Concentrations of Tramadol and *O*-desmethyltramadol Enantiomers in Different *CYP2D6* Genotypes

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The influence of *CYP2D6* genotype and CYP2D6 inhibitors on enantiomeric plasma levels of tramadol and *O*-desmethyltramadol as well as response to tramadol was investigated. One hundred and seventy-four patients received one hundred intravenous tramadol 3 mg/kg for postoperative analgesia. Blood samples drawn 30, 90, and 180 min after administration were analyzed for plasma concentrations of the enantiomers (+)-, (−)tramadol and (+)-, (−)*O*-desmethyltramadol by liquid chromatography-tandem mass spectrometry. Different *CYP2D6* genotypes displaying zero (poor metabolizer (PM)), one (heterozygous individual (HZ)/intermediate metabolizer (IM)), two extensive metabolizer (EM), and three (ultra rapid metabolizer (UM)) active genes were compared. Concentrations of *O*-desmethyltramadol differed in the four genotype groups. Median (1/3 quartile) area under the concentration–time curves for (+)*O*-desmethyltramadol were 0 (0/11.4), 38.6 (15.9/75.3), 66.5 (17.1/118.4), and 149.7 (35.4/235.4) ng · h/ml for PMs, HZ/IMs, EMs, and UMs ($P<0.001$). Comedication with CYP2D6 inhibitors decreased (+) *O*-desmethyltramadol concentrations ($P<0.01$). In PMs, non-response rates to tramadol treatment increased fourfold compared with the other genotypes ($P<0.001$). In conclusion, CYP2D6 genotype determined concentrations of *O*-desmethyltramadol enantiomers and influenced efficacy of tramadol treatment.

Cytochrome *P*450 (CYP) 2D6 genetic variability is supposed to be a major variable of adverse drug reaction, influencing hospital stay and total costs.[1] About 40 commonly used drugs are metabolized by this enzyme (www.medicine.iupui.edu/flockhart/table.htm). Polymorphisms within CYP2D6 have been associated with altered enzyme activity. In contrast to carriers of two wild-type alleles (extensive metabolizers (EMs)), heterozygous individuals (HZ) with only one functionally active allele, intermediate metabolizers (IMs) with two variant alleles known to decrease enzymatic capacity, and poor metabolizers (PMs) with no functionally active alleles are predicted to have either reduced or no enzyme activity, respectively. PMs display a frequency of about 7–10% in Caucasian populations.[2,3] They are at increased risk to suffer from adverse drug effects by overdose from β-blockers, antiarhythmics, tricyclic antidepressants, antipsychotics, etc. Blood levels might exceed the therapeutic range because of the lack of catalytic activity of CYP2D6. In contrast, ultra rapid metabolizers (UMs) display a *CYP2D6* gene duplication or multiduplication which significantly increases enzyme activity.[4,5] This can result in therapeutic failure owing to drug levels below the therapeutic range.

In the case of pro-drugs like codeine and tramadol, PMs experience no or reduced analgesia, respectively.[6,7] Tramadol hydrochloride (T) is a weak opioid with its ì-opioid receptor analgesic properties preferentially mediated by its M1 metabolite.[6,7] Furthermore, the clinically used racemic mixture of the transisomer inhibits noradrenaline and serotonin reuptake, which also contributes to its analgesic properties.

This study was conducted in a clinical setting, enrolling patients under concomitant medication who had recovered from major abdominal surgery. It describes the course of sequential enantiomer concentrations of tramadol and its major metabolite *O*-desmethyltramadol (ODT), which mediates tramadol efficacy within the most important 3 h

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following tramadol administration. Dependence of enantiomer concentrations on genotype uncovers further the impact of genetic variation on efficacy of this pain medication.

RESULTS

Demographic data and genotypes

Of the 187 patients genotyped, 68 were categorized as EMs (**Table 1**). Genotyping revealed 18 individuals with no active CYP2D6 allele. One of 11 patients with a gene duplication was heterozygous for the **4* polymorphism and was categorized as an individual with only one active *CYP2D6* allele (**1/*4xN*) because of reduced ODT levels. Two further patients presenting a gene duplication and an additional heterozygous **41* single-nucleotide polymorphism (**1/*41xN*) showed a phenotype with reduced enzyme activity and were allocated to the HZ/IM group.

