# *OPRM1* and *CYP2B6* Gene Variants as Risk Factors in Methadone-Related Deaths

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**Methadone is a medication valued for its effectiveness in the treatment of heroin addiction; however, many fatal poisonings associated with its use have been reported over the years. We have examined the association between *CYP2B6* and μ-opioid receptor (*OPRM1*) gene variations and apparent susceptibility to methadone poisoning. Genomic DNA was extracted from postmortem whole blood of 40 individuals whose deaths were attributed to methadone poisoning. The presence of CYP2B6*4,*9, and *6 alleles and the *OPRM1* A118G variant was determined by SNP genotyping. CYP2B6 *4, *9, and *6 alleles were found to be associated with higher postmortem methadone concentrations in blood ($P \leq 0.05$). *OPRM1* A118G was also associated with higher postmortem methadone concentrations in blood but not to a level of statistical significance ($P = 0.39$). In these methadone-related deaths, *OPRM1* 118GA was associated with higher postmortem benzodiazepine concentrations ($P = 0.04$), a finding not associated with morphine-related deaths. The risk of a methadone-related fatality during treatment may be evaluated in part by screening for CYP2B6*6 and A118G.**

Methadone is a μ-receptor agonist valued for its effectiveness in the treatment of opioid dependency and pain management. Administered in a racemic mixture, methadone has a long plasma elimination half-life (between 13 and 55 h) and high bioavailability when administered orally (70–90%).[1–5] Extensively metabolized in the liver, methadone is converted into its primary metabolite, 2-ethylidene-1, 5-dimethyl-3, 3-diphenylpyrrolidine, by the cytochrome P450 enzymes *CYP3A4*, *CYP2B6*, and, to a lesser extent, *CYP2D6* (ref. 6).

Interindividual variation in blood methadone concentrations and toxic drug accumulation have been increasingly reported[3,7–11] and might be explained by pharmacogenomics.[3–4,11] In drug-tolerant individuals, blood methadone concentrations can reach >0.84 mg/l, whereas in cases of fatality blood methadone concentrations typically range from 0.4 mg/l to 1.8 mg/l. However, many fatalities occur with concentrations as low as 0.05 mg/l—significantly lower than the average concentration in blood.[12] This interindividual variation may be explained on the basis of alterations in drug pharmacokinetics brought about by the presence of genetic variations in the cytochrome enzymes responsible for methadone metabolism. Although several *CYP3A4* variants have been identified, and *CYP3A4* gene expression levels demonstrate interindividual differences of a magnitude of up to 40-fold,[13,14] no significant association between *CYP3A4* variants and methadone metabolism has been documented to date.[15]

*CYP2B6* is of interest because it is involved in the metabolism of a number of drugs, including midazolam,[16] ketamine,[17] bupropion,[18] and methadone.[6,11] Although it is expressed predominantly in the liver, *CYP2B6* can also be found at lower levels in the brain, stomach, lung, kidney and heart.[19] *CYP2B6* is highly polymorphic, and several genotypes have been identified.[16] CYP2B6*4/*4 is associated with allele *4 (A785G single-nucleotide polymorphism (SNP)), and this genotype produces a rapid metabolizer phenotype. When allele *9 (G516T SNP) is found in combination with allele *4, they form a haplotype corresponding to allele *6. The CYP2B6*6/*6 genotype produces a slow or poor metabolizer phenotype and has a prevalence of ~6% in Caucasian populations.[6,11,16] Slow metabolizers have lower enzymatic activity, which can result in drug accumulation and increased toxicity.

Genetic variations within the opioid receptors may also affect drug pharmacodynamics, thereby influencing the response to methadone. The μ-opioid receptor (*OPRM1*) is of particular interest because it is the preferential binding target of methadone. Several *OPRM1* variants have been identified, and the A118G missense SNP in exon 1 has been linked with significant reductions in β-endorphin binding,[20] increased morphine requirements,[21] protection from morphine-6-glucuronide-induced toxicity,[22] and susceptibility to drug addiction.[23] Therefore, gene variations such as the *OPRM1* A118G variation

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could affect drug binding and drug response. The association between A118G and methadone is unclear and requires further investigation.

