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Abstract: Tramadol concentrations and analgesic effect are dependent on the CYP2D6 enzymatic activity. It is well known that some genetic polymorphisms are responsible for the variability in the expression of this enzyme and in the individual drug response. The detection of allelic variants described as non-functional can be useful to explain some circumstances of death in the study of post-mortem cases with tramadol. A Sanger sequencing methodology was developed for the detection of genetic variants that cause absent or reduced CYP2D6 activity, such as *3, *4, *6, *8, *10 and *12 alleles. This methodology, as well as the GC/MS method for the detection and quantification of tramadol and its main metabolites in blood samples was fully validated in accordance with international guidelines. Both methodologies were successfully applied to 100 post-mortem blood samples and the relation between toxicological and genetic results evaluated. Tramadol metabolism, expressed as its metabolites concentration ratio (N-desmethyltramadol/O-desmethyltramadol), has been shown to be correlated with the poor-metabolizer phenotype based on genetic characterization. It was also demonstrated the importance of enzyme inhibitors identification in toxicological analysis. According to our knowledge, this is the first study where a CYP2D6 sequencing methodology is validated and applied to post-mortem samples, in Portugal. The developed methodology allows the data collection of post-mortem cases, which is of primordial importance to enhance the application of these genetic tools to forensic toxicology and pathology.

Lisbon, 30th September of 2015

Dear Prof. Drummer,

We are submitting our paper "**Sequencing CYP2D6 for the detection of poor-metabolizers in post-mortem blood samples with tramadol**" for peer review and subsequent publication in the Forensic Science International, as a contribution for the special issue "TIAFT 2015" in the form of an **Original Research Article**.

We further state that this paper reports original work and is not under consideration for publication elsewhere.

Yours sincerely,

Suzana Fonseca

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Este e-mail pretende ser amigo do ambiente. Pondere antes de o imprimir!

Original Research Article

Sequencing CYP2D6 for the detection of poor-metabolizers in *post-mortem* blood samples with tramadol

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Sequencing CYP2D6 for the detection of poor-metabolizers in *post-mortem* blood samples with tramadol

Highlights:

- Validation of a Sanger sequencing method to detect null alleles of CYP2D6 in *post-mortem* samples
- Application to 100 samples of forensic cases with tramadol
- Poor-metabolizers correlated with the *post-mortem* concentrations of tramadol and metabolites
- CYP2D6 inhibitors must be included in the toxicological screening in cases with tramadol

1 Original Research Article

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3 ***post-mortem* blood samples with tramadol**

4
5 **Abstract**

6 Tramadol concentrations and analgesic effect are dependent on the CYP2D6 enzymatic
7 activity. It is well known that some genetic polymorphisms are responsible for the
8 variability in the expression of this enzyme and in the individual drug response. [The](#)
9 [detection of allelic variants described as non-functional can be useful to explain some](#)
10 [circumstances of death in the study of *post-mortem* cases with tramadol.](#) A Sanger
11 sequencing methodology was developed for the detection of genetic variants that cause
12 absent or [reduced](#) CYP2D6 activity, such as *3, *4, *6, *8, *10 and *12 alleles. This
13 methodology, as well as the GC/MS method for the detection and quantification of
14 tramadol and its main metabolites in blood samples was fully validated in accordance
15 with international guidelines. Both methodologies were successfully applied to 100
16 *post-mortem* blood samples and the relation between toxicological and genetic results
17 evaluated. Tramadol metabolism, expressed as its metabolites concentration ratio (N-
18 desmethyltramadol/O-desmethyltramadol), has been shown to be correlated with the
19 [poor-metabolizer](#) phenotype based on genetic characterization. It was also demonstrated
20 the importance of enzyme inhibitors identification in toxicological analysis. According
21 to our knowledge, this is the first study where a CYP2D6 sequencing methodology is
22 validated and applied to *post-mortem* samples, in Portugal. [The developed methodology](#)
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25

26

27 **Keywords:** pharmacogenetics; CYP2D6; poor metabolizers; tramadol; *post-mortem*;
28 forensic toxicology

29

30

31 **1. Introduction**

32 Tramadol is a centrally acting opioid analgesic commonly prescribed for treatment of
33 postoperative, dental, cancer, neuropathic and acute musculoskeletal pain control , with
34 high clinical efficacy, low incidence of adverse effects and low abuse potential.
35 Tramadol is administrated in a racemic mixture and undergoes extensive phase I and II
36 metabolization to 23 metabolites, mostly excreted in the urine. The main metabolites
37 resulting from the phase I metabolization are O-desmethyltramadol (ODT), catalyzed by
38 CYP2D6 enzyme, and N-desmethyltramadol (NDT), catalyzed by CYP3A4 and
39 CYP2B6 enzymes. Tramadol acts as a norepinephrine and serotonergic re-uptake
40 inhibitor, possesses low affinity for μ opioid receptors and no affinity for δ or κ opioid
41 receptors. The main opioid analgesic effect is attributed to ODT because it has
42 approximately 300 times more affinity to μ -opioid receptors than the parent compound
43 [1–3].

44 *Post-mortem* concentrations of tramadol are difficult to compare with reference values
45 of therapeutic and toxic levels. There are variables as *post-mortem* redistribution,
46 variations on the sample collection site and the time between the administration and the
47 death that can influence the *post-mortem* concentrations. Tolerance in chronic users,
48 drug interactions and individual genetic factors are also specific aspects of each case
49 that must be considered in the interpretation of toxicological results. Genotyping can be
50 a useful tool to *post-mortem* toxicology to explain some unexpected concentrations of
51 tramadol and parent/metabolite ratios.

52 1.1. CYP2D6

53 CYP2D6 enzyme is coded by a gene with the same name that is located on the human
54 chromosome 22 (22q13.1) and is part of a cluster with 2 pseudogenes, CYP2D7 and
55 CYP2D8. These three genes have a high genetic homology but can be distinguished by
56 some well characterized sequence variants that are responsible for the incapacity of the
57 pseudogenes to produce a functional enzyme [4]. CYP2D6 gene is highly polymorphic
58 and more than 20 allelic variants have been already correlated with the enzyme
59 inactivation, usually called null alleles (<http://www.cypalleles.ki.se/cyp2d6.htm>), some
60 of them with high prevalence in Caucasian population. When these genetic variants are
61 present the metabolic activity is compromised. The characteristics of CYP2D6 gene
62 locus, possible genotypes and phenotypes, as well as the difficulties of the genetic

63 analysis and interpretation were extensively reviewed by Andrea Gaedigk in 2013 [4].

