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Methods

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

Filipa Ferreira [a,1], Sofia Esteves [b,1], Lígia S. Almeida [b], Ana Gaspar [c], Cláudia Dias da Costa [c], Patrícia Janeiro [c], Anabela Bandeira [d], Esmeralda Martins [d], Elisa Leão Teles [e], Paula Garcia [f], Luísa Azevedo [g], Laura Vilarinho [a,b,*]

[a] Newborn Screening, Metabolic and Genetics Unit, Department of Human Genetics, National Institute of Health Dr Ricardo Jorge, 4000-055 Porto, Portugal
[b] Research and Development Unit, Department of Human Genetics, National Institute of Health Dr Ricardo Jorge, 4000-055 Porto, Portugal
[c] Metabolic Diseases Unit, Pediatric Department, CHLN EPE, Santa Maria Teaching Hospital, 1649-035 Lisbon, Portugal
[d] Metabolic Diseases Unit, Pediatric Department, Santo António Hospital — CHP, 4099-001 Porto, Portugal
[e] Metabolic Diseases Unit, Pediatric Department, São João Hospital — HSJ, 4200-319 Porto, Portugal
[f] Inherited Metabolic Diseases Outpatient Clinic of Pediatric Hospital, Hospital and University Center of Coimbra, 3000-602 Coimbra, Portugal
[g] IPATIMUP — Institute of Molecular Pathology and Immunology of the University of Porto, 4200-465 Porto, Portugal

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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.

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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.

* Corresponding author at: Newborn Screening, Metabolic and Genetics Unit, Department of Human Genetics, National Institute of Health Dr Ricardo Jorge, Rua Alexandre Herculano, No. 321, 4000-055, Portugal. Tel.: +351 223 401 171.
E-mail address: laura.vilarinho@insa.min-saude.pt (L. Vilarinho).

[1] These authors contributed equally to the study.

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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.

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