disease development. Furthermore, we will discuss means to modify gut microbiome composition in humans to help establish causality and discuss systems biology approaches that might hold the key to unravel the role of gut microbiota in obesity and diabetes.

Factors Shaping the Gut Microbiota

The human gut microbiota is a complex ecosystem consisting of an estimated 10^{14} bacteria (11). This number equals the number of human cells (12). The combined genetic material of the gut microbiota, collectively called the gut microbiome, exceeds the human genome ~100 times (13–15). The gut microbial community is dominated by five bacterial phyla: Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria, and

Verrucomicrobia. Variable pH (pH increases from proximal to distal intestine) and oxygen concentration (decreases from proximal to distal intestine) affect both relative and absolute abundance of bacteria across the gastrointestinal (GI) tract. The proximal GI tract is enriched in bacteria belonging to the phyla Firmicutes and Proteobacteria and the genus *Lactobacilli*, whereas the distal GI tract mainly comprises bacteria belonging to the phyla Bacteroidetes, Firmicutes, and the Akkermansia muciniphila species (16).

Although a definition of what a healthy gut microbiome comprises still has to be defined, it is clear that in healthy individuals, the composition of the intestinal microbiota is highly diverse (17). Interestingly, together with increased industrialization, an overall decline in gut microbiota diversity can be observed (18). This decline likely is a consequence of modern lifestyle and driven by introduction of new medication and increased availability of processed foods. Importantly, gut microbial composition is highly variable between individuals and is continuously modified by both endogenous and exogenous factors. This interindividual variability already starts at birth and is mainly determined by the microbiota composition of the mother (19). Interestingly, the intestine of a newborn is not sterile; it has been suggested that intrauterine exposure to the mother's microbiome is one of the first shaping factors of the gut microbiome (20). The intestine is further colonized by bacteria as soon as the amniotic fluid disappears (21) and is predominantly determined by the mode of delivery. Children born through natural (vaginal) delivery have a gut microbiota composition resembling the vaginal microbiota composition of the mother. Children born through Caesarean section on the other hand, have a gut microbiota composition that resembles the skin microbiota composition of the mother's (22). These first determinants of gut microbiota composition persist for months, potentially even longer (23). Whether differences in gut microbiota this early in life affect disease development later in life remains to be determined.

A recent study that combined whole genome sequencing (WGS) with 16S rRNA sequencing showed that there are significant interindividual differences in gut microbial diversity and richness depending on age and ethnicity of the host (24). In addition to interindividual changes in microbiota composition, functional analysis of the gut microbiota of children and adults indicated age-related differences in the abundance of genes involved in amino acid metabolism, lipopolysaccharide (LPS) biosynthesis, RNA degradation, and steroid hormone biosynthesis (25). Although these results indicate that gut microbiota output differs between subjects in an age-dependent manner, these results have to be interpreted with caution. WGS is indicative only of potential functions but does not assess the actual gene expression levels. Rather, these results indicate that age and ethnicity are associated with differences in functionality of the gut microbiome.

One of the most important modulators of the gut microbiota is diet. Intervention studies in humans have revealed the extent to which the microbiota can be modulated by dietary changes (26, 27). The influence of diet on gut microbiome composition and functionality can be described in three different themes (28). First, the response of the gut microbiota to (major) changes in dietary composition is very fast.

Several studies have shown an acute shift in gut microbial composition and functionality as soon as 2 days after the start of a dietary intervention (26, 27). Switching from plant- and meat-based diets to a diet with a daily add-on of 30 g of dietary fibers induced both compositional and functional changes in the gut microbiota (26). In addition, compositional and functional changes were observed in subjects who followed either a high-fiber, low-fat or low-fiber, high-fat diet for 10 days (26, 27). Second, despite rapid changes in composition and function following (major) changes in dietary composition, long-term dietary habits are required to induce major changes in gut microbiota composition. This is most clearly exemplified by observations that certain microbial taxa found in traditional populations that stick to a plants-only diet [unique abundance of bacteria from the genus *Prevotella* and *Xylanibacter*] are absent in Western populations (29). Furthermore, several studies have shown acute effects of diet on microbiome composition soon after the start of a dietary intervention but failed to show major changes at later time points. One study, for example, reported that dietary intervention for 10 days did not mediate major compositional changes in gut microbiota composition, whereas changes were detectable 24 hours after the start of the dietary intervention (27). Third, there is high interindividual variability in response of microbiome composition to changes in dietary composition (30–32). The fact that dietary interventions to treat obesity have variable effects could therefore potentially be due to differences in microbiota composition at the start of the diet (33). Increased intake of fibers and decreased total caloric intake have been shown to increase microbial diversity in subjects with low microbial gene richness, at baseline. In contrast, subjects with high microbial gene richness at baseline remain unaffected by this dietary intervention (31).

Medication also significantly influences the gut microbial composition. Antibiotics treatment in particular, is well-known for influencing the gut microbiota (34, 35). Moreover, antibiotics use early in life has been associated with weight gain later in life (36). A recent study showed that oral antibiotic treatment leads to specific expansion of Firmicutes (37), which might have unfavorable effects since an increased abundance of Firmicutes has been associated with obesity (38) and T2DM (39). A single-blinded randomized controlled trial in 20 male obese subjects who received either vancomycin or amoxicillin for 7 days showed that vancomycin-treated subjects had significantly decreased peripheral insulin sensitivity compared with amoxicillin-treated subjects (40). Vancomycin treatment, which specifically eradicates gram-positive bacteria, shifts the gut microbial community to a community dominated by gram-negative bacteria, which might negatively affect host

metabolism, including insulin sensitivity. In another study, however, these metabolic effects of vancomycin treatment were not observed (41). In this randomized double-blind, placebo-controlled trial, 57 obese human subjects were treated (oral) with vancomycin, amoxicillin, or placebo for 7 days (41). Amoxicillin treatment did not significantly affect microbiota composition, whereas treatment with vancomycin had major impact on microbial diversity and composition with a decrease of gram-positive bacteria and a compensatory increase in gram-negative bacteria. Although this was accompanied by changes in microbiota-mediated metabolic processes [*i.e.*, reduced conversion of primary to secondary bile acids and reduced production of short chain fatty acids (SCFAs)], insulin sensitivity, energy metabolism, and systemic low grade inflammation were unaffected (41). The discrepancy in metabolic outcome in these two studies is potentially due to differences in fecal bacterial richness at baseline. In a recent study from our group, we show that microbiota composition at baseline is indeed an accurate determinant of the effect of fecal microbiota transplantation (FMT) on insulin sensitivity. We were able to predict with high accuracy if obese, insulin-resistant recipients should be categorized as responders or nonresponders following an allogenic FMT from a lean donor (42). The fact that FMT-mediated improvements in metabolism do not sustain in recipients with high bacterial diversity at baseline (prior to FMT) can be attributed to the presence of a highly personal core microbiome (43). In this subset of recipients, intestinal microbiota composition following FMT quickly returns to baseline composition, potentially driven by resilience of the host immune system (44). It cannot be excluded, however, that lifestyle (particularly dietary habits) of these subjects plays a large part in this phenomenon. Nevertheless, higher bacterial diversity is likely accompanied by a more pronounced personal core microbiome composition that is difficult to change with FMT or antibiotics treatment. Therefore, microbiota-mediated effects on metabolism following FMT or antibiotic treatment are more challenging and less sustainable in subjects with high bacterial diversity.

Metformin is currently the most prescribed oral antidiabetic medication and known to affect intestinal microbiota composition (45, 46). In a recent double-blind, placebo-controlled trial in patients with T2DM, it was indeed shown that metformin-treated subjects had significantly altered gut microbiome composition compared with patients receiving placebo (47). Interestingly, germ-free (GF) mice that received an FMT from metformin-treated subjects had improved glucose tolerance compared with mice that received an FMT from placebo-treated controls suggesting that metformin-induced changes in gut microbiome

composition mediate part of the beneficial effects of this drug on glucose homeostasis (47). It has been suggested that the beneficial effects of metformin are, at least in part, mediated by the production of SCFAs by the gut microbiota (46). Functional shifts in LPS biosynthesis and SCFA metabolism in patients treated with metformin were observed (46). Interestingly, known adverse events of metformin such as diarrhea, nausea, vomiting, and bloating were associated with a relative increase in abundance of *Escherichia* species (46). In a recent study assessing the gut metagenome in fecal samples of 748 human subjects with and without T2DM, it was shown that metformin is a strong confounding factor in metagenomic analysis (46). Thus, when assessing microbiota composition in T2DM subjects, it is of critical importance to correct for metformin use.

