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Association between Cytochrome P450 2D6 genetic polymorphisms and tramadol metabolism in Egyptian tramadol-intoxicated subjects

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ABSTRACT

There is substantial evidence for a causal relationship between genetic variability of the cytochrome P450 2D6 (CYP2D6) gene and changes in the pharmacokinetics of drugs. Polymorphic CYP2D6 activity has been shown to be a determinant of the pharmacokinetics and pharmacodynamics of tramadol via hepatic phase I O-demethylation of (+)-tramadol to (+)-O-desmethyltramadol. Several studies have demonstrated the impact of CYP2D6 polymorphism on the pharmacokinetics of tramadol. Hence, the aim of this study was to determine if the well documented pharmacokinetics of tramadol regarding CYP2D6 could be verified in a group of Egyptian abusers. The genotype-phenotype relationships were also assessed. A total of 83 tramadol intoxicated subjects who were referred to the Poison Control Center (PCC), Ain Shams University Hospitals, were enrolled in the present study. Urinary concentrations of tramadol (TMD), and its metabolites, O-desmethyltramadol M1 (ODT) and N-desmethyltramadol M2 (NDT) were determined using the Trace GC-TSQ mass spectrometer. CYP2D6 genotyping was performed using PGX-CYP2D6 Strip Assay. Through the use of CYP2D6 phenotyping, 10 patients (12.1 %) were classified as CYP2D6 poor metabolizers (PMs), and 73 (87.9 %) were genotyped as CYP2D6 extensive metabolizers (EMs), including 57 (68.7 %) homozygous EMs and 16 (19.2 %) heterozygous EMs. Median TMD level didn't differ significantly between PMs and EMs (p = 0.356). Median M1 level was significantly higher in EMs than that in PMs (p = 0.001), while median M2 level didn't differ significantly between PMs and EMs (p = 0.597). There were statistically significant differences in TMD/M1, TMD/M2 and M1/M2 ratios between PMs and EMs (p = 0.001). M1/M2, with an area under the ROC curve of 0.976, performed better than TMD/M1 (AUC = 0.724) and TDM/M2 (AUC = 0.656), in differentiating between EMs and PMs. The impact of the CYP2D6 polymorphism on the pharmacokinetics of tramadol was clearly demonstrated in a group of tramadol-intoxicated Egyptian subjects.

Introduction

Tramadol (2-[(dimethylamino) methyl]-1- (3-methoxyphenyl)-cyclohexanol) is a synthetic opioid analgesic of the amino cyclohexanol type. Tramadol is a centrally acting analgesic drug with a dual mechanism of action that includes low agonistic effects for the μ-opioid receptor that are exclusively mediated by its M1 metabolite (O-desmethyltramadol) because it has a higher affinity for opioid receptors than the parent drug, as well as inhibition of monoamine (serotonin, norepinephrine) reuptake that is preferentially mediated by tramadol itself [1].

The superfamily of cytochrome P450 (CYP) enzymes is the most important metabolic system in Phase I [2]. Human CYP forms are divided into families and subfamilies on the basis of similarities in amino acid sequence. Families CYP1, CYP2, and CYP3 participate extensively in drug metabolism, with three of the major isozymes (CYP2C9, CYP2C19, and CYP2D6) being

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polymorphic to a clinically significant degree [3]. The human CYP2D locus consists of the three highly homologous genes, CYP2D8P, CYP2D7, and CYP2D6. In humans, the 4.2-kb region containing the CYP2D6 gene resides on the long arm of chromosome 22 (22q13.1). CYP2D6 has been estimated to participate in the metabolism of more than 70 common drugs and 20-25 % of all drugs in clinical use. The major reaction types catalyzed by CYP2D6 appear to be ring oxidation and O-demethylation [4].

According to current knowledge, CYP2D6 is the most polymorphic CYP gene, with more than 80 allelic variants have been identified. Polymorphisms within CYP2D6 have been associated with altered enzyme activity. Individuals are classified with regard to metabolic activity of the CYP2D6 enzyme into four phenotypes: 1) Extensive metabolizers (EMs; those who have normal enzyme activity) are defined as individuals carrying two functional alleles; 2) Intermediate metabolizers (IMs; those who have reduced enzyme activity) are defined as individuals carrying two decreased-activity (*9, *10, *17, *29, *36, *41) alleles or carrying one active (*1, *2, *33, *35) and one inactive (*3–*8, *11–*16, *19–*21, *38, *40, *42) allele, or carrying one decreased-activity (*9, *10, *17, *29, *36, *41) allele and one inactive (*3–*8, *11–*16, *19–*21, *38, *40, *42) allele; 3) Poor metabolizers (PMs; those who lack a functional enzyme) are defined as individuals carrying two inactive (*3–*8, *11–*16, *19–*21, *38, *40, *42) alleles; 4) Ultra-rapid metabolizers (UMs; those who have increased enzyme activity) are defined as individuals carrying three or more CYP2D6 functional alleles (gene duplication or multi-duplication) in the absence of inactive (*3–*8, *11–*16, *19–*21, *38, *40, *42) or decreased-activity (*9, *10, *17, *29, *36, *41) alleles [5].

Tramadol is rapidly and extensively metabolized phase I in the liver by two principal pathways: O-demethylation to O-desmethyltramadol (M1) by CYP2D6 and N-demethylation to N-desmethyltramadol (M2) by CYP2B6 and CYP3A4. CYP2D6 is the major isoform responsible for tramadol's oxidation; nonetheless CYP2B6 and CYP3A4 are minor contributors [6].The N-demethylation product N-desmethyltramadol (M2) is further N-demethylated to N, N-didesmethyltramadol (M3) by CYP3A4 and O-demethylated to O, N-didesmethyltramadol (M5) by CYP2D6, possibly followed by formation of O, N, N-didesmethyltramadol (M4) from M3 via CYP2D6 as well as from M5 *via* CYP3A4. In the phase II, the O-demethylated metabolites are excreted in urine by glucuronic acid and sulfate conjugation [7].

UMs and PMs are those most at risk for pain treatment failure or dose-dependent drug toxicity, respectively. PMs may exhibit, through the accumulation of a parent drug or its metabolites, toxic side effects or a lack of response during drug treatment. By contrast, UMs whose metabolism is substantially accelerated require higher than usual doses to achieve therapeutic parent drug levels in the blood [8]. In addition to inter-individual variation, the CYP genes show inter-ethnic variation. Since the discovery of the CYP2D6 genetic polymorphism, numerous studies have investigated the Pharmacogenetics effects in various ethnic groups [9].

