Trimethylamine and trimethylamine N-oxide, a flavin-containing monooxygenase 3 (FMO3)-mediated host-microbiome metabolic axis implicated in health and disease

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ABBREVIATIONS: cnt, carnitine utilization gene cluster; cut, choline utilization gene cluster; DMA, dimethylamine; FMO, flavin-containing monooxygenase; LIRKO, liver insulin receptor-knockout; MA, methylamine; TMA, trimethylamine; TMAO, trimethylamine N-oxide; TMAU, trimethylaminuria.
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ABSTRACT

Flavin-containing monooxygenase 3 (FMO3) is known primarily as an enzyme involved in the metabolism of therapeutic drugs. However, on a daily basis we are exposed to one of the most abundant substrates of the enzyme, trimethylamine, which is released from various dietary components by the action of gut bacteria. FMO3 converts the odorous trimethylamine to non-odorous trimethylamine N-oxide, which is excreted in urine. Impaired FMO3 activity gives rise to the inherited disorder primary trimethylaminuria. Affected individuals cannot produce trimethylamine N-oxide and, consequently, excrete large amounts of trimethylamine. A dysbiosis in gut bacteria can give rise to secondary trimethylaminuria. Recently, there has been much interest in FMO3 and its catalytic product trimethylamine N-oxide. This is because trimethylamine N-oxide has been implicated in various conditions affecting health, including cardiovascular disease, reverse cholesterol transport and glucose and lipid homeostasis. In this review, we consider the dietary components that can give rise to trimethylamine, the gut bacteria involved in the production of trimethylamine from dietary precursors, the metabolic reactions by which bacteria produce and utilize trimethylamine and the enzymes that catalyze the reactions. Also included is information on bacteria that produce trimethylamine in the oral cavity and vagina, two key microbiome niches that can influence health. Finally, we discuss the importance of the trimethylamine/trimethylamine N-oxide microbiome-host axis in health and disease, considering factors that affect bacterial production and host metabolism of trimethylamine, the involvement of trimethylamine N-oxide and FMO3 in disease and the implications of the host-microbiome axis for management of trimethylaminuria.
Introduction

Flavin-containing monooxygenases (FMOs) (EC 1.14.13.8) catalyze the NADPH-dependent oxidative metabolism of a wide array of foreign chemicals, including drugs, dietary-derived compounds and environmental pollutants (Krueger and Williams, 2005). Humans possess five functional FMO genes, FMO1, 2, 3, 4 and 5 (Hernandez et al., 2004; Phillips et al., 2007). The main site of expression of FMO3 is the liver (Dolphin et al., 1996), however, high expression has also been observed in the skin of certain individuals (Janmohamed et al., 2001). The FMO3 gene is switched on in human liver at birth and it can take several years for the gene to attain maximum expression, as the liver develops to its full functional capacity (Koukouritaki et al., 2002).

Drug substrates for FMO3 include the antipsychotic olanzapine (Söderberg et al., 2013), the antiestrogen tamoxifen (Parte and Kupfer, 2005), the gastroprokinetic agent itopride (Mushiroda et al., 2000) and the H2-receptor antagonist cimetidine (Cashman et al., 1995). Substrates undergo N- or S-oxygenation; generally, this is a detoxification route and the products are excreted in urine, but some chemicals are converted to a more active or toxic form (Krueger and Williams, 2005). Retro-reduction of the oxygenated product can occur, creating a pool of drug that can be recycled (Cashman, 2008). The importance of FMO3 in detoxification and bioactivation of xenobiotics has recently been reviewed (Cruciani et al., 2014). The FMO3 gene is highly polymorphic (Phillips et al., 2007) and genetic variants that are common in the general population are known to influence drug metabolism (Phillips et al., 2007; Söderberg et al., 2013; Zhou et al., 2014).

FMO3 has an important relationship with the gut microbiome, with an abundant non-drug substrate, trimethylamine (TMA), being derived from dietary components by the action of gut microbes (Seim et al., 1985; Al-Waiz and Smith, 1992). TMA is rapidly absorbed and is converted in the liver to trimethylamine N-oxide (TMAO) (Higgins et al., 1972). Of the
five functional FMOs of humans (FMOs 1-5), only FMO3 effectively catalyzes the conversion of TMA to TMAO (Lang et al., 1998). FMO3 is, thus, an excellent example of a protein that participates in host-gut microbiome metabolic interactions (Nicholson et al., 2012). Rare genetic variants of the FMO3 gene that abolish or severely impair activity of the enzyme give rise to the inherited disorder primary trimethylaminuria (TMAU) (Phillips and Shephard, 2008), because of inefficient conversion of microbiome-derived odorous TMA to non-odoriferous TMAO.

Recently, there has been much interest in FMO3 and its catalytic product trimethylamine N-oxide. This is because trimethylamine N-oxide has been implicated in various conditions affecting health, including cardiovascular disease, reverse cholesterol transport and glucose and lipid homeostasis (Wang et al., 2011; Bennett et al., 2013; Tang et al., 2013; Koeth et al., 2013; Gao et al., 2014; Obeid et al., 2016). The microbial origin of TMA is of great interest, as we begin to fully appreciate the importance of our microbiomes for health and wellbeing. Knowledge of the microbial species that give rise to TMA would contribute greatly to our understanding of the consequences for human health of the complex interrelationships of diet, the microbiome and the capacity for FMO3-catalyzed conversion of TMA to TMAO.

In this review, we first describe the origins and metabolic fate of TMA and outline the basis and consequences of TMAU. We then consider the dietary components that can give rise to TMA, the gut bacteria involved in the production of TMA from dietary precursors, the metabolic reactions by which bacteria produce and utilize TMA and the enzymes that catalyze the reactions. Also included is information on bacteria that produce TMA in the oral cavity and vagina, two key microbiome niches that can influence health, and on non-commensal bacteria that produce TMA. Finally, we discuss the importance of the TMA/TMAO microbiome-host axis in health and disease, considering factors that affect
bacterial production and host metabolism of TMA, the involvement of TMAO and FMO3 in disease and the implications of the host-microbiome axis for management of TMAU.

