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ORIGINAL RESEARCH

ABCB1 haplotype and *OPRM1* 118A > G genotype interaction in methadone maintenance treatment pharmacogenetics

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Background: Genetic variability in *ABCB1*, encoding the P-glycoprotein efflux transporter, has been linked to altered methadone maintenance treatment dose requirements. However, subsequent studies have indicated that additional environmental or genetic factors may confound *ABCB1* pharmacogenetics in different methadone maintenance treatment settings. There is evidence that genetic variability in *OPRM1*, encoding the mu opioid receptor, and *ABCB1* may interact to affect morphine response in opposite ways. This study aimed to examine whether a similar gene-gene interaction occurs for methadone in methadone maintenance treatment.

Methods: Opioid-dependent subjects (n = 119) maintained on methadone (15–300 mg/day) were genotyped for five single nucleotide polymorphisms of *ABCB1* (61A > G; 1199G > A; 1236C > T; 2677G > T; 3435C > T), as well as for the *OPRM1* 118A > G single nucleotide polymorphism. Subjects' methadone doses and trough plasma (R)-methadone concentrations (C_{trough}) were compared between *ABCB1* haplotypes (with and without controlling for *OPRM1* genotype), and between *OPRM1* genotypes (with and without controlling for *ABCB1* haplotype).

Results: Among wild-type *OPRM1* subjects, an *ABCB1* variant haplotype group (subjects with a wild-type and 61A:1199G:1236C:2677T:3435T haplotype combination, or homozygous for the 61A:1199G:1236C:2677T:3435T haplotype) had significantly lower doses (median ± standard deviation 35 ± 5 versus 180 ± 65 mg/day, $P < 0.01$) and C_{trough} (78 ± 22 versus 177 ± 97 ng/mL, $P < 0.05$) than *ABCB1* wild-type subjects. Among subjects with the most common *ABCB1* haplotype combination (wild-type with 61A:1199G:1236T:2677T:3435T), the *OPRM1* 118 A/G genotype was associated with a significantly higher C_{trough} than 118 A/A (250 ± 126 versus 108 ± 36 ng/mL, $P = 0.016$). No *ABCB1* haplotype group or *OPRM1* genotype was associated with dose or C_{trough} without taking into account confounding genetic variability at the other locus. Therefore, two interacting pharmacogenetic determinants of methadone maintenance treatment response were identified, ie, *ABCB1*, where variants are associated with lower methadone requirements, and *OPRM1*, where the variant is associated with higher methadone requirements.

Conclusion: These opposing pharmacogenetic effects therefore need to be considered in combination when assessing methadone maintenance treatment pharmacogenetics.

Keywords: methadone, opiate substitution treatment, *ABCB1*, P-glycoprotein, *OPRM1*, receptors, opioid, mu

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Introduction

Long-term opioid maintenance remains the most cost-effective approach for managing opioid dependence.[1] However, the safe and effective use of substitution opioids, such as methadone, relies heavily on optimal dosing that minimizes both withdrawal and adverse opioid side effects, whilst reducing heroin use. This is made difficult by the

narrow therapeutic index and large interindividual variability observed in the dose-response and plasma concentration-response relationship for methadone.[2–5] Despite application of individualized treatment strategies that titrate dose against patient symptoms, attrition rates in Australian methadone maintenance therapy programs remain high (62%–74% at 12 months).[6,7] Therefore, a better understanding of the factors underlying individual responses to methadone is required in order to improve treatment individualization and enhance clinical efficacy.

In addition to pathology and environmental factors such as diet, smoking, drug-drug, and food-drug interactions, there is increasing recognition that genetic factors may play a role in shaping the pharmacokinetic and pharmacodynamic aspects of the individual response to opioids.[8] Thus, patient genetics could be used to guide dose individualization further, helping to reduce the incidence of withdrawal and adverse effects, particularly during the induction phase of treatment, and improve treatment retention.

Some investigations into the pharmacogenetics of methadone metabolism have suggested a role for *CYP2D6* and *CYP2B6* genetic variability in influencing systemic methadone exposure,[2,8–11] whilst others have not been able to confirm some of these findings.[12] Even when variability in plasma methadone pharmacokinetics is accounted for, there remains a substantial (five-fold) range of methadone concentrations required to suppress opioid withdrawal.[3] Therefore, methadone is clearly influenced by additional factors affecting central nervous system distribution and pharmacodynamic response.