Benzodiazepines are often administered to patients on methadone maintenance therapy to treat anxiety associated with drug addiction and heroin withdrawal;[24] however, the concomitant use of benzodiazepines and methadone can increase the risk of lethal respiratory depression.[25]

We explored the possibility that genetic variations in the μ-opioid receptor *OPRM1* and *CYP2B6* may be linked with susceptibility to methadone toxicity. For this purpose, we analyzed the prevalence of the *OPRM1* A118G variation and CYP2B6*4, *9, and *6 alleles in 40 cases of fatality attributed to methadone toxicity.

**Table 1 Drugs detected at postmortem in 40 cases of fatalities attributed to methadone as a cause of death**

| Psychoactive substance | Total |
|---|---|
| *Blood* | 40 (100%) |
| Ethanol | |
| Negative | 25 (62.5%) |
| ≤100 mg/100ml | 11 (27.5%) |
| 100–200 mg/100ml | 2 (5%) |
| 200–300 mg/100ml | 2 (5%) |
| Antidepressants | 11 (27.5%) |
| Benzodiazepines | 20 (50%) |
| Morphine | 15 (37.5%) |
| Dihydrocodeine | 2 (5%) |
| Quetiapine | 2 (5%) |
| Amphetamine | 1 (2.5%) |
| Propranolol | 1 (2.5%) |
| *Urine* | |
| Codeine | 3 (7.5%) |
| 6-Monoacetylmorphine | 2 (5%) |
| Cannabinoids | 11 (27.5%) |
| Cocaine and/or metabolites | 1 (2.5%) |

## RESULTS

The 40 postmortem cases in which methadone had been implicated in the cause of death included 34 men and 6 women, the majority of whom were Caucasian (97.5%). The mean age of the case subjects was 31 ± 1.6 (range: 17–60). Methadone, together with benzodiazepines, was present in 20 of the 40 subjects. Other drugs detected in the postmortem blood samples are listed in Table 1.

### CYP2B6 alleles and postmortem methadone blood concentrations

Of the 40 methadone-related fatalities, 14 (35%) were genotyped as heterozygous carriers of allele *9, and 1 fatality (2.5%) was genotyped as a homozygous carrier. These frequencies are higher than those reported previously[6,26,27] but within the expected range detailed on the National Center for Biotechnology Information website for Caucasian populations (http://www.ncbi.nlm.nih.gov/SNP). Heterozygous carriers of allele *4 were identified in 16 cases—a frequency of 40%, which is noticeably higher than that in other reports from Caucasian populations.[6,26–28] Allele *6 was identified in 15 cases; the frequency of 37.5% is, again, higher than that found in other studies.[26–28] As expected, the postmortem methadone blood concentrations (mean 0.56 mg/l) for the CYP2B6*1/*1 (wild type) genotype were lower to a statistically significant extent ($P < 0.05$) as compared with those of the other genotypes identified (Table 2). The frequency of CYP2B6*1/*1 (57.5%) was higher than those reported previously (43%, 22%) for living subjects;[16,26] however, only three alleles were observed in the subjects in this study.

All the CYP2B6 alleles tested (*4, *9, and *6) were associated with higher mean postmortem methadone blood concentrations ($P < 0.05$, independent *t*-test), reflecting poor methadone metabolism (Figures 1 and 2, Table 2).

