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Novel Variants of the Human Flavin-Containing Monooxygenase 3 (*FMO3*) Gene Associated with Trimethylaminuria

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Abstract

The disorder trimethylaminuria (TMAu) often manifests itself in a body odor for individuals affected. TMAu is due to decreased metabolism of dietary-derived trimethylamine (TMA). In a healthy individual, 95% or more of TMA is converted by the flavin-containing monooxygenase 3 (*FMO3*, EC 1.14.13.8) to non-odorous trimethylamine *N*-oxide (TMA *N*-oxide). Several single nucleotide polymorphisms (SNPs) of the *FMO3* gene have been described and result in an enzyme with decreased or abolished functional activity for TMA *N*-oxygenation thus leading to TMAu. Herein, we report two novel mutations observed from phenotyping and genotyping two self-reporting individuals. Sequence analysis of the exon regions of the *FMO3* gene of a young woman with severe TMAu revealed heterozygous mutations at positions 187 (V187A), 158 (E158K), 308 (E308G), and 305 (E305X). Familial genetic analysis showed that the E158K/V187A/E308G derived from the same allele from the mother, and the E305X was derived from the father. *FMO3* variants V187A and V187A/E158K were characterized for oxygenation of several common *FMO3* substrates (i.e., 5- and 8-DPT, mercaptoimidazole (MMI), TMA, and sulindac sulfide) and for its thermal stability. Our findings show that with the combination of V187A/E158K mutations in *FMO3*, the enzyme activity is severely affected and possibly contributes to the TMAu observed. In another study, genotyping analysis of a 17 year old female revealed a mutation that caused a frame shift after K415 and resulted in a protein variant with only 486 amino acid residues that was associated with severe TMAu.

Keywords

flavin-containing monooxygenase; *FMO3*; novel variants; novel mutations; V187A; K415 frameshift; trimethylaminuria; TMAu

Introduction

The family of flavin-containing monooxygenases (FMOs, EC 1.14.13.8) represents, after the cytochrome P450s, the most important drug-metabolizing monooxygenase enzymes in adult human liver. FMOs catalyze the oxygenation of various nucleophilic nitrogen-, sulfur-, and phosphorous-containing xenobiotics [1]. In humans, five isoforms (*FMO1* - 5) and 6 pseudogenes (*FMO6P* - *11P*) have been described [2,3] but *FMO3* is considered to be the major drug-metabolizing FMO isozyme in adult human liver.

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The disorder trimethylaminuria (TMAu) often manifests itself in a body odor for the individual affected. TMAu is caused by the accumulation and excretion of unmetabolized trimethylamine (TMA) a substance derived from foodstuffs including choline. In a healthy individual, 95% or more of TMA is converted by FMO3 to non odorous TMA *N*-oxide. Two different major forms of TMAu have been described [4]: a primary genetic form that causes decreased FMO3 enzyme function and a secondary form that is due to TMA or a TMA-precursor overload. The two forms could be associated with each other, because individuals with a slightly decreased enzyme activity (primary TMAu) might not exhibit TMAu symptoms until the affected individual is challenged with increased amounts of TMA as a result of diet, liver disease, or bacterial overgrowth (secondary form). In addition, minor forms of TMAu including an acquired TMAu with no obvious genetic background, a transient childhood form, and a transient form in women associated with menstruation have been described [5-11]. Nevertheless, in general, the majority of TMAu cases reported are associated with single nucleotide polymorphisms (SNPs) of the *FMO3* and therefore categorized as the primary genetic form of TMAu. More than 300 SNPs of the human *FMO3* have been reported (<http://www.ncbi.nlm.nih.gov/projects/SNP/>) and over 40 of these polymorphisms have been linked to TMAu due to decreased or abolished TMA *N*-oxygenation ability of the *FMO3* variant [4]. Depending on the *FMO3* SNP, the incidence and severity of the disorder varies [12]. By themselves, several common polymorphic variants do not significantly decrease TMA *N*-oxygenation activity, but in combination with other SNPs may have a more deleterious impact (e.g., the common SNPs E158K and E308G) [6,13,14]. For the affected individual, accurate diagnosis of TMAu can relieve concerns and provide an impetus to obtain medical advice on dietary restriction to limit the intake of TMA precursors and thereby decrease the TMAu condition [15]. Diagnosis includes phenotype analysis (i.e., measurement of the urinary ratios of TMA *N*-oxide to TMA that in healthy individuals should be $\geq 95\%$), and genotyping of the affected individuals in order to identify *FMO3* gene mutations that cause TMAu.

In genotyping studies of two individuals, we identified two novel mutations associated with TMAu. In a 33 year old woman, in addition to the common polymorphisms E158K and E308G, we observed a SNP at position 187 (i.e., V187A) that had not been described to date and a truncation mutation E305X reported previously. Examination of both biological parents showed that the biological mother carried the E158K/V187A/E308G allele, and the biological father carried the E305X allele. While it is known that E305X will abolish the *FMO3* function, the V187A mutation has not been reported nor previously characterized. In order to characterize this new and unusual variant, we cloned, expressed, and purified the V187A and the V187A/E158K variants of *FMO3* as maltose-binding fusion proteins. The triple mutant E158K/V187A/E308G reflecting the genotype of one allele of the affected individual examined was not studied because of the difficulty in expression and characterization of the enzyme. The variants were tested *in vitro* for the oxygenation of selective functional substrates for the *FMO3* enzyme (i.e., 5- and 8-DPT, mercaptoimidazole (MMI), TMA, and sulindac sulfide). The thermal stability of the variant *FMO3*s were also examined and compared to wild-type enzyme. In a separate study, genotype analysis of a 17 year old female revealed a mutation that caused a frame shift after K415 and resulted in a variant protein with only 486 amino acid residues.

