The gut microbiome, diet, and links to cardiometabolic and chronic disorders

Judith Aron-Wisnewsky and Karine Clément

Abstract | Cardiometabolic diseases (CMDs) have been associated with changes in the composition of the gut microbiota, with links between the host environment and microbiota identified in preclinical models. High-throughput sequencing technology has facilitated in-depth studies of the gut microbiota, bacterial-derived metabolites, and their association with CMDs. Such strategies have shown that patients with CMDs frequently exhibit enrichment or depletion of certain bacterial groups in their resident microbiota compared to healthy individuals. Furthermore, the ability to transfer resident gut microbiota from mice or humans into germ-free mouse models, or between human patients, has enabled researchers to characterize the causative role of the gut microbiota in CMDs. These approaches have helped identify that dietary intake of choline, which is metabolized by the gut microbiota, is associated with cardiovascular outcomes in mice and humans. Trimethylamine *N*-oxide (TMAO) — a metabolite derived from the gut microbiota — is also associated with poor cardiovascular outcomes in patients with cardiovascular disease and is elevated in patients with chronic kidney disease (CKD). TMAO might represent a biomarker that links the environment and microbiota with CKD. This Review summarizes data suggesting a link between the gut microbiota and derived metabolites with food intake patterns, metabolic alterations, and chronic CMDs.

Institute of Cardiometabolism and Nutrition (ICAN), Assistance Publique–Hôpitaux de Paris, INSERM, Sorbonne Université, Paris 6, Pitié–Salpêtrière hospital, F-75013 Paris, France. Correspondence to J.A.-W and K.C.
judith.aron-wisnewsky@psl.aphp.fr; karine.clement@psl.aphp.fr

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Cardiometabolic diseases (CMDs) are becoming a worldwide epidemic, with a dramatic increase in the prevalence of obesity[1] and closely interrelated diseases, such as metabolic syndrome, type 2 diabetes mellitus (T2DM), cardiovascular disease (CVD), and chronic kidney disease (CKD)[2]. The global financial burden of CMDs is huge, with an estimated cost of US$6.3 trillion in 2010, and a projected doubling by 2030[3]. Treatments are available to manage some CMDs, but are not curative, thus a critical need exists to identify novel potential targets and pathways that are shared between CMD entities. Studies of the microbiota have rapidly progressed over the past 10 years, particularly with regard to metabolic health[4,5]. The intestine harbours >10^{14} microorganisms with critical physiologic roles, and the microbial composition differs along the digestive tract[6,7]. As only 30% of these bacteria can be cultured, researchers have pioneered the development of new culture-independent methods[8]. A large reference catalogue of gut bacterial genes[9] has been generated from high-throughput sequencing data[10] and has indicated that dysbiosis (microbial imbalance) is associated with CMDs[11]. The addition of various 'omics' technologies, bioinformatics, and modelling approaches[12] that are capable of integrating large datasets with sequencing data has enabled comprehensive insights into the links between the gut microbiota, the environment, and various diseases. Investigators must, however, be careful when designing or analysing studies that evaluate the gut microbiota. Factors such as patient selection, sampling methods, sample handling and storage, and methods of DNA extraction should be standardized to avoid potential bias (BOX 1). Furthermore, these methodological aspects should be carefully assessed when trying to generalize data across studies. The methods used to analyse the composition of the gut microbiota differ between studies, and each methodology carries its own advantages and limitations (TABLE 1). International initiatives aim to encourage use of standardized methodologies[13,14], and data processing of the obtained results also requires standardization[15]. This Review discusses current data that support a link between the gut microbiota, obesity, and associated metabolic diseases. We also summarize the latest advances in our understanding of how the gut microbiota and its metabolites associate with more advanced disease stages of CVD and CKD.

Gut microbiota and weight modulation

Faecal transfer in germ-free mice

Pioneering experiments that demonstrated a role for the gut microbiota in weight regulation originally came from

Key points

- Dietary modifications (in particular high-fat feeding) can induce changes in the microbiota that might impact on host-associated metabolic disorders, such as insulin resistance
- Germ-free mice exhibit reduced adiposity and fat pads compared to conventionally housed mice, thus implicating the gut microbiota in energy storage
- An obese phenotype is transmissible via faecal transfer from obese mice or humans to germ-free mice
- Metabolites that are derived from the gut microbiota, such as trimethylamine *N*-oxide (TMAO), are associated with poor cardiovascular outcomes in patients with cardiovascular disease
- TMAO is increased in patients with chronic kidney disease (CKD) before haemodialysis and decreases after kidney transplantation
- TMAO is associated with poor cardiovascular outcomes in patients with CKD

studies performed in germ-free mice. Germ-free mice have no resident microorganisms and are raised in an environment that prevents exposure to any parasitic agent. Germ-free mice fed a high-fat diet (HFD) have reduced adiposity compared to conventionally raised animals also fed a HFD, despite increased caloric consumption and decreased energy expenditure. After colonization of germ-free mice with faecal content from conventionally raised mice, the originally germ-free mice rapidly gain weight and increase fat mass without any change in calorie ingestion[16–18], thus illustrating crosstalk between the gut microbiota and host tissue homeostasis (FIG. 1)[16]. These breakthrough findings demonstrated that the gut microbial ecosystem is transmissible, and paved the way for a new field of research that would explore how the gut microbiota contributes to the efficiency of energy storage, can disrupt metabolic homeostasis, and confer cardiometabolic risk.

Another milestone was reached when faeces from human twin pairs discordant for obesity were transferred into germ-free mice[19]. The mice that received faeces from the obese twin developed metabolic alterations and gained more weight compared to those that received faeces from the lean twin. When the two groups of mice were co-housed, microbial transfer could occur by coprophagia (ingestion of faeces), and consequently the mice that received the microbiota from the obese twin showed changes in their microbiota profile that were accompanied by improvements in their metabolic phenotype. This improved phenotype in the obese mice was, however, only achieved upon receipt of a healthy diet that was high in fibre and low in saturated fat, thus emphasizing the importance of dietary components. The microbiota from the lean twin had a high efficiency to ferment short-chain fatty acids (SCFA), thus promoting good metabolic health[20,21].

The effect of the gut microbiota on host metabolism is mediated in part by its capacity to process indigestible complex dietary carbohydrates[17,22,23] via numerous hydrolytic enzymes that facilitate the intestinal absorption of SCFA, such as acetate, propionate, and butyrate[24]. Some researchers have hypothesized that the gut microbiota present in obese individuals is more efficient at extracting energy from the diet, which subsequently promotes fat storage in the host[25]. One study found that obese patients exhibit higher levels of SCFA and lower residual food calories in their faeces than do lean individuals[24]. This hypothesis has been challenged, however, as the microbiota from lean individuals can in some cases produce more SCFA without marked calorie loss in stools[26]. Another important link between gut microbiota and its host is the crosstalk that occurs between the microbiota and host immunoinflammation, which affects the signalling of metabolic sensors that control energy homeostasis[27].