Two subjects had atypical melting curves in the LightCycler assay and thus, additional sequence analysis was performed. One of these patients was of black African origin and had a homozygous G1716A base exchange, which is typical for the **45* or **46* polymorphism. The other individual with wild-type alleles for all polymorphisms investigated displayed low (+)ODT concentrations. Additional sequencing revealed a heterozygous *T1678C* base exchange within intron 2. No comedication was reported in the patient's history and the reason for reduced enzyme activity remains unexplained. For statistical analysis, this patient was allocated to the HZ/IM group.

Eleven patients were heterozygous for **10*. As CYP2D6-dependent metabolite levels were slightly reduced in these individuals, they were allocated to the HZ/IM group. In parallel, heterozygous **41* individuals were also categorized to the HZ/IM group with the exception of one patient presenting an additional homozygous **4* polymorphism (*2D6*4/*4*41*) and concentrations of (+)ODT below the detection limit. The **7* and **8* alleles were not detected. Allele frequencies amounted to 0.019 for allele **3*, 0.198 for allele **4*, 0.019 for allele **5*, 0.008 for allele **6*, 0.029 for allele **10*, and 0.083 for allele **41* and 0.029 for the gene duplication. There was no deviation from Hardy–Weinberg equilibrium with *P*-values ranging between 0.8 and 0.99.

Demographic and surgery related data as well as tramadol-loading doses before emergence of anesthesia did not vary between the four different genotype groups (**Table 2**).

Concentrations of tramadol and ODT enantiomers

Complete laboratory results with enantiomeric tramadol and ODT concentrations were available in 174 patients. Logistic reasons made blood sampling or measurement of drug concentrations at all time points impossible in 14 patients (eight HZ and six EM subjects).

Quantification of tramadol enantiomers revealed high plasma levels, with both enantiomers ranging between 55 and 1,100 ng/ml. Tramadol concentrations were highest 30 min after infusion and decreased during the following 2.5 h, with UM showing the most pronounced metabolism.

Table 1 Number/percentage of patients allocated to the different genotypes

Genotype *2D6*	Active genes	Enzyme activity	Assigned to group	Number of patients	% of patients
**1/*1*	2	Normal	EM	68	36.36
**1/*3*	1	Reduced	HZ/IM	2	1.07
**1/*4*	1	Reduced	HZ/IM	41	21.92
**1/*5*	1	Reduced	HZ/IM	5	2.67
**1/*6*	1	Reduced	HZ/IM	1	0.53
**1/*10*	2	Reduced	HZ/IM	8	4.28
**1/*41*	2	Reduced	HZ/IM	23	12.3
**10/*41*	2	Reduced	HZ/IM	2	1.07
**3/*41*	1	Reduced	HZ/IM	2	1.07
**4/*41*	1	Reduced	HZ/IM	2	1.07
**6/*10*	1	Reduced	HZ/IM	1	0.53
**6/*41*	1	Reduced	HZ/IM	1	0.53
**45/*45* or **46/*46*	2	Reduced	HZ/IM	1	0.53
**1/*1* and *T1678C*[a]	?	Reduced	HZ/IM	1	0.53
**3/*4*	0	No	PM	1	0.53
**3/*5*	0	No	PM	2	1.07
**4/*4*	0	No	PM	14	7.48
**4/*4*[b]	0	No	PM	1	0.53
**1/*4xN*	1	Reduced	HZ/IM	1	0.53
**1/*41xN*	1	Reduced	HZ/IM	2	1.07
**1/*1xN*	⩾3	Increased	UM	8	4.28

EM, extensive metabolizer; HZ/IM, heterozygous individual/intermediate metabolizer; PM, poor metabolizer; UM, ultra rapid metabolizer. [a]The *T15678C* SNP is not listed by the 2D6 nomenclature committee. Reduced enzyme activity cannot be explained sufficiently in this subject without any CYP2D6-inhibiting comedication. [b]The patient had an additional heterozygous **41* SNP.

ODT was found in much lower concentrations than the parent drug. Concentrations at 30, 90, and 180 min for all patients amounted to 22.1 ± 3.1, 22.8 ± 2.0, and 30.3 ± 2.8 ng/ml for (+)ODT and 51.9 ± 7.3, 52.1 ± 4.8, and 57.0 ± 4.9 for (−)ODT, respectively. Concentration of ODT showed an increase over 180 min in carriers with at least one active allele (**Figure 1**). ODT levels were significantly higher in these genotypes compared with PMs 90 and 180 min after tramadol administration ($P < 0.001$).