Materials and Methods

Reagents

Chemicals and reagents used in this study were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO) in the highest purity commercially available. Buffers and other reagents were purchased from VWR Scientific, Inc. (San Diego, CA). The synthesis of the phenothiazines 5-DPT and 8-DPT has been previously described [16-18].

Genomic DNA Preparation and Polymerase Chain Reaction (PCR) Amplification

Genomic DNA preparation and PCR amplification was done as described before [19]. Briefly, blood samples from individuals with self-reported TMAu symptoms were collected by their primary care physicians and sent to the HBRI laboratory for genotype analysis. All human samples were approved by the Independent Review Consultant Inc. (San Anselmo, CA) institutional review board. The ethnicity of the individuals was defined by a self-report questionnaire that indicated the race of both biological parents. The individuals tested in this study were U.S. citizens from Northern European descent. Genomic DNA was prepared from whole blood using the Qiagen QIAmp Blood Kit (Valencia, CA) following the manufacturer's protocol. Eight coding exons of *FMO3* and neighboring flanking intronic regions were amplified from genomic DNA and sequenced using the primers listed in Table 1 and PCR conditions reported previously [19]. DNA sequences were analyzed with Sequencher Software (Gene Code Corporation, Ann Arbor, MI) by procedures that could resolve heterozygotes under reliable quality-control conditions. To verify the heterozygous frame shift mutation of the second individual, PCR products of exon 7 were subcloned into the TA vector (Invitrogen, San Diego, CA) and multiple individual clones were sequenced. Genebank sequence [NT 004487](#) was used as the wild-type *FMO3* reference sequence.

FMO3 Phenotyping by urinary TMA and TMA-N-Oxide Analysis

A selective functional method was used to characterize the *FMO3* phenotype in humans by determining the amount of TMA and TMA *N*-oxide from urine. After collection of first void morning urine sample the TMA and TMA *N*-oxide concentrations following a normal diet were determined. The urine was cooled to 4 °C with ice and acidified to pH 1 with 6 N HCl and immediately stored at -80 °C until thawing for assay. TMA and TMA *N*-oxide concentration was determined by electrospray ionization mass spectrometry with a deuterated TMA internal standard as previously described [20].

Cloning and cDNA Expression

The expression vector for FMO V187A and E158K/V187A constructs were cloned into pMal-2c (New England Bio Labs, Ipswich, MA) by site directed mutagenesis methods as described previously [19,21]. Briefly, wild-type *FMO3*, *FMO3* V187A, and *FMO3* V187A/E158K were expressed as *N*-terminal maltose-binding fusion proteins (i.e., MBP-*FMO3*, MBP-*FMO3* V187A, and MBP-*FMO3* V187A/E158K). After transformation of *E. coli* DH 1 α cells with pMal-MBP-*FMO3* plasmid, cells were grown at 37 °C in SOC medium to an absorbance of 0.4-0.5 at 600 nm and then 0.2 mM IPTG, 0.05 mM riboflavin, and 100 μ g/ml ampicillin were added and the cells were further shaken at room temperature overnight and harvested by centrifugation for 10 min at 6000 $\times$ g.

Purification of MBP-FMO Fusion Proteins

All of the following procedures including the purification process were carried out at 4 °C. The cell pellet was resuspended in lysis buffer as described previously [22]. After stirring the cell suspension on ice for 30 minutes, the cells were disrupted by sonication (i.e., five 8-seconds bursts separated by periods of cooling) on a Sonics Vibracell ultrasonic processor. The solution was centrifuged and the resulting supernatant was loaded onto an amylose column (New England Bio Lab, Beverly, MA). In order to obtain a higher yield of protein, the pellets were extracted a second time as described above and the resulting supernatant of the second extraction was also loaded onto the amylose column. Protein purification was carried out on a low pressure chromatography system, Biologic LP (Bio-Rad, Hercules, CA). After loading the supernatants at 0.75 ml/min onto an amylose column that was equilibrated with 10 column volumes of buffer A (i.e., 50 mM Na₂HPO₄ pH 8.4 and 0.5 % Triton X-100) the column was washed with at least 10 column volumes of buffer A. Bound *FMO3* protein was then eluted

with a linear maltose gradient: 0-100 % buffer B (buffer A with 10 mM maltose). Eluted fractions (5 ml each) were analyzed and the fractions with the highest enzyme activity were pooled and concentrated with a centrprep tube (30,000 molecular weight cut-off).