Germ-free mice are a popular and interesting model to examine the contribution of the gut microbiota in the development of various diseases, and the use of gnotobiotic models (where only certain strains of microorganisms are present) has further enabled the causal role of bacteria in host-associated disorders to be determined. These models do, however, present some limitations and data obtained using this approach should be interpreted with caution. The physiology of germ-free mice is substantially altered compared to that of conventionally housed mice due to life-long housing under aseptic conditions. Germ-free mice also display differences in immune capacity and intestinal physiology compared to that of conventionally housed mice[28], which might affect the development of metabolic diseases. Furthermore, gut microbiota regulate enzyme activity in the digestive tract and produce metabolites that are important for host signalling[29]. These important limitations should be taken into account before translating findings from these mice to humans. The combined use of other models, such as germ-free-like mice that display robust microbiota depletion after antibiotic treatment[30], might help overcome these described limitations by accounting for the relationship between nutrients, the microbiota, intestinal function, immunity, produced metabolites, and host physiology. Even with this model, however, the direct effects of antibiotics on the host cannot be excluded. Overall, numerous differences exist between humans and mice regarding bacterial composition and the physiology of the gastrointestinal tract[31], and caution is warranted when translating discoveries from studies in mice to human pathophysiology.

The effect of food-intake patterns on microbiota

The gut microbiota not only metabolizes ingested food, but is itself shaped by the mode of food consumption (for example, food intake patterns) and even the general environment. Differences in microbiota composition at the phylum level have been identified between populations residing in different geographic regions, such as a higher abundance of Bacteroidetes and a decreased abundance of Firmicutes and Proteobacteria in African children compared to children living in Western Europe[32]. The bacteria found in African children were more efficient at metabolizing fibre and could subsequently produce greater amounts of SCFA[32]. The increased consumption of carbohydrates and fibre in the African diet might explain these differences and promote richness and diversity of gut microbiota. Differences in the richness of gut microbiota have also been observed between American, Malawi, and American-Indian populations[33], resulting from geographic differences and dietary dissimilarities.

Box 1 | **Critical methodology that can induce bias in gut microbiota analyses**

Patient selection

Age

- The age of the cohort deserves close attention
- The gut microbiota is progressively shaping its future composition from birth to 2–3 years of age, when it is then considered to be relatively stable until ~65 years of age[33]
- The diversity and composition of the gut microbiota declines in the elderly[36,146,147]

Effects of treatments

- Numerous drugs affect the gut microbiota, such as antibiotics that reduce microbiota diversity but also have specific effects on certain genera[148,149]
- Depending on the antibiotic, the complete recovery of gut microbiota can take several weeks and up to several months
- Patients should not have been on antibiotic treatment for at least 3 months before inclusion in any study, and the history of previous antibiotic treatments should be recorded
- Other drugs, such as metformin[150] used in type 2 diabetes mellitus, or proton pump inhibitors[151], can modify gut bacteria composition

Smoking

- Smoking habits must be considered when designing a study to decipher the role of the gut microbiota in cardiovascular disease
- Smoking is a known cardiovascular risk factor, but can also modulate the gut microbiota composition[152]

Faeces sampling

Source of sampling

- The composition of the gut microbiota differs along the digestive tract, with the highest number and diversity of species in the large intestine in humans[6] and mice[153]
- For faecal transfer studies, care must be taken as to the part of the gut from which microbiota are transferred, as transfers from different regions might account for differences in results across studies
- Some teams transfer microbiota obtained from the caecum[73] of mice after sacrifice whereas other studies use stool samples[154]
- Different outcomes in association studies might also originate owing to differences in sampling location along the digestive tract

Faeces storage

- Faeces must be frozen immediately after collection to avoid DNA degradation
- If samples are not immediately frozen, they should be kept in an anaerobic atmosphere until later freezing at –80 °C
- Methods to standardize faeces collection, as well as storage methods, have been previously described[155]

DNA extraction

- Currently no reference technique exists for DNA extraction from the microbiota[155]
- Differences in DNA extraction methods have been shown to induce variations in the overall outcome[156]
- The same method of DNA extraction should be used throughout a study
- Caution is required when comparing results between studies that have used different DNA extraction techniques

Microbiota richness is also associated with different dietary patterns in overweight or obese individuals from Western countries[34,35]. Finally, a healthy diet (defined as high in fibre and low in fat) is associated with increased microbiota diversity in elderly individuals, whereas both moderate-to-low fibre diets and HFDs were associated with reduced microbiota diversity[36].

Enterotypes. The concept of enterotypes was developed from an analysis that aimed to understand and visualize a large metagenomic dataset by segregating individuals on the basis of their gut microbiota composition. Enterotypes are clusters of faecal metagenomic sequences associated with networks of dominant genera that can be used to separate individuals into clusters[37–39]. Enterotypes are linked to usual food intake habits that are evaluated by food frequency questionnaires in large populations[40], and are independent of age, sex, and BMI. Three enterotypes were initially proposed, with enterotype 1 (Bacteroides), which was frequently observed in patients who consumed a diet rich in saturated fats, and enterotype 2 (Prevotella), which was usually found in individuals who consumed a carbohydrate-enriched diet showing strong associations to food intake habits. One study demonstrated that wild mice displayed two different enterotype-like clusters that were associated with either plant-derived or animal-derived food intake[41]. These two enterotypes share some similarities with the described human clusters. When transferring the wild mice to laboratory housing, the gut microbiota switched within 1 week towards the enterotype that was able to process the usual chow diet given to laboratory mice[41], illustrating a key link between food intake and gut microbiota composition.

The concept of enterotypes has been debated since their initial description in 2011[42,43]. Some studies that have confirmed the existence of enterotypes[37–39] have only been able to distinguish two clusters[40,44], whereas other studies have been unable to detect any enterotypes or have instead observed a community gradient[45]. A new concept has, therefore, been developed that proposes a continuum or gradient of bacterial species with functional properties rather than discontinuous segregated enterotypes. This gradient of species reflects correlated genera (or co-abundance groups) that are frequently found together, which probably reflects the co-colonization of the species and their nutritional cross-feeding (i.e. dependence on similar nutritional sources)[36]. This categorization of the gut microbiota seems to better classify patients from healthy controls[36,43]. Whatever the means to describe the microbiota composition (using enterotypes or genera gradients), the importance of food intake habits in shaping the gut microbiota is widely accepted.