Proton pump inhibitors are frequently used oral antacid medication that have also been implicated to modulate gut microbiota composition (48). Although fecal microbial diversity did not change significantly, certain taxa known to have high potential to overgrow (*e.g.*, *Clostridium difficile*) were increased after 4 weeks of omeprazole (40 mg/d) treatment. These results suggest that proton pump inhibitor treatment might predispose to *C. difficile* infection (48).

The role of human genetics in shaping the composition of the gut microbiota remains largely associative. A recent study in monozygotic twins suggested heritability of a number of microbial species (49), in part based on the association between the human gene locus that encodes lactase and the *Bifidobacterium* genus. Other associations between human genetic make-up and microbiome composition were found in genome-wide association studies in which genetic loci, microbial taxa, and functional pathways were linked (50–52). Recently, a novel, computational method applied on cross-sectional data sets from two large metagenomic studies was used to investigate regulatory factors driving individual microbial composition (53). Interestingly, it was suggested that gut microbial composition, at least at the species level, was independent of host genetics (53). This conclusion challenges the assumption that along with host genetics, host immunity has a smaller role in shaping the gut microbiome than was previously considered. If true, this conclusion will have major influence on the development of successful generic procedures and products to manipulate microbiota composition (54).

Although the hierarchy of factors that drive gut microbial composition remains largely unknown, it is evident that a complex interplay between ethnicity, host genetics, mode of delivery, dietary habits, and (history of) medication use all play an important role in shaping the microbial community. We will discuss in the next paragraphs available evidence that implicate a role for the gut microbiota in development of metabolic diseases such as obesity and diabetes in humans.

Gut Microbiome Composition and T2DM Development in Humans: Why It Is Challenging to Determine Causality in Humans

Interest in the role of the gut microbiota in development of metabolic disturbances such as obesity and T2DM in humans has risen substantially over the past decade. This is in part due to the introduction of novel and more affordable next-generation sequencing techniques combined with increased availability of fecal samples and tissue biopsies obtained from human subjects. Nevertheless, studies reporting a link between gut microbiome composition and metabolic disease development in humans are still largely associative/correlative in nature and mostly based on the differences in relative abundance of bacterial strains in the accessible fecal compartment. Furthermore, reproducibility of results from studies in humans has been shown to be fairly low (55), which challenges a one-directional interpretation of the role of the gut microbiota in disease development. Discrepancies in study set up, geographical location of sample preparation, and inconsistencies in data analysis all play part in low reproducibility. Full transparency of study details including open access to methodology and raw data in online repositories and collaborative initiatives between research groups (e.g., exchange of samples and analysis on different sites) will enhance the reproducibility of data in the field.

Scarcity of biopsies or luminal/mucosal material from proximal parts of the intestine leaves the microbial composition and function of this important part of the GI tract relatively unexplored. In addition, gut microbiota composition has been mainly linked to clinical parameters obtained from observational (retrospective) studies. Often times, it cannot be concluded if gut microbiota composition was affected prior to disease development (causal) or whether the microbiota composition is a reflection (consequence) of the disease itself. This chicken-egg situation can in part be clarified in large, prospective studies such as the Dutch Life Lines (56) and HELIUS (57) cohorts. Although prospective studies will provide insight in the timeline of disease development linked to changes in gut microbiota composition, a causal contribution (*i.e.*, microbiota as driving factor for disease development) can only be concluded from intervention studies. However, controlled intervention studies with significant effect on microbiota composition in humans are rare and have thus far been limited to FMT, antibiotic treatment, diet, and probiotic therapy. Although FMT in particular holds potential to serve as efficient intervention strategy to study causality in humans (58), other intervention studies in humans have thus far shown limited causal evidence for a role of the gut microbiota in metabolic disease development (41). A top-down approach to determine a causal role of the gut microbiome in the development of (cardio)metabolic disease is presented in Fig. 1.

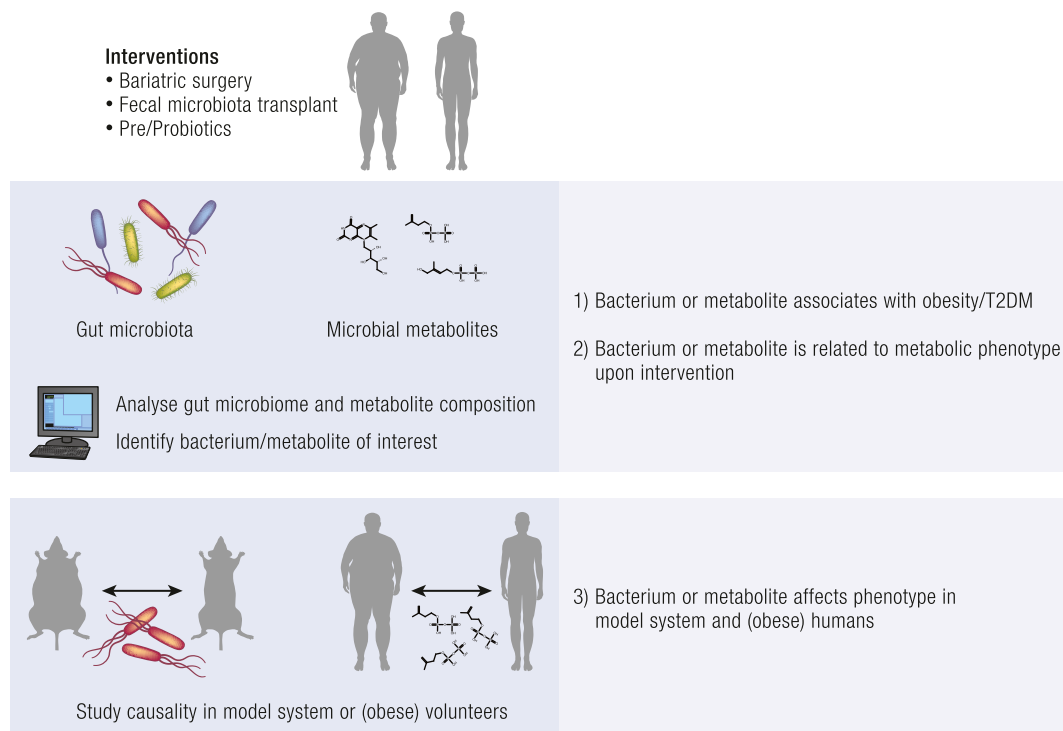
Causality: insight from studies in mice

Causal evidence that link the intestinal microbiota to host health and development of metabolic disease mostly originates from rodent studies (59). The GI tract of humans and mice are anatomically, genetically, and physiologically quite similar. Composition of sectional tissue of small and large intestine from mice resembles sections from humans. In addition, Goblet and Paneth cells fulfill the same unique role in intestinal integrity and host-microbiota equilibrium in both humans and mice (60). Nevertheless, important differences exist and therefore, care must be taken to draw direct parallels between mice and human studies. An important difference between human and mouse GI tract is that the mouse cecum is relatively large in comparison with the size of the total GI tract. Moreover, the cecum is an important site for fermentation in mice. Increased fermentation capacity in mice significantly affects gut microbial diversity, composition, and functionality (60). In contrast, the human cecum, is relatively small and does not have a clear function (61). Genetic background is one of the main drivers of the metabolic phenotype in mice, whereas in humans, obesity and insulin resistance are driven by a complex interaction of genetics, diet, and lifestyle (4). Furthermore, and in sharp contrast to most human studies, mouse studies can be strictly controlled to minimize confounding factors that often times complicate data interpretation in humans (e.g., food intake, dietary composition, history of medication use). The ability to genetically modify mice provides valuable mechanistic insight in how the gut microbiota affects host metabolism and augments metabolic disease development.