Area under the concentration–time curves (AUCs) of tramadol did not differ between the (+)- and (−)enantiomer. Owing to the high variability of the enantiomer concentrations, specifically in PMs and HZ/IM individuals, there was no significant difference for concentrations between the genotypes (**Figure 2**). AUCs of (+)- and (−)ODT both clearly separated the PM subjects from the other genotypes, with PMs displaying the lowest values (Kruskal–Wallis test; $P < 0.001$) (**Figure 2**). There was no difference between HZ/IMs and EMs as well as EMs and UMs. In general, concentrations of (−)ODT were higher than of (+)ODT.

Table 2 Demographic and perioperative data in PM, HZ/IM, EM, and UM groups

	PM	HZ/IM	EM	UM
Number of patients	18	93	68	8
Age (years)	58.6±14.4	58.9±11.5	55.5±12.5	52.6±15.4
Body weight (kg)	73.6±15.9	77.4±18.1	78.6±16.3	78.8±15.3
Height (cm)	172.4±10.7	172.6±8.6	173.7±15.4	172.0±9.5
Duration of surgery (min)	172.5±74.3	170.6±60.3	169.1±66.1	163.8±55.5
Duration of anesthesia (min)	211.0±72.2	210.4±64.4	208.0±68.2	205.0±74.3
Sex (male/female)	11/7	58/35	49/19	5/3
ASA classification I/II/III[a]	0/16/2	10/63/20	13/41/14	0/6/2
Trauma of surgery				
Small	2 (11.1%)	10 (10.7%)	4 (5.9%)	1 (12.5%)
Medium	15 (83.3%)	75 (80.7%)	56 (82.4%)	6 (75.0%)
Large	1 (5.6%)	8 (8.6%)	8 (11.7%)	1 (12.5%)
Tramadol-loading dose (mg)	219.4±45.8	218.7±42.9	225.9±38.5	221.2±32.2

EM, extensive metabolizer; HZ/IM, heterozygous individual/intermediate metabolizer; PM, poor metabolizer; UM, ultra rapid metabolizer. [a]American Society of Anesthesiologists physical status classification: I, healthy patient, no medical problems; II, mild systemic disease; III, severe systemic disease, but not incapacitating.

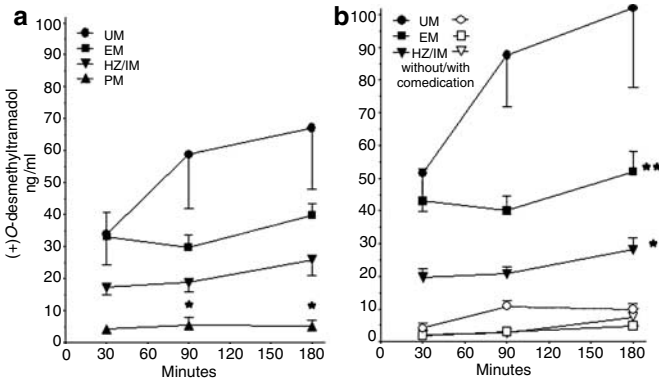

Figure 1 Concentration of (+)ODT (mean±SEM) at 30, 90, and 180 min after i.v. bolus dose of tramadol. (**a**) Allocated to genotype groups UM ($n=8$), EM ($n=62$), HZ/IM ($n=85$), and PM ($n=18$); *$P<0.001$ at 90 and 180 min. (**b**) Allocated to genotype groups without and with comedication: UM (six patients without/two patients with); EM (47 patients without/15 patients with), and HZ/IM (77 patients without/eight patients with). *$P<0.05$ for group HZ/IM without comedication *versus* HZ/IM with comedication; **$P<0.001$ for group EM without comedication versus EM with comedication.

