Biochemical and Clinical Aspects of the Human Flavin-Containing Monooxygenase Form 3 (FMO3) Related to Trimethylaminuria

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Abstract: Trimethylaminuria is a rare metabolic disorder that is associated with abnormal amounts of the dietary-derived trimethylamine. Excess unmetabolized trimethylamine in the urine, sweat and other body secretions confers a strong, foul body odor that can affect the individual's ability to work or engage in social activities. This review summarizes the biochemical aspects of the condition and the classification of the disorder into: 1) primary genetic form, 2) acquired form, 3) childhood forms, 4) transient form associated with menstruation, 5) precursor overload and 6) disease states. The genetic variability of the flavin-containing monooxygenase (form 3) that is responsible for detoxication and deodoration of trimethylamine is discussed and put in context with other variant forms of the flavin-containing monooxygenase (forms 1-5). The temporal-selective expression of flavin-containing monooxygenase forms 1 and 3 is discussed in terms of an explanation for childhood trimethylaminuria. Information as to whether variants of the flavin-containing monooxygenase form 3 contributes to hypertension and/or other diseases are presented. Discussion is provided outlining recent bioanalytical approaches to quantify urinary trimethylamine and trimethylamine N-oxide and plasma choline as well as data on self-reporting individuals tested for trimethylaminuria. Finally, trimethylaminuria treatment strategies and nutritional support are described including dietary sources of trimethylamine, vitamin supplementation and drug treatment and issues related to trimethylaminuria in pregnancy and lactation are discussed. The remarkable progress in the biochemical, genetic, clinical basis for understanding the trimethylaminuria condition is summarized and points to needs in the treatment of individuals suffering from trimethylaminuria.

INTRODUCTION

History of Trimethylaminuria and the First Clinical Reports

Sprinkled throughout human history and hidden among folktales, myths and other narratives are pointers to the existence of an unusual and unwelcome condition called trimethylaminuria. Trimethylaminuria was not unknown in the past and the incidence of such sufferers was probably not rare. In all cases, the individuals so affected have been the subject of distaste, social exclusion, and even ridicule. Anecdotal reports of such individuals are to be found in the Indian epic, '*Mahabharata*', (compiled around 400AD), Thai folklore of the Sukhothai period (*c*.1250AD) and in the play the '*Tempest*' by William Shakespeare (1564-1616) [1]. An early scientific description can be found in the scholarly treatise, '*Nature of Ailments*', by the physician John Arbuthnot (1667-1735) and two early reports in the Lancet allude to this condition [2,3]. The first clinical description has been attributed to Humbert and colleagues [4] and since that time several reports have occurred in the literature [5].

Description

The ailment is characterized at the biochemical level by the excretion of excessively large amounts of trimethylamine (TMA) in all of the bodily fluids including the urine, breath and sweat. The presence of volatile TMA in sweat is the critical issue as it imposes upon the individual a pungent odor, emanating from the skin and surrounding the patient in an unpleasant aura. These exaggerated amounts of TMA arise from its failure to be removed via the usual enzymatic N-oxygenation route that produces a non-odorous metabolite, trimethylamine N-oxide (TMA N-oxide). Operationally, the definition is an unaffected individual as having 0-9% unmetabolized free TMA, an individual suffering from mild trimethylaminuria as having 10-39% unmetabolized TMA and a severely affected individual as having > 40% unmetabolized TMA in their urine. Another definition is for an individual suffering from severe trimethylaminuria as one with urinary concentrations of free TMA of 10 mg/ml (18-20 mmol/mmol creatinine). Today, it is recognized that in humans, the prominent enzyme responsible for TMA N-oxygenation is the flavin-containing monooxygenase, form 3 (FMO3). The underlying reasons for a failed FMO3 enzyme reaction may be complex. Regardless, the clinical phenotype results from a mismatch

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between the enzyme's normal capacity to undertake the TMA N-oxygenation reaction and the substrate concentration.

Classification of the Disorder

As more cases of this condition have been uncovered it has become apparent that there are several different sub-types of this disorder, falling into two major categories, and this understanding has permitted a clearer picture to emerge. Firstly, there are those forms that are related to a dysfunction of the normal human FMO3 enzyme activity owing to genetic, hormonal or inhibitory chemical influences. Secondly, there are those forms arising from substrate overload of the enzyme activity (either in a normal or depressed state) such as an excess of dietary precursors or variations in the gut microflora resulting in enhanced liberation of TMA. Clearly, certain aspects of these two categories are intimately entwined and factors from each may act together to give rise to the disorder. An appraisal of cases within the literature and other information gathered has enabled the system of classification described below to be proposed.

1. Primary Genetic Form

This is probably the best understood of the various forms of the disorder and accounts for the majority of the reported cases. In recent years, there has been rapid progress in the elucidation of TMA N-oxygenation enzymology. As described above, it is presently accepted that, out of the five human FMO enzymes, FMO3 is the enzyme that appears to be most responsible for the N-oxygenation of TMA in humans. Recent studies have shown the human *FMO3* to be highly polymorphic and some mutations, either alone or in combination, are associated with dysfunctional enzyme activity whereas some genetic variation appear to be benign [6-9]. In addition, it seems that there may be combinations of intragenic polymorphisms within *FMO3* that determine a modified and less severe form of the condition [10, 11].

2. Acquired Form

There are at least three cases where the condition (i.e., biochemically diagnosed) appeared to emerge for the first time during adult life. These individuals had neither a familial background of the problem nor any previous history of the disorder during their childhood. However, one commonality was evidence of an episode of hepatitis in adult life, possibly viral in nature. This infection may have initiated the condition, perhaps via a genetic mechanism, and although subsequent examinations of these individuals have not been undertaken, the alterations observed may have been permanent [1, 12].

3. Childhood Forms

The feeding of a choline-containing infant formula to a preterm infant (i.e., 29 weeks old) resulted in the appearance of a strong, foul odor that disappeared when the choline source was discontinued. This phenomenon also occurred in one of three other premature infants who were similarly treated. When the choline-containing formula was reintroduced at 8 weeks of age, the odor failed to reappear and the problem was assigned to the initial immaturity of the N-oxygenating enzyme [13]. Other cases of the pediatric condition have been described recently and molecular analyses have revealed a 'predisposition' in these individuals of a compound heterozygosity for several mutations [14], a situation that may be more common than previously recognized [11]. Below, is a description of the molecular basis for the 'predisposition' of the postnatal individual to trimethylaminuria.

4. Transient Form Associated with Menstruation

Several female patients have stated, anecdotally, that their odor problems seem to intensify at the onset of menstruation [15]. A biochemical study in a single female patient showed that her trimethylaminuria was indeed exacerbated just before the onset of menstruation, relating closely to her own subjective description of the problem [16]. There is evidence in the literature for the influence of steroid hormones on the activity of FMO in both rodents and human [17]. In concordance with this, some patients have also reported that their problems emerged during puberty, or became exacerbated at this time [15, 18].

5. Precursor Overload

A few cases of a transient form of the condition have been attributed to precursor overload thereby saturating the existing levels of FMO3 enzyme capacity (or possibly an enzyme variant). Patients have complained of a pungent odor following oral therapeutic doses of choline (8-20 g/day) administered to treat Huntington's chorea. This was attributed to an overwhelming of their ability to convert the dietary choline to the non-offensive TMA N-oxide [19]. A patient receiving choline therapy for Alzheimer's disease also complained of a foul odor [20]. It is worthy of note that precursors of TMA, including choline, are sometimes recommended in quite high doses in health foods, food supplements and alternative diets.

6. Disease States

Impaired hepatocellular function or the existence of portosystemic shunts in patients with liver cirrhosis may interfere considerably with the clearance of TMA absorbed from the gut. Consequently, blood TMA concentrations increase in these patients, perhaps playing a role in the development of hepatic encephalopathy and coma, with the volatile amine being excreted in both urine and breath [21-23]. In uremic patients the abnormal overgrowth of bacteria in the small intestine greatly increases the liberation of TMA from dietary precursors. This event, compounded by reduced renal clearance, leads to an increase of amine concentrations within the circulatory system and their escape via the breath and sweat [24-26].

Biochemical and Genetic Aspects of the Human FMO

Common FMO Polymorhphisms in Healthy Individuals

The flavin-containing monooxygenases (FMOs) (EC 1.14.13.8) are important for the oxygenation of a wide variety of dietary compounds, synthetic environmental toxicants, natural plant alkaloids, and therapeutics. The FMOs are widely known for their ability to carry out

heteroatom-oxygenation reactions with substrates that share the common property of containing soft nucleophilic nitrogen, sulfur, selenium, or phosphorous centers [27, 28]. In most mammalian species, five FMO enzymes have been identified (FMO1-5), each encoded by a distinct gene. A comparison of non-orthologous forms between or within a mammalian species shows sequence identities between 48 and 58%, consistent with the evolution of this gene family prior to speciation. In contrast, sequence identities of orthologous forms range between 76 and 86%, suggesting a relatively high degree of conservation once these different genes evolved. In the human, *FMO1, 2, 3* and *4* are localized in a single, 257 kbp cluster on chromosome 1q23-25; human *FMO5* is localized at 1q21. A putative 6th member of this gene family, *FMO6*, was identified as part of the human genome project (accession no. AL021026), but recent studies suggest this gene fails to encode a functional FMO due to multiple alternative processing events [29].

In view of the numerous ways that individuals can manifest trimethylaminuria, it is important to characterize *FMO3* of healthy populations so that any genetic variation attributed to the trimethylaminuria condition can be placed in the proper perspective. It is important to understand the underlying genetic variability in human *FMO3*, because it is possible that combinations of common variants could produce a less severe form of the condition [11]. In addition, human *FMO3* allellic variation may eventually be shown to contribute to interindividual and interethnic variation in FMO3-mediated drug metabolism.

Traditionally, insight into the possible variability of drug metabolism has been ascertained by examining the microsomal metabolism of a drug in vitro [30]. Considerable interindividual variability in adult human microsomal FMO3 activity has been observed in vitro (i.e., approximately 7-fold variability in amine N-oxygenation and 3-fold variability in sulfide S-oxygenation) [6]. Because human FMO3 is apparently not induced by administration of low molecular weight chemicals or drugs, differences in FMO3-selective metabolism is more likely due to genetic and not environmental effects. Before determining whether variation in the gene in the population contributes to the susceptibility to disease in the population at large, the amount and organization of genetic variability in the gene should be examined in samples from individuals ascertained without regard to their health status. Considerable statistically significant variation in the relative frequencies of single and multiple site alleles, haplotypes and genotypes of *FMO3* have been observed from samples of healthy individuals [31]. Some of the common *FMO3* variants are listed in Table **1**.