Studies in GF mice, which lack microbiota, provided first important evidence that the gut microbiota potentially plays a causal role in development of obesity and -related diseases. It was demonstrated that, despite a higher food intake, GF mice are leaner compared with conventionally raised mice (62). In addition, GF mice are fairly resistant to HFD-induced obesity (63). GF mice allow for generation of gnotobiotic models: GF mice colonized with a specific microbe of interest or harboring a strictly defined microbial community. FMT using a fecal transplant from conventionally raised mice, increased body fat by 60% and reduced food intake in GF recipients (62). The gut microbiota thus increases the ability to derive energy from food (particularly from indigestible carbohydrates) thereby fueling energy metabolism of the host. A follow-up study, in which fecal microbiota was transplanted from conventionally raised obese mice to GF mice further accelerated establishment of a causal role for the gut microbiota in development of obesity (38). Interestingly, GF recipient mice that received a transplant from an obese donor gained more weight on the same diet compared with recipients that received a transplant from a lean donor. These results

"It was demonstrated that, despite a higher food intake, GF mice are leaner compared with conventionally raised mice."

Figure 1. How to determine causality of the gut microbiome in cardiometabolic disease: a top-down approach to determine a causal role of the microbiome/microbial metabolite in obesity and T2DM in humans. (1) Microbiome and microbial metabolite composition is determined in lean and obese/T2DM subjects. Bacteria or metabolites of interest have to associate with these conditions. (2) Following interventions that impact on microbiome composition of functional output and affect metabolic phenotype of the host, the bacterium/metabolite of interest has to correlate with the changes in phenotype. (3) Bacterium or metabolite of interest accelerates or improves phenotype in a model system (e.g., mouse models for obesity and T2DM) and in healthy or obese, insulin-resistant volunteers, respectively.



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suggested that the microbiome of obese mice harvest more energy from dietary components. Additionally, these data implicated that an obese phenotype can be transferred from donor to recipient indicating causality. In line, a study where fecal microbiota from twins discordant for obesity was transplanted to GF mice showed that recipients of the fecal microbial transplant from the obese cotwin gained significantly more weight gain compared with counterparts that received a transplant from the lean cotwin (49). Although studies in GF mice have provided crucial insight in the contribution of the gut microbiota to host metabolism, there are substantial differences in metabolism of GF vs conventionally raised mice (60). For example, GF mice have the tendency to consume more calories, excrete more lipids, and weigh less than conventionally raised mice (64). Importantly, lack of microbiota has significant consequences for maturation and capacity of the immune system and intestinal physiology (65). Because immune system and intestinal function are crucial players in development of (microbiome-mediated) obesity and T2DM, results obtained from GF mice should be interpreted with some restraint.

Despite convincing evidence from studies in mice, data implicating a causal role for the gut microbiota in

obesity development in this model system cannot be projected on humans. To exemplify difficulties in interpreting mouse and human data and to underscore the challenges of translational research approaches, a recent study in mice reported that a membrane protein of the mucin-degrading bacterium *Akkermansia muciniphila* improved obesity and T2DM (66). This was conflicting with a study in humans where both *Akkermansia muciniphila* and *Desulfovibrio* were enriched in samples of patients with T2DM (39) thus underscoring the question whether decreased relative abundance of specific strains is a driving factor or merely a reflection of the disease. It is therefore relevant to ask where we currently stand in our understanding and evidence of the role of the gut microbiome in cardiometabolic disease development in human disease and look into strategies to tackle these challenges.

Gut Microbiome Composition and Function in Cardiometabolic Disease Development: Evidence From Human Studies

In line with studies in rodents, an increased ratio of Firmicutes/Bacteroidetes, which reduces with weight

loss (67), has been associated with obesity in humans. Increased abundance of Firmicutes was suggested to extract more energy from food (38). In contrast, other research groups were not able to find differences in the ratio between Firmicutes/Bacteroidetes in obese vs lean subjects (68, 69). It is important to point out that technical difficulties and methodological discrepancies have been suggested to facilitate underrepresentation of bacterial groups, in particular of *Bacteroides* (70), thereby incorrectly indicating affected abundance between phyla. Furthermore, despite interesting findings on differences in the Firmicutes/Bacteroidetes ratio, it remains to be determined if this is a reflection of dietary intake or a driving factor of obesity. The relevance of these findings is therefore debatable.

Insulin resistance precedes development of T2DM and several metabolic markers thereof have been associated with *Lactobacillus* and *Clostridium* species (45). Fasting glucose and HbA_{1c} levels showed a positive correlation with *Lactobacillus*, whereas *Clostridium* showed a negative correlation with these parameters (45). Additionally, it has been shown that patients with T2DM had reduced abundance of bacteria that produce the presumably beneficial SCFA butyrate (39).

Interestingly, in three (independent) metagenomic studies (17, 32, 71), obesity was associated with a reduced bacterial gene richness. Subjects with a less diverse gut microbiota composition were shown to have higher body mass index (BMI), increased fat mass, reduced insulin sensitivity, dyslipidemia, and increased markers of inflammation (17). In addition, low bacterial richness was predictive for weight gain in a 10-year follow-up in which subjects with low bacterial richness had gained more weight compared with subjects with higher bacterial richness. As is the case for reported associations between improved ratio of Firmicutes/Bacteroidetes following weight loss, it remains to be determined if increased bacterial richness is a mere reflection of a healthy and varied diet, or that it directly contributes to the protection from obesity. Nevertheless, bacterial richness was simultaneously reported to have predictive potential for dietary interventions aiming to lose weight (31). Metagenomics studies should be interpreted with caution, because a recent meta-analysis indicated that the reproducibility of metagenomics studies in humans is limited (72). The authors concluded, after pooling data sets from several separate studies (67, 71, 73), that there was no association between BMI and taxonomic composition.

Bacterial metabolites and bacteria-derived components as modifiers of human metabolism

Changes in gut microbial output (metabolite production) or host exposure to bacterial-derived components (*e.g.*, endotoxin) have been suggested to play a larger role in metabolic disease development than

microbial composition on the genome level *per se* (74). A chronic, low-grade inflammatory state is often found in patients with obesity, insulin resistance, and T2DM (75). This increased inflammatory state has been proposed to be a driving factor in development of insulin resistance. In particular, by reducing insulin sensitivity in muscle and adipose tissue (75) and by impairing pancreatic islet function (76). Although increased inflammatory tone in obesity is likely driven by multiple factors, studies in mouse models indicate that the gut microbiota is a causal factor in increasing inflammatory tone in obesity (77–79). These findings lead to the hypothesis of metabolic endotoxemia in obesity and T2DM; a low-grade inflammatory state resulting from translocation of toxic, bacteria-derived components of mainly gram-negative bacteria (*e.g.*, endotoxin) (77). Significantly higher concentrations of LPS have indeed been measured in plasma from patients with T2DM compared with nondiabetic subjects (80–82). Nevertheless, the risk of exogenous LPS contamination during analysis remains a topic of debate (83). Blood from the GI tract drains into the portal vein; highest concentrations of LPS are therefore likely to be found in this compartment of the circulation. The portal vein is the main (75%) supplier of blood to the liver. High LPS influx into the liver potentially has significant consequences for inflammatory signaling pathways and insulin signaling in the liver. This hypothesis remains to be addressed because portal vein blood and liver biopsies are difficult to obtain. Increased translocation of endotoxin is potentially facilitated by a diet-induced increase in gut permeability and subsequent reduction in protective gut barrier function (84). In line with increased gut permeability, humans predisposed to develop T2DM had increased circulating levels of bacterial DNA (85).

The gut microbiota produces numerous organic compounds such as nitric oxide, ammonia, carbon oxide, indole, and hydrogen sulfide that possess pro- and anti-inflammatory properties and might be able to alter gut permeability (86). Hydrogen sulfide has specifically gained interest in the past decades for its role in GI diseases (87) and CVD (88). However, the role of these organic compounds in cardiometabolic disease is still under debate, partially due to the numerous conflicting studies that have been published. For example, H₂S can potentially alter gut permeability (87) and increased levels of H₂S are found in patients with ulcerative colitis (89), whereas H₂S could have a protective role against nonsteroidal anti-inflammatory drug-induced gastritis (90). Interestingly, a recent paper showed that H₂S possess cardioprotective effects during the cardiac remodeling process post myocardial infarction in rats by increasing macrophages infiltration into the infarcted myocardium and thus antagonizing hypoxia-induced damage of cardiomyocytes (91, 92). In addition, H₂S might have a beneficial role in the immune–inflammatory

processes in atherosclerosis by inhibiting the macrophage-derived foam cell formation (93). However, these studies are performed in murine models, *in vitro* or *ex vivo*, therefore the (causal) role of H₂S in CVD and GI diseases has to be defined. The conflicting results regarding the inflammatory properties of H₂S suggests that H₂S may be a double-edged sword. Future research therefore needs to be focused on resolving these discrepancies and further investigate the role of this gaseous molecule on immune–inflammatory responses in CVD and GI disease.