The effect of dietary switches on the microbiota

Although the composition of the microbiota is quite resilient over an individual's life span[46,47], dietary interventions that induce rapid changes in certain nutrients (such as altering fibre intake) can modify the microbiota composition[48,49]. This concept has been confirmed using various techniques to analyse the gut microbiota, such as 16S rRNA sequence analysis and quantitative PCR (TABLE 1). One study reported that in cases where no marked variations as a result of dietary modification were seen at the phylum level, modifications to the microbiota were predominantly observed at finer taxonomic levels for specific phylotypes such as *Roseburia* spp. and *Eubacterium rectale,* which are known to have a role in the digestion of dietary carbohydrates[50]. Changes in *Roseburia* and *E. rectale* have been observed with changes in the proportion of carbohydrate content in the diet[51,52]. These data suggest a strong link between the dietary environment and gut microbiota composition and suggest that

Table 1 | **Techniques to analyse the gut microbiota**

Technique	Function	Advantages	Limitations
Quantitative PCR	Investigates specific bacterial groups	Simple to perform Affordable Results obtained rapidly No need for sophisticated bioinformatics	Not representative of the overall complexity of the gut microbiota Not representative of the overall ecosystem Can evaluate specific bacteria or a group of bacteria
16S pyrosequencing	Amplification and sequencing of the 16S rRNA gene — part of a ribosome that is present in every bacterial and archea species	Relatively rapid Profiles the gut microbiota (that is, gut bacteria composition) Affordable	Unspecific — some bacteria contain several copies of 16S rRNA, which is a pitfall when examining the link between gene copy number and bacteria groups Different hyper-variable regions are analysed across studies, making comparisons between studies difficult Some species share an almost comparable 16S rRNA region, which leads to identification issues Technical bias where some species are amplified more than others Limitations in functional analyses Limited to explore diversity
Shot-gun metagenomics	Breaks the full DNA content into small constant fragments that can be sequenced and aligned to a reference catalogue	Enables direct quantification of gene abundance and bacterial genome reconstruction Captures genetic information, including that of unknown bacteria Assesses bacterial functionality Assesses the virome, eukaryotic genome, and fungome	High expertise required High costs High quality and high molecular DNA required for library preparations Massive amounts of data produced and need for accurate bioinformatics and biostatistic pipelines (storage and analysis)

in the context of specific diseases, food intake should be carefully monitored to potentially assess links between the gut microbiota and host biology.

Upon metagenomic sequence analysis, representative species from the dominant phyla can be extracted and an algorithm (known as CASINO[53]) can predict metabolite production that reflects the interaction between the gut microbiota composition and the consumed diet of the individual. This algorithm has been validated both *in vitro* and in a diet intervention study with metabolomic analyses of faecal and blood samples[53]. Interestingly, this tool enables the prediction of the specific nutrient from which a particular metabolite is derived. The necessary dietary changes needed to modify the gut microbiota from a low microbiota richness (and metabolically unhealthy) to a high microbiota richness have subsequently been modelled in order to direct improvements in metabolic health status[53].

Gut microbiota and metabolic diseases

Lessons from animal models

Studies performed in mice fed a HFD led to the concept that metabolic endotoxaemia — increased systemic lipopolysaccharide (LPS) concentration — can drive the development of insulin resistance. Mice that underwent 4 weeks of a HFD exhibited an increase in lipopolysaccharide (LPS) levels and developed insulin resistance concomitant with modifications to the composition of the gut microbiota (FIG. 1)[54]. This phenotype was mimicked when mice underwent LPS infusion. LPS forms part of the gut bacterial membrane and binds to the CD14 receptor that is expressed on immune cells and adipocytes[55]. *Cd14*$^{-/-}$ mice exposed to a HFD or LPS infusion were protected from weight gain and metabolic impairments, such as the insulin resistance and systemic and adipose tissue inflammation that was observed in similarly treated wild-type mice. These results indicate the importance of the LPS–CD14 pathway in mediating the metabolic consequences of a HFD[54].

Antibiotic interventions. Antibiotics that affect both metabolic endotoxaemia and gut microbiota composition improve insulin resistance, decrease systemic inflammation, and reduce weight gain in genetically obese (ob/ob) mice and mice fed a HFD[56,57]. Metabolic alterations induced by a HFD involve a disruption of the gut barrier (as demonstrated by increased intestinal permeability in mice), which can be reversed in part by antibiotic treatment. These findings further support a link between the gut microbiota and host-related metabolic phenotypes in response to a HFD, which requires thorough investigation in the context of CMDs. Data from a human observational study have suggested that abdominal obesity is associated with increased visceral permeability[58], but this finding remains to be confirmed using different techniques[59].

Bacterial translocation in the blood is increased at the onset of HFD-induced insulin resistance. Mice display a high systemic level of *Escherichia coli* 1 week

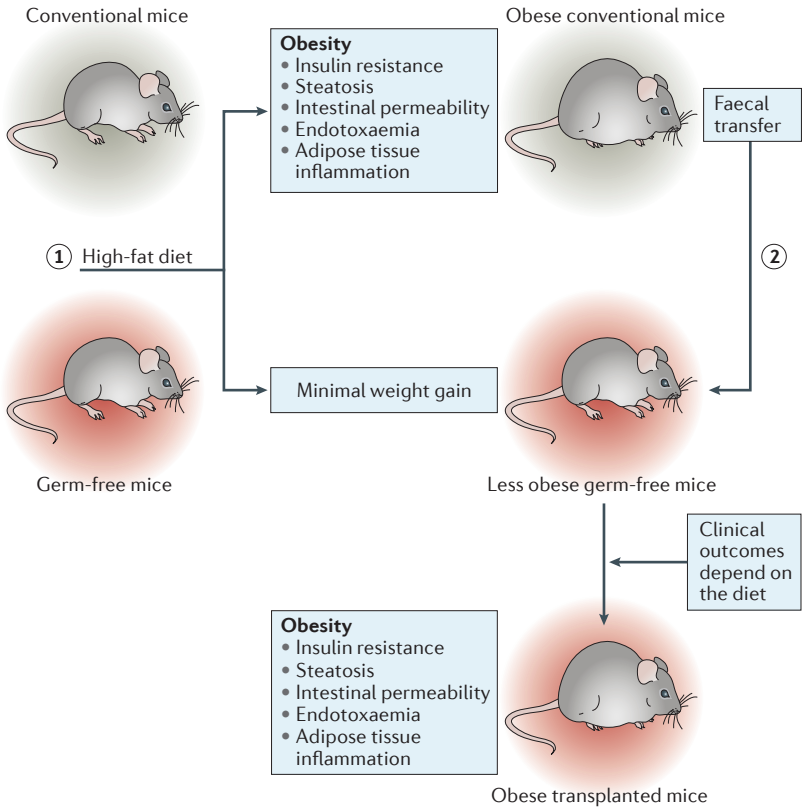

Figure 1 | **Insights into the role of the gut microbiota from germ-free mice and faecal transfer experiments.** Administration of a high fat diet (HFD) induces a notable difference in weight gain between conventional mice and germ-free mice (1). Conventional mice develop several metabolic alterations in response to a HFD, such as weight gain, insulin resistance, steatosis, and systemic and adipose tissue inflammation, whereas germ-free mice show minimal weight gain. The transfer of faeces from obese, conventionally housed mice to germ-free mice induces weight gain in germ-free mice that reaches the level of conventional mice fed a HFD, in the absence of increased food intake (2). Concomitantly, these mice display metabolic alterations. The metabolic effects of faecal transfer are also dependent on diet.

