# Association of *ABCB1/MDR1* and *OPRM1* Gene Polymorphisms With Morphine Pain Relief

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**The pharmacokinetics and pharmacodynamics of morphine are under the control of several polymorphic genes, which can account for part of the observed interindividual variation in pain relief. We focused on two such genes: *ABCB1/MDR1*, a major determinant of morphine bioavailability, and *OPRM1*, which encodes for the μ-opioid receptor, the primary site of action for morphine. One hundred and forty-five patients of Italian origin undergoing morphine therapy were genotyped for the single-nucleotide polymorphism (SNP) C3435T of *ABCB1/MDR1* and for the A80G SNP of *OPRM1*. Pain relief variability was significantly ($P<0.0001$) associated with both polymorphisms. Combining the extreme genotypes of both genes, the association between patient polymorphism and pain relief improved ($P<0.00001$), allowing the detection of three groups: strong responders, responders, and non-responders, with sensitivity close to 100% and specificity more than 70%. This study provides a good example of the possible clinical use of pharmacogenetics.**

Severe pain is therapeutically addressed by administration of opioid analgesics. Morphine is one of the most important and widely used opioids in clinical medicine, but appreciable interindividual differences in its effectiveness are a major disadvantage for clinical use. Several factors have been hypothesized or identified as the cause of this interindividual variability. For example, variable opioid bioavailability and differences in the intensity of pain stimuli and perception have been invoked as an explanation.[1–4] However, with recent advances in genetic research, the inherited causes of the variability of opioid efficacy have been investigated.[5]

Clinical studies indicate that the extensive interindividual variability in drug response occurs as a result of molecular alterations at the level of drug transport proteins (pharmacokinetics) and drug receptors (pharmacodynamics).[6]

The efflux transporter P-glycoprotein (P-gp), encoded by the *ATP-binding cassette B1 (ABCB1)/multiple drug resistance 1 (MDR1)* gene, is a major determinant of the bioavailability of many opioids of importance to anesthesiologists, such as fentanyl, sufentanil, and alfentanil, and also morphine-6-glucuronide and morphine-3-glucuronide. P-gp is capable of limiting the entry of some opiates into the brain[7] and actively pumping a variety of drugs out of the central nervous system. It thus represents an important component of the blood–brain barrier.[8]

Polymorphisms in genes encoding for these proteins have been described, and several mutations in the *ABCB1/MDR1* gene have been demonstrated. One major site of interest, the single-nucleotide polymorphism (SNP) C3435T, affects the bioavailability of many drugs such as digoxin[9] and antiretrovirals.[10] Moreover, this SNP has been frequently associated with different P-gp expression levels and activities.[11,12] Opiates are potential candidates for P-gp activity, with some compounds, such as methadone, completely suppressing P-gp activity, while others, such as fentanyl, partly inhibit its activity. Several experimental findings with knockout mice lacking functional P-gp have shown a significant impact on analgesia for some opioids, especially morphine.[13,14] Taken together, these findings demonstrate that the genetic variability of P-gp may affect morphine disposition in the central nervous system.[15]

The μ-opioid receptor, encoded by the *opioid receptor μ 1 (OPRM1)* gene, is the primary site of action for the most commonly used opioids. Therefore it represents the first-line candidate for evaluating the role of polymorphisms in the clinical effects of morphine. The most prevalent SNP in the *OPRM1* gene is a nucleotide substitution at position 118 (A118G), predicting an amino-acid change (Asn40Asp) at a putative N-glycosylation site in the extracellular receptor region.

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Subjects carrying one or two copies of the variant G allele were found to have a reduced response to morphine treatment and a reduced analgesic response to alfentanil[16–18] and morphine-6-glucuronide.[19] Chou *et al.*[20,21] studied patients who underwent total knee arthroplasty and abdominal total hysterectomy, and they observed that G/G homozygotes have a poorer response to morphine for postoperative pain control than A/A homozygotes or heterozygotes.

Zhang *et al.*[22] documented a significant functional change attributable to the substitution of *A118* by *G118* in *OPRM1*, involving lower mRNA expression in human brain tissue and in transfected cells, and lower translation into functional protein, *in vitro*. Klepstad *et al.*[23] showed that cancer patients homozygous for the G allele required higher doses of morphine to relieve pain.

For these reasons, it is of great importance to investigate whether polymorphic variation of the *OPRM1* and *ABCB1/MDR1* genes, as well as their possible combinations, may have an impact on opiate pharmacokinetics and pharmacodynamics.