Gut microbiota produce a large number of (yet to be defined) small molecules through primary (direct) or secondary (indirect) metabolic pathways (94). It has therefore been suggested that the composition of gut-derived metabolites (as a measure for microbial output and functionality) is largely dependent on the diet of the host (18). Although some of these metabolites might be retained within the gut ecosystem, others might be released in the circulation of the host and exert a diverse array of metabolic effects (95, 96). The bacterial metabolite trimethylamine (TMA) is an example of one of many gut-derived metabolites that has been associated with CVD development in humans (97). TMA is converted to trimethylamineoxide (TMAO) in the liver. High concentrations of TMAO were shown to accelerate atherosclerosis development in mice, and high concentrations of TMAO are correlated with a higher incidence of CVD in humans (98). Several studies have observed increased levels of TMAO in patients with T₂DM than in healthy subjects (99–101). Interestingly, increased levels of TMAO were observed in hepatic insulin receptor knockout mice (LIRKO mice) via upregulation of the TMAO-producing enzyme FMO₃ in the liver (102). Furthermore, knockdown of FMO₃, the enzyme responsible for conversion of TMA to TMAO, prevented hyperglycemia, hyperlipidemia, and atherosclerosis, suggesting that TMAO might be a potential player in diabetes-associated atherosclerosis, at least in mice (102). The first mechanistic link between TMAO and cardiovascular risk was provided in a study that showed that TMAO mediates blood platelet hyperresponsiveness and subsequent thrombosis (98).

SCFAs and (human) metabolic disease

SCFAs are produced by bacterial fermentation of nondigestible dietary fibers in the large intestine and mainly comprise acetate, propionate, and butyrate. Studies in mice have shown that SCFA supplementation improves insulin-sensitivity and dyslipidemia, prevents weight gain, and increases energy expenditure in diet-induced obese mice (103, 104). SCFA-mediated activation of G-protein coupled receptor-mediated signaling pathways are involved in several metabolic processes including enteroendocrine regulation (105); glucagonlike peptide (GLP)-1 secretion (106, 107); inflammatory response (108, 109); glucose uptake and fatty acid oxidation (110); and energy metabolism (39).

Murine and *ex vivo* experiments have shown that SCFA improve intestinal barrier function by a SCFA-mediated increase in transcription of mucin genes (111, 112). Improved gut barrier function prevents overt exposure to the innate immune system of the host, potentially reducing inflammatory tone. SCFA are inhibitors of histone deacetylases (HDACs). SCFA-mediated inhibition of HDAC in regulatory T cells (Treg) was shown to increase Forkhead box P₃ expression thereby affecting Treg generation (113, 114). In line, SCFA-mediated inhibition of HDAC has been shown to have anti-inflammatory properties by regulating intestinal macrophages (115) and dendritic cells (116). Depletion of SCFA, might therefore contribute to the increased inflammatory tone often found in patients with obesity and diabetes. The beneficial anti-inflammatory effects of SCFA in humans however remain to be further elucidated.

Through a complex intestine-brain-neural circuit, SCFA have been suggested to increase intestinal gluconeogenesis, thereby improving peripheral glucose production and insulin sensitivity (117). In a recent study in rats, however, it was shown that the SCFA acetate increased food intake and promoted glucose-stimulated insulin secretion (104). In humans, acetate supplementation was reported to facilitate short-term satiety (118) and reduce weight gain (119). In line, SCFA reduced food intake and prevented weight by activating anorectic pathways in the brain (103). Direct colonic delivery of propionate reduced weight gain in a randomized controlled study in 60 overweight subjects (120). In addition, fecal acetate levels have been inversely correlated to insulin resistance (121).

Despite these positive correlations between SCFA and metabolic health in humans, the fecal microbiota composition of obese subjects has been reported to be shifted toward increased numbers of SCFA-producing species compared with lean subjects (71, 122). In line, increased fecal concentrations of SCFA, especially butyrate, have been observed in obese subjects (38, 95, 122). Interestingly, it was shown that in twins discordant for obesity, the gut microbiota of the obese twin was relatively enriched in SCFA-producing bacteria compared with the lean twin (49). It has been proposed that an increased capacity to extract energy (in the form of bacterial SCFA production) from fibers might be a driving factor in obesity development. Thus, despite increased relative abundance of SCFA-producers and increased fecal SCFA content in obesity, it is difficult to interpret potential health benefits of SCFA in obese subjects. Following production, SCFA are rapidly absorbed by the host (at least in healthy subjects) where they regulate glucose and lipid metabolism. In addition, SCFA can be absorbed and converted by the gut microbiota itself. It can therefore be speculated that, despite increased SCFA production, the gut microbiota of obese subjects has reduced capacity to handle SCFA.

In contrast to obese subjects without diabetes, fecal microbiota from obese patients with T2DM has been shown to be relatively depleted in SCFA-producing bacterial species (39, 45). In line, vancomycin treatment of patients with metabolic syndrome reduced insulin sensitivity with a coinciding reduction in butyrate-producing bacteria (40). A study in which the fecal microbiota of lean, insulin-sensitive donors was transplanted to recipients with insulin-resistant metabolic syndrome demonstrated that improved insulin sensitivity following FMT correlated positively with abundance of butyrate-producing bacteria (58). A metagenomic study showed that metformin-naïve patients with T2DM could be associated with a decrease in genera of butyrate-producers (*e.g.*, *Roseburia* spp, *Subdoligranulum* spp) (46). In the same study, it was shown that the gut microbiota of metformin-treated patients with T2DM contains significantly more butyrate and propionate-producers compared with patients with T2DM not treated with metformin.

Bile acid signaling in host metabolism

Bile acids play a pivotal role in human health and metabolic disease development, mainly by their role as signaling molecules that can activate receptors in the gut, liver, and adipose tissue (123). Primary bile acids (cholic and chenodeoxycholic acids) are produced from cholesterol in the liver via a complex pathway including at least 17 enzymes and is under control of the nuclear Farnesoid X receptor (FXR) and its downstream targets FGF15/19 (in intestine) and small heterodimer partner (in liver) (124). Mice also produce α - and β -muricholic acids in addition to the primary bile acids found in humans (125). Upon secretion into the intestine, bile acids are subject to modifications by the gut microbiota (125, 126). Primary bile acids are metabolized into secondary bile acids (deoxycholic and lithocholic acids) following 7α -dehydroxylation, which compromises numerous reactions carried out by bacteria that mainly belong to the Firmicutes (127, 128). It was shown that in mice, gut microbiota regulates expression of several key enzymes in bile acid formation including CYP7A1 and CYP27A1 by changing the composition of the bile acid pool, thereby alleviating FXR inhibition (129). In addition to bile acid synthesis and modification, bile acid uptake in the gut has been suggested to be regulated by the microbiota. Expression of the apical sodium dependent bile acid transporter, a transporter found in the small intestine responsible for the uptake of bile acids, is reduced in conventionally raised mice compared with their GF counterparts (129).

Data underscoring a role for bile acids in metabolic disease development originate in large from mouse studies. For example, cholic acid supplementation reduced HFD-induced weight gain and attenuated insulin resistance in mice, coinciding with increased circulating levels of bile acids (130). FXR and

downstream target FGF15/19 have been shown to regulate glucose and lipid metabolism (131). Synthetic inhibition of FXR reduced bile acid pool size and attenuated weight gain and glucose intolerance in HFD-fed mice (132). Furthermore, by acting on the G-protein coupled receptor TGR5, bile acids have been shown to promote (antidiabetic) GLP-1 secretion (133) and increase energy metabolism (130). Higher concentrations of deoxycholic acid have been associated with obesity in mice (134). Tauro- β -muricholic acid, an endogenous FXR antagonist (129, 131), is metabolized by the gut microbiota. Therefore, GF mice are not able to metabolize this bile acid. This ability has been shown to be a prerequisite to induce obesity, hepatic steatosis, impaired glucose tolerance, and reduced insulin sensitivity (129). The bile acid receptors FXR and TGR5 might play an important role in the development of metabolic diseases and have become major targets in translational and intervention studies (123). Bile acids generated by the gut microbiota can modulate signaling through these bile acid receptors and therefore might have the potential to alter lipid and glucose metabolism in humans.