after initiation of a HFD. The HFD induces a continuous increase in *E. coli* over the course of 4 weeks, which continues to rise until a diabetic status is established. These results suggest that dietary factors affect glucose homeostasis[60]. Mice fed a HFD also exhibited a marked increase in the presence of bacterial DNA in various tissues, including mesenteric adipose tissue and mesenteric lymph nodes, from low baseline levels[60]. This effect was blunted in *Cd14*$^{-/-}$ mice fed a HFD[54]. One month of probiotic *Bifidobacterium animalis* (sp. *lactis 420*) treatment decreased bacterial translocation and improved insulin sensitivity, suggesting that probiotic intervention could reverse the adverse metabolic phenotype induced by a HFD[60].

Prebiotic interventions. Prebiotics modify the gut microbiota composition and subsequently host biology by selectively stimulating the growth and/or activity of a number of bacteria. Ob/ob mice treated with prebiotics displayed reduced metabolic endotoxaemia, lower fasting glucose levels, reduced insulin-resistance, decreased fat mass, and increased fat-free mass, and improved intestinal permeability and integrity compared to untreated ob/ob mice[61]. A shift in the microbiota composition was observed in the animals that received prebiotics, associated with their improved metabolic phenotype. An increase in Bacteroidetes and a decrease in Firmicutes was observed at the phylum level and a large increase in the abundance of *Akkermansia muciniphila* (*A. muciniphila*), at the species level[62]. When *A. muciniphila* was added to a HFD, mice were protected from developing insulin resistance, had reduced metabolic endotoxaemia and adipose tissue inflammation, and showed improvements in gut integrity compared to those fed a HFD without added *A. muciniphila*[63]. Heat-killed *A. muciniphila* was inefficient in eliciting these protective effects, thus confirming that this species contributed to the improved phenotype.

A. muciniphila is a mucin-degrading gram-negative member of the Verrucomicrobia phylum that produces SCFA and other metabolites[64]. The mechanisms of action of *A. muciniphila* are not fully understood, but are thought to induce changes in the production of lipids involved in the endocannabinoid system that trigger the secretion of gut peptides, such as GLP-1[63]. Metformin treatment (an insulin sensitizing agent) increased the level of *A. muciniphila*, improved the glycaemic profile, and increased the number of mucin-producing intestinal goblet cells in mice fed a HFD[65]. Oral gavage of *A. muciniphila* alone was able to recapitulate the beneficial effects of metformin on the glycaemic profile and number of goblet cells. Administration of both metformin and *A. muciniphila* by oral gavage induced beneficial modifications on visceral adipose tissue inflammatory tone, again suggesting a link between the gut microbiota and host metabolic biology[65].

Overall, these murine studies demonstrate a role of the gut microbiota in insulin resistance[66] and the development of metabolic diseases. Some association and mechanistic studies have also been performed in humans[67,68] and are discussed in more detail below (FIG. 2).

Lessons from human studies

Observational studies. The composition of the gut microbiota is associated with metabolic phenotypes in overweight and obese populations, as well as with weight-loss, which is known to improve cardiometabolic risk[69]. Studies using shot-gun sequencing have found that 20–40% individuals from populations in Denmark and France have low microbiota richness and a disturbed metabolic profile, including insulin-resistance, dyslipidaemia, and systemic and adipose tissue inflammation[34,70]. These metabolic alterations might contribute to increased cardiovascular risk. Those with a low gene count gained more weight during the study period than those with a high gene count.

Studies performed in the French cohort described above found that low and high microbial gene counts are associated with differential gut microbiota signatures, with 18 gene clusters being more abundant in individuals with a high gene count than in those with a low gene count, whereas other bacterial groups were diminished in individuals with a high gene count[34].

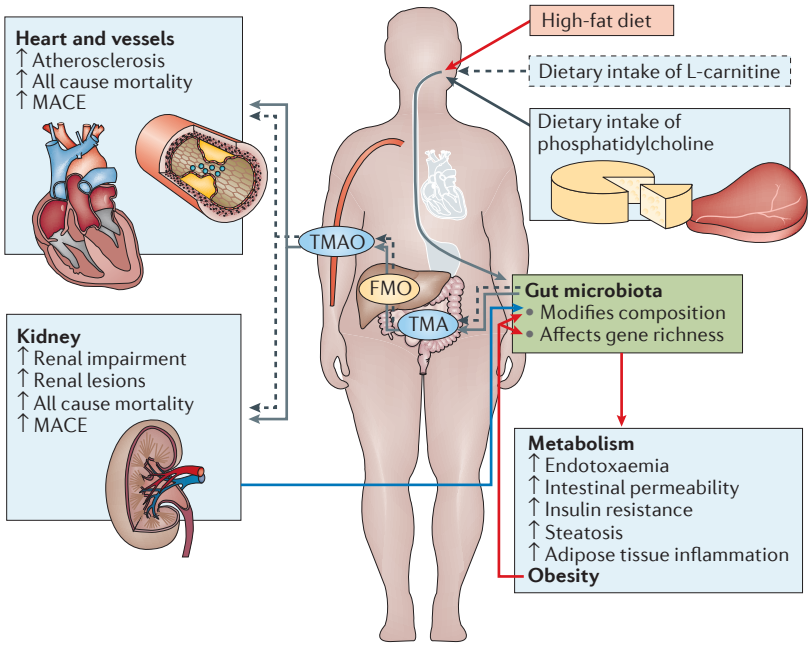

Figure 2 | **Links between dietary intake, gut microbiota, host biology and tissue damage.** Dietary phosphatidylcholine is converted by the gut microbiota into trimethylamine *N*-oxide (TMAO), which is associated with host diseases such as cardiovascular disease or kidney disease. The solid grey lines indicate the direction of the associations between diet and host damage. Dietary L-carnitine is also converted into TMAO but a link between dietary L-carnitine and cardiovascular outcomes is debated. This uncertainty is represented by the dotted lines. The gut microbiota is modified during obesity or as a result of a high fat diet, which subsequently leads to adverse metabolic outcomes. The red lines represent observations seen in obesity or with a high fat diet and the links between diet, gut microbiota modifications, and host biology. Renal impairment can also affect the composition of the microbiota (blue line). FMO, flavin monoxygenase; MACE, major adverse cardiac events; TMA, trimethylamine.