64 1.2. Genotype and metabolism

65 The metabolic capability can be distinguished in four different groups: poor (PM),
66 intermediate (IM), extensive (EM) and ultra-rapid metabolizers (UM). Poor
67 metabolizers have very low metabolic capacity and higher metabolic ratios. Genetically
68 they are characterized by the presence of 2 null alleles and 5 to 10% of the European
69 population are considered to be PM [5]. The alleles CYP2D6 *3, *4, *5 and *6 are
70 responsible for 93-98% of the PM [6]. The relationship between CYP2D6 genetic
71 variation and enzymatic activity has already been studied by many authors and recently
72 reviewed by Zanger and Schwab [5].

73 In tramadol positive cases, the relation between low metabolism phenotype and PM
74 genotype of CYP2D6 has already been demonstrated by several studies: PM have a
75 lower concentration of ODT, the main active metabolite, reducing the opioid analgesic
76 effect as well as the opioid related adverse effects [2,7–13]. The half-life and
77 concentration of tramadol can be higher in PMs [11] and the alternative metabolic ways
78 can be stimulated. CYP2B6 and CYP3A4 enzymes catalyze the biotransformation of
79 tramadol to N-desmethyltramadol (NDT), an inactive metabolite. The inhibition of the
80 CYP2D6 metabolism can conduce to an increment of the concentration of NDT
81 [1,7,10].

82 High blood concentrations of tramadol, due to accumulation or to increasing dosage,
83 can lead to adverse reactions, not directly related with the opioid depression of the
84 central nervous system, but specially with the inhibition of serotonin and
85 norepinephrine reuptake [1].

86 1.3. Forensic application

87 Forensic pharmacogenetics is a relatively new and growing area of research [9,14–16].
88 The application of genotyping methodology to *post-mortem* forensic cases is dependent
89 on the level of the DNA degradation, the existence of reliable methodologies that can be
90 applied to routine analysis and on the gene characteristics. The interpretation of the
91 results depends on the knowledge based on scientific research with statistical coverage,
92 reason why further studies and the compilation of *post-mortem* data are needed to fully
93 understand the relation between toxicological and genetic results. This information is
94 important to enable the use of genotyping in the evaluation of some cases, specially
95 when the concentration of drugs and metabolites can be considered suspect of an acute

96 intoxication.

97

98 To aid the interpretation of unexpected high concentrations of tramadol in *post-mortem*
99 cases, it is important to have a method capable to detect genetic variants of CYP2D6
100 responsible for the enzyme inactivation. The purpose of this study was to develop and
101 validate a Sanger sequencing method to detect the more prevalent null variants in *post-*
102 *mortem* blood samples, using the technology usually existent in the forensic genetics
103 laboratories without further costs.

104

105 **2. Materials and methods**

106 2.1.Samples

107 100 *post-mortem* peripheral blood samples, positive for tramadol, were selected from
108 forensic toxicological cases that were analyzed between 2012 and 2015 in the south
109 branch of the National Institute of Legal Medicine and Forensic Science (INMLCF), in
110 Portugal. In the selected cases, 56 were male and the mean age of the subjects was 65
111 years old (range: 30 to 93). The probable cause of death mentioned in the toxicological
112 request was: violent traumatism (34 cases); intoxication (10 cases); natural death (16
113 cases) and unknown (40 cases).

114 2.2.CYP2D6 genetic analysis

115 Blood spots were collected in *Whatman*[®] *FTA* cards according to manufacturer's
116 recommendations. DNA was extracted using Chelex100[®] method [17] and quantified by
117 Real-Time PCR using the *Quantifiler*[®] *Trio DNA Quantification kit* from *Applied*
118 *Biosystems* (AB) [18], according to the standard protocol .

119 The PCR amplification method was modified and optimized from Levo *et al* [7] and
120 Hersberger *et al* [6]. For each sample, three fragments of 200bp, 437bp and 736bp were
121 directly amplified, to detect the main null alleles CYP2D6 *3 (2549delA); CYP2D6 *4
122 (100C>T and 1846G>A) and CYP2D6 *6 (1707delT), but also other variants located in
123 these fragments, such as CYP2D6 *8 (1758G>T), *10 (100C>T), *12 (124 G>A), *14
124 (1758G>A), *15 (137_138insT), *40 (1863_1864 ins), *43 (77G>A), *44 (82C>T), *47
125 (73C>T), *49 (1611T>A) and *50 (1720A>C). PCR was prepared to a final volume of
126 25 µL, using *Multiplex PCR Master Mix 2x* (QIAGEN); dimethylsulfoxide 5%
127 (DMSO); 200nM of *primers* and approximately 5 ng of DNA. Thermocycling
128 conditions were adjusted to obtain the better results for each fragment (Table 1). After

129 purification with *ExoSAP-IT*[®] (*Affymetrix*) the amplification was confirmed by SDS-
 130 PAGE Electrophoresis with Silver Staining in a *Phastsystem* (*GE Healthcare*).
 131 The modified Sanger sequencing reaction was performed with *BigDye Terminator v.3.1*
 132 *Cycle Sequencing Kit* of AB, using 4 µL of *Better Buffer* (*Microzone*); DMSO; 500nM
 133 of each of the PCR primers and 1µL of amplified template, to a final volume of 10 µL.
 134 Thermocycling conditions used are in Table 1. After purification with the *BigDye*
 135 *XTerminator Purification Kit* (AB), the sequencing products were analyzed by capillary
 136 electrophoresis in a *Genetic Analyzer 3130* of AB.

137
 138

Table 1. Thermocycling conditions for the PCR and sequencing methods.

| <u>PCR</u> | <u>[-173 to 264]</u> | <u>[1299 to 2035]</u> | <u>[2369 to 2569]</u> |
|----------------------|----------------------|-----------------------|-----------------------|
| Initial denaturation | 95°C/15 min | 95°C/15 min | 95°C/15 min |
| Cycles | 40 | 40 | 35 |
| denaturation | 94°C/30s | 94°C/30s | 94°C/30s |
| annealing | 55°C/30s | 55°C/30s | 57°C/30s |
| extension | 72°C/30s | 72°C/30s | 72°C/30s |
| Final extension | 72°C/7 min | 72°C/7 min | 72°C/7 min |
| <u>Sequencing</u> | | | |
| Initial denaturation | 95°C/3 min | 95°C/3 min | 95°C/3 min |
| Cycles | 35 | 35 | 35 |
| denaturation | 95°C/20s | 95°C/20s | 95°C/20s |
| annealing | 55°C/20s | 55°C/20s | 57°C/20s |

139

140 The results were verified using the *Sequencing Analysis v.5.2* software. The sequence
 141 alignment was done according to the Human Cytochrome P450 Allele Nomenclature
 142 Committee (<http://www.cypalleles.ki.se>) and the allelic variants were detected
 143 comparing the sequences obtained with the reference sequence (entry M33388.1 at
 144 Genbank) using *SeqScape v.3* software.