In humans, bile acids have been implicated in regulation of food intake (135). Furthermore, increased circulating levels of bile acids have been reported in obese subjects with T2DM (136) and were shown to correlate with BMI (135). Rectal administration of taurodeoxycholic acid improved glucose homeostasis and lowered food intake in obese subjects with T2DM (137). Particular interest in a role for bile acids in regulation of host (energy) metabolism, however, arose from observations that (postprandial) bile acid metabolism is severely affected following bariatric surgery (138). Circulating levels of primary and secondary bile acids are increased after bariatric surgery and correlate with improved glucose control (138–140). Bile-acid mediated signaling events have been reported to be increased in post-Roux-en-Y gastric bypass (RYGB) subjects: this correlated with the release of satiety-promoting gut hormones such as GLP-1 and PYY (141–143). Furthermore, supporting an important role for bile acids in RYGB-mediated improvements in glucose homeostasis: metabolism of FXR knockout mice is not improved following vertical sleeve gastrectomy (VSG) (142). The beneficial effects of RYGB on energy metabolism were reproduced by diverting the biliary flow from duodenum to ileum in rats, suggesting that bile acids play an important role in adiposity, liver steatosis, and lipid and glucose metabolism (144). The animals in this study lost ~20% of their body weight; therefore, these results have to be interpreted with caution because these results can be partially explained by weight loss.

Crosstalk between the gut microbiota and bile acids affect host metabolism. However, most of the studies that mechanistically assess pathways involved in this crosstalk were performed in animal models. The

"In humans, bile acids have been implicated in regulation of food intake."

human and rodent bile acid pools have major compositional differences. This has significant consequences for bile acid signaling properties, and conclusions derived from rodent studies have to be interpreted with caution. In humans, causal evidence supporting a role for changes in microbiota composition with subsequent bile acid-mediated changes in host metabolism remains largely unknown.

Gut Microbiome Alterations After Bariatric Surgery

Bariatric surgery is a last resort for the treatment of morbid obesity and -related complications such as T2DM and is superior to any other treatment regimen aiming to reduce weight (145, 146). The rapid improvement in metabolic parameters such as fasting glucose (147) and fasting insulin (148) (usually within days after surgery) can be explained in large by calorie restriction (149, 150). Bariatric surgery has significant effects on gut microbiome composition, induced by considerable alterations in the GI tract [*i.e.*, reduced caloric intake, reduced gastric emptying, alterations in gastric acid production and bile acid (151)]. Tremaroli *et al.* (141) showed that two distinct bariatric surgery procedures [*i.e.*, VSG (no intestinal diversion) and RYGB (with intestinal diversion)] have similar effects on gut microbiome composition. Nevertheless, and despite small sample size, functional shifts were apparent and differed between the two surgical procedures and between the control group (141). RYGB has significant effects on gut microbial composition, the abundance of Firmicutes, which is generally high in obesity, decreases and Proteobacteria increases following RYGB (152). These effects differ strongly from effects of diet-mediated weight loss (68). However, a recent meta-analysis showed that there is a high discrepancy in human studies investigating gut microbial alterations after bariatric surgery (153); therefore, these results have to be validated in larger cohorts.

Altered microbiome composition and microbial metabolic output (*e.g.*, metabolite production) after bariatric surgery was hypothesized to add to the long-term beneficial effects of this surgical procedure on weight loss, diabetes remission, and cardiovascular risk (154). In support of this hypothesis, microbiota of murine RYGB donors augmented weight gain in GF recipients compared with GF mice that had received microbiota from sham-operated donors (154). Similar effects were observed in GF mice that received fecal microbiota transplants from human RYGB or VSG donors compared with GF mice transplanted with feces from obese controls (141). Mice colonized with microbiota from RYGB-treated mice had higher lean mass and lower respiratory quotient (ratio between CO₂ produced and O₂ consumed) compared with

VSG and control group, indicating decreased utilization of carbohydrates and increased utilization of lipids in the RYGB recipient mice (154).

Bariatric surgery is associated with significant changes in gut microbial composition and functionality (141, 153). However, large prospective studies are needed to validate these alterations and to further investigate whether the gut microbiome contributes to the beneficial metabolic effects of bariatric surgery. Bearing in mind the great dissimilarities in metabolic outcome (155) (responder, nonresponder), it would be interesting to have follow-up data available of the gut microbiome composition, diversity, and functionality years after the initial surgery.

The Gut Microbiome and T1DM

Although the main focus of this review is on the role of the gut microbiome in development of T2DM, the gut microbiome has also been implicated in the pathogenesis of T1DM. Both disorders are characterized by alterations in host immune response and have been linked to an immune system–gut microbiota interaction (156). Interestingly, enhanced systemic inflammation and autoimmunity can be detected years before disease onset. This suggests that environmental factors, including changes in gut microbiota composition and output (*e.g.*, LPS, SCFA production), are determinants of disease progression and can have predictive value for those at risk to develop T1DM. It has been suggested that shifts in gut microbial communities indeed precede disease development (157). Nevertheless, in humans it is difficult to determine whether an altered microbiota, as observed in patients with T1DM, is causal to or a consequence of compromised immune function. In addition, studies performed in humans are often subject to major confounding factors.

T1DM is generally considered to be driven by an (auto)immune-associated destruction of insulin-producing pancreatic β cells (158, 159). Approximately 70% to 90% of patients with T1DM show features of an immunological contribution (*e.g.*, self-reactive autoantibodies such as IA2 and GAD, genetic associations with genes controlling immune response) (160). The remainder of T1DM cases can be classified as monogenic forms of T1DM, including certain types of maturity-onset diabetes of the young (161) or have a yet-to-be-determined pathogenesis. T1DM generally manifests early in life. Interestingly, most children are diagnosed in autumn and winter (162), and being born in spring is associated with a higher chance of developing T1DM (163). This suggests that the pathogenesis of T1DM is heterogeneous and environmental (seasonal) influences might initiate or even drive the pathogenic processes in T1DM. A plethora of environmental factors such as vitamin D deficiency (164,

165), infant and adolescent nutrition (166), and early enterovirus infection (9) all have been postulated to contribute to the development of T₁DM (6). Improved sanitation and decreased incidence of childhood infections over the past decades are associated with an increased incidence of autoimmune diseases such as T₁DM and led to the hygiene hypothesis (167, 168). According to this hypothesis, infants may benefit from early exposure to specific microorganisms and parasites; this stimulation of the immune system early in life was indeed associated with lowered risk to develop allergies and autoimmune diseases later in life (167–169). Removing microbes from an individual's living environment therefore has consequences for gut microbiome composition and development of the immune system. These associative studies have increased interest in the role of the gut microbiome in the development of T₁DM in the past decade. As for T₂DM, however, mechanistic evidence for a role of the gut microbiota in the pathophysiology of T₁DM is mainly derived from studies in rodents.

Studies in BioBreeding Diabetes Prone rats (170) and nonobese diabetic (NOD) mice (171) that were treated with antibiotics indicated that the subsequent alterations in gut microbial composition reduced the risk of T₁DM development. In 2008, a landmark paper by Wen *et al.* (172) showed that MyD88, which functions as a critical signal transducer in interleukin-1 and TLR signaling pathway, deficient NOD mice are protected from the development of T₁DM (172). Interestingly, the protection of developing T₁DM is lost when deficient MyD88 mice are housed under GF conditions, suggesting that an interaction between the gut microbiota and the innate immune system has a role in the development of T₁DM. In addition to shifts in gut microbial composition as contributing determinant for development of T₁DM, microbial output in the form of SCFAs has been implicated to elevate the number and enhance the function of intestinal Treg cells and T helper (Th) 17 cells (113, 114, 173). Treg cells and Th17 are lymphocyte subsets with opposing actions (174). An imbalance between Treg cells (anti-inflammatory) and Th17 cells (pro-inflammatory) has been shown to contribute to the pathophysiology of autoimmune diseases (174). Because T₁DM is a T-cell-mediated disease associated with a reduced number of dysfunctional Treg cells (175, 176), an imbalance between Treg cells and Th17 cells could therefore augment an inflammatory response (174). Interestingly, Th17 cells are important in maintenance of intestinal barrier function (177). In a recent study, it was shown that antibiotic treatment reduced the number of Th17 cells in the lamina propria and increased T₁DM incidence in NOD mice (178). This result corresponds with earlier findings (179) and strengthens the hypotheses that an increased intestinal permeability might precede the clinical onset of T₁DM (180). The importance of a gut microbiome

capable of producing sufficient SCFA was underscored by a study in which mice were fed diets supplemented with acetate and/or butyrate (181). The acetate yielding diet decreased the number of activated diabetogenic T cells in lymphoid tissue. The butyrate-supplemented diet markedly increased the number and function of Treg cells and increased the expression of the tight junction protein occludin in the colon thereby preserving gut integrity. An intriguing interplay between genetics, altered gut microbiome/metabolites, and immunity might play a role in the development of T₁DM. Rodent studies have provided insight in this interplay, however, human data are scarce. In the next paragraph, we will discuss studies involving the gut microbiome in the development of T₁DM in humans.