One possible pharmacogenetic determinant of methadone central nervous system distribution is the *ABCB1* (*MDR1*) gene encoding the P-glycoprotein efflux transporter, for which methadone is a substrate.[13,14] P-glycoprotein has a wide tissue distribution, but its most important role relating to opioid drugs is likely at the blood–brain barrier, where it can directly influence central nervous system exposure to substrates.[15–17] We have previously investigated the relationship between *ABCB1* haplotypes [consisting of the 61A > G (rs9282564); 1199G > A (rs2229109); 1236C > T (rs1128503); 2677G > T (rs2032582); 3435C > T (rs1045642) single nucleotide polymorphisms] and methadone maintenance therapy doses (15–110 mg/day) in a South Australian treatment center, demonstrating a link between variant haplotypes and significantly lower (approximately 50%) methadone doses.[18] Specifically, subjects carrying the variant AGCTT (61A; 1199G; 1236C; 2677T; 3435T) haplotype (underlined nucleotides here and throughout remainder of paper are variant) required only 62% of the dose of other subjects, presumably due to decreased P-glycoprotein function at the blood–brain barrier and thus higher brain methadone concentrations (hence effect) relative to dose.[18] However, a later study in Swiss subjects receiving a much larger range of methadone doses (3–430 mg/day) failed to replicate this association between *ABCB1* haplotypes and dose.[19] Alternatively, a study in Israeli subjects receiving 30–280 mg/day found that the frequency of homozygous 1236TT; 2677TT; 3435TT variant haplotypes was significantly higher in high-dose (>150 mg/day) methadone maintenance therapy subjects than those designated as low-dose (<150 mg/day),[20] a finding seemingly contrary to the majority of *ABCB1* pharmacogenetic studies in opioids.[8]

Therefore, there is conflicting evidence regarding the role of variant *ABCB1* haplotypes in determining the methadone maintenance therapy dose, depending on the clinical context (eg, treatment population, clinical policy, philosophy, and/or dosing practice). One notable difference between studies has been the range of methadone doses that subjects received. Not only were wider ranges of doses encompassed by the Swiss and Israeli studies when compared with the Australian study (3–430 and 30–280 versus 15–110 mg/day, respectively), on average the doses in these patient populations were also higher (107 and 160 versus 59 mg/day, respectively). Therefore, it seems plausible that the dose range investigated may impact on whether *ABCB1* genetic variability significantly influences the response to methadone maintenance therapy. Indeed, we have previously proposed that as doses of methadone increase, the potential impact of genetic variability of *ABCB1* haplotypes on requirements may decrease, and that the impact of pharmacogenetics at other loci, eg, genes encoding for target receptors and signal transduction or reward pathways, will become more significant factors in determining methadone response.[21] However, prior to this study, the impact of *ABCB1* genetic variability in higher dose ranges (>150 mg/day) in Australian methadone maintenance therapy settings was unknown.

One such genetic locus potentially altering opioid pharmacodynamics is the 118A > G (Asn40 Asp, rs1799971 recently renamed c.335A > G, Asn102 Asp, http://www.ncbi.nlm.nih.gov/SNP/) polymorphism of the mu opioid receptor gene, *OPRM1*.[8,22,23] Clinical studies have provided strong evidence that the G variant is associated with reduced analgesic response and side effects to opioids, and have been reviewed elsewhere.[24] Regarding methadone, whilst the 118A > G variant has been associated with decreased miotic potency in healthy controls,[25] it has not been shown to relate to methadone

dose or response in methadone maintenance therapy[22] when analyzed in isolation. However, there is evidence that variants of *OPRM1* and *ABCB1* interact to affect morphine pain relief,[26] and it is possible that a similar gene-gene interaction may occur in methadone maintenance therapy.

Therefore, the aims of this study were to investigate the impact of *ABCB1* and *OPRM1* genetic variability on dose requirements and pharmacokinetics in an opioid-dependent population maintained on a wide range of methadone doses, and the potential gene-gene interaction between *ABCB1* and *OPRM1* in this context.

It was hypothesized that, in this cohort, methadone requirements would significantly differ between *ABCB1* haplotypes and *OPRM1* genotypes when taking into account confounding genetic variability (from *OPRM1* and *ABCB1*, respectively), but not when these genetic factors were analyzed in isolation.