Data obtained from the Danish cohort found that 46 genera differed between the low and high gene count groups[70]. At the phylum level, a higher abundance of Proteobacteria and Bacteroidetes were identified in the low gene count individuals whereas Verrucomicrobia (*A. muciniphila*), Actinobacteria, and Euryarchaeota were increased in individuals with high gene counts[70]. Individuals with high levels of *A. muciniphila* had a better metabolic profile with improved insulin sensitivity, less dyslipidaemia[70], and better metabolic outcomes upon weight loss intervention compared to those with lower levels of *A. muciniphila*[71]. Levels of *A. muciniphila* have been shown to increase after bariatric surgery[72,73] and could contribute to associated improvements in insulin sensitivity[74]. Metagenomic approaches have found that the abundance of *A. muciniphila* in the gut is tightly associated with other bacterial groups (measured as clusters of bacterial genes termed 'metagenomic species') that co-vary with *A. muciniphila* during weight loss. Indeed, individuals with a high gene count and high levels of *A. muciniphila* display a better metabolic profile compared to those with a low gene count or low levels of *A. muciniphila*[71]. *A. muciniphila* produces propionate, which affects intestinal L-cells and GLP-1 production[75] via receptors expressed in the gut, such as GPR43[76], and thus act directly in the lumen. The individual gut microbiota ecosystem and associated functional aspects are probably important to consider when examining the role of putative bacterial targets in improved cardiometabolic health.

The importance of specific bacterial targets and their links with the ecosystem has been supported by a twin study that showed that some microbiota taxa are shared by individuals with the same genome and some of these heritable taxa, namely the Christensenellaceae family, form a coexpressed network with other shared bacteria, such as methanogenic Archaea. This study found that Christensenellaceae and associated bacteria are more abundant in individuals with a decreased BMI[77].

A low microbiota gene count profile is also associated with reduced levels of *Faecalibacterium* spp. and worse systemic inflammation, compared to individuals with a high gene count[70]. *Faecalibacterium prausnitzii* is a butyrate-producing bacteria that exerts anti-inflammatory effects by blocking the activation of NF-κB[78], and induces a reduction in bacterial-produced proteins that can modulate the NF-κB pathway[79]. *F. prausnitzii* is decreased in patients with intestinal inflammatory diseases, such as Crohn disease[79]. Furthermore, abundance of *F. prausnitzii* has been associated with glycaemic status in humans, with a reduction of *F. prausnitzii* reported in patients with metabolic syndrome or overt T2DM compared levels in healthy controls[38,80,81]. In a study that compared 345 individuals with normoglycaemia or T2DM, three enterotypes were identified but a significant association between these three groups and T2DM disease status was not observed[38]. T2DM was, however, associated with a moderate degree of dysbiosis. At a functional level, the microbiota from patients with T2DM was characterized by an increased level of bacteria involved in sulphate reduction, resistance against oxidative stress, and a decrease in butyrate-producing bacteria compared to those of normoglycaemic individuals, thus confirming previous reports[38]. Finally, *F. prausnitzii* levels are reduced in obese patients with T2DM, and increase after bariatric surgery[82] — a procedure that promotes weight-loss[83] and improves glycaemia and inflammation[84,85].

Interventional studies. Weight loss, even if moderate, drastically improves cardiometabolic risk and kidney function in those who are overweight or obese[86], and caloric restriction with enriched fibre improves microbial gene richness. Patients with a low microbial gene count together with metabolic disturbances show a reduced ability to improve their metabolic profile and risk of cardiovascular events compared to patients with a high microbial gene count[34]. This finding suggests that future diagnostics could use the microbiota profile as a predictive marker of dietary intervention outcomes. Concordantly, a small human study demonstrated that a strict vegetarian diet enriched in fibre was able to induce weight loss and improve insulin resistance and the lipid profile of patients with T2DM or other metabolic disturbances. This diet induced a reduction in gut inflammation, as measured by decreased expression of intestinal lipocaline-2[87], and was associated with

changes in the gut microbiota composition at the phylum level and a decrease in SCFA production[88]. Of note, no change in microbiota diversity was observed by 16S pyrosequencing of the microbiota genome

Bacterial DNA has also been detected in the bloodstream[89], which could provide indirect support for the intestinal permeability hypothesis previously discussed. The DESIR study — which aimed to identify characteristics that can predict the future development of T2DM using variables available in the clinic — followed 3,000 patients without T2DM for 9 years and evaluated the incidence of T2DM in this cohort. Baseline circulating bacterial DNA was increased in the patients who developed T2DM during the follow-up period compared to those who did not develop T2DM, again suggesting the possible predictive role of the microbiota in CMD progression[89]. The bacterial DNA in the blood could, however, originate from body sites other than the gut, but researchers have demonstrated that microbial richness is 10-fold higher in the gut compared to the mouth, throat, and gums[45].

Microbiota transfer to treat CMDs

Gut microbiota transfer is now viewed as a possible therapeutic tool to treat various diseases. This therapeutic approach is growing and is already well-established for the treatment of *Clostridium difficile* infection, where faecal transplantation induces an 80% remission rate[90–92]. Gut microbiota transfer has also been tested in humans in the context of insulin resistance. Overweight patients with metabolic syndrome were transferred microbiota from either their own faeces (autologous transfer) or from lean healthy controls (allogeneic transfer). After 6 weeks of follow-up, the allogeneic faecal transfer had improved hepatic and peripheral insulin sensitivity by 119% and 176%, respectively, as shown by a euglycaemic–hyperinsulinaemic clamp technique[93]. This metabolic improvement was independent of any weight variations. The allogeneic faecal transfer induced an increase in overall gut microbial richness, and more specifically, increased the abundance of butyrate-producing bacteria, such as *Roseburia,* confirming previous results that showed an association between *Roseburia* and glucose homeostasis[38,80]. Whether this method of faecal transfer could be extended to other facets of CMDs remains an open question.