The origins and metabolic fate of trimethylamine

The discovery and properties of TMA have been recently reviewed (Mitchell and Smith, 2016). TMA is an indicator of food spoilage, particularly of fish and milk. As early as the 1930s Beatty showed that TMA was produced during fish spoilage (Beatty, 1938). The TMA is derived from bacterially mediated reduction of TMAO (Barrett and Kwan, 1985), which is present in large quantities in marine fish (Zhang et al., 1999).

The requirement of bacterial action for the production of TMA from the diet in vivo is demonstrated by the failure of germ-free rats fed carnitine (Seim et al., 1985), or of germ-free or antibiotic-treated mice fed a normal diet, to excrete TMA in their urine (Al-Waiz and Smith, 1992). TMA is derived from the diet by microbial degradation of precursors found in, for example, marine fish, eggs, offal, soya beans, peas and red meat (Zhang et al., 1999; Zeisel et al., 2003). Once liberated, TMA is rapidly absorbed through the gut wall and transported to the liver, where it is converted to non-odorous TMAO, which is then rapidly cleared in the urine (Al-Waiz et al., 1987a). Analysis in vitro of heterologously expressed FMOs revealed that, at physiological pH, N-oxygenation of TMA is catalyzed by FMO3, with a $K_M$ of 28 μM and an apparent $k_{cat}$ of > 30 min$^{-1}$ (Lang et al., 1998). Other FMOs are far less effective in catalyzing this reaction: FMO1, FMO2 and FMO4, none of which are expressed in adult human liver (Phillips et al., 2007), exhibit apparent $k_{cat}$s of 0.1, 1 and 0.1 min$^{-1}$, respectively, whereas FMO5, the only other form of FMO expressed in adult human liver (Phillips et al., 2007), is unable to catalyze the reaction (Lang et al., 1998). FMO1, which is expressed in human kidney (Dolphin et al., 1991), can catalyze TMA N-oxygenation, with an apparent $k_{cat}$ of 5 min$^{-1}$, but only at substrate concentrations of 5 mM.
(Lang et al., 1998), which are unlikely to be relevant for metabolism of TMA in vivo. The importance of FMO3 for the metabolism of TMA in vivo is demonstrated by the marked reduction in the ability to N-oxygenate TMA of individuals homozygous or compound heterozygous for mutations that severely impair FMO3 activity (Dolphin et al., 1997, 2000; Treacy et al., 1998).

In addition to N-oxygenation, rat liver microsomes carry out demethylation of TMA, but the latter reaction is much less efficient (Gut and Conney, 1991). Demethylation of TMA in rat liver was shown to be catalyzed by a different FMO from that which catalyzes production of the N-oxide (Gut and Conney, 1993). This FMO is probably FMO1, whose gene is expressed in the liver of rodents and other mammals, but not in adult human liver (Dolphin et al., 1991). In human volunteers, the demethylation product was found in low amounts and only in those dosed with high amounts of TMA (Al-Waiz, et al., 1987b). Consequently, in humans FMO3-catalyzed N-oxygenation is by far the most important route of metabolism of TMA.

Since microbial diversity studies have entered the era of high-throughput sequencing and in silico analysis, more insight has been gained into the composition of the human gut microbiota. However, studies based on bacterial cultivation are still the main source for information about the metabolic capacities of the microbiota. Combining what is being uncovered about the composition of the human microbiota with what is known of the functional activity of its components, enables identification of microbial metabolic pathways by which TMA can be produced or metabolized in the human gut.

### Trimethylaminuria, a deficiency of FMO3

In humans, mutations that abolish or severely impair the activity of FMO3 cause the inherited disorder primary TMAU (Dolphin et al., 1997; Hernandez et al., 2003). Affected
individuals have a severely reduced ability to convert TMA to TMAO and, consequently, excrete large amounts of odorous TMA in their urine, sweat and breath (Ayesh et al., 1993). Although the disorder has no overt physiological effects on patient health, it can have profound psychological and social consequences, resulting in a severe loss of quality of life, in extreme cases giving rise to clinical depression and suicidal tendencies (Mitchell and Smith, 2001; Shephard et al., 2015, Phillips and Shephard, 2015). In terms of drug metabolism, TMAU individuals have impaired metabolism of the FMO3 drug substrate, benzydamine (Mayatepek et al., 2004). Since the first discovery of a mutation known to cause TMAU (Dolphin et al., 1997), many different causative mutations have been identified (Phillips et al., 2007; Yamazaki and Shimizu, 2013). A catalogue of variants of the FMO3 gene and their effect on the ability of FMO3 to catalyze the oxygenation of TMA and drug substrates of the enzyme can be accessed at the FMO3 locus-specific mutation database (http://databases.lovd.nl/shared/genes/FMO3). 

Production of large amounts of TMA, as a result of gut microbial action, exacerbates the symptoms of primary TMAU. In addition, overproduction of TMA, as a consequence of a dysbiosis of the gut microbiome, can give rise to a non-genetic form of the disorder, known as secondary TMAU (Mitchell and Smith, 2001). Therefore, a better understanding of the bacterial species that produce TMA in the gut may provide insights into why some individuals develop secondary TMAU in the absence of impaired N-oxygenation of TMA (Shimizu et al., 2014) and offers the potential to develop improved strategies for the management and treatment of both primary and secondary forms of the disorder.

Dietary precursors of TMA and the bacteria involved in its production in the gut

TMAO. TMAO, the oxygenated product of TMA, is itself a dietary constituent that can give rise to TMA in the gut. Marine fish contain the highest amounts of TMAO (up to 3
mg/g) of any food source (Mitchell et al., 2002). The TMAO is thought to act as an osmolyte that allows adaptation to changes in salinity (Pang et al., 1977) and hydrostatic pressure (Zerbst-Boroffka et al., 2005). It is estimated that about 50% of consumed TMAO is not metabolized and passes through the body unchanged to be excreted in the urine (Zhang et al., 1999). In the human gut, the remainder of the TMAO is reduced to TMA by bacterial TMAO reductase (EC 1.7.2.3) (Kwan and Barrett, 1983) (Figure 1). Examples of bacteria that produce TMA from TMAO are given in Table 1.