Materials and methods

Subjects

In total, 119 subjects were included in this retrospective study. All were Caucasian and not pregnant. They consisted of 38 methadone maintenance therapy patients from our previously published *ABCB1* pharmacogenetic study[18] (having excluded all pregnant and non-Caucasian subjects from that study) and a further 45 methadone maintenance therapy patients who had taken part in clinical studies conducted by the Discipline of Pharmacology at The University of Adelaide since 2006 (all approved by the Royal Adelaide Hospital research ethics committee). All of these 83 subjects were originally recruited from South Australian clinics and private prescribers operating under the guidelines of Drug and Alcohol Services South Australia.[27] A further 36 methadone maintenance therapy subjects, originally part of a clinical study from the Byrne Surgery, a specialist drug and alcohol medical practice in Redfern, New South Wales (approved by the South East Sydney Area Health Service ethics committee), were also included. Treated under New South Wales Department of Health clinical guidelines that allow dosing up to and above (with state approval) 200 mg/day,[28] these 36 subjects represent a cohort of subjects who require relatively high (>150 mg/day) doses to reduce withdrawal symptoms and heroin use. Written informed consent for genotyping analysis was obtained for all subjects.

Genotyping

Genomic DNA was isolated from whole blood samples using a QIAamp® DNA mini kit according to the manufacturer's instructions (Qiagen Pty Ltd, Doncaster, Australia).

ABCB1

61A > G, 1199G > A, 1236C > T and 3435C > T genotyping was performed using polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) as described previously.[18] 2677G > T genotyping differed from previously because it was also detected by PCR-RFLP based on the method of Cascorbi et al.[29] Briefly, PCR primers were: forward – 5′-TGCAGGCTATAGGTTCCAGG-3′; reverse – 5′-TTTAGTTTGACTCACCTTCCC-3′. PCR reactions were performed in 30 µL total volume containing 100 ng DNA, 50 µM dNTPs, 0.1 µM each primer, 2.5 U Taq DNA polymerase and 1 × reaction buffer (New England Biolabs Inc, distributed by Genesearch Pty Ltd, Arundel, Australia). PCR cycling conditions were: 5 minutes at 94°C; 45 cycles of 30 seconds at 94°C, 30 seconds at 60°C, and 1.5 minutes at 72°C; and 5 minutes at 72°C. Twenty microliters of PCR product was digested with 4 U of *BanI* (New England Biolabs Inc) for 16 hours at 37°C, then 20 minutes of heat inactivation at 65°C. Digested PCR products were visualized by agarose gel electrophoresis as described previously.[18] Restriction fragments were: wild-type (digested), 198 and 26 base pairs (bp); variant (undigested), 224 bp. An example of this 2677G > T PCR-RFLP assay is provided in Supplementary Material A.

OPRM1

118A > G genotyping was performed using allele-specific PCR as previously described.[30]

For all genotyping assays, genotypes of random samples were confirmed via sequencing using BigDye Terminator v3.0 chemistry and analysis on an ABI Prism 3700 DNA Sequencer (Applied Biosystems, Australia). All assays contained both negative (no DNA template) and positive (DNA of known genotype confirmed by sequencing) controls to ensure accuracy.

Demographic, dose, and pharmacokinetic data

Demographic, dose, and pharmacokinetic data were obtained from original study case notes and are summarized in Table 1. Because dose as a measure of methadone requirements can be confounded by significant variability in absorption, distribution, metabolism, and elimination unrelated to P-glycoprotein, trough plasma (R)-methadone concentrations (C_{trough}, ng/mL), were also investigated. Used here as an indicator of plasma concentrations required to suppress withdrawal, C_{trough} should not be confused with dose-adjusted C_{trough} (C_{trough}/dose), which is used here as an indicator of the potential influence of P-glycoprotein on the intestinal absorption and elimination

Table 1 Subject demographic data

	All subjects (n = 119)	Subjects with PK data (n = 84)
Male:female	100:19	69:15
Age, median ± SD (range)	35 ± 8 (20–54)	36 ± 8 (23–54)
Body weight (kg), median ± SD (range)	75 ± 14 (48–127)	76 ± 15 (48–127)
Time in treatment (months), median ± SD (range)	12 ± 58 (1–276)	13 ± 63 (1–276)
Methadone dose (mg/day), median ± SD (range)	80 ± 72 (15–300)	90 ± 80 (15–300)
C_{trough} (ng/mL), median ± SD (range)	–	153 ± 139 (18–669)
C_{trough}/dose ((ng/mL)/mg), median ± SD (range)	–	1.7 ± 0.7 (0.5–3.5)

Abbreviations: PK, pharmacokinetic; C_{trough}, trough plasma (R)-methadone concentration; C_{trough}/dose, dose-adjusted trough plasma (R)-methadone concentration; SD, standard deviation.

of methadone. Eighty-four subjects had C_{trough} data available and were included in pharmacokinetic analyses (the demographic, dose, and pharmacokinetic data for this subset of 84 subjects are also summarized in Table 1).