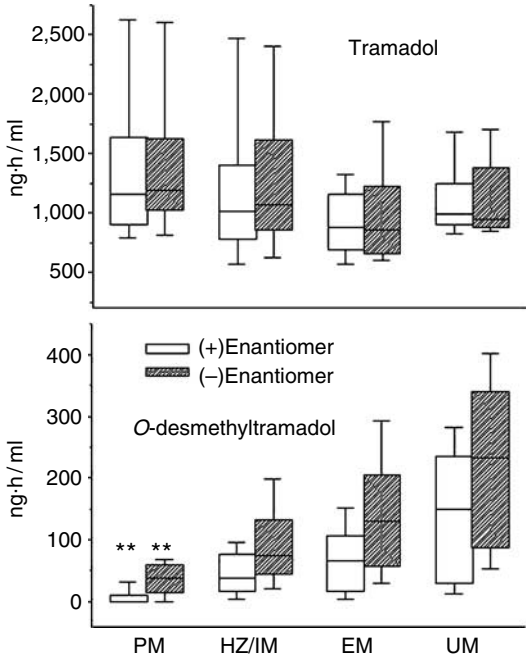

Figure 2 AUC for (+)T and (−)T as well as (+)ODT and (−)ODT according to the number of active *CYP2D6* genes. Box plots show medians (black line), interquartile range (boxes), and 5/95% percentile. **$P<0.001$ for (+)ODT and (−)ODT. Kruskal–Wallis test.

Comedication

Variability of drug and metabolite concentrations was high. This was in part due to pretreatment with comedication, which inhibits cytochrome activity. Of the patients enrolled in this study, 128 were under concomitant medication. Of these, 26 individuals (HZ/IMs: nine, EMs: 15, UMs: two) were verified for taking long-term medication including antidepressants, metoclopramide, ranitidine, or other medication and revealed a blocked enzyme activity. **Figure 1b** differentiates patients with at least one functional *CYP2D6* gene in subgroups with or without comedication. In patients with one and two active genes and long-term comedication with CYP2D6 inhibitors, concentrations for both ODT enantiomers were significantly lower compared to the respective genotype without comedication.

AUCs for EMs with or without concomitant CYP2D6 inhibitors amounted to 88.7 (49.3/130.6) versus 6.6 (1.8/

14.9) ng · h/ml ($P<0.001$) for (+)ODT and 176.3 (97.2/272.4) versus 44.3 (28.9/55.3) ng · h/ml ($P<0.001$) for (−)ODT. The respective data for the HZ/IM group were 44.1 (20.8/80.0) versus 8.0 (3.9/11.6) ng · h/ml ($P<0.001$) for (+)ODT and 83.2 (51.0/136.7) versus 46.0 (23.0/54.1) ng · h/ml ($P=0.01$) for (−)ODT. Owing to the small number of patients, the difference was not significant for comparison of UM without (+)ODT: 200.0 (155.0/270.9); (−)ODT: 412.5 (286.3/554.5) versus UM taking CYP2D6 inhibitors. AUC of (+)ODT in these two subjects with concomitant comedication amounted to 18.3 and 46.4 ng · ml h, AUC for (−)ODT amounted to 9.5 and 68.6 ng · h/ml.

However, variability of ODT concentrations remained unexplained in a few other patients. Although genotype indicated CYP2D6 activity and medication history revealed no inhibiting concomitant drug, concentrations of (+)ODT were low in one UM, three EMs, and three HZ/IMs.

Response to pharmacologic treatment

A total of 177 patients completed the 48-h patient-controlled analgesia (PCA) period and analgesic response could be evaluated. In 10 individuals (PM: two, HZ: four, EM: four), outcome could not be assessed; in four patients because of prolonged postoperative intubation and mechanical ventilation; in three because postoperative pain treatment by PCA was not necessary owing to an unplanned short surgical procedure and low pain scores. Violation of the study for protocol was the reason for exclusion of three other patients.

In the postanesthesia care unit, 86 patients (PM: 10, HZ: 24, EM: 18, UM: six) had pain scores >40 and received a second dose of tramadol 50–100 mg. In 12 patients, this dose was given before the third blood sample was drawn. As most of these patients were PMs, influence on ODT concentrations was negligible and calculation of drug concentrations was not corrected for this additional tramadol application. Ten PMs needed rescue medication piritramide in the postoperative care unit, significantly more than patients with at least one wild-type allele ($P<0.001$) (**Figure 3**).

Cumulative analgesic consumption allocated to genotypes is displayed in **Figure 4**. PMs needed more analgesics compared with HZ/IM subjects and EM subjects after 24 and 48 h ($P=0.002$). Owing to the small number of patients in the UM group, statistical analysis of the analgesic consumption in this group was not feasible.