Determination of MBP-FMO3 Concentration by SDS-PAGE and Commassie Blue Staining

MBP-FMO3 were quantified by SDS-PAGE and Commassie Blue staining and compared with a bovine serum albumin (BSA) standard. Briefly, MBP-FMO3 proteins and different quantities of standard BSA (2, 1.5, 1.0, 0.5, and 0.1 μ g per lane) were fractionated by electrophoresis on a 10 % polyacrylamide gel under denaturing conditions and stained with Commassie Blue. After destaining, FMO quantification was done by densitometry analysis employing Kodak molecular imaging software (Eastman Kodak Company, Rochester, NY).

Enzyme Assays

Oxygenation of MMI was determined spectrophotometrically by measuring the rate of MMI *S*-oxygenation via the reaction of the oxidized product with nitro-5-thiobenzoate (TNB) to generate 5,5'-dithiobis(2-nitrobenzoate) (DTNB). The assay contained 50 mM sodium phosphate buffer (pH 8.5), 0.5 mM NADP⁺, 0.5 mM glucose-6-phosphate, 1.5 IU/ml glucose-6-phosphate dehydrogenase, 0.06 mM DTNB, 0.04 mM dithiothreitol, and 45 - 360 μ g/ml MBP-FMO3. Reactions were initiated by the addition of 2 mM final concentration of substrate and the disappearance of the yellow color was followed spectrophotometrically at 412 nm. Kinetic parameters (i.e., k_{cat} and $K_{m\ app}$) for FMO3-mediated MMI *S*-oxygenation were determined by initiating the enzyme reaction with different amounts of substrate. The final substrate concentrations were 800, 400, 200, 100, 40, 10, and 1 μ M for wild-type FMO3, 800, 200, 100, 40, 20, 10, 5, and 1 μ M for the MBP-FMO3 V187A variant, and 200, 100, 40, 10, and 5 μ M for the MBP-FMO3 V187A/E158K variant.

Kinetic parameters (i.e., k_{cat} and $K_{m\ app}$) for FMO3-mediated TMA *N*-oxygenation were determined by spectrophotometrically monitoring the oxidation of NADPH associated with TMA *N*-oxygenation. The assay medium contained 50 mM sodium phosphate buffer (pH 8.5), 0.5 mM diethylenetriaminepentaacetic acid (DETAPAC), 0.2 mM NADPH, and 160 - 640 μ g/ml MBP-FMO3. Incubations were initiated by the addition of different amounts of substrate and monitored at 340 nm for NADPH depletion. The final substrate concentration was 100, 40, 20, 10, and 5 μ M for wild-type FMO3 and the MBP-FMO3 V187A variant and 800, 400, 200, 100, and 40 μ M for the MBP-FMO3 V187A/E158K variant.

N-Oxygenation of 5- and 8-DPT was determined by HPLC analysis as previously described [22]. Briefly, a standard incubation mixture contained 50 mM potassium phosphate buffer (pH 8.4), 0.4 mM NADP⁺, 0.4 mM glucose-6-phosphate, 4 U glucose-6-phosphate dehydrogenase, 0.25 mM DETAPAC, and 40 μ g/ml wild-type MBP-FMO3 or its variant at 4 °C. Incubations were initiated by the addition of substrate to a final concentration of 200 μ M at 37 °C. After incubation for 20 min shaking under aerobic conditions, enzyme reactions were stopped by the addition of 4 volumes of cold dichloromethane. About 20 mg of Na₂CO₃ was added and the incubations were mixed and centrifuged to partition metabolites and remaining substrate into the organic fraction. The organic phase was collected and evaporated under a stream of argon. Metabolites and remaining substrate were dissolved in methanol, mixed thoroughly, centrifuged and analyzed with an Hitachi HPLC system (Hitachi L-7200 autosampler and L-7100 pump interfaced to a Hitachi L-7400 UV detector). Chromatographic separation of analytes was done on an Axxi-Chrom normal phase analytical column (250 $\times$ 4.6 mm 5 μ m, silica) with a mobile phase of 80 % MeOH/20 % isopropanol/0.025 % HClO₄. The flow rate was 1.6 ml/min and the total run time was 10 min for 5-DPT and 8.5 min for 8-DPT. The wavelength for UV detection was set at 243 nm. The retention times for 5-DPT and 5-DPT *N*-oxide, and 8-DPT and 8-DPT *N*-oxide were 6.4, 4.6, 5.8, and 4.1 min, respectively.

The *S*-oxygenation of sulindac sulfide was determined by HPLC analysis as previously described with slight modifications [11,23]. Briefly, a standard incubation mixture contained 50 mM potassium phosphate buffer (pH 8.4), 0.4 mM NADP⁺, 0.4 mM glucose-6-phosphate, 4 U glucose-6-phosphate dehydrogenase, 0.25 mM DETAPAC, and 40 µg/ml wild-type MBP-FMO3 or MBP-FMO3 variant. Incubations were initiated by the addition of sulindac sulfide to a final concentration of 200 µM. After incubation for 20 min with continuous shaking under aerobic conditions at 37 °C, the incubation was stopped by addition of 20 µl cold 25 % phosphoric acid and 4 volumes of ethyl acetate. The incubations were mixed and centrifuged to partition metabolites and remaining substrate into the organic fraction. The organic fraction was collected and evaporated under a stream of argon. Metabolites and remaining substrate were dissolved in methanol, mixed thoroughly, centrifuged, and analyzed with a Hitachi HPLC system (Hitachi L-7200 autosampler and L-7100 pump interfaced to a Hitachi L-7400 UV detector). Chromatographic separation of analytes was performed on an Axxi-Chrom reverse phase analytical column (250 × 4.6 mm 5 µm, Supelco) with a mobile phase consisting of 70 % acetonitrile and 30 % phosphate buffer, pH 3. The flow rate was 1.0 ml/min and the total run time was 12 min. The wavelength for UV detection was set at 360 nm. The retention times for sulindac sulfide and sulindac *S*-oxide were 9.6 and 5.0 min, respectively.