**Choline.** Choline is an essential dietary nutrient, which can function as a precursor for the synthesis of phospholipids, including phosphatidylcholine, that are essential components of membranes, and of the neurotransmitter acetylcholine. It is present in high quantities in a variety of foodstuffs, for example, beef liver, cauliflower and peanuts (Zeisel et al., 2003) and high concentrations of free choline are present in human milk and in soya bean-derived milk formulae (Zeisel, 1990). However, choline is usually ingested as lecithin (also known as phosphatidylcholine), in which the choline moiety is covalently bound to a phosphatidate (a phosphoglycerol attached to two fatty acids) (Hirsch et al., 1978). Interconversion of lecithin and choline is bidirectional, conversion of lecithin to choline being catalyzed by phospholipase D (EC 3.1.4.4) and the first step in the conversion of choline to lecithin by choline kinase (EC 2.7.1.32) (Figure 1).

Free choline is absorbed throughout the small intestine and is subsequently integrated into cell membranes, or actively taken up by the liver, where it can be converted to betaine, phosphocholine or lecithin (Zeisel, 1990). However, high amounts of choline may exceed the absorptive capacity and pass through to the large intestine, where it is metabolized to methylamines by microbial action (Zeisel et al., 1983). Choline is a quaternary ammonium compound, containing a trimethylammonium moiety. Thus, it can act as a precursor for TMA (Zeisel et al., 1989; Chalmers et al., 2006). The bacterial conversion of choline to
TMA involves the cleavage of the carbon-nitrogen bond of choline, producing TMA and acetaldehyde (Hayward and Stadtman, 1959). Craciun and Balskus (2012) proposed that a glycycl radical enzyme CutC (EC 4.3.99.4), encoded by the bacterial choline utilization gene cluster (cut), might act as a choline TMA-lyase to catalyze this initial step in choline degradation (Figure 1). This was confirmed by demonstrating that deletion of cutC in *Desulfovibrio desulfuricans* abolished the ability of the organism to produce TMA from choline. Bioinformatics analysis revealed cutC homologues in 89 bacterial genomes. The homologues are not distributed evenly among the major bacterial phyla of the human gut, being present in *Firmicutes, Actinobacteria* and *Proteobacteria*, but absent from *Bacteroidetes* (Craciun and Balskus, 2012). Table 1 shows bacteria known to be associated with formation of TMA via choline degradation.

**Carnitine.** Carnitine plays a key role in metabolism, being involved in the transport of long-chain fatty acids from the cytosol into mitochondria. Carnitine is a quaternary ammonium compound that can be synthesized in the body from methionine and lysine, with its immediate precursor being γ-butyrobetaine (Feller and Rudman, 1988). L-carnitine is present in red meat and dairy products (Feller and Rudman, 1988) and it is estimated that the average non-vegetarian American consumes 100-300 mg/day (Feller and Rudman, 1988). Of dietary carnitine, about half is absorbed from the intestine, while the other half is metabolized by gut flora, eventually resulting in the excretion of TMAO and γ-butyrobetaine in urine and faeces, respectively (Rebouche and Chenard, 1991). Gut bacteria are thought to cleave the 3-hydroperoxybutyryl moiety from L-carnitine to produce TMA (Meadows and Wargo, 2015). This pathway has been observed in several bacteria including *Serratia marasescens* and *Acinetobacter calcoaceticus* (Meadows and Wargo, 2015). Using bioinformatics approaches, Zhu *et al.* (2014) identified a two-component oxygenase/reductase Rieske-type enzyme, encoded by *cntAB*, that catalyzed the formation of
TMA from carnitine (see Figure 1). Both components are required for catalysis of electron transfer and the degradation of carnitine to TMA. Deletion of \textit{cntAB} in \textit{Acinetobacter calcoaceticus} rendered the microbe incapable of producing TMA. As is the case for \textit{cutC} in choline metabolism (Craciun and Balskus, 2012), \textit{cntAB} is distributed unevenly among the major bacterial phyla, being present in \textit{Proteobacteria} (mainly \textit{Gammaproteobacteria}) and \textit{Firmicutes} and absent from \textit{Bacteroidetes} (Zhu et al., 2014).

In addition to generating TMA directly from L-carnitine, it has been suggested that gut bacteria can also convert $\gamma$-butyrobetaine to TMA. Most studies consider $\gamma$-butyrobetaine to be an intermediary metabolite produced during the biosynthesis of L-carnitine, by a reaction catalyzed by $\gamma$-butyrobetaine hydroxylase (EC 1.14.11.1). However, the gut microbiota may also be able to produce $\gamma$-butyrobetaine from catabolism of L-carnitine, by a reaction catalyzed by $\gamma$-butyrobetainyl-CoA: carnitine-CoA transferase (EC 2.8.3.21) (Rebouche and Seim, 1998; Caspi \textit{et al.}, 2014). Koeth \textit{et al.} (2014) showed that $\gamma$-butyrobetaine, obtained through oral $\gamma$-butyrobetaine or L-carnitine supplementation, can be metabolized by microbes to produce TMA, and identified the gene pair \textit{yeaW/yeaX} as encoding an enzyme that catalyzes this reaction \textit{in vitro}. The enzyme, termed a carnitine TMA lyase (see Figure 1), can also catalyze production of TMA directly from choline, betaine and carnitine (Koeth \textit{et al.}, 2014). Table 1 shows bacteria known to be associated with the formation of TMA via carnitine degradation.

**Betaine.** Betaine, or trimethylglycine, plays an important role as a methyl donor in the betaine homocysteine methyltransferase pathway (Obeid, 2013). It is present in high concentrations in various foodstuffs, for example, wheat bran, wheat germ and spinach (Zeisel \textit{et al.}, 2003). The daily intake of betaine varies from $\sim 30 – 400$ mg/day (Obeid, 2013). Betaine can also be biosynthesized, in both the human host and in gut bacteria, by oxidation of choline or carnitine. The oxidation of choline to betaine involves an
intermediate conversion to betaine aldehyde, catalyzed by choline dehydrogenase (EC 1.1.99.1), which in turn is converted to betaine in a reaction catalyzed by betaine aldehyde dehydrogenase (EC 1.2.1.8) (Andresen et al., 1988) (Figure 1). L-carnitine can be converted to betaine via a multistep pathway, the first reaction of which is catalyzed by L-carnitine dehydrogenase (EC 1.1.108) (Meadows and Wargo, 2015) (Figure 1).