After the 48-h study period, more PMs (81.25%) were categorized as non-responders compared to patients with CYP2D6 activity ($P<0.001$). There was no difference in response rates between patients of the HZ/IM group, EMs, and UMs (**Figure 3**).

DISCUSSION

Altered CYP2D6 enzyme activity is a major variable of pharmacologic response. Previous studies demonstrated the influence of genetic polymorphisms on the phenotype of drug response to antipsychotics, antidepressants, codeine, tramadol, and others.[5,6,8–11]

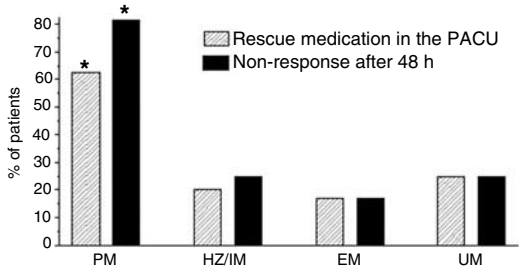

Figure 3 Non-responders to tramadol treatment allocated to genotypes. Grey columns: % of patients needing rescue medication in the postanesthetic care unit (PACU); black columns: % of non-responders after the 48-h study period. *$P<0.001$ for the PM group compared with the HZ/IM, EM, and UM groups.

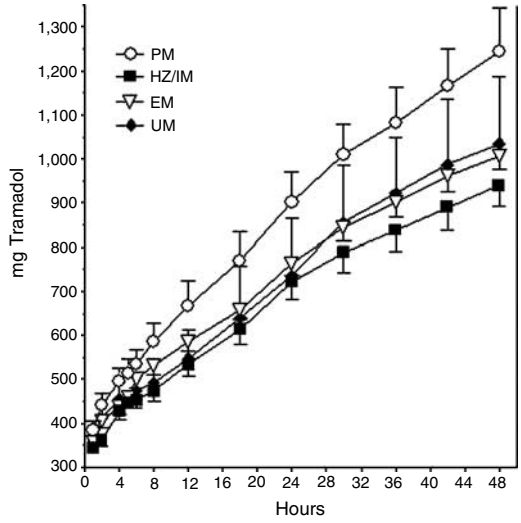

Figure 4 Cumulative analgesic consumption (mean ± SEM) over the 48-h study period in the PM ($n=16$), HZ/IM ($n=89$), EM ($n=64$), and UM groups, ($n=8$). Tramadol loading dose, tramadol bolus application via PCA, and rescue medication piritramide (conversion piritramide: tramadol = 1:10) are considered.

There is considerable need to improve clinical outcome in pharmacotherapy. In pain management, therapy remains insufficient in numerous patients, although a wide range of drugs and techniques are available. Besides lack of compliance, poor timing of drug administration, prescription on an "as-needed basis", insufficient dosing as well as organizational and logistic reasons, which are major pitfalls, the genetic background of a patient is a further variable influencing individual analgesic needs.

Several studies report the contribution of CYP2D6 to tramadol metabolism and the μ-opioid receptor-mediated analgesic effects of M1.[6,7,10,12] Concentrations of (+)ODT are of specific interest, as this enantiomer has the highest μ-opioid receptor affinity and intrinsinc efficacy, thus predominantly contributing to opioid analgesia.[13] In addition, the monoaminergic effect of the parent compound itself also mediates analgesic effects.[10,14,15] In this prospective study, the influence of *CYP2D6* genotype was studied on tramadol pharmacokinetics and clinical outcome in patients

recovering from major abdominal surgery. In contrast to most previous investigations, where healthy volunteers were included,[10,15,16] this trial investigates a representative patient cohort suffering from various medical conditions and taking additional drugs, mainly because of pre-existing cardiovascular disease, lung disease, or cancer. Thus, this study design provides an everyday clinical setting and no highly selected study population.

Blood sampling was restricted to three time points during the patients' stay in the operating and recovery room. Logistic reasons made it impossible to draw additional blood samples, as patients were discharged to various peripheral wards of the University Hospital. Additionally, further pain management was provided by PCA, which allowed no standardized analgesic regimen, but individual application of tramadol bolus doses according to the patients' needs.