FMO3 is not the only *FMO* gene that possesses significant genetic variation. To date, significant variation in the genomic *FMO* DNA and in the coding region has been observed. For example, *FMO1, FMO2, FMO3, FMO4* and *FMO5* has 34, 57, 40, 30 and 40 genomic DNA variants on the basis of inspection of the *Homo sapiens* chromosome 1 working draft sequence from locuslink of Genebank [6]. The genomic variants for *FMO1, FMO2, FMO3, FMO4* and *FMO5* translate into 2, 9, 19, 1 and 2 variants in the coding region, Tables **1**, **2**. It may be that the relatively large amount of variation in the *FMO3* gene is a consequence of the possibility that more sequence information has been deposited for that gene. Regardless, significant variation exists for the other *FMO* genes as well. The relative allelic frequencies for prominent human *FMO3* polymorphisms in the DNA of healthy male and female Caucasians, Hispanics, Asians and African Americans has been determined and significant differences exist among the ethnic groups for human *FMO3* allelic frequencies. It is not known whether the genetic heterogeneity in various ethnic subdivisions predispose certain populations to abnormal or adverse drug metabolism for drugs metabolized predominantly by FMO3 but this possibility could exist [31, 32].

Table 1. A List of Amino Acid Changes and Relative Activity from the Coding Region of Human FMO3[a]

Amino Acid Change	Apparent Enzyme Activity (*in vitro*/*in vivo*)
D132H	Decreased?
S147S	Normal
E158K	Decreased?
V257M	Normal
N285N	Normal
E308G	Decreased

[a]Common variation in the coding region from African Americans and Caucasians

Table 2. A List of Amino Acid Changes Predicted from the Coding Region of Human FMOs

Gene	Amino Acid Change
FMO1	T249T V396V
FMO2	D36G D71deletion F81S F182S S195L S195S R249X E314G N413K
FMO4	V323A
FMO5	P337P S351P

Although it is the human FMO3 that is responsible for TMA N-oxygenation, characterization of other *FMO* genes can also contribute to an understanding of trimethylaminuria. For example, in prenatal human liver tissue where other FMOs are prominent, a clear understanding of the molecular mechanisms regulating the temporal- and tissue-selective expression of FMO1 and FMO3 has provided insight into possible childhood forms of trimethylaminuria.

Human FMO1 and FMO3 Hepatic Temporal-Selective Expression: An Explanation for Pediatric Trimethylaminuria?

The *FMO* gene family appears to exhibit a pronounced tissue- and temporal-selective expression pattern (Table **3**). Thus, FMO3 predominates in the adult liver where it attains expression levels approaching 60% of the major cytochrome P450, CYP3A [33, 34], but has been reported to be non-detectable in fetal liver. In contrast, FMO1 expression has been described in human fetal liver with protein levels approximately 25% of those seen in the adult [35]. However, these conclusions are drawn from a minimal number of tissue samples representing limited windows of time. Given the dynamic changes that occur in gene expression during development that extend into the early years of life, it is unclear how accurate and/or complete this picture of human hepatic FMO developmental expression is.

Table 3. Human FMO and CYP Expression Levels[a]

Tissue	FMO1	FMO3	CYP3A
Fetal Liver	14.4 ± 3.5	< 0.1	~45.0[b]
Adult Liver	< 1.0	60.3 ± 43.1[c]	98.9 ± 74.8[d]
Adult Kidney	47.0 ± 9.0[e]	< 1.0	?
Adult Intestine	2.9 ± 1.9	< 1.0	?

[a] Expression levels expressed as pmol/mg of microsomal protein
[b] CYP3A7, estimated based on relative activity
[c] Data from Overby *et al*. [33]
[d] Data from Wrighton *et al*. [34]
[e] Data from Yeung *et al*. [35]

To gain a better understanding of human hepatic FMO expression during development, a tissue bank was assembled with 240 samples. The ages of tissue donors ranged from 8 weeks gestation to 18 years of life. Gender information was available for 221 samples, 137 being male and 84 female. Microsomal fractions prepared by differential centrifugation were fractionated by SDS-polyacrylamide gel electrophoresis along with known amounts of recombinant FMO1 and FMO3 protein. Specific content was determined by western blot analysis, integrating the optical density of immunoreactive protein signals followed by linear regression. The data are summarized in Fig. (**1**) and described more fully in a previous report [36]. FMO1 was expressed at the highest level during the first trimester, and declined in the second and third trimesters. Within three days after birth, FMO1 expression was essentially extinguished. Low levels of FMO3 expression also was detectable in approximately 30% of the individuals during the first trimester, but was undetectable throughout the remaining periods of gestation. The onset of expression in most individuals was observed between 21 and 304 days after birth, but at levels approximately 8% of that seen in adults. Between 304 days and 11 years after birth, FMO3 expression was elevated to 20% of adult levels. At puberty, there was a gender-independent increase in FMO3 expression, but even in the oldest samples in this data set (18 years), FMO3 expression still had not attained the adult values reported by Overby *et al*. [33] in 5 individuals ranging in age from 39 to 58 years (Table **3**). Further analysis of tissue samples from donors that were born prematurely, but lived for defined periods of time after birth, suggested that the suppression of FMO1 is tightly linked to the birth process, but not gestational age. In contrast, birth is clearly necessary, but not sufficient for the onset of FMO3 expression (data not shown, see [36]). Finally, given that other forms of FMO are not expressed to any appreciable degree in the human liver, these data suggest that in neonates, hepatic FMO expression is absent or low in the majority of infants.

The *FMO1* developmental- and tissue-specific expression pattern is consistent with data obtained on the regulatory elements controlling this gene. Two functional and overlapping hepatic nuclear factor (HNF) 1 sites have been mapped between positions -132 and -105 on rabbit *FMO1* that play a dominant role in controlling FMO1 transcription. In addition, two distal HNF4 sites at positions -195 to -182 and positions -467 to -454 also contribute to *FMO1* transcription. Both HNF1 sites, as well as the more proximal HNF4 site also are highly conserved on the orthologous human gene and likely play a similar regulatory role [37]. During organogenesis (first trimester), HNF1 expression is activated, largely in response to HNF4 . HNF1 expression then declines during the second and third trimesters [38]. This cascade of hepatic HNF expression during development closely matches the ontogeny of *FMO1*, consistent with HNF1 , and to a lesser extent, HNF4 , being important for the regulation of this expression pattern. However, it is unlikely either of these transcription factors is responsible for the sharp, birth-dependent suppression of *FMO1* expression. Further, the elements that may be important for postnatal *FMO3* expression are just beginning to be understood.

As described above, it is now well established that null variants at the *FMO3* locus are responsible for the disorder, trimethylaminuria. It also has been shown that human FMO1 is incapable of catalyzing the N-oxygenation of TMA [39, 40]. Together with the FMO1/FMO3 temporal-specific expression described above (see Fig. (**1**)), these observations offer a plausible explanation for the transient trimethylaminuria described by Mayatepek and Kohlmüeller [14] in children (Table **4**). These authors described two patients, one

Table 4. Transient Trimethylaminuria in Childhood[a]

Patient	Age	%TMA/%TMA N-Oxide
1	2 mo	0.148
1	6 mo	0.065
2	4 yr	0.241
2	5 yr	0.014
Trimethylaminuria		0.852 to 9.000 (3.000)[b]
Control		0.002 to 0.099 (0.045)[b]

[a] Data on patients 1 and 2 from Mayatepek and Kohlmüller [14]
[b] Data from Mitchell and Smith [1]