Data analysis

Chi-square tests were used to test for genotype deviations from Hardy-Weinberg equilibrium. *ABCB1* haplotypes were inferred from genotype data using PHASE (version 2.1) software.[31,32] Subjects whose haplotypes were inferred with low confidence (PHASE call confidence probability less than 0.75) were excluded from haplotype analyses (n = 5). Dose, C_{trough} and C_{trough}/dose data are presented as the median ± standard deviation. For differences between *ABCB1* haplotypes, four haplotype combination groups were compared based on observed frequency and our previous significant findings,[18] ie, homozygous wild-type ("AGCGC/AGCGC"); combination of wild-type with variant at 1236C > T, 2677G > T and 3435C > T only ("AGCGC/AGTTT"); homozygous variant at 1236C > T, 2677G > T, and 3435C > T only ("AGTTT/AGTTT"); and combination of wild-type with variant at 2677G > T and 3435C > T only, or homozygous variant at 2677G > T and 3435C > T only ("AGCTT"). These groups were compared simultaneously using Kruskal-Wallis tests (with Dunn's post hoc comparing variant groups with AGCGC/AGCGC). This was first done for all subjects, regardless of *OPRM1* genotype, and then only within subjects who were *OPRM1* 118A > G wild-type (A/A) genotype (for dose and C_{trough} only).

Differences in daily dose and C_{trough} between *OPRM1* genotypes were first investigated in all subjects, regardless of *ABCB1* haplotype, using Kruskal-Wallis tests (with Dunn's multiple comparisons post hoc). Then, within subjects with the most common *ABCB1* haplotype combination (AGCGC/AGTTT), *OPRM1* 118A > G A/G and G/G (n = 1 and 0 for dose and C_{trough}, respectively) genotype subjects were combined and compared with the A/A genotype using the Mann-Whitney U test.

The influence of demographic covariates on the relationship between genetic variability and methadone dose, C_{trough} and C_{trough}/dose was also examined by multiple linear regression analysis (see Supplementary Material B). $P < 0.05$ was considered statistically significant for all analyses.

Results

Genetic variability

OPRM1 (see Supplementary Material C, Table SC1) and *ABCB1* genotype frequencies were in Hardy-Weinberg equilibrium ($P \geq 0.40$). *ABCB1* haplotypes could be confidently determined for 114 subjects, with 12 different haplotypes observed (see Supplementary Material C, Table SC1).

Methadone requirements and pharmacokinetics

Not taking into account *OPRM1* genetic variability, there were no significant differences between *ABCB1* haplotype groups for either dose ($P = 0.51$), C_{trough} ($P = 0.97$), or C_{trough}/dose ($P = 0.60$, Table 2). Not taking into account *ABCB1* genetic variability, there were no significant differences between *OPRM1* genotypes for either dose ($P = 0.40$) or C_{trough} ($P = 0.064$, Table 2).

Among *OPRM1* 118A/A subjects, there was a significant difference in dose and C_{trough} between *ABCB1* haplotype groups, with the AGCTT variant haplotype group associated with a significantly lower dose (median ± standard deviation 35 ± 5 versus 180 ± 65 mg/day, $P < 0.01$) and C_{trough} (78 ± 22 versus 177 ± 97 ng/mL, $P < 0.05$) than the wild-type *ABCB1* subjects (Figure 1). Among subjects with the *ABCB1* AGCGC/AGTTT haplotype combination, the *OPRM1* 118A/G genotype was associated with significantly higher C_{trough} than the 118A/A genotype (250 ± 126 versus 108 ± 36 ng/mL, respectively, $P = 0.016$), but with no significant difference for dose (133 ± 89 versus 75 ± 75 mg/day, respectively, $P = 0.37$). Results of multiple linear regression analyses are provided in Supplementary Material B.

Discussion

Conflicting findings of previous pharmacogenetic studies of methadone maintenance therapy suggest the relative impact

Table 2 Comparison of methadone dose, (R)-methadone C_{trough} and dose-adjusted (R)-methadone C_{trough} between common *ABCB1* haplotype combinations and *OPRM1* genotypes of methadone maintenance therapy patients

	Median ± SD (n)		
	Dose (mg/day)	C_{trough} (ng/mL)	C_{trough}/dose ((ng/mL)/mg)
ABCB1 haplotype group			
AGCGC/AGCGC	180 ± 60 (9)	181 ± 113 (6)	1.5 ± 0.5 (6)
AGCGC/AGTTT	78 ± 80 (24)	201 ± 144 (13)	1.6 ± 0.7 (13)
AGTTT/AGTTT	105 ± 67 (9)	173 ± 143 (7)	1.8 ± 0.8 (7)
AGCTT	40 ± 99 (7)	220 ± 193 (6)	1.9 ± 0.3 (6)
P value[a]	0.51	0.97	0.60
OPRM1 118A > G genotype			
A/A	80 ± 63 (77)	124 ± 115 (52)	
A/G	73 ± 82 (34)	214 ± 135 (26)	
G/G	93 ± 101 (8)	262 ± 259 (6)	
P value[a]	0.40	0.064	

