



HHS Public Access

Author manuscript

Pain. Author manuscript; available in PMC 2021 March 01.

Published in final edited form as:

Pain. 2020 March ; 161(3): 619–629. doi:10.1097/j.pain.0000000000001750.

A functional polymorphism in the ABCB1 transporter predicts pharmacologic response to combination of nortriptyline and morphine in neuropathic pain patients

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Abstract

Many genetic markers have been associated with variations in treatment response to analgesics, but none have been assessed in the context of combination therapies. In this study, the treatment effects of nortriptyline and morphine were tested for association with genetic markers relevant to pain pathways. Treatment effects were determined for single and combination therapies. A total of 24 functional Single Nucleotide Polymorphisms (SNPs) were tested within the gene loci of OPRM1, ABCB1, CYP2C19 and CYP2D6, COMT and HTR2A. Genotyping was performed in a population of neuropathic pain patients that previously participated in a clinical trial. For monotherapy, neither nortriptyline nor morphine responses were associated with SNPs. However, for nortriptyline + morphine combination therapy, the SNP rs1045642, within the drug efflux pump ABCB1 transporter significantly predicted analgesic response. The presence of the C allele accounted for 51% of pain variance in this subgroup in response to combination treatment. The T-allele homozygotes demonstrated only 20% improvement in pain scores, while the C-allele homozygotes 88%. There was no significant contribution of rs1045642 to the medication side-effects under all treatment conditions. The UK Biobank dataset was then used to validate this genetic association. Here, patients receiving similar combination therapy (opioid + tricyclic antidepressant) carrying the C allele of rs1045642 displayed 33% fewer body pain sites than patients without that allele, suggesting better pain control. In all, our results show a robust effect of

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Conflict of interest

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

the rs1045642 polymorphism on response to chronic pain treatment with a nortriptyline + morphine combination.

Introduction

Chronic neuropathic pain is a debilitating condition that affects 7 to 10% of the general population and requires challenging multidisciplinary treatment strategies.[11,22,87] Because first line medications usually provide only partial relief, an effort has been made to determine if combination therapies deliver more effective symptom control.[8,22] However, the great variability amongst individual patient response to analgesics has been a limiting factor in predicting treatment response and individualizing therapy. One important cause of this variability is related to the genetic variants associated with pain pathways and the metabolism of analgesic drugs.[1,63,84,85,94]

A recent crossover clinical trial involving patients being treated for neuropathic pain compared a morphine + nortriptyline combination to either drug alone, and found improved outcomes with combination therapy versus monotherapy.[24] Even though patients receiving combination therapy had significant improvement in pain scores overall, there were patients who responded very well and those who demonstrated little to no response. A question remains whether it is possible to distinguish which patients could potentially respond better to combination drug therapy. The current study is a secondary analysis of the results from this clinical trial, in which the treatment effects are examined in the context of different genetic variants.

Many genetic markers have been associated with variation in treatment response to opioids or tricyclic antidepressants. These mainly consist of single nucleotide polymorphisms (SNPs) within the mu opioid receptor (*OPRM1*) gene locus, Cytochrome P450 gene family (*CYP2C19* and *CYP2D6*), catecholamine inactivator Catechol-O-Methyl Transferase (*COMT*), ATP-Binding Cassette B1 Transporter (*ABCB1*), and serotonin receptor 2A (*HTR2A*).[28,39,85,94] From this group of candidate markers, the *OPRM1* SNP rs1799971 has demonstrated strong evidence linking it to opioid dose requirements, risk of dependence and pain intensity in conditions such as diabetic neuropathy and low back pain.[9,10,27,35,44,49] Genetic markers in drug metabolizing enzymes such as *CYP2C19* and *CYP2D6* have also been associated with altered drug plasma levels, treatment effect and incidence of side effects with medications including amitriptyline, nortriptyline and duloxetine. However, this evidence was obtained from studies focusing on psychiatric treatment response rather than pain.[30,31,80] Genetic variations in the *COMT* gene have been implicated in the risk of developing pain conditions, and in treatment response to analgesics, including opioids and antidepressants.[14,43,59,82] SNPs within the *COMT* and *HTR2A* genes have also been implicated in the incidence of chronic pain conditions and the risk of depression associated with pain.[13,28,45,77,93] The *ABCB1* gene codes for a transporter (ABC) of a wide variety of drugs, and polymorphisms within its gene have been shown to have an effect on morphine concentrations in the central nervous system, and incidence of side effects and treatment effects with amitriptyline and nortriptyline.[33,36,65,68] Despite this growing awareness of the clinical implications of genetic

variability, most genetic markers have not been tested in the setting of combination therapies or in relation to treatments in chronic pain. In this study, we describe a novel effect of the *ABCB1* gene SNP rs1045642 in the treatment response to nortriptyline and morphine combination therapy for patients with neuropathic pain.

Materials and Methods

The neuropathic pain discovery cohort

The secondary genetic analysis was performed in a cohort of neuropathic pain patients from a double-blind crossover clinical trial by Gilron *et al.*[24] The study was conducted with the approval of the Queen's University Health Sciences & Affiliated Teaching Hospitals Research Ethics Committee and all study subjects consented to participate. In brief, participants were recruited from January 2010 to May 2014, and randomized, using a double-dummy design, to receive a sequence of oral study medications: nortriptyline, morphine, and combination nortriptyline/morphine. The trial started with a 7-day baseline period with no antidepressant or opioid treatment, and then each of the three 6 week treatment periods consisted of a 3.5 week titration phase in which the study medication was titrated to reach the maximum tolerated dose (MTD), then a maintenance treatment phase where participants were treated with the MTD of the medication for 7 days, and finally a 7-day dose taper followed by a 4-day washout period prior to starting the next treatment period. Outcomes documented in this trial included, amongst others, the average daily pain intensity at MTD (0-10 numerical rating scale), percentage change in average daily pain from baseline to MTD, the Stanford Sleepiness Scale (SSS), the Patient Assessment of Constipation Symptoms (PAC-SYM), and postural changes in heart rate and blood pressure. [19,32,38]

The 36 participants who completed all three treatment periods from the original clinical trial were contacted and all consented to this secondary genetic analysis study. Thirty-two participants returned the saliva sample kit (Oragene DNA OG-500, DNA Genotek, Ontario, Canada) as requested. Four participants did not return the saliva sample kit by mail and were lost to follow up. These four participants were eliminated from the study. A total of 27 DNA samples were successfully obtained for genetic analysis, with five deemed not adequate for DNA extraction (inadequate volume, damaged container). DNA purification was performed in accordance with the manufacturer recommendation (DNA Genotek, Ontario, Canada) and genotyping for a total of 34 SNPs was performed by the Genome Quebec Innovation Center with Sequenom IPLEX Gold technology and using pre-designed TaqMan® assays (Applied Biosystems; Foster City, CA). Allele-specific fluorescence signals were distinguished by measuring endpoint 6-FAM or VIC fluorescence intensities at 508 nm and 560 nm, respectively, and genotypes were generated using Genotyper® Software V 1.3 (Applied Biosystems; Foster City, CA). The DNA Elution Buffer was used as a negative control, and K562 Cell Line DNA (Promega Corporation; Madison, WI) was included in each batch of samples tested as positive control. Results of two participants were further removed from the analysis because their analgesic responses were missing for morphine and combination therapy medications, resulting in a final sample of 25 participants.

