Maja Matić

Personalized Pain Therapy

Is the answer in the genes?

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Personalized Pain Therapy

Is the answer in the genes?

Individuele pijnbehandeling

Zijn de genen het antwoord?

Proefschrift

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Maja Matić
geboren te Zenica (Joegoslavië)

Erasmus University Rotterdam

PROMOTIECOMMISSIE

Promotoren:	Prof.dr. R.H.N. van Schaik
	Prof.dr. D. Tibboel
	Prof.dr. S.N. de Wildt
Overige leden:	Prof.dr. K. Allegaert
	Prof.dr. C.C.D. van der Rijt
	Prof.dr. T. van Gelder

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Part I

Introduction


SLC22A1
COMT
UGT2B7

Chapter 1

General Introduction

PAIN

The 'International Association for the Study of Pain' (IASP) defined pain in 1979 as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage." [1]. Recently this definition has been debated and revised in "a distressing experience associated with actual or potential tissue damage with sensory, emotional, cognitive, and social components." [2]. Worldwide, 1 in 5 individuals is experiencing acute, intermittent or chronic pain. This is leading to employment issues, depression and chronic comorbidities, especially in a prolonged and inadequately controlled setting [3]. The experience of pain is very subjective and multidimensional, displaying substantial variability between individuals even when exposed to an identical pain-evoking stimulus. Differences in gender, age, ethnicity and anxiety level [4, 5] are among the known factors that contribute to this interindividual variability in the experience of pain.

In the present setting, with the exception of age, neither the choice of analgesics nor the doses selected are based on these factors. In recent years, genetic variation in genes involved in the disposition of analgesics as well as genes responsible for the analgesic effect itself has been suggested to play an important role in variation in response.

ASSESSMENT OF PAIN

Pain experience is very subjective. Due to the lack of objective markers for pain [6], self-reports remain the gold standard. This is particularly challenging for specific patient groups, such as young children (< 5–6 years) or cognitive impaired individuals, who are generally less able to adequately communicate their pain to health care practitioners. Critically ill patients admitted to an intensive care unit are frequently receiving respiratory support and are sedated, which also complicates pain assessment. To sufficiently manage pain in specific groups (e.g. neonates, infants, children, critically ill adults) and circumstances (e.g. procedures, postoperative and prolonged pain), observational pain assessment instruments have been developed and validated in pediatrics (COMFORT-B, FLACC, MAPS and PIPP-R) [7] and critically ill adults (CPOT) [8].

Although procedural pain is decreasing in children admitted at an intensive care, still on average they are exposed to 11.4 (SD = 5.7) painful/stressful procedures per day [9, 10]. Adequate analgesia is needed from an ethical perspective as well as to avoid negative neurodevelopmental consequences due to pain experience during life [11], in particular in pre-terms although still under debate [12–14]. As a consequence 33.3% of the children is exposed to analgesics and sedative drugs, with up to 89.3% in tracheal ventilated children [15]. In the adult population, adequate pain management is of importance because 20% of

the individuals develop chronic pain after a surgical procedure. This may lead to reduced quality of life, a rise in unemployment and concordantly an increase in healthcare costs [16]. Because inadequate treatment of pain is non-ethical and may have serious mental, physical and economic consequences, there is a need for predictive markers in the management of pain.

PHARMACOLOGICAL TREATMENT

The WHO pain scale (Pain Ladder) was initially developed to tailor the treatment of cancer related pain in adults, but the basic principle of this strategy is now also applied in other non-malignant pain conditions. Mild pain is controlled with anti-inflammatory drugs (e.g. acetaminophen, ibuprofen). When pain relief with these analgesic agents is insufficient, or when patients present initially with mild to moderate pain, weak opioids are prescribed (e.g. codeine, tramadol). Further escalation in pain intensity or the occurrence of moderate to severe pain at presentation is treated with strong opioids such as morphine and fentanyl [17]. Cancer related pain or chronic pain with a neuropathic component are inadequately controlled with these analgesic agents and require a different pharmacological approach (e.g. antidepressants, anticonvulsants) [18]. These analgesics are, however, outside the scope of this thesis.

Opioids are generally an effective approach in relieving acute moderate to severe pain, especially by use of compounds with a short half-life or by rapid absorption due to administration via non-oral route. As with most pharmacological interventions, the use of opioids is not without limitations. In the chronic pain setting, 5.8–10.3% of the patients report poorly controlled pain and 12.1–22% discontinue pain medication as a consequence of adverse events (AEs) [19].

Young children are more prone to develop AEs, such as respiratory depression, as a consequence of immature organ function and dosing challenges [20]. In addition to the non-life threatening AEs (e.g. constipation, vomiting, sedation, pruritus) that either resolve over time or are treated symptomatically, serious toxic effects can increase morbidity and mortality risk in this vulnerable group [21]. Several cases from the pediatric population with severe toxicity and fatal outcomes while exposed to opioids have been reported in the literature [22–24].

Currently, the pharmacological management of pain is reflected by a trial-and-error approach in providing adequate analgesia, with periods of remaining pain or the occurrence of toxicity. The starting dose is similar for patients, with some adjustments based on age and/or weight. This can lead to delayed or inadequate pain relief in some or toxicity in others. Ideally, one would want to distinguish these patients pre-emptively before the first drug and dose are administered.

GENETIC IMPACT

The variability observed in pain experience and opioid response is a complex interplay of several clinical and environmental factors. Twin studies assessing pain phenotypes and analgesia highlight genetics as one of the contributing factors [25, 26]. The use of genetic information in guiding pharmacological treatment (drug selection and dosing) is the focus of the Pharmacogenetics (PGx) science field. The PGx term is used interchangeably with pharmacogenomics, although the latter field covers a broader range including the effect of the genome (combination genes) on drug therapy [27]. Currently the relevance of PGx is mainly recognized outside the pain area, in e.g. psychiatry, cancer, cardiology and internal medicine [28].

The implementation of PGx in a clinical setting remains a slow process. Challenges encountered in the clinical translation process are unawareness of available analysis among health care providers, lack of harmonization in laboratory results, translating genotype in phenotype difficulties [29], lack of appropriate cost-effectiveness analysis and clear reimbursement policies [30]. To overcome the translational gap between genetic research and clinical use, the Clinical Pharmacogenomics Implementation Consortium (CPIC) has developed evidence-based guidelines that report gene-drug interactions and give actionable decision tools for the physician in the prescription process [31]. In the Netherlands, it is the Royal Dutch Association for the Advancement of Pharmacy - PGx Working Group (KNMP) who performs these analyses since 2005, with recommendations for drug dosing. These evidence-based dosing recommendations are taken over by the USA (www.pharmgkb.org), demonstrating the leading position of the Netherlands in this particular field.