To determine the thermal stability of wild-type FMO3, FMO3 V187A, and FMO3 V187A/E158K, all three FMO enzymes were incubated at 40 °C for 0, 1, and 5 minutes in the presence or absence of an NADPH-regenerating system prior to the addition of the completed reaction mixture. *N*-Oxygenation of 8-DPT was determined using the HPLC method described above. Mean velocity calculated for 0 min incubation at 40 °C was designated as 100 % for each recombinant enzyme, and the velocities for heat-treated enzymes were normalized accordingly.

Data analysis

The kinetic parameters for TMA and MMI were determined by examining the data from incubation of at least five different substrate concentrations with MBP-FMO3 variants. Incubations were done in triplicate and for data analysis a nonlinear regression curve fit tool using a Michaelis-Menten model in Graphpad software (Graphpad Prism, Version 3.00, San Diego, CA) was utilized. Data obtained was presented as the best fit value ± standard error. Statistical analysis was also done using Graphpad Prism software. Statistical significance was judged at $P < 0.05$.

Results

Phenotyping and Genotyping Results

Diagnosis of TMAu included measurement of the urinary ratios of TMA *N*-oxide to TMA and genotyping of the affected individuals. A urine and blood sample from a young woman of Northern European descent with a history of unpleasant body odor was examined and the phenotype as well as genotype was determined. When determining TMA and TMA *N*-oxide levels in the urine of this sample, TMA could be detected whereas no TMA *N*-oxide was detectable indicating severely abnormal TMA metabolism.

Genotyping of the 33 year old female showed several mutations at a number of different loci. One heterozygous missense mutation identified was Val (GTT) to Ala (GCT) at position 187 in exon 4 of the *FMO3* gene. This is the first time we have detected this mutation after genotyping over 100 individuals with self-reported body odor. To our knowledge, the V187A mutation has not been reported in the literature by any other group genotyping and phenotyping individuals for FMO3. The individual was also identified as heterozygous for E305X, a known mutation leading to TMAu due to abolished FMO3 function. In addition, two common polymorphisms (i.e., E158K and E308G) were also identified and these SNPs have been shown

to generally decrease FMO3 activity when observed together [6,14,24]. Sometimes, TMAu observed from individuals harboring the 158/308 mutations was modest and only observable under challenge situations such as conditions of large dietary intake of TMA or TMA precursors. Other mutations were observed in the intronic regions with unknown biological significance and are not described here. Both biological parents of the young woman were also genotyped. The father was found to be heterozygous for E305X whereas the mother was heterozygous at three positions E158K/V187A/E308G. Thus, it was clear that E158K/V187A/E308G is on the same chromosome and inherited from the mother, and E305X is on the other chromosome inherited from the father. Unfortunately, the urine samples from the parents were not available for analysis.

For another individual genotyped, a 17 year old female, sequence analysis of the *FMO3* coding exons amplified from genomic DNA revealed that the individual was heterozygous for V257M and a g.23612-23613AG>T mutation in exon 7 of the *FMO3* gene. The mutation caused a frame shift after K415 and continued for 71 mutant amino acid residues and resulted in a protein with only 486 amino acid residues (Figure 1). Family pedigree analysis revealed that the deletion mutation was inherited from the biological father of this individual in combination with V257M, a common polymorphism that is not generally a pathological or defective gene by itself, on the same allele. Further phenotype analysis revealed significantly abnormal TMA:TMA-*N*-oxide 48:52 for the father who had the same genotype.

Cloning, Expression and Purification of wild-type FMO3, FMO3 V187A, and FMO3 V187A/E158K

To study the effect of the novel V187A mutation on FMO3 enzyme function, and its effect on FMO3 enzyme activity in combination with the common E158K polymorphism, wild-type, V187A mutant, and V187A/E158K double-mutant *FMO3*s were expressed in *E. coli* as MBP-fusion proteins and assayed for their ability to catalyze the *N*- and *S*-oxygenation of various typical FMO substrates. When expressing the protein, we observed that the expression of both mutants (i.e., FMO3 V187A and FMO3 V187A/E158K) was less efficient than that of wild-type FMO3, yielding only about 20 % of the wild-type protein expressed. Due to the significant decrease in expression and enzyme activity for both, V187A/E158K and E158K/E308G, the triple mutant E158K/V187A/E308G reflecting the genotype of one allele of the affected individual examined was not studied because of the difficulty in expression and characterization of the enzyme.