In this study, we report on an association study between the A118G SNP in the *OPRM1* gene and the C3435T SNP in the *ABCB1/MDR1* gene and severe pain relief, in 145 patients of Italian origin treated with morphine. The study was conducted to evaluate the influence of these two genes in determining individual responsiveness to morphine, with the aim of improving the efficacy of drug treatment and reducing adverse drug reactions. To the best of our knowledge, previous studies provide no clear evidence on the clinical relevance of ABC's polymorphisms for morphine therapy.

Our *a priori* hypothesis was that patients having both good efflux pump functionality (*ABCB1/MDR1* homozygous C/C) and a defective morphine receptor (*OPRM1* homozygous G/G) would be the worst responders to pain relief treatment. By contrast, patients with ineffective efflux pump (*ABCB1/MDR1* homozygous T/T) and a functional receptor (*OPRM1* homozygous A/A) were expected to be the best responders.

## RESULTS

### Pain relief

Complete data were available for 137 patients who underwent morphine therapy. The treatment was defined according to the standard protocols, taking into account several variables, including body mass index, age, health status, and pain level evaluated by Numerical Rating Scale (NRS) and Present Pain Intensity scale (PPI).

The effect of pain relief therapy was monitored for a period of 2 months as shown in Figure 1. Correlation between the two pain indexes, NRS and PPI, was very high (Spearman test = 0.936, $P < 0.0001$). In this study, we describe only the analysis for NRS, not for PPI, as the results obtained with PPI are superimposable.

The major effect of pain relief therapy, in terms of decrease in pain (ΔNRS), was experienced by patients immediately after the first week of treatment. By contrast, only a moderate further decrease in pain was obtained in the following weeks of therapy, even though significant modifications (mainly an increase) in morphine dosage were introduced, according to patient response (Figure 1). Thus, we analyzed in the models only the ΔNRS between baseline and 7 days of morphine treatment as the phenomena of saturation and adaptation and disease progression may modify individual responsiveness during the late course of therapy, masking the genotype effect. It is worth noting that during the second week, the morphine dose was increased on average by 37%, and by 82% during the third week to achieve the maximum effect. This dose modification was required to balance individual variability, possibly related to factors like opioid tolerance or disease progression, but at least in part, it was also related to individual genetic makeup.

The average pain level reported by patients at $T_0$ was 8.15 NRS units (SD = ±1.2). The mode and median was 8,

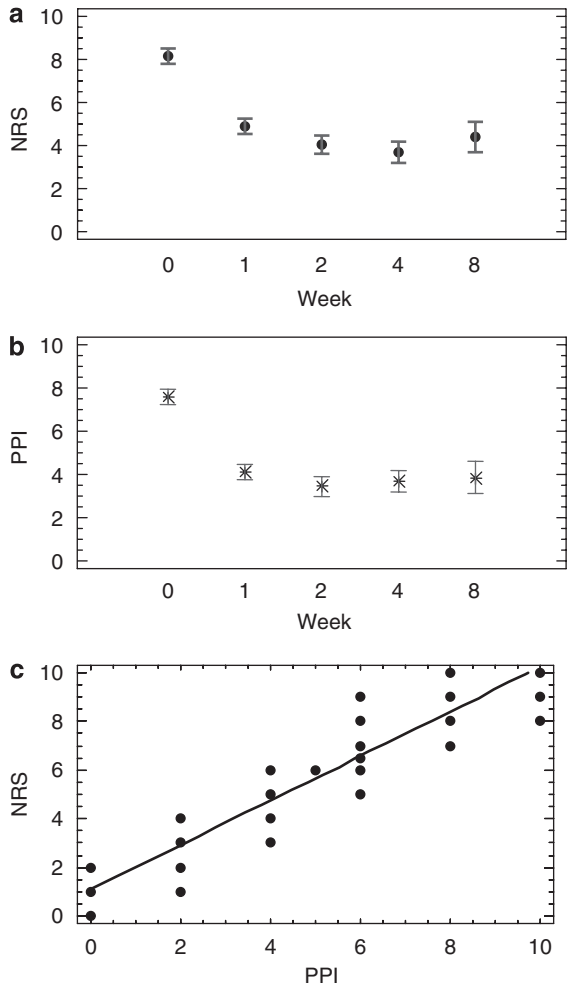


**Figure 1** Time course (weeks) of pain intensity decrease experienced by patients under morphine therapy. The decrease of pain was assessed by means of (**a**) NRS and (**b**) PPI scale values. Vertical bars represent confidence intervals (CIs) (95%). (**c**) The correlation between PPI and NRS pain index (Spearman test = 0.936, $P < 0.0001$).