Genetic variability within the cytochrome P450 (CYP) enzymes has been extensively studied, characterized and translated to genotype predicted phenotypes. For the highly polymorphic CYP2D6 enzyme, over 100 allelic variants have been reported in the Human Cytochrome P450 Allele Nomenclature Database (http://www.cypalleles.ki.se/cyp2d6.htm). These variant alleles can be translated to predict phenotypes, such as poor metabolizer (PM), intermediate metabolizer (IM), normal/extensive metabolizer (EM) or ultrarapid metabolizer (UM) status [32]. The frequencies of these predicted phenotypes may vary between ethnic populations. Where up to 5–10% of the individuals is CYP2D6 PM in the European population, CYP2D6 PM status in Asians and Africans is less frequent (< 2%). In contrast, approximately 2–5% of the Caucasian population is a CYP2D6 UM whereas the percentages in the African and Asian population are up to 40% and 2%, respectively [33].

Considering the management of pain, a CPIC guideline on codeine addressing the genetic variability in the metabolizing enzyme CYP2D6, responsible for activating codeine into morphine, has been published [34]. The guideline states that both codeine and tramadol should be avoided in individuals with CYP2D6 genotypes predicting UM or PM

status. These patients are more prone to either morphine toxicity (UMs) or inefficacy of treatment (PMs). A case report in Lancet in 2006 described the death of a newborn due to codeine intoxication in a mother with the CYP2D6 UM status [35]. Due to increased conversion of codeine into morphine by CYP2D6, high levels of morphine did reach the neonate through the breast milk, thus causing respiratory depression as a result of exposure to high morphine concentrations. Codeine toxicity in relation to CYP2D6 genotype was also the cause of three additional deaths in children aged between 4–10 years [36]. Also for tramadol, an alternative opiate, severe respiratory depression has been observed in a child with obstructive sleep apnea syndrome that had the CYP2D6 UM status [24].

For pain PGx, it is thus far only a small subset of findings that is translated to the clinic. In contrast, a large number of genetic variants has been described in the literature regarding the relation to pharmacokinetic (PK) profiles and response of opioids [37, 38]. Examples are the genes encoding metabolizing enzymes (*UGT2B7)* and transporters (*ABCB1, ABCC3, SLC22A1*), but also the mu-opioid receptor (*OPRM1*) and its second messenger system (*KCNJ6, ARRB2)* or other enzymes involved in pain such as the catechol-O-methyltransferase enzyme (*COMT*).

Thus, there seems to be a large potential of DNA markers available, yet, clinical implementation is not achieved and it is difficult to assess which of these potential markers are suitable for further exploration in a clinical setting.

INTERPLAY DEVELOPMENTAL PATTERN AND GENETICS

While most PGx studies have been performed in adult patients, the evidence in the pediatric population is scarce. Ethical concerns, lack of data on PGx utility, lack of cost effectiveness information in combination with developmental aspects also affecting drug metabolizing capacity in this population have been mentioned as obstacles [39]. Children are subject to physiological maturation and developmental changes affecting the absorption, distribution, metabolism and elimination (ADME) of drugs. The absorption of drugs is altered in children as a consequence of pH changes in the gastrointestinal tract, increased gastric emptying and lower intestinal surface area compared to adults. Renal elimination is affected by maturation of the glomerular filtration rate and tubular secretion process. Adult rates are reached between 8 to 12 months. Also the distribution of drugs is different in children due to changes in body (more water vs. fat tissue) and plasma proteins composition but also due to ontogeny of transporters such as *P*-glycoprotein (ABCB1). [40].

The metabolizing enzymes in the PK process are also highly subjective to an age-dependent maturation. For the most important cytochrome P450 enzyme with respect to drug metabolism, CYP3A4, adult activity levels are not reached until the age of 1–2 years

[41–43]. In children drugs metabolized by this enzyme will have a lower degradation as compared to adults, and thus will lead to higher drug concentrations. CYP2D6 matures more rapidly with 90% expressed after the first postnatal week [44]. A CYP2D6 genotype predicted phenotype can be accurately predicted by 2 weeks of postnatal age (PNA) [44, 45]. The effect of a CYP2D6 genotype has already been observed in the neonatal population, when looking at tramadol disposition [46].

The developmental pattern of these drug metabolizing enzymes and transporters leads to alterations in the formation and elimination of active compounds and metabolites, hereby affecting efficacy and drug toxicity risk. Findings on genetic markers for analgesia in adults predicting pharmacokinetics of analgesics cannot always be simply translated to the pediatric population [47]. For instance, low protein expression and thus activity of an enzyme or transporter will disrupt any genotype-phenotype correlation in this population since all individuals will be a poor metabolizer, irrespective of genetic composition [48].

SUMMARY

Numerous genetic variants have been implicated in pain and analgesia. An overview with the most potential candidate polymorphisms for application in clinical practice is needed. The role of candidate genes needs to be validated in adult postoperative and cancer cohorts. As opposite to adults, data on the relevance of PGx for pain treatment in the pediatric population is limited. Since found genotype-phenotype associations are not directly translatable to (the youngest) children due to the influence of developmental changes on the phenotypic activity, research addressing this topic is highly required.

AIMS AND OUTLINES OF THIS THESIS

The aims of this thesis are:

- To investigate which genetic variants are potential candidates
- To validate whether selected genetic variants are related with the observed variability in opioid response in an adult postsurgical situation and in adults treated for cancer-related pain
- To assess in healthy children associations between experimentally induced pain and these candidate genes
- To investigate the effect of known genetic variants in DME's and drug transporters on the PK of opioids in the neonatal population
- To explore whether the most commonly addressed candidate genes are correlated with opioid response in the pediatric population

This thesis starts in **Part I** with an overview of the available PGx literature for their correlation with effectiveness of opioids and relation with side effects in adults and children (**Chapter 2**). **Part II** (**Chapter 3–5**) is addressing the role of PGx in the adult population: the role of genetics on thermal, postsurgical acute and chronic pain of patients undergoing cardiac surgery is discussed in **Chapter 3** whereas the clinical and genetic factors in oncological populations is investigated in **Chapter 4** and **5. Part III (Chapter 6–10)** focusses on the role of PGx on pain and its treatment in the pediatric population. **Chapter 6** describes the genetic contribution in an experimental pain setting in children. **Chapter 7** and **8** are addressing the effect of ADME gene polymorphisms in relation to morphine and tramadol PK. The effect of *OPRM1* and *COMT* genetic variants on morphine efficacy is described in **Chapter 9** and withdrawal during opioid treatment in children admitted to the neonatal and pediatric intensive care unit is presented in **Chapter 10**. Finally, in **Part IV**, a discussion on the main findings of this thesis in a broader perspective is included (**Chapter 11**), taking into account recent data from literature and speculating on future directions. The thesis is concluded with a summary (**Chapter 12**).