Comparison of *N*- and *S*-Oxygenation Functional Activity of FMO3 V187A and FMO3 V187A/E158K with wild-type FMO3

To compare the *N*- and *S*-oxygenation of wild-type FMO3 with FMO3 V187A and FMO3 V187A/E158K, selective functional substrates (i.e., 5- and 8-DPT) were used to examine *N*-oxygenation and sulindac sulfide was used as substrate to investigate differences in *S*-oxygenation. The results (Figure 2) showed that wild-type FMO3 had the highest *N*-oxygenation activity followed by FMO3 V187A with a specific activity of 69 % of wild-type *N*-oxygenation activity for 5- and 8-DPT. FMO3 V187A/E158K had the lowest specific activity with only 15 and 17 % of wild-type activity for 5- and 8-DPT, respectively. FMO3 V187A had an *S*-oxygenation activity of 102 % for sulindac sulfide compared to wild-type FMO3. For FMO3 V187A/E158K the *S*-oxygenation was decreased to only 31 % of wild-type values for sulindac sulfide *S*-oxygenation.

Kinetic Parameters for TMA *N*-Oxygenation by FMO3 V187A and FMO3 V187A/E158K

To determine the kinetic parameters for TMA *N*-oxygenation by FMO3 V187A and FMO3 V187A/E158K, the enzymes were incubated with different concentrations of TMA. The single mutant V187A had a similar k_{cat} and K_m value as the wild-type enzyme. However, the

double mutant FMO3 V187A/E158K had a much lower k_{cat} value and a K_m that was 25-fold greater than that of FMO3 V187A or wild-type FMO3, causing a 65-fold decrease in catalytic efficiency for TMA (Table 2).

Kinetic Parameters for MMI S-Oxygenation by FMO3 V187A and FMO3 V187A/E158K

To determine the kinetic parameters for MMI S-oxygenation by FMO3 V187A and FMO3 V187A/E158K, the enzyme was incubated with different concentrations of MMI. All three enzymes had similar K_m values. The single mutant V187A also had a similar k_{cat} value as the wild-type enzyme, but the k_{cat} value of the double mutant FMO3 V187A/E158K was only 24 % of that of the wild-type enzyme (Table 3).

Thermal Stability of wild-type FMO3, FMO3 V187A, and FMO3 V187A/E158K

The N-oxygenation of 5-DPT was determined by HPLC analysis as described under enzyme assays after incubation of the three FMO enzymes at 40 °C for 0, 1, 2, and 5 min in the presence or absence of an NADPH-regenerating system. In Figure 3 only wild-type FMO3 and FMO3 V187A are shown, because the functional activity of FMO3 V187A/E158K was non-detectable after incubation at 40 °C regardless of the presence or absence of an NADPH regeneration system.

While no significant difference between the relative loss of activity for the wild-type enzyme compared to FMO3 V187A was observed after pre-incubation at 40 °C, a distinct difference was notable when both enzymes were pre-incubated at elevated temperature in the presence of NADPH for 1 and 5 minutes (Figure 3, $P < 0.001$). About 70 % of the wild-type FMO3 activity was retained whereas the enzyme activity of FMO3 V187A increased to 140 and 170 % of its original activity after 1 and 5 minutes preincubation with NADPH, respectively.

Discussion

Comprehensive biochemical characterization of recombinant variant FMO3 enzymes based on data from genotype and phenotype analysis of individuals with self-reported symptoms of TMAu can potentially reveal important new information about structure and function of human FMO3 [5,11,13,25-28]. Findings from the studies herein provide important new information to our understanding of factors contributing to the primary genetic form of TMAu, and identify functionally important residues of human FMO3. For the samples examined from the 33 year old woman, the novel mutation V187A in combination with the two common polymorphisms, E158K and E308G, were causative for decreased TMA metabolism and the resultant severe TMAu was confirmed by phenotyping studies.

Despite the subtlety of the V187A mutation, in combination with the E158K and E308G polymorphisms, we observed a major impact on FMO3 enzyme functional activity that led to an enzyme with significantly decreased activity. To confirm this, we expressed and purified not only the FMO3 V187A mutant enzyme, but also the double mutant, FMO3 V187A/E158K, and characterized the enzyme function of both enzymes compared with wild-type FMO3 using selective functional substrates. As shown in Figure 2, FMO3 enzyme activity observed from wild-type FMO3 confirmed that all substrates examined were efficiently oxygenated by human FMO3. The V187A mutation decreased the catalytic efficiency of the enzyme for 5-DPT and 8-DPT, and the double mutant V187A/E158K further decreased enzyme activity significantly for all substrates tested. Kinetic parameters for TMA N-oxygenation by FMO3 V187A and FMO3 V187A/E158K (Table 2) showed that although k_{cat}/K_m for FMO3 V187A did not differ from wild-type FMO3, the k_{cat}/K_m for FMO3 V187A/E158K was significantly decreased (65-fold). The kinetic parameters of MMI S-oxygenation by FMO3 (Table 3) showed

similar $k_{cat}/K_{m\ app}$ values for wild-type and FMO3 V187A whereas the $k_{cat}/K_{m\ app}$ for V187A/E158K for MMI *S*-oxygenation was about 4-fold lower than that of wild-type FMO3.

It has been reported previously that mutations that did not have a significant impact on specific activity of FMO3 by themselves decrease its oxygenation activity significantly in combination with other SNPs [6,13,14]. Similarly, the effect of the V187A mutation on FMO3 functional activity is not very distinct, but in combination with the common mutation E158K the enzymes oxygenation activity is drastically decreased for all substrates tested.