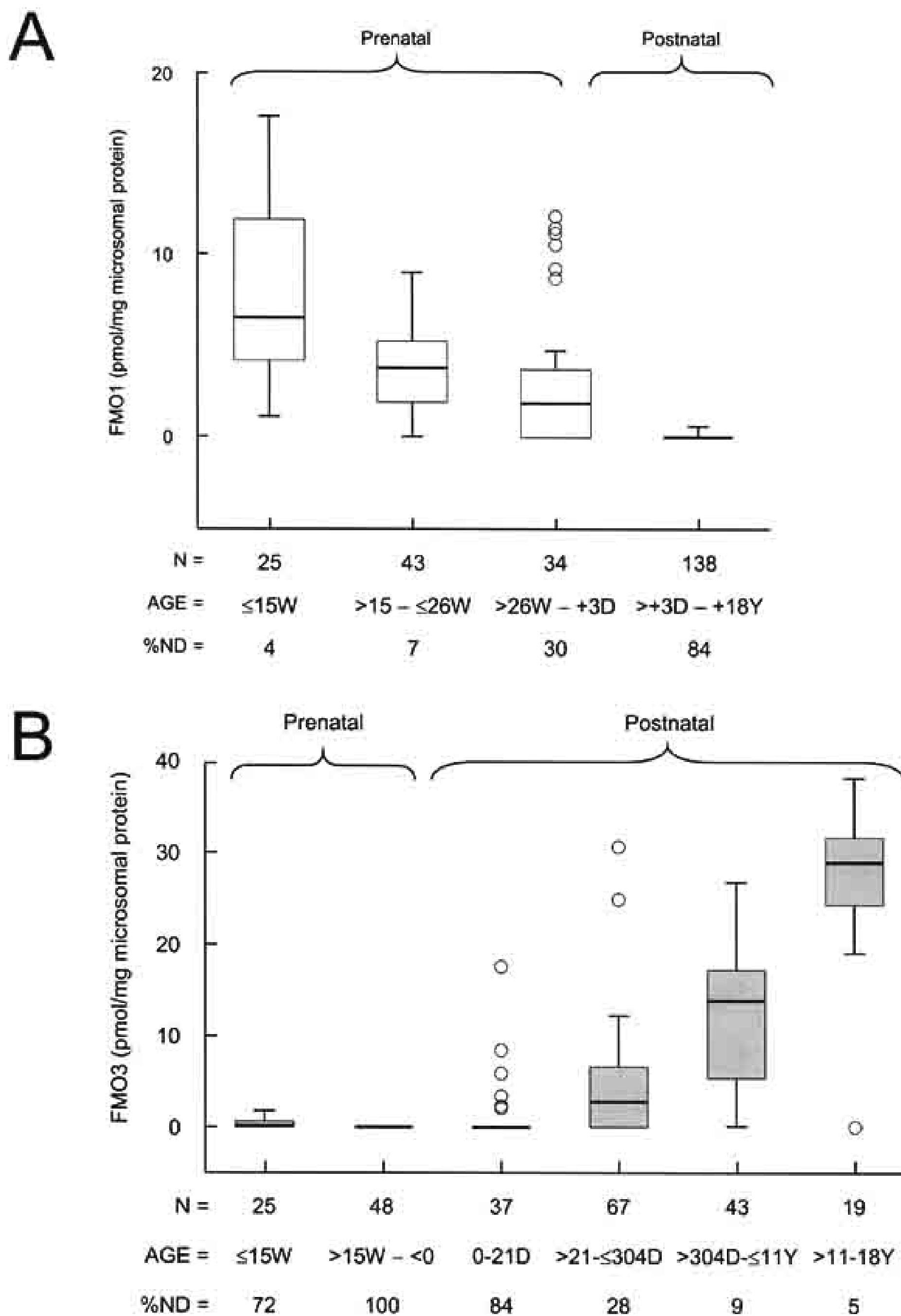


Fig. (1). Temporal-Specific Expression of Human Hepatic FMO1 and FMO3. Hepatic FMO1 (A) and FMO3 (B) specific content was determined by western blot analysis of microsomal preparations using monospecific antibodies obtained from Gentest, Inc. (Woburn, MA) with detection by enhanced chemiluminescence (Amersham Biosciences, Arlington Heights, IL). Age brackets resulting in the maximum variance between groups and the least deviance within groups were determined by regression tree analysis using S-Plus 2000 (Insightful Corp., Seattle, WA). The data are depicted as a box and whisker plot where the horizontal bars represent the median, the boxes the 25th to 75th percentiles and the vertical bars the 5th to 95th percentiles. Outliers (depicted as circles) were defined as samples having specific contents outside 1.5 times the 25th or 75th percentiles. The sample size (N), age ranges (D, days; W, weeks; M, months; and Y, years) and the fraction of samples with nondetectable FMO1 or FMO3 protein (% ND) for each age bracket also are shown.

a full term infant at 2 months of age and the second at 4 years of age, that presented with trimethylaminuria. Although the patients did not show a %TMA N-Oxide/%TMA urinary ratio typically observed in confirmed trimethylaminuria patients, it was significantly different than seen in normal controls [8, 32]. Generally, the normal urinary %TMA N-oxide is > 95%. A genetic analysis of both patients revealed them to be heterozygous for *FMO3* mutants known to be associated with trimethylaminuria, but such patients normally do not present with the disorder unless challenged [11]. Interestingly, the disorder resolved spontaneously in both patients within a year and a repeat urinary analysis revealed a %TMA/%TMA N-Oxide ratio in the normal range for both patients. The explanation for these observations is likely the delayed onset of FMO3 expression, compounded by their heterozygosity for a deficient *FMO3* allele.

In summary, human hepatic *FMO1* and *FMO3* exhibit a novel, temporal-specific expression pattern. Hepatic *FMO1* is only expressed during fetal development and is suppressed within the first 3-days of life in a process tightly coupled to birth. The transcription factor HNF1 is critically important for the *FMO1* expression pattern, but other factors yet to be elucidated also play a significant role. Hepatic *FMO3* expression is largely restricted to postnatal time periods. Birth is necessary, but not sufficient for the onset of *FMO3* expression - a phenomenon that is highly variable. The delayed onset of *FMO3* expression is most likely responsible for mild, transient trimethylaminuria in children and is an excellent example of an important principal of developmental pharmacology. Delayed expression of drug metabolizing enzymes can be the underlying mechanism whereby children may present with a "poor metabolizer" phenotype, despite a normal genotype. With further development of the individual, the phenotype matures to more closely match that predicted by genotype.

Some Examples of Rare FMO Genetic Variants

FMO2 is the major FMO in the lung of all mammals examined, with the exception of the domestic rat [41] and humans [42]. Genetic polymorphisms affecting expression have been characterized for *FMO2* [42] and *FMO3* [7, 8]. FMO2 is expressed at relatively high levels in the lung of non-human primates [42, 44] and presumably plays an important role in xenobiotic metabolism in this organ. In humans, FMO2 expression is characterized by an ethnic dependent genetic polymorphism. All Caucasians and Asians genotyped to date are homozygous for a C T transition mutation that results in a Q472X truncated (*FMO2*2* allele) and non-functional protein (FMO2.2) [42, 43]. Previous genotype analysis has shown that approximately 26% of African-Americans possess at least one CAG allele (*FMO2*1* allele) and express full-length, functional protein (FMO2.1) [43, 45].

Recently, an allele-specific PCR genotyping approach was used to examine DNA from 290 Hispanic individuals. The majority of the Hispanic individuals genotyped were either of Mexican or Puerto Rican descent (Table **5**). Although the genotype frequency for FMO2*1/2 is higher in Puerto Ricans (i.e., 9.5%) than Mexicans (i.e., 2.7%), there were too few samples to reach statistical significance. Overall, among the 290 individuals from different regions, the FMO2*1/2 genotype frequency was 4.5%. Expression of FMO2.1 in insect cells utilizing a baculovirus system was used to examine activity toward potential sulfur-containing xenobiotic substrates.

Rabbit, monkey and human full-length FMO2 were expressed to high specific contents (5-14% of the total protein) in Sf9 insect cell microsomes (Table **6**). A construct of monkey FMO2 with the same truncation at AA472 was also expressed, but the specific content as measured by FAD levels was very low and the enzyme was catalytically inactive. This observation was consistent with results from lung microsomes isolated from humans genotyped as *FMO2*2/2* in which no protein or activity could be detected

Table 5. Allelic-Specific Genotype of Individuals of Hispanic Descent for FMO2*1 (Full-Length) and FMO2*2 (Truncated)

Origin	FMO2*1/2 (%)	FMO2*2/2 (%)
Mexico	6/223 (2.7)	217/223 (97.3)
Argentina	0/1 (0)	1/1 (100)
Belize	0/1 (0)	1/1 (100)
Columbia	0/1 (0)	1/1 (100)
Costa Rica	0/1 (0)	1/1 (100)
Honduras	1/5 (20)	4/5 (80)
Philippines	0/1 (0)	1/1 (100)
Puerto Rico	4/42 (9.5)	38/42 (90.5)
Unknown	2/15 (13.3)	13/15 (86.7)
TOTAL	**13/290 (4.5)**	**277/290 (95.5)**

Table 6. Specific Content of Expressed FMO2 in Sf9 Insect Cell Microsomes

Species	[a]nmol FMO2/mg microsomal protein	% Total microsomal protein
Rabbit	1.25-1.91	7.7-11.7
Monkey	1.46-1.93	8.9-11.8
Human (full length)	0.85-2.27	5.2-13.8
Monkey (truncated)[b]	ND[c]	ND

[a] The specific content was determined by nmol of FAD [55] per mg microsomal of protein [56].

[b] The monkey FMO2 was truncated at AA471 by insertion of a stop codon. The truncated protein was expressed to a specific content of approximately 0.44 nmol/mg of protein as estimated by western blotting, but apparently failed to incorporate FAD. These data are taken from Krueger *et al.,* [46, 47]

[c] ND, not detected

Table 7. Expressed Human, Monkey and Rabbit FMO2 Activity toward *S*-Oxygenation of Selected Substrates

FMO2[a]	Methimazole		Phorate		Thiourea		Phenylthiourea		Ethylenethiourea	
	K_m[b]	V_{max}[c]	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
Rabbit[d]	756	37.4	-	-	23	220	-	-	-	-
Monkey[d]	744	31.2	-	-	-	-	-	-	-	-
Human[e]	1,235	23.2	52	42	25	51	4	25	14	50

[a] Activity was determined by substrate-dependent NADPH oxidation as previously described [46, 47] using Sf9 microsomal protein and a pH of 8.5, except for the rabbit FMO2 K_m toward thiourea which was measured as substrate-dependent O_2 consumption at pH 7.5 with purified rabbit FMO2 [50].
[b] The apparent K_m was determined from double-reciprocal plots of velocity versus substrate concentration and is reported in units of μM.
[c] The V_{max} is in units of nmol NADPH oxidized/min/nmol of FMO2 (min^{-1}).
[d] Taken from Krueger *et al*., 2001 [46]. The incubations were performed at pH 8.5.
[e] Taken from Krueger *et al*., 2002 [47]. The incubations were performed at pH 9.5.

[43, 46, 47]. The organophosphate insecticide phorate was a good substrate for expressed human FMO2.1 with an apparent K_m of 52 μM and a V_{max} of 42 min^{-1} (Table **7**). FMO2-mediated S-oxygenation of the thioether sulfur of phorate produced the sulfoxide, a presumed detoxication pathway [48]. In contrast, cytochrome P450 (CYP) bioactivates phorate as it does other organophosphates through production of the oxon [48].

Methimazole (MI) and N,N'dimethylaniline (DMA), are both prototypical FMO substrates and represent S- and N-oxygenation, respectively [49]. MI, DMA and nicotine are oxygenated by FMO2, but the K_m values for the rabbit, monkey and human enzyme are fairly high (mM range, data not shown). TMA is N-oxygenated by FMO2 (33.8 min^{-1} at 500 μM), but we have not as yet determined the K_m. Small molecular weight thioureas, on the other hand, have previously been shown to be excellent substrates for FMO2 [50]. Thiourea, ethylenethiourea and 1-phenylthiourea are efficiently S-oxygenated by human FMO2.1 with apparent K_m values of 25, 14 and 4 μM, respectively. The K_m for thiourea is nearly identical to that previously determined with rabbit FMO2 (23 μM) [50]. S-Oxygenation of thioureas produce the sulfenic acid followed by a second oxygenation to the sulfinic acid [51, 52]. Both of these S-oxygenated metabolites are chemically reactive. The sulfenic acid tends to react rapidly with reduced glutathione that in turn can be reduced back to the parent compound with generation of oxidized glutathione. The net result of this futile cycle is depletion of reduced glutathione and NADPH leading to oxidative stress and toxicity [53, 54]. Other S-containing compounds S-oxygenated by FMO2*1 include thioacetanilide (79.1 min^{-1} at 200 μM) and thiobenzamide (49.6 min^{-1} at 200 μM) (unpublished data).

