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Methods

Trimethylaminuria (fish odor syndrome): Genotype characterization among Portuguese patients

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ABSTRACT

Trimethylaminuria (TMAu) or "fish odor syndrome" is a metabolic disorder characterized by the inability to convert malodorous dietarily-derived trimethylamine (TMA) to odorless TMA N-oxide by the flavin-containing monooxygenase 3 (FMO3). Affected individuals unable to complete this reaction exude a "fishy" body odor due to the secretion of TMA in their corporal fluids leading to a variety of psychosocial problems. Interindividual variability in the expression of *FMO3* gene may affect drug and foreign chemical metabolism in the liver and other tissues. Therefore, it is important to screen for common TMAu mutations but also extend the search to other genetic variants in order to correlate genotype and disease-associated phenotypes. In this study, 25 Portuguese patients with phenotype suggestive of TMAu were evaluated for molecular screening of the *FMO3* gene. Herein, we found 16 variants in the *FMO3* coding region, some of which had not been previously documented (Gly38Trp, Asp232Val, Thr307Pro, Ser310Leu). Whenever common variants (Glu158Lys, Glu308Gly) were considered in combination a distinct pattern between the control population and patients was observed, mainly in what concerns the presence of Lys158 and Gly308 in homozygous state. Further studies are necessary to clarify the pathogenicity of novel variants identified in this study, as well as the effect of the common single nucleotide polymorphisms, which may play an important role in disease presentation and/or protective mechanism to xenobiotics drugs or environment.

1. Introduction

Trimethylaminuria [TMAu; MIM 602079] (Cashman et al., 2001; Christodoulou, 2012; Mitchell and Smith, 2005; Motika et al., 2009; Treacy et al., 1998), also known as "fish odor syndrome", is an autosomal recessive metabolic disorder (Cashman and Zhang, 2002; Chalmers et al., 2006; Mackay et al., 2011) caused by a defect in the normal production of flavin containing monooxygenase 3 (FMO3; EC 1.14.13.8) enzyme. FMO3 is a NADPH-dependent enzyme that catalyzes the oxidation of a wide range of foreign chemicals including therapeutic drugs, dietary components and pesticides (reviewed in Cashman, 2002; Shimizu et al., 2007; Zhang et al., 1996). TMAu is characterized by the presence of abnormally high levels of trimethylamine (TMA) in urine, sweat, breath, and other body excretions with a powerful aroma of rotting fish due to defects in the regulation of the biotransformation pathway that converts the malodorous TMA into the non-odorous N-oxide (TMAO). TMAu is not associated with mortality or morbidity, but psychosocial consequences may be devastating (Christodoulou, 2012). Two major forms of TMAu have been described (Motika et al., 2007): a primary genetic form that causes decreased FMO3 function, and a secondary one that is due to TMA or to a TMA-precursor overload. The two forms can be detected in the same individual, as a slightly decreased enzyme activity (primary TMAu) that might not lead to TMAu symptoms until increased amounts of TMA occurs (as a result of diet, liver

Abbreviations: TMAu, Trimethylaminuria; TMA, Trimethylamine; FMO3, Flavin-containing monooxygenase 3 enzyme; FMO3 gene, flavin-containing monooxygenase 3 gene; TMAO, N-oxide Trimethylamine; PCR, Polymerase chain reaction; MIM, Mendelian Inheritance in Man; NMR, Nuclear Magnetic Resonance.

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disease, or bacterial overgrowth; secondary form). In addition, minor forms of TMAu including an acquired TMAu with no obvious *FMO3* background, a transient childhood form, and a transient form in women associated with menstruation have been described (Cashman and Zhang, 2002; Cashman et al., 2003; Mitchell and Smith, 2001; Shimizu et al., 2007; Zhang et al., 2003). The diagnosis of TMAu is based on clinical symptoms, biochemical assays of urine samples (free TMA plus the non-odorous metabolite TMAO) and by molecular screening of the *FMO3* gene (Mitchell and Smith, 2001). Early diagnosis is important so that appropriate dietary therapy may be introduced as soon as possible. Although initial indications of the disorder may be obtained by analysis of a urine sample, the approach is not always informative, especially when infants ingest a diet poor in trimethylamine precursors. In these cases, confirmation of the diagnosis is important to exclude transient secondary TMAu and other malodor syndromes.