indicating a tendency of the distribution to be symmetrical. After a week, $T_7$, of morphine treatment, patients experienced significant pain relief, showing an average NRS value of 4.9 (SD = ±2.1), with a mode and median of 5. The average NRS decrease (ΔNRS) was 3.25 (SD = ±2.03), which was found to be highly significant ($P<0.0001$) in a *t*-test for repeated measures after analysis of variance.

### Association of pain relief with gender, age, dose, and genotype

At $T_0$, no factor was significantly associated with variability of initial pain. Pain relief (ΔNRS) was used as a therapy efficacy measure.

No significant effect of gender ($P=0.42$), age ($P=0.15$), and dosage on ΔNRS was observed following univariate analysis (Table 1). To assess the possible functional effect of each allele of the *ABCB1/MDR1* and *OPRM1* genes, the co-dominant model, *i.e.*, the three genotypes separated, was used in univariate analysis. Genetic variability in the *ABCB1/MDR1* gene showed a highly significant association (F = 12.8; $P<0.0001$) with pain relief variability, accounting for 16% of total deviance. Homozygous T/T carriers were significantly ($P<0.001$) associated with greater pain relief (ΔNRS = 4.39, SD = ±2.21) than homozygous wild-type C/C (ΔNRS = 2.31, SD = ±1.73) (Table 1). Heterozygous carriers, T/C, showed no significant difference from the homozygous C/C, thus allowing use of the recessive model in the following analysis (Figure 2a).

Genetic variability in the *OPRM1* gene was even more significantly associated (F = 23.94; $P<0.0001$) with pain relief variability, accounting for 22.6% of total deviance (Table 1). A/A homozygotes were associated with a significantly higher decrease in pain (ΔNRS = 3.73, SD = ±1.72; $P<0.001$) than G/G homozygotes, whose response was virtually undetectable (ΔNRS = 0.30, SD = ±1.77). A/G heterozygotes showed no significant difference compared to G/G homozygotes, thus allowing use of the dominant model in the following analysis (Figure 2b).

Multivariate analysis was performed to assess both the effect of the genotypes under the assumption of a dominant model for the *OPRM1* gene and a recessive model for the *ABCB1/MDR1* gene, and the possible interaction between them. The deviance of ΔNRS explained by the complex model was as high as 30%. The effect of the genes, individually, was highly significant (*ABCB1/MDR1*: F = 19.8, $P<0.00001$ and *OPRM1*: F = 38.5, $P<0.00001$), whereas the interaction analysis, which was not significant (F = 1.17, $P=0.28$), exhibited an additive effect (Figure 3).

**Table 1 Univariate analyses for gender, age, and morphine dosage, genotypes *ABCB1* and *OPRM1* as factors on ΔNRS in the first week of therapy**

| Factor | ΔNRS | F-ratio (*P*) | Multiple range test[a] | *P*-value |
|---|---|---|---|---|
| *Gender* | | 0.66 (0.42) | | |
| Female (*n*=76) | 3.07 (±2.12) | | *X* | |
| Male (*n*=62) | 3.35 (±1.91) | | *X* | |
| *Age (terziles)* | | 2.07 (0.13) | | |
| 43–62 (*n*=44) | 3.41 (±2.01) | | *X* | |
| 63–72 (*n*=48) | 3.48 (±1.99) | | *X* | |
| 73–94 (*n*=46) | 2.70 (±2.03) | | *X* | |
| *Dose (mg/die)* | | 0.08 (0.92) | | |
| 10–30 (*n*=50) | 3.19 (±2.05) | | *X* | |
| 31–60 (*n*=45) | 3.09 (±2.01) | | *X* | |
| 61–500 (*n*=42) | 3.26 (±2.00) | | *X* | |
| *ABCB1 (C3435T)* | | 12.81 (<0.0001) | | |
| C/C (*n*=49) | 2.31 (±1.73) | | *X* | 0.001 |
| C/T (*n*=50) | 3.15 (±1.72) | | *X* | |
| T/T (*n*=38) | 4.39 (±2.21) | | *X* | |
| CC+TT (99) | 2.73 (±1.77) | 20.94 (<0.0001) | | |
| *OPRM1* (A118G) | | 23.94 (<0.0001) | | |
| A/A (*n*=106) | 3.73 (±1.72) | | *X* | 0.0001 |
| A/G (*n*=22) | 1.95 (±1.73) | | *X* | 0.0001 |
| G/G (*n*=10) | 1.77 (±1.77) | | *X* | |
| A/G+G/G (32) | 1.44 (±1.81) | 40.29 (<0.0001) | | |