Genetic Variant Selection

The following genetic variants were selected for our analysis based on previous literature linking genetic polymorphisms with analgesia/pain and drug metabolism (Table 1). This method has been chosen over the potential functionality of SNPs (or gene sequencing approach) because of the small sample size of the discovery cohort. We decided to apply the smallest list of candidate SNPs that might show association with the highest probability to reduce the burden of multiple testing correction. At the *OPRM1* locus, we tested the single functional non-synonymous C>T variation at rs1799971. For the *COMT* locus, a set of four *COMT* SNPs (rs6269, rs4633, rs4818 and rs4680) were grouped to form three pain haplotypes: low pain sensitive (LPS), average pain sensitive (APS) and high pain sensitive (HPS) as previously described.[14,56,88] Then, the number of LPS alleles expressed by each subject was counted, creating three different risk groups: highly pain responsive group (0 LPS alleles), medium pain responsive group (1 LPS allele) and low pain responsive group (2 LPS alleles) following the method previously described by Tchivileva et al.[82] For the serotonin receptor pathway, rs6313 and rs7997012 were selected because they were shown to be associated with pain and with antidepressant response.[3,77] Two SNPs, rs1045642 and rs2032582 were selected from the *ABCB1* gene. Finally, a functional grouping was used to assess variants within *CYP2D6* and *CYP2C19*. Nortriptyline is metabolized in the liver by CYP2D6. Genetic variations of the gene coding for this enzyme have been shown to alter the drug bioavailability depending on the level of activity of the enzyme [29]. CYP2C19 metabolism is a secondary path for nortriptyline, and thus it was included as a gene variant of interest as it is commonly associated with therapeutic and side effect profile of tricyclic antidepressants in general [66]. SNPs within the *CYP2C19* and *CYP2D6* gene locus were tested as diplotypes denoting enzymatic metabolic rate activity (PharmGKB), based on which patients can be categorized as poor (PM), intermediate (IM), extensive (EM) and ultra-rapid (UM) metabolizers. Among 25 patients, with respect to *CYP2C19* genotypes, 3 were identified as IM, 11 as EM and 11 as UM. With respect to *CYP2D6* genotypes, one patient was identified as PM, one patient was identified as IM, and the remaining 23 as EM. Thus, *CYP2D6* genotypes have been removed from subsequent analyses due to low number of genetic variants carriers.

Validation analysis using the UK Biobank

The UK Biobank (UKBB) data (UKBB application number 20802) was used to validate our results from the neuropathic pain cohort. This validation step was considered for significant associations found in the discovery cohort. Thus, only the rs1045642 effect on combination treatment response was tested. The side effect profile or associations with other genotypes such as metabolic enzyme activity were not tested in the UK Biobank cohort. The UKBB is a database with genetic and phenotypic information of 40 to 69 year old people living in the United Kingdom [81]. A total of 502,000 participants were recruited between 2006 and 2010 and informed consent was obtained from all.

For the UKBB validation analysis, data were extracted from the data-field 20003, which captured the patient's answers provided at an assessment center through a verbal interview with a trained nurse, regarding which prescription medications they were taking on a regular basis (not including short term medication taken for less than 1 week). Participants were

divided into the following treatment groups: Treatment Group 1, consisting of patients taking amitriptyline or nortriptyline monotherapy; Treatment Group 2, consisting of participants taking morphine or fentanyl monotherapy; or Treatment Group 3, consisting of participants taking a combination of one of the tricyclic antidepressants (amitriptyline or nortriptyline) along with one of the opioids (morphine or fentanyl). The second set of data was obtained using a touchscreen question delivered during the assessment center visit: “In the last month have you experienced any of the following that interfered with your usual activities?” (UKBB data-field 6159). Participants could choose all that applied from the following options: headache, facial pain, neck or shoulder pain, back pain, stomach or abdominal pain, hip pain, knee pain, pain all over the body, none of the above, or prefer not to answer. From these options, a score between 0 and 8 was calculated with one point allocated to each painful site. Participants that answered “pain all over the body” were given the maximum score of 8, and the ones that marked “none of the above” or “prefer not to answer” were given a score of 0. Lastly, genotyping data that passed quality control was available for 487,409 participants.

Data analysis

The discovery analyses focused on genetic factors associated with better response to combination pain therapy versus mono- pain therapy. We selected the primary outcome, for the purposes of this secondary genetic analysis, as the “% reduction in pain”, defined as “change in pain/treatment period baseline” $\times 100\%$ by Gilron et al.[24] The rationale for using % change from baseline rather than using actual pain ratings is that this controls for the wide variation of start-of-treatment and end-of-treatment pain scores across all trial patients. The “% reduction in pain” during each study period was calculated as the difference between treatment period baseline and average pain during MTD. We fitted a linear mixed-effects model to the “% reduction in pain” with age, sex, treatment type, genetic factor and an interaction between drug and genetic factor as the fixed effects and patient ID as the random effect. Under this model, we evaluated an association between the genetic factor and “% reduction in pain” under specific therapy type by assessing statistical significance of a contrast, $H_0 : \beta_{gf} + \beta_{gf:tr} = 0$, where β_{gf} represents the baseline effect of the genetic factor and $\beta_{gf:tr}$ represents the interaction term between genetic factor and treatment type. To determine if a genetic factor was associated with an increased risk of side effects, we fit a linear mixed effects model with the same set of predictors but with the self-reported side effect as an outcome. To determine if a genetic factor was associated with an increased risk of side effects, we fit a linear mixed effects model with the same set of predictors but with the self-reported side effect as an outcome. All analyses were performed using R statistical software (version 3.5.1). Linear mixed-effects models were fit using the “lme4” package [3] and linear contrasts were evaluated using the “multcomp” package. [34]

For the UKBB replication analyses of genetic factors associated with benefits of either mono- or combination therapy, an overdispersed Poisson model was fit to the total number of painful body sites from UKBB data as the outcome with age, sex, genetic factor, treatment group, an interaction between treatment group and genetic factor, and 40 first principal components as linear predictors. The main predictors were chosen to be the same as in the discovery analysis. We included all 40 principal components provided by Bycroft et

al. [6] as co-variables to correct for UKBB population structure, self-reported sex, and genotyping array. However, since a majority (88.26%) of participants in the UKBB cohort report their ethnic background as “British,” within the broader-level group “white,” one can assume that this cohort has relatively homogeneous ancestry. Therefore, we repeated our analysis with adjustment for only the first 3 principal components instead of 40 (results are not presented here). The estimated coefficients for the key variables (i.e., genetic factor and treatment groups) and their corresponding P-values did not differ appreciably between the two models.