Genetic variants associated with the *FMO3* gene range from those associated with the most severe symptoms to those associated with mild symptoms that are polymorphic at the population level (Cashman et al., 2003; Phillips and Shephard, 2008; Yeung et al., 2007). According to Cashman et al. (2001), the incidence of TMAu may range from 1% to 10%. More than 300 *FMO3* single nucleotide polymorphisms are documented (http://www.ncbi.nlm.nih.gov/projects/SNP) (Hernandez et al., 2003) and over 40 of these polymorphisms have been associated with the TMAu phenotype (Online database, http://human-fmo3.biochem.ucl.ac.uk/Human_FMO3) by disturbing TMA N-oxygenation (Motika et al., 2007) and contributing to inter-individual differences in the phenotypic spectrum of the disease (Cashman, 2002; Cashman and Zhang, 2002; Dolphin et al., 1997; Park et al., 2002; Shimizu et al., 2007). Synergistic epistatic interactions between common polymorphisms also contribute to the deleterious impact as in the case of Glu158Lys and Glu308Gly (Akerman et al., 1999; Cashman et al., 2003; Zschocke et al., 1999).

In this study we present the molecular data of the *FMO3* gene of 25 patients with clinical suspicions of TMAu from different regions of our country.

2. Material and methods

2.1. Patients and control subjects

We investigated 25 Portuguese patients (13 men and 12 women), with a phenotype suggestive of TMAu, referred to our center from several hospitals around the country. Individuals' ages range from the first year of life to up to 50 years. As a control population, we studied 100 healthy (200 alleles) unrelated individuals of Portuguese origin.

2.2. Molecular characterization

Genomic DNA was automatically extracted from whole blood using an automated method (EZ1 DNA Blood 350 μl, QIAGEN). The eight protein-coding exons and flanking intronic sequences of *FMO3* gene (NM_006894.5) were directly sequenced after PCR amplification. Primer sequences and detailed PCR conditions are provided in supplementary material. Sequencing reactions by the Sanger method were prepared using Big Dye Terminator sequencing kit following the manufacturer's protocol and reactions run on an ABI PRISM™ 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

The conservation degree of mutation-sites in vertebrates was assessed by the comparison of 17 FMO3 orthologous sequences obtained from the Ensembl database [Ensembl 67, http://www.ensembl.org/index.html]. Only complete sequences were considered. Protein sequences were aligned in Geneious v5.4 using the default options (Drummond et al., 2011). *In silico* predictions were made using Polyphen and Sift database [http://genetics.bwh.harvard.edu/pph2; http://sift.jcvi.org/www/SIFT_BLink_submit.html, respectively].

3. Results and discussion

3.1. Genetic characterization of the TMAu patients

The results of the molecular screening of the *FMO3* gene are presented in Table 1.

Among the 25 patients studied, a total of 16 variants were found in the *FMO3* coding region. With the exception of Ser147 and Asn285, all the remaining variants were non-synonymous. Of those, four (Gly38Trp, Asp232Val, Thr307Pro, Ser310Leu) were not reported before this study. None of these variants were detected in 200 alleles of the control population, which suggests a disease-associated effect. The conservation pattern of these variants in mammals is depicted in Fig. 1, reinforcing the putative deleterious effects of these replacements.

Table 1
Identification of *FMO3* gene variations in the 25 patients studied for TMAu.