Concentrations of tramadol enantiomers

Large interindividual variations were found in concentrations of tramadol enantiomers after an i.v. bolus dose of tramadol 3 mg/kg. Tramadol doses administered to the patients were high. However, in other clinical trials, equivalent doses were used in postoperative patients.[17,18] Compared with previous data, racemic concentrations were within the range reported by other authors.[19,20] Lower serum levels of 590 ± 410 ng/ml were described under PCA therapy with blood samples drawn before a demand dose was requested.[21] Lehmann *et al.*[22] reported the minimum effective serum concentrations of the racemate ranging between 20.2 and 986.3 ng/ml (median 287.7 ng/ml). Concentrations of enantiomers in relation to genotypes have not been studied after i.v. tramadol in a clinical setup until now.

Concentrations of M1

(+)ODT concentrations were of specific interest as this enantiomer is the main substrate-producing μ-opioid receptor-mediated analgesia. The metabolism of tramadol is stereoselective for the *O*-demethylation with PM hardly forming any (+)ODT, but does form detectable, albeit lower, levels than EM of (−)ODT,[23] a finding which is in line with the present results. Whereas CYP2D6 is practically the only enzyme forming (+)M1, other enzymes also contribute to the formation of (–)M1.[22] This study confirms the (–)ODT levels being generally higher than (+)ODT levels. In PM, no (+)ODT was measurable in all individuals presenting the **4/*4* genotype. Concentrations of (+)ODT were at the limit of detection (2–8 ng/ml) in **3/*4* and **3/*5* genotypes. In contrast, (–)ODT could also be detected in patients with CYP2D6 deficiency, thus suggesting that an additional enzyme is involved in the formation of (−)ODT. In general, concentrations of (−)ODT were about twofold of (+)ODT, an observation already reported after oral tramadol application.[24]

Genotyping and clinical outcome

Allele frequencies for *2D6*3*, **4*, **5*, **6*, **7*, *8, and **41* were within the range reported previously for a Caucasian population.[25,26] However, the predictive power of genotyping can be compromised by the number of variant alleles that remain uncharacterized, misclassified, or unidentified by the assay used.[7] In this study, some rare mutations which could not be identified by the real-time PCR approach remained undetected. Although this inherent bias cannot be precluded and misclassification of some subjects probably has occurred, results considering clinical outcome were distinct, with a significantly higher rate of non-responders in PM compared to patients with at least one wild-type allele. This strongly confirms previous results of experimental and clinical trials, finding an impact of *CYP2D6* genotype on analgesic efficacy of tramadol.[5,8] Biotransformation of (+)T to (+)ODT is crucial for the opioid effect; however, serotonergic and monoaminergic interactions also contribute to the overall analgesic efficacy.[10,12,14] Genotyping for *CYP2D6* alleles resulting in deficient catalytic activity can identify those patients at fourfold increased risk for non-response to tramadol treatment.

Comedication

Comedication may compromise drug safety by increasing the risk of drug interactions and adverse events, a fact often underestimated in patients.[27] Comedication can produce enzyme induction or inhibition mimicking genetic defects,[23,28,29] which also contributes to the variable response to drugs. Well known CYP2D6 inhibitors are paroxetine, amiodarone, cimetidine, and ranitidine. In some patients with at least one wild-type allele and low (+)ODT concentrations, CYP2D6 inhibitors were taken. The subgroup analysis considering comedication revealed that some of the high variability in drug and metabolite concentrations is a result of this comedication. This is in line with previous studies reporting an influence of paroxetine on stereoselective *O*-demethylation of tramadol and a reduction of its hypoalgesic effect in healthy volunteers.[23] In women suffering from breast cancer, CYP2D6 inhibitors influenced tamoxifen metabolism and an impact on response to tamoxifen therapy was supposed.[29]

Although comedication can have a profound influence on tramadol metabolism and some genotypes were turned to pharmacologically induced "poor" or "low" metabolizers; this did not compromise the overall findings. Notwithstanding this bias, the results considering genotype, (+)ODT concentrations, and response to tramadol were unequivocal.

CONCLUSION

Enantioselective analysis of tramadol and ODT was performed in a clinical setting enrolling patients recovering from major abdominal surgery. Variability of ODT concentrations was closely correlated to *CYP2D6* genotypes. Concomitantly used CYP2D6 inhibitors also contributed to variability of tramadol metabolism. Non-response rates to pain medication increased fourfold in PM and thus this genotype was associated with poor efficacy of tramadol analgesia.