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Part II

Genetics, Pain and Analgesia in Adults

Chapter 4

Opioid treatment failure in cancer patients: the role of clinical and genetic factors

Astrid W. Oosten*
Maja Matic*
Ron H.N. van Schaik
Maxime P. Look
Joost L. Jongen
Ron H.J. Mathijssen
Carin C.D. van der Rijt

*These authors contributed equally to this manuscript.

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ABSTRACT

Aim: To identify clinical and genetic factors associated with outcome of opioid treatment.
Patients & methods: We performed an exploratory analysis in a cohort of 353 patients treated with fentanyl, morphine, oxycodone and/or hydromorphone for cancer-related pain, exploring selected clinical and pharmacogenetic factors for a correlation with treatment failure for all and per type of opioid.
Results: Use of adjuvant pain medication, intensity of pain at rest and age were associated with treatment failure in the various cohorts. Only the genetic variants rs12948783 (*RHBDF2*) and rs7016778 (*OPRK1*) correlated statistically significant in univariate, but not in multivariable analysis.
Conclusion: Several clinical and genetic factors were identified that warrant further study to clarify their role and use in opioid treatment.

INTRODUCTION

Opioids are the cornerstone of treatment for moderate to severe cancer-related pain. Although treatment is successful in the majority of patients, 25–40% does not achieve sufficient pain control and/or experiences serious side effects limiting dose escalation [1, 2). In these cases, opioid rotation is successful in about two-thirds of patients. However, opioid rotation is time-consuming, which is unwanted in a population with limited life-expectancy (3, 4). As we are currently unable to predict the clinical response to specific opioids for an individual patient, finding the right type and dose of opioid is still a matter of trial and error. The effects on pain and the occurrence of side effects are the result of a complex interplay between clinical / demographic, pharmacokinetic and -genetic factors (5, 6). So far, factors related to treatment-failure of individual opioids that can be used to guide treatment decisions (2, 7–10) have not been identified.

Studies in twins, separating environmental from genetic influences, have demonstrated that up to 60% of the inter-individual variation in pain perception and analgesia can be attributed to a person's genetic predisposition (11, 12). In the last decade, a large number of studies have found associations between genetic variants of drug metabolizing enzymes (*CYP3A4, CYP2D6*), membrane drug transporters (*ABCB1, ABCC3, OCT1*), molecules involved in opioid receptor signaling (*OPRM1, OPRK1, OPRD1, KCNJ6*) and pain modulators (*COMT*) on the one hand and opioid efficacy, required dose, and toxicity on the other hand (5, 13). These studies mostly had a small sample size, focused solely on morphine, or data from various types of opioids were pooled. The European Pharmacogenetic Opioid Study (EPOS) included a large number of cancer patients and studied the influence of genetic variability on opioid dose, during opioid treatment. No statistically significant associations were found between 112 single nucleotide polymorphisms (SNPs) in 25 candidate genes and opioid dose (14). Sub studies from the EPOS patient cohort focused on pharmacokinetics of fentanyl (15), pharmacokinetics of oxycodone (16), occurrence of nausea and vomiting (17) and constipation (18). The study on pharmacokinetics of fentanyl reported that the *CYP3A4*22* and *CYP3A5*3* variants accounted for a small proportion of the variability in pharmacokinetics of fentanyl (15). For oxycodone, *CYP2D6* genotypes were shown to influence the pharmacokinetics of oxycodone, but not the pharmacodynamics (16). For nausea and constipation, although a correlation was found with 8 and 5 SNPs, respectively, only two SNPs (rs1672717 in the 5-Hydroxytryptamine (Serotonin) Receptor 3B (*HTR3B*) for nausea (17); and rs2020917 in the enzyme Catechol-O-methyltransferase (*COMT*) for constipation (18)) passed the Benjamini-Hochberg criterion for a 10% false discovery rate. However, EPOS was a cross-sectional study, in which outcomes were studied at a random time point during opioid treatment. To our knowledge, no studies have assessed whether a combination of clinical and genetic factors is related to the efficacy

or failure of treatment with individual opioids, whereas this information could help to personalize pain management in cancer patients (19).

With the aim to identify clinical and genetic factors related to treatment failure of opioids, we performed an exploratory prospective study in patients treated with morphine, oxycodone, fentanyl, or hydromorphone for cancer-related pain.

PATIENTS AND METHODS

Patients admitted to the department of Medical Oncology of Erasmus MC Cancer Institute (Rotterdam, the Netherlands), who were treated with opioids for moderate-severe nociceptive cancer-related pain (with or without a neuropathic component) were included in this prospective study. Patients with an expected duration of hospitalization < 72 h and patients unable to give informed consent were excluded from the study. Patients were admitted to our specialized acute palliative care unit (PCU) or one of two general oncology wards. Pain was treated stepwise following the World Health Organization analgesic ladder (20) and only patients treated with strong-acting (step III) opioids were eligible. Treatment was given in line with our institutional protocol for the treatment of oncological pain, which is based on (inter-) national guidelines. Of note, because many patients on the PCU are admitted with complex pain problems; high doses of opioids, opioid rotation, parenteral administration of opioids and/or adjuvant analgesics were often necessary. In general, the type of opioid used before hospital admission was continued unless dose escalation was not possible due to side effects or problems related to administration. In patients with severe pain, we generally used subcutaneous morphine or fentanyl for titration. Doses were titrated while closely monitoring the effect on pain (by numeric rating scale 0–10 twice daily) and side-effects (10 most common side effects assessed using a 4-point Likert scale twice daily). Opioid rotation was performed in case of insufficient pain control despite adequate dose escalation and/or dose limiting side-effects and/or the occurrence of other dose limiting events, such as volume related problems with subcutaneous infusions. Adjuvant pain medication was started in case of an insufficient effect of opioids in patients with mixed nociceptive-neuropathic pain. Selection of the opioid of first, second or third choice was based on clinical factors (i.e. renal function, possibility for use of oral route) and treatment history. In opioid naive patients, our protocol advises oxycodone as a first choice.