**Table 2 CYP2B6*4,*9, and *6 alleles and associated postmortem methadone blood concentrations**

| | Frequency | Mean (mg/l) | SD | SEM | Median | *P* value |
|---|---|---|---|---|---|---|
| *CYP2B6* | | | | | | |
| A785G (allele *4) | | | | | | |
| AA | 23 | 0.56 | 0.296 | 0.062 | 0.56 | |
| GA and GG | 17 | 0.95 | 0.608 | 0.148 | 0.97 | 0.024* |
| G516T (allele *9) | | | | | | |
| GG | 25 | 0.58 | 0.353 | 0.071 | 0.56 | |
| TG and TT | 15 | 0.96 | 0.598 | 0.154 | 0.97 | 0.039* |
| Allele *6 | 15 | 0.96 | 0.598 | 0.154 | 0.97 | 0.039* |
| *1/*1 | 23 | 0.56 | 0.296 | 0.618 | 0.56 | 0.024* |
| *1/*4 | 2 | 0.86 | 0.948 | 0.670 | 0.86 | 0.690 |
| *1/*6 | 14 | 0.93 | 0.609 | 0.163 | 0.91 | 0.041* |
| *6/*6 | 1 | 1.38 | | | | 0.153 |

*Significance at <0.05.

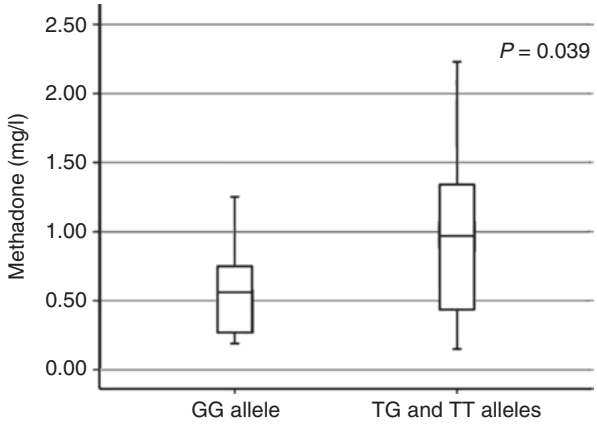


**Figure 1** Distribution (median and interquartile range) of postmortem methadone concentrations associated with the 516GG, 516TG, and 516TT genotypes.

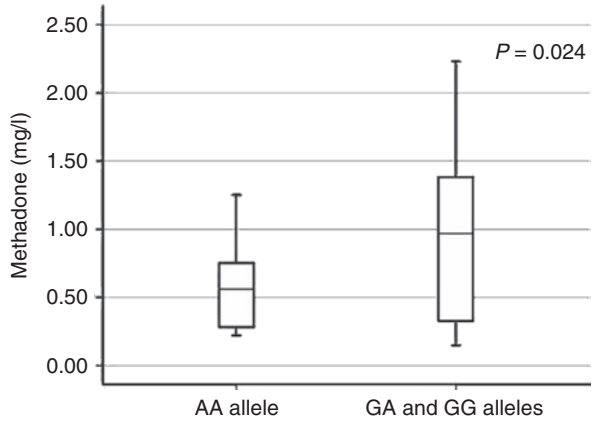


**Figure 2** Distribution (median and interquartile range) of postmortem methadone concentrations associated with the 785AA, 785GA, and 785GG genotypes.

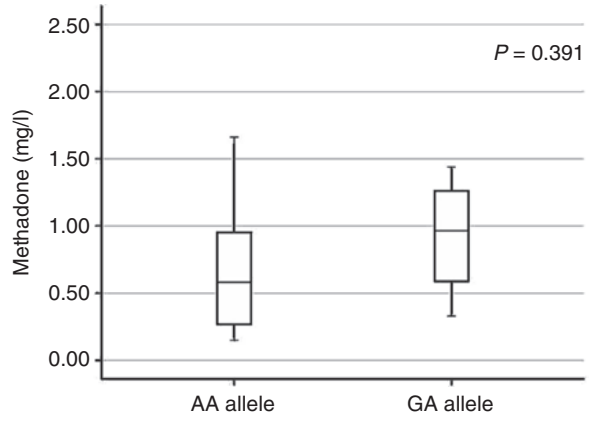


**Figure 3** Distribution (median and interquartile range) of postmortem methadone concentrations associated with the 118AA and 118GA genotypes.