145 2.3.Validation of the genetic method

146 The method was validated according to the general SWGDAM guidelines, with a
 147 special approach for Sanger sequencing methodology [21], and fulfilling the ENFSI
 148 recommended minimum criteria for the following parameters: specificity, accuracy,
 149 repeatability, reproducibility and sensitivity [19–23].

150 Reference materials were selected from the Coriell Cell Repositories (National Institute
 151 of General Medical Sciences) based on genotypic characterization with the more
 152 prevalent variants (ref: NA17226; NA17280 and NA17300) that included: 100C>T;
 153 1707 del T; 1846G>A, 2549delA [24]. The genotypes were correctly assessed and the

154 peak balance ratios of heterozygote alleles were above 60%. These samples were used
155 as positive controls in the analysis.

156 All the sequences obtained were well aligned with the reference sequence. NCBI's
157 BLAST (basic local alignment search tool) analysis for sequence similarity was also
158 used in the primers and in the sequences of 3 different samples to evaluate the
159 specificity and to check for homology to other genes or pseudogenes that may interfere
160 with the analysis. The search was made using "*Standard Nucleotide BLAST*", in the
161 "*nucleotide collection nr/nt; human (taxid:9606)*", with *Megablast* [29]. The 18
162 sequences verified had matches of 99-100% with the "*Homo sapiens CYP2D6*
163 (*CYP2D6*) gene, complete cds, Sequence ID: gb/JF307778.1|Length: 6587", depending
164 on the variants of each sample. The search using NCBI Genomes (chromosome)
165 database only match with the *Homo sapiens Chromosome 22 Primary Assembly* with
166 100% identity.

167 The Limit of Detection was determined by performing dilution experiments of a high-
168 quality genomic DNA with a known concentration for the following final
169 concentrations: 10, 1, 0.5, 0.1, 0.05, 0.01ng/uL. Signal/Noise and variant identification
170 were the parameters evaluated. The minimum concentration at which was possible to
171 correctly identify the genotype in all the fragments was 0.1ng/μL. The limits of
172 detection of the smaller fragments were lower (0.05ng/μL for the fragment with 437bp
173 and 0.01ng/μL for the fragment with 200bp), as expected. Nevertheless one postmortem
174 sample with a DNA concentration of 0.06ng/μL was successfully analyzed.

175 The precision of the method was verified by repeatability and reproducibility
176 experiments. To test the repeatability, three replicates of five samples with different
177 genotypes were simultaneously analyzed, only varying the location in the thermocycling
178 equipment and in the sequencing plate. With 2 sequences for each of the 3 fragments, a
179 total of 30 replicates for each variant were evaluated, with 100% of success.

180 To test the reproducibility, 15 samples were analyzed in three different days using the
181 same technique, including the normal variables of the routine work in the lab, such as
182 room temperature, reagents and equipments. With 2 sequences for each of the 3
183 fragments, a total of 90 replicates in each variant were evaluated for quality and the
184 correct variant identification. Results are given in the Table 2.

185

186

Table 2. Reproducibility results of the genetic method validation

| Allele | *4, *10 | *12 | *6 | *8 | *4 | *3 |
|---------|---------|--------|--------------------|--------------------|--------------------|----------|
| Variant | 100C>T | 124G>A | 1707delT | 1758G>T | 1846G>A | 2549delA |
| Day 1 | 15/15 | 15/15 | 15/15 | 15/15 | 14/14 ² | 15/15 |
| Day 2 | 15/15 | 15/15 | 14/14 ¹ | 14/14 ¹ | 14/14 ¹ | 15/15 |
| Day 3 | 15/15 | 15/15 | 15/15 | 15/15 | 15/15 | 15/15 |

¹ – Failure in the amplification of the fragment. ² – variant not sequenced.

187

188

189 Most fragments were consistently amplified and sequenced, with only two failures: one
 190 in the sequence (day1) and one in the amplification (day2). The validation was made
 191 with routine *post-mortem* samples. The reported failures are in the larger fragment (with
 192 736bp) and the 2 samples with problems had a degradation index of 1.3 and 1.6, which
 193 are above 1 (the cut-off of the *Quantifiler trio kit*).

194 The quality of the sequences was evaluated with the following criteria, as referenced in
 195 the Userguide for DNA Sequencing by Capillary Electrophoresis of the Applied
 196 Biosystems: Signal > 50, Signal/noise > 25 and Sample score between 20 and 50.
 197 Selecting 5 samples of one day of the reproducibility study, the values were calculated
 198 for 30 sequences and fulfilled the criteria: minimum Signal was 278, minimum
 199 signal/noise was 120 and minimum Sample score was 23.

200 2.4. Toxicological analysis

201 All the samples were analyzed by a general toxicological screening for pharmaceutical
 202 drugs as antidepressants, antipsychotics, opioids and others. The screening comprised
 203 the more prescribed compounds that are known to inhibit CYP2D6 enzymatic action,
 204 such as fluoxetine, paroxetine, sertraline, citalopram, haloperidol, methadone or
 205 ticlopidine [5,25]. The confirmation analysis of tramadol and its metabolites, O-
 206 desmethyltramadol (ODT) and N-desmethyltramadol (NDT) was done in 500 µL of
 207 peripheral blood stored at -10°C in test tubes containing 1% of sodium fluoride.

208 Blood samples were prepared by solid phase extraction using *Oasis® HLB* 3cc 60 mg
 209 cartridges (Waters) and GC-MS analysis was performed using an Agilent 6890 Gas
 210 Chromatograph equipped with a HP-5MS (30mx0.25mmx0.25mm) capillary column
 211 and a 5973 Mass Detector. The injector was set a 280°C and the injection (1 µL) was
 212 made in split mode with 10:1 split ratio. The oven temperature was held at 150°C for 1
 213 min, increased to 290°C at a rate of 5°C/min with a final hold time of 8 min. Data was
 214 acquired using selected ion monitoring mode (**Error! Reference source not found.**).