There is substantial evidence for a causal relationship between genetic variability of the CYP2D6 gene and changes in the pharmacokinetics of tramadol. Therefore, knowledge of single-nucleotide polymorphisms (SNPs) prior to drug administration is highly desired for assisting in the development of individualized pharmacotherapy [10].

The aim of this study is investigating the association between cytochrome P450 2D6 (CYP2D6) genetic polymorphisms and the metabolism of tramadol (tramadol to tramadol metabolites ratios) in Egyptian abusers in order to assess the genotype-phenotype relationships.

Subject and Methods

Patients

A total of 83 tramadol intoxicated subjects who were referred to the Poison Control Center (PCCC, Ain Shams University Hospitals) were enrolled in the present study. An informed consent was taken from every subject before inclusion in the study. Data were collected from all patients including demographic data (age and sex). In addition, clinical examination was done systematically and included vital data (respiratory rate, heart rate, blood pressure, body temperature and any systemic abnormalities). Then, 3 ml of heparinized arterial blood were collected from each patient for measuring arterial blood gases, and 10 ml venous blood were collected from each patient for routine investigations analysis. Complete blood picture was performed using an automated cell counter. Random blood glucose as well as serum levels of aspartate transaminase (AST), alanine transaminase (ALT), creatine phosphokinase (CPK), urea, creatinine, Na+ and K+, were determined using the cobas c 311 analyzer (Roche diagnostics, Basel, Switzerland). The level of consciousness was assessed by the Reed's classification and Glasgow Coma Scale (GCS). Reed's classification has been used for assessment of the level of consciousness of patients by their response to painful stimulus and reflexes and is graded into 5 degrees; 0, I, II, III and IV. APACHE II (Acute Physiology and Chronic Health Evaluation II) is a severity-of-disease classification system with a final score of 0 to 71, with higher scores corresponding to more severe disease and a higher risk of death. It is determined within 24 hours of admission to an intensive care unit (ICU). Urine samples were collected for urine toxicological screening of (benzodiazepine, cannabis, barbiturate, opiates, cocaine, and amphetamine).

Determination of tramadol and its metabolites O-desmethyltramadol and nortramadol (N-desmethyltramadol)

Urinary concentrations of tramadol (TMD), and its major metabolites, O-desmethyltramadol M1 (ODT)

and N-desmethyltramadol M2 (NDT) were determined using the Trace GC-TSQ mass spectrometer (Thermo Scientific, Austin, TX, USA).

CYP2D6 genotyping

Blood was collected into EDTA-containing vacutainers (Becton-Dickinson, Franklin Lakes, NJ, USA) and genomic DNA was isolated using the Gentra Puregene Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. CYP2D6 genotyping was performed using PGX-CYP2D6 Strip Assay (Vienna Lab Diagnostics GmbH, Vienna, Austria) as per the manufacturer's instructions. The procedure includes 2 steps: (1) PCR amplification of DNA using biotinylated primers, (2) hybridization of amplification products to a test strip containing allele-specific oligonucleotide probes immobilized as an array of parallel lines. Bound biotinylated sequences were detected using streptavidin-alkaline phosphatase and color substrates. The assay covers 3 CYP2D6 polymorphic loci, detecting the allelic variants CYP2D6*3 (2637delA), CYP2D6*4 (1934 G > A) and CYP2D6*6 (1795delT).

Statistical analysis

IBM SPSS statistics (V. 25.0, IBM Corp., USA, 2017-2018) was used for data analysis. Date were expressed as median and percentiles for quantitative non-parametric measures in addition to both number and percentage for categorized data. The following tests were done: Comparison between two independents for non-parametric data using Wilcoxon Rank Sum test. Comparison between more than 2 patients for non-parametric data using Kruskall Wallis test. Chi-square test to study the association between each 2 variables or comparison between 2 independents as regards the categorized data. Diagnostic validity test It includes the diagnostic sensitivity; it is the percentage of diseased cases truly diagnosed (TP) among total diseased cases (TP+FN). The diagnostic specificity; it is the percentage of non-diseased truly excluded by the test (TN) among total non-diseased cases (TN+FP). The predictive value for a +ve test: It is the percentage of cases truly diagnosed among total positive cases. The predictive value for a -ve test; it is the percentage of cases truly negative among total negative cases. The efficacy or the diagnostic accuracy of the test; it is the percentage of cases truly diseased plus truly non-diseased among total cases. Variables required for calculating the APACHE II score were collected for each patient and entered into a computer program designed to provide an estimate.

Results

Social and Demographic data

Social and Demographic data show that 67 male (80.7 %) and 16 females (19.3 %) have a median age of 30 (21.75 – 35) years old. Regarding route and mode of poisoning, most of cases were intoxicated by oral route (n = 82, 98.8 %) and intravenous (IV) (n = 1, 1.2 %). Tramadol overdose in addicts represented 11 cases (13.3 %), attempted suicides were evident in 70 cases (84.3 %), while accidental ingestions were observed in 1 case (1.2 %) and iatrogenic cause in one more case (1.2 %) **Table 1.**

Table 1: Social and Demographic data

Studied Parameters		Groups				
		Total-EMs (n = 73)	Homo-EMs (n = 57)	Hetero-EMs (n = 16)	PMs (n=10)	Total (n=83)
Sex	**Female**	15 (20.5 %)	12 (21.1 %)	3.0 (18.8 %)	1.0 (10.0 %)	16 (19.3 %)
	Male	58 (79.5 %)	45 (78.9 %)	13 (81.3 %)	9.0 (90.0 %)	67 (80.7 %)
Route of administration	**IV**	1.0 (1.4 %)	1.0 (1.8 %)	0.0 (0.0 %)	0.0 (0.0 %)	1 (1.2 %)
	Oral	72 (98.6 %)	56 (98.2 %)	16 (100.0 %)	10 (100.0 %)	82 (98.8 %)
Mode of poisoning	**Accidental**	1.0 (1.4 %)	1.0 (1.8 %)	0.0 (0.0 %)	0.0 (0.0 %)	1 (1.2 %)
	Overdose	7.0 (12.3 %)	7.0 (12.3 %)	2.0 (12.5 %)	2.0 (20.0 %)	11 (13.3 %)
	Suicidal	62 (84.9 %)	48 (84.2 %)	14 (87.5 %)	8 (80.0 %)	70 (84.3 %)
	Therapeutic error	1.0 (0.4 %)	1.0 (1.8 %)	0.0 (0.0 %)	0.0 (0.0 %)	1 (1.2 %)
Age (years)		30.00 (22.25-35.00)	30.00 (23.00-34.50)	31.00 (21.25-35.75)	28.00 (18.50-42.00)	
Duration of hospitalization (days)		2.00 (1.00-2.00)	2.00 (1.00-3.00)	2.00 (2.00-3.50)	2.00 (1.00-3.00)	
Delay time (hrs.)		5.5 (4.75-8.0)	5.0 (4.0-7)	5.5 (4.0-6.75)	7.0 (4.0-7.0)	

(Total-EMs): Total extensive metabolizers. (Homo-EMs): Homozygous extensive metabolizers (Hetero-EMs): Heterozygous extensive metabolizers. (PMs): Poor metabolizers, Qualitative variables were expressed as count and Percentages (%). Quantitative data are represented as median (interquartile range, IQR: 25th quartile to 75th quartile* indicates a statistically significant difference. Statistical significance at p value < 0.05.