In all the cases in which allele *9 was identified, allele *4 was also found. This is in agreement with previous reports pointing out that allele *9 acts as a marker of allele *4 (refs. 6,11). A linkage between the presence of allele *9 and that of allele *4 was identified with a $\chi^2$ (32.471), 1 d.f., $P$ <0.001. Only one subject was identified with the CYP2B6*6/*6 genotype; this subject had a postmortem blood methadone concentration of 1.38 mg/l, which is 3.45-fold higher than the normally recognized fatal threshold, but this result was not statistically significant (Table 2). CYP2B6*1/*4 was identified in two cases (5%). By itself, allele *4 has been linked with rapid metabolism;[16] however, our results did not show this trend, and the mean postmortem concentration associated with this allele was 0.86 mg/l (Table 2). CYP2B6*4/*4 genotypes were not found in any of the subjects.

**Table 3 A118G genotypes and postmortem methadone and benzodiazepine concentrations in fatalities attributed to methadone (population 1), and A118G genotypes and postmortem morphine and benzodiazepine concentrations in fatalities attributed to morphine (population 2)**

| A118G genotype | Frequency | Mean (mg/l) | SD | SEM | Median |
|---|---|---|---|---|---|
| *Population 1* | | | | | |
| Methadone | | | | | |
| AA | 36 | 0.70 | 0.493 | 0.082 | 0.58 |
| GA | 4 | 0.93 | 0.465 | 0.233 | 0.97 |
| Two-tailed significance | 0.391 | | | | |
| Benzodiazepines | | | | | |
| AA | 18 | 0.69 | 0.363 | 0.086 | 0.66 |
| GA | 2 | 1.66 | 0.749 | 0.530 | 1.66 |
| Two-tailed significance | 0.004* | | | | |
| *Population 2* | | | | | |
| Morphine | | | | | |
| AA | 25 | 0.45 | 0.86 | 0.172 | 0.22 |
| GA | 2 | 0.12 | 0.05 | 0.035 | 0.16 |
| Two-tailed significance | 0.593 | | | | |
| Benzodiazepines | | | | | |
| AA | 25 | 1.62 | 1.57 | 0.315 | 0.58 |
| GA | 2 | 1.05 | 0.10 | 0.705 | 0.97 |
| Two-tailed significance | 0.626 | | | | |

Population 2 consists of 27 postmortem cases in which death was attributed to morphine.

*Significance at <0.05.

## OPRM1 A118G and postmortem methadone blood concentrations

The A118G SNP demonstrated an allelic frequency of 10%, a result similar to that reported in other studies conducted in living subjects.[22,29] No significant association was seen between A118G and postmortem methadone blood concentrations ($P$ > 0.05, Table 3). However, the median methadone concentration for 118 GA carriers is higher than for 118 AA carriers (0.97 mg/l vs. 0.58 mg/l) (Figure 3). No homozygote for the G allele was identified in this study. Using Spearman's rank correlation, significant associations were found between the 118G allele and

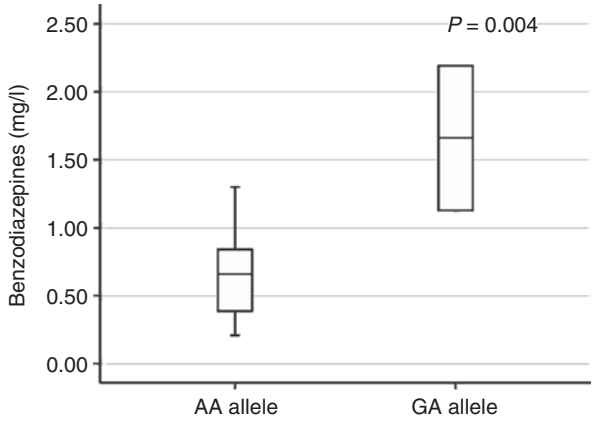


**Figure 4** Distribution (median and interquartile range) of postmortem benzodiazepine concentrations associated with the 118AA and 118GA genotypes.

both the CYP2B6 *9 allele and the CYP2B6 *4 allele ($P < 0.01$ and $P < 0.05$, respectively).