Patient	Age (years)	Sex	Genotype (protein)	Reference
1	6	M	[a]	–
2	8	M	Ser147Ser	Chalmers et al. (2006))
			Glu158Lys	Akerman et al. (1999)
			Asn285Asn	rs909530
3	3	F	Glu158Lys/Glu158Lys	Akerman et al. (1999)
			Glu308Gly/Glu308Gly	
4	3	M	Glu158Lys	Akerman et al. (1999)
			Gln373GlnfsX11	This study
5	4	F	Glu180Val	Dolphin et al. (2000)
			Asn285Asn	rs909530
6	3	M	Glu158Lys	Akerman et al. (1999)
			Ser310Leu	This study
7	4	F	Glu158Lys	Akerman et al. (1999)
8	4	F	Ser147Ser/Ser147Ser	Chalmers et al. (2006)
			Glu180Val	Dolphin et al. (2000)
			Asn285Asn	rs909530
9	4	F	Glu158Lys/Glu158Lys	Akerman et al. (1999)
			Glu308Gly	Akerman et al. (1999)
			Arg417Leu	rs149551557
			Thr428Ser	rs147245760
10	3	M	Glu158Lys	Akerman et al. (1999)
			Val257Met	Furnes et al. (2003)
11	3	F	Glu158Lys/Glu158Lys	Akerman et al. (1999)
			Glu308Gly/Glu308Gly	
12	50	F	Pro153Leu	Dolphin et al. (1997)
			Glu158Lys	Akerman et al. (1999)
			Arg417Leu	rs149551557
			Thr428Ser	rs147245760
13	6	F	Glu158Lys	Akerman et al. (1999)
14	3	F	Val257Met	Furnes et al. (2003)
15	2	M	Glu158Lys	Akerman et al. (1999)
			Glu308Gly	
16	2	M	Gly38Trp	This study
			Glu158Lys/Glu158Lys	Akerman et al. (1999)
			Trp388Leu	rs199975586
17	1	F	Pro153Leu	Dolphin et al. (1997)
			Glu158Lys	Akerman et al. (1999)
18	6	F	Gly38Trp	This study
			Glu158Lys/Glu158Lys	Akerman et al. (1999)
19	2	M	Glu158Lys/Glu158Lys	Akerman et al. (1999)
			Glu308Gly/Glu308Gly	
20	2	M	Glu158Lys	Akerman et al. (1999)
			IVS5+10 C>G	Chalmers et al. (2006)
21	2	M	Glu158Lys	Akerman et al. (1999)
			IVS5+10 C>G	Chalmers et al. (2006)
22	1	F	Glu158Lys	Dolphin et al. (1997)
			Asp232Val	This study
23	2	M	Glu158Lys/Glu158Lys	Akerman et al. (1999)
24	1	M	Glu158Lys/Glu158Lys	Akerman et al. (1999)
			IVS5+10 C>G	Chalmers et al. (2006)
25	7	M	Thr307Pro	This study

[a] The disease was confirmed in urine by Nuclear Magnetic Resonance (NMR) but no mutation or polymorphisms were found in *FMO3* gene.

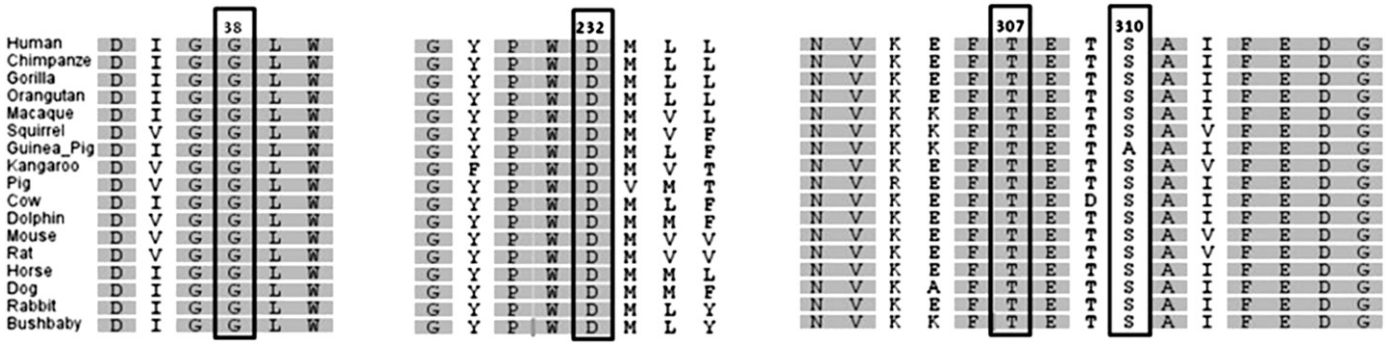

Fig. 1. Cross-species comparison of homologous positions and neighboring sequence to those novel mutations variants found in this work (G38, D232, T307, S310).