The genetic polymorphism of *FMO2* is different in that the dominant allele *FMO2*2*, is non-functional. The variant allele (*FMO2*1*), present in 26% and 5% of individuals of African and Hispanic descent, respectively, is functional and may be associated with a reduced risk upon exposure to compounds detoxicated by FMO (phorate), but enhanced toxicity in instances, such as the thioureas, where FMO produces a toxic metabolite.

Is There a Relationship Between Urinary TMA Levels and Hypertension?

Previous studies have indicated an evolutionary relationship between FMO and osmoregulation [57, 58]. Studies using euryhaline fish as models have indicated the induction of FMO by TMA N-oxide and urea (i.e., presumably as organic osmolytes) during periods of exposure to hypersaline environments [59]. In humans, diets high in salt content (hypersaline) cause increasing peripheral resistance within blood vessels and have been associated with cardiovascular disease particularly in African Americans. If TMA N-Oxide and other organic osmolytes are not produced to counter-balance increases in osmotic pressure and peripheral resistance, it is possible that individuals deficient in the formation of TMA N-Oxide may have a higher prevalence of hypertensive diseases. Because human FMO3 is the primary enzyme system involved in the formation of urinary TMA N-Oxide, individuals with diminished enzymatic activities might be susceptible to hypertension or other cardiovascular diseases. In addition, recent in vitro studies have indicated a role for FMO3 in the metabolism and detoxication of catecholamines which may also contribute to this potential phenomenon [60].

To test whether a relationship between hypertension and urinary TMA:TMA N-Oxide levels existed, urine samples were obtained from 104 African Americans visiting the Tutwiler Clinic in Tutwiler, MS, located approximately 20 miles from Clarksdale, MS. Participating individuals were screened to eliminate pregnant or menstruating females; any individual undergoing steroid therapy; or patients with hepatic and/or renal dysfunction. Samples were separated into controlled (i.e., receiving drug therapy for hypertension); uncontrolled (i.e., not receiving drug therapy for hypertension); and normal (i.e., not presenting with hypertension symptoms). Hypertension was defined according to World Health Organization values such that normal values (systolic/diastolic) were considered 130/70 or less; mild hypertension values requiring life-style changes were indicated with 140/80 to 150/90 with obvious hypertension and requiring pharmaceutical therapy had initial readings greater than or equal to 150/90 prior to initiation of therapy. Mean blood pressure was calculated

using the equation: Mean blood pressure = Diastolic pressure + Pulse pressure/3.

In this population, thirty five individuals screened for urinary TMA and TMA N-Oxide possessed TMA:TMA N-Oxide ratios equal to or greater than 10%. Four individuals possessed TMA:TMA N-Oxide ratios greater than 20%. Overall, males had a statistically higher incidence (i.e., 46%, 16/35) of TMA:TMA N-Oxide ratios $\geq$ 10% than females (i.e., 29%, 20/69) (unpublished data).

No consistent relationship was observed between urinary TMA content and any specific genotype (Table **8**). The *FMO3* E158K variant was the most common polymorphism identified in this group (10 individuals were heterozygous and 4 were homozygous). The FMO3 E308G polymorphism was identified in 3 heterozygous individuals. Other previously reported *FMO3* rare variants, including M66I, P153L, V257M, E305X, and R492W, were not observed in the population examined. Two silent mutations (S147S and N285N), and substitutions, deletions, and additions within intron regions were also identified. However, all mutations identified were randomly distributed among the individuals. The D132H substitution was a novel polymorphism observed in 2 out of 32 chromosomes, but this variant was observed in one subject with normal TMA levels and in another subject with 13% urinary TMA. The substitution E308G was observed in two out of 28 chromosomes; one heterozygote was a normal subject (2% urinary TMA) and one heterozygote was an individual initially having 32% urinary TMA that presented with normal urinary TMA following treatment with choline (unpublished data).

No significant differences in urinary TMA were observed between hypertensive females and hypertensive males (Fig. **2**). Linear regression analyses of all individuals (N =104) comparing mean blood pressure; diastolic or systolic metrics failed to show a significant relationship between TMA:TMA N-Oxide ratios in control, uncontrolled or normal individuals (data not shown). However, when the groups were segregated into controlled, uncontrolled and normal individuals, a statistically higher number of cases having TMA:TMA N-Oxide ratios (i.e., ratios $\geq$ 10%) were observed in uncontrolled female subjects (i.e., 33%, 7/21) compared with controlled females (i.e., 25%, 4/16). Likewise, more cases of elevated urinary TMA (i.e., TMA:TMA N-Oxide ratios $\geq$ 10%) were observed in uncontrolled male patients (i.e., 56%, 5/9) compared with normal males (i.e., 46%, 6/13) and controlled males (i.e., 38%, 5/13) (unpublished data).

In summary, a relationship appears to exist between urinary TMA and hypertension, particularly in males not receiving therapy for diagnosed hypertension. A genetic

Table 8. Human *FMO3* Genotyping of Sixteen African American Individuals

			D132H	**S147S**	**E158K**	**del CTT**				**V257M**	**N285N**	**E308G**	**add T**		
gDNA #	192	11063	15089	15136	15167	15241	15366	15399	15573	21351	21375	21443	23516	23783	
WT	A	G	G	C	G	CTT	G	G	C	G	C	A	: :	C	
intron/exon	~ in 2	in 3~	ex 4	ex 4	ex 4	~in 4	in 5~	in 5~	~in 5	ex 5	ex 7	ex 7	in 8~	~in 8	%TMA
176	AG	GG	GG	CC	GG	CTT	GG	AG	CC	GG	CT	AA	T:		48
182	AG	GT	GG	CC	AG	CTT	AG	AG	CC	GG	CC	AG	T :	CC	32
227	GG	GG	GG	CC	AA	CTT	GG	AA	GG	GG	CC	AA	: :	CC	26
203	AA		GG	CC	AG	CTT	GG	AA		GG	TT	AA	TT	CC	20
234	GG	GG	GG	CC	AA	CTT	GG	AA	CC	GG	TT	AA	TT	CC	16
270	GG	GG	GG	CT	AG	del CTT	GG	AA		GG	CC	AA	: :	CC	16
237	AA	GG	GC	CC	AG	CTT	GG	AA	CC	GG	TT	AA	TT	CT	14
254	AG	GG	GG	CC	AG	CTT	GG	AG	CC	GG	CT	AA	T :	CC	14
219	AG	GG	GG	CC	AG	CTT	GG	AA	CC	GG	TT	AA	TT	CC	13
222	AG	GG	GG	CC	AG	CTT	GG	AA		GG	TT	AA	TT	CT	13
251	AA	GG	GG	CC	GG	CTT	GG	GG		GG	CC	AA	: :	CC	13
252	AG	GT	GG	CC	AG	CTT	AG	AG		GG	CC	AA	T:	CC	3
245	GG	GT	GG	CC	AA	CTT	AA	AA		GG	CT	AG	T:	CC	2
258	GG	GT	GG	CC	AA	CTT	AG	AA		GG	CT	AG	T:	CC	2
275	GG	GG	GG	CC	AG	CTT	GG	AA	CC	GG	CT	AA	T:	CC	2
257	AG	GG	GC	CC	AG	CTT	GG	AA		GG	CT	AA	T:	CC	0.1

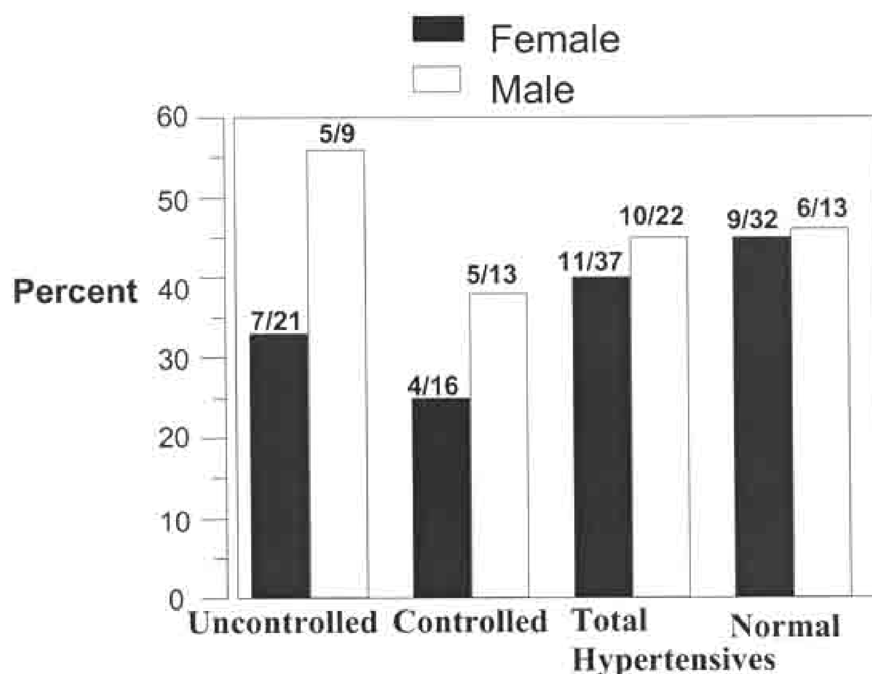


Fig. (2). Percentage of Normal and Hypertensive African Americans with urinary TMA:TMA N-oxide ratios greater than or equal to 10%.

basis for a single variant causing trimethylaminuria was not observed in this population, however. A more thorough genetic analysis with larger sample size to evaluate FMO3 variation and hypertension is necessary before ruling out a genetic basis for this relationship.

Data from Individuals Self-Reporting Symptoms of Trimethylaminuria

Trimethylaminuria is a gas at body temperature and possesses a strong, unpleasant odor at concentrations that exceed the olfactory threshold; at lower concentrations it may be described as foul, unpleasant or garbage-like. In addition, a 7% specific anosmia for TMA has been reported in the population at large [61]; and this will render those individuals unable to smell this compound (albeit having normal olfactory abilities with respect to other odorants). Further, people may not recognize the presence of TMA in a body fluid [62] and thus the need for an objective, analytical test for diagnosis. As described below, the test reliably determines an individual's capacity to metabolize TMA to TMA N-oxide.