### OPRM1 A118G and postmortem benzodiazepine blood concentrations

Benzodiazepines are concurrently administered with methadone to addicts for the treatment of substitute-related anxiety. The additive effect of benzodiazepines and methadone in causing respiratory depression has been well documented.[25,30] Concomitant use of methadone and benzodiazepines was demonstrated in 50% of the subjects in our study (**Table 1**). There was a significant association between the A118G SNP and mean postmortem benzodiazepine concentrations ($P < 0.01$, **Table 3**). Heterozygous individuals demonstrated a 2.4-fold higher mean benzodiazepine concentration as compared with homozygous wild-type carriers (**Figure 4**).

## DISCUSSION

The use of methadone in de-addiction-related maintenance programs worldwide[8,9] has been associated with methadone-associated fatalities. Patients who are prescribed methadone for heroin withdrawal are reported to be 6.7 times more likely to experience an adverse drug reaction in the drug's induction phase,[12] during which either drug tolerance may be overestimated or other drugs are in use concomitantly.[3] In this study, a significant association was revealed between the CYP2B6*4, *9, and *6 alleles and high methadone concentrations in postmortem blood, characteristic of the slow metabolizer phenotype. This is the first time such a genetic association has been demonstrated in methadone-related fatalities (**Table 2**), that is, in deaths for which the autopsy pathologist assessed methadone toxicity to be a probable significant factor. Furthermore, the allelic frequencies of these target alleles were considerably higher than those reported in live patients,[6,11] supporting the concept that there is linkage between these gene variants and methadone toxicity. This observation is in agreement with the results of a study examining the association between *CYP2D6* SNPs and methadone metabolism in postmortem cases, in which the prevalence rate of poor metabolizers exceeded the reported prevalence rates in general-population reports.[31] Therefore, the risk of a methadone-related fatality may be predetermined, in part, by screening for *CYP2B6* variants, in particular CYP2B6*6. There is potential value in screening for *CYP2B6* variants before prescribing methadone for drug addiction, and possibly also during palliative care, when methadone may be used as an alternative to morphine for analgesia.[32] It would be interesting to examine the *CYP2B6* allele frequencies in an opioid-abusing population, in order to identify whether these alleles occur more frequently among opioid abusers as compared with the general population.

The CYP2B6*4 allele has been linked with increased enzymatic activity[16,27] and rapid metabolizer status, but the anticipated lower drug levels were not observed in this study. Only two subjects were CYP2B6*1/*4 carriers, which limited further interpretation (**Table 2**). Future studies that take into account individual drug histories, the time period between drug administration and death, and the time period between death and postmortem might strengthen our findings, which so far only identify a trend. The inevitable interval between death and sampling could potentially increase blood drug concentrations because of postmortem redistribution.[33] The sampling site and the manner of sample collection used in this study should ameliorate this effect. Nevertheless, even femoral venous blood samples are subject to postmortem increase.[34]