Gut microbiota and CVD

Emerging data suggest associations between the gut and oral microbiota and several facets of CVD, including atherosclerotic plaque formation, myocardial infarction, and heart failure. The contribution of environmental factors to CVD has also been explored in this context (FIG. 2). Analyses of gut microbiota composition by shot-gun sequencing has been used to successfully discriminate between patients with symptomatic atherosclerosis (with stenotic plaques in the carotid artery) and age-matched and sex-matched controls without plaques, as evaluated by ultrasonography[39]. Patients with atherosclerosis displayed reduced faecal abundance of butyrate-producing *Roseburia*, which is also known to be decreased in patients with T2DM compared to healthy controls, as previously discussed[38,80]. Changes in bacterial gene composition were indicative of alterations in microbiota metabolic function, such as an enrichment in butyrate–acetoacetate CoA-transferase genes in patients with atherosclerosis. These genes negatively correlated with systemic inflammation, confirming a negative association between butyrate and inflammation[94]. By contrast, healthy control patients had an enrichment of bacterial groups with phytoene dehydrogenase functions involved in lipid-soluble antioxidant metabolism and serum β-carotene production[39]. Strikingly, bacterial DNA was discovered in atherosclerotic plaques, and some of the species identified were similar to those found in the microbiota of the oral cavity, such as high levels of Proteobacteria and low levels of Firmicutes[95]. Abundance of bacterial DNA in the atherosclerotic plaque correlated with CVD risk factors[96], such as serum LDL-cholesterol[95]. Overall, both the gut and oral microbiota could contribute to CVD development. This field has now taken a further step forward by combining high-throughput sequencing with metabolomic analysis to provide deeper insight into the putative links between the gut microbiota and CVD, and the role of ingested nutrients and bacterial-derived metabolites following metabolism of these compounds[97,98].

Phosphatidylcholine and TMAO production

Dietary phosphatidylcholine-derived gut bacteria metabolites are involved in the development of atherosclerosis, progression of heart failure, and mortality[99]. Trimethylamine *N*-oxide (TMAO) is produced from the metabolism of dietary phosphatidylcholine (mostly originating from meat, eggs, and fish) by commensal microbes. Phosphatidylcholine is hydrolysed by trimethylamine (TMA) lyase into TMA, which is further oxidized in the liver by flavin monooxygenase (FMO) into circulating TMAO[100,101]. FMO3 enzymatic activity is regulated by bile acids via the nuclear hormone farnesoid X receptor expressed in the liver[102]. TMAO was markedly increased in patients with cardiac insufficiency[103,104], but interestingly was decreased in patients with ischaemic heart insufficiency compared to levels in patients with stable coronary artery disease without cardiac insufficiency[103]. Furthermore, circulating TMAO was a robust prognostic marker of adverse cardiac events during a 5 year follow-up, even after adjustment for age, kidney function, and NT-proBNP hormone levels[104]. These findings were later confirmed in a larger cohort by the same investigators[105].

TMAO concentrations were analysed in a large cohort of cardiac stable patients with atherosclerosis. TMAO was increased in patients who developed notable cardiac events during follow up compared to those who remained stable[99]. Furthermore, an increase in TMAO concentration was associated with an increased risk of death and major adverse cardiovascular events, even after adjustment for traditional cardiovascular risk factors[106]. These observations were further substantiated in preclinical models of CVD. A TMAO enriched diet administered to atherosclerosis prone (C57BL/6J *ApoE*$^{-/-}$) mice

induced aortic lesions[99]. Furthermore, dietary intake of phosphatidylcholine induced a progressive increase in TMAO that was abolished by broad spectrum antibiotics and was absent in germ-free mice[99]. These data demonstrate the obligatory role of the gut microbiota in processing phosphatidylcholine into circulating TMAO. These data were confirmed in humans who showed increased systemic TMAO concentration after an oral load of phosphatidylcholine[106].

High concentrations of choline or betaine (sources of TMAO) are also associated with increased major adverse cardiac events, even after accounting for traditional cardiovascular risk factors, such as smoking, age, hypertension, dyslipidaemia, and T2DM. This association, however, only holds true for patients with concomitant elevations of TMAO, suggesting the contribution of common pathways[107]. Importantly, mice fed an enriched choline or betaine diet displayed increased aortic lesions containing cholesterol-laden foam cells, which was rescued by antibiotic treatment[99]. Further studies confirmed that higher susceptibility to atherosclerosis could be transmitted by caecal transfer from mice on a permissive background producing high levels of TMAO[108].

Overall, these results suggest the relevance of TMAO in the development of atherosclerosis. Phosphatidylcholine intake seems to be involved in these described deleterious outcomes, whereas the dietary choline-dependent differences in TMA and TMAO levels are not concordant in all published studies. Although convincing, these results need to be confirmed in other populations worldwide. In particular, they need to be reproduced in countries other than the USA where markedly different dietary habits are encountered. The complex links between food diversity, specific nutrients, the microbiota, and CVDs warrant further studies with careful monitoring of food intake.

Characterizing food intake patterns in large cohorts might provide further insights into still unexplained paradoxes. Indeed, phosphatidylcholine also originates from seafood which is, by contrast, associated with a reduced risk of death from CVD[109]. Of note, a beneficial effect on CVD reduction has been documented after substantial daily fish consumption[109]. However, seafood is not only rich in phosphatidylcholine but is also a source of polyunsaturated fatty acids (PUFAs), which have beneficial effects on CVD outcome[110]. PUFAs might, therefore, elicit a superior effect over phosphatidylcholine intake to induce the beneficial health outcomes that have been reported with fish consumption. These considerations indicate the need to have a thorough food diary with detailed food composition data to quantify the intake of choline-related nutrients. These details would enable stratification of patients according to their precise nutrient intake and food patterns. Furthermore, studies comparing the effects of fish versus meat intake on TMAO concentration and related cardiovascular outcomes might help clarify this issue.

L-carnitine and TMAO production

L-carnitine found in red meat induces TMA production via the same pathways as phosphatidylcholine, and is associated with an increased risk of CVD and major adverse cardiac events[111]. The systemic level of L-carnitine is increased in omnivores compared to vegan subjects, and also increases upon meat ingestion[110]. L-carnitine is suppressed by antibiotic treatment in humans and different rodent models, and increases after antibiotic wash-out. Importantly, an L-carnitine-enriched diet is also associated with modifications to the microbiota. Patients with high systemic levels of TMAO tend to have a prevalent *Prevotella* enterotype. Of note, several genera, such as *Peptostreptococcaceae incertae sedis,* are significantly associated with an omnivorous diet and increased TMAO levels. Likewise, modifications to the microbiota composition have been observed in mice following L-carnitine supplementation[111], with specific taxa, such as *Prevotella* and unclassified *Prevotellaceae*, associated with increased TMAO levels, thus confirming results obtained from human studies.