There are several catabolic pathways for betaine (Figure 1). One involves demethylation of betaine to dimethylglycine, in a reaction catalyzed by glycine betaine transmethylase (EC 2.1.1.5) (Wood et al., 2010; Caspi et al., 2014). Dimethylglycine could be metabolized to TMA, via a hypothetical decarboxylation (Wood et al., 2010). Betaine can also function as an electron acceptor in a coupled reduction-oxidation reaction, i.e., Stickland reaction, catalyzed by betaine reductase (EC 1.21.4.4), in which it is reduced and cleaved to produce TMA and acetate (Naumann et al., 1983). Table 1 shows bacteria associated with the formation of TMA through betaine degradation.

**Ergothioneine.** Ergothioneine, a biogenic amine, is a derivative of histidine. Production of ergothioneine is common among fungi and in certain bacteria, such as Actinobacteria, Cyanobacteria, Bacteroidetes and Proteobacteria (Seebeck, 2010; Pfeiffer et al., 2011). Ergothioneine is also present in mammals, where it is concentrated in specific tissues or cells such as kidney, liver and erythrocytes (Cheah and Halliwell, 2012). However, mammals lack the ability to synthesize ergothioneine and humans derive the compound exclusively from dietary sources such as mushrooms (bolete and oyster), some meat products (kidney and liver) and some plant products (black beans, red beans and oat bran) (Ey et al., 2007). The biological role of ergothioneine in humans is unknown, but it is thought to have antioxidant and cytoprotectant properties (Cheah and Halliwell, 2012). When grown on ergothioneine, some bacteria are able to use the compound as a sole source of nitrogen, carbon and/or energy. The degradation of ergothioneine, catalyzed by
ergothionase (EC 4.3.3.-), yields TMA and thiolurocanic acid (Muramatsu et al., 2013) (Figure 1). Table 1 shows bacteria associated with the production of TMA from ergothioneine.

**Metabolism of TMA by gut bacteria**

Two pathways for microbial metabolism of TMA have been proposed (Colby and Zatman, 1973). One involves the sequential oxygenation of TMA and demethylation of its oxygenated form to yield dimethylamine and formaldehyde (Figure 2). This pathway is mediated by a trimethylamine monooxygenase (EC 1.14.13.148) and a trimethylamine-oxide aldolase (EC 4.1.2.32) and has been demonstrated to occur in *Pseudomonas aminovorans* (Large et al., 1972; Boulton et al., 1974), *Bacillus* (Myers and Zatman, 1971) and in methylotrophs such as *Hyphomicrobium* (Meiberg et al., 1980; Barrett and Kwan, 1985). The other pathway involves an energetically more favorable direct N-demethylation of trimethylamine to give dimethylamine and formaldehyde, catalyzed by trimethylamine dehydrogenase (EC 1.5.8.2) (Colby and Zatman, 1973) (Figure 2). In methylotrophs, dimethylamine is further metabolized, to methylamine and then ammonia, each step producing formaldehyde, by reactions catalyzed by dimethylamine dehydrogenase (EC 1.5.8.1) and methylamine dehydrogenase (amicyanin) (EC 1.4.9.1), respectively (Asatoor and Simenhoff, 1965; Colby and Zatman, 1973; Barrett and Kwan, 1985) (Figure 2).

**Oral and vaginal microbiota**

The gut, although the major site of TMA production, is not the only niche with resident bacterial species capable of producing TMA. Patients with TMAU report a strange taste in their mouth and may suffer from bad breath (Mitchell, 2005; Wise et al., 2011). Halitosis is primarily the result of microbial production of volatile sulfur compounds, short-
chain fatty acids and diamines (Scully and Greenman, 2012). However, TMA can also contribute to foul breath odour, either being produced in situ from choline (Chao and Zeisel, 1990) or as a result of TMA circulatory overload (Mitchell, 2005). One specific member of the oral microbiota, *Streptococcus sanguis I*, has been shown to form TMA from choline, presumably due to enzymatic action on the carbon-nitrogen bond of choline (Chao and Zeisel, 1990).

In bacterial vaginosis, the vaginal discharge has a prominent fishy odour due to elevated amounts of TMA (Brand and Galask, 1986; Oakley *et al*., 2008). Vaginal TMA can arise from degradation of carnitine and choline, or from reduction of TMAO produced by some methylotrophs (Barrett and Kwan, 1985) (see above). Normally, *Lactobacillus* and *Atopobium* are the predominant members of the vaginal microbiota. In cases of bacterial vaginosis, *Lactobacillus* appears to be replaced by genera such as *Gardnerella*, *Pseudomonas*, *Mobiluncus*, *Bacteroides*, *Prevotella*, *Porphyromonas* and *Streptococcus* (Cruden and Galask, 1988; Hyman *et al*., 2005; Armougom and Raoult, 2009). Thus, compared with a healthy vaginal microbiota, bacterial vaginosis is associated with a higher proportion of *Actinobacteria* and *Bacteroidetes* phyla and a lower proportion of *Firmicutes* (Oakley *et al*., 2008). *Mobiluncus* strains isolated from patients with bacterial vaginosis can produce TMA by reduction of TMAO (Cruden and Galask, 1988; Wolrath *et al*., 2002) and, to a lesser extent, by degradation of choline (Cruden and Galask, 1988). One *Bacteroides* isolate has also been shown to produce TMA from choline (Cruden and Galask, 1988).

As described above, methylotrophs can produce methyamine, their main source of energy, from TMA (Barrett and Kwan, 1985). Thus, excess production of TMA in the mouth and vagina in halitosis and bacterial vaginosis, respectively, would provide environments favourable to methylotrophs. Several methylotrophic strains have been shown to be present in the microbiota of the human mouth (Anesti *et al*., 2005).
Non-commensal bacteria able to produce TMA

In humans, TMA-producing bacteria are not limited to the commensal flora. Some non-commensal bacteria, including pathogens such as *Aeromonas, Burkholderia, Campylobacter, Salmonella, Shigella* and *Vibrio*, are able to form TMA (Wood and Keeping, 1944; Lerke *et al.*, 1965; Kwan and Barrett, 1983; Muramatsu *et al.*, 2013) from TMAO, carnitine, choline or ergothioneine. Non-commensal bacteria that produce TMA are shown in Table 2.