Notes: [a]*P* value of Kruskal-Wallis test for differences between *ABCB1* haplotype groups or *OPRM1* 118A > G genotypes.
AGCGC/AGCGC: Subjects homozygous wild-type for *ABCB1* single nucleotide polymorphisms (61A > G; 1199G > A; 1236C > T; 2677G > T; and 3435C > T. AGCGC/AGTTT: Subjects with one wild-type haplotype and one 61A:1199G:1236T:2677T:3435T haplotype. AGTTT/AGTTT: Subjects homozygous for 61A:1199G:1236T:2677T:3435T haplotype. AGCTT: Subjects with one wild-type haplotype and one 61A:1199G:1236C:2677T:3435T haplotype, or homozygous for 61A:1199G:1236C:2677T:3435T haplotype.
Abbreviations: C_{trough}, Trough plasma (R)-methadone concentration; C_{trough}/dose, Dose adjusted trough plasma (R)-methadone concentrations.

of *ABCB1* genetic variability on methadone dose may be dependent on the clinical context.[18,19] More specifically, we have previously published an association between the *ABCB1* variant AGCTT (61A; 1199G; 1236C; 2677T; 3435T) haplotype and significantly lower methadone doses in a methadone maintenance therapy population receiving doses up to approximately 150 mg/day.[18] However, a subsequent larger study by Crettol et al[19] in patients receiving a much higher range of doses (up to 430 mg/day) failed to replicate these findings.

To investigate further the basis of these discordant findings, this study first set out to refine (by excluding all pregnant and non-Caucasian subjects) and expand upon (adding a further 81 subjects) our existing methadone maintenance therapy study population, to re-examine *ABCB1* pharmacogenetics over a dose range more akin to other studies in methadone maintenance therapy.[19,20]

In agreement with the findings of Crettol et al,[19] no significant association between *ABCB1* haplotypes and methadone dose was observed when *ABCB1* genetic variability was investigated in isolation. Similarly, and in line with a separate study by Crettol et al,[22] the *OPRM1* 118A > G polymorphism had no significant influence on methadone dose, despite strong clinical evidence that it can influence the pharmacodynamics of other opioids.[24]

Given the complex nature of opioid dependence and the heterogeneity within the methadone maintenance therapy population, particularly over such wide ranges of doses, it is not surprising that single genetic factors cannot individually explain variability in dose requirements. However, we have been able to show that by taking into account at least part of the confounding genetic variability, the potential influence of *ABCB1* and *OPRM1* on methadone response can be revealed.

For *ABCB1*, the AGCTT haplotype group was associated with a significantly lower median dose (<20% of wild-type) when controlling for *OPRM1*. Alternatively, when controlling for *ABCB1* variability, *OPRM1* 118G variant allele carriers had a nearly 1.8-fold higher median dose than 118A/A genotype subjects, although this difference was not significant, with doses within each genotype group still varying greatly (5–8-fold).

The degree of variability in dose within the *OPRM1* genotype groups is not entirely surprising, given that methadone dose requirements can also be significantly influenced by additional variability in intestinal absorption, peripheral distribution, metabolism, and elimination (unrelated to P-glycoprotein activity).

Downstream of variability in pharmacokinetics affecting the dose-plasma concentration relationship, C_{trough} was examined as a potentially more direct indicator of variability in pharmacodynamics due to the *OPRM1* 118A > G polymorphism, as well as the impact of *ABCB1* haplotype on the distribution of methadone in the central nervous system. For *ABCB1*, as with dose, the AGCTT haplotype group was associated with a significantly lower median C_{trough} (44% of wild-type) when controlling for *OPRM1*. In addition, variant *OPRM1* 118G allele carriers had significantly higher (2.3-fold) median C_{trough} than 118A/A subjects, when controlling for *ABCB1*.