215 Using a positive control prepared and analyzed simultaneously to the samples, the

216 identification criteria for positivity was: retention time within 2% or ± 0.1 min; the
217 presence of 3 ionic fragments per compound with $S/N > 3$; the maximum allowed
218 tolerances for the relative ion intensities were as required by the World Anti-Doping
219 Agency [26].

220 2.5. Validation of the analytical method

221 The method was fully validated according to international parameters. Experiments
222 were conducted as described in the SWGTOX guidelines in terms of selectivity,
223 interference studies, recovery, limit of detection, limit of quantification, linearity and
224 calibration model, repeatability, reproducibility, accuracy and carryover [27,28]. All
225 validation experiments were conducted using fortified samples of blank *post-mortem*
226 blood using LGC and Lipomed standards.

227 Selectivity was evaluated by analyzing 40 blank samples pooled. Two aliquots of each
228 of the 10 pools were prepared: one was analyzed as blank and the other was spiked with
229 all the analytes (100ng/mL). The chromatograms were compared, the identification
230 criteria applied and the existence of interferences by matrix constituents was checked in
231 the blank chromatograms. The method proved to be selective, fulfilled the criteria for all
232 the samples and without interferences. For the recovery studies, six replicates were
233 prepared at three concentrations (150, 500 e 850 ng/mL), three of them were spiked
234 before extraction and the others 3 after. The internal standard was only added after the
235 extraction procedure. The obtained peak area ratios were compared and the results are in
236 the Table 3 . Five calibration curves were measured over a period of 15 days, using
237 seven levels of spiked blood samples in the working range (between 50 and
238 1000ng/mL) and three independent controls were prepared each day with the
239 concentrations of 150, 500 and 850ng/mL. The calibration model was chosen as
240 explained by Almeida et al [28] using as criteria the correlation coefficient higher than
241 0.99 and the best calibrators' accuracy (obtained by back calculating their
242 concentrations). The method was linear over the working range using a weighting factor
243 of $1/x^2$. Repeatability (within-day precision) was determined by analyzing six spiked
244 samples at the low, medium and high concentration levels simultaneously. The accuracy
245 and the precision were determined by the calculation of BIAS and the coefficient of
246 variation (% CV), using the concentration obtained for the triplicates of controls (see
247 Table 3). The limit of detection (LOD) was determined by the analysis of blood samples
248 spiked with decreasing amounts of the analytes, being the lowest concentration that

249 fulfilled the identification criteria, with the signal/noise of all the peaks above 3, in the
 250 replicates (Table 3). The limit of quantitation (LOQ) was validated by analyzing six
 251 replicates of spiked samples with a concentration of 50ng/mL (the first point of the
 252 calibration curve) and verifying the coefficient of variation (<10%). Dilution of the
 253 sample was tested for 1:2 and 1:5 using 10 real samples, covering a concentration range
 254 between 50ng/mL to 5000ng/mL. The main results of the method validation are
 255 summarized in Table 3 **Error! Reference source not found.**

256

257 Table 3. Summary of the main results obtained in the validation of the confirmation method for
 258 tramadol (TMD), N-demethyltramadol (NDT) and O-demethyltramadol (ODT).

| Compound | RT (min) | Ions (m/z) | Mean Recovery | LOD (ng/mL) | CV | BIAS |
|----------|----------|---------------|---------------|-------------|------|------|
| NDT | 12.1 | 58, 135, 263 | 88% | 10 | 11% | 2,0% |
| ODT | 12,5 | 249, 188, 135 | 95% | 12.5 | 5,2% | 1,0% |
| TMD | 13,4 | 58, 249, 121 | 93% | 10 | 6,6% | 0,7% |

259

260 2.6. Statistical analysis

261 The genetic and toxicological results were graphically and statistically compared, using
 262 SPSS 17.0 software. The distribution of the results was tested for normality with
 263 Kolmogorov-Smirnova test and the hypothesis was rejected. Non-parametric tests were
 264 then used and the statistical differences between the medians of the genotype groups
 265 were calculated using the Mann–Whitney test with 95% of confidence interval.

266

267 3. Results and discussion

268 *Post-mortem* blood samples were studied searching for CYP2D6 genetic variants
 269 responsible for the enzyme inactivation, and the results obtained were then compared
 270 with the concentration of tramadol and its main metabolites: NDT and ODT.

271 3.1. Genetic Results

272 Among the 100 *post-mortem* samples analyzed in this study, amplification failed only in
 273 3 samples, which is comparable to other studies [9]. The DNA degradation was
 274 probably the main limitant factor as was demonstrated by the degradation index (DI)
 275 obtained with the *Quantifiler trio kit*. The degradation index of these samples was 4.2,
 276 6.5 and 12.1 whereas it was under 1.6 for all the others samples.

277 The sequencing methodology allowed the detection of 4 different alleles: CYP2D6*3
 278 (2549delA); CYP2D6*4 (100C>T and 1846G>A); CYP2D6*6 (1707delT) and
 279 CYP2D6*10 (100C>T). Sanger sequencing methodology can't detect the copy number
 280 variation (CNV) of the gene, so it was not possible to identify the gene complete
 281 deletion (allele *5). Nevertheless, in cases with allele *5 the PM phenotype assignment
 282 is not necessarily compromised. In heterozygotes, this methodology will assign the
 283 individual as if he was homozygote for the other allele: if it is null, the individual will
 284 be designated as PM; if it's functional, he will not. On the other hand, in *5/*5
 285 homozygotes, there will be no amplification product because there is no gene. So, when
 286 the amplification fails, there may be two main explanations: the low quantity or the
 287 degradation of the DNA in the sample, which can be evaluated using the degradation
 288 index given by *Quantifiler trio kit*; or maybe the individual is homozygote for the
 289 CYP2D6*5 allele, and this genotype should then be confirmed by a suitable method.

290

291 The allele with the higher prevalence was CYP2D6 *4, with a frequency of 19.6%. The
 292 allele *10 was detected with a prevalence of 16,5%, which is higher than the expected
 293 based on large European population studies and according to CYP2D6 Allelic Variation
 294 Summary Table in <http://www.cypalleles.ki.se/cyp2d6.htm> [30,31] but is in accordance
 295 with a recent study for Portuguese population [32]. CYP2D6*10 isn't a null allele but it
 296 can decrease the enzymatic activity and change the substrate specificity of the enzyme
 297 [13,33–35]. Only one allele *3 and one allele *6 have been detected. All the alleles that
 298 hadn't any variant in the studied fragments were considered as wild type (WT).