Comparison of the fecal bacterial composition of four pairs of infants with T₁DM and controls revealed a higher ratio of Bacteroidetes/Firmicutes ~6 months after birth in infants who developed T₁DM compared with the controls (10). This corresponds to other studies that reported increased ratio of Bacteroidetes/Firmicutes in children with T₁DM (182, 183). In addition, the diversity of the gut microbiome was less diverse in subjects with T₁DM compared with the controls (10). Seroconversion is the time between development of a specific antibody till moment of detection of this antibody in the circulation (184). In infants who later developed T₁DM, detectability of anti-islet autoantibodies coincided with reduced abundance of bacterial genes associated with SCFA production and with gut integrity (183). This indicates that changes in early autoantibody production is related to changes in microbiome functional output.

In line, infants who expressed at least two diabetes-associated autoantibodies had low abundance of lactate and butyrate producing species compared with autoantibody-negative infants (185). In the BABY-DIET study (186), infants with first-degree relatives with T₁DM and HLA genotypes associated with increased risk to develop T₁DM at 6 or 12 months had similar gut microbial composition and diversity compared with controls (187). Interestingly, however, alterations in microbial interactions networks were observed in infants who developed anti-islet cells autoantibodies (187). In a longitudinal prospective cohort of 33 HLA-matched infants followed from birth until 3 years of age, decreased microbial diversity correlated with seroconversion, thus prior to the diagnosis of T₁DM (188). Furthermore, levels of human β -defensin 2 were increased in infants who later developed T₁DM (188). Because human β -defensin 2 is an antimicrobial product produced by colonic epithelial cells during inflammation (189, 190), this finding supports the hypothesis that development of T₁DM is accompanied by intestinal inflammation. A case-control study in 10 infants who were at risk to develop T₁DM (*i.e.*, positive for at least two

"An intriguing interplay between genetics, altered gut microbiome/metabolites, and immunity might play a role in the development of T₁DM."

diabetes-associated autoantibodies) reported higher intestinal permeability as assessed by a lactulose/mannitol test in those infants compared with controls (191). A possible mechanistic explanation for the contribution of the gut microbiome in the development of T1DM comes from a recent prospective study in Finland, Estonia, and Russia (169). Finland and, albeit to lesser extent, Estonia have higher autoimmune disease prevalence, including T1DM, compared with neighboring Russia. Gut microbiome development was followed from birth until the age of three in 222 infants and differed markedly between infants from Finland and Estonia compared with Russian infants. Of particular interest was a marked reduction in *Bacteroides* species in Russian infants compared with infants from Finland and Estonia. Functional pathway analysis suggested that early microbial communities of infants from Finland and Estonia produced more LPS compared with their Russian counterparts. However, LPS produced in this cohort was mainly derived from *Bacteroides* species; *Bacteroides*-derived LPS differs structurally and functionally from LPS derived from, for example, *E. coli* and has been shown to be nonimmunogenic in mice (169). Furthermore, in contrast to *E. coli* LPS, *Bacteroides*-derived LPS did not decrease incidence of autoimmune diabetes in NOD mice. Although a clear link between *Bacteroides*-derived LPS and T1DM could not be made in this study, these data raise the interesting hypothesis that the nature and composition of different LPS subtypes might determine the level of immune activation and serve protective roles in autoimmune disease development (169). In line, based on the relation between celiac disease and T1DM, the intestine and its inhabitants might be a shared risk factor (192). These findings, however, have to be interpreted with caution because geographically, gut microbial composition varies considerably among young children at risk to develop T1DM (193). A framework for the potential role of the gut microbiome in the development in T1DM is given in Fig. 2.

Interventions in Humans: Diet, Pro and Prebiotics, and FMT

As mentioned previously, gut microbiota composition as well as gut microbial function is highly related to dietary intake of the host (26). A relative deprivation in plant-based dietary fibers in industrialized nations has been suggested to be a driving force behind the widespread change in functional capacity of the gut microbiota potentially contributing to the increasing prevalence of obesity and -related complications (39, 45, 194). In addition to macronutrient intake, it has been shown that food additives such as artificial sweeteners induce both compositional and functional changes in gut microbiota and augment features of the

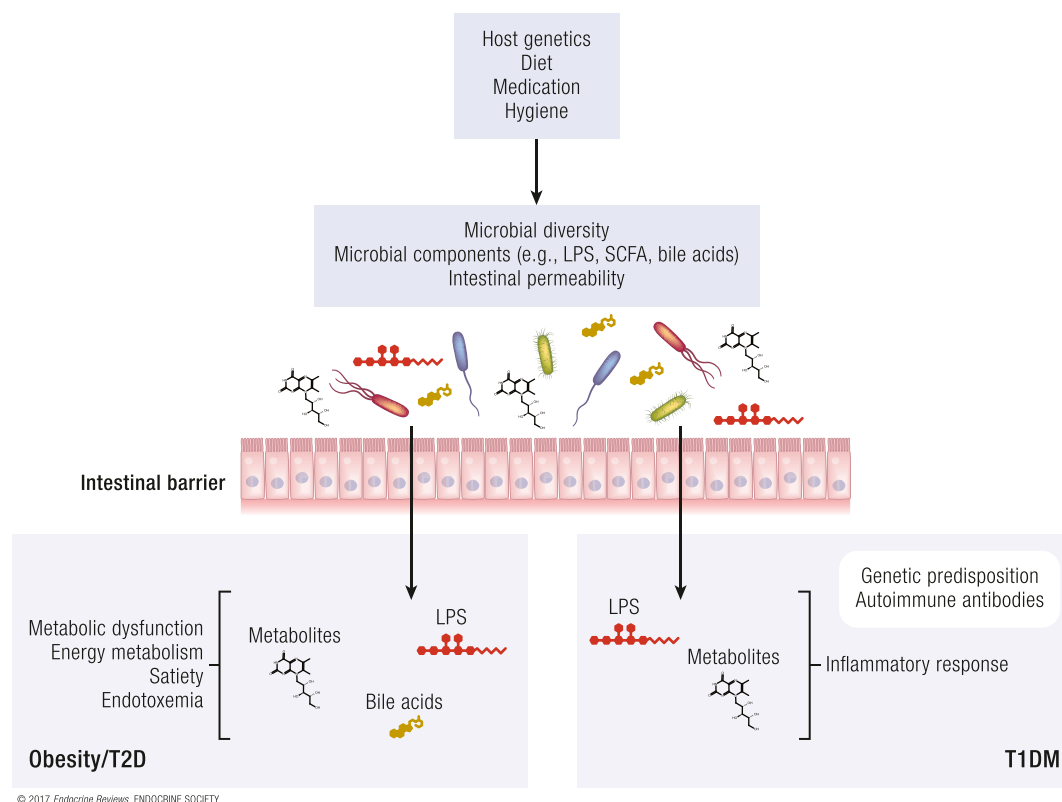
metabolic syndrome (195, 196). Because the gut microbiota is easily accessible and responds rapidly to changes in nutrient composition, dietary reinforcements have been put forward as an attractive therapeutic target for obesity. However, in addition to low overall adherence to diets, high interindividual differences in response to diet makes this a challenging endeavor. For example, complex carbohydrate supplementation increased starch-degrading taxa in some but not all subjects who participated in a strictly controlled 10-week dietary intervention (30). Another study showed that a low calorie, high fiber diet increased diversity only in subjects with high-gene count at baseline (31). Subjects with improved glucose metabolism after a 3-day intervention with whole grain bread had higher ratio of Prevotella/Bacteroidetes after the interventions than nonresponders (32).