ANOVA, analysis of variance; ΔNRS, decrease in Numerical Rating Scale. All values are reported as mean±SD compared to reference group from two-factor ANOVA with *post hoc* testing. [a]The vertical column of *Xs* (multiple range test) indicates the variables in homogeneous group that are not significantly different.

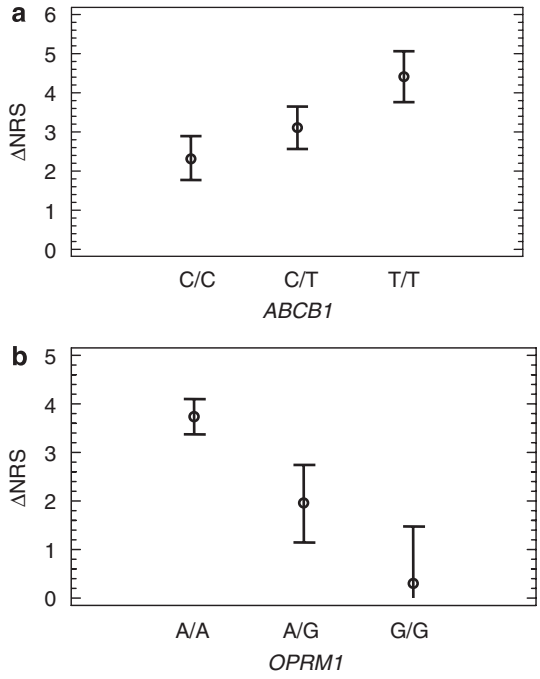


**Figure 2** Average decrease of pain according to patients genotype. Average decrease of pain (ΔNRS; ±CI 95%) after a week of morphine therapy in patients according to (**a**) *ABCB1* genotype and (**b**) *OPRM1* genotype.

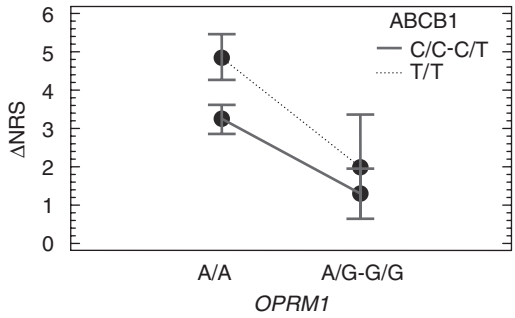


**Figure 3** Multivariate analysis and interaction plot between the two genes: the average pain intensity decrease (ΔNRS ± CI 95%) is reported according to patient's genotype.

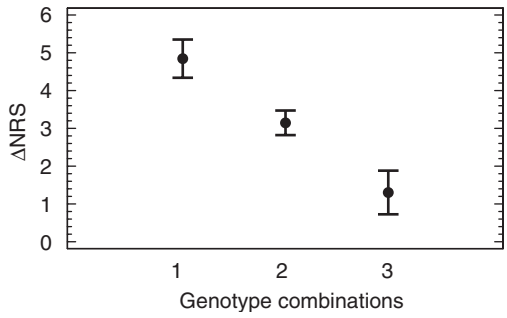


**Figure 4** Pain intensity decrease experienced by patients according to their combined genotypes for the two genes: 1 = *OPRM1*: A/A and *ABCB1*: T/T; 2 = *OPRM1*: A/A and *ABCB*1: C/C or C/T; 3 = all other allele combinations. ΔNRS (± CI 95%) as computed by means of multifactor analysis of variance.