Mechanistic insight into the role of L-carnitine on CVD outcomes was gained by the aforementioned study whereby addition of L-carnitine to the diet of mice increased TMAO levels and worsened aortic lesions[111]. These effects were prevented by antibiotic treatment[111]. L-carnitine induced a reduction in reverse cholesterol transport[112,113], which was again prevented by antibiotics[111]. Together, these results suggest a potential role for the microbiota metabolizing L-carnitine into TMA and TMAO. The bacterial groups and interactions that are involved in this process need further investigation. The data obtained to date suggest a strong link between dietary phosphatidylcholine intake and TMAO, which could represent a new non-traditional cardiovascular risk factor that could eventually be used for patient stratification[101].

Despite these data, the link between phosphatidylcholine intake, TMAO formation, atherosclerotic plaque formation, and all-cause mortality is still debated and some caution is required regarding the relevance of L-carnitine intake to cardiovascular effects[114]. Although the results produced from mouse studies are convincing, the amount of L-carnitine supplementation added to the diet was much higher than humans would typically ingest, which raises concerns regarding the translational relevance of these data. As well as L-carnitine supplementation, red meat was also consumed by the mice, which would also raise TMAO levels. These confounds render it difficult to identify the metabolite that modifies CVD risk or determine whether a combination of both L-carnitine and red meat intake can increase CVD risk[40].

Other beneficial effects of L-carnitine have been demonstrated. L-carnitine has been shown to improve insulin sensitivity[115]. For example, supplementation of L-carnitine[115] for 6 months induced an improvement in the insulin resistance index of elderly patients with insulin resistance at baseline. Furthermore, metabolic flexibility — the ability to switch back and forth between glucose and fat oxidation based on availability and need — was enhanced in the muscle tissue of mice with muscle-specific carnitine acetyltransferase deletion as well as in humans following L-carnitine supplementation[116]. Other examples are provided by the exploration of heart function recovery after ischaemia, which returned to baseline

levels after transcardial perfusion of L-carnitine in rats. Rats that were not perfused with L-carnitine displayed a marked decrease in recovery of heart function[117]. Finally, a meta-analysis of 13 controlled trials indicated that L-carnitine supplementation was associated with a 27% reduction in all-cause mortality, a 65% reduction in ventricular arrhythmias, and a 40% reduction in symptoms of angina after acute infarction compared to placebo treatment[118]. Importantly, supplementation with 3 g L-carnitine per day conferred beneficial outcomes, and a greater dose had no additional effect on improving outcomes and induced no secondary effects[119]. Overall the effects of L-carnitine remain debated and more studies that include comprehensive food diary reports are needed across different populations and diseases.

Finally and further complicating this picture, TMA and TMAO metabolites have been studied in patients with HIV infection[120]. TMA but not TMAO was associated with calcium score and with the number and volume of total and calcified atherosclerotic plaques in patients with HIV infection[121], independent of the Framingham risk score. No data on food intake was available for this population. These data suggest that choline and derived metabolites deserve further attention, and that further studies with thorough documentation of food intake are warranted in populations at risk of CMDs, such as those with HIV infection.

Gut microbiota and CKD

Microbiota signatures in CKD

The majority of risk factors for CVD are also risk factors for CKD[122,123]; therefore, certain changes in the gut microbiota that affect CVD might also be expected to be associated with CKD. Although data remain limited, studies employing 16S pyrosequencing have started to investigate the microbiota–kidney disease axis. Marked differences in the composition of the microbiota were found using phylogenetic microarrays to compare the microbiota of healthy controls with that of patients with end-stage renal disease (ESRD)[124]. A total of 190 bacterial operational taxonomic units (OTU) were increased in patients with ESRD, including some from the major bacterial phyla Firmicutes (especially subphylum Clostridia), Actinobacteria, and Proteobacteria (primarily Gammaproteobacteria). These phyla are also associated with other chronic and common diseases[124]. Using phylogenetic microarrays, the relative richness of the microbiota did not differ between the groups, but this approach might not be optimal to characterize microbiota richness and diversity. Microbiota richness relates to total bacterial gene number obtained by metagenomic sequencing. At this level, the composition of the different species in gut microbiota might differ between individuals while the overall number of bacterial gene will not. Furthermore, the bacterial gene number can be assessed by metagenomic sequencing that evaluates the overall microbiota, including the unknown species. By contrast, phylogenetic microarrays can only assess the known sequences of bacterial genes. For this reason, a difference in gene richness might not have been detected using this technique. Further investigations in rodent models identified differences in the gut microbiota signature between nephrectomized rats and controls, in which 175 operational taxonomic units differed between the two groups. Indeed, rats with ESRD displayed a lower abundance of certain families of Bacteroidetes and Firmicutes, especially Lactobacillaceae and Prevotellaceae[124]. These findings, however, could be due in part to uraemia.

In order to determine the influence of uraemia on the microbiota, 16S pyrosequencing has been used to delineate the differences in the microbiota composition between patients with ESRD and healthy individuals[125]. Patients with ESRD exhibited a gut microbiota that was enriched in bacteria possessing urease and uricase enzymatic activity, which could contribute to the increased metabolism of urea associated with CKD. Importantly, dietary interventions are recommended for patients with ESRD to reduce hyperkalaemia, and as a consequence, patients consume less fruits, vegetables, and fibres. Accordingly, the gut microbiota becomes depleted in bacteria that can produce SCFA[125]. Another study using denaturing gradient gel electrophoresis (DGGE) found no marked differences in the gut microbiota profiles between a small cohort of healthy control individuals and patients with CKD[126]. Large inter-individual differences were, however, noted, which suggested limited power of the study design. Upon use of quantitative PCR to analyse the products obtained by DGGE, differences in levels of bacterial phyla were identified, including an increase in Firmicutes in patients with CKD, whereas bacteria belonging to the Bacteroidetes phyla were more frequent in healthy controls[126].