CYP2D6 genotypic distribution

As shown in **Table 2**, subjects who did not possess one of the common inactivating alleles were considered to be homozygous extensive metabolizers (homo-EMs, n = 57, 68.7 %, CYP2D6 *1/*1 genotype). Carriers of one variant allele were considered to be heterozygous extensive metabolizers (hetero-EMs, n = 16, 19.2 %, CYP2D6 *1/*4 genotype). Carriers of two variant alleles were classified as poor metabolizers (PMs, n = 10, 12.1 % CYP2D6 *4/*4 genotype).

Urinary concentration of tramadol (TMD) and its major metabolites, O-desmethyl-tramadol M1 (ODT) and N-desmethyl-tramadol M2 (NDT)

The urinary concentration of the parental form of tramadol and its metabolites O-desmethyl-tramadol M1 (ODT) and N-desmethyl-tramadol M2 (NDT) were determined. The tramadol/O-desmethyl-tramadol concentration ratio was also calculated. When the number of functional alleles increased, the median of TMD/M1 decreased. Moreover, the median TDM/M1 of PMs was significantly increased from that of homozygous extensive metabolizers EMs and heterozygous extensive metabolizers EMs ($P < 0.001$). To further characterize the effect of CYP2D6 polymorphism on tramadol metabolism, we analyzed the urinary concentration of nortramadol and calculated the TMD/M2 concentration ratio. The median TMD/M2 correlated with the number of functional alleles, inversely. The median TMD/M2 in PMs was significantly decrease from the median in homo extensive metabolizers EMs and heterozygous extensive metabolizers EMs ($p < 0.001$). The median M1/M2 of PMs was significantly decrease from that of Total extensive metabolizers EMs and PMS ($p < 0.001$) **Table 3.**

Table 2: CYP2D6 genotypic distribution

CYP2D6 genotyping	Phenotype		No	Prevalence (%)
***1/*1**	(homo-Ems)	Homozygous extensive metabolizers	57	68.70%
***1/*4**	(hetero-Ems)	Heterozygous extensive metabolizers	16	19.20%
***4/*4**	(PMs)	Poor metabolizers	10	12.10%
***3**			0	0%
***6**			0	0%

*1/*1 genotype: Carriers who did not possess one of the common inactivating alleles.
*1/*4 genotype: Carriers of one variant allele.
*4/*4 genotype: Carriers of two variant alleles.

Table 3: Urinary concentration of tramadol (TMD) and its major metabolites, O-desmethyl-tramadol M1 (ODT) and N-desmethyl-tramadol M2 (NDT)

Studied Parameters	Groups				*P* value
	Total EMs (n = 73)	Homo-EMs (n = 57)	Hetero-EMs (n = 16)	PMs (n = 10)	
TMD ng/ml	430 (220-985)	420 (190-980)	600 (282.5-1082.5)	380 (215-960)	0.356 [a] 0.182 [b] 0.840 [c]
M1 ng/ml	170 (90-95)	160 (75-365)	300 (140-480)	50 (17.5-77.5)	0.001* [a] 0.122 [b] 0.001* [c]
M2 ng/ml	70 (40-150)	60 (30-135)	120 (45-355)	165 (115-217.5)	0.597 [a] 0.044* [b] 0.014* [c]
TDM/M1	2.33 (1.64-2.85)	2.3 (1.60-2.85)	2.55 (1.71-2.88)	6.55 (3.75-16.87)	0.001* [a] 0.23 [b] 0.001* [c]
TDM/M2	6.16 (0.62-7.14)	6.33 (3.62-7.29)	5.95 (2.27-6.91)	2.0 (1.31-3.0)	0.001* [a] 0.001* [b] 0.001* [c]
M1/M2	2.33 (2.0-3.33)	2.3 (1.83-3.42)	2.36 (2.23-3.0)	0.33 (0.14-0.43)	0.001* [a] 0.001* [b] 0.001* [c]

EMs, extensive metabolizers; Homo-EMs, homozygous extensive metabolizers; Hetero- EMs, heterozygous extensive metabolizers; PMs, poor metabolizers. Quantitative data are represented as median (interquartile range, IQR: 25th quartile to 75th quartile* indicates a statistically significant difference. Statistical significance at p value < 0.05. [a] Difference in distribution between Total EMs and PMs, [b] difference in distribution between Homo-EMs and PMs, [c] difference in distribution between Hetero-EMs and PMs.

Vital sign

As regards clinical manifestation, the majority of cases had unstable vital signs on admission. The median systolic blood pressure (SBP) and Diastolic blood pressure (DBP) were significantly increased in Homo-Ems, hetero-Ems compared to PMs. There is significance decreased in respiratory rate in Total-Ems compare to PMs. There was non-significance difference between groups in Temperature and pulse **Table 4**.

General manifestations

As regards the skin examination, cyanosis was seen in patients of group Homo-Ems and Hetero-EMs (n = 17, 23.3 %), (n = 14, 24.6 %) respectively while it was not observed in any patient of group PMs. sweating was found in patients of Homo-EMs (5.3 %), Hetero-EMs (6.3 %) and PMs (10 %) **Table 5**.

Central nervous system manifestations

Table 6 shows that the (n = 10, 12 %) of studied cases showed no abnormal neurological manifestations while seizures were observed in group Homo-EMs (n = 14, 24.6 %), in group Hetero-EMs (n = 1.0; 6.3 %), and in group PMs (n = 3.0; 30 %). Agitation was noticed only in patients of group Homo-EMs (n = 5.0, 8.8 %).