Results

Association analysis in the discovery cohort

A set of 34 preselected SNPs were used for the discovery association analysis. This selection was based on previous reports demonstrating an association with morphine or nortriptyline response or those that have been shown to modify levels of morphine, serotonin, or norepinephrine (Table 1). As the test cohort was small (n=25), the number of genotyped SNPs was conservative (n=34). Furthermore, when possible, SNPs were grouped based on their known functionality (see Materials and Methods). With this grouping, we now performed eight independent tests, bringing our correction for multiple testing threshold to 6.25×10^{-3} .

All twenty-five genotyped participants (7 females; 18 males) were Caucasian, with average age of 67 ± 9 years old (mean \pm standard deviation) and average weight of 90 ± 16 kg. In line with the previous report [28], the effect of combination therapy in this subgroup (n=25) of genotyped participants demonstrated an average % reduction in pain with nortriptyline monotherapy that was 10% lower than with combination therapy ($P = 0.085$) and 14% lower with morphine monotherapy than with combination therapy ($P = 0.015$) (Figure 1).

We then examined whether SNPs were associated with the % reduction in pain and thus underpin changes in the benefits of mono- or combination therapy. Thirty-four SNPs were combined into eight genetic test groups based on functionality (Table 2). The association between the C allele of *ABCB1* rs1045642 (C/T genotype) with the percent change in pain was the only association that reached statistical significance ($P = 4.89 \times 10^{-5}$) and continued to be significant after Bonferroni correction for multiple testing. In the mixed-effects model, the presence of the C allele accounted for 21% of the variance in pain score, while only 7% of the pain score variance was accounted for by the rest of the predictors. The C allele homozygotes showed an improved response under all pain treatment conditions but this improvement had the greatest magnitude (and was the only one that reached statistical significance) under the combination therapy treatment. Specifically, in the combination treatment group only, the C allele of rs1045642 accounted for 51% of the variance in pain scores. Moreover, in the combination therapy group, the homozygotes for the T allele demonstrated only 20% improvement in pain scores, while the homozygotes for the C allele demonstrated an 88% improvement (Figure 2). Furthermore, the probability of having more than 30% improvement in pain rating given that a subject was taking the combination therapy and had the C allele was 88% (i.e., positive predictive values (PPV)), while that among subjects under combination therapy with the T allele was 54%. As none of the other

genotyped SNPs demonstrated an association, our results suggest that the C allele of *ABCB1* rs1045642 may significantly improve neuropathic pain treatment management and specifically with morphine/nortriptyline combination therapy.

Association of *ABCB1* rs1045642 with adverse drug events

The original study by Gilron *et al.* collected self-reported side effects, such as The Stanford Sleepiness Scale, Patient Assessment of Constipation Symptoms (PAC_SYM) and blood pressure changes during combination treatment of neuropathic pain.[19,24,32] Here, we tested the association of *ABCB1* rs1045642 with side effects in the three treatment groups described above. We questioned if the increased therapeutic efficacy of C allele carriers of rs1045642 was also associated with stronger side effects. There was no statistically significant contribution of *ABCB1* rs1045642 with the observed side-effects in any of the treatment groups (Table 3). These results suggest that only the therapeutic effects described herein but not the observed side effects of morphine-nortriptyline combination therapy are related to *ABCB1* genotype.

Validation analysis in the UK Biobank

Next, we analyzed the UKBB dataset to validate associations found between *ABCB1* rs1045642 and therapeutic effect based in the discovery cohort findings. In the UKBB, we included participants who were also taking amitriptyline and fentanyl in order to increase sample size due to the extensive use of these drugs and their similarities to nortriptyline and morphine respectively, leading to identical interactions with the *ABCB1* transporter.

There were 1,624 participants taking amitriptyline or nortriptyline (Treatment Group 1), 689 taking morphine or fentanyl (Treatment Group 2), and 37 taking a combination of amitriptyline or nortriptyline along with morphine or fentanyl (Treatment Group 3). There were no significant sex differences, except there were more females than males taking amitriptyline or nortriptyline (Treatment Group 1), and more males than females taking morphine or fentanyl (Treatment Group 2, Table 4). The *ABCB1* rs1045642 genotype was also obtained for each study participant. We used the number of painful body sites as a proxy for clinically relevant chronic pain intensity, as a strong correlation between these two phenotypes has been reported previously. [16,17,67,74,79,89] (Table 4)

Our primary outcome of interest was the relationship between the C allele of rs1045642 and the total number of painful body sites (Figure 3). Subjects in Treatment Group 3 (combination therapy) reported the highest number of painful body sites (4.04 ± 1.11). The number of pain sites were 2% lower ($P = 0.896$) in Treatment Group 2 (a proxy for nortriptyline monotherapy) and 29% lower ($P = 0.003$) in Treatment Group 1 (a proxy for morphine monotherapy) relative to group 3 (Figure 3A).

As with the discovery cohort analysis, the results of the Poisson model, fitted to the number of painful sites, suggest that rs1045642 of *ABCB1* is associated with reduced pain in the therapy group (Treatment Group 3, $P = 0.022$) but not either of the monotherapies (Treatment Group 1, $P = 0.138$; Treatment Group 2, $P = 0.530$) (Table 5). We considered one-sided P -values at this replication stage with a prior effect direction based on the discovery cohort (Treatment Group 3, $P = 0.011$). The target SNP, rs1045642, showed a

concordant direction of effect size in the UKBB data. Specifically, in the combination therapy group (Treatment Group 3), the T/T homozygotes demonstrated, on average, 6 painful sites, whereas the C/C homozygotes averaged 2 painful sites (Fig. 3B). Furthermore, the probability of having 8 pain sites given that a subject was taking the combination therapy (Treatment Group 3) and had the C allele was 13.8% (i.e., PPV), while that among subjects under combination therapy with the T allele was 37.2%; the probability of having more than 3 pain sites among people with the C allele and combination therapy was 20.68%, while that among people with the T allele was 51.1%. The presence of the C allele of rs1045642 in the Poisson model explained about 2% of the variance in the number of painful sites, while the rest of the predictors (including 40 first principal components) accounted for an additional 7% of the variance. The weaker effect of rs1045642 genotype on the treatment effect in the replication cohort has several possible explanations. It may be due to an overestimation of the effect in the discovery cohort and/or a difference in the quality of the phenotypic information collected in the discovery cohort through the randomized control clinical trial versus self-report in the UKBB with a proxy measure for pain levels. Another explanation may be the inclusion of fentanyl as a proxy for morphine. While morphine and fentanyl interact with ABCB1 in a similar manner, they are metabolized differently.

Associations between other *ABCB1* functional SNPs and the number of pain sites in the UK Biobank

As *ABCB1* has a number of functional polymorphisms and several are in linkage disequilibrium (LD) [42], we tested whether the rs1045642 polymorphism is driving the efficacy of combination therapy as described above, or if it is a marker for a different functional allele. We re-fitted Poisson regression models to the number of pain sites with other functional *ABCB1* SNPs as genetic predictors. The results of these additional analyses are summarized in Table 5 and unequivocally demonstrate that other *ABCB1* polymorphisms do not appreciably alter the efficacy of the three treatments considered.