Several factors contribute to the odors that are created on different parts of the human body including the type and number of cutaneous bacteria, amount of available oxygen as well as moisture and the type and number of skin gland secretions [61]. These factors may all contribute to normally produced body odors and are the reason the underarms, mouth, groin, scalp and chest all smell differently.

Individuals suffering from an odor-producing genetic disorder such as trimethylaminuria may present with body and/or oral malodors with no apparent cause, despite good hygiene. Research into the nature and origin of human odors has received considerable attention in the media and this has resulted in referrals from clinicians and dentists concerning individuals coming to their practices with idiopathic malodor production. To determine the cause for odor production in these referred patients, a multi-step analytical procedure has been used: 1) examine for odor production from the oral cavity and upper body [64] and 2) use of a choline challenge test for trimethylaminuria [65].

Evaluation of TMA and TMA N-oxide levels from individuals that self-report symptoms of trimethylaminuria is difficult to do because confounding factors related to the age, gender and ethnicity of the population studied are problematic. As discussed above, functional activity of human FMO3 is dependent on the age and ethnicity of the individual from whom the sample was obtained. The episodic nature of malodor (i.e., the transient forms of

trimethylaminuria described above) also contributes to make a difficult diagnosis. It is possible that these factors alone could complicate any detailed analysis of the data. However, it is useful to describe the type of information available even from a limited number of laboratories to gain some appreciation for the "clinical situation."

In one study conducted from 1980 until the end of 2001 approximately 550 urine samples were screened for trimethylaminuria using a gas chromatography mass spectrometry method [65]. The number of requested tests each year ranged from 1 to 15 from 1980 to 1990 and 20 to 40 until the end of 2001. The subjects were largely from the United States, but a considerable number of individuals from Canada, England, Argentina and Equador also were studied. From this population, 100 subjects were detected with excessive urinary TMA. The individuals with excess urinary TMA are listed in Table **9** according to ethnicity. From the self-reporting population examined, approximately 18% were found to be affected with the trimethylaminuria disorder, 14% were in the intermediate range and 68% had normal levels of TMA.

Patient Presentation and Diagnosis

Diagnosis of trimethylaminuria is based upon both the concentration of TMA (relative to creatinine) and the % TMA N-oxide in urine. Many individuals who report symptoms fall into a borderline area of 85-89% metabolized TMA. Any differences may become more apparent when more genotyping information is obtained and associated with genetic changes in the *FMO3* gene.

Table 9. Comparision of Ethnicity of the United States with that of Individuals Self-Reporting and Confirmed with Trimethylaminuria

% US Population	% Total Trimethylaminuria Individuals
75 Whites	51
12 African American	33
9 Hispanics	9
4 Asians	7

One might expect the severity of symptoms to match the level of excreted TMA; however, this is not always the case. Patient self-reports of symptoms may be tainted by the amount of trauma an individual has experienced because of sporadic/idiopathic odor production. Symptoms also vary greatly in trimethylaminuria-positive populations (see Fig. **3**) and seldom fit the often-cited classical definition of a foul-smelling, unpleasant odor.

Trimethylaminuria-affected individuals come from diverse ethnic backgrounds as seen in Table **9**. African-American patients are present in greater numbers than their representation in the population. The ethnic distribution of trimethylaminuria estimated from the above noted study population was compared with the ethnic distribution in the United States population from the most recent United States census. From this comparison there appears to be a disproportionate percentage (i.e., 33%) of African Americans with this disorder compared to the same ethnic group in the

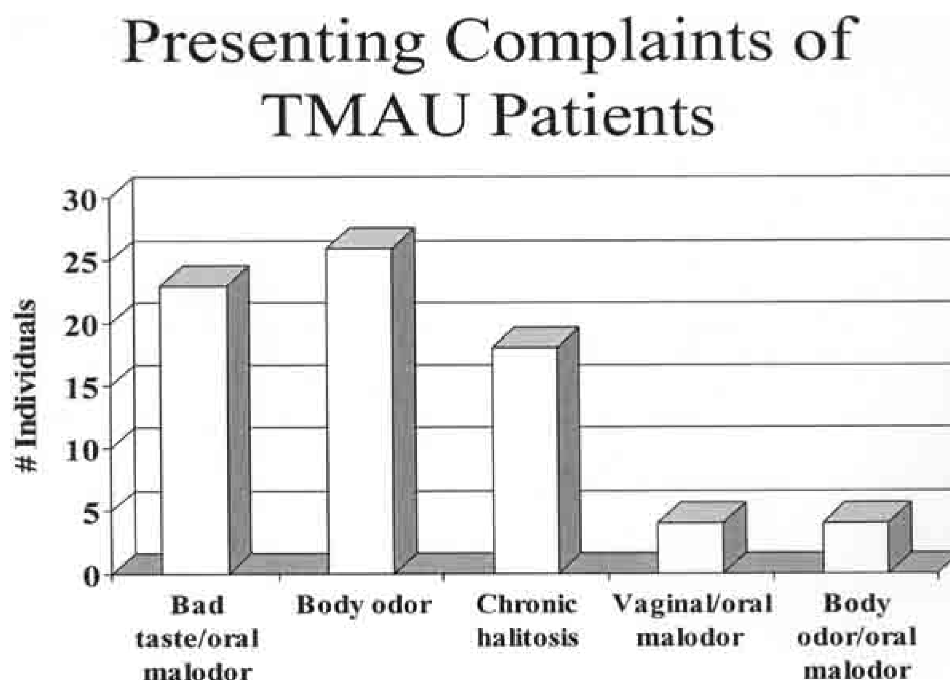


Fig. (3). Presenting symptoms of self-reporting individuals that were trimethylaminuria-positive.

general United States population (i.e., 12%). However, as only a relatively small number of individuals (i.e., 100) were available for this survey, the suggestion is that African Americans may be more affected for the trimethylaminuria condition. The data for Whites, Hispanics and Asians are not remarkable. In the population examined, most patients do not have affected parents or siblings. However, periodically, a trimethylaminuria individual is encountered where one parent and/or one or more siblings are affected. Examination of families is very useful to gain insight into genetic factors associated with trimethylaminuria. Females dominate the population examined, regardless of ethnicity (Fig. **4**). Among the female patients, symptoms are reported to be worse in the premenstrual time-frame. In the population examined, there was the suggestion of higher concentrations of TMA in females tested within ± 5 days of menses onset: TMA levels within ± 5 days of menses were 0.500 ± 0.160 mg TMA/mg creatinine; other days in the cycle were 0.400 ± 0.060 mg TMA/mg creatinine. However, this difference did not reach statistical significance (unpublished data).

Many patients examined have long-standing halitosis as the main reason for their malodor symptoms. Both trimethylaminuria-positive and trimethylaminuria-negative individuals may present with oral malodor caused only by a bacterial plaque on the posteria dorsal surface of the tongue [66]. The latter individuals may emit more than 200-600 ppb of sulfurous/fecal smelling volatile sulfur compounds (i.e., VSC: hydrogen sulfide; methylmercaptan; dimethylsulfide) with each exhalation, creating a "malodorous cloud" in their vicinity. Approximately 53% of the trimethylaminuria-affected population examined also had VSC levels above the 200 ppb level considered objectionable (Fig. **5**). Bad breath was more common in the non-trimethylaminuria population examined.

In summary, the data shows that many patients with trimethylaminuria have a varied phenotype with little overt odor being present at the time of their examination: only 7 of the trimethylaminuria patients had the "classic" pungent-odor presentation following choline challenge. These results show that it is not prudent to simply rely upon olfactory abilities in diagnosing trimethylaminuria. Objective analytical and genetic testing are available and can provide answers. African-American females make-up the majority of trimethylaminuria-positive patients examined to date (unpublished data). However, it is uncertain whether this is because females are more likely to present for clinical help when faced with an unusual problem compared with men, or whether there is a still-to-be-discovered sex steroid regulatory area on the *FMO3* gene.

Several unexplored factors may also contribute to symptom severity in both trimethylaminuria-positive and -negative individuals and these include other genetic anomalies, such as dimethylglycinuria that cause odor production; liver disorders, viral disorders as well as the intake of drugs that may require the FMO3 enzyme for metabolism [1]. Increased knowledge of patient genotype may help to elucidate some of these unknowns.

In a complementary genotyping study, 56 of the above-noted subjects self-reporting malodor were examined. Of these, 26 (46%) displayed a phenotypic abnormality in their capacity to oxidize TMA in response to a choline challenge. These subjects fell into the mild (i.e., 10-39% unmetabolized

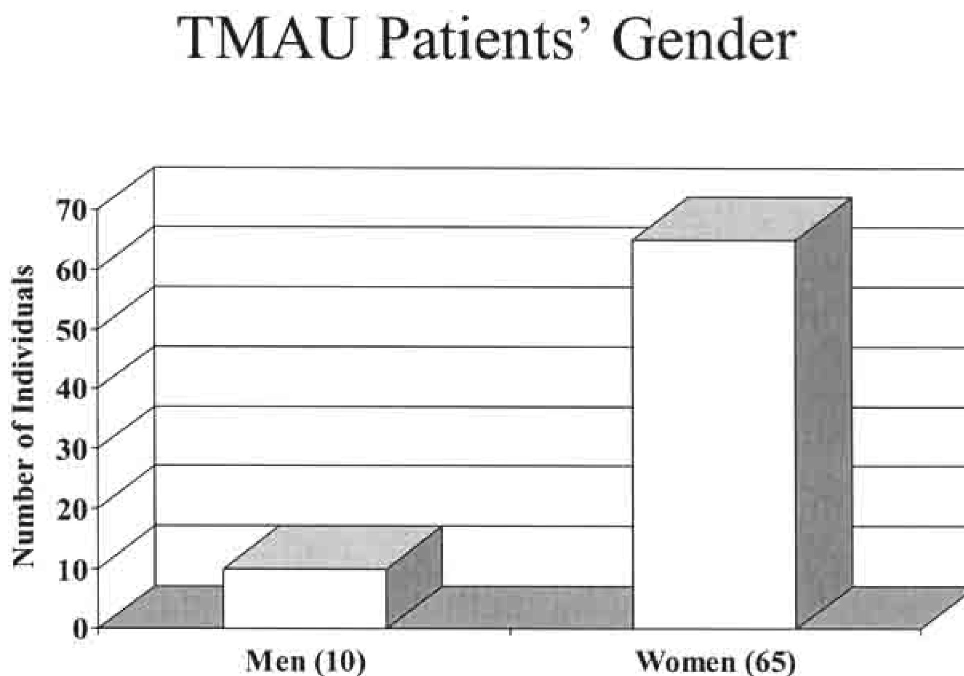


Fig. (4). The gender of the self-reporting individuals that were trimethylaminuria-positive.