A number of studies have examined the association between *OPRM1* variants and interindividual variability in morphine metabolism,[21,22] but there is limited information on the relationship between this genotype and response to methadone. The *OPRM1* A118G SNP has been shown to affect the efficacy of opioids.[21] This SNP, located in exon 1, results in an amino acid change from asparagine to aspartic acid, causing the loss of a putative *N*-linked glycosylation site; this might be associated with changes in receptor trafficking to the membrane.[35] *In vitro* expression studies at the transcription level that examined this SNP also noted significantly higher expression levels in wild-type carriers as compared with 118 GA carriers.[36] Given that *OPRM1* 118AA receptors have 10 times as many binding sites as 118GA,[36] it was postulated that the residue of 118 may be attributed to a *cis*-acting factor. Reductions in cell surface μ-receptors may limit the availability of drug binding sites. No significant increases in postmortem methadone concentrations were found in association with *OPRM1* 118 GA (**Table 3**); however, there is a distinct difference between the median postmortem methadone concentrations for 118 AA carriers and 118 GA carriers (0.70 mg/l vs. 0.93 mg/l) (**Figure 3**), without considering the data from two outliers (1.66 and 1.44 mg/l), which would have misleadingly elevated the mean drug concentration related to 118 AA carriers. The fact that A118G reduces the receptor binding sites[36] might account for the increased drug concentrations observed in this study. Therefore, the possibility of an association between A118G and methadone blood concentration cannot be excluded. A larger sample size would be necessary to explore this potential relationship.

When benzodiazepines were found in conjunction with methadone (**Table 3**, population 1), carriers of *OPRM1* 118 GA

had a 2.4-fold higher postmortem mean blood benzodiazepine concentration ($P < 0.01$). Although this study has a limited sample size, it must be noted that a clear difference was observed. Interestingly, this was not seen in morphine-associated fatalities (**Table 3**, population 2), suggesting the existence of a specific link between methadone and benzodiazepines. Given that both methadone and morphine bind to the μ-opioid receptor in a similar manner, this raises the question of why the interaction varies for different opioids. It may be postulated that this is linked to receptor endocytosis, which is induced by methadone but not by morphine.[37] However, the reason for this interaction remains unknown, and it would be interesting to study this further. A potential pharmacokinetic mechanism for interactions between methadone and benzodiazepines may involve a shared CYP metabolic pathway. A number of benzodiazepines are metabolized by *CYP3A4* and *CYP2B6*, including diazepam,[38] midazolam,[16] and flunitrazepman.[39] Also, some benzodiazepines have been hypothesized to act as weak inhibitors of *CYP3A4* (ref. 40). Increased methadone concentrations in liver and brain have been reported when diazepam was administered after methadone exposure.[41] It has been reported that the inhibition of benzodiazepine by *CYP3A4* is weak and unlikely to be of clinical significance.[4] Other drugs known to inhibit methadone metabolism include ketoconazole,[42] nelfinavir,[39] paroxetine,[43] and sertraline.[43]The concomitant use of any of these drugs with methadone may lead to increased methadone concentrations postmortem. Therefore, drug interactions cannot be excluded as additional factors in the increased methadone concentrations reported in this study.

It is well documented that the coadministration of methadone and benzodiazepines can result in lethal respiratory depression.[25,30,44] Because benzodiazepines bind to $GABA_A$ receptors and not to opioid receptors, this does not involve a direct association.[44] However, both receptor systems share common signal transduction pathways.[45] Animal model studies have indicated the occurrence of associations between the opioid and $GABA_A$ receptor systems, in which, for example, benzodiazepines have been shown to potentiate the respiratory effects of fentanyl.[46] In the rat, concomitant use of benzodiazepines with the partial μ-opioid agonist buprenorphine resulted in the increased recruitment of μ-opioid receptors.[47] Consequently, it may be postulated that benzodiazepines affect μ-opioid receptor regulation through signal transduction and regulatory pathways. Our findings suggest that susceptibility to methadone and benzodiazepines is associated with the A118G genotype through a presently unknown mechanism.