Discussion

Responses to the evolving opioid crisis in many parts of the world have emphasized tremendous caution around the use and prescribing of opioids for chronic noncancer pain, and this has included recommendations to limit the daily opioid dose prescribed. [5,15] In addition to careful screening, intensive education, and close follow-up of patients receiving opioids for chronic pain, strategies to limit prescribed opioid doses may include combination pharmacotherapy whereby additive or synergistic analgesic interactions may facilitate an opioid sparing effect. [5,21,24,51] Thus, understanding the genetic basis of therapeutic responses to various analgesics may serve to better guide new pain management strategies.

In this manuscript, we selected a group of SNPs to be tested as potential predictors of treatment response in neuropathic pain patients. Despite convincing findings on their role in opioid or tricyclic antidepressant pharmacology [14,29,35,39,85,94], little has been published of their value as markers of treatment response for chronic pain conditions, such as neuropathic pain, and in the setting of mixed medication regimens. From all the genetic variations tested, the *ABCB1* SNP rs1045642, but none of the other *ABCB1*

polymorphisms, demonstrated a replicable association with the efficacy of nortriptyline/morphine combination treatment as compared to that of either nortriptyline or morphine as monotherapy.

The *ABCB1* gene, also known as the MDR1 or multidrug resistance 1 gene, encodes for P-glycoprotein, which is a transmembrane ATP dependent efflux pump that is expressed in many tissues, including the renal tubules, small and large intestinal epithelium, hepatocytes, in the endothelial cells of the blood-brain barrier, and spinal cord.[12,70,72,83] P-glycoprotein recognizes and transports a broad variety of structurally different and functionally unrelated drugs, including tricyclic antidepressants amitriptyline and doxepin, selective serotonin reuptake inhibitors (SSRIs) such as citalopram, and opioids such as morphine and fentanyl.[36,50,57] . Relevant to our study, nortriptyline has been classified as a moderate affinity substrate of P-glycoprotein, with a transmembrane transport ratio determined between 0.9 to 1.1[58]. On the other hand morphine is classified as a weak P-glycoprotein substrate with an efflux: influx ratio of 1.2 to 1.5[55]. Pharmacokinetic interactions exist among drugs transported by P-glycoproteins [78,91]. Combination treatments with drugs that either inhibit or induce P-glycoproteins might alter the amount of drug available in the brain.

Polymorphisms in the *ABCB1* gene have been associated with altered P-glycoprotein activity and expression levels, which have been demonstrated to alter plasma drug levels, side effect profiles and clinical response.[33,41,76] Although the first report by Hoffmeyer et al. showed that the T allele is associated with reduced mRNA expression of P-glycoprotein, subsequent studies on the role of ABCB1 polymorphisms SNP rs1045642 on P-glycoprotein functionality and drug response are controversial, with evidence on both sides.[33,47] When we extend this to drug concentrations and resulting plasma levels, once again, there is evidence to support higher plasma levels and, therefore, greater efflux for both CC [61] and TT [93] carriers.

There is also equivocal evidence surrounding the downstream effect of the SNP rs1045642 on opioid response.[47] A study by Campa et al. identified an increased drug effect in patients treated with morphine carrying the T allele as opposed to C. [7] However, in some studies, there is no reported effect of the SNP on response to opioids, including fentanyl [69], morphine [46,73], or oxycodone. [95] On the other hand, individuals with TT genotype as part of a haplotype required more methadone. [48] In the same study, the TT genotype individually was associated with the higher methadone dose requirement, although the association did not reach statistical significance.[48] Specifically for nortriptyline, rs1045642 has been associated with changes in the incidence of postural hypotension as a side effect, in which the T homozygote population is more at risk of postural hypotension compared to the C homozygote, but reports have been conflicting and no changes have been found in its therapeutic effect.[37,65]

Although a mechanism to explain the functional effect of this SNP in our study still needs to be demonstrated, several hypotheses can be offered. Importantly, this is the first published evidence to demonstrate the genotypic effect of the *ABCB1* transporter variant for combination therapy of two analgesics, but neither morphine nor nortriptyline separately, so

this effect can be different from what is expected based on the single drug response versus combination. Furthermore, existing controversy on the functional effect of rs1045642 at molecular genetic level may result from a complex epistatic interaction of different allelic variants of *ABCB1*. It has recently been reported that the rs1045642 polymorphism affects mRNA stability as part of haplotype, but this may not necessarily translate directly to reduced protein levels.[90] There is often an inverse correlation between secondary-structure dependent RNA stability and translation efficiency, and this translation efficiency is often tissue-dependent. [56,86] Furthermore, there is evidence to suggest the haplotype of *ABCB1* tagged by rs1045642 controls the translation efficiency, and, more importantly, co-translational folding and insertion of P-glycoprotein into the membrane, thereby altering substrate specificity. [36] Thus, first, a better understanding of the impact of rs1045642 polymorphism on P-glycoprotein protein expression and its specificity to nortriptyline and morphine should be established.

Furthermore, it is well documented that a decreased expression of P-glycoprotein increases bioavailability of many drugs in plasma and in the CNS through the blood brain barrier. [33,70,72,83] From this perspective, we should expect that the C allele of rs1045642 should be associated with decreased expression of P-glycoprotein protein. However, there is also evidence of a reverse effect in which P-glycoprotein in the choroid plexus increases the transport of molecules from the circulation into the cerebrospinal fluid [64] and hence could augment the influx of drugs from the choroid plexus into the CSF. The degree to which these mechanisms specifically influence the concentration and pharmacologic effect of morphine or nortriptyline in the CNS has yet to be determined.

Finally, transporters are known to be dynamic structures and their substrate specificity is influenced by a series of factors, including the size of the molecules to be transported and their chemical composition.[76] The effect of a single genetic variant in the *ABCB1* gene can be tissue specific, and compensatory transport pathways may also be activated. These compensatory transport pathways may have a greater impact on transporter activity when two or more drugs with different mechanisms of action are combined.[33,54,92]

An obvious limitation of this study is the number of subjects that were available for genetic testing. Despite the small sample, the controlled settings of the clinical trial allowed the detection of an effect of *ABCB1* rs1045642 in treatment response. The detection was possible in a small sample size due to the strong effect of the C allele. To determine if this association was relevant in more heterogeneous populations, the secondary analysis was pursued using UKBB data. The phenotypic outcome selected for the study was the number of self-reported painful sites within the past month, and even though the scale of characterization in the UKBB is not at the level of a controlled clinical trial for chronic pain, the number of painful sites is well understood to have a direct relationship with perceived intensity of pain, and with other pain-related outcomes, such as risk of pain chronicity, sleep disorders and limited functional capacity.[16,18,26,60,79] The drugs studied in this secondary analysis were expanded to include amitriptyline, which is a tricyclic antidepressant structurally similar to nortriptyline, and fentanyl, an opioid like morphine, both of which are substrates of *ABCB1*. [40,58,62] The results from our analysis show that rs1045642 is related to a decrease in the number of painful sites in participants that take

nortriptyline or amitriptyline in combination with morphine or fentanyl. Thus, although the UK Biobank cohort used for validation phenotypically different than the discovery clinical trial cohort, and the drug combination is extended rather than direct replication, the genetic association results are congruent.