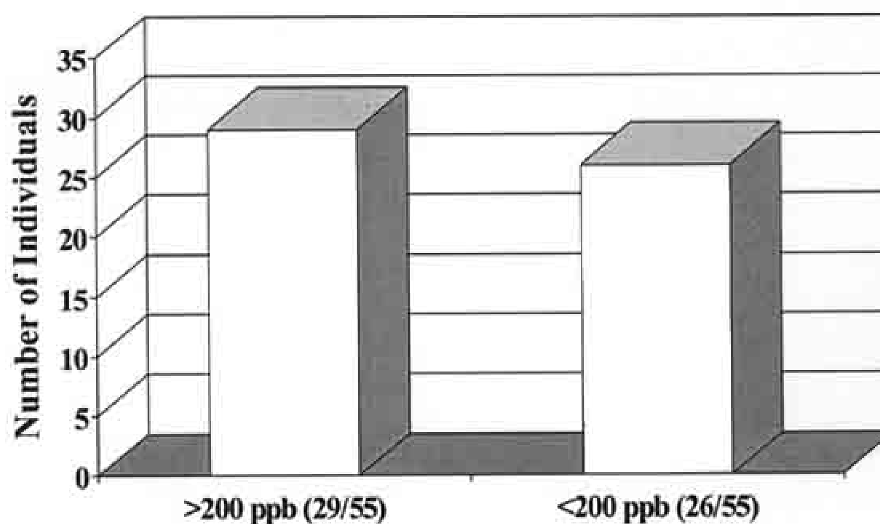


Fig. (5). Volatile Sulfur Compound (VSC) concentration in self-reporting individuals that were trimethylaminuria-positive.

TMA) to severely affected (i.e., >40% unmetabolized TMA) categories, making them candidates for further genetic testing to assess for *FMO3* gene defects. *FMO3* gene analysis was done on 19 of these 26 subjects.

The candidates for *FMO3* gene analysis in this cohort were of a variety of ethnic/racial backgrounds and displayed a wide range of capacity to oxidize TMA. Ten of the 19 individuals studied were African-American, whereas the remainder were Caucasian (5 subjects), Hispanic (2 subjects), or of East Asian extraction (2 subjects). Direct sequence analysis of all *FMO3* coding exons was done on genomic DNA samples from each subject to identify potential mutations that cause trimethylaminuria.

Within the group of 10 African-American subjects, one patient who was homozygous for the novel D132H alteration in exon 4 of the *FMO3* gene was identified. No other specific amino acid coding changes were detected other than the E308G polymorphism in exon 7. Choline challenge testing revealed this individual to have moderate to severe trimethylaminuria based on a value of 52.7% unmetabolized TMA (unpublished data). That this patient had a somewhat intermediate capacity to generate TMA N-Oxide, despite harboring a homozygous D132H alteration in *FMO3*, suggested that the D132H change impedes but does not ablate FMO3 activity.

Among another 87 African-American healthy individuals (174 chromosomes) screened, two were found to harbor the D132H variant. Neither individual complained of symptoms of trimethylaminuria, however, and one was available for further testing. Sequence analysis showed that the mutation giving rise to the D132H variant was present in the homozygous state in this subject. In addition, choline challenge testing revealed a decreased capacity to oxidize TMA (i.e., 27% unmetabolized TMA) indicating a mild deficiency in FMO3 activity despite this subject's lack of symptoms. Conceivably, any of a number of determinants including environmental factors, differences in modifier genes, or presence of additional changes, such as the E308G polymorphism, could influence FMO3 activity and account for the discrepancy in symptoms and relative FMO3 activity between these subjects. None of 42 Caucasian controls (84 chromosomes) were found to carry the D132H change (unpublished data). This is in keeping with other previously reported data [30].

Among the remaining African-American subjects studied, specific *FMO3* gene changes were identified in only two individuals. One was homozygous for both the E158K and E308G polymorphisms. This combined E158K/E308G variation has been implicated in cases of mild trimethylaminuria either when present in the homozygous state or in association with other known "severe" mutations which ablate FMO3 activity [11]. In contrast to these mild cases, this subject had severe trimethylaminuria based on the markedly diminished capacity to oxidize TMA (i.e., 81% unmetabolized TMA upon choline challenge testing). The other subject was found to be heterozygous for a novel valine to alanine change at codon 277 (V277A) in exon 7 of the *FMO3* gene. No other coding alterations were detected in this patient's *FMO3* gene. However, this individual had severe trimethylaminuria based on a value of 67.4% unmetabolized TMA upon choline challenge testing. Moreover, the identical heterozygous V277A change was observed in two unaffected control subjects. The V277A alteration most likely represents a rare variant of *FMO3* that

does not exert a significant influence on FMO3 activity. Strikingly, in both cases only *FMO3* gene changes that are unlikely to account for the severity of trimethylaminuria in the respective patients were detected. Similarly, none of a multitude of *FMO3* mutations previously identified in subjects of different racial/ethnic background and no novel *FMO3* gene changes were detected in the remaining seven African-American subjects. Among these, five had values of >45% unmetabolized TMA, including one subject with a value of 98.2%, upon choline challenge testing (unpublished data).

Of the nine other Caucasian, Hispanic and Asian patients in this study, coding mutations that accounted for diminished FMO3 activity were detected in just two. One Caucasian individual, with 79.8% unmetabolized TMA upon choline challenge testing, was found to be homozygous for the well-characterized P153L mutation. The other subject was of mixed Caucasian/Asian background and upon choline challenge testing had 75% unmetabolized TMA. This patient was a compound heterozygote for two different *FMO3* mutations. One allele carried a previously identified R492W mutation [10]. The other allele was found to harbor a novel arginine to glutamine mutation at codon 223 (R223Q). The arginine at position 223 is highly conserved across species and among all human FMO family members, suggesting that it is likely vital to FMO3 structure and function. Moreover, the R223Q substitution has not been detected in 79 Caucasian and Asian control subjects (i.e., 158 chromosomes), indicating that it unlikely represents a polymorphism. Thus, in conjunction with each other, the R492W and R223Q mutations decrease FMO3 activity sufficiently to cause severe trimethylaminuria in this individual (unpublished data).

A number of observations may be made from these studies. First, relatively few *FMO3* alterations were detected in this cohort overall. That a paucity of mutations was detected suggested that mechanisms other than mutations in *FMO3* coding sequence may account for a portion of cases of trimethylaminuria. Some may represent examples of cases other than primary genetic trimethylaminuria. However, some may harbor alternative gene changes such as mutations in non-coding, regulatory regions of the *FMO3* gene, or structural alterations in the *FMO3* gene that would not necessarily be detected by direct sequence analysis. To date, only one case of trimethylaminuria associated with deletion of *FMO3* coding exons has been reported [67]. Alternatively, mutations in other/modifier genes may account for some cases of trimethylaminuria. This may be particularly relevant in mild cases of trimethylaminuria, in which subjects display only a modest decline in %TMA N-Oxide upon choline challenge testing and experience transient or relatively mild symptoms. Conceivably, defects in such genes may exert only a mild influence on TMA metabolism that may become apparent only under stress conditions such as choline challenge testing or a large dietary bolus of foods rich in TMA precursors.

Second, certain *FMO3* gene variants may be restricted to certain racial/ethnic populations. Although several mutations in the *FMO3* gene that cause trimethylaminuria have been reported mostly in Caucasian subjects, none of these were detected in the group of African-American trimethylaminuria patients examined. However, two of these (i.e., P153L and R492W) were detected in two different subjects with trimethylaminuria of Caucasian descent. Conversely, the D132H change was observed only in African-American subjects. This suggests that founder effects may account for the presence of certain *FMO3* variants in specific population groups.

Third, some insights into *FMO3* genotype-trimethylaminuria phenotype correlations may be drawn from these studies. The D132H alteration, when present in the homozygous state, may give rise to mild to moderate trimethylaminuria. The wild type D132 codon is poorly conserved across species and it is likely that the D132H mutation causes relatively mild disruption of FMO3 structure and function. In light of this, the D132H mutation is somewhat analogous to the E158K/E308G dual polymorphism that may be associated with mild cases of trimethylaminuria when present in the homozygous state or in conjunction with a severe mutation. In contrast, the vast majority of previously characterized *FMO3* mutations associated with trimethylaminuria occur in highly conserved codons, abolish FMO3 activity, and cause severe trimethylaminuria.

In another unpublished study of self-reporting individuals from the United States that were tested for trimethylaminuria using infusion electrospray mass spectrometry as a detection method [31], approximately 25% of the individuals tested had urinary TMA/TMA N-oxide ratios that exceeded 15:85. In some cases, the individual possessed severe trimethylaminuria. Genetic analysis of DNA samples by resequencing from these severely-affected individuals showed that the *FMO3* gene was invariably defective. Oftentimes the causative mutation observed was the same as that seen previously (i.e., P153L, E305X) that explained the incidence of the condition in a large percentage of Caucasian individuals with severe trimethylaminuria [7, 8]. In other cases, extremely rare mutations were observed and the functional properties of the cDNA-expressed proteins revealed that the genotype alteration was consistent with the phenotype information. Clearly, individuals with elevated urinary TMA and diagnosed with trimethylaminuria should also be tested for changes in the *FMO3* gene. Finally, it is clear that a large number of other affected individuals in the United States and elsewhere should be diagnosed. Information about this disorder needs to be taught to medical students and medical care providers.

Biochemical Analytical Chemistry and Diagnosis of Trimethylaminuria.

Mass spectrometric (MS) biochemical methods have been developed to determine urinary TMA, TMA N-oxide and plasma choline in patients with trimethylaminuria [68]. A stable isotope dilution MS assay method is summarized below, and typical results will be illustrated. Previously, another method has been reported [31] that outlines a direct infusion electrospray MS method for the determination of urinary TMA and TMA N-oxide. A number of advantages are embodied in the latter assay method including determination of both analytes simultaneously and directly

with a minimum of sample handling or derivatization steps. Using TMA-^{9}D as an internal standard, acidified urine can be directly analyzed for TMA and TMA N-oxide by electrospray MS in the positive ion mode with great sensitivity [31].