As shown in Figure 4A, position V187 is a highly conserved residue within the *FMO* gene family (i.e., *FMO1* to 5) and across species (i.e., chimpanzee, rhesus monkey, dog, cattle, rabbit, chicken, rat, and mouse). From the primary sequence, the mutation is immediately upstream of the essential FMO3 NADPH binding domain (GXGXXG) (Figure 4). According to the human FMO3 homology structure model we developed based on four related proteins [29], the residue V187 resides at the beginning part of a β -sheet leading to the NADPH binding domain. The residue is also conserved in FMO from *Schizosaccharomyces pombe* and *Methylophaga* sp. strain SK1, two FMO related enzymes with crystal structures recently solved [30, 31], and phenylacetone monooxygenase from *Thermobifida fusca*, the first Baeyer-Villiger monooxygenase crystallized [32] (Figure 4B). Based on the crystal structure of these related enzymes, we hypothesize that the Val resides in the second Rossmann fold involved in NADP⁺/NADPH binding. From the structure of *Methylophaga* FMO with NADP⁺ (2vqb), the Val side chain does not directly interact with NADP⁺. We hypothesize that the mutant amino acid of FMO3 V187A interferes with NADPH and NADP⁺ binding indirectly through affecting the Rossmann fold conformation. Alternatively, it is possible that the enzyme adopts large conformational changes during catalytic processes to accommodate substrate binding and hydroperoxyflavin formation [31]. However, no crystal structure is available yet to illustrate those conformations. During such putative conformational changes, the Val could directly interact with NADPH and/or NADP⁺ binding, and therefore the V187A can possibly have direct interference with NADPH and/or NADP⁺ binding as well. As expected, the data from heat treatment (Figure 3) showed that wild-type FMO3 is not stable under elevated temperature when incubated in the absence of NADPH. Interestingly, we observed a significantly higher activity after pre-incubation of the V187A mutant enzyme with NADPH at 40 °C. This also points to a decreased or slower interaction with the enzyme's cofactor. Based on the literature, the departure of NADP⁺ is proposed to be the rate-limiting step in the catalytic cycle of FMO enzymes [33-37]. It is possible that the V187A mutation interferes with NADPH binding and the enzyme binds the NADPH less efficiently. We attempted to verify the possibility that wild-type FMO3 and FMO3 V187A kinetics differ from each other by examining the effect of NADP⁺ on the selective functional activity of both enzymes. NADP⁺ has been reported in the literature to be a non-competitive inhibitor of FMO1 against the xenobiotic substrate and a competitive inhibitor of NADPH binding to FMO1 [34, 36]. We examined the effect of NADP⁺ on the selective functional activity of wild-type FMO3 and FMO3 V187A. For FMO3 V187A, increasing concentrations of NADP⁺ not only led to a decrease in k_{cat} , but also resulted in a significant decrease in $K_{m\ app}$ (data not shown), suggesting that this variant follows an uncompetitive model rather than a non-competitive model as proposed for pig-liver FMO1. Unfortunately, the photometric assay readouts in the presence of NADP⁺ reduced the range of assay detection limit and we cannot reliably conclude if the inhibition mechanism is significantly different for the V187A variant compared to wild-type FMO3. Alternative stopped-flow kinetic analysis will be necessary to clarify this issue. Thus, we could not confirm NADP⁺ as a non-competitive inhibitor of MMI *S*-oxygenation in the presence of wild-type FMO3. Characterization of the V187A mutant in this report may prompt additional studies to test the hypothesis of cofactor interaction once additional FMO3 structural information becomes available and stopped-flow kinetic studies are performed.

It is also notable that enzyme expression for the recombinant protein is significantly lower for the V187A containing variants, suggesting that protein folding may not be as efficient as the wild-type enzyme. Whether this is occurring in the *in vivo* situation and results in lower overall FMO3 protein concentration in the adult human liver of affected individuals remains to be determined.

The other *FMO3* mutation we reported herein is associated with severe TMAu and was found in genotyping analysis of a 17 year old female. This mutation caused a frame shift after K415 and resulted in a truncated protein variant with only 486 amino acid residues, of which the C-terminal 71 amino acids differed from the wild-type enzyme. Yamazaki *et al.* [38] reported an Arg500Stop mutation that lacked detectable functional activity when expressed as a recombinant protein. Five other recombinant FMO3 proteins truncated after different amino acids (i.e., F510X, S467X, L437X, E403X, D339X, and E305X) were analyzed and showed no (E305X - S467X) or severely decreased (F510X) functional enzyme activity with the probe substrate 5-DPT. Thus, a prematurely truncated enzyme such as the one described above with only 486 amino acids lacks functional activity and explains the TMAu observed in the young woman.

It is surprising that for the second individual, although only one allele is affected, the young woman suffers from TMAu. It has been reported previously that patients carrying heterozygotic FMO3 mutations show the TMAu phenotype, [14,19,39]. We cannot exclude the possibility that other mutations in intronic regions of the allele that encode for the functional enzyme can affect the enzyme expression level. However, we recently observed that purified recombinant human FMO3 forms stable and catalytically active oligomeric forms in the presence of different detergents (data not shown) and similar observations were reported earlier [40]. Whether FMO3 mutants can interfere with self oligomer-formation and/or hetero oligomer formation with wild-type enzymes and hence lead to lower overall enzyme activity is not known. The characterization of these effects in the *in vivo* setting that may affect oligomerization of FMO3 expressed from different alleles remains to be investigated.