A significant association between the *OPRM1* A118G genotype and CYP2B6 *4, *9, and *6 alleles was revealed in this study. Both genes are vital to methadone action *in vivo*, and this assemblage of gene variants may reflect the nature of coordinated action of the enzyme and receptor in contributing to susceptibility to methadone. Agonist occupancy at μ-receptors leads to receptor phosphorylation and rapid receptor desensitization.[48] This in turn facilitates receptor internalization, thereby reducing agonist response,[49] and is thought to be a mechanism involved in the acquisition and development of drug tolerance.[49] Receptor internalization should reduce the number of potential drug binding sites, protecting against methadone toxicity in the presence of poor drug metabolism. However, concomitant use of methadone and benzodiazepines may upregulate μ-receptors,[47] neutralizing the reduction in available binding sites and leading to methadone toxicity. The A118G genotype of *OPRM1* has also been linked to susceptibility to heroin addiction.[23] As demonstrated in our study, CYP2B6*9 and *6 alleles may be linked to poor drug metabolism, as suggested by higher postmortem blood drug concentrations. The combination of *OPRM1* A118G genotype and CYP2B6*9 and *6 variants could lead to predisposition to opioid addiction and greater susceptibility to methadone fatality. The sensitivity of the μ-opioid receptor to methadone would be lower in 118 GA subjects, and therefore methadone concentrations in these subjects may reach higher levels before toxic effects appear.

In this retrospective review of fatalities, potential confounding factors such as the presence and respective concentrations of all other concomitant drugs, mode of drug intake, previous opiate use or recent abstinence, and manner of death (rapid or delayed) cannot be excluded. In summary, *CYP2B6* variants, specifically the CYP2B6*6 allele, are associated with higher methadone concentrations in the postmortem blood of persons who died of methadone toxicity, probably as a consequence of "poor" or "slow" drug metabolism. This genotype clearly correlates with higher methadone concentrations; we can therefore postulate that, in a normal population, subjects with this genotype will experience higher methadone accumulation and are therefore at higher risk of methadone toxicity. This suggests that the CYP2B6*6 allele may be a suitable risk marker for screening for an individual's susceptibility to methadone toxicity. The 118 GA genotype may have a similar use as a marker, but this needs to be explored in a larger study. The presence of the A118G SNP on the *OPRM1* gene may also be of forensic value for interpreting the potential toxic relationship between methadone and benzodiazepines. Given that methadone maintenance therapy has been effective in reducing heroin-associated mortalities for many years,[50] it would be beneficial to reduce methadone-associated mortality by assessing and monitoring its adverse effects on individuals with slow-metabolizer genotypes. Genetic screening for "susceptibility" variations prior to maintenance therapy could therefore be used to identify patients who may be at increased risk because it is not routine practice for patients undergoing methadone maintenance treatment to be subjected to therapeutic-drug monitoring. Typically, therapeutic-drug monitoring is employed for substances with a low therapeutic index, such as cardiotoxic, neuroleptic, and immunosuppressive drugs for which the difference between beneficial therapeutic and toxic concentrations in the blood/plasma is small. Although therapeutic-drug monitoring offers a scientific approach to selecting a drug regime to optimize therapy, it involves regular clinic visits and can entail costly analysis. Also, in many instances, clinical response does not correlate with plasma drug concentration. Genetic screening of subjects prior to methadone maintenance treatment would involve a single rapid diagnostic test. Specific screening to identify CYP2B6*6 and *OPRM1* A118G carriers

prior to addiction treatment could therefore be valuable as part of a cost-effective risk-management strategy.

## METHODS

**Case selection.** A retrospective review was conducted (2007–2008) of methadone-associated deaths from a geographically defined area of Scotland (Tayside, Fife, and Central regions).

**Toxicological analyses.** The toxicological analysis for each subject was conducted at the Centre for Forensic and Legal Medicine, Dundee University. After cross-clamping of the femoral vein, blood samples were collected by needle and syringe distally. Methadone was extracted from postmortem blood specimens using liquid/liquid extraction and estimated using high-pressure liquid chromatography with a diode array detector. Postmortem blood specimens were rendered alkaline with 0.2 mol/l carbonate buffer and extracted with 1-chlorobutane. A Waters Spherisorb 5-µm OD/CN high-pressure liquid chromatography column (4.6 × 150mm) and a guard column were used with acetonitrile (25% acetonitrile in aqueous triethylammonium phosphate buffer) as the mobile phase.