Clinical and demographic data were collected. All data were registered in an electronic database (©2004–2012 OpenClinica, LLC and collaborators). Patients were categorized in treatment groups according to the type of opioid(s) they received. In case of rotations between different types of opioids, patients were included in all the specific treatment groups.

For the analysis, we defined T_0 as the start of the clinical titration period for the opioid of study. In case of pain requiring opioid titration at admission, T_0 was set at the time of hospitalization irrespective of the use of opioids before referral. When an opioid was started after hospitalization, T_0 was set at the moment of that start. In case of an opioid rotation, a new titration period started. Therefore, at T_0, patients could be opioid naive, already using the respective opioid (before hospitalization) or starting a new opioid after rotation. For every patient, the treatment response per opioid was classified as failure or non-failure. The response was classified as failure in case of: 1) a rotation to another type of opioid because of insufficient pain control and/or side effects, 2) a treatment with intrathecal opioids because of persistent pain and/or side effects, *or* 3) the use of palliative sedation because of refractory symptoms associated with opioid treatment in the dying phase. In all other patients the response was classified as non-failure. A rotation from oxycodone to another type of opioid given parenterally was considered as failure only if the reason for rotation included adverse events. We excluded patients rotating solely because of a need for (fast) parenteral titration, as oxycodone for parenteral use is not available in our hospital. The study was approved by the Erasmus MC medical ethics review board (study ID: MEC 09.332) and conducted in accordance with the Declaration of Helsinki. The trial was registered at the Dutch Trial Registry (Trial registration ID: NTR4369). Written informed consent was obtained from all participants, with separate informed consent for the DNA analysis.

Analyses of SNPs

Blood samples for pharmacogenetic analysis were collected concurrent with the first venipuncture for blood sampling for a medical indication and after obtaining informed consent. DNA was isolated from 1 mL EDTA blood on the MagNA Pure LC 2.0 instrument (Roche Diagnostics®). Genetic variants were selected based on evidence from literature, taking into account allele frequency, clinical impact and reproducibility of effect. The analysis was performed with the TaqMan allelic discrimination method on the 7500 Real-Time PCR System (Life Technologies®). *CYP2D6* duplication and deletion (*5 allele) were determined on the ProFlexTM PCR system (Life Technologies®) and visualized via gel electrophoresis on 1% agarose gel.

Violation of Hardy-Weinberg (HW) equilibrium was calculated for all genetic variants with the chi-squared – test. Additionally, the observed minor allele frequency (MAF) was compared with the MAF from HapMap in dbSNP (National Centre for Biotechnology Information). The *COMT, CYP2D6 and OCT1* haplotypes were estimated based on the expectation-maximization (EM) logarithm with R (version 3.1.1) haplo.stats package, using a posterior probability > 0.98. Patients genotyped GGC (rs4680, rs4818, rs4633 resp.) for *COMT* were categorized in the low pain sensitivity (LPS) group, ACT genotype in average pain sensitivity (APS) group and GCC in high pain sensitivity (HPS) group, as

previously this haplotype has been related with experimental pain sensitivity (21) and opioid consumption (22{Tan, 2016 #5836, 23, 24). The LPS group consisted of patients with the LPS/LPS or LPS/APS alleles, APS from APS/APS or LPS/HPS alleles and HPS from the alleles HPS/HPS or APS/HPS.

Statistical analysis

Data were analyzed using STATA® version 13. Descriptive statistics were used to summarize patients' characteristics. Statistical analyses were performed for the whole group of patients and per type of opioid. For the analysis of all patients, the first opioid that was used for titration during admission for which an observation period of at least 24 hours was available, was selected. Logistic regression analysis was used with treatment failure as the dependent variable and the SNPs described in the previous paragraph and clinical/demographic factors as covariates. For the analysis in the whole group of patients only SNPs in genes related to pharmacodynamics or pain sensitivity were tested. The following clinical/demographic factors were explored: gender, age, radiotherapy on any tumor localization related to the pain for which opioid treatment was initiated (either 1–8 weeks before T_0 *or* 1 week before - during hospitalization), use of adjuvant pain medication (pregabalin, gabapentin, or amitriptyline) or use of corticosteroids started before T_0 and continued *or* started on T_0 or during hospitalization, pain at rest and worst pain at T_0 (rated using Numeric Rating Scale (NRS 0–10) and divided into categories mild (NRS 0–4), moderate (NRS 5–6) and severe pain (NRS 7–10) and opioid dose at T_0. For the analysis of all patients, doses were recalculated to the median oral morphine equivalent daily dose (MEDD) according to published equi-analgesic dose tables: oral morphine 60mg/d = parenteral morphine 20 mg/d = transdermal fentanyl 25 mcg/h = oral oxycodone 40 mg/d = parenteral hydromorphone 4 mg/d (25). For the whole group, as well as the opioid specific groups, doses were divided into 2, 3 or 4 equally sized dose level groups based on the appropriate quantiles (Q)). For the ordinal factors (pain, opioid dose) each category was analyzed using the first category as reference. Patients already using opioids before admission, who were rotated within 24 hours after admission were excluded for the analysis of time-dependent variables for the opioid used at admission. Reported *p*-values are two-sided and because of the exploratory nature of this analysis, factors with a *p*-value < 0.10 in univariate analysis were entered into the multivariable analysis. The 'backward elimination' method was used to find the combination of clinical and genetic factors associated with treatment failure. Again, we used a threshold of 0.1 for significance. Resulting *p*-values were not corrected for multiple testing because of the exploratory nature of this analysis. The multivariable analysis was performed twice, with and without adjustment for opioid dose at T_0.

RESULTS

Between January 2010 and April 2014, a total of 356 individual patients were included in this study. Three patients were not evaluable because they used different opioids simultaneously during the entire study period and therefore 353 patients were analyzed. The median age of the patients was 61.5 years (range 24–86) and 168 (48%) patients were male. The most frequent tumor origins were the urinary tract (20%), gastro-intestinal tract (18%) and breast (16%). Most patients had advanced stages of cancer (80%) and the median WHO performance status was 2. The median duration of hospitalization – and therefore follow-up – was 9 days (range 1–48). For all but 2 patients the duration of follow-up exceeded 72 hours.