In summary, we report two novel *FMO3* gene mutations associated with TMAu: one frame-shift truncation mutation that completely abrogates enzyme activity and one functional mutation that influences substrate oxygenation characteristics. The novel mutation of human *FMO3*, V187A, in combination with the common polymorphism E158K leads to an enzyme that has less than 3 % TMA *N*-oxygenating activity compared with wild-type FMO3. Generally, the common polymorphisms E158K and E308G alter FMO3 enzyme activity only slightly [41], but often, if the two polymorphisms occur together, they can lead to an FMO3 enzyme with decreased activity [6,14,24,41] and this has been reflected in *in vivo* functional activity leading to elevated unmetabolized TMA [14,42]. Similarly, the novel V187A mutation in conjunction with the common polymorphisms E158K and E308G significantly impairs FMO3 resulting in an enzyme with drastically decreased function that manifests itself in severe TMAu.

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Abbreviations

BSA	bovine serum albumin
DETAPAC	diethylenetriaminepentaacetic acid
5-DPT	10-[(N,N-dimethylaminopentyl)-2-(trifluoromethyl)]phenothiazine
8-DPT	10-[(N,N-dimethylaminooctyl)-2-(trifluoromethyl)]phenothiazine
DTNB	5,5'-dithiobis(2-nitrobenzoate)
FMO	flavin-containing monooxygenases
IPTG	isopropyl β -D-1-thiogalactopyranoside
MBP-FMO3	maltose binding protein-FMO3 fusion protein
MMI	mercaptoimidazole
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNPs	single nucleotide polymorphisms
TMA	trimethylamine
TMA N-oxide	trimethylamine N-oxide
TMAu	trimethylaminuria
TNB	nitro-5-thiobenzoate

S M E D M M N D I N E K M E K I A N G L 420aa
 TCTATGGAAG ACATGATGAA TGATATTAAT GAGAAAATGG AGAAAATCGC AAATGGTTTG
 (AG)
 A K A R P Y R Q I T L F I W M N S P P S 440aa
 GCAAAAGCGA GACCATACAG ACAGATTACA TTGTTTATAT GGATGAACTC TCCTCCTTCA
 L G Q S P T S H G C F S Q I P N W P W K 460aa
 TTGGGGCAAA GCCCAACATC CCATGGCTGT TTCTCACAGA TCCCAAATTG GCCATGGAAG
 F I L A L V V P T S L G W W A Q G S G Q 480aa
 TTTATTTTGG CCCTTGTA GT CCCTACCAGT TTAGGCTGGT GGGCCCAGGG CAGTGGCCAG
 E P E M P Y * 486aa
 GAGCCAGAAA TGCCATACTGA

Figure 1.

FMO3 sequence segment affected by the g.23612-23613AG>T mutation in exon 7. The mutation of *FMO3* causes a frame shift after K415 and continues for 71 mutant amino acid residues (underlined) and resulted in a protein variant with 486 amino acid residues.

Figure 2.

Specific activity for *N*-oxygenation of 5-DPT and 8-DPT and *S*-oxygenation of sulindac sulfide by wild-type FMO3 (white bars), FMO3 V187A (hatched bars), and FMO3 V187A/E158K (solid black bars) in nmol/min/mg of FMO3. Statistically significant differences between wild-type FMO3 and FMO3 V187A or wild-type FMO3 and FMO3 V187A/E158K are identified with * for $P<0.05$, ** for $P<0.01$, and *** for $P<0.001$.

Figure 3.

N-Oxygenation of 5-DPT by wild-type FMO3 (white bars) and FMO3 V187A (hatched bars) after incubation at 40 °C for 1 and 5 minutes with or without cofactor added expressed as % remaining activity. The 0 min time point is set at 100 % and equals 72 and 19 nmol/min/mg of FMO3 for wild-type FMO3 and FMO3 V187A, respectively. Statistically significant differences between wild-type FMO3 and FMO3 V187A are identified with * for $P<0.05$, ** for $P<0.01$, and *** for $P<0.001$.

A

			187	NADPH-binding ↓ motiv:GXGXXG
gi_6166183	FMO3_HUMAN	(179)	PGVFNGKRVLVVGLGNSGCDIA	
gi_57114053	FMO3_CHIMPANZEE	(179)	PGVFNGKRVLVVGLGNSGCDIA	
gi_74136341	FMO3_RHESUS MONKEY	(179)	PGVFKGKRVLVVGLGNSGCDIA	
gi_50978720	FMO3_DOG	(179)	PGIFKGKRVLVI GLGNSGCDIA	
gi_74355026	FMO3_CATTLE	(179)	PGIFKGKRVLVI GLGNSGCDIA	
gi_544325	FMO3_RABBIT	(179)	PGIFKGKRVLVI GLGNSGCDIA	
gi_45383027	FMO3_CHICKEN	(179)	PEKFRGKKVLVVGLGNSGCDIA	
gi_148539991	FMO3_RAT	(179)	PGTWKGKRVLVI GLGNSGCDIA	
gi_2494585	FMO3_MOUSE	(179)	PGIWKGKRVLVI GLGNSGCDIA	
gi_399505	FMO1_HUMAN	(179)	PDIFKDKRVLVI GMGNSGTDIA	
gi_6225373	FMO2_HUMAN	(179)	PDGFEGKRILVI GMGNSGSDIA	
gi_399506	FMO4_HUMAN	(179)	PEGFQGKRVLVI GLGNTGGDIA	
gi_1346021	FMO5_HUMAN	(180)	PEGFTGKRVI I I I GIGNSGGDLA	