The interindividual response to diet was particularly exemplified in a landmark study by Zeevi and coworkers (33) who showed that the individual postprandial glycemic response to a high glycemic meal was highly variable. This response correlated with individual microbiota composition. Interestingly, using a machine-learning approach, the individual response to and success of a particular dietary regimen could be predicted based on existing microbiota composition. This work provided crucial insight in the role of the gut microbiota in responsiveness to dietary strategies. Using novel, personalized prebiotics or probiotics to modify the gut microbiota composition of people predicted to have low response rate to diet-induced weight loss regimens into a more responsive composition might optimize the effectiveness of dietary strategies.

Probiotics are living microorganisms that either have potential to improve host metabolism directly (e.g., by improving gut barrier function or increasing SCFA-production) or have the capacity to re-establish a more favorable intestinal balance by modulating pH, antibacterial compound production, and competing with pathogens (197, 198). In mice, the probiotic strains *Akkermansia muciniphila* (66) and *Lactobacillus planetarium* (199) were both shown to lower endotoxemia and weight gain in HFD-fed mice. In humans, administration of *Lactobacillus reuteri* was associated with increased insulin secretion in obese, insulin-resistant subjects (200). Furthermore, a double-blinded, randomized, placebo-controlled intervention trial in overweight subjects showed beneficial effects of *Lactobacillus gasseri* on weight loss compared with fermented milk use only (201).

Prebiotics are nonmicrobial entities (usually dietary fibers) that elicit a favorable impact on microbial composition and function. Prebiotics might therefore be a feasible tool to modulate gut microbiota. Supplementation of prebiotics has been associated with improved plasma lipid levels and improved glycemic control in both humans (66, 202) and mice (203). Oligofructose was shown to increase release of the

Figure 2. The role of the gut microbiome in the pathogenesis of cardiometabolic disease. The human gut microbial community is shaped by a complex interplay between host genetics, diet, and (history of) medication use. Alterations in gut microbiota composition (e.g., reduced diversity) or microbial output (e.g., LPS subtypes, SCFA production, or bile acid conversion) have been implicated in development of metabolic diseases such as obesity and T2DM in humans. Although mechanistic evidence for a causal role of the gut microbiota in the pathophysiology of these diseases in humans is scarce, currently available data suggest that an altered microbiota composition affects gut barrier function and induces (low-grade) inflammatory events, either locally in the intestine or systemically. Furthermore, bacterial metabolites including SCFAs and secondary bile acids, which serve important regulatory roles in energy homeostasis and regulation of peripheral glucose and lipid metabolism, have been hypothesized to be drivers of T2DM development. T1DM is generally considered to be driven by autoimmune antibodies that specifically destroy insulin-producing β cells in the pancreas. It has been hypothesized that autoimmune antibody generation is in part consequential to removal of particular microbes, with a crucial role for maturation of the immune system, from our living environment (hygiene hypothesis). In addition, some studies have observed increased autoimmune antibodies prior to T1DM diagnosis and have suggested that altered microbiota composition or microbial output and subsequent initiation of inflammatory events accelerates onset of T1DM. Please see text for details.



satiety promoting hormone PPY and GLP-1 in mice (204). In humans with T2DM, oligofructose proved to be a useful prebiotic: supplementation for 3 months increased weight loss and improved glucose control compared with patients receiving placebo (205).

Despite focus of (food) industry on development of novel prebiotics and probiotics to modulate microbiota composition and/or functional output to subsequently improve host metabolism, thus far, only minimal beneficial effects of gut microbiota modulation on metabolism have been obtained. Improved engraftment of probiotic strains might help improve effectiveness of probiotic strain administration (206).

Fecal microbiota transplantation

FMT has a long medical history and has been used for treatment of several GI illnesses. As long as 1700 years ago, FMT was used to treat patients with food

poisoning and diarrhea in China (207). After the realization that hygiene plays an important role in preventing infectious disease, FMT became obsolete. In 1958, after a long period of silence, FMT garnered interest again following a description of its use in treating fulminant enterocolitis (208). The real breakthrough of FMT as treatment modality was after publication of an open-label, randomized, controlled trial, which demonstrated that the resolution of *C. difficile* infection was 94% after FMT compared with 31% efficacy of conventional vancomycin treatment (209). FMT is now the method of choice for treatment of recurrent *C. difficile* infection. However, FMT is also of interest as therapeutic modality for a wide range of diseases including inflammatory bowel disease (210), obesity (58), and metabolic syndrome (58). In addition, neurologic (211) and psychological disorders (212) might benefit from FMT if correlations with

altered gut microbiome composition are indeed valid. In two separate studies in humans, our group has shown that FMT has beneficial effects on insulin sensitivity (42, 58). Although effects are temporal and variable, FMT might have merit as intervention option for metabolic syndrome.

Peripheral insulin sensitivity of obese, insulin-resistant subjects was significantly improved 6 weeks after receiving a transplant from a lean, insulin-sensitive donor (allogenic transplant) (58). Transplantation of one's own fecal microbiota (autologous transplant) did not affect insulin sensitivity. In a second, larger cohort of obese, metabolic syndrome subjects, we were able to reproduce these findings. Allogenic FMT improved insulin sensitivity compared with autologous FMT in metabolic syndrome recipients 6 weeks after transplantation. Interestingly, 12 weeks after transplantation, this beneficial effect could no longer be observed. Engraftment of donor microbiota in the gut of recipient was negatively associated with the metabolic outcome of FMT, suggesting that specific donor-host interactions are important determinants of FMT efficacy. In line, based on baseline microbiota composition of the recipient, the metabolic response to FMT could be predicted.

Variation in experimental protocols, preparation of fecal samples, and diurnal oscillations of the gut microbiota (213) are additional explanations for variable efficacy of FMT. This underscores the need for development of stringent standard operation procedures (54). In addition to bacteria, viruses, fungi, and bacteriophages that all reside in the fecal compartment are cotransplanted (214). Although it has yet to be determined if and how these components contribute to FMT efficacy, it was recently shown that the virome (bacteriophages) has an important role in host health by modulating bacterial community and by direct interaction with host cells (215, 216). In addition, eukaryotic viruses and bacteriophages have been shown to modulate bacterial metabolism (e.g., amino acid, lipid, and carbohydrate metabolism) and to affect signal transduction pathways and transcriptional regulation (215, 217).

Modulation of the gut microbial composition and functionality by FMT only partly affects the intrinsic and complex pathophysiology of obesity and T2DM. Gut microbiome composition and function is influenced by many factors and therefore, it is unlikely that a single FMT can cure obesity or T2DM. Nevertheless, a combination of FMT with personalized prebiotics or treatment with "missing" intestinal bacterial strains (drugging the microbiome) might enhance the effects of conventional treatment strategies (54). Furthermore, early intervention in patients who are at risk to develop T2DM or patients who were recently diagnosed with these pathologies might benefit from gut microbial modulation in a personalized manner, such as microbiota-based dietary strategies or personalized FMT.

Systems Approach Potentially Holds the Key to Establish Driving Role of the Gut Microbiota in Obesity and T2DM

The introduction of DNA sequencing technologies substantially boosted the study of complex microbial communities and allowed for taxonomic identification of individual microbes. Nevertheless, early sequencing technologies were slow and expensive because large genomic fragments had to be cloned into plasmid vectors and transformed into suitable hosts for amplification prior to being sequenced (218).

Polymerase chain reaction (PCR)-based, massive parallel sequencing now allows for identification of previously undetectable bacteria within complex communities (218). In addition, shotgun WGS approaches have significantly enhanced detection of diversity, and increased prediction of genes and taxa at species level can be identified (219). Although WGS is currently more expensive and requires more extensive data analysis, this method is preferred above PCR-based sequencing.