These findings suggest that when methadone potency at the mu opioid receptor is not compromised, *ABCB1* haplotype (influencing exposure to methadone in the central nervous system) can have a significant effect on methadone requirements, but has less impact when higher doses/concentrations are required to overcome deficiencies in mu opioid receptor activation. Conversely, when variability in peripheral pharmacokinetics and *ABCB1* genetics are controlled for, an association between the *OPRM1* 118G variant, and higher than normal plasma methadone concentrations to suppress withdrawal, is revealed. This effect is most likely as a result of decreased methadone potency at the mu opioid receptor, a hypothesis supported by previous

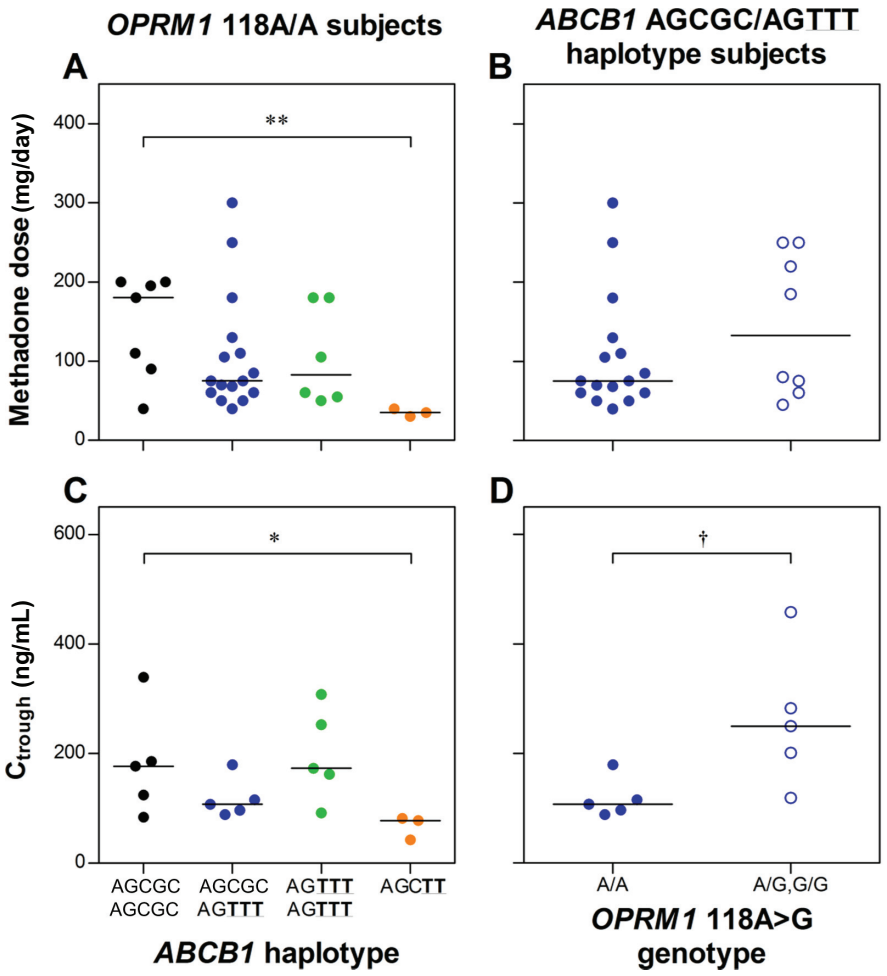

Figure 1 Influence of *ABCB1* haplotype group on (**A**) methadone dose and (**C**) trough plasma (R)-methadone concentrations (C_{trough}) among *OPRM1* wild-type (118A/A) subjects; and *OPRM1* 118A > G genotype on (**B**) methadone dose and (**D**) C_{trough} among *ABCB1* AGCGC/AGTTT haplotype combination subjects.
Notes: Lines are medians. *$P < 0.05$, **$P < 0.01$ versus *ABCB1* AGCGC/AGCGC haplotype group, †$P < 0.05$ *OPRM1* 118A/A versus A/G,G/G genotype in *ABCB1* AGCGC/AGTTT haplotype group.

studies in healthy subjects.[25] Such an interaction between *ABCB1* and *OPRM1* genetic variability is mechanistically expected, and corresponds with that reported for morphine analgesia,[26] but requires confirmation within a larger cohort of subjects.

It is interesting that, as in our previous study,[18] the AGCTT, but not the AGTTT, haplotype was associated with a lower methadone dose. Certainly, *in vitro* evidence would suggest that these variant haplotypes should have similar phenotypes (either decreased[33,34] or no effect[35–37] on P-glycoprotein expression and/or function). However, findings from Levran et al[20] in methadone maintenance therapy, linking the homozygous 1236TT; 2677TT; 3435TT variant haplotype to high-dose requirements (>150 mg/day), indicate that the situation *in vivo* may be quite different. These studies highlight the importance of examining *ABCB1* haplotypes, rather than individual single nucleotide polymorphisms, at least for methadone maintenance therapy. This, along with the confounding effect of *OPRM1* genetics, may explain why a significant association between individual *ABCB1* single nucleotide polymorphisms and methadone requirements has not been observed in previous studies.[2,11,18]