The majority of patients (n = 214) was treated with a single type of opioid, whereas 113 patients were treated with 2 opioids, 22 patients with 3 and only 4 patients with 4 opioids (**figure 1**). In the fentanyl group, most patients (66%) already used fentanyl before hospital admission, while in the morphine group patients were mostly rotated from another type of opioid (58%). In the oxycodone cohort most patients were either opioid naive (38%) or already used oxycodone (43%) before study entry. As expected, the hydromorphone cohort contained mostly patients in whom treatment with other opioids had failed (89%). A

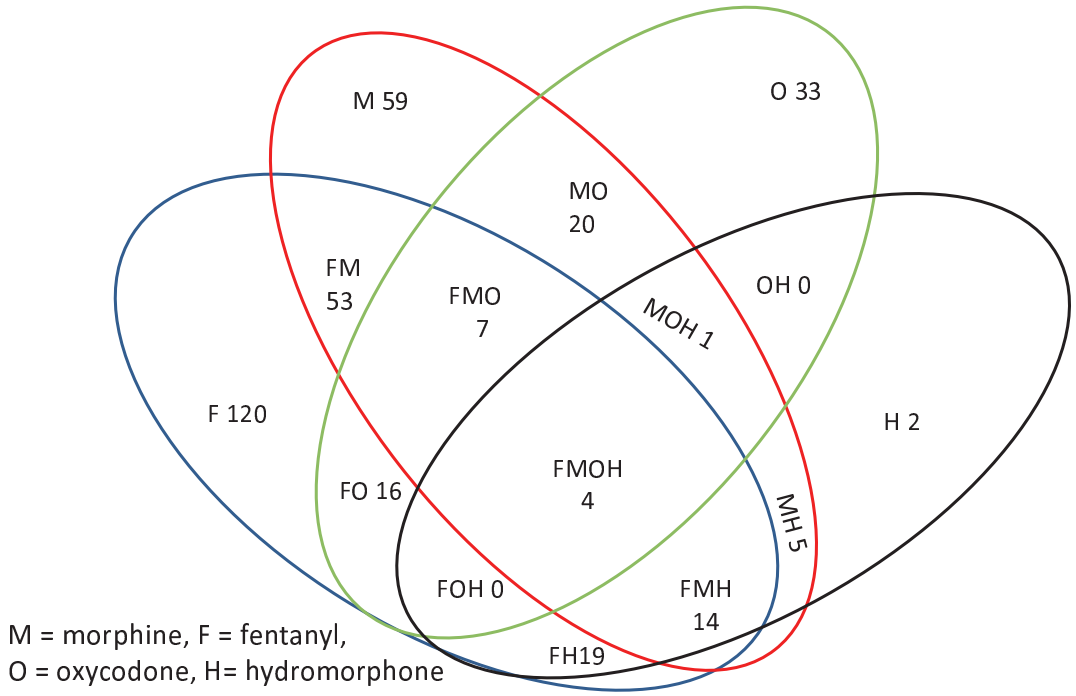

Figure 1. A Venn diagram showing the numbers of patients treated with the specified opioid consecutively throughout the study. For example, 20 patients were treated with both morphine and oxycodone and studied in both cohorts.

wide range of treatment doses was observed for all types of opioids. Regarding the median morphine equivalent daily dose (MEDD), doses were quite similar in the fentanyl (120 mg) and morphine group (101 mg) but lower treatment doses were given in the oxycodone cohort (30 mg), whereas, as expected, MEDD was highest in the hydromorphone group (504 mg). The number of patients in whom treatment failed divided by the total number of patients per group was 81/353 (23%) for all patients, 59/233 (25%) for fentanyl, 56/163 (34%) for morphine, 27/81 (33%) for oxycodone and 9/45 (20%) for hydromorphone.

Genotype distributions

From the total cohort, written informed consent for DNA-analysis and a blood sample were available for 346 patients. The undetermined genotype results ranged from 0.9–2.3% per assessed genetic variant. None of the SNPs violated HW equilibrium ($p > 0.05$), nor were there large differences observed between the study and MAFs reported in the literature (**Supplementary table 1**).

Association of treatment failure with clinical and genetic factors in univariate and multivariable analysis

All patients: In univariate analysis, factors associated with failure of treatment were age (Odds ratio (OR) 0.58, 95% Confidence Interval (CI) 0.39–0.97, $p = 0.039$), use of adjuvant pain medication started on T0 or later (OR 3.04, 95% CI 1.76–5.24, $p = 0.000$), use of corticosteroids started on T0 or later (OR 1.95, 95% CI 1.15–3.29, $p = 0.012$), pain at rest (category severe pain OR 3.13, 95% CI 1.29–7.56, $p = 0.011$) and worst pain at T0 (category severe pain OR 3.21, 95% CI 1.19–8.69, $p = 0.022$), the MEDD at T0 (Q3: OR 2.27, 95% CI 1.04–4.92, $p = 0.038$ and Q4: OR 3.10, 95% CI 1.45–6.63, $p = 0.004$) and the rs12948783 SNP in RHBDF2 (OR 0.55, 95% CI 0.32–0.96, $p = 0.035$). Of these, the use of adjuvant pain medication (OR 3.49, $p = 0.000$), severe pain at rest (OR 2.67, $p = 0.048$) and the rs12948783 SNP in RHBDF2 (OR 0.37, p 0.056) were (possibly) independent as shown in multivariable analysis (tables 1–3). When the analysis was corrected for opioid dose, results were unchanged.

Fentanyl: In univariate analysis, factors associated with failure of fentanyl treatment were age (Odds ratio (OR) 0.98, 95% Confidence Interval (CI) 0.95–1.00, $p = 0.071$), use of adjuvant pain medication started on T_0 or later (OR 2.45, 95% CI 1.20–5.00, $p = 0.013$), use of corticosteroids started on T_0 or later (OR 2.88, 95% CI 1.42–5.87, $p = 0.004$), pain at rest (category severe pain OR 5.72, 95% CI 1.61–20.37, $p = 0.007$) and worst pain at T_0 (category severe pain OR 7.67, 95% CI 0.98–59.84, $p = 0.052$), the dose of fentanyl at T_0 (Q3: OR 3.41, 95% CI 1.16–10.09, $p = 0.026$ and Q4: OR 2.90, 95% CI 0.91–9.29, $p = 0.072$) and the rs1799971 SNP in *OPRM1* (OR 0.44, 95% CI 0.19–1.06, $p = 0.066$). Of these, age (OR 0.95, $p = 0.081$), the use of adjuvant pain medication (OR 1.83, $p = 0.067$),