High through-put amplicon sequencing of isolated DNA samples or PCR amplification of regions within universally conserved 16S rRNA genes has generated an enormous amount of data on microbiome composition from different environments and conditions. Reference metagenomes of microbes were published in 2012 by the Human Microbiome Consortium and showed that the dominant microbial taxa in the human gut include Bacteroidetes, Firmicutes, and Proteobacteria, and that species including *Bacteroides fragilis*, *B. melaninogenicus*, *Enterococcus faecalis*, and *E. coli* are present in the majority of healthy human subjects (220). It is important to note that taxonomic characterization of intestinal microbiota is based on relative (and not absolute) abundance and does not always translate into function. To effectively understand the impact of the microbiome on the host, it is critical to connect compositional to functional studies. This can be undertaken with a systems biology approach.

Systems biology approaches can be used to integrate omics data to untangle driving factors underlying gut microbiota composition (Fig. 3). Additionally, these approaches can provide insight in the hierarchy of mechanisms underlying the development of metabolic diseases. Taxonomic profiling can identify who's there, and complementary with metagenomic profiling: what are they capable of? Nevertheless, it is important to emphasize that the vast amount of data generated by high-throughput sequencing currently surpasses the ability to analyze those data with currently available bioinformatics tools.

A study in which the overlap between metagenomics (which microbes are there, and what genetic potential do they have) and metatranscriptomics

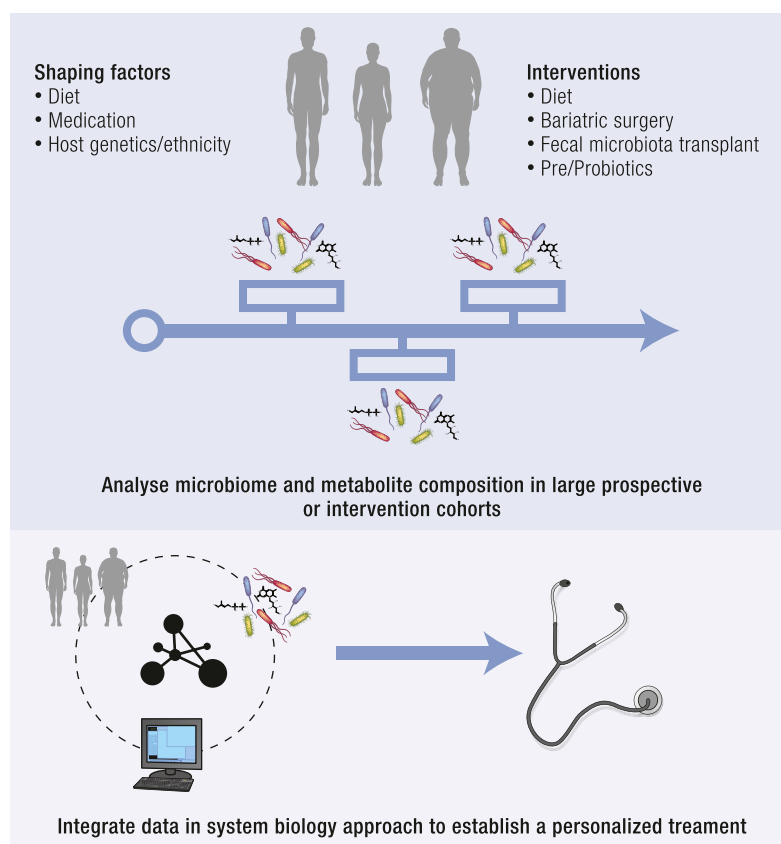
(which genes are most highly expressed) of the human gut's microbiome community was systematically compared revealed that only 41% of microbial transcripts corresponds to microbial genomic abundance (221). This underscores the importance to move beyond metagenomics to understand "what the community is really doing." Moreover, the importance of post-transcriptional and translational regulation and the fact that protein abundance does not correspond with gene expression in either eukaryotes or bacteria has to be taken into account (222, 223). Metaproteomics and metabolomics might be the solution to better understand the functional capacities of the microbiota. The interpretation of metatranscriptomic and proteomic data are challenging due to incomplete information on the gut microbial genomes and proteomes and, hence, lack of (gut-specific) reference databases (218). Moreover, metatranscriptomics provides only a snapshot of the dynamic interactions between host, gut microbiota, and environment (224). An example of the question "who's there" and more important, "who's active," comes from a study where metaproteomics was combined with taxonomic profiles of gut microbiome obtained from obese and nonobese individuals (225). This study demonstrated that, despite a lower abundance, Bacteroidetes had higher metabolic activity in obese individuals than in nonobese individuals. In addition, insulin sensitivity, as estimated by homeostasis model assessment index, was positively associated with peptides originating from a group of proteins derived from bacteria from the genus *Ruminococcus* (225).

A complimentary approach for the study of microbiota functionality and host-microbiota co-metabolism is provided by metabolomics, which analyzes the small-molecule composition of host fluids and tissues to detect metabolites derived from bacteria or organisms other than the host (225). Metabolomics can be grouped in targeted and untargeted methodologies. With untargeted metabolomics, up to 10,000 independent spectral features can be measured (226). Thus far, however, only one-third of these spectra can be identified, because translating the signals obtained by mass spectrometry to a specific/known chemical structure is still low throughput (226). Targeted metabolomics is a quantitative technology because it measures known metabolites in clusters with similar chemical structure (227). This approach has revealed that several microbial metabolites are associated with the metabolic syndrome in humans and experimental models (227). Targeted metabolomics can also be applied to test whether shifts in microbial gene functions are coupled to shifts in community functionality, as has been done by profiling SCFAs (228) and bile acids (229, 230) in obesity and after bariatric surgery-mediated weight loss (141, 142). A systems biology approach with combined input of different

omics data sets will accelerate our understanding of the contribution of the microbiome to human health and metabolic disease.

Conclusions

Since the introduction of next-generation sequencing techniques, a plethora of studies has shown striking associations between the composition of the gut microbiota or gut microbial metabolites in the development of obesity and diabetes. Nevertheless, only a few studies have provided mechanistic or causal evidence of the pivotal role of the gut microbiota in the development of metabolic diseases in humans. The complex interplay between ethnicity, host genetics, dietary habits, and medication use all play an important role in shaping the microbial community and therefore makes it an intriguing yet challenging research field. Inconsistent application or lack of corrective measures for



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Figure 3. The role of systems biology approach in gut microbiome research. Systems biology approaches that combine patient data with microbiome and microbial metabolite composition in preintervention and postintervention settings and in large prospective cohorts of initially healthy subjects will reveal crucial insight in the role of the gut microbiome in metabolic disease development. Moreover, this strategy will allow for development of tools to predict metabolic disease development and to specify optimal treatment strategies to tackle these pathologies.

confounding factors that might underlie changes in the gut microbial composition is a challenging aspect of data interpretation in humans. It likely is one of the main causes for the low reproducibility of research results between studies. Large prospective studies will be of critical importance to answer whether gut microbial composition is a reflection of the disease itself or the microbial composition was affected prior to disease development and hence was a driving factor. Although studies using antibiotic therapy or FMT are suggestive of causal linkage between the gut microbiota and metabolic disease development, effect size and evidence for causality are still marginal. Furthermore, these studies do not provide mechanistic insight into the interplay between the gut microbiota and host metabolism.

Prospective and intervention studies in large human cohorts combined with dedicated mechanistic studies in model systems are required to understand if and how gut microbiota affects metabolic disease development. Using a multiomics approach, a deeper understanding of host–microbe, microbe–microbe, and diet–microbe interactions can be achieved. This will provide insight into the hierarchy of mechanisms underlying the development of metabolic diseases and lead to identification of a personalized intestinal microbiota signature. This will accelerate development of strategies to predict cardiometabolic disease development and, importantly, establish means to develop personalized, microbiota-based interventions to tackle metabolic pathologies in humans.

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Abbreviations

BMI, body mass index; CVD, cardiovascular disease; FMT, fecal microbiota transplantation; FXR, Farnesoid X receptor; GF, germ-free; GI, gastrointestinal; GLP, glucagonlike peptide; HDAC, histone deacetylase; LPS, lipopolysaccharide; NOD, nonobese diabetic; PCR, polymerase chain reaction; RYGB, Roux-en-Y gastric bypass; SCFA, short chain fatty acid; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; Th, T helper; TMA, trimethylamine; TMAO, trimethylamine-oxide; Treg, regulatory T cell; VSG, vertical sleeve gastrectomy; WGS, whole genome sequencing.