**Table 2 Patient's group by *OPRM1* and *ABCB1* genotypes**

| Genotype | | | |
|---|---|---|---|
| ***OPRM1*** | ***ABCB1*** | Group | ***n*** |
| A/A | T/T | 1 | 32 |
| A/A | C/C or C/T | 2 | 79 |
| G/G or A/G | T/T | | |
| G/G or A/G | C/C or C/T | 3 | 26 |

We found that patients sharing at least one G allele for the *OPRM1* gene were the poorest morphine responders, regardless of *ABCB1/MDR1* polymorphism. In contrast, patients homozygous for the A/A allele for the *OPRM1* gene were found to be good responders. Among the latter group, stratifying for *ABCB1/MDR1* genotypes, we were able to discriminate two statistically different groups, with *ABCB1/MDR1* T/T homozygotes being better responders (ΔNRS = 4.39, SD = ± 2.21) than *ABCB1/MDR1* C/C or C/T homozygotes (ΔNRS = 2.73, SD = ± 1.77).

These findings suggest that the C3435T polymorphism of the *ABCB1/MDR1* gene and the A118G polymorphism of *OPRM1* strongly and independently affect morphine responsiveness.

This prompted us to evaluate how the possible genotypic combination of the two genes may modulate individual response to morphine therapy. To assess better the effect of these polymorphisms, patients were grouped according to their possible genotype combinations, considering the dominant model for *OPRM1* and the recessive model for *ABCB1/MDR1* (Table 2).

The first group was composed of patients who were simultaneously homozygous for both *OPRM1* and *ABCB1/MDR1* (A/A and T/T, respectively, $n = 32$). These patients were found to be the best responders, with a ΔNRS of 4.8, SD = ± 1.62. The second group was composed of A/A carriers for *OPRM1* and C/C or C/T for *ABCB1/MDR1* or T/T carriers for *ABCB1/MDR1* and G/G or A/G for *OPRM1*, $n = 79$. These patients were referred to as "normal responders" with an average ΔNRS of 3.1, SD = ± 1.68. Finally, we included in the third group the following patients: first, carriers of G/G or A/G alleles for the *OPRM1* gene who were, in addition, also carriers of C/C; and second, carriers of G/G or A/G alleles for the *OPRM1* gene who were, in addition, also carriers of C/T for the *ABCB1/MDR1* gene, $n = 26$. These patients were found to be the poorest responders with a ΔNRS = 1.3, SD = ± 1.47 (Figure 4).

Then, we calculated the sensitivity and specificity of genotyping to predict "non-responders" (*i.e.*, ΔNRS ⩽ 3.1) by applying the standard formula and counting how many non-responders could be identified by means of the above genotype classification.

### Morphine side effects

In the first week of treatment, two individuals suffered from dizziness, six had vomiting events, six diarrhea, five anxiety, three constipation, and four pruritus.

Multivariate analysis identified that the *OPRM1* and *ABCB1/MDR1* polymorphisms forming the object of this study were not associated with a higher or lower occurrence of the above-mentioned effects.

## DISCUSSION

The hypothetical effects of two polymorphic genes, one involved in pharmacokinetics, *i.e.*, the extent of drug concentration at the site of action, and another involved in pharmacodynamics, *i.e.*, sensitivity of the drug receptor, have been described in a comprehensive review.[6] In particular, the review showed that drug effect heterogeneity may be explained by the possible combination of the polymorphisms of two genes. The aim of this study was to assess the effect on pain relief of a polymorphic gene involved in pharmacokinetics, *ABCB1/MDR1*, and a gene involved in pharmacodynamics, *OPRM1*, and their possible combined effect. A wide range of opioids such as morphine are known as *ABCB1/MDR1* substrates.[15,24,25] As stated in the Introduction section, our "*a priori* hypothesis" was that patients having both good efflux pump functionality (*ABCB1/MDR1* homozygous C/C) and a defective morphine receptor (*OPRM1* homozygous G/G) would be the poorest responders to pain relief treatment. In contrast, patients with ineffective efflux

pump (*ABCB1/MDR1* homozygous T/T) and a functional receptor (*OPRM1* homozygous A/A) were expected to be the best responders. If the effects of the two genes are independent, it would be reasonable to predict that individuals carrying other allelic combinations will show intermediate responses.

Although a functional effect of C3435T polymorphism of the *ABCB1/MDR1* gene has already been reported for some chemotherapeutical agents such as irinotecan[26] and anti-inflammatory drugs,[27–31] in a very recent study, Crettol *et al.*[32] found that *ABCB1/MDR1* genetic polymorphisms also contribute slightly but significantly to the interindividual variability of methadone kinetics. To the best of our knowledge, there has been no clear evidence for a functional effect of this polymorphism in chronic pain morphine therapy.