3.1.1. Novel variants

The novel mutation Gly38Trp (Table 1) is located in a highly conserved region of *FMO3* gene (Fig. 1) and the two *in silico* approaches (Polyphen and Sift) predict that glycine substitution for a tryptophan is a deleterious/damaging mutation and alter protein function (PolyPhen score: 1.0). In this study, in patient 16, Gly38Trp and Glu158Lys were segregated in the maternal chromosome and the father segregates the Trp388Leu (rs199975586) and Glu158Lys. The other novel mutation, Asp232Val, was found in heterozygosity along with Glu158Lys (patient 22). Asp232Val mutation is located in a highly conserved region of *FMO3* where the substitution of aspartic acid for valine might not be tolerated (PolyPhen score: 0.997). In this case, the mutation Asp232Val is linked to Glu158Lys, since the mother is homozygous for this polymorphism, and the father heterozygous. The other two mutations, Gln373GlnfsX11 and Ser310Leu (PolyPhen score: 0.976), found in two different patients (patient 4 and 6 respectively), also affect a conserved region of *FMO3*, indicating that these two novel mutations are probably pathogenic. It is interesting to observe that besides these mutations, these two patients also carry the Glu158Lys polymorphism in heterozygosity. This fact is in accord with previous studies that reported that patients carrying heterozygotic *FMO3* mutations exhibit the TMAu phenotype (Dolphin et al., 2000; Fujieda et al., 2003; Furnes et al., 2003). Moreover, some mutations can diminish FMO3 activity in combination with some single nucleotide polymorphisms. These polymorphisms have a minor effect on FMO3 activity, but in combination with other single nucleotide polymorphisms or mutations could markedly decrease its activity (synergistic effect) leading to the TMAu phenotype (Akerman et al., 1999; Cashman et al., 2003; Zschocke et al., 1999). Possibly, this is the case of those two patients carrying the Glu158Lys together with Gln373GlnfsX11 and Ser310Leu mutations reported here. In addition to these novel variants, one patient carried Arg417Leu (rs149551557) and Thr428Ser (rs147245760) polymorphisms in heterozygosity, along with Glu158Lys in homozygosity and Glu308Gly in heterozygosity (patient 9). Further studies will be necessary to evaluate the effect of these variants on the catalytic performance of FMO3 activity. In this study we also found in heterozygosity the Pro153Leu variant (patient 12), a previously reported pathogenic mutation (Dolphin et al., 1997). This variant in combination with Arg417Leu, Thr428Ser and the Glu158Lys, a common functional polymorphism, could be responsible for TMAu phenotype. In fact, the proline residue in codon 153 is normally conserved in all mammalian FMO isoforms (Treacy et al., 1998) and the Pro153Leu mutation has previously been shown to abolish FMO3 catalytic activity *in vitro* (Dolphin et al., 1997; Treacy et al., 1998). Therefore, these three single nucleotide polymorphisms (Arg417Leu, Thr428Ser and Glu158Lys) in combination with the Pro153Leu variant may be responsible for the TMAu phenotype observed in this patient. The presence of Glu158Lys polymorphism in homozigosity in combination with the intronic variation IVS5 + 10 C > G, in heterozygosity was detected in one patient (patient 24). The common polymorphism Glu158Lys in *cis*, associated with this intronic variation could lead to a discrete reduction in enzyme activity, which could be responsible for the malodorous phenotype of TMAu.

3.1.2. Association between TMAu and Glu158Lys and Glu308Gly polymorphisms

The common single nucleotide polymorphisms, Glu158Lys and Glu308Gly were found in three patients in homozygosity. According to Zschocke et al. (1999) in some carrier individuals these variants have decreased TMA N-oxygenation after oral TMA challenge when they are compound heterozygotic or homozygotic. Although these polymorphisms only have a slight effect on FMO3 enzyme activity (Cashman et al., 2000) in combination with *FMO3* gene mutations or in a *cis* form, they can lead to an FMO3 enzyme with decreased activity. These findings have already been demonstrated *in vivo* functional studies leading to increase of unmetabolized TMA (Cashman et al., 2000; Dolan et al., 2005; Fujieda et al., 2003; Lambert et al., 2001; Zschocke et al., 1999). Probably, in some cases for these individuals, the TMAu will be a transitory condition exacerbated under certain conditions (*i.e.* dietary conditions, elevated dietary precursors of TMA or elevated diets of *Brassica* vegetables, or even during menstruation (Fenwick et al., 1983) that inhibit human FMO3 activity (Cashman et al., 1999). In fact, sometimes young children or even adults have been reported to suffer from "transient" trimethylaminuria (Mayatepek and Kohlmu, 1998) that often is resolved over the years. These differences in *FMO3* variant alleles may explain some examples in the literature of adverse interaction with drugs and/or chemicals mediated by P450 cytochrome. Besides the scarce literature about the evidence of FMO3 in clear-cut adverse drug interaction, some examples have emerged. In a recent study (Hisamuddin et al., 2004), individuals with Glu158Lys and Glu308Gly seemed to have more protection to sulindac effects than those without these single nucleotide polymorphisms. Fig. 2