The TMA/TMAO host-microbe metabolic axis in health and disease

TMA is produced from dietary precursors by the action of gut bacteria and is metabolized to TMAO in the liver by a reaction catalyzed by FMO3 (Figure 3A).

Factors affecting the production of TMA from dietary precursors. Production of TMA by microbial gut action is dependent on the type and amount of TMA precursors in the diet, the foodstuffs in which they are present, and the identity and relative abundance of bacterial species present in the gut.

A study of healthy male volunteers found that, in each individual, there were striking differences in the amount of TMA produced from various dietary precursors (15 mmol) administered orally in pure form, as judged by urinary analysis of TMA and TMA N-oxide (Zhang *et al.*, 1999). TMAO was the best source, with almost 80% of the dose being excreted in urine as TMA or TMA N-oxide, followed by choline (about 60%) and carnitine (about 30%). However, betaine, creatinine and lecithin, although containing a trimethylamino moiety, elicited no significant increase in urinary excretion of either TMA or TMAO in any of the individuals studied. In addition, comparison of the amounts of TMA
released by chemical hydrolysis of foods in vitro with those obtained from biological
digestion of the foods in vivo revealed that marine fish and seafood are by far the best source
of dietary-derived TMA, with up to 85% of the TMA content of the food being liberated by
biological digestion. In contrast, red meat, despite its high content of carnitine, a good source
of TMA when ingested in its pure form, is a relatively poor source of TMA, with only 5 and
12% of the TMA content of lamb and beef, respectively, being released by biological
digestion (Zhang et al., 1999; Mitchell et al., 2002). These studies, although on a relatively
small number of individuals, indicate that amounts of TMA derived from digestion of a
particular food cannot be accurately predicted on the basis of the content of the
trimethylamino moiety in the food.

The human gut microbiome displays considerable inter- and intra-individual
variation (Caporaso et al., 2011; Yatsunenko et al., 2012). Such variation would be expected
to influence the relative yield of TMA produced from various foodstuffs. The identity and
relative abundance of gut microbes can be investigated by bacterial cultivation. However,
this approach is limited due to the inability to culture some members of the microbiota
(Zoetendal et al., 2008). Although sequence determination of 16S rDNA is able to identify
most major phyla, it is thought to underestimate Actinobacteria (Tottey et al., 2013), which
are known to produce TMA from TMAO and choline (Table 1). The development of next-
generation deep-sequencing methods will provide more accurate knowledge of the identity
and relative abundance of bacterial species present in the human gut.

In addition to the identification of bacteria, it is necessary to establish their function
with respect to their ability to produce or metabolize TMA. There are several pathways
resulting in the formation of TMA (Figure 1) and production of TMA is not exclusive to any
one bacterial phylum. Current taxonomic classification is based on DNA sequence rather
than on bacterial function. Thus, it is possible for a bacterial species belonging to one
phylum to share metabolic activity with species belonging to other phyla, and not with other members of the same phylum. Based on DNA sequence analysis, *Bacteroidetes* lack the \texttt{cutC} and \texttt{cntAB} genes (Craciun and Balskus, 2012; Zhu \textit{et al.}, 2014) and, thus, would appear unlikely to produce TMA from choline or carnitine. However, \textit{in vivo} cultivation studies show that some members of the *Bacteroidetes* phylum are able to produce TMA from choline (Cruden and Galask, 1988). In addition, not all strains that encode \texttt{CutC} are able to produce TMA from choline and, even within the same species, strains may differ in the ability to convert choline to TMA (Romano \textit{et al.}, 2015). Thus, one should be cautious when using phylogeny as the sole predictor of microbial TMA production or as an indicator of metabolic capacity in general.

The lack of increase in urinary TMA or TMAO, in response to ingestion of betaine, in any of the individuals studied (Zhang \textit{et al.}, 1999; Mitchell \textit{et al.}, 2002) (see above) indicates that the metabolic reactions that produce TMA from betaine, either directly or via dimethylglycine (Figure 1), or the bacteria in which these reactions occur, are not prevalent, at least in healthy British males. The inability to generate TMA from betaine also indicates that the potential routes for production of TMA from choline or carnitine, via their initial conversion to betaine (Figure 1), do not make significant contributions to TMA production.

Factors affecting the production of TMA in the gut are summarized in Figure 3B.

\textbf{Factors influencing TMA metabolism in the host.} Individuals that consume a ‘typical’ western diet will produce, via the action of gut bacteria, about 50 mg TMA/day (Walker and Wevers, 2012). The majority of this (~95%) is converted to TMAO, which is excreted in the urine (Mitchell and Smith, 2001). Thus, FMO3 is efficient in dealing with a relatively high substrate load. However, even individuals with good FMO3 catalytic capacity can become overloaded when challenged with large amounts of TMA precursors (Al-Waiz
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et al., 1989; Mitchell and Smith, 2016). Dietary components such as indoles, found in members of the Cruciferare family of vegetables, have been shown to inhibit the activity of FMO3 in vitro and, in a human study, to decrease the amount of TMAO produced as a percentage of total TMA (TMA+TMAO) excreted (Cashman et al., 1999).

Gender-specific effects in humans also influence the amount of urinary TMA. In a study of males over a 30-day period there was only a slight variation in the amount of TMA excreted in the urine, when expressed as a percentage of total TMA (TMA+TMAO) (Mitchell and Smith, 2010). In contrast, females excreted increased urinary TMA around the time of menstruation and this increase lasted for ~7 days (Mitchell and Smith, 2010). This increase in TMA excretion is the result of a reduction in its metabolism to TMAO, which is thought to be due to the action of female sex hormones decreasing the expression of the FMO3 gene (Coecke et al., 1998). However, in males high levels of testosterone also have been found reduce FMO3 capacity (Ayesh et al., 1995).