299 The genotype distribution is presented in the Table 4.

300

301

Table 4. Genotype distribution for the 97 *post-mortem* samples.

| Genotype | n | Prevalence |
|----------|----|------------|
| *4/*4 | 5 | 5,1% |
| *3/*4 | 1 | 1,0% |
| *4/*10 | 15 | 15,5% |
| *10/*10 | 1 | 1,0% |
| *4/WT | 12 | 12,4% |
| *6/WT | 1 | 1,0% |
| *10/WT | 15 | 15,5% |
| WT | 47 | 48,5% |

302

303 Six persons were predicted as poor metabolizers (PM) according to their CYP2D6
 304 genotype: 5 individuals were found as homozygotes for the allele *4 and one was
 305 heterozygote with one allele *3 and one allele *4.

306 3.2. Toxicology results

307 Among the *post-mortem* samples selected for this study, half presented tramadol
 308 concentration below 800 ng/mL, within the therapeutic range according to the published
 309 reference tables [36,37]. 27 of these cases had a negative result for at least one of the
 310 metabolites: 4 of them in the second group (tramadol concentrations above 800 ng/mL).
 311 Descriptive statistics of the concentration of tramadol and metabolites are presented in
 312 the Table 5.

313

314 Table 5. Descriptive statistics for the concentration of tramadol (TMD), N-desmethyltramadol
 315 (NDT) and O-desmethyltramadol (ODT) in 100 *post-mortem* blood samples.

| TMD < 800ng/mL | TMD | NDT | ODT | TMD > 800ng/mL | TMD | NDT | ODT |
|--------------------------|------------|------------|------------|--------------------------|------------|------------|------------|
| mean | 439 | 312 | 114 | mean | 3070 | 497 | 363 |
| median | 421 | 77 | 75 | median | 1514 | 161 | 222 |
| max | 789 | 464 | 482 | max | 34000* | 3863 | 3127 |
| min | 94 | 12 | 29 | min | 813 | 20 | 18 |

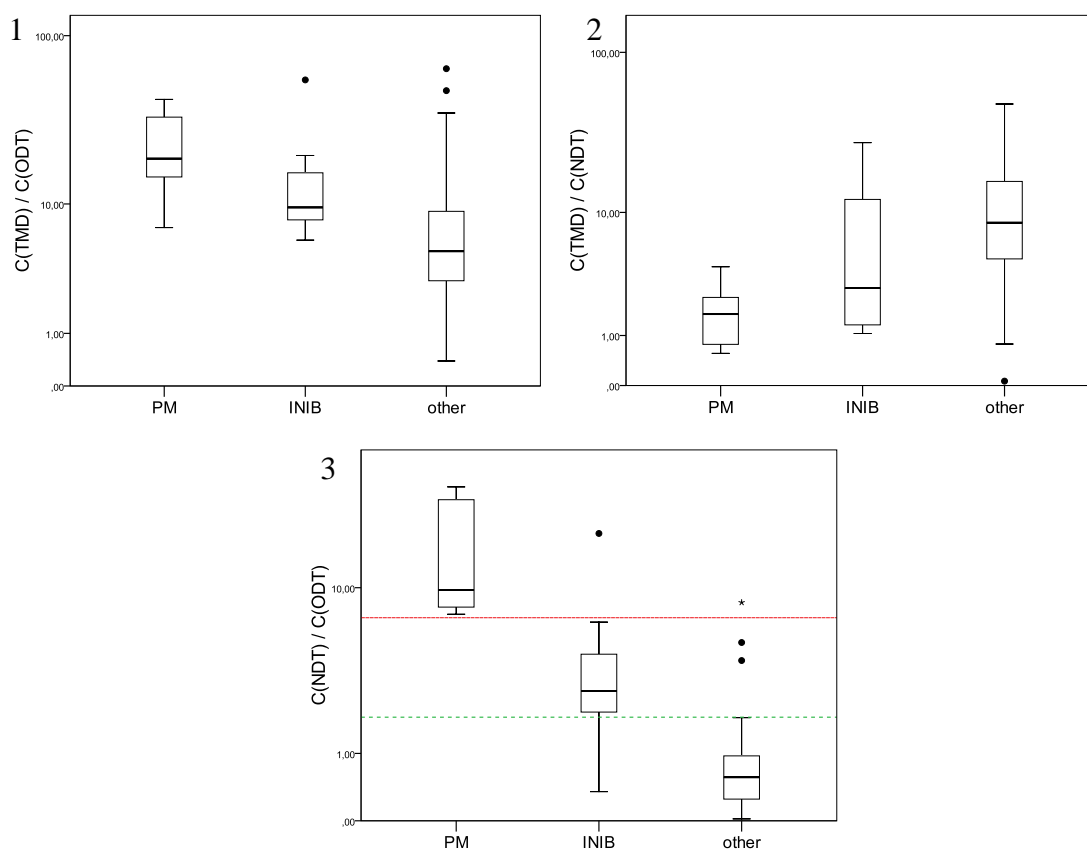
316 * values < 50 ng/mL and > 5000 ng/mL were obtained by extrapolation of de calibration curves.

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318 3.3. Correlation results

319 The genetic and toxicological results were correlated. Cases where was not possible to
 320 confirm the presence of at least one of the metabolites were excluded, remaining a total
 321 of 73 cases. The concentration ratio of TMD and its metabolites was plotted in a
 322 boxplot graphic with a decimal logarithmic scale and the samples were grouped in two
 323 categories according to the genotypes: PM and others. A third group of cases with
 324 positive result to inhibitors compounds, such as fluoxetine, paroxetine, sertraline,
 325 citalopram, ticlopidine and methadone [25,38,39] was also plotted (INIB). Graphics are
 326 presented in Fig. 1.

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Fig. 1. Distribution of the concentration ratios tramadol/O-demethyltramadol (graphic 1), tramadol/N-demethyltramadol (graphic 2) and N-demethyltramadol/O-demethyltramadol (graphic 3) according to the poor-metabolizer predicted phenotype (PM; n=6), the cases that were positive for substances that are considered enzymatic inhibitors (INIB; n=10) and the other cases (n=57).

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The metabolic ratios TMD/ODT or TMD/NDT used by other authors [7,9], as well as the concentration ratio NDT/ODT were tested. The best correlation was obtained using NDT/ODT ratio, as is shown in the graphics presented in Fig. 1. This observation can be explained by the complementarity of the two tramadol metabolic pathways [1,7,10]. 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 NDT may explain the increase in its concentration. So, in these cases the ratio between the two metabolites will be higher, allowing to differentiate the PM phenotype and the possible presence of a CYP2D6 inhibitor.