Table 1. Distribution of clinical factors between patients failing and not-failing treatment for all patients and per type of opioid

	All - NF n = 249 (%)	**All - F n = 104 (%)**	**Fe - NF n = 174 (%)**	**Fe - F n = 59 (%)**	**Mo - NF n = 107 (%)**	**Mo - F n = 56 (%)**	**Ox - NF n = 54 (%)**	**Ox - F n = 27 (%)**	**H - NF n = 36 (%)**
Male	124 (50)	44 (42)	84 (48)	26 (44)	49 (46)	25 (45)	25 (46)	11 (41)	23 (64)
Median age	61	59 *	63	58 ‡	63	60 ‡	61	61	55
Excluding patients rotating < 24 h	*n = 249 (%)*	*n = 104 (%)*	*n = 172 (%)*	*n = 40 (%)*	*n = 106 (%)*	*n = 49 (%)*	*n = 54 (%)*	*n = 16 (%)*	*n = 36 (%)*
Radiotherapy									
○ 1–8 weeks before T_0	43 (17)	11 (11)	33 (19)	6 (15)	15 (14)	7 (14)	8 (15)	1 (6)	7 (19)
○ up to 1 week before T_0 / during treatment	40 (16)	19 (18)	34 (20)	11 (28)	20 (19)	9 (18)	4 (7)	2 (13)	6 (17)
Adjuvant pain medication									
○ Used before T_0 - continued	4 (2)	2 (2)	11 (6)	4 (10)	8 (8)	5 (10)	5 (9)	1 (6)	12 (33)
○ Started on T_0 or later	55 (22)	47 (45)*	43 (25)	18 (45)*	26 (25)	22 (45)*	6 (11)	7 (44)*	14 (39)
Corticosteroids									
○ Used before T_0 continued	7 (3)	3 (3)	11 (6)	2 (5)	7 (7)	3 (6)	7 (13)	1 (6)	8 (22)
○ Started on T_0 or later	97 (39)	51 (49)*	63 (37)	25 (63)*	39 (37)	26 (53) ‡	24 (44)	7 (44)	13 (36)
Pain at rest (NRS)									
○ Mild (NRS 0–4)	80 (32)	25 (24)	54 (68)	11 (55)	35 (60)	17 (53)	19 (66)	3 (50)	11 (58)
○ Moderate (NRS 5–6)	27 (11)	11 (11)	19 (24)	2 (10)	16 (28)	9 (28)	6 (21)	1 (17)	8 (42)
○ Severe (NRS 7–10)	16 (16)	13 (13)*	6 (8)	7 (35)*	7 (12)	6 (19)	4 (14)	2 (33)	0
○ Missing	126	55	93	20	48	17	25	10	17
Worst pain (NRS)									
○ Mild (NRS 0–4)	35 (14)	8 (8)	23 (17)	1 (3)	16 (19)	3 (7)	10 (24)	3 (21)	3 (12)
○ Moderate (NRS 5–6)	53 (21)	13 (13)	44 (32)	6 (19)	16 (19)	10 (24)	10 (24)	3 (21)	7 (27)
○ Severe (NRS 7–10)	109 (44)	61 (59)*	72 (52)	24 (77) ‡	51 (61)	28 (68)	22 (52)	8 (57)	16 (62)
○ Missing	52	22	33	9	23	8	12	2	10
Opioid dose on T0									
○ Q1: 1st quarter/third/half	71 (29)	18 (17)	48 (28)	5 (13)	59 (56)	19 (39)	35 (65)	7 (44)	17 (47)
○ Q2: 2nd quarter/third/half	92 (37)	32 (31)	46 (27)	9 (23)	33 (31)	15 (31)	19 (35)	9 (56)	19 (53)
○ Q3: 3rd quarter/third	38 (15)	20 (19) *	45 (26)	16 (40)*	14 (13)	15 (31)*			
○ Q4: 4th quarter	48 (19)	33 (32) *	33 (19)	10 (25) ‡					

Abbreviations: NF = not failing; F = failing, Fe= fentanyl, Mo= morphine, Ox = oxycodone, H = hydromorphone; * = $p < 0.05$, ‡ = $p < 0.1$

Table 2. Distribution of genotypes between patients failing and not-failing treatment for all patients and per type of opioid

	All – NF n = 272 n (%)	**All – F n = 81 n(%)**	**Fe - NF n = 174 n (%)**	**Fe - F n = 59 n (%)**	**Mo - NF n = 107 n (%)**	**Mo - F n = 56 n (%)**	**Ox - NF n = 54 n (%)**	**Ox - F n = 27 n (%)**	**H - NF n = 36 n (%)**	**H – F n = 9 n (%)**
OPRM1 (rs1799971)										
○ 118AA	206 (80)	63 (79)	126 (76)	51 (88)	87 (84)	40 (73)	43 (81)	21 (81)	26 (72)	9 (100)
○ 118G carrier	53 (20)	17 (21)	39 (24)	7 (12) ‡	17 (16)	15 (27)	10 (19)	5 (19)	10 (28)	0
COMT haplotype										
○ LPS	137 (54)	37 (46)	82 (50)	30 (53)	56 (54)	30 (54)	25(47)	11 (42)	22 (65)	5 (56)
○ APS	85 (33)	32 (40)	57 (35)	20 (35)	35 (34)	20 (36)	20 (38)	12 (46)	12 (35)	2 (22)
○ HPS	33 (13)	11 (14)	25 (15)	7 (12)	12 (12)	5 (9)	8 (15)	3 (12)	0	2 (22)*
KCNJ6 (rs2070995)										
○ 1032GG	164 (64)	51 (64)	106 (64)	35 (61)	66 (63)	29 (54)	33 (67)	17 (65)	22 (61)	7 (78)
○ 1032A carrier	91 (36)	29 (36)	59 (36)	22 (39)	38 (37)	25 (46)	16 (32)	9 (35)	14 (39)	2 (22)
RHBDF2 (rs12948783)										
○ GG	178 (68)	65 (81)	116 (69)	44 (76)	77 (75)	43 (78)	34 (64)	23 (88)	23 (64)	8 (89)
○ A carrier	82 (32)	15 (19)*	51 (31)	14 (24)	26 (25)	12 (22)	19 (36)	3 (12)*	13 (36)	1 (11)
HTR3B (rs1672717)										
○ TT	97 (37)	35 (44)	61 (37)	25 (43)	40 (38)	23 (42)	21 (40)	10 (38)	16 (44)	5 (56)
○ TC	116 (45)	34 (43)	80 (49)	23 (40)	41 (39)	25 (45)	21 (40)	11 (42)	14 (39)	3 (33)
○ CC	46 (18)	11 (14)	23 (14)	10 (17)	23 (22)	7 (13)	11 (20)	5 (19)	6 (17)	1 (11)
OPRK1 (rs7016778)										
○ AA	191 (74)	63 (78)	123 (76)	46 (79)	81 (78)	42 (78)	33 (62)	22 (85)		
○ T carrier	66 (26)	17 (21)	39 (24)	12 (21)	23 (22)	12 (22)	20 (38)	4 (15)*		
OPRK1 (rs7824175)										
○ CC	206 (80)	65 (81)	131 (79)	42 (72)	81 (79)	47 (85)	46 (88)	20 (77)		
○ G carrier	52 (20)	15 (19)	34 (21)	16 (28)	22 (21)	8 (15)	6 (12)	6 (23)		
CYP3A4 (rs2242480)										
○ *1/*1			130 (78)	45 (82)			38 (72)	23 (88)		