METHODS

Patients. Approval of the study design was obtained from the local Ethics Committee. After providing written informed consent, 187 patients (ASA classification I–III) scheduled for elective, larger abdominal surgery were instructed of the details of the study, the use of the PCA device, and the numeric rating scale for pain intensities (NRS: 0 denotes no pain, 100 denotes worst pain imaginable). Reasons for exclusion from the study were alcoholism, drug dependence, psychic diseases, epilepsy, known opioid intolerance, serious perioperative complications, and changes in anesthetic procedure. Pre-existing medication was documented and discontinued only for the day of surgery with the exception of antihypertensive and cardiac disease drugs. The majority of patients were of central European origin; one patient was from Asia, six were of Arabian origin (Tunesia, Morocco, Syria, Turkey), one of black African origin, and two from Eastern Europe.

Clinical study protocol. General anesthesia for abdominal surgery and postoperative analgesia were conducted using a standardized protocol: propofol 2 mg/kg, fentanyl 0.2 mg, and *cis*-atracurium for induction and remifentanil, isoflurane 1 MAC, and *cis*-atracurium for maintenance of anesthesia. About 90 min before termination of anesthesia, an i.v. loading dose of tramadol (Tramal®, Grunenthal GmbH, Aachen, Germany) 3 mg/kg (maximum loading dose 250 mg) and dipyrone 1 g i.v. was infused via infusion pump over 15 min. After emergence of anesthesia, about 90 min after the another infusion of the study medication, patients were transferred to the recovery room where they were monitored for another 90 min. When patients had recovered from anesthesia, they were asked for pain intensities. At pain scores >40 at rest on the NRS, they received an additional dose of tramadol 50 mg, which could be repeated after 30 min. If analgesia remained insufficient in the postanesthetic care unit, rescue medication piritramide in 2-mg increments was given i.v.

For further analgesic treatment in the general ward, a PCA device (Injektomat®-CP PACOM, Fresenius AG, Bad Homburg, Germany) was provided and patients could self-administer i.v. bolus doses of tramadol 20 mg and dipyrone 200 mg. The delivery time of one bolus dose was 1.5 min and the lock-out time was 8 min.

In case of prolonged elevated pain scores, either additional rescue medication piritramide was administered or the analgesic regime was changed to piritramide via PCA.

Analgesic efficacy was assessed by pain intensities under rest and exercise/coughing by means of NRS and need for rescue medication piritramide in the postanesthetic care unit and during the further 48-h study period. Variables were recorded before the initial bolus and hourly up to the 8th hour, at 12 h and every 6 h up to the 48th hour. Patients were categorized as responders if they experienced sufficient analgesia during the 48-h study period without the need for rescue medication and expressed satisfaction with pain management. A patient was assessed as a non-responder if he/she needed rescue medication during the 48-h study period and/or gave a negative rating for pain management.

Analysis of drug concentrations. Blood samples were obtained with ethylenediaminetetraacetic acid as anticoagulant 30, 90, and 180 min after tramadol infusion. The whole blood was centrifuged and plasma and cells were separated and frozen at −80°C. Laboratory analysis was performed after completion of the clinical part of the study.

Quantification of (+)T and (−)T as well as (+)ODT and (−)ODT was performed by a liquid chromatographic-mass spectrometric assay with atmospheric pressure chemical ionization.[30] A mixture of plasma 0.5 ml, internal standard solution (10 μl of (+/−)Td$_4$ in a concentration of 10 μg/ml methanol) and 0.1 ml of 2 M NaOH were extracted with 1 ml dichloromethane/iso-propanol (85:15 v/v). After centrifugation (4,000 *g* for 8 min), the organic phase was evaporated to dryness under a stream of nitrogen at 50°C. The residue was dissolved in 0.1 ml of high-performance liquid chromatography mobile phase and a 10 μl-aliquot was used for chromatography (LC-MS/MS system consisting of an Agilent, Waldbronn, Germany) 1100 high-performance liquid chromatography system (binary pump, degasser and autosampler) coupled with an Applied Biosystems (Darmstadt, Germany) API 2000 triple quadrupole mass spectrometer. The enantiomeric separation was achieved on a Chiralpak AD column (10 μm, 250 × 4.6 mm; Daicel Chemical Industries, Illkirch Cedex, France) containing amylose tris-(3,5-dimethylphenylcarbamate) as chiral selector using a mobile phase (*n*-hexane/ethanol 97:3v/v, 5 mM triethylamine (TEA)) under isocratic conditions with a flow of 1 ml/min. From the molecular ions ($[M+H^+]$), the following transitions were detected in multiple reaction monitoring: (+/−)T *m/z* 264.1 → 57.9, (+/−)ODT *m/z* 250.1 → 58.0, (+/−)T-d$_4$ *m/z* 268.1 → 58.00.