Regarding the potential influence of *ABCB1* genetic variability on methadone pharmacokinetics altering the dose-plasma concentration relationship, Crettol et al[2] have previously reported significant differences in dose-adjusted trough (R)-, (S)- and (R,S)-methadone concentrations between *ABCB1* 61A > G and 3435C > T genotypes. However, in this study, *ABCB1* haplotypes had no significant influence on C_{trough}/dose. This is not surprising, given that for drugs such as methadone, P-glycoprotein is expected to have a relatively minor impact on intestinal absorption and elimination when compared with its influence on central nervous system distribution, especially at high doses.[15,16]

Multiple linear regression analyses incorporating demographic covariates did not add significantly to the interpretation

of the influence of *ABCB1* and *OPRM1* genetic factors on methadone dose, C_{trough}, or C_{trough}/dose (see Supplementary Material B).

Frequencies of the common *ABCB1* haplotypes and the *OPRM1* 118G allele in subjects on methadone maintenance therapy were similar to those reported in non-opioid-dependent Caucasians (AGCGC: 30% versus 28%; AGCGT: 10% versus 8%; AGCTT: 6% versus 3%; AGTTT: 32% versus 35%; GGTTT: 8% versus 6%; *OPRM1* 118G: 21% versus 20%), in line with previous findings that these genes are not associated with risk of opioid dependence.[18,30]

Some limitations to this study, largely brought about by the number of subjects, need to be discussed. Firstly, only a small subset of *ABCB1* haplotypes were able to be analyzed, therefore it is possible that further, less common, haplotype combinations may also influence methadone distribution, and could not be analyzed here. Secondly, only the most basic of comparisons were able to be performed regarding gene-gene interactions, which encompassed only a third of the study population. Given sufficient numbers, a two-way analysis of variance or regression analysis, with inclusion of other clinical covariates, would be ideal. Thirdly, this study was limited to examination of only two genes in what is becoming increasingly recognized as a polygenetic trait. In addition to *ABCB1* and *OPRM1*, there is evidence that genetic variants in the dopamine D_2 receptor/ankyrin repeat and kinase domain containing 1 (*DRD2/ANKK1*),[22,38] nerve growth factor β polypeptide (*NGFB*),[39] myocardin (*MYOCD*),[40] metabotropic glutamate receptor (*GRM6*),[40] beta-Arrestin2 (*ARRB2*),[41] and potassium inwardly rectifying channel (*KCNJ6*),[42] genes may also contribute to interindividual variability in methadone maintenance therapy response in Caucasians. A recent study in Han Chinese subjects on methadone maintenance therapy has also demonstrated that *ABCB1*, *CYP2B6*, *OPRM1*, and *ANKK1-DRD2* genetic polymorphisms jointly correlate with methadone dose.[43] Therefore, consideration of multiple genetic and environmental factors will be central to the enhanced individualization, and ultimately improvement, of the treatment of opioid dependence.

Finally, no adjustments were made for multiple testing. Therefore, whilst an attempt was made to keep the number of cross-analyses to a minimum, there is a risk of type 1 error in these findings, and they require replication. A prospective, large-scale, multicenter study is currently being conducted that will be able to address the issues discussed above.

In conclusion, neither *ABCB1* nor *OPRM1* genetic variants independently predicted methadone requirements in methadone maintenance therapy. However, a significant association between an *ABCB1* variant and lower methadone requirements was revealed when controlling for *OPRM1* genetic variability. Likewise, a significant association between an *OPRM1* variant and higher methadone requirements was revealed when controlling for *ABCB1* genetic variability. Therefore, these two opposing pharmacogenetic effects need to be considered in combination when assessing their impact on methadone maintenance therapy.

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Disclosure

The authors have no conflicts of interest to report in this work.

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Supplementary material A

ABCB1 2677G > T genotype determination by restriction fragment length (Figure SA1).

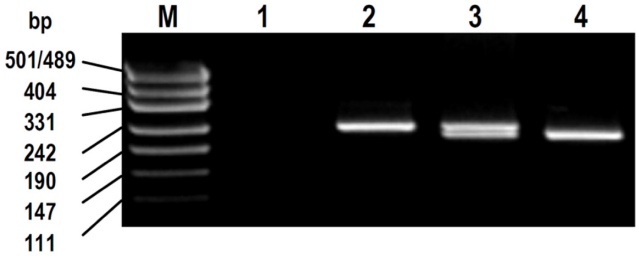

Figure SA1 *BanI* restriction fragment patterns for 2677G > T genotyping.
Notes: bp: size of marker bands in base pairs; M: pUC19/*Hpa*II DNA molecular weight marker; 1, negative control; 2, homozygous variant genotype (T/T) with 224 bp undigested polymerase chain reaction product; 3, heterozygous genotype (G/T) with 224 bp undigested + 198 bp digested fragment; and 4, homozygous wild-type genotype (G/G) with 198 bp digested fragment. 26 bp digested fragments not visible on gel.