In a recent study, Coulbault *et al.*[5] attempted to determine the contribution of genetic factors to interindividual morphine response variability in post-operative pain treatment. None of the SNPs of the candidate genes could be associated with morphine dose requirements. However, a linear (but statistically nonsignificant) trend was observed for higher morphine dose requirements among carriers of the G allele of the *OPRM1* A118G SNP. The absence of a statistically significant link is probably related, as the authors suggest, to a lack of statistical power due to the small sample size used in their study.

Our sample was almost twice the size of that examined by Coulbault *et al.* This may explain why we were able to find a statistically significant ($P<0.001$) functional effect of C345T polymorphism on morphine pain relief. In particular, the univariate analysis with a recessive model allowed us to detect a significant difference in morphine response between the two genotype groups: patients sharing C/C and C/T alleles proved to be moderate responders ($\Delta NRS = 2.73$), whereas T/T carriers were good responders ($\Delta NRS = 4.39$). The biological plausibility of such an association relies on the assumption that a functional membrane transporter determines an effective drug flux from the cell, reducing morphine absorption, permeability of the blood–brain barrier, and thus bioavailability of morphine for brain receptors. Therefore, these findings lead to the conclusion that the *ABCB1/MDR1* gene may play a relevant role in morphine pharmacokinetics as well.

The *OPRM1* gene is known to be the major player in morphine pharmacodynamics. We found that carriers of at least one G allele were poor responders (A/G and G/G, $\Delta NRS = 1.44$) compared to individuals homozygous for the A allele, who were good responders ($\Delta NRS = 3.73$, $P<0.001$).

Analysis of interaction between the two genes revealed a simple additive effect of single allele combinations, with poor or non-responders being double homozygotes for *ABCB1/MDR1* (C/C) and for *OPRM1* (G/G). It is likely that in these patients, morphine is quickly excreted, determining a low concentration at the defective receptor site and thus eliciting a scarce or null therapeutic effect ($\Delta NRS \approx 1$). By contrast, we found that strong responders were characterized by an opposite genotype, being double homozygotes for *ABCB1/MDR1* (T/T) and for *OPRM1* (A/A). As expected, in these patients, due to less effective drug extrusion from cells, a larger amount of morphine will be absorbed and will cross the blood–brain barrier. In addition, wild-type receptors are easily bound, thereby determining effective pain relief ($\Delta NRS \geqslant 4$). It is worth noting that "intermediate" genotype combinations are associated with intermediate phenotypes. Therefore, the extent of the genotype effect is appreciated by considering that this factor accounts for almost 30% of variance in pain decrease.

In our study, we found that using two allelic variants instead of one led to an improvement in sensitivity of the test. When one marker was used, we obtained a sensitivity of 87% (28 non-responders of 32 G/G or A/G for *OPRM1*) and a specificity of 50% (53 responders of 106 A/A for *OPRM1*), and a sensitivity of 68% (67 non-responders of 99 C/C for *ABCB1/MDR1*) and a specificity of 63% (24 responders of 38 T/T for *ABCB1/MDR1)*. Using both markers combined, sensitivity increased to 75% (47 non-responders of 63 GG/AG for *OPRM1* and C/C *ABCB1/MDR1*), whereas specificity was 72% (23 responders of 32 A/A for *OPRM1* and T/T for *ABCB1/MDR1*).

*ABCB1/MDR1* is actively expressed throughout the intestine. Therefore, it is very likely that polymorphisms influencing P-gp activity will undermine the analgesic efficacy of morphine, diminishing its absorption in the gut or increasing its efflux from the cerebral endothelium. In our study, we focused attention on the final end point, morphine analgesic efficacy, but we did not measure morphine metabolites. Thus, we cannot assess which of the two mechanisms exerted greater influence in the measured pain intensity decrease. It is unclear, though, whether the lack of a pharmacodynamic response in the patients is due to efflux from the cerebrospinal fluid or poor absorption of the original dose.

However, this study shows that genotyping patients for these two polymorphic genes may allow patient responsiveness to morphine to be predicted with very high sensitivity and good specificity. This means that poor responders can be easily predicted and, consequently, directed to more effective treatment, with greater benefit for the patients and reduction of costs for health care institutions. In this respect, one promising way to deal with *ABCB1/MDR1* opioid resistance seems to be the use of *ABCB1/MDR1* inhibitors. One illustrative study in human subjects assessed the effect of co-treatment with loperamide (an *ABCB1/MDR1* substrate) and quinidine (an *ABCB1/MDR1* inhibitor), and suggested a possible role for P-gp inhibitors as modulators to increase central nervous system opioid concentration.[33] However, the genetic variability in the *ABCB1/MDR1* gene is likely to influence the potential efficacy of *ABCB1/MDR1* inhibitors themselves. For this reason, further research into the therapeutic use of inhibitors is warranted.