Most of the cases with disturbed conscious levels were found the total percent of cases presented with coma grade I was (n = 13, 15.7 %), coma grade II (n = 17, 20.4 %), coma grade III (n = 16, 19.3 %), and coma grade IV (n = 12, 14.5 %) with The median Glasgow coma score (GSC) was between 5.5 and14.5 in group Total-EMs while it was between 3.00 and 15 in group PMs. Regarding the pupil size, Constricted pupil was observed in patients of all groups, while Dilated pupil was observed in patients of group Homo-EMs (7 %) and in group Hetero-EMs (18.8 %).

Gastrointestinal manifestations

Concerning gastrointestinal manifestations, vomiting, nausea and diarrhea was noticed only in patients of group Total -EMs while Hematemesis was observed in patients of group PMs **Table 7**.

Respiratory manifestations

Respiratory system revealed that respiratory distress is observed in Homo-Ems (7.0 %) and in hetero-Ems (25 %), while pulmonary edema was noticed only in patients of group Homo-Ems and PMs. Apnea and Coarse crepitation were observed in patients of all groups **Table 8**.

Table 4: Vital sign

Studied Parameters		Groups			
		Total EMs (n = 73)	**Homo-EMs (n = 57)**	**Hetero-EMs (n = 16)**	**PMs (n = 10)**
Respiratory rate (16-24 breaths/min)		8.00*[a] (4.00-14)	9.00*[b] (4.5-15)	7.00*[c] (4.00-13)	14.00 (7.00-17)
Blood pressure	**Systolic (SBP) (120 mmHg)**	115.00*[a] (110-120)	115.00*[b] (110-120)	120.00 *[c] (110-125)	90.00 (80-120)
	Diastolic (DBP) (80 mmHg)	70.00*[a] (70-80)	70.00*[b] (70-80)	70.00*[c] (70-80)	60.00 (47.5-72.5)
Temperature (°C)		37.00 (37-37)	37.00 (37-37)	37.00 (37-37)	37.00 (37-37.2)
Pulse (60-100 beats/min)		80.00 (81.5- 88.25)	79.00 (70-89.25)	80.50 (73.5-86.75)	70.00 (68-112.5)

EMs, extensive metabolizers; Homo-EMs, homozygous extensive metabolizers; Hetero- EMs, heterozygous extensive metabolizers; PMs, poor metabolizers. Quantitative data are represented as median (interquartile range, IQR: 25th quartile to 75th quartile* indicates a statistically significant difference. Statistical significance at p value < 0.05 [a] Difference in distribution between Total EMs and PMs, [b] difference in distribution between Homo-EMs and PMs,[c] difference in distribution between Hetero-EMs and PMs.

Table 5: General manifestations

Studied Parameter		Groups			
		Total EMs (n = 73)	**Homo-EMs (n = 57)**	**Hetero-EMs (n = 16)**	**PMs (n = 10)**
Dermal	**Normal**	51 (69.9 %)	39 (68.4 %)	12 (75.0 %)	9.0 (90.0 %)
	Cyanosis	17 (23.3 %)	14 (24.6 %)	3.0 (18.8 %)	0.0 (0.0 %)
	Sweating	4.0 (5.5 %)	3.0 (5.3 %)	1.0 (6.3 %)	1.0 (10.0 %)

(Total-EMs): Total extensive metabolizers. (Homo-EMs): Homozygous extensive metabolizers (Hetero-EMs): Heterozygous extensive metabolizers. (PMs): Poor metabolizers, Qualitative variables were expressed as count and Percentages (%).

Table 6: Central nervous system manifestations

Characteristics		Groups			
		Total EMs (n = 73)	Homo-EMs (n = 57)	Hetero-EMs (n = 16)	PMs (n = 10)
Neurological Manifestations	Normal	8.0 (11.0 %)	6.0 (10.5 %)	2.0 (12.5 %)	2.0 (20.0 %)
	Seizures	15 (20.5 %)	14 (24.6 %)	1.0 (6.3 %)	3.0 (30.0 %)
	Agitation	5.0 (6.8 %)	5.0 (8.8 %)	0.0 (0.0 %)	0.0 (0.0 %)
	Coma I	13 (17.8 %)	11 (19.3 %)	2.0 (12.5 %)	0.0 (0.0 %)
	Coma II	15 (20.5 %)	10 (17.5 %)	5.0 (31.3 %)	2.0 (20.0 %)
	Coma III	15 (20.5 %)	11 (9.3 %)	4.0 (25.0 %)	1.0 (10.0 %)
	Coma IV	8.0 (11.0 %)	6.0 (10.5 %)	2.0 (12.5 %)	4.0 (40.0 %)
	Constricted pupil	28 (38.4 %)	19 (33.3 %)	9.0 (56.3 %)	5.0 (50.0 %)
	Dilated pupil	7.0 (9.6 %)	4.0 (7.0 %)	3.0 (18.8 %)	0.0 (0.0 %)
	Disequilibrium	5.0 (6.8 %)	5.0 (8.8 %)	0.0 (0.0 %)	0.0 (0.0 %)
	GCS	10 (5.5 – 14.5)	11 (6 – 15)	8.5 (4.25 – 12.75)	7.5 (3 – 15)

(Total-EMs): Total extensive metabolizers. (Homo-EMs): Homozygous extensive metabolizers (Hetero-EMs): Heterozygous extensive metabolizers. (PMs): Poor metabolizers, Qualitative variables were expressed as count and Percentages (%). Quantitative data are represented as median (interquartile range, IQR: 25th quartile to 75th quartile* indicates a statistically significant difference. Statistical significance at p value < 0.05.

Table 7: Gastrointestinal manifestations

Studied Parameters		Groups			
		Total EMs (n = 73)	Homo-EM b (n = 57)	Hetero-EMs (n = 16)	PMs (n = 10)
Gastrointestinal manifestations	Normal	64 (87.7 %)	52 (91.2 %)	12 (75.0 %)	9 (90.0 %)
	Vomiting	8 (11.0 %)	4 (7.0 %)	4 (25.0 %)	0 (0.0 %)
	Diarrhea	1 (1.4 %)	1 (1.8 %)	0 (0.0 %)	0 (0.0 %)
	Hematemesis	0 (0.0 %)	0 (0.0 %)	0 (0.0 %)	1 (10.0 %)
	Nausea	8 (11.0 %)	4 (7.0 %)	4 25.0 %)	0 (0.0 %)

(Total-EMs): Total extensive metabolizers. (Homo-EMs): Homozygous extensive metabolizers (Hetero-EMs): Heterozygous extensive metabolizers. (PMs): Poor metabolizers, Qualitative variables were expressed as count and Percentages (%).