For quantification, peak area ratios of the analytes to the corresponding deuterated standards were calculated as a function of the concentration of the substances. The (+)- and (−)enantiomers were calculated using (+)T-d$_4$ and (−)T-d$_4$, respectively.

Genotyping. For genotyping, genomic DNA was isolated from whole blood using the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN). A first PCR reaction was performed to amplify a 1,700-bp fragment of *CYP2D6*, using primers specifically designed to avoid amplification from the pseudogenes *CYP2D7* and *CYP2D8*. The 1,700-bp fragment was used as template for a real-time PCR (LightCycler™, Roche Diagnostics, Mannheim, Germany) using fluorescence-labeled hybridization probes specific for the *CYP2D6 *3 (2549 delA)*, **4 (G1846A)*, **6 (1707 delT)*, **7 (A2935C)*, **8 (G1758T)*, **10 (C100T)*, **14 (G1758A)*, and **41 (G2988A)* mutation (www.cypalleles.ki.se/cyp2d6.htm, gene accession no. M33388). Real-time PCR was performed as described previously with small modifications.[17] For analyses of the *CYP2D6*10 (C100T)* allele, the following primers and probes were used: forward primer 5′-CCATTTggTAgTgAggCAggT-3′, reverse primer 5′-gTCCTTCATgCCATgTATAAATgC-3′, sensor 5′-gCTgCACgCTACC-CACCA X-3′, and anchor 5′-LC Red640-gCCCCCTgCCACTgCCCg p-3′ (TIB Molbiol, Berlin, Germany). The PCR conditions were denaturation with 95°C for 30 s and amplification for 35 cycles (95°C for 3 s, 60°C for 10 s, 72°C for 12 s). Melting curves were obtained using a denaturation step (95°C for 20 s) and a final hybridization (40°C for 20 s) was performed from 40 to 95°C with a temperature transition of 0.2°C/s.

For the *CYP2D6*41 (G2988A)* allele, the following real-time PCR assay was established: forward primer 5′-gATggTgACCACCTCgAC-3′, reverse primer 5′-ggTgTCCCAgCAAAgTT-3′, sensor 5′-gCCTgTACCCTTCCTCCCTCg-FL, and anchor 5′-LC Red640-CCCCTgCACTgTTTCCCAgATgggC p-3′ (TIB Molbiol, Berlin, Germany); PCR conditions: 95°C for 3 s, 57°C for 10 s, 72°C for 12 s.

Furthermore, a long-range PCR assay was employed to detect a deletion affecting the entire gene (*CYP2D6*5* allele).[17,31] Duplication or multiduplication was detected by the method published by Løvlie *et al.*[4] Duplication assignment of either functional *2D6*1xN*, *2D6*41* or non-functional *2D6*4xN* was performed by considering results of duplication PCR, single-nucleotide polymorphism-PCRs, and phenotype (ODT concentrations) in parallel.

Selected PCR products of each genotype as well as samples with atypical melting curves were sequenced bidirectionally (ABI 377, Perkin Elmer, Weiterstadt, Germany) to confirm real-time PCR results.

Statistics. According to the number of active *CYP2D6* genes, subjects were assigned to one of four genotype groups presenting no (PM group), one (HZ group), two (EM group), or more than two (UM) functionally active genes. IM groups carrying two alleles moderately reducing CYP2D6 capacity were allocated together with the heterozygous subjects to the HZ/IM group. All patients in whom no single-nucleotide polymorphism was detected

were assigned to the EM group assuming two wild-type alleles (*1/*1). Hardy–Weinberg equilibrium was tested using χ^2 goodness of fit test.

Drug concentrations were expressed as mean ± SEM. Median (1/3 quartile) AUC was calculated for (+)T, (−)T, (+)ODT, and (–)ODT and compared between the genotypes using the Kruskal–Wallis test. χ^2 test was performed for comparison of response between the four genotypes. Differences were considered statistically significant for $P < 0.05$; adjusted for multiple testing.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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