In summary, our results suggest that the rs1045642 polymorphism has a strong clinical effect on combination treatment responses and may represent one example of the utility of genetic testing in the effective treatment of chronic pain. The genetic effect size of the C allele on treatment response is impressive and has little precedence on the pharmacokinetics field. [52] The increase in therapeutic efficacy of C allele carriers of rs1045642 does not correlate with increased adverse drug events, which suggest this combination of morphine and nortriptyline may be of benefit for neuropathic patients with this genotype. The results of this study increase the understanding of molecular genetic pathophysiology of outcomes in combination therapies, and the potential development of genetically targeted treatments for neuropathic pain. The standard implementation of genetic testing to future clinical trials studying treatment effects will speed the translation of knowledge, and fast-track the control of symptoms in chronic pain patients.

Acknowledgments

Financial support:

Canada Excellence Research Chairs Program (Grant # CERC08) to Luda Diatchenko

Alan Edwards Fellowship in clinical pain research to Rodrigo Benavides

Intramural Research Program of the NIH, National Institute of Environmental Health Sciences to Dmitri Zaykin

Clinical trial funding: Canadian Institutes of Health Research (CIHR) Grant #MCT-94187, Ethypharm (morphine study drug) and Apotex (nortriptyline study drug) to Ian Gilron

Gilron research salary support: CIHR-Pfizer Rx&Dc Collaborative Research Investigator Program (CIHR Grant #MSH-55041) and the Queen's University Department of Anesthesiology & Perioperative Medicine to Ian Gilron. Ian Gilron has received industry support from Adynxx, Biogen, Eupraxia, Novaremed and Teva.

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Characteristic	Females (n = 7)	Males (n = 18)	<i>P</i> -value
Age (Mean)	67.00	67.09	0.956
Weight	80.87	94.77	<0.0001
% reduction in pain			
Nortriptyline	41.19 (44.89)	45.86 (27.44)	0.788
Morphine	47.67 (34.73)	40.08 (33.24)	0.490
Combination	53.42 (41.29)	55.86 (26.85)	0.927

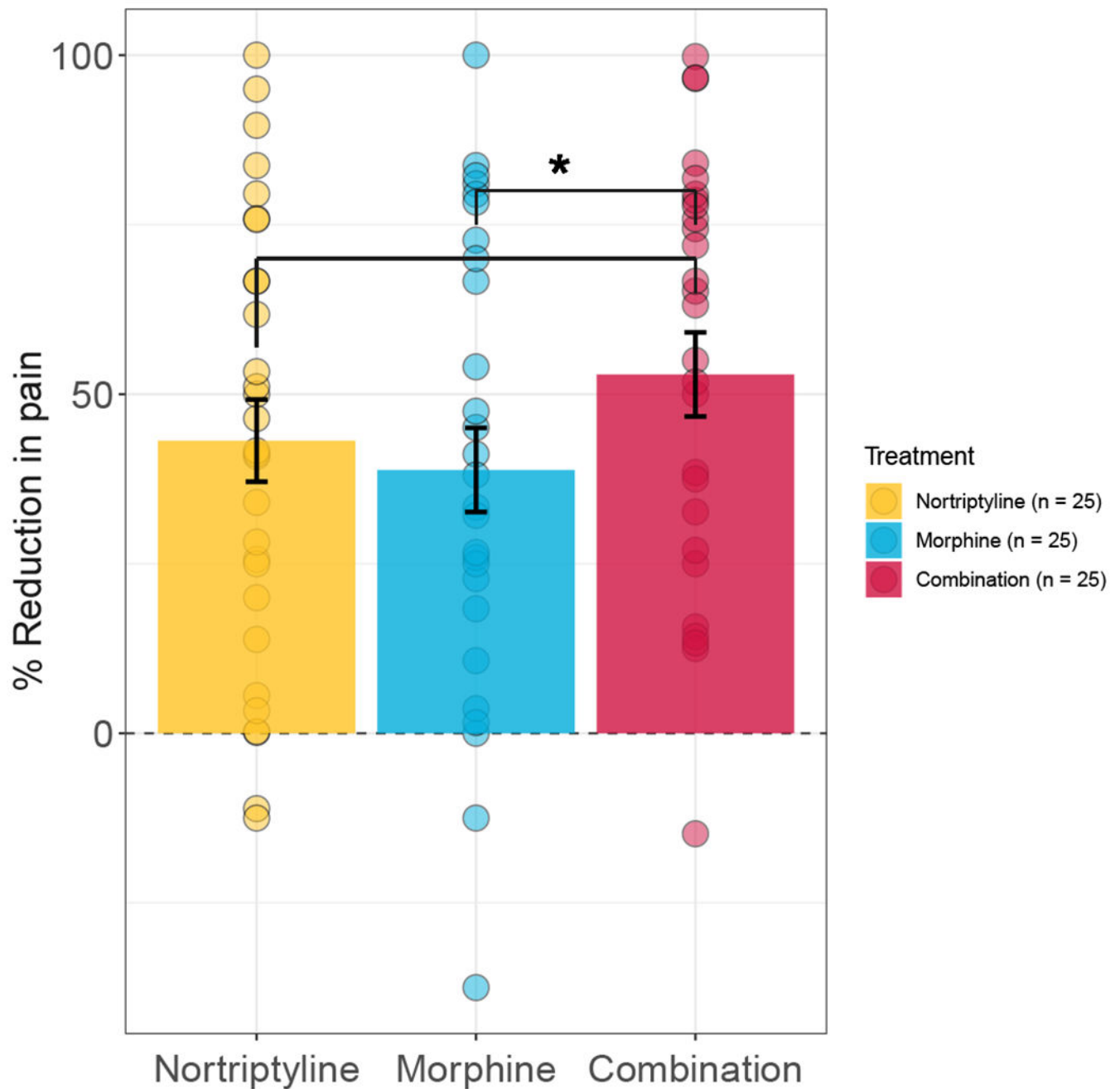


Figure 1. Pain score characteristics in the discovery cohort.

(A) Participant demographics and percent change in pain scores characteristics by sex. None of the observed differences were statistically significant. (B) Percent change in pain score under mono- (i.e., nortriptyline or morphine) or combination therapies. Dots represent individual observations, bars represent means, and error bars represent standard deviations.