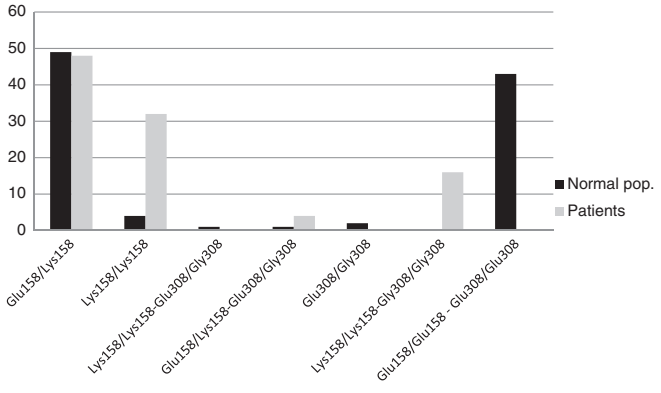

Fig. 2. Distribution of the allelic combinations for Glu158Lys and Glu308Gly in control population (N = 100 individuals) and in the TMAu studied patients (N = 25).

shows the haplotypic distribution of Glu158Lys and Glu308Gly in control and in the TMAu patients in this study. An important fraction of individuals in normal population are not carriers of either Glu158Lys or Glu308Gly whereas no patients showed the same pattern. Moreover, the frequency of individuals carrying the Glu158Lys replacement in heterozygosity is similar in both populations (48% in patients *versus* 49% in control population, Fig. 2), but homozygous individuals for Glu158Lys are more frequently found among patients (32% *versus* 4% in control population). Another major difference between both populations is the frequency of individuals carrying both variants in homozygosity. In this case, homozygosity for both alleles was not detected in the normal population. Such haplotypic distribution reflects the functional role of these two variants in TMAu condition. It is therefore expected that the severity by which FMO3 protein is affected varies according to the combination of linked variants. In this sense, the presence of these common variants *per se* or in combination with other, less frequent replacements would have a higher impact on enzyme activity.

Another polymorphism very common in the Caucasian population and also linked to Glu158Lys and Glu308Gly is Val257Met (Cashman and Zhang, 2002). Two of our patients present Val257 replacement (one of this also with Glu158Lys polymorphism in heterozigosity) and both show a mild phenotype in agreement with the results showed in Fig. 2. It seems that *FMO3* 257Met and 308Gly contribute to decreased N-oxygenation activity (Cashman et al., 2000), so it is possible that homozygous individuals possessing these alleles will detoxicate amines less efficiently and be at greater risk of exaggerated clinical response compared with individuals with wild type human *FMO3* (Cashman and Zhang, 2002).

According to Cashman and Zhang (2002), six *FMO3* variants (*i.e.*, Glu158Lys, Val257Met, Glu308Gly, Gly180Val, Ser147Ser and Phe239Phe) have been observed to be associated with normal or slightly reduced TMA N-oxygenation activity. Three of those (Glu158Lys, Val257Met and Glu308Gly) are common polymorphisms and the remaining (Gly180Val, Ser147Ser and Phe239Phe) are somewhat rarer and code for synonymous substitutions. In fact, in our study, one patient carries the Ser147Ser, Asn285Asn and Glu158Lys polymorphisms in heterozygosity (patient 2). The same is true for the heterozygous Gly180Val and Asn285Asn found in another patient (patient 5), but not for another patient with the Ser147Ser polymorphism in homozigosity and Gly180Val and Asn285Asn in heterozigosity (patient 8). Again, the described variants alone do not appear to have a significant effect on FMO3 activity, but when in combination with other polymorphisms, may have a more deleterious impact (Motika et al., 2009). For three patients, the presented polymorphisms were inconclusive and possibly the fish-smell would be a "transient form" of trimetylmaminuria. It is possible to observe the variability of these common polymorphisms in different populations (Cashman et al., 2000, 2001; Cashman and Zhang, 2002; Hao et al., 2007; Lattard et al., 2003; Park et al., 2002; Sachse et al., 1999) and they show significant heterogeneity in the relative frequencies of single- and multiple-site alleles, haplotypes, and genotypes (Allerston et al., 2007; Zhou and Shepard, 2006).

Unfortunately, for one patient (patient 1), the molecular study of *FMO3* gene was inconclusive despite the undoubtedly fish odor phenotype which was according to the biochemical findings of urine TMA/TMAO ratio. For those patients whose molecular characterization was not possible to achieve, we cannot exclude the possibility of a mutation in a region of the gene that we have not sequenced, for instance, in the intronic region, a gross gene deletion or a mutation in the 5′ or 3′ untranslated regions.