Supplementary material B

Regression analysis

Methods

Multiple linear regression analysis (IBM SPSS Statistics 19) was used to investigate the effects of demographic covariates on methadone dose, C_{trough}, and C_{trough}/dose. For these analyses, C_{trough} and C_{trough}/dose data were normalized by log-transformation (dose data could not be normalized by any standard transformation). Because the contributions of *ABCB1* haplotype groups AGCGC/AGTTT and AGTTT/AGTTT were unclear from this study, only the AGCGC/AGCGC and AGCTT groups were included in regression analysis, and coded 0 and 1, respectively. *OPRM1* 118A/A, 118A/G, and 118G/G genotypes were coded 0, 1, and 2, respectively.

Firstly, gender, age, and weight (log-transformed) were submitted to stepwise inclusion (F probability < 0.05) regression for each dependent variable. Based on these results, any significant covariate was then automatically entered for all regression analyses of relevant dependent variable (dose, C_{trough}, or C_{trough}/dose) prior to stepwise inclusion (F probability < 0.05) of: *OPRM1* genotype then *ABCB1* haplotype; *ABCB1* haplotype in *OPRM1* 118 A/A subjects; or *OPRM1* 118A > G genotype in *ABCB1* AGCGC/AGTTT subjects. A subsequent conditional removal (F probability > 0.1) step for weight was also included for comparison.

Results

Of the demographic variables examined, weight was the only significant predictor of methadone dose by regression analysis (positive correlation, model $P = 0.013$). Subsequent regression analyses identified *ABCB1* haplotype in *OPRM1* 118A/A subjects (negatively correlated), and *OPRM1* 118A > G genotype in *ABCB1* AGCGC/AGTTT subjects (positively correlated), but not *ABCB1* haplotype group or *OPRM1* 118A > G genotype, as significant predictors of dose (Table SB1).

Table SB1 Multiple linear regression models for estimation of methadone dose based on weight and *ABCB1* and *OPRM1* genetic variability

Model variables	Model adjusted R^2	*P* value
Weight	0.054	0.013
Weight, *ABCB1* haplotype in *OPRM1* 118A/A subjects	0.478	0.022
ABCB1 haplotype in *OPRM1* 118A/A subjects	0.482	0.007
Weight, *OPRM1* 118A > G genotype in *ABCB1* AGCGC/AGTTT subjects	0.201	0.082
OPRM1 118A > G genotype in *ABCB1* AGCGC/AGTTT subjects	0.229	0.03

No demographic variable was a significant predictor of C_{trough} or C_{trough}/dose. *ABCB1* haplotype in *OPRM1* 118A/A subjects (model adjusted $R^2 = 0.481$, $P = 0.023$), and *OPRM1* 118A > G genotype in *ABCB1* AGCGC/AGTTT subjects (model adjusted $R^2 = 0.458$, $P = 0.019$) were the only significant predictors of C_{trough}. Neither *ABCB1* haplotype group, nor *ABCB1* haplotype group in *OPRM1* 118A/A subjects, were significant predictors of C_{trough}/dose.

Supplementary material C

Supplementary tables

Table SC1 Frequencies of *ABCB1* haplotypes[a] and *OPRM1* 118A > G minor allele and genotypes in opioid-dependent methadone maintenance treatment patients

ABCB1 haplotype[b]	% (n)	*OPRM1* c.118 A > G	% (n)
AGCGC	29.8 (68)	MAF	21.0 (50)
AGCGT	9.6 (22)	A/A	64.7 (77)
AGCTC	3.1 (7)	A/G	28.6 (34)
AGTGC	4.8 (11)	G/G	6.7 (8)
AACGC	2.2 (5)		
AGCTT	5.7 (13)		
AGTGT	1.8 (4)		
AGTTC	2.6 (6)		
AACTC	0.4 (1)		
GGCGT	0.4 (1)		
AGTTT	32.0 (73)		
GGTTT	7.5 (17)		

Notes: Variant nucleotides in *ABCB1* haplotypes are underlined. [a]Excluding subjects with PHASE call confidence probabilities less than 0.75. [b]Haplotypes consisting of 61A > G, 1199G > A, 1236C > T, 2677G > T, and 3435C > T single nucleotide polymorphisms of *ABCB1*.
Abbreviation: MAF, minor allele frequency.