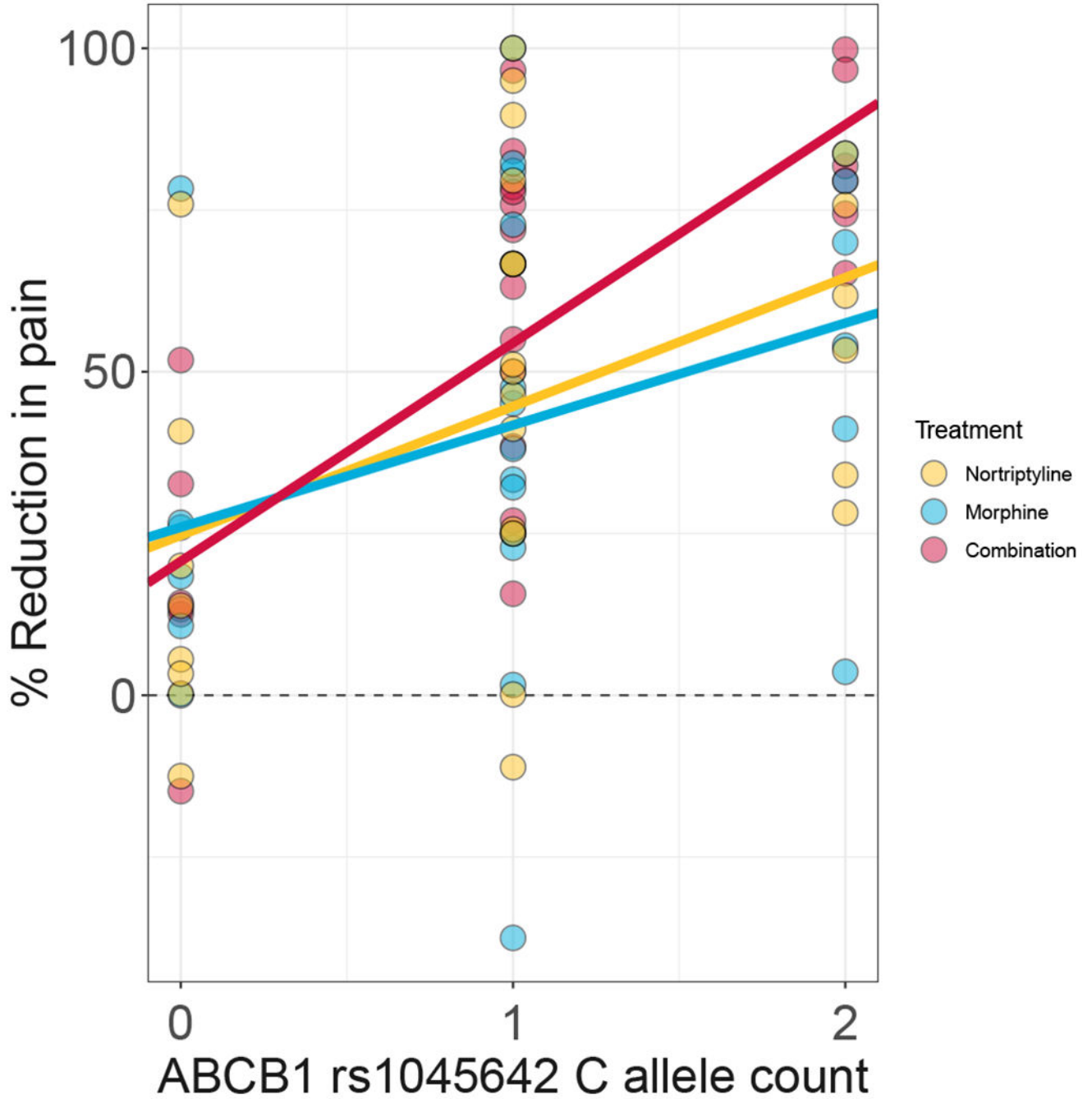
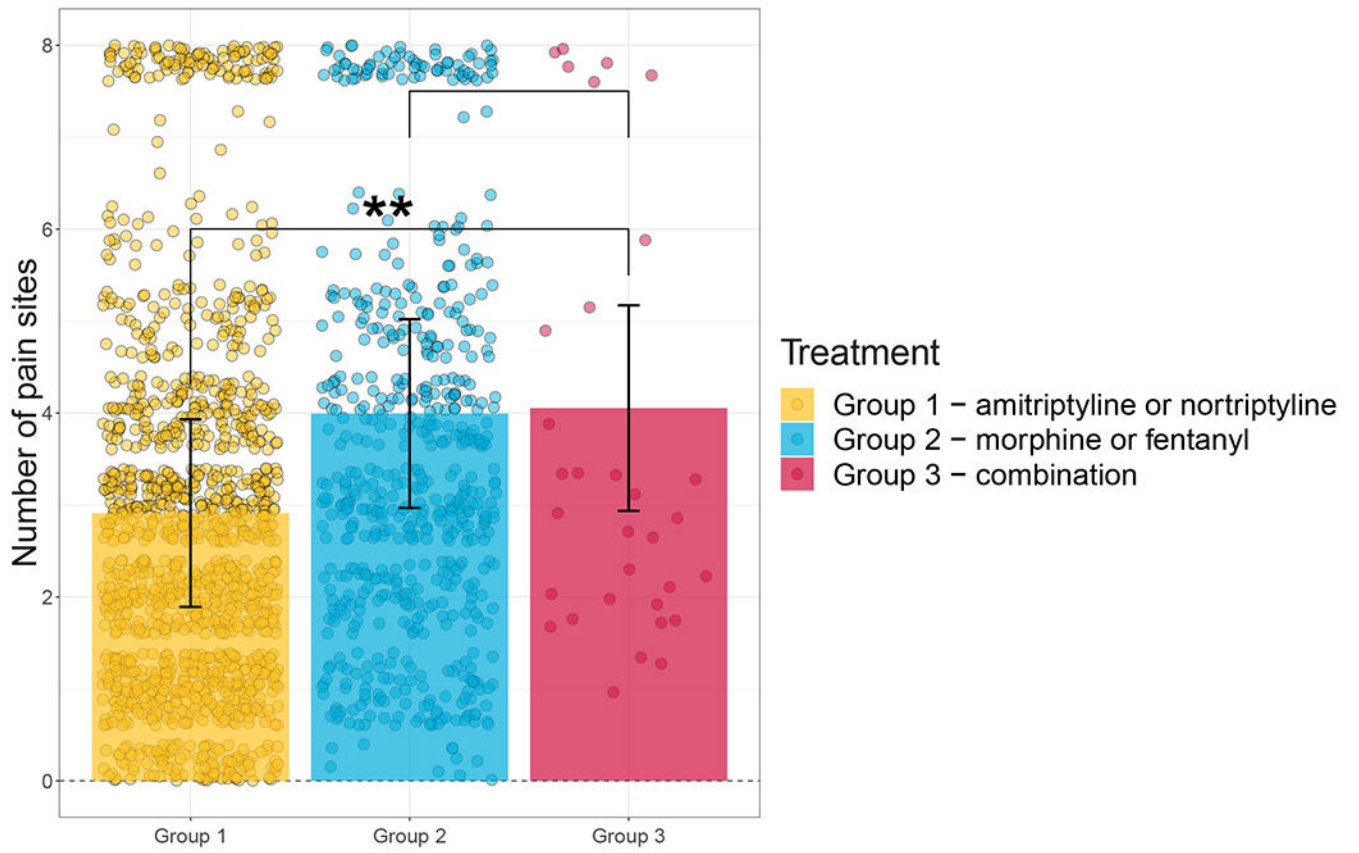


Figure 2. Percent reduction in pain stratified by C allele of *ABCB1* rs1045642 and treatment type in the discovery cohort.

Dots represent individual observations and lines represent fitted values from a linear mixed-effect model. Percent values highlight differences in treatment type efficacy between the homozygotes for the T allele and the homozygotes for the C allele. Under combination therapy, the homozygotes for C allele, on average, reported 88% improvement for pain reduction while the homozygotes for T allele reported 20% improvement for pain reduction. The improvement for pain reduction for C allele homozygotes was 64% in nortriptyline treatment group and 57% morphine treatment group.