Primary TMAU is known to be due to mutations that result in impaired catalytic activity of FMO3 and, hence, increased urinary excretion of TMA. However, other situations also cause an increase in TMA excretion (Mitchell and Smith, 2016), for example, impaired liver function (Mitchell et al., 1999), impaired renal function (Bain et al., 2006; Hur et al., 2012; Hao et al., 2013) and pancreatic cancer (Navaneethan et al., 2014).

The expression of the FMO3 gene in human liver is switched on between birth and two years of age and increases during childhood and adolescence, reaching its maximum level in adults (Koukouritaki et al., 2002). Thus, in comparison with adults, children, especially infants, have lower amounts of FMO3. Consequently, they are less able to metabolize TMA to TMAO and, thus, may exhibit symptoms of transient childhood TMAU, particularly if they are homozygous for two common polymorphic variants of FMO3, c.472G>A (p.(Glu158Lys)) and c.923A>G (p.(Glu308Gly)), which moderately reduce
catalytic activity (Zschocke and Mayatepek, 2000). One polymorphic variant of FMO3, c.1079T>C (p.(Leu360Pro)), increases catalytic activity (Lattard et al., 2003). However, the variant has been detected only in African populations and then at low frequency.

Polymorphic promoter-region variants of the FMO3 gene have been identified that severely reduce transcription in vitro, whereas others increase transcription (Koukouritaki et al., 2005), but the impact of these variants on expression of FMO3 in vivo has not been validated.

Factors influencing the metabolism of TMA to TMAO in the liver are summarised in Figure 3C.

**Potential involvement of TMAO and FMO3 in disease.** Recently, TMAO has been implicated in a number of disease states, as a cause, consequence or biomarker of the disease. It has been suggested that an increase in the plasma concentration of TMAO, as a consequence of production of TMA from dietary supplements of choline, carnitine or TMAO by gut bacteria and its subsequent oxygenation by FMO3 in the liver, increases the risk of cardiovascular disease (Wang et al., 2011; Bennett et al., 2013; Tang et al., 2013; Koeth et al., 2013; Obeid et al., 2016). TMAO has also been implicated in chronic kidney disease (Tang et al., 2015), colorectal cancer (Xu et al., 2015) and in impaired glucose tolerance in mice fed a high-fat diet and a TMAO supplement (Gao et al., 2014). However, lower plasma concentrations of TMAO have been associated with inflammatory bowel disease and with active versus inactive ulcerative colitis (Wilson et al., 2015).

A number of roles for FMO3 itself in health and disease have been suggested, based on studies using mice. Knockdown of FMO3 mRNA, by antisense oligonucleotides, in female low-density lipoprotein receptor-knockout mice suggests a role for FMO3 in modulating glucose and lipid homeostasis (Shih et al., 2015). FMO3 has also been identified
as a target of insulin (Miao et al., 2015): Fmo3 expression was more than 1,000-fold higher in the livers of male liver insulin receptor-knockout (LIRKO) mice compared with littermate controls, whereas knockdown of FMO3 in LIRKO mice reduced expression of the transcription factor forkhead box O1 and prevented the development of hyperglycaemia, hyperlipidaemia and atherosclerosis. Other suggested roles for FMO3 include involvement in cholesterol metabolism and reverse cholesterol transport (Bennett et al., 2013; Warrier et al., 2015).

The implication that TMAO itself is a causative factor for cardiovascular and other diseases is controversial. A recent review considers in detail the physiological roles of TMAO and the evidence for and against TMAO being detrimental for health (Ufnal et al., 2015). The link between TMAO levels and atherosclerosis seems counterintuitive when we consider that consumption of marine fish, the richest dietary source of TMAO (Zhang et al., 1999), is firmly associated with a reduction in the incidence of cardiovascular disease (Takata et al., 2013). Other studies found no correlation of TMAO levels with biomarkers of cardiovascular disease. For instance, elevated levels of TMAO caused no increase in C-reactive protein or low-density lipoprotein, in either mouse or human plasma (Miller et al., 2014) and a study of oral carnitine supplementation of patients undergoing haemodialysis showed that, although TMAO was increased, markers of vascular injury were reduced and, consequently, supplementation with carnitine is thought to be beneficial for these patients (Fukami et al., 2015). Indeed, carnitine has proven efficacious for treatment of cardiovascular disease (Flanagan et al., 2010).

Many of the studies that implicate TMAO as a causative factor in cardiovascular disease are based on studies of mice and involve chronic administration of pure TMA precursors in amounts that far exceed those present either in a normal diet or in therapeutic supplements. Indeed, in the case of carnitine, significant increases in dietary-derived TMA in
humans are obtained only with high chronic dose regimens (Holmes et al., 1997).

Consequently, normal dietary conditions or moderate supplementation are unlikely to elicit the production of the amounts of TMAO that have been associated with cardiovascular disease.

When designing experimental studies on mice and extrapolating the results of these experiments to humans, it is important to appreciate that there is a species-specific gender difference in the expression of FMO3 between humans and mice. At about 6 weeks of age, expression of the Fmo3 gene is switched off in the liver of male, but not female, mice (Falls et al., 1995; Janmohamed et al., 2004). After this age, male mice produce no hepatic FMO3 protein and, thus, in comparison with females, produce far less TMAO and, consequently, excrete larger amounts of TMA in their urine. Despite this marked gender-specific difference, there is no obvious difference in the propensity of male and female wild-type mice to develop cardiovascular disease. A study of adult C57BL/6 wild-type mice found no difference in plasma glucose between male and female mice and showed that females, which express hepatic FMO3 and, thus, produce TMAO, have lower plasma cholesterol than males (Gonzalez Malagon et al., 2015). A further complication is that humans do not express FMO1 in liver (Dolphin et al., 1991; Phillips et al., 1995; Koukouritaki et al., 2002), in marked contrast to all other mammals investigated, in which FMO1 is a major form of FMO in this tissue (Hernandez et al., 2004).