Using the concentration ratio NDT/ODT, the poor metabolizers (PM) are completely separated from the others (INIB and Others), with a NDT/ODT concentration ratio

349 above 7. The INIB group has a wide concentration ratio interval, but more than 3
 350 quarters are between 2,5 and 7. In these cases, regardless of genotype, the interaction of
 351 the inhibitors leads to a different metabolic behavior. These results are in accordance
 352 with other previous studies [15,38,40,41]. The medians of the groups were statistically
 353 compared using a Mann-Whitney test with a level of significance of 0.05 and proved to
 354 be significantly different using the NDT/ODT ratio.

355

356 In *post-mortem* cases, the information about the administration is oftentimes unknown,
 357 like the time, dosage, route and the time until death [9]. The concentration range is very
 358 wide for both the parent compound and the metabolites and is not correct to compare it
 359 with the results obtained in clinical studies. A high TMD/ODT does not necessarily
 360 mean that there is a deficient metabolism. Many factors can explain it, like co-
 361 medication, existence of pathologies, or if the death occurred right after the
 362 administration. NDT/ODT ratio can be useful to reduce the impact of those unknown
 363 variables, as the degree of metabolism at the time of death. Further evaluation of
 364 these data might be important and should be considered in future studies.

365 3.4. Case Results

366 In this study, six individuals were found to be poor-metabolizers (PM). All the available
 367 information concerning these six cases is presented in the Table 6.

368

369 Table 6. Case information: Age, gender, probable cause of death, toxicological findings and
 370 genotype of the 6 PM cases.

| CASE | age | gender | Probable cause of death | TMD | NDT (ng/mL) | ODT | Other substances (ng/mL) | Ethanol (g/L) | GEN |
|------|-----|--------|-------------------------|--------|-------------|-----|---|---------------|-------|
| 1 | 66 | male | Unknown | 1137 | 1479 | 161 | Pethidine (12) Sertraline (212) Benzodiazepines (< therap) | negative | *4/*3 |
| 2 | 64 | male | Unknown | > 5000 | 1338 | 167 | Ticlopidine (401) Trazodone (156) Bromazepam (54) Flurazepam (291) | 0.13 | *4/*4 |
| 3 | 71 | male | Natural (Neoplasia) | 2130 | 1502 | 50 | negative | negative | *4/*4 |

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|---|----|------|-------------------------|-----|-----|-----|------------------------------------|----------|-------|
| 4 | 71 | male | Accident (traumatic) | 528 | 221 | 30 | negative | negative | *4/*4 |
| 5 | 55 | male | Unknown | 205 | 103 | <25 | negative | negative | *4/*4 |
| 6 | 83 | male | Suicide (hanging) | 147 | 262 | <25 | Paroxetine (160) Alprazolam (6) | negative | *4/*4 |

(in the table: TMD, NDT and ODT are tramadol, N-desmethyltramadol and O-desmethyltramadol concentrations; GEN is the genotype)

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374 The first three cases have tramadol concentrations higher than the therapeutic range
375 according to reference tables [36,37] but the ODT concentrations are comparatively
376 low, considering the data published by Grond *et Sablotzki* and Stamer *et al* [1, 11, 12].
377 Namely in the case number 3 the ODT concentration is comparable to the obtained as a
378 therapeutic concentration in the pharmacokinetics studies. In this particular case, the
379 individual was a cancer patient. The absence of a functional genetic variant for CYP2D6
380 can explain the concentrations found in *post-mortem* peripheral blood. The higher
381 concentration of tramadol may be due to accumulation or to an increment of the dosage,
382 which can be related with a decrease in the opioid analgesic effect associated with the
383 lower ODT concentration. However, we cannot exclude the possibility of the lack of
384 analgesic effect be due to the development of tolerance in a chronic user. Only one pill
385 was found at the stomach content and the cause of death was determined as natural.

386

387 Predicting the analgesic effect of tramadol based on the pharmacogenetics results is
388 tempting but there are multiple factors, some of them still unknown, that can influence
389 the interpretation [42,43]. Additional fundamental research and collection of routine
390 data is still needed before using pharmacogenetics results as evidence in court.
391 However, in particular *post-mortem* cases, these approaches, together with all the
392 autopsy findings and clinical information, can be very useful in the investigation of
393 cause of death.

394

395 4. Conclusions

396 This study proved that Sanger sequencing methodology can be successfully applied to
397 the detection of genetic polymorphisms at CYP2D6 in *post-mortem* blood samples. The
398 method proved to be specific, accurate, with a good precision and limit of detection for
399 the null variants analyzed.

400 The results showed a good correlation between the PM genotype and the toxicology
401 results of the tramadol metabolic ratio NDT/ODT, appearing to be an alternative
402 parameter in the evaluation of the degree of metabolization of tramadol in *post-mortem*
403 cases.

404 The presence of enzymatic inhibitors affects significantly the degree of metabolization,
405 which can be seen in the results obtained. By this reason, is very important to include
406 that compounds in the toxicology screening.

407 The detection of allelic variants described as non-functional can be useful to explain
408 some circumstances of death in the study of tramadol positive cases and the results
409 obtained demonstrate the importance of this genetic tool to forensic toxicology and
410 pathology.

411 Sanger sequencing methodology applied in this study can also be applied to cases with
412 other substances with the same metabolic pathway (CYP2D6), such as codeine,
413 antidepressants and neuroleptics.

414

415 5. References

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- 559

We would like to thank both reviewers for the deep appreciation of our manuscript and for all the suggestions. We tried to fulfill all the requirements and we will answer point-by-point to the comments

Reviewer #1:

1. The introduction of this manuscript oversimplifies the metabolism and pharmacological properties of tramadol. The metabolism of tramadol is complex and mediated by several polymorphic cytochrome P450 enzymes [Gong Li et al. Pharmacogenetics and genomics (2014)].

* Secondly, tramadol analgesia is mediated not only by the polymorphic mu opioid receptor but also through modulation of norepinephrine and serotonergic re-uptake.

A: I have reformulated the introduction as suggested.

* Thirdly, tramadol is not always given in controlled doses (as stated), but commonly prescribed in outpatient settings. Therefore, the assumption that higher than expected tramadol doses would arise from malpractice or neglect is short-sighted and should probably be removed from the manuscript altogether as it is outside the scope of this work.