Recently, a stable isotope dilution assay method for urinary TMA, TMA-N-oxide, and plasma choline has been developed utilizing MS detection [68]. It is important to monitor plasma choline in patients with trimethylaminuria that are receiving a choline and choline-precursor restricted diet. Choline is an essential dietary component in humans, and while deficiencies in the general population are rare, they can be associated with fatty liver, anemia and uremia. At the end of this report we describe the nutritional aspects of choline use.

Stable Isotope Dilution Assays for Urinary TMA and TMA-N-oxide, and Plasma Choline by Mass Spectrometry

Mass spectral methods to determine urinary TMA and TMA N-oxide and plasma choline concentrations in patients with trimethylaminuria are summarized as follows.

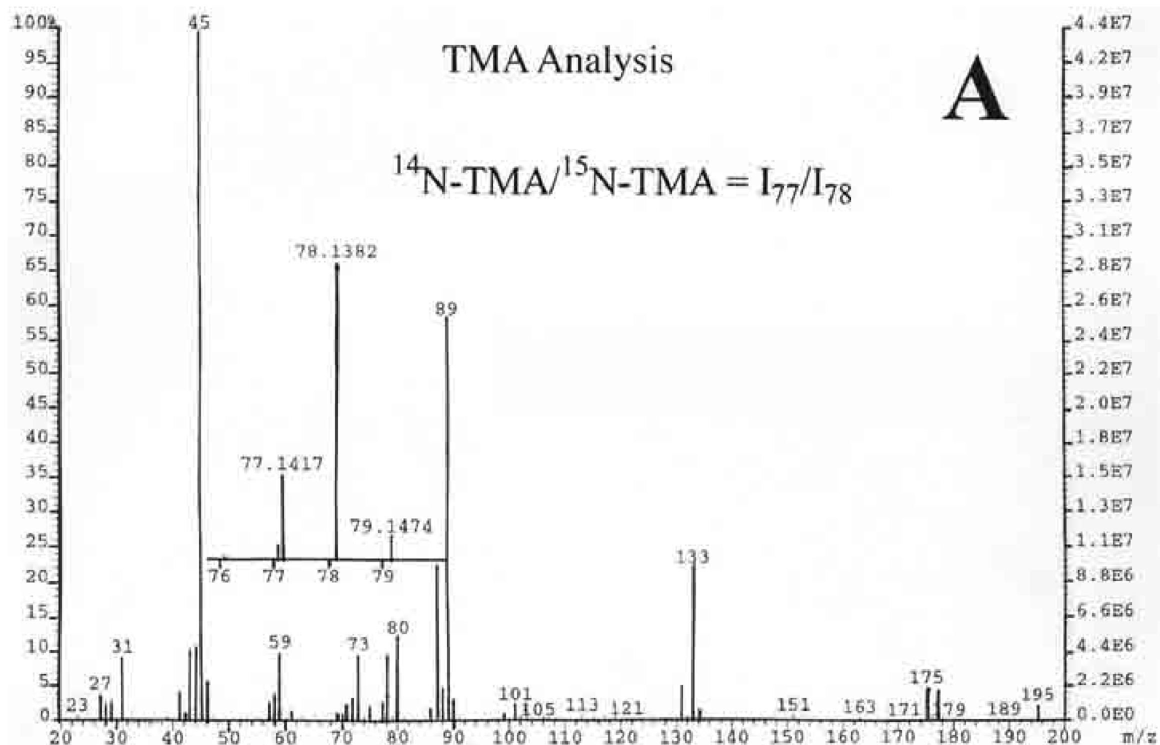
1. Trimethylamine

Immediately after collection, urine is acidified to pH 2 or less with hydrochloric acid. The urine may be frozen at this point for storage or transport. To the thawed urine is added ^{15}N-TMA HCl as the internal standard and the solution is saturated with sodium chloride and extracted twice with diethyl ether. This extract is discarded. The aqueous phase is cooled in an ice bath and made basic with 5N sodium hydroxide to pH 12 or higher. While cold, the solution is extracted with diethyl ether. To the ether extract is added trideuteromethyl iodide. This solution is maintained at room temperature for 20 min, and then evaporated to dryness. The residue is reconstituted in water, and an aliquot is evaporated onto the stage of a fast atom bombardment (FAB) probe. Hexaethylene glycol (HEG, 200 nl) is added as matrix, and a dozen 8 kV xenon bombardment spectra are collected with a ZAB mass spectrometer at 3000 resolution and averaged. The relative intensities of *m/z* 77 and 78 for $[^{14}N(CH_3)_3C^2H_3]^+$ and $[^{15}N(CH_3)_3C^2H_3]^+$ respectively, are measured and used to calculate the TMA concentration in the normal manner of a stable isotope dilution assay.

2. Trimethylamine-N-Oxide

To the same urine sample described above is added ^{15}N-TMA N-oxide internal standard, made by oxidation of ^{15}N-TMA by hydrogen peroxide [68]. A portion is evaporated to dryness on the FAB probe stage and FAB spectra in HEG matrix are collected as described above. The ratio of *m/z* 76 and 77 for ^{14}N- and ^{15}N-TMA N-OxideH$^+$ respectively, is measured and used to calculate the TMA N-Oxide concentration. Finally, the ratio of the concentrations of TMA N-Oxide to TMA is calculated.

The FAB spectra obtained for TMA and TMA N-Oxide analyses in a normal control is shown in Fig. (**6**). The difference in the masses of the endogenous and internal standard forms is due to the difference in the masses of ^{15}N and ^{14}N. Relatively small intensities of interfering ions at the same nominal masses of interest are eliminated by measuring the spectra at a resolving power of 3000. TMA N-



(Fig. 6) contd….

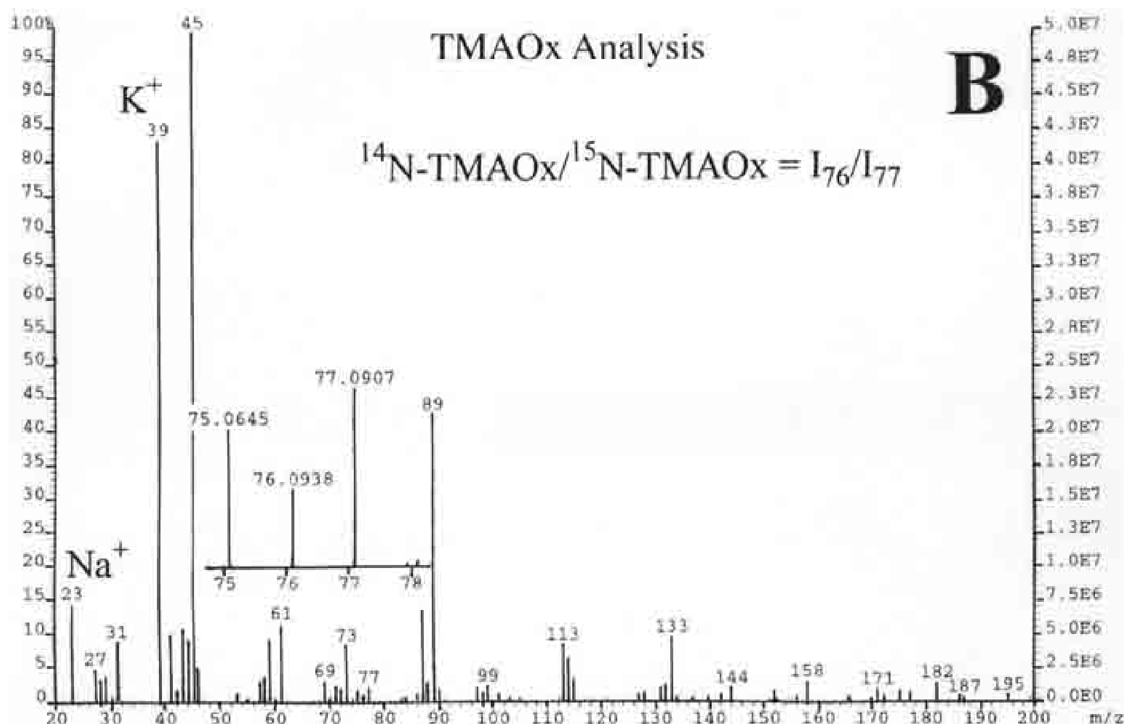


Fig. (6). FAB spectra obtained for a normal control urine: **A**: Analysis for TMA. The inset portion of the spectrum illustrates the *m/z* 77 and 78 ions used in the quantitation of TMA. The mass difference (78.1382 – 77.1417), 0.9965, is due to the difference between the masses of ^{14}N and ^{15}N (0.9971). **B**: Analysis for TMA N-Oxide. The inset portion of the spectrum illustrates the *m/z* 76 and 77 ions used in the quantitation of TMA. The mass difference (77.0907 – 76.0938), 0.9969, again is due to the difference between the masses of ^{14}N and ^{15}N.

Oxide/TMA ratios less than 2 are consistent with trimethylaminuria, ratios greater than 20 are usually normal.

3. Plasma Choline

To plasma is added 2,2,3,3-$^2H_4$-choline chloride. The plasma is then frozen at -80° for storage. After thawing, formic acid and cold methanol are added, and the mixture is thoroughly mixed and then centrifuged to precipitate protein. The supernatant is refrozen at -80°, thawed and re-centrifuged to remove the last traces of insoluble protein. The supernatant is infused directly into the electrospray source of a Quattro II triple quadrupole mass spectrometer, and scans from *m/z* 40 to 110 are collected in MCA mode. The quaternary ammonium ions *m/z* 104 and 108 are fragmented in argon at 10^{-6} bar at 25 eV collision energy (Fig. **7**). The transitions 104 to 45 (endogenous choline) and 108 to 49 (2,2,3,3-$^2H_4$-choline) are measured and the product ion intensity ratio calculated and used to determine the plasma choline concentration. This ratio is used to calculate the endogenous choline concentration in the normal manner of a stable isotope dilution assay. An example of the data obtained for this assay as applied to a normal control plasma is illustrated in Fig. (**8**). The plasma choline concentration determined in this example was 6.2 mg/L. A patient in a choline restricted dietary regime was found by this analysis to have a plasma choline concentration of 2.4 mg/L (unpublished data).