Table 8: Respiratory manifestations

Studied Parameters		Groups			
		Total EMs (n = 73)	Homo-EMs (n = 57)	Hetero-EMs (n = 16)	PMs (n = 10)
Respiratory manifestations	Normal	53 (72.6 %)	44 (77.2 %)	9.0 (56.3 %)	6.0 (60.0 %)
	Pulmonary Edema	1.0 (1.4 %)	1.0 (1.8 %)	0.0 (0.0 %)	1.0 (10.0 %)
	Respiratory distress	8.0 (11.0 %)	4.0 (7.0 %)	4.0 (25.0 %)	0 (0.0 %)
	Apnea	7.0 (9.6 %)	6.0 (10.5 %)	1.0 (6.3 %)	1.0 (10.0 %)
	Coarse crepitation	5.0 (6.8 %)	3.0 (5.3 %)	2.0 (12.5 %)	3.0 (30.0 %)

(Total-EMs): Total extensive metabolizers. (Homo-EMs): Homozygous extensive metabolizers (Hetero-EMs): Heterozygous extensive metabolizers. (PMs): Poor metabolizers, Qualitative variables were expressed as count and Percentages (%).

Arterial Blood Gas analysis among the studied Cases

Table 9 shows that there was a difference in percentage between the normal arterial blood gas analysis (n = 16, 19.3 %) and the abnormal findings (80.7 %) among studied cases. Respiratory acidosis represented the majority of the abnormal findings, in Total-EMs (n = 42, 57.5 %) and in PMs (n = 4.0, 40 %) then metabolic acidosis (n = 15, 20.5 %), (n = 3.0, 30.0 %) in both groups respectively. Respiratory alkalosis (n = 3.0, 4.1 %), Metabolic Alkalosis **(**n = 1, 1.4 %) and Hypercapnia (n = 1.0, 1.4 %), were noticed only in patients of group Total-EMs.

Mechanical ventilation

In this study, 60.4 % of cases needed mechanical ventilation divided into 50.9 % in Homo-EMs, 62.5 % in Hetero-EMs and70 % in PMs **Table 10.**

Outcomes

This table shows that (n = 72, 86.7 %) of studied cases fully recovered and (n = 11, 13.3 %) was died, divided into 5.3 % in Homo-EMs, 18.8 % in Hetero-EMs and 70 % in PMs **Table 11**.

Severity score of tramadol (APACHE II score)

The APACHE II score of PMs was significantly different from that of homozygous extensive metabolizers EMs and heterozygous extensive metabolizers EMs ($P < 0.001$) **Table 12**.

Table 9: Arterial Blood Gas analysis among the studied Cases

Studied Parameters		Groups			
		Total EMs (n = 73)	Homo-EMs (n = 57)	Hetero-EMs (n = 16)	PMs (n = 10)
Arterial blood gases (ABG)	Normal	13 (17.8 %)	9.0 (15.8 %)	4.0 (25.0 %)	3.0 (30.0 %)
	Respiratory Acidosis	42 (57.5 %)	31 (54.4 %)	11 (68.8 %)	4.0 (40.0 %)
	Respiratory Alkalosis	3.0 (4.1 %)	3.0 (5.3 %)	0.0 (0.0 %)	0.0 (0.0 %)
	Hypercapnia	1 (1.4 %)	1 (1.8 %)	0 (0.0 %)	0.0 (0.0 %)
	Metabolic Acidosis	15 (20.5 %)	13.0 (22.8 %)	2.0 (12.5 %)	3.0 (30.0 %)
	Metabolic Alkalosis	1.0 (1.4 %)	1.0 (1.8 %)	0.0 (0.0 %)	0.0 (0.0 %)

(Total-EMs): Total extensive metabolizers. (Homo-EMs): Homozygous extensive metabolizers (Hetero-EMs): Heterozygous extensive metabolizers. (PMs): Poor metabolizers, Qualitative variables were expressed as count and Percentages (%).

Table 10: Mechanical ventilation

Studied Parameters		Groups			
		Total EMs a (n = 73)	Homo-EMs b (n = 57)	Hetero-EMs c (n = 16)	PMs d (n = 10)
mechanical Ventilation	No	34 (46.6 %)	28 (49.1 %)	6 (37.5 %)	3 (30.0 %)
	Yes	39 (53.4 %)	29 (50.9 %)	10 (62.5 %)	7.0 (70.0 %)

(Total-EMs): Total extensive metabolizers. (Homo-EMs): Homozygous extensive metabolizers (Hetero-EMs): Heterozygous extensive metabolizers. (PMs): Poor metabolizers, Qualitative variables were expressed as count and Percentages (%).

Table 11: Outcome

Studied Parameters		Groups			
		Total EMs (n = 73)	Homo-EMs (n = 57)	Hetero-EMs (n = 16)	PMs (n = 10)
Death	No	67 (91.8 %)	54 (94.7 %)	13 (81.3 %)	5.0 (50.0 %)
	Yes	6.0 (8.2 %)	3.0 (5.3 %)	3.0 (18.8 %)	5.0 (50.0 %)

(Total-EMs): Total extensive metabolizers. (Homo-EMs): Homozygous extensive metabolizers (Hetero-EMs): Heterozygous extensive metabolizers. (PMs): Poor metabolizers, Qualitative variables were expressed as count and Percentages (%).

Table 12: Severity score of tramadol (APACHE II score)

Median					
studied parameters	**Total-Ems (n = 73)**	**Homo-Ems (n = 57)**	**Hetero-Ems (n = 16)**	**PMs (n = 10)**	***P* value**
APACHEII Score	18.00 (17.00-19.00)	18.00 (16.50-19.00)	18.00 (17.00-19.00)	26.00 (17.75-34.00)	0.007* [a] 0.006* [b] 0.006* [c]

EMs, extensive metabolizers; Homo-EMs, homozygous extensive metabolizers; Hetero- EMs, heterozygous extensive metabolizers; PMs, poor metabolizers. Quantitative data are represented as median (interquartile range, IQR: 25th quartile to 75th quartile). * indicates a statistically significant difference. Statistical significance at p value < 0.05. [a] Difference in distribution between Total EMs and PMs, [b] difference in distribution between Homo-EMs and PMs, [c] difference in distribution between Hetero-EMs and PMs.