A: Removed as suggested.

2. Please provide a reference to support the statement (page 3) that PM of tramadol have reduced analgesic effects with tramadol and reduced adverse effects with tramadol.

A: Done.

3. The postmortem concentrations should not, in general, be compared to those in clinical studies/therapeutic studies. Please discuss at length the various issues with postmortem findings (post-mortem redistribution, variances in drug collection sites (femoral blood, etc), death interval) as they pertain to the population in this study, and as they pertain to tramadol specifically.

A: We have added the information required.

4. The methods section should clearly state which concomitant medications were considered in this study as CYP2D6 substrates. Furthermore, not all CYP2D6 substrates are "inhibitors". Please refer to the page by David Flockhart in Indiana University for correct classification of medication as CYP2D6 substrate or inhibitor.

A: The analytical method has the most common medication, including anti-depressants, antipsychotic and other opioids. For the INIB group we have focused in the substances that are reported as inhibitors (also in the Flockhart chart). The outliers of the "others" group on the NDT/ODT graphic are, curiously,

positive for compounds that are also substrates of CYP2D6, as venlafaxine and nortriptyline. Done as suggested.

5. There are over 100 allelic variants of CYP2D6. In this study, the author's method only categorizes 4 of these variants. The authors then subscribe individuals to either poor, intermediate, or extensive metabolizers based on only 4 variants and make several assumptions on their tested population to rationalize this approach. As their testing approach is not the gold standard in pharmacogenetic testing, given its limited scope of testing, they need to compare their methodology with a platform that tests for the majority of CYP2D6 alleles in order to justify these assumptions. A false positive and false negative rate of attributing an individual to the poor metabolizer phenotype based on only 4 variants needs to be provided.

A: We have clarified in the manuscript the aims of our work.

The focus was to detect the PM. We have tried to explain better the exceptions. We changed also the assignment of the genotypes based in the variants searched to restrict it to the PM.

On the other hand, we described in more detail the validation of the method. It is not possible for us to compare our results with other methodology as suggested, but we had used reference materials to evaluate the accuracy of the method.

We would like to thank both reviewers for the deep appreciation of our manuscript and for all the suggestions. We tried to fulfill all the requirements and we will answer point-by-point to the comments

Reviewer #2:

1. The present manuscript describes a Sanger sequencing method for detection of some CYP2D6 polymorphisms causing a reduced or absent enzyme function, as well as a GC/MS method for the quantitation of tramadol, O-desmethyltramadol and N-desmethyltramadol. The methods were applied to 100 tramadol positive post-mortem samples. The purpose of doing that is however not clearly stated. Several papers on CYP2D6 sequencing methods and quantitative tramadol methods have been published previously. Furthermore, many of those are better described in terms of validation and performance and are using techniques able to detect also the CYP2D6 ultra-rapid metabolizers and quantifying the enantiomers of tramadol and its metabolites. If the authors clearly state the aim of identifying poor metabolizers and further describe and discuss the six cases being poor metabolizers the present manuscript could however add on to the current knowledge within this field.

A: We have clarified in the manuscript the aims of our work.

2. Abstract:

* "A Sanger sequencing methodology was developed for the detection of genetic variants that cause absence of CYP2D6 activity, such as *3, *4, *6, *8, *10 and *12 alleles". As mentioned later in the manuscript *10 causes a decreased function of the enzyme, not a total absence. Consider to reformulate, for example "...genetic variants that cause absent or reduced CYP2D6 activity".

A: Done.

* Why is not all alleles possible to identify with the present method mentioned?

A: The amplification of the entire gene is very difficult (> 5000pb), specially in post-mortem samples with high degradation index. On the other hand, Sanger sequencing only allows the analysis of amplicons with a maximum of 1000 pb. To detect copy number variation is necessary to use other methods, with other equipments such as real-time PCR or platforms as AmpliChip CYP450 test from Roche. The method that we have used is simple, rapid, low cost and available in forensic genetics labs and can detect the main variants. We had clarified it on the manuscript as required.

3. Introduction:

* The aim of the study is not clearly stated. Why was the study conducted? What was the research question? Any hypothesis?

A: We have clarified in the manuscript the aims and scope of our work.

* In the first paragraph it is written that "The information about therapeutic concentrations of ODT is scarce". There are however several publications covering both tramadol and ODT concentrations.

A: The sentence was removed. There are several publications with clinical studies but the toxic or lethal concentrations aren't well defined, to our knowledge.

* UMs are usually referred to as ultra-rapid metabolizers but in this paper the term ultra-metabolizers are used instead. Consider changing that.

A: Changed

* I also suggest that explanations of abbreviations, for example "poor metabolizer (PM)", are given the first time the word is written. Subsequently only the abbreviation is used.

A: Changed

4. Materials and methods:

* If the toxicological analysis has been published previously a reference referring to that publication could be added to section 2.2. If there is no previous publication the sample preparation and validation could be further described. The validation parameters investigated are given but no information on how the experiments were conducted. What chemicals, reagents and reference compounds were used? What quality control levels were used? How many replicates were utilized? What kind of blank blood was used? What were the predetermined acceptance criteria for each validation parameter? Please clarify in the manuscript.

* It is also important that results are given for all the validation parameters investigated, and they could with advantage be given in the results and discussion section.

* It is stated that the coefficient of variation is under 11% for the three compounds. Clarify what parameters that are referred to.

* Was dilution integrity part of the validation? Since calibration ranges between 0.05-1 mg/L and some samples have significantly higher concentrations I assume some dilution was made? Please clarify in the manuscript.

A: we described in more detail the validation of the method.

* What transitions were used in SIM mode? Add those to the manuscript.

A: added as requested.

* The two first comments for this section are applicable also to the validation of the sequencing method. There is no information on how the validation experiments were conducted and results are scarce. "The quality of the sequences was considered good", in terms of what? Accuracy is an especially important parameter when it comes to Sanger sequencing; how was the accuracy experiment conducted and what was the result of it? Please describe in the manuscript.

A: we described in more detail the validation of the method.

* Should pb be bp instead, bp for base pairs?

A: Changed

* Which polymorphisms were used for the identification of allele *8, *12, *14, *15, *40 etc., mentioned on page 5? Add those to the manuscript.

A: Done

* The thermocycling conditions used for each fragment could be described in the manuscript.

A: Done

5. Results and discussion:

* The first sentence on page 6 is in my opinion not necessary.

A: Removed

* Check the maximum concentration for NDT in table 1, should it be 5889?