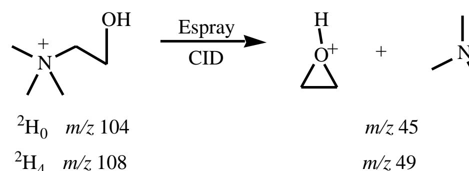


Fig. (7). The collision-induced fragmentation employed in the quantitation of choline. The intensity of the *m/z* 45 fragment derived from *m/z* 104 is related to endogenous unlabelled choline. The *m/z* 49 fragment derived from *m/z* 108 is related to choline labelled on carbon atoms 2 and 3, and this fragment bears the labelling intact. In both cases, trimethylamine is eliminated in the collision as an uncharged and unlabelled fragment.

Treatment and Nutritional Support

Nutrition support of trimethylaminuria is based on a restriction of dietary choline. Exclusion of foods high in dietary choline can significantly diminish TMA production and ameliorate symptoms in patients with trimethylaminuria [69, 70]. However, the correlation of dietary choline to TMA production is not absolute. Polymorphisms in the defective hepatic FMO3 enzyme can influence patients' tolerance to dietary choline [1, 71]. Moreover, currently known and unknown environmental and physiologic influences,

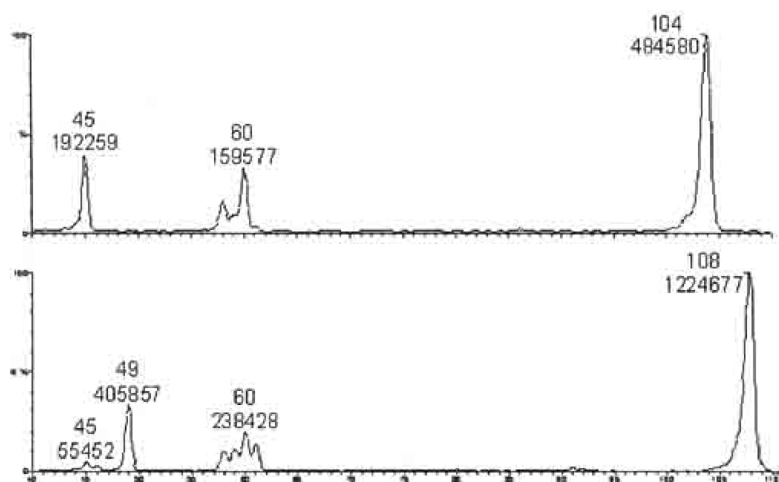


Fig. (8). Product ion spectra obtained for internal standard labelled and endogenous unlabelled choline (*m/z* 108 and 104 respectively). The intensity ratio of the product ions *m/z* 45 and 49 are related to the ratio of the endogenous and internal standard cholines in the sample, and is used to determine the concentration of the former.

including gut microflora, stress, onset of puberty, and in women, menses, can aggravate symptoms associated with excess TMA, regardless of dietary restriction.

Before discussing the role of a choline-restricted diet in patients with trimethylaminuria it is important to provide a brief review on choline. In animals and humans, choline is an important component in cellular function, and is required for synthesis of phospholipids, transfer of methyl groups and synthesis of acetylcholine, a neurotransmitter. Based on its functionality, choline plays a major role in liver function and cardiovascular health, reproduction, physical and brain development [72]. Choline has been considered a "conditionally-essential" amino acid because it can be endogenously synthesized via methylation of phosphatidylethanolamine and because of the lack of a true deficiency in humans. Some support for choline's essentiality can be found in studies that showed decreased concentrations of plasma choline concentrations concomitant with fatty liver in humans fed choline deficient total parenteral solutions [73, 74].

Free choline is absorbed throughout the small intestine [75]. At excess concentrations choline is absorbed passively. Large doses of dietary choline will overload the absorptive capacity of the lumen and be metabolized to methylamines by colonic bacteria [76-78]. Consequently, patients with trimethylaminuria who consume choline in quantities beyond their absorptive capacity will have significant production of unoxygenated TMA and concomitant malodor.

Goals of Nutrition Support

For patients with trimethylaminuria, the goal of nutrition support includes maintenance of trace to low concentrations of urinary TMA while conserving normal nutrition status. The general approaches to nutrition support include:

1. restriction of dietary precursors of TMA [e.g., choline, carnitine, and phosphatidylcholine (i.e., lecithin)].
2. enhancement of residual hepatic FMO3 activity by pharmacologic supplementation of riboflavin.
3. elimination of excess intestinal TMA using oral antibiotics (e.g., metronidazole) to decrease gut flora and/or increasing transit time using laxatives (i.e., lactulose).

Choline and Other Sources of Trimethylamines

Historically, nutrition support for patients with trimethylaminuria was directed towards limiting major dietary sources of choline, including whole egg and egg yolks, organ meats, liver, most fish, legumes and whole soybeans. Minor, but significant, dietary precursors of TMA (e.g., betaine, carnitine and creatinine) are generally not restricted. Dietary choline restriction was based on qualitative and not quantitative modifications. However, with the knowledge of choline essentiality and recent Dietary Recommended Intakes for choline [79] it is possible to plan a precise dietary regimen based on age and gender (Table **10**). Using a qualitative approach to diet restriction, dietitians can titrate the amount of dietary free choline to prevent deficiency while helping reduce symptoms associated with excess choline intake.

Whereas animal and human research has established choline as a major TMA precursor [75, 77], the extent to which each of the minor precursors (listed above) contributes to TMA production has not been fully elucidated. Zhang and coworkers [80] have reported in humans (non-trimethylaminuria individuals) that bolus doses (15 mmol) of betaine (1.76 g), creatinine (1.7 g) and lecithin (11.65 g) did not significantly increase urinary TMA and TMA N-oxide excretion. In contrast, free choline (2.1 g) and D,L-carnitine (2.97 g) produced significant increases in urinary TMA and its N-oxide. However, dietary sources of carnitine, as found in red meat did not increase TMA excretion. These authors also reported a muted response to urinary TMA excretion with certain choline-containing foods.

Table 10. Dietary Reference Intakes (DRIs) for Choline and Folate[a]

Life Stage Group	Choline (mg/day)		Folate (mg/day)	
Infants				
0 – 6 months	125		65	
7 – 12 months	150		80	
Children				
1 – 3 years	200		150	
4 – 8 years	250		200	
	Male		**Female**	
	Choline	**Folate**	**Choline**	**Folate**
Older Children/Adults				
9 – 13 years	375	300	375	300
14 – 18 years	550	400	400	400
> 19 years	550	400	425	400
Pregnancy (all child-bearing years)	N/A	N/A	450	600
Lactation	N/A	N/A	450	500

[a]Adequate intake is a value based on observed or experimental estimates of intake by a group of healthy people.

Lecithin, when used as an emulsifier in processed foods, should not readily increase TMA production. This is important to note because the major source of dietary choline is lecithin, which, by weight, contains 13% choline. Regardless, patients with trimethylaminuria must avoid lecithin and lecithin-containing fish oil supplements, which can convert to TMA with increased shelf time. A standard lecithin capsule contains approximately 250 mg of free choline. Egg yolks, and organ meats are generous sources of lecithin and choline and should be avoided by patients with trimethylaminuria. A list of foods containing choline and choline phospholipids has been published [81].

Dietary indoles or glucosinolates found in brussels sprouts, broccoli, cabbage and cauliflower) may inhibit hepatic FMO3 activity and exacerbate urinary TMA excretion [82, 83]. Ten adult healthy males given 300 grams/day of cooked brussels sprouts for 3 weeks, showed a significant increase in the ratio of urinary TMA to TMA N-oxide. Based on this preliminary research, it may be medically prudent for patients with severe trimethylaminuria to limit their intake of glucosinolate-containing vegetables.

Vitamin Supplementation

Folate supplementation is suggested in patients with trimethylaminuria on a choline-restricted diet. A dietary choline restriction increases the requirement of folate, a methyl donor, which interacts with choline and methionine in metabolic processes [84]. In addition, choline deficiency may interfere with assimilation of newly synthesized folate by hepatic tissue [85].

Theoretically, individuals with a defect in FMO3 may benefit from riboflavin supplementation. Riboflavin, a coenzyme of the FMO3 enzyme, might help stabilize residual enzyme activity and increase its half-life, thereby muting symptoms in patients with this disorder. Recommended intake is 30-40 mg three to five times per day with food. Children that are given riboflavin supplementation should be monitored closely as high doses could cause GI distress or other medical problems.

Drug Treatment

Metronidazole, an antibiotic, has been used to modulate and reduce intestinal anaerobic bacteria in patients with different medical disorders [86, 87]. Case reports in patients with trimethylaminuria showed that oral administration of metronidazole successfully reduced urine TMA excretion [88]. However, that this was not always associated with a decrease in malodor suggests a possible delay in response or other extenuating factors. Chronic antibiotic therapy is not feasible as a long-term therapy due to bacterial resistance, diarrhea, and other potential side effects and use of antibiotics should be done with the consent of a physician.

Issues in Pregnancy and Lactation

Women with trimethylaminuria should not restrict dietary choline during pregnancy and lactation. Choline is critical for nerve and brain development in the fetus and young infant [72, 89]. During pregnancy, large amounts of choline are transferred across the placenta to the fetus

resulting in a ten-fold increase in concentration in amniotic fluid compared to maternal blood [90]. Increased choline flux across the placenta may result in depletion of maternal choline reserves. In rodents, choline depletion resulted in depressed cognition due to changes in the hippocampal memory center [91]. The requirement for increased amounts of choline persists in the newborn period and large amounts are found in human milk. This increased need is reflected in the Dietary Reference Intake for choline for pregnant and lactating women (Table **10**).

In summary, many issues remain regarding the choline-restricted diet. Published research on choline content of foods is severely limited and done years ago using antiquated analytical methods. Questions remain as to the optimal choline intake required to reduce symptoms in patients with trimethylaminuria. A careful balance between science and patient needs is required for successful dietary intervention and clinical outcome. A sound nutrition regimen should be tailored to each person's requirements and monitored under medical supervision. Medical supervision is required to prevent over-restriction of dietary choline and to prescribe and monitor the use of metronidazole. While individual patients have various tolerances to choline-containing foods, they should be encouraged not to overly restrict their diets based on unproven or anecdotal information.

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ABBREVIATIONS

FMO	=	Flavin-containing monooxygenase
HNF	=	Hepatic nuclear factor
TMA	=	Trimethylamine
TMA N-Oxide	=	Trimethylamine N-oxide

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