**DNA extraction and quantitation.** Genomic DNA was isolated from leukocytes (in sodium fluoride–anticoagulated blood) using the DNeasy Blood and Tissue Kit (Qiagen, Crawley, UK) and quantified using the Human Quantifiler Kit (Applied Biosystems, Warrington, UK) in accordance with manufacturer instructions.

**OPRM1 and CYP2B6 genotyping using conventional PCR.** Samples were amplified by PCR to identify homozygous wild types, homozygous variants, and heterozygous controls. For the A118G SNP in exon 1 of *OPRM1*, primer EX1F_HB (forward: 5′ ATGCCTTGGCGTACTCAAGTTG) and primer EX1R_HB (reverse: 5′ CTAACTCCCAAGGCTCAATGTTG) were used. G516T in exon 4 was amplified using primer C2B6E4F (forward: 5′ GTACATAATTAGCTGTTACGG) and primer C2B6E4R (reverse: 5′ AAGTCTGGTAGAACAAGTTCA). A785G in exon 5 of *CYP2B6* was amplified using primer C2B6E5F (forward: 5′ AGGAGATATAGAGTCAGTGAG) and primer C2B6E5R (reverse: 5′ AGTTCCTCCTCCCTATTTTCT). PCRs were performed with a reaction volume of 50 µl, consisting of 27.5 µl PCR-grade water, 10 µl of 5× GoTaq buffer, 3.0 mmol/l $MgCl_2$, 1 µl of 10 mmol/l deoxynucleoside triphosphates (final concentration of 0.25 mmol/l), 1 µl forward and reverse primers, 10 ng/µl DNA, and 0.5 µl GoTaq DNA polymerase. PCRs were performed with a Primus 96 advanced machine (Alpha Laboratories, Eastleigh, UK). The cycling conditions were as follows: initial denaturation at 95 °C for 5 min; subsequent denaturation at 95 °C for 1 min; annealing at 65.3 °C (118 A>G), 59.5 °C (516 G>T), and 62.1 °C (785 A>G) for 30 s; primer extension at 72 °C for 2 min repeated for 30 cycles, followed by final extension at 72 °C for 5 min. PCR products were visualized by electrophoresis with a 2% Cyber Green agarose gel on blue light. Samples were purified using the QIAquick PCR Purification Kit (Qiagen, Crawley, UK) in accordance with manufacturer instructions. DNA sequencing was conducted by COGENICS (COGENICS, Essex, UK).

**OPRM1 genotyping using real-time PCR.** Samples were genotyped for the A118G variation using the commercial TaqMan SNP genotyping assay (product no. 4351379; Applied Biosystems) in accordance with the manufacturer's instructions.

**CYP2B6 genotyping using real-time PCR.** Samples were genotyped for the G516T variation using the commercial TaqMan drug-metabolism genotyping assay (product no. 4362691; Applied Biosystems). A custom-designed TaqMan assay (product no. 4331349; Applied Biosystems) was used to genotype for A785G. Genotyping was performed in accordance with the manufacturer's instructions.

**Statistical analysis.** Data are presented as medians and means ± SE of the mean. The statistical significance of the differences between methadone mean concentrations and the G516T and A785G variants and between methadone/benzodiazepine mean concentrations and A118G was determined using the two-tailed independent *t*-test. Linkage between gene variants was determined using Pearson's $\chi^2$-test and Spearman's rank correlation. A $P$ value ≤0.05 was considered to indicate statistical significance. All analyses were performed using SPSS software (version 14.0; SPSS, Chicago, IL).

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## CONFLICT OF INTEREST

The authors declared no conflict of interest.



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