Table 2. Distribution of genotypes between patients failing and not-failing treatment for all patients and per type of opioid (continued)

	All – NF n = 272 n (%)	**All – F n = 81 n(%)**	**Fe - NF n = 174 n (%)**	**Fe - F n = 59 n (%)**	**Mo - NF n = 107 n (%)**	**Mo - F n = 56 n (%)**	**Ox - NF n = 54 n (%)**	**Ox - F n = 27 n (%)**	**H - NF n = 36 n (%)**	**H – F n = 9 n (%)**
○ *1/1G			31 (19)	10 (18)			12 (23)	3 (12)		
○ *1G/*1G			6(4)	0			3 (6)	0		
CYP3A4 (rs35599367)										
○ *1/*1			146 (88)	50 (91)			46 (87)	24 (92)		
○ *1/*22			20 (12)	5 (9)			7 (13)	2 (8)		
OCT1 phenotype										
○ EM					56 (54)	32 (58)				
○ IM					42 (40)	21 (38)				
○ PM					6 (6)	2 (4)				
ABCC3 (rs4793665)										
○ -211CC					26 (25)	12 (22)				
○ -211CT					51 (50)	24 (44)				
○ -211TT					25 (25)	19 (35)				
○ -900GG					34 (33)	15 (27)			11 (31)	5 (56)
○ -900GA					49 (47)	28 (51)			16 (44)	3 (33)
○ -900AA					21 (20)	12 (22)			9 (25)	1 (11)
CYP2D6 phenotype										
○ PM							4 (8)	2 (8)		
○ IM							23 (47)	9 (35)		
○ EM							20 (41)	14 (54)		
○ UM							2 (4)	1 (4)		

Where numbers do not add up to the numbers indicated per column, data are missing (supplemental table 2). Abbreviations: NF = not failing; F = failing, Fe= fentanyl, Mo= morphine, Ox = oxycodone, H = hydromorphone, LPS = low pain sensitivity, APS = average pain sensitivity, HPS = high pain sensitivity; EM = extensive metaboliser, IM = intermediate metaboliser, PM = poor metaboliser, UM = ultra-rapid metaboliser; * = $p < 0.05$, ‡ = $p < 0.1$

Table 3. Results of univariate and multivariable analyses for all patients and per type of opioid

	Univariate analysis			Multivariable analysis		
	Odds ratio	**95% Confidence Interval**	**P**	**Odds ratio**	**95% Confidence Interval**	**P**
All						
- Age	0.58	0.35 – 0.96	0.035			
- Adjuvant pain medication ≥ T_0	2.49	1.48 – 4.19	0.001	2.61	1.25–5.44	0.011
- Corticosteroids ≥ T_0	2.24	1.35 – 3.72	0.002			
- Pain at rest						
○ Mild pain	1			1		
○ Moderate pain	1.06	0.44 – 2.53	0.898	1.11	0.45–2.72	0.819
○ Severe pain	3.5	1.49–8.24	0.004	3.27	1.31–8.14	0.011
- Worst pain						
○ Mild pain	1					
○ Moderate pain	1.57	0.46 – 5.36	0.474			
○ Severe pain	4.14	1.41 – 12.19	0.010			
- Dose at T_0						
○ Q1	1					
○ Q2	1.91	0.91 – 4.02	0.086			
○ Q3	2.27	1.04 – 4.92	0.038			
○ Q4	3.10	1.45 – 6.63	0.004			
- rs12948783 (*RHBDF2*)						
○ A carrier	0.50	0.27–0.93	0.029	0.44	0.24–0.99	0.083
Fentanyl						
- Age	0.98	0.95–1.00	0.071	0.95	0.91–1.01	0.081
- Adjuvant pain medication ≥ T_0	2.45	1.20–5.00	0.013	2.82	0.93–8.54	0.067
- Corticosteroids ≥ T_0	2.88	1.42–5.87	0.004			
- Pain at rest						
○ Mild pain	1			1		
○ Moderate pain	0.52	0.10 – 2.55	0.42	0.53	0.10–2.74	0.448
○ Severe pain	5.72	1.61–20.37	0.007	5.68	1.52–21.28	0.010
- Worst pain						
○ Mild pain	1					
○ Moderate pain	3.14	0.36–27.64	0.30			
○ Severe pain	7.67	0.98–59.84	0.052			
- Dose at T_0						
○ Q1	1					
○ Q2	1.87	0.59–6.02	0.289			
○ Q3	3.41	1.16–10.09	0.026			
○ Q4	2.90	0.91–9.29	0.072			

Table 3. Results of univariate and multivariable analyses for all patients and per type of opioid (continued)

	Univariate analysis			Multivariable analysis		
	Odds ratio	**95% Confidence Interval**	**P**	**Odds ratio**	**95% Confidence Interval**	**P**
- rs1799971 *OPRM1*						
○ 118G carrier	0.44	0.19–1.06	.066			
Morphine						
- Age	0.97	0.94–1.00	0.081	0.96	0.93–0.99	0.047
- Adjuvant pain medication ≥ T_0	2.50	1.23–5.13	0.012	2.51	1.22–5.19	0.013
- Corticosteroids ≥ T_0	1.94	0.98–3.86	0.058			
- Dose at T_0						
○ Q1	1.00	-				
○ Q2	1.41	0.63 – 3.14	0.40			
○ Q3	3.33	1.36–8.13	0.008			
Oxycodone						
- Adjuvant pain medication ≥ T_0	6.22	1.69–22.88	0.006	11.18	2.21–56.40	0.003
- rs12948783 (*RHBDF2*)						
○ A carrier	0.23	0.06 - .88	0.032	0.19	0.03–1.12	0.066
- rs7016778 (*OPRK1*)						
○ T carrier	0.30	0.09–1.00	0.050	0.20	0.04–1.09	0.063

≥ T_0: started on T_0 or during hospitalization, Q1 first quantile, Q2 second quantile, Q3 third quantile, Q4 fourth quantile

and severe pain at rest (OR 5.68, p = 0.010) were independent as shown in multivariable analysis (tables 1–3). When the analysis was corrected for opioid dose the use of adjuvant pain medication was no longer significant (p = 0.106).