Data to describe the gut microbiota signature of ESRD at the phyla level are needed. Studies have shown differences in the gut microbiota in bacteria belonging to the Bacteroidetes and Firmicutes phyla between patients with ESRD and healthy controls. Marked correlations between these gut microbiota profiles and indirect markers of CVD risk in ESRD, such as VCAM-1 serum concentrations, have also been found[127,128]. Differences in the number of bacterial species have been identified in patients with ESRD and healthy controls by pyrosequencing, with an increased total number of bacterial species in patients with ESRD. Some species were common between the two groups, some species were dramatically increased in patients with ESRD (namely *Klebsiella*, *Proteus*, *Escherichia*, and *Pseudomonas*), and the *Enterobacter* species was unique to those with ESRD[129]. An increased concentration of systemic D-lactate in patients with ESRD was also detected, which was hypothesized to be caused by increased gut permeability in these patients. In agreement with this hypothesis, bacterial DNA was also present in the blood of a subset of patients with ESRD, and was not detected in healthy controls[129,130]. The circulating bacterial DNA matched that present in the gut microbiota of patients with ESRD. Patients with ESRD who had circulating bacterial DNA exhibited increased systemic inflammation, as assessed by high sensitivity test for C-reactive protein and IL-6[130]. From these data, it was proposed that bacterial translocation occurs in

ESRD, which is further responsible for increased systemic inflammation that could hasten the progression of kidney disease[131]. This hypothesis requires confirmation in larger cohorts and might benefit from direct evaluation of gut permeability, for example with the urinary lactulose:mannitol ratio test[132], or with specific permeability assays[59]. Previous data obtained from permeability assays in both humans[133] and rats[134] have already suggested increased gut permeability during ESRD, at least for large sized molecules.

Gut-derived TMAO in CKD

A link between dietary choline exposure, systemic TMAO concentrations, CKD, and CVD has been demonstrated in humans and mice[135]. TMAO is cleared by the kidney, and levels are, therefore, increased in patients with CKD compared to levels in healthy controls[104]. Patients with ESRD also display higher levels of TMA and TMAO before haemodialysis compared to levels in healthy controls. The elevated levels of these metabolites were drastically reduced after only one session of haemodialysis and recovered to the levels detected in healthy controls[136]. The elevated levels of TMAO in patients undergoing haemodialysis[137] can also markedly reduce after kidney transplantation[138]. No information regarding food intake consumption was provided for the patients undergoing haemodialysis who were included in these studies. Interestingly, one study has evaluated the concentration of TMAO in patients undergoing haemodialysis after oral supplementation of L-carnitine. Supplementation induced an increase in both L-carnitine and TMAO levels that were reduced following haemodialysis sessions[139].

Increased levels of TMAO have been shown to be predictive of an increased risk of all-cause mortality in patients with CKD[140], even after adjustment for kidney dysfunction by cystatin C[141]. Patients with increased TMAO and cystatin C levels had a threefold increase in mortality risk even after adjustment for traditional risk factors, such as smoking, age, hypertension, dyslipidaemia, and T2DM. High TMAO concentrations predicted long-term cardiac death in patients with CKD and coronary atherosclerosis[138]. These data failed to be replicated in a separate cohort of haemodialysis patients; however, the coronary burden was not specifically assessed in that setting[137]. Concordantly, mice fed a TMAO-enriched or choline-enriched diet had increased circulating TMAO levels compared to mice on a normal diet and subsequently developed tubulointerstitial fibrosis with collagen deposition after only 6 weeks, which suggests a pathophysiologic role for TMAO in kidney fibrosis. When followed for a longer period of 16 weeks[140], mice fed the enriched diets displayed elevated cystatin C levels compared to controls — an indirect marker of glomerular filtration[142]. Together, these data suggest a strong link between high dietary phosphatidylcholine, changes in the gut microbiota, and subsequent TMAO production in CKD. As in CMDs, high levels of TMAO could predict cardiovascular outcomes in patients with CKD[140]. The levels of TMA and TMAO should probably be measured in patients with different kidney diseases and the link between the amount and quality of food intake in different countries should be examined. Because of the importance of reduced gut bacterial richness in chronic diseases, exploring gut microbial richness during the progression of kidney disease and related disorders would be of interest.

Conclusions

During obesity or in the context of a HFD, changes in the gut microbiota contribute to metabolic disorders in mice. In humans, changes in the gut microbiota have been associated with several metabolic diseases. These observational associations, however, warrant controlled intervention studies to determine the underlying mechanisms. Although the use of germ-free mouse models is convenient to test mechanistic hypotheses, one must be mindful that these models do not fully reflect a normal physiology due to the absence of a mature intestine and immune system. The use of other models (such as antibiotic-treated mice) and/or human faecal transfer might complement the results obtained thus far. Data from murine studies are somewhat difficult to translate to humans as the environment has such an important role in shaping the composition of the gut microbiota. Well-controlled intervention studies in humans that are paired before and after an intervention with an appropriate control population, are needed. The question of what constitutes a healthy microbiota requires further investigation in order for a definition to be agreed and for a control population to be identified. Importantly, thorough phenotypic analyses of patients, both clinically and of food intake is critical to decipher the potential role of the microbiota in disease and to mitigate confounding environmental factors. When designing such studies, investigators need to be mindful of standardizing methodologies (BOX 1; TABLE 1).

Metagenomic sequencing and metabolomic profiling have opened avenues to find new gut microbiota-derived diagnostic markers to stratify complex co-related diseases, and to identify new pathways associated with developing or established diseases. Many microbes (such as *A. muciniphila* and *F. prausnitzii*) are altered in several CMDs, and represent targets to be tested in associated diseases. Gut-derived metabolites, such as TMAO, produced from the metabolism of dietary phosphatidylcholine have been proposed to be involved in the development of atherosclerosis and serious cardiovascular outcomes, such as major adverse cardiac events and all-cause mortality, but also seem to be implicated in kidney diseases. Performing metabolomic analyses on serum, urine, and eventually faeces will help confirm the functionality of results obtained by metagenomic or pyrosequencing analyses of the gut microbiota. This approach might also help raise new hypotheses. Computational predictive tools[53] have also been validated, which when associated with multi-omic studies might help corroborate the observed results. These new 'omic' techniques have enabled better characterization of the virome[143] and fungome that are also present in the digestive tract. These data have raised the notion

that viruses are not only pathogens but also have a role in the gut[143], in particular an interaction with the gut microbiota to facilitate host physiological processes, such as metabolism, the killing of bacteria that promote antibiotic resistance, and host–immune system interactions[144]. The local environment, which includes food intake[145] and gut bacteria, shapes the virome as a pathogen or commensal. A new field of research is now open to decipher the potential role of the virome in diseases such as those discussed in this Review. Potential therapeutic options might be generated in the future through the use of modified viruses that will enable the gut microbiota to be shaped to improve host health status.

Overall, these described observations open new avenues to examine the pathways that link diet, the microbiota and its derived metabolites, and various diseases in populations affected by CMDs and kidney diseases from different countries, environments, and risk exposures. Since lifestyle modifications are somewhat difficult to achieve and/or maintain in the long term, these data could help initiate the development of effective targets towards the gut microbiota and their derived-metabolites, eventually in combination with other therapies targeting host biology. The ability to use modified viruses in the future to manipulate the gut microbiota is also an attractive possibility.

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Competing interests statement
The authors declare no competing interests.