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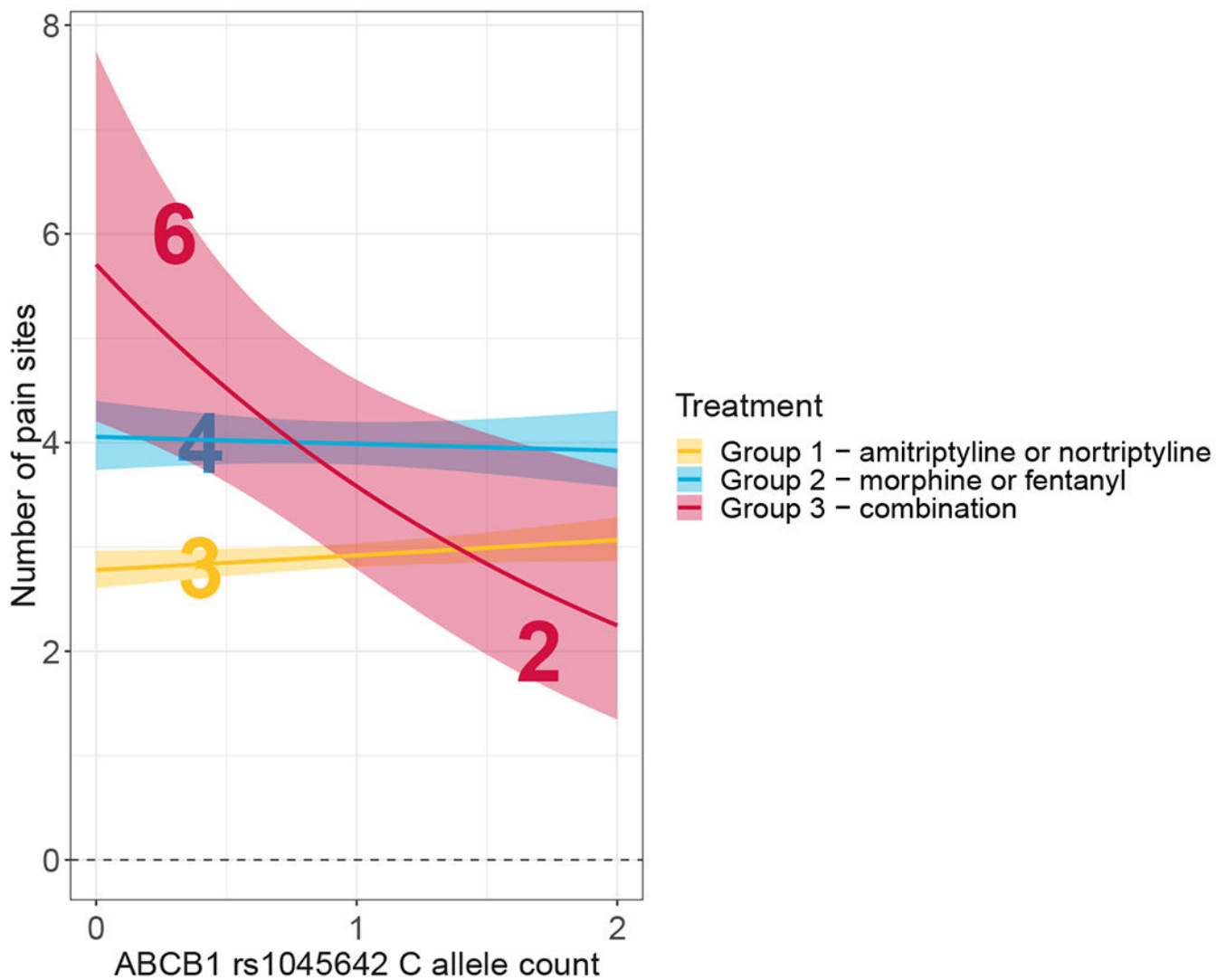


Figure 3. The number of pain sites by treatment group in the UK Biobank cohort (UKBB).

(A) The number of total painful body sites reported by subject in the UKBB cohort stratified by treatment group. Dots represent individual observations, bars represent means, error bars represent standard errors. (B) The reduction in number of reported pain sites stratified by C allele of rs1045642 and treatment group. Under combination therapy, the homozygotes for T allele, on average, reported 6 painful sites while the homozygotes for C allele reported 2 painful sites. The number of painful sites did not vary appreciably between the homozygotes for C or T alleles under either monotherapy treatment.

Table 1.

SNPs selected for the genetic association analysis.

Gene or metabolic pathway	rs number	Location	Functional change	Gene symbol	Gene name	Reference
OPRM1 gene	rs1799971	Exon 1	Asn40Asp	OPRM1	Opioid Receptor Mu 1	[4,8–10,24,27,35,44,49]
COMT gene	rs6269	Intron 2		COMT	Catechol-O-Methyl Transferase	[14,43,59,82]
	rs4633	Codon 62				
	rs4818	Exon 4				
	rs4680	Exon 4	Val158Met			
Serotonin receptor	rs6313	Exon 2	T102C	HTR2A	5-Hydroxytryptamine Receptor 2A	[28,45,77,94]
	rs7997012	13:46837850				
ABCB1 gene	rs1045642	Exon 26	C3435T	ABCB1	ATP Binding Cassette Subfamily B Member 1	[33,36,65]
	rs2032582	Exon 21	Ala893Thr			
CYP2C19 metabolism	rs12248560	Promoter		CYP2C19	Cytochrome P450 Family 2 Subfamily C Member 19	[25,30,31,71,80]
	rs4244285	Exon 5				
	rs4986893	Exon 4				
	rs28399504	Exon 1				
	rs56337013	Exon 9				
	rs7252267	Exon 3				
	rs7258186	Intron 5				
	rs41291556	Exon 3				
CYP2D6 metabolism	rs1065852	22:42130692	P34S	CYP2D6	Cytochrome P450 Family 2 Subfamily D Member 6	[25,30,31,80]
	rs201377835	22:42129910				
	rs5030862	22:42130668	G42R			
	rs72549357	22:42526657				
	rs28371706	22:42129770	T107I			
	rs1080985	22:42132375				
	rs72549354	22:42128815				
	rs59421388	22:42127608	V338M			
	rs35742686	22:42128242	259Frameshift			

Gene or metabolic pathway	rs number	Location	Functional change	Gene symbol	Gene name	Reference
	rs769258	22:42130761				
	rs3892097	22:42128945	Splicing defect			
	rs28371725	Intron 6	Splicing defect			
	rs1135840	22:42126611	S486T			
	rs5030655	22:42129084	118Frameshift			
	rs5030867	22:42127856	H324P			
	rs5030865	22:42129033	G169R			
	rs5030656	22:42128174	K281del			

Table 2.

Association results between the selected SNP/metabolic pathway and % reduction in pain by therapy type in the discovery cohort.