B

			187	NADPH-binding ↓ motiv:GXGXXG
gi_6166183	HUMAN FMO3	(179)	PGVFNGKRVLVVGLGNSGCDIA	
gi_185177618	BACTERIAL FMO	(197)	ALEFKDKTVLLV GSSYSAEDIG	
gi_109158094	YEAST FMO	(207)	PELFVGESVLVVGGASSANDLV	
gi_123629491	PAMO	(181)	PVDFSGQRVGV I GTGSSGIQVS	

Bacterial FMO, *Methylophaga* sp.; yeast FMO, *Schizosaccharomyces pombe*; PAMO, Phenylacetone monooxygenase (Baeyer-Villiger monooxygenase)

Figure 4.

An alignment of amino acids 179 to 200 for various FMOs. V187 is highly conserved in FMO family members (FMO1, 3, 4, and 5) and across species lines. Amino acids adjacent to V187 including the nearby NADPH-binding domain (GxGxxG). Human FMO family members (FMO1-5) and FMO3 from different species including chimpanzee, rhesus monkey, dog, cattle, rabbit, chicken, rat, and mouse, are aligned based on homology. The GenBank accession number for each gene is indicated. The NADPH binding domain (GxGxxG) located downstream of V187 is marked. The V187 residue is conserved in all FMO genes listed except for human FMO2. **B** Alignment of FMO3 amino acids 179 to 200 with the bacterial FMO from *Methylophaga* sp., a yeast FMO from *Schizosaccharomyces pombe* as well as a Bayer Villiger monooxygenase, PAMO (Phenylacetone monooxygenase). V187 is highly conserved in all aligned flavoprotein monooxygenases. The GenBank accession number for each gene is indicated. The NADPH binding domain (GxGxxG) located downstream of V187 is marked.

Table 1

Polymerase chain reaction and sequencing primers for coding exons of FMO3

Exon 2	498 bp	Forward	tcaaaactcctgggctcaagt
		Reverse	tttccaacctgctcttgaca
Exon 3	581 bp	Forward	cagattcaaccaccattga
		Reverse	ttcttcagcattatgacaagagc
Exon 4/5	651 bp	Forward	atctgccaaaaccatttgct
		Reverse	acgagagtcacccgagtacc
Exon 6	408 bp	Forward	ggggtgctcaccagaatc
		Reverse	aaaagccagcaggcatatca
Exon 7	429 bp	Forward	tccaataattgtctctgtttcca
		Reverse	ttcatcttcgcaatccatga
Exon 8	473 bp	Forward	ggaaaattacaggctggctct
		Reverse	cattccaatgatgtcattcagg
Exon 9	475 bp	Forward	gcgagccattttctctgttc
		Reverse	cccctgtctgggtattgtca

Table 2*N*-Oxygenation of trimethylamine (TMA) by FMO3 V187A and FMO3 V187A/E158K.

	WT FMO3	FMO3 V187A	FMO3 V187A/E158K
$K_{m\ app}$ (μ M)	15.3 ± 5.1	17.5 ± 5.3	430 ± 150
k_{cat} ($\text{nmol min}^{-1} \text{mg}^{-1}$)	11.9 ± 1.3	19.3 ± 2.0	6.3 ± 1.1
$k_{cat}/K_{m\ app}$ ($10^{-3} \text{ min}^{-1} \text{mg}^{-1}$)	0.8 ± 0.2	1.1 ± 0.2	0.02 ± 0.01

Data are best fit values $\pm$ standard error. WT FMO3 data was calculated from one or two assays from two separate enzyme preparations. Data of FMO3 V187A was calculated from four assays from pooled enzyme of three enzyme preparations. FMO3 V187A/E158K data was calculated from two assays from pooled enzyme of four enzyme preparations

Table 3

S-Oxygenation of mercaptoimidazole (MMI) by FMO3 V187A and FMO3 V187A/E158K.

	WT FMO3	FMO3 V187A	FMO3 V187A/E158K
$K_{m\ app}$ (μM)	12.7 ± 2.0	9.7 ± 1.9	11.7 ± 1.7
k_{cat} ($\text{nmol min}^{-1} \text{mg}^{-1}$)	27.8 ± 0.9	30.3 ± 1.4	6.8 ± 0.2
$k_{cat}/K_{m\ app}$ ($10^{-3} \text{ min}^{-1} \text{mg}^{-1}$)	2.2 ± 0.3	3.1 ± 0.5	0.6 ± 0.1

Data are best fit values $\pm$ standard error. WT FMO3 data was calculated from two enzyme preparations. Data of FMO3 V187A was calculated from two assays from pooled enzyme of three enzyme preparations. FMO3 V187A/E158K data was calculated from pooled enzyme of four enzyme preparations.