We are aware of the possible limitations of the study. As the individuals enrolled are cancer patients, our findings may

be limited to oncological pain. Enrollment of cancer patients could introduce some uncontrolled bias, but in this study, we considered the data obtained within the first week of pain therapy, thereby minimizing possible confounding factors that are more likely to act in the long term, such as tolerance and pathology progression. Another confounding factor could be represented by the presence of metastasis. Stratified analysis for this variable was therefore performed, but no significant difference between the two groups (metastatic patients vs. non-metastatic patients) was detected.

To consider other factors or confounders, this study should be replicated in a more extensive population, as our results could be false positives. In contrast, compared to other available studies on this topic, our investigation was based on a fairly broad sample size and good statistical power.

Although additional data are required, from a pharmacological point of view, this study could be seen as a first step toward achieving the ultimate goal of personalized morphine therapy. The next step, given an adequate sample size, will be performed using a hybrid tagging/functional approach to cover the total range of genetic variability of the candidate genes.

## METHODS

**Study subjects.** One hundred and forty-five Italian patients of Caucasian origin, undergoing opioid-based cancer pain relief therapy, were recruited by the Analgesic Therapy Division of St Chiara Hospital in Pisa between 2004 and 2006. Relevant characteristics of the study population are summarized in Table 1. None of the patients declined to participate in the project, resulting in an overall participation rate of 100%.

Of 137 cancer patients, 77 presented metastases. Morphine was the first opioid for patients enrolled in the study. Previously, they had been treated with non-steroidal anti-inflammatory drugs and acetaminophen. None of the patients underwent surgery immediately before enrollment in the study. Subjects were individually interviewed by trained health personnel using questionnaires to record demographic characteristics and pain severity. Information on age, gender, and pain relief treatment was collected. All participants gave their informed written consent to the project and agreed to undergo genotyping. Approval for the study was given by the Regional Ethics Committee. Exclusion criteria were age less than 18 years, inability to understand the questions, inability to give informed consent, and altered level of consciousness.

All patients presenting with chronic nociceptive pain were eligible for entry into the study. All patients were also asked about their prescribed analgesics before enrollment in the study. None of the patients used opioid analgesics before entering the study. Pharmacological therapy was administered according to clinical need as assessed by the treating doctor. Oral twice-daily controlled-release morphine sulfate was administered to outpatients according to the standard protocols, taking into account several variables, including body mass index, age, health status, and pain level evaluated by NRS and PPI. Pain management was not delayed or withheld by participation in this study. Interviews and data collection were performed at the first examination ($T_0$), after 1 ($T_7$), 2, 4, and 8 weeks ($T_{14}$, $T_{28}$, and $T_{56}$).

**Pain intensity assessment.** Pain intensity markers are based on patients' pain intensity scores. In this study, we used a Verbal Rating Scale according to Lund *et al.*[34] (PPI) and an NRS. We decided to use both scales because we thought that using a Verbal Rating Scale and an NRS would achieve a more accurate pain assessment. In particular, older patients may have difficulty expressing pain intensity by numbers, whereas the majority can use a categorical scale. All questionnaires were administered by trained personnel.

NRS requires patients to rate their pain from 0 to 10 (11-point scale), with the understanding that 0 represents the absence of pain and 10 represents the opposite extreme of pain intensity (*i.e.*, pain as bad as it could be). The number selected by the patient represents his or her pain intensity score.[35–37]

PPI, which is included in the Italian Pain Questionnaire and represents the validated Italian version of the McGill Pain Questionnaire,[38] consists of five anchor words taken from the Verbal Intensity Scale and a 5-point verbal scale derived from these words. On the 5-point scale, mild pain was represented by a score of 1, moderate pain a score of 2, distressing pain a score of 3, exhausting pain a score of 4, and unbearable pain a score of 5. The number associated with the adjective chosen by the patient would constitute his or her pain intensity score. Pain was measured as resting pain.

The two scales for self-rated pain intensity were submitted to patients at the first examination ($T_0$), and they were asked to rate their pain intensity at that moment. The assessment procedure was repeated after 1 ($T_7$), 2, 4, and 8 weeks ($T_{14}$, $T_{28}$, and $T_{56}$) for intraindividual pain intensity evaluation.