Laboratory findings in tramadol-intoxicated subjects on admission

The results showed that there was significant decrease in platelets in group Total EMs, Homo-EMs, Hetero-EMs groups compared to PMs (p < 0.01). Sodium, urea and BUN showed that there were significance differences between cases in group Hetero-EMs and PMs (p < 0.05). Results also, show a significance increase in BUN in Homo-PMs group compared to Hetero-EMs group **Table 13**.

Table 13: Laboratory findings in tramadol-intoxicated subjects on admission.

Studied Parameters	Groups				
	Total EMs (n = 73)	**Homo-EMs (n = 57)**	**Hetero-EMs (n = 16)**	**PMs (n = 10)**	***P* value**
Glucose (60-110mg/dl)	99 (85 - 145)	99 (88.50 - 117.50)	91.5 (81 - 107.75)	82.5 (53.75-111.50)	0.119[a] 0.115[b] 0.246[c]
Sodium (135-145meq/l)	139 (137 - 140)	138 (137 - 140)	139.00 (138 - 142)	137 (136.75 - 139)	0.113[a] 0.188[b] 0.043[c]
Potassium (3.5-5.0meq/l)	3.8 (3.0 - 4.1)	3.8 (3.05 - 4.10)	3.8 (2.93 - 4.15)	3.8 (2.58 - 4.05)	0.649[a] 0.597[b] 0.548[c]
CPK (20 – 200 U/l)	97 (86.5 - 117.5)	98 (86.50 - 124.50)	97 (87.00 - 100.75)	100 (87.75 - 127.50)	0.629[a] 0.731[b] 0.428[c]
Platelet (150 – 450) $10^3/m^3$	312 (267 - 367.5)	312.00 (265.00-367.00)	301.00 (283.00-373.25)	240.5 (36.00-287.25)	0.003*[a] 0.004*[b] 0.006*[c]
Hb (12 – 18 g//dL)	13 (12.30 - 14.00)	13.1 (12.50 - 14.10)	12.2 (11.90 - 13.10)	12.35 (12.00 - 14.10)	0.405[a] 0.218[b] 0.578[c]
WBCs (4,500 - 10,000)/ mm3	8 (6.80 - 10.00)	8.2 (6.55 - 10.00)	7.85 (7.00 - 8.85)	6.4 (4.70 - 14.00)	0.284[a] 0.36[b] 0.196[c]
Urea (15 – 45 mg/dL)	30 (24.00 - 40.0)	33 (26.00 - 40.50)	24.00 (16.00 - 32.25)	37.5 (27.25-120.00)	0.109[a] 0.231[b] 0.014*[c]
Creatinine (0.6-1.4 mg/dl)	0.8 (0.65 - 1.00)	0.8 (0.60 - 0.95)	0.85 (0.70 - 1.00)	0.9 (0.50 - 3.90)	0.507[a] 0.483[b] 0.96[c]
BUN	14.0187 (11.2 - 18.69)	15.42 (12.15 - 18.93)	11.22 (7.48 - 15.07)	17.52 (12.73 - 56.07)	0.109[a] 0.231[b] 0.014*[c]
AST 5-40 (U/l)	31 (22.0 - 48.5)	31 (21.00 - 60.00)	30.5 (26.50 - 39.75)	25 (18.00 - 133.50)	0.538[a] 0.692[b] 0.256[c]
ALT 5-40 U/L	25 (19.00 - 43.5)	25 (18.00 - 47.50)	22.5 (20.00 - 26.75)	24 (21.00-115.50)	0.397[a] 0.444[b] 0.369[c]

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CPK, creatine phosphokinase, hemoglobin, WBCs: white blood cells (Total-EMs): Total extensive metabolizers. (Homo-EMs): Homozygous extensive metabolizers. (Hetero-EMs): Heterozygous extensive metabolizers. (PMs): Poor metabolizers. Quantitative data are represented as median (interquartile range, IQR: 25th quartile to 75th quartile* indicates a statistically significant difference. Statistical significance at p value < 0.05. [a] Difference in distribution between Total EMs and PMs, [b] difference in distribution between Homo-EMs and PMs, [c] difference in distribution between Hetero-EMs and PMs.

Diagnostic validity test of TMD/M1, TMD/M2 and M1/M2 in the discrimination between EMs and PMs

Diagnostic validity test showed that M1/M2 exhibited the best AUC (0.976), followed by TDM/M1 (AUC = 0.724) and TDM/M2 (AUC = 0.656), in differentiating between EMs and PMs **Table 14**.

Diagnostic validity test of TMD/M1, TMD/M2 and M1/M2 in the discrimination between Homo-EMs and Hetero-Ems

Diagnostic validity test revealed that M1/M2 exhibited the best AUC (0.532), followed by TDM/M1 (AUC = 0.451) and TDM/M2 (AUC = 0.43), in differentiating between Homo-EMs and Hetero-EMs **Table 15**.

Diagnostic validity test of TMD/M1, TMD/M2 and M1/M2 in the discrimination between PMs and Homo-Ems

Diagnostic validity test revealed that M1/M2 showed the best AUC (0.986), followed by TDM/M1 (AUC = 0.717) and TMD/M2 (AUC = 0.66), in differentiating between PMs and Homo-EMs **Table 16.**

Diagnostic validity test of TMD/M1, TMD/M2 and M1/M2 in the discrimination between PMs and Hetero-EMs

Diagnostic validity test showed that M1/M2 revealed the best AUC (0.938), followed by TDM/M1 (AUC = 0.788) and TDM/M2 (AUC = 0.679), in differentiating between PMs and Hetero-Ems **Table 17 & Fig. 1**.

Table 14: Diagnostic validity test of TMD/M1, TMD/M2 and M1/M2 in the discrimination between EMs and PMs

Biomarker	Cut-off point	EMs (n = 73)	PMs (n = 10)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	AUC (95% CI)	Accuracy (%)	X2	*P*
TMD/M1	≤ 4	5	1	93.2	90	98.6	64.3	0.724	92.8	0.13	0.718
	> 4	68	9								
TMD/M2	≤1.9	9	5	87.7	50	92.8	35.7	0.656	83.1	8.901	0.003
	>.9	64	5								
M1/M2	≤0.6	2	0	97.3	100	100	83.3	0.976	97.6	67-272	0.001
	> 0.6	71	10								

PPV, positive predictive value; NPV, negative predictive value; AUC, area under the curve; 95% CI, 95% confidence interval.