There is evidence that the FMO3 gene of humans has been the subject of balancing natural selection (Allerston et al., 2007). Three alleles appear to be targets for selection: the ancestral allele, an allele encoding an enzyme with moderately reduced catalytic activity and one associated with increased promoter activity. Heterozygotes for a loss-of-function mutation of FMO3, despite having a 50% reduction of FMO3 activity, are able to convert ~95% of a normal dietary load of TMA to TMAO and, in this respect, are indistinguishable
from individuals homozygous for a functional FMO3 gene (Al-Waiz et al., 1987c). This indicates that it is unlikely that the decreased capacity to produce TMAO was the evolutionary advantage that drove selection of the allele encoding a FMO3 with reduced activity.

An alternative explanation for high levels of TMAO associated with cardiovascular disease is that the concentrations are indicative of dysfunction(s) elsewhere and that TMAO is not the mediator, but merely a marker of the disease (Ufnal et al., 2015). In mice, TMAO increases in response to a high-fat diet (Yang et al., 2014). High-fat diets change the composition of the gut microbiome to one associated with obesity and its related health problems. These changes increase the relative proportion of Firmicutes (Ley et al., 2006), a phylum whose members contribute to TMA production, and decrease that of Bacteriodetes, a phylum that contributes very little to TMA production (Table 1). Elevated plasma and urinary TMAO may, therefore, be a marker for a gut microbiome that has deleterious effects on health and not itself a causative factor. Indeed, atherosclerosis indices were increased in mice in response to a high-fat diet, but dietary supplementation with TMAO had the opposite effect, significantly decreasing atherosclerosis indices (Gao et al., 2014). It is important to note that no health benefits have been reported for TMAU individuals, who excrete very low amounts of TMAO because of their impaired FMO3 activity.

**Implications for TMAU.** One disorder known unambiguously to be associated with FMO3 is primary TMAU, caused by mutations in the FMO3 gene (Dolphin et al., 1997) (see above), a condition characterized by secretion of excessive amounts of odorous TMA (Mitchell and Smith, 2001). There is no cure for the disorder and current treatment is aimed at management of the symptoms. The main approach is restriction of dietary precursors of TMA. Although humans may possess gut bacteria capable of producing TMA from choline
and carnitine when administered in pure form, they are much less able to access these precursors from foodstuffs such as red meat and peanuts (Zhang et al., 1999; Mitchell et al., 2002). In contrast, the precursor TMAO is efficiently extracted in the gut from marine fish and seafood (Zhang et al., 1999; Mitchell et al., 2002). These results have implications for dietary management of TMAU, indicating that the most important foodstuffs to avoid are marine fish and seafood and those that contain free choline, such as soya bean-derived milk, whereas red meat would contribute less to TMA load.

Another strategy would be manipulation of gut flora to reduce the proportion of bacteria that produce TMA and increase the proportion of those that metabolize TMA. Although TMA-producing species are widely distributed across bacterial phyla, they are more common in Firmicutes and relatively scarce in Bacteriodetes (Table 1). Consequently, decreasing the ratio of Firmicutes to Bacteriodetes would be expected to reduce production of TMA from dietary precursors. A low ratio of Firmicutes to Bacteriodetes is associated with a healthy microbiome (Ley et al., 2006) and, thus, such alteration of the gut microbiome would promote general health as well as aiding in the management of TMAU.

One approach to altering the composition of the gut microbiome is exogenous supplementation with beneficial species (probiotics). However, a recent study found that the multi-strain probiotic VSL#3 had no effect on the increase in plasma concentration of TMAO in individuals fed a high-fat diet (Boutagy et al., 2015). Another proposal is the therapeutic use of methylotrophic strains of archaea (archaebiotics) that utilize TMA as an energy source (Brugère et al., 2014), although this has yet to be tried.

A further possibility is the selective inhibition of bacterial enzymes that catalyze reactions involved in the production of TMA. Candidates are TMA reductase, CutC and CntAB, which respectively catalyze TMA production from TMAO, choline and carnitine (Figure 1). For instance, 3,3-dimethyl-1-butanol, a structural analogue of choline, inhibits
microbial CutC and reduces the plasma concentration of TMAO in mice fed diets rich in choline or carnitine (Wang et al., 2016). However, most of the choline eaten by humans is in the form of lecithin, which is not a good dietary source of TMA (Zhang et al., 1999), and, thus, the efficacy of the inhibitor for reducing TMA production in human gut is unclear.

**Conclusions**

TMA is derived from dietary precursors via the action of gut bacteria. The amount of TMA absorbed by humans is determined by the type and quantity of dietary precursors and the composition of the gut microbiome, in particular with respect to bacteria that produce or consume TMA. TMAO, choline and carnitine, but not betaine, creatinine and lecithin, are good sources of TMA in their pure form. Marine fish and seafood are the best food sources of TMA, but red meat, despite its high content of carnitine, is a poor source. Consequently, it is not possible to predict the amount of TMA that will be derived from a particular food on the basis of the content of the trimethylamino moiety in the food. Bacteria from several phyla produce TMA from dietary precursors, but are more common in *Firmicutes* and scarce in *Bacteroidetes*.

FMO3, its microbiome-derived substrate TMA and the product of FMO3 catalysis, TMAO, have been implicated in a number of disease states. That mutations of *FMO3* cause the inherited disorder primary TMAU, through a failure to convert TMA to TMAO, is well established. However, TMAO has attracted increasing notoriety as a molecule that might be harmful for health. The evidence for this is controversial and there are studies for and against the importance of TMAO in conditions affecting health. No known health benefits have been reported as a consequence of the lack or low production of TMAO in primary TMAU patients. Large, well-designed clinical studies are needed to examine whether changes in the plasma concentration of TMAO in non-TMAU patients are a consequence of the diet of an
individual or of the bacterial species that populate their gut, which may well change in response to the health status of an individual. It is intriguing that consumption of marine fish, the richest dietary source of TMAO, is beneficial, not harmful, to cardiovascular health.

In addition, FMO3 has a wide range of drug substrates. This, together with its intimate relationship with the microbiome-produced FMO3 substrate TMA, indicates that changes in composition of the gut microbiome might influence both the metabolism and efficacy of therapeutic drugs.