Gene or metabolic Pathway	SNP rsID	Genotype frequency		Morphine		Nortriptyline		Drug Combination		
		$f(AA)$	$f(aa)$	β (SE)	P-value	β (SE)	P-value	β (SE)	P-value	
OPRM1 gene	rs1799971	0.77	0.07	0.16	-0.97 (9.18)	0.999	2.15 (9.14)	0.988	-5.51 (9.18)	0.854
COMT metabolism	0 LPS (n = 5)				-7.13 (42.9)	0.973	-0.33 (42.9)	1.000	5.03 (42.9)	0.989
	1 LPS (n = 17)				-32.2 (41.9)	0.504	-28.9 (41.8)	0.555	-18.3 (41.9)	0.737
	2 LPS (n = 3)				-35.8 (49.3)	0.600	-46.6 (49.3)	0.507	-28.4 (49.3)	0.705
Serotonin receptor	rs6313	0.35	0.38	0.27	-0.88 (8.14)	0.999	2.12 (7.96)	0.985	-2.33 (8.15)	0.981
	rs7997012	0.54	0.42	0.04	2.92 (11.5)	0.986	-2.38 (11.5)	0.992	8.26 (11.5)	0.786
ABCB1 gene	rs1045642	0.23	0.54	0.23	14.5 (8.33)	0.193	18.2 (8.13)	0.065	32.5 (8.33)	4.89e-05*
	rs2032582	0.35	0.50	0.15	-13.4 (9.11)	0.307	-14.4 (9.10)	0.253	-21.2 (9.11)	0.052
CYP2C19 metabolism	PM (n = 0)				NA	NA	NA	NA	NA	NA
	IM (n = 3)				-30.4 (46.7)	0.586	-14.6 (46.7)	0.838	-8.50 (46.7)	0.939
	EM (n = 11)				-0.54 (51.1)	1.000	0.46 (51.1)	1.000	5.74 (51.1)	0.993
	UM (n = 11)				-27.2 (44.8)	0.624	-34.5 (44.6)	0.512	-20.8 (44.8)	0.729
CYP2D6 metabolism	PM (n = 1)				25.8 (46.9)	0.833	19.8 (46.9)	0.911	-1.90 (46.9)	1.000
	IM (n = 1)				23.2 (53.2)	0.886	-20.1 (53.2)	0.919	-3.46 (53.2)	0.999
	EM (n = 23)				-35.7 (44.9)	0.473	-45.0 (44.9)	0.355	-50.6 (44.9)	0.295
	UM (n = 0)				NA	NA	NA	NA	NA	NA

The results are based on linear mixed-effect models fitted to % reduction in pain with age, sex, treatment type, genetic factor, and an interaction between treatment type and genetic factor. A: major allele, a: minor allele. LPS: Low Pain Sensitivity; PM= poor metabolizers; IM= intermediate metabolizers; EM= extensive metabolizers and UM= ultra-rapid metabolizers.

* Statistical significant set at $\alpha=6.25e-03$ level.

Table 3.

Association results for SNP rs1045642 and side effects by treatment group in the discovery cohort.

Side effect	Morphine		Nortriptyline		Drug Combination	
	β (SE)	P-value	β (SE)	P-value	β (SE)	P-value
Epworth Sleepiness Scale (1-7)	0.07 (0.26)	0.988	-0.08 (0.24)	0.974	-0.11 (0.26)	0.956
Patient Assessment of Constipation Symptoms (0-4)	0.02 (0.19)	0.999	-0.02 (0.19)	0.999	0.07 (0.19)	0.970
Systolic Blood Pressure	3.54 (3.68)	0.657	0.55 (3.58)	0.997	-0.26 (3.67)	0.999
Diastolic Blood Pressure	-4.17 (3.27)	0.475	2.68 (3.27)	0.783	-0.49 (3.38)	0.998

The results are based on linear mixed-effect models fitted to the side effects with age, sex, treatment type, SNP, and an interaction between treatment type and SNP. *P*-values are for contrast coding that evaluate statistical significance of a genetic factor on a side effect under identified treatment group.

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Table 4.

Participant demographics in the UK biobank.

	Females (n = 1569)	Males (n = 792)	P-value
Age in years, mean (S.E.)	58 (7)	59 (8)	0.266
Treatment			<0.0001
Group 1 *	1165	472	
Group 2 *	378	314	
Group 3	26	12	
Number of pain sites			0.478
0	174	74	
1	265	143	
2	278	129	
3	287	151	
4	179	99	
5	104	51	
6	43	13	
7	6	3	
8	222	129	

Treatment Group 1 - amitriptyline or nortriptyline; Treatment Group 2 - morphine or fentanyl; Treatment Group 3 – combination of amitriptyline or nortriptyline along with morphine or fentanyl.

* Significant difference between females and males ($P < 0.05$). A two-sample t-test was used to assess differences between continuous variables and a chi-square test was used to compare counts.

Table 5.

Association results for functional SNPs within the *ABCB1* gene locus and the number of pain sites by therapy group in the UK Biobank.

<i>ABCB1</i> SNP	Genotype frequencies			Morphine		Nortriptyline		Drug Combination	
	<i>f</i> (AA)	<i>f</i> (Aa)	<i>f</i> (aa)	β (SE)	<i>P</i> -value	β (SE)	<i>P</i> -value	β (SE)	<i>P</i> -value
rs10248420	0.71	0.26	0.03	-0.02(0.05)	0.643	-0.02 (0.04)	0.511	-0.52(0.28)	0.062
rs1045642	0.30	0.48	0.22	-0.02 (0.04)	0.530	0.04 (0.03)	0.138	-0.37 (0.16)	0.022*
rs1128503	0.32	0.49	0.19	0.02 (0.04)	0.595	-0.05 (0.03)	0.061	0.11 (0.17)	0.500
rs11983225	0.77	0.21	0.02	-0.007 (0.06)	0.891	-0.04 (0.04)	0.367	-0.51 (0.32)	0.109
rs12720067	0.77	0.21	0.02	-0.0003 (0.06)	0.995	-0.05 (0.04)	0.258	-0.51 (0.32)	0.109
rs2032583	0.77	0.21	0.02	-0.007 (0.06)	0.891	-0.04 (0.04)	0.367	-0.51 (0.32)	0.109
rs2235040	0.77	0.21	0.02	-0.007 (0.06)	0.891	-0.04 (0.04)	0.406	-0.51 (0.32)	0.109
rs2235067	0.77	0.21	0.02	-0.001 (0.05)	0.983	-0.03 (0.04)	0.431	-0.52 (0.32)	0.105
rs4148739	0.77	0.21	0.02	-0.007 (0.05)	0.891	-0.03 (0.04)	0.367	-0.51 (0.32)	0.109
rs4148740	0.77	0.21	0.02	-0.007 (0.05)	0.894	-0.04 (0.04)	0.320	-0.51(0.31)	0.109
rs7787082	0.71	0.26	0.03	-0.02 (0.05)	0.633	-0.03 (0.04)	0.481	-0.52 (0.28)	0.062

The results are based on a Poisson model fit to the number of pain sites with age, sex, SNP, treatment group, an interaction between SNP and treatment group, and the first 40 principal components as linear predictors.

* Statistically significant result at $\alpha = 0.05$ level.