Apart from a single exception, all our patients are young children. Previous data demonstrate that FMO3 enzyme do not achieve full expression before age eleven (Koukouritaki et al., 2002), this fact can indeed explain the severity of TMAu phenotypes presented here. Also, it explains some transient forms of the condition that are observed in older individuals (Shimizu et al., 2007; Zhang et al., 1996).

4. Conclusions

Our findings indicate that FMO3 deficiency is not merely a rare recessive disorder, but rather a spectrum of phenotypes that causes the malodorous rotten fish smell, transient or mild forms of TMAu in which some factors such as diet or environmental exposures can play an important role as the triggering symptoms of this disease. The knowledge of these phenotype spectra is of great importance not only to clarify the role of FMO3 in the TMAu but also to investigate the participation of the human FMO3 in the oxygenation of a great variety of drugs, xenobiotics and endogenous materials. There are limited reports about allele genotype frequencies in some populations (*i.e.* in Africans) (Hao et al., 2007) but we now know that there is an inter-individual and interethnic variability in *FMO3* expression and enzyme activity (Cashman et al., 2001; Hao et al., 2007; Krueger and Williams, 2005; Lattard et al., 2003; Mackay et al., 2011) which may delineate correlations expression of wild type *FMO3* or particular *FMO3* allelic variants to disease susceptibility, drug-induced side effects or even drug response.

Until now, studies have been concentrated on those polymorphisms that are present in the protein-coding sequences of the gene and these variants do not encompass the full extent of *FMO3* variation. It is likely that more *FMO3* variants exist, and these will undoubtedly be population-specific (Cashman et al., 2001). However, nonexon genetic variability may also play a role in determining the functional activity of human *FMO3*. Mutations in promoter or intronic regions may alter the transcription of human *FMO3*, influence the functional activity and are likely to contribute to interindividual differences in *FMO3* expression (Koukouritaki and Hines, 2005; Koukouritaki et al., 2007). Both synonymous and nonsynonymous polymorphisms can also potentially affect mRNA splicing by altering the efficiency of exon splice enhancer motifs (Fairbrother et al., 2002) and hence affect the way in which a particular FMO3 substrate is cleared from the body. Further studies are required to identify the distribution of *FMO3* variation and to study the consequences of both rare and common substitutions of this gene (Zhou and Shepard, 2006). Moreover, larger sample analysis will be needed to understand the possible contribution of these variants to FMO3 pharmacogenetics. It is important to understand if *FMO3* variants are linked and how these variants are associated with specific ethnic groups, and population-specific metabolism. It is also important to establish fundamental information of *FMO3* polymorphisms present within a healthy population and then apply this information to achieve a relationship with the disease.

Defective trimethylamine N-oxygenation causes trimethylaminuria or "fish-like odor syndrome" and several molecular studies on human *FMO3* mutations and polymorphisms have provided knowledge that underlined the molecular mechanisms for TMAu. Upon this, the variability of the common polymorphism observed in human *FMO3* as a function of different populations may portend population differences in the susceptibility of humans to abnormal metabolism or adverse drug reactions to chemicals or drugs metabolized by human *FMO3* (Cashman and Zhang, 2002; Poetsch et al., 2010; Sachse et al., 1999). Knowledge on *FMO3* gene could open new research areas in the molecular role of these variants associated to drug metabolism. In fact, human F*MO3* could be an example of an environmental gene that participates in a protective mechanism to help humans ward off potentially toxic exposure of chemicals as was demonstrated by Hisamuddin et al. (2004).

Finally, the possibility of idiopathic reactions related to rare or private human *FMO3* gene mutations cannot be excluded and arises from the observation that symptoms including hypertension, adverse tyramine reactions, depression, and other central nervous system (CNS) effects are manifested in TMAu individuals (Hisamuddin et al., 2005; Hukkanen and Dempsey, 2005; Poetsch et al., 2010). On the other hand, some cases of TMAu may have no genetic component, having developed due to liver or kidney

disease (Chen and Aiello, 1993; Mitchell and Smith, 2001; Mitchell et al., 1999).

The present study contributes to the enhancement of the genetic variant database of the *FMO3* gene and, consequently, to a more accurate understanding of possible correlations between a genotype and disease-susceptibility phenotypes. Further studies are necessary to clarify the pathogenicity of novel variants identified in this study, as well as the effect of the associated single nucleotide polymorphisms, which may play an important role in the expression of the disease and or protective mechanism to xenobiotics drugs or environment.

Conflict of interest

The authors do not have any conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2013.05.025.

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