Authorship contributions. Contributed to the writing of the manuscript: Fennema, Phillips, Shephard.
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Ishimoto M, and Shimokawa O (1978) Reduction of trimethylamine N-oxide by Escherichia


monooxygenase 3 (FMO3) and their drug oxidation activities. *Biochem Pharmacol* **85**:1588-1593.


Footnotes

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Figure Legends

Figure 1: Metabolic pathways for the production and metabolism of TMA by the
human microbiota. TMA can be produced from TMAO in a reaction catalyzed by TMAO
reductase. Choline, either in its free form or released from lecithin (phosphatidylcholine),
contributes to the formation of TMA directly, via the action of choline-TMA lyase, encoded
by the choline utilization cluster (cutC), or potentially indirectly, via conversion to betaine.
Similarly, L-carnitine, present in red meat or derived from γ-butyrobetaine, contributes to
TMA formation directly, via the action of a Rieske-type carnitine reductase/oxidase (cntAB),
or potentially indirectly, via conversion to betaine or to γ-butyrobetaine. Betaine can
potentially contribute to TMA formation directly, via the action of a betaine reductase, or
indirectly, via conversion to dimethylglycine, which could be metabolized to TMA via a
hypothetical decarboxylation. TMA can be produced from ergothioneine via the action of
ergothionase. TMA itself can be oxidized to TMAO, via the action of TMA monooxygenase.
Asterisks represent multistep pathways. Bold text, TMA and its precursors and metabolites.
Plain text, enzymes that catalyze reactions in the pathways. Based on (Zeisel, 1990; Kleber,
1997; Serra et al., 2002; Wood et al., 2010; Craciun and Balskus, 2012; Caspi et al., 2014;
Zhu et al., 2014).

Figure 2: Metabolism of TMA by gut bacteria. TMA can be metabolized to TMAO,
which is subsequently converted to dimethylamine (DMA) and formaldehyde. TMA can be
converted by direct N-demethylation to DMA and formaldehyde. In methylotrophs DMA
can be further converted to methylamine (MA) and then ammonia, each step producing
formaldehyde. Reactions are catalyzed by 1, trimethylamine monooxygenase; 2,
trimethylamine-oxide aldolase; 3, trimethylamine dehydrogenase; 4, dimethylamine
dehydrogenase and 5, methylamine dehydrogenase (amicyanin). Based on Colby and
Zatman (1973). Names of enzymes are those currently recommended by the Enzyme Commission and some differ from those given in the original papers.

Figure 3: The origin of TMA and TMAO and factors that influence their production.

A. The production and metabolic fate of TMA. TMA is produced from dietary precursors by the action of gut bacteria. TMA can be metabolized by gut bacteria to TMAO and dimethylamine (DMA) (see Figure 2) or absorbed and converted in the liver to TMAO in a reaction catalyzed by FMO3. TMAO and any unmetabolized TMA will enter the plasma and, subsequently, will be excreted in urine. B. Factors affecting the production of TMA in the gut. The amount of TMA produced is dependent on the nature and quantity of dietary precursors and the relative abundance of TMA-producing and TMA-metabolizing bacteria. C. Factors influencing TMA metabolism in the host. The metabolism of TMA to TMAO in liver is dependent on the amount and activity of FMO3. The abundance and activity of FMO3 can be increased as a consequence of genetic variation, resulting in an increase in the ratio of TMAO:TMA. The amount and activity of FMO3 can be decreased by genetic variation, hormones, inhibitors and disease, resulting in a decrease in the ratio of TMAO:TMA.
### Table 1. Bacteria that produce TMA from various substrates

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<td><em>Micrococcus</em></td>
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<td><em>Clostridium</em></td>
<td>(Robinson et al., 1952)</td>
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<td><em>Staphylococcus</em></td>
<td>(Robinson et al., 1952)</td>
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<td>(Robinson et al., 1952)</td>
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<td></td>
<td><em>Streptococcus</em></td>
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<td></td>
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### Choline

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<td></td>
<td><strong>Desulfitobacterium</strong></td>
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Enterococcus (Simenhoff et al. 1976)

Streptococcus (Robinson et al., 1952; Simenhoff et al., 1976; Chao and Zeisel, 1990; Craciun and Balskus, 2012; Martínez-del Campo et al., 2015)

Proteobacteria

Desulfovibrio* (Hayward and Stadtman, 1959, 1960; Baker et al., 1962; Bradbeer, 1965; Fiebig and Gottschalk, 1983; Craciun and Balskus, 2012)

Edwardsiella (Romano et al., 2015)

Enterobacter** (Eddy, 1953; Craciun and Balskus, 2012)

Escherichia (Craciun and Balskus, 2012; Martínez-del Campo et al., 2015; Romano et al., 2015)

Klebsiella** (Eddy, 1953; Craciun and Balskus, 2012; Kuka et al., 2014; Kalnins et al. 2015; Martínez-del Campo et al., 2015)
Proteus

(Seim et al. 1982a; Craciun and Balskus, 2012; Kuka et al., 2014; Martínez-del Campo et al., 2015; Romano et al., 2015)

Providencia

(Craciun and Balskus, 2012; Romano et al., 2015)

Pseudomonas

(Robinson et al., 1952; Kleber et al., 1978)

Yokenella

(Craciun and Balskus, 2012)

* synonym: Vibrio cholincus

** initially observed as Aerobacter but reassigned to genera Enterobacter and Klebsiella

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### Ergothioneine

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Table 2. Non-commensal bacteria that produce TMA from various substrates

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Shaw and Shewan, 2008;
Easter et al., 1982)

* basionym: *Alteromonas putrefaciens*

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<td><em>Stigmatella</em></td>
<td>(Muramatsu et al., 2013)</td>
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<td><em>Sinorhizobium</em></td>
<td>(Muramatsu et al., 2013)</td>
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DMD # 70615

* basionym: *Rhizobium meliloti*
Figure 1
Figure 2
Figure 3

A. Dietary precursors → GUT TMA → LIVER FMO3 → TMAO → plasma → urine

B. Dietary precursors of TMA → GUT TMA → TMA-producing bacteria → TMA → TMA-metabolizing bacteria

C. genetic variation → FACTORS LIVER FMO3 amount or activity → PLASMA/URINE TMAO/TMA