Questionnaires were explained during an interview that took place before pain assessment. The questionnaires were explained again each time pain intensity was measured.

**Morphine side effects.** Morphine side effects were recorded using the Italian Pain Questionnaire,[38] a part of which is dedicated to collecting data on nausea, diarrhea, pruritus, constipation, anxiety, dizziness, and vomiting.

Data were collected on a qualitative (yes/no) but not quantitative basis, as the questionnaire does not allow side effects to be rated on a numeric scale.

**SNP selection.** We selected one SNP in the μ-opioid receptor gene (*OPRM1*) and one in the efflux transporter P-gp (*ABCB1/MDR1*). SNP selection was based on the criteria of frequency $\geqslant 5\%$ in Caucasians and proven biological activity.

**DNA extraction.** DNA was extracted upon sample arrival from whole-blood samples, with standard proteinase K digestion followed by phenol/chloroform extraction and ethanol precipitation. DNAs were then stored at −20°C, according to the standard procedure in our laboratory.

**Genotyping.** Genotyping was carried out using the Taqman assay (Applied Biosystems, Foster City, CA). The MGB Taqman probes were designed using the Primer Express software and synthesized by Applied Biosystems. The reaction mix included 20 ng of genomic DNA, 10 pmol of each primer, 2 pmol of each probe, and 5 μl of 2 × master mix (Applied Biosystems) in a final volume of 10 μl. Thermocycling involved 40 cycles with 30 s at 95°C followed by 60 s at 60°C. PCR plates were read on an ABI PRISM 7900HT instrument (Applied Biosystems). Genotype discrimination was performed using SDS software (Applied Biosystems), version 2.2. The PCR profile and reaction conditions were tested and optimized to ensure equal contents of template DNA, probes, and primers and to allow running with unique thermal conditions. All samples that did not give a reliable result in the first round of genotyping were resubmitted for up to three additional rounds of genotyping. Data points that still remained unfilled after this procedure were left blank.

To ensure quality control, DNA samples were randomly distributed on PCR plates, and all genotyping was conducted by

personnel blinded to sample identity. Only genotype calls scored concordantly by two independently trained operators were retained. Finally, a random 10% of the samples were re-genotyped blindly. The labeling and sequences of probes and primers were as follows: *OPRM1*: A118G (**rs1799971**); FAM: 5′-CTTAGATGGCGACCT GT-3′; VIC: 5′-CTTAGATGGCAACCTGT-3′; forward primer: 5′-CG GTTCCTGGGTCAACTTGTC-3′; reverse primer: 5′GTTCGGACC GCATGGGT-3′; *ABCB1/MDR1*: C3435T(**rs1045642**); FAM: 5′-CC CTCACAATCTCTT-3′; VIC: 5′-CCCTCACGATCTCTT-3′; forward primer: 5′-CTGTTTGACTGCAGCATTGCT-3′; reverse primer: 5′-ATGTATGTTGGCCTCCTTTGCT-3′.

The reaction mixture included 10 pmol of each primer, 2 pmol of each probe, and 5 µl of 2 × master mix (Applied Biosystems) in a final volume of 10 µl. Thermocycling involved 40 cycles with 30 s at 95°C followed by 60 s at 60°C.

**Statistical analysis.** One-factor analysis of variance was used to assess the significance of the effect of several variables on pain indicator; SEMs are also given in parentheses. A significance cutoff of $P < 0.20$ was used to select variables to be included in multivariate analysis of variance. The multiple range test, with Bonferroni's conservative correction, was used for multiple, planned sample comparisons when more than two groups were considered. Analyses and plots were performed by the statistical package Statgraphics 2.0 Plus.

Analyses were initially performed under a co-dominant inheritance model (three genotypes separated). Simplified models were then fitted: a dominant model (heterozygotes grouped with the homozygotes for the allele determining the poor responsive phenotype (for the minor allele)) and a recessive model (heterozygotes grouped with the homozygotes for the responsive phenotype).

**Statistical power.** Our study had 90% power to detect an association with a decrease of 2 in pain intensity for the *ABCB1/MDR1* SNP and it had 82% power for the *OPRM1* SNP, assuming $\alpha = 0.05$, a two-sided test, and a co-dominant model.


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## CONFLICT OF INTEREST

The authors declared no conflict of interest.




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