Table 15: Diagnostic validity test of TMD/M1, TMD/M2 and M1/M2 in the discrimination between Homo-EMs and Hetero-EMs

Biomarker	Cut-off point	Homo-EMs (n = 57)	Hetero-EMs (n = 16)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	AUC (95% CI)	Accuracy (%)	X2	*P*
TMD/M1	≤ 2.6	24	8	57.9	50	80.5	25	0.451	56.2	0.316	0.874
	> 2.6	33	8								
TMD/M2	≤ 5.9	28	6	50.9	62.5	82.9	26.3	0.43	53.4	0.678	0.42
	> 5.9	29	10								
M1/M2	≤ 2.3	21	8	63.2	50	81.8	27.6	0.532	60.3	0.903	0.342
	> 2.3	36	8								

PPV, positive predictive value; NPV, negative predictive value; AUC, area under the curve; 95% CI, 95% confidence interval.

Table 16: Diagnostic validity test of TMD/M1, TMD/M2 and M1/M2 in the discrimination between PMs and Homo-EMs

Biomarker	Cut-off point	Homo-EMs (n = 57)	PMs (n = 10)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	AUC (95% CI)	Accuracy (%)	X2	P
TMD/M1	≤ 4	4	1	93	90	98.1	69.2	0.717	92.5	0.11	0.741
	> 4	53	9								
TMD/M2	>1.9	52	5	91.2	50	91.2	50	0.66	85.1	11.38	0.001
	≤1.9	5	5								
M1/M2	>0.6	56	0	98.2	100	100	90.9	0.986	98.5	59.84	0.0001
	≤ 0.6	1	10								

PPV, positive predictive value; NPV, negative predictive value; AUC, area under the curve; 95% CI, 95% confidence interval.

Table 17: Diagnostic validity test of TMD/M1, TMD/M2 and M1/M2 in the discrimination between PMs and Hetero-EMs

Biomarker	Cut-off point	Hetero-EMs (n = 16)	PMs (n = 10)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	AUC (95% CI)	Accuracy (%)	X2	*P*
TMD/M1	≤ 3.9	1	1	93.8	90	93.8	90	0.788	92.3	0.122	0.727
	> 3.9	15	9								
TMD/M2	>3.1	12	1	75	90	92.3	69.2	0.679	80.8	10.4	0.001
	≤ 3.1	4	9								
M1/M2	>0.6	15	0	93.8	100	100	90.9	0.938	96.2	22.159	0.0001
	≤ 0.6	1	10								

PPV, positive predictive value; NPV, negative predictive value; AUC, area under the curve; 95% CI, 95% confidence interval.

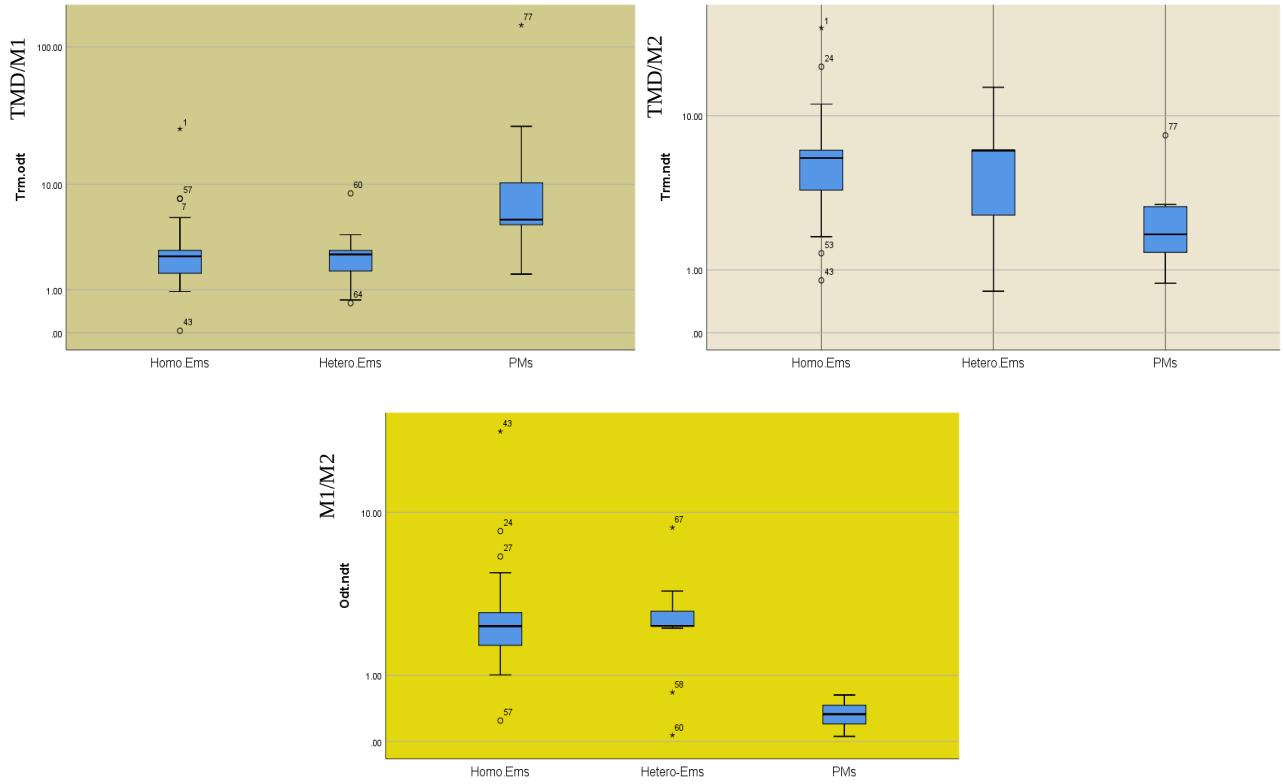

Fig. 1: Distribution of the concentration ratios TMD/M1 (graphic 1), TMD/M2 (graphic 2) and M1/M2 (graphic 3) according to homozygous extensive metabolizers (n = 57); heterozygous extensive metabolizers (n = 16); PMs, poor metabolizers (PM; n = 10).

Discussion

In the present study, males represented the majority of cases 67 (80.7 %) in comparison to females 16 (19.3 %). This can be explained by the increased tramadol abuse in males and its alleged enhancement of sexual performance as reported [11]. On contrary, another study [12], found that female intoxicated patients have been reported represented at a higher rate than males. This observation was also reported by [13], who found that females were highly represented in their study, which might reflect a gender preference of women to rely on drug overdose as a mean of self-harm, whereas men may be more likely to inflict physical self-harm.