A: Corrected

* It does not emerge from table 1 that 27 cases had metabolite concentrations below the detection limit (is the limit of detection and the limit of quantitation the same?), as it does in the text. Consider if it should since minimum values for the metabolites are presented in the table, and the table text says descriptive statistics for all the 100 cases.

A: Corrected.

Values < 50 ng/mL and > 5000 ng/mL were obtained by extrapolation of de calibration curves.

* Concerning the first and second sentence in section 3.2: What was the degradation index of the three samples that failed amplification in comparison to the degradation index of the other samples? That information would be of interest to add to the manuscript.

A: added as requested.

as suggested * If it is desirable to shorten the manuscript figure 1 and 2 could be deleted since the information is given in the text as well.

A: Removed

* In figure 2 and table 2 the text implies results for all the 100 post-mortem samples. However, since 3 samples failed amplification the results are only for 97 samples, right? Prevalences in table 2 as well as allele frequencies in figure 2 need to be slightly adjusted in case of calculating with 100 samples, which seems to be the case since the sum of prevalences in table 2 should be 100% but is only 97%.

A: Corrected.

* The allele frequency of the null allele *5 (which was not searched for) is higher than that of *3 and *6 (which was searched for) in a Caucasian population. Therefore consider to reformulate the following sentences on page 7 "All the alleles that hadn't any variant in the studied fragments were considered as wild type. This assumption was considered acceptable because the prevalence of the other null alleles that weren't searched for is very low in Caucasian population".

A: The sentence was removed and we have tried to explain better the exceptions, focusing in the identification of the PM, which is the main purpose of the study.

* In section 3.2 it is written "Since this method is not able to detect CNV events, the ultra-metabolizers were included in the group of EM. This assumption was considered acceptable once the final objective of the present work was to detect the PM". What was the purpose of constructing figure 3 if only PMs were important to find? Consider to reformulate.

A: We changed the assignment of the genotypes based in the variants searched to restrict it to the PM. Thank you for your suggestions.

* A further description of the INIB group would be valuable. Were those cases PM, IM or EM? What was the concentrations of the inhibitors? To be able to draw conclusions about the significance of the inhibitors a comparison between for example EMs with and without inhibitors seems more valuable than putting all cases with inhibitors in one group, regardless of genotype.

A: Since we had focused in the PM cases, we removed the EM and IM assignment. On the other hand, and unfortunately, the available information of the cases is scarce. We were not able to do this.

* Table 3 is in my opinion not necessary. It would be more interesting to compare all the measured values for each group, than just comparing medians between groups. Statistically significant differences based on all measured values for each group could be indicated in figure 3, a separate table is not necessary. The number of individuals that are given in table 3 could be given in the text below figure 3.

A: Done as suggested.

* Abbreviations are not explained in the text below figure 3 which would be good.

A: corrected as suggested.

* Why does the authors conclude that the NDT/ODT ratio is a better measure of TMD to ODT metabolism than the TMD/ODT ratio? The correlation seemed better for the NDT/ODT ratio, yes, although other factors than the CYP2D6 genotype might affect this ratio. A discussion concerning the impact of CYP2B6 and CYP3A4 genotype for the formation of NDT would be meaningful. If the authors think that the correlation between the TMD/ODT ratio and CYP2D6 genotype was less than expected a discussion concerning reasons for this would be highly valuable. Could for example the inability of the sequencing method to detect allele *5 and multiple copies of the gene have had an impact on the results? Or could the classification into IMs and EMs have had an impact? The present classification is not wrong although according to other definitions EMs have two functional alleles. The present EM group includes individuals with both one, two and multiple copies of CYP2D6 alleles.

A: We have tried to explain it better, especially for post-mortem cases.

* The information in table 4 is interesting, as well as the measured concentrations in table 5, although the concentration unit must be given in table 5. This information could however be compiled in one table, while DNA-concentration, degradation index and polymorphisms are left

out. The important thing is that all individuals are PMs and that information is given in the table text.

A: Done as suggested.

* The results of table 4 and 5 are only discussed with a few sentences on page 11, saying that in three cases the tramadol concentration was higher than the therapeutic range but ODT concentrations were acceptable. What does that mean, what are the conclusions drawn? Case 2 and 3 have "unknown" and "natural" stated as the cause of death, respectively, in spite of high tramadol concentrations. Was the tramadol concentrations not considered toxic (because of low ODT concentrations)? What were the circumstances of death? Any comments regarding the other cases? Please discuss the results in more detail.

* What substances were included in the toxicological screening? It could be interesting to describe case 3,4 and 5 (without other substances present) in more detail.

* It could perhaps be of interest to show the concentrations and CYP2D6 genotypes for the 10 intoxication cases mentioned in section 2.1.

A: Done as suggested, although we cannot discuss better the results because of the lack of information. The study of the autopsy results of these cases together with the pathologists would be indeed very interesting but it was not possible yet.

6. Conclusions:

* "The Sanger sequencing method proved to be specific, accurate, with a good precision and limit of detection". As mentioned previously results for all validation parameters are not presented in the manuscript, for what reason it is difficult for the reader to be able to draw the same conclusion.

A: We have reformulated the manuscript and included the validation parameters.

* "The results of genotypes showed a good correlation with toxicology results of the tramadol metabolic ratio NDT/ODT, appearing to be a better parameter to know the degree of metabolization of TMD to ODT, than the usual metabolic ratio as TMD/ODT". See also the comments above in the "results and discussion" section. In my opinion this is not a correct conclusion.

A: We have reformulated the results and discussion and also the conclusions in the manuscript.

* "The presence of enzymatic inhibitors affects significantly the degree of metabolization"
See also the comments above in the "results and discussion" section. It is known that some drugs acts as inhibitors of CYP2D6 and therefore affect the metabolism of tramadol. In my opinion it is however not satisfactory shown in this manuscript.

A: We have reformulated the conclusions in the manuscript.

* "The detection of allelic variants described as non-functional were useful to explain some circumstances of death in the study of tramadol positive cases and demonstrate the importance of this genetic tool to forensic toxicology and pathology". What circumstances were clarified in

this study? A more profound discussion regarding the results is desirable.

A: As said above, it was not possible yet. Nevertheless, the cases number 1 and 3 can be good examples of the future applications of this approach.

7. References:

* Is reference 8, 12 and 13 complete? Where can one find them?

A: Corrected

* Where is reference 19 published?

A: Corrected

* I am not sure if user guides (reference 20 in this case) are appropriate to refer to in the reference list?

A: Removed