In the present study, the majority of cases were young adults in their 3rd decade of life, with a median age of 30 years (21.75 – 35 years), which is in agreement with the results of Ahmadi *et al.* [14]. This can be explained by the fact that adolescence is one of the most vulnerable stages of life, a time typified by puberty and stresses at home, community and in personal relationships. On the contrary, Tja¨derborn *et al.* [15], reported that the mean age was 44 years (range 18 – 78 years).

Our study highlights the CYP2D6 allelic variants' distribution among Egyptian tramadol intoxicated subjects. Depending on which alleles are present in an individual, a wide range of clinical manifestations and tramadol level were observed. Approximately 68.7 % of the tramadol addicts had a wild type of CYP2D6 allelic variants (*1/*1) and the most common mutant allelic variants were (*4). Our results are in line with [16-17], who studied CYP2D6 allelic variants among the Egyptian cases.

The CYP2D6*3, CYP2D6*4 and CYP2D6*6 null alleles are the major variants associated with loss of activity in PMs [18]. The results of the present study showed that the most frequent null allele in Egyptian is CYP2D6*4, which occurs with an allele frequency of about 12.1 % versus 20 % – 25 % in Caucasians and it is responsible for 70 % – 90 % of all PMs.

The metabolic ratios TMD/M1 and TMD/M2 [19] as well as the concentration ratio M1/M2 were tested. The concentrations of tramadol and its metabolites strongly indicated significantly different pharmacokinetics in CYP2D6 PMs and EMs. In accordance with our results Halling *et al.* [10] found that individuals with mutant CYP2D6 alleles (e.g. *3,*4,*4xn,*10,*17or*5) had minimal or absent enzymatic activity with higher tramadol concentration [10].

When the frequency distributions of TMD/M1, TDM/M2 and M1/M2 in the genotype groups were calculated, the distribution of M1/M2 was the best correlation obtained to discriminate between EMs and PMs, as is shown in the graphics presented in **Fig. 1**. This observation can be explained by the information about the administration is often not accurate, like the time, dosage, route and the time until death [19]. In some cases, the ingested doses reported by the patients or their relatives were not absolutely reliable. Moreover, there was considerable inter-subject variability in tramadol metabolism. Therefore, it was hard to find a Logical relationship between the determined urine concentrations and the tramadol dose consumed.

In addition, the complementarity of the two tramadol metabolic pathways [20]. In the presence of high substrate concentrations, low CYP2D6 concentrations or when this enzyme is inhibited, a metabolic switch in favor of enhanced N-demethylation can be observed. On the other hand, the possible involvement of CYP2D6 in the elimination process of M2 may explain the increase in its concentration. So, in these cases the ratio between the two metabolites will be higher, allowing to differentiate the PMs and the other groups. Generally found no connection between the presence of known CYP2D6 inhibitors or substrates [3] and exceptionally high TDM/M1 or low TDM/M2 values therefor M1/M2 ratio can be useful to reduce the impact of those unknown variables, as the degree of metabolization.

Using the concentration ratio M1/M2, the PMs are completely different from the other groups, with an M1/M2 concentration ratio < 1. On the other hand, the EMs group has a wide concentration ratio interval, between 1 and 7.

In this study, three subjects were characterized as PMs according to M1/M2 but their genotypes were two CYP2D6*1/*4 and one *1/*1, respectively, giving rise to a classification of EMs. The patient genotyped as CYP2D6*1/*4 was, in addition to tramadol, may receive blockade of M1 formation (for example, concomitant consumption of antidepressants / CYP2D6 inhibition) or a diversion of the metabolism of tramadol to M2 (for example, concomitant consumption of carbamazepine / CYP3A4 stimulation) could explain, even in cases of acute poisoning, a preferential formation of M2 over M1. In these instances, a longer time lapse is required to reach high levels of M1, especially in those subjects who developed tolerance. Consequently, this subject most likely had changed from EMs to PMs a phenomenon termed phenocopying [10].

For the second patient, also PMs according to TMD/M2 and EM genotype, the explanation could be attributed to mutations/deletions not tested in the present study. An example could be the CYP2D6*5 allele appearing with considerable frequency in Caucasians and resulting in the PM phenotype. The applied genotyping assay in the present study does not detect this or other mutations different from CYP2D6 *3, *4, and *6. With an expanded genotyping test detecting CYP2D6*5 among other mutations, this subject perhaps would appear as a PM.

Moore *et al.* [21] proposed that the M1/M2 ratio could be useful as an indicator of the time lapse between ingestion of tramadol and death. A preferential formation of M1 over M2 (M1/M2 ratio > 1) is indicative of acute death, whereas preferential formation of M2 over M1 (M1/ M2 ratio < 1) would suggest a longer time lapse after ingestion. This hypothesis is in contradiction with the results. As regards the results of the current study, the median APACHE II Score in PMs was showed statistically significant highest worse score 26 (17.75 – 34) and M1/M2 ratio < 1 when compared with EMs groups 18 (17 – 19), and this significantly higher median APACHE II score with 100 % deaths. This result agrees with a study by Chen *et al.* [22] found that the post-ICU non-survivors had greater severity of illness on admission with a mean admission APACHE II score of 22.9 ± 5.5, compared to 18.6 ± 6.1 for post-ICU survivors. Haidri *et al.* [23] demonstrated that the APACHE II score of patients who were successfully discharged from ICU has lower score as compared to patients who died.

No statistical difference was noted between poor and extensive metabolizers concerning the clinical consequences or laboratory parameters despite the significantly higher active metabolites and significantly lower ratios of tramadol / M1 and tramadol / M2 in extensive metabolizer. This might be explained by the multiple actions of tramadol on different non-opioid receptors.

Although tramadol is recognized as a safe drug for the treatment of moderate to severe pain, without any major opioid-type side effects, a CYP2D6 UM phenotype,

could be a major risk factor for potentially life-threatening tramadol cardiotoxicity. Accordingly, dosage of tramadol and its main metabolite M1, advantageously completed by CYP2D6 genotyping, could be applied to patients with excessive morphinomimetic effects in order to identify individuals at risk of tramadol-related cardiotoxicity. The detection of allelic variants described as non-functional can be useful to explain some circumstances of death in the study of tramadol positive cases and the results obtained demonstrate the importance of this genetic tool to forensic toxicology and pathology. Genetic screen can be applied to cases with other substances with the same metabolic pathway (CYP2D6), such as codeine, anti-depressants and neuroleptics Tramadol treatment could then be optimized in these at-risk individuals and patient outcome and safety could consequently be improved.

Conflict of interest

The authors declare that they have no conflict of interest .

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