Morphine: For morphine, age (OR 0.97, 95% CI 0.94–1.00, p = 0.081), use of adjuvant pain medication started on T_0 or later (OR 2.50, 95% CI 1.23–5.13, p = 0.012), use of corticosteroids started on T_0 or later (OR 1.94, 95% CI 0.98–3.86, p = 0.058) and the dose of morphine at T_0 (Q3 OR 3.33, 95% CI 1.36–8.13, p = 0.008) were found to be correlated with treatment failure in univariate analysis. None of the genetic variants correlated with failure of treatment (all: p > 0.10). In multivariable analysis use of adjuvant pain medication (OR 2.51, p < 0.013) and age (OR 0.96, p = 0.047) were found to correlate with treatment failure (tables 1–3). As for fentanyl, when the analysis was corrected for opioid dose, the use of adjuvant pain medication was no longer significant (p = 0.10).

Oxycodone: For oxycodone, use of adjuvant pain medication started on T_0 or later (OR 6.22, 95% CI 1.69–22.88, p = 0.006) and the SNPs rs12948783 (*RHBDF2*) (OR 0.23, 95%

CI 0.06–0.88, $p = 0.032$) and rs7016778 (*OPRK1*) (OR 0.30, 95% CI 0.09–1.00, $p = 0.050$) were identified in univariate analysis. In multivariable analysis, all three variables remained in the model, without and with correction for opioid dose on T_0. (adjuvant pain medication OR 11.18, $p = 0.003$); rs12948783 in *RHBDF2:* OR 0.19 $p = 0.066$; rs7016778 in *OPRK1:* OR 0.20, $p = 0.063$) (tables 1–3).

Hydromorphone: The number of patients in the hydromorphone group was considered too small for further analyses and to draw conclusions.

DISCUSSION

In this cohort of cancer patients treated with opioids, we found that in 20–34% treatment with fentanyl, morphine, oxycodone or hydromorphone failed because of insufficient pain control and/or dose limiting side effects. This is in line with previously reported data (1, 2, 26, 27). Clinical factors associated with treatment failure were the use of adjuvant pain medication started after T_0 and severity of pain at rest at T_0. In the morphine and fentanyl cohorts, younger age was also associated with a worse outcome. For the selected SNPs, we identified rs12948783 (*RHBDF2*) and rs7016778 (*OPRK1*) as factors to be explored further in a future study. Previous studies assessing clinical risk factors for the need of opioid rotation have yielded variable results. In a large and prospective study, 118/345 (34.2%) patients underwent opioid rotation and no association between the need for rotation and pain type, use of adjuvant drugs or opioid doses was found (28). In another, retrospective, analysis, 103/273 patients (37.5%) rotated from their first line opioid. Although no correlation with age, type of pain or co-analgesics was found, the use of corticosteroids was associated with a significantly lower rate of opioid rotation (29). In our study, the use of corticosteroids was correlated with higher rates of failure, but only in the univariate analyses. A possible explanation is that in our cohort of patients, corticosteroids were given to patients with severe complex pain. It is also possible that corticosteroids may alter pharmacokinetics (e.g. by induction of CYP3A4) and pharmacodynamics of opioids. The association with the use of adjuvant pain medication is complex. Adjuvant drugs are preferentially used when a neuropathic mechanism may contribute to the clinical presentation. Neuropathic pain is more difficult to treat, as was shown in a validation study of the Edmonton Classification System for Cancer Pain. In that study, neuropathic pain and initial severity of pain were found to be significant predictors of pain complexity and positively correlated with the number of days needed to achieve stable pain control, the use of more adjuvants and higher doses of opioids (30). In another study in cancer patients using morphine, neuropathic pain was associated with a higher opioid escalation index (31). In our study, all patients had nociceptive pain but patients with a neuropathic component, were eligible.

Adjuvant drugs were prescribed in case of a suspected neuropathic pain component, which were usually more complex pain syndromes. Further studies should assess neuropathic pain using validated tools. The correlation with age has been observed before. Ericson *et al.* reported a decrease in risk of treatment failure of 3% per 10-year increase in age. Above the age of 65 the risk decreased even 13% per 10-year increase (32). In the present analysis, the correlation between age and failure to morphine and fentanyl remained unchanged when the multivariable analysis was corrected for opioid dose at T_0 and therefore the association cannot be explained by lower treatment doses in elderly patients. We can speculate that differences in opioid metabolism play a role or even that elderly patients and/or their doctors are less likely to report insufficient pain control or severe side effects because they are less demanding and/or more often fear dose escalation. Finally, the association with pain intensity at rest was not unexpected and was reported before (30).

The genetic analysis was also set-up as an exploratory analysis in order to identify candidate SNPs associated with treatment failure of (specific) opioids. While the frequencies of the studied SNPs followed widely reported prevalence rates, none of the selected genetic variants were found to be significantly correlated with failure of treatment in the entire cohort and the fentanyl, morphine and oxycodone cohorts in the multivariable analysis. We did however find an association between the variant upstream of the *RHBDF2* gene (rs12948783) and treatment outcome ($p < 0.10$). In a previous genome wide association study (GWAS), this SNP was found to be significantly associated with decreased pain relief from opioids (33). As this gene is coding for inactive rhomboid protease, an enzyme that has been associated with cancer growth (34, 35), the found hit could be due to cancer demographics of the analyzed cohort, which were not specified in the GWAS study. In our cohort we observed a trend in the opposite direction, i.e. a lower rate of treatment failure. The distribution of tumor types might have been different which, combined with low number of patients, may explain these seemingly contradictory findings. Although the genetic variation (rs1672717) in the *HTR3B* gene, coding for the serotonin receptor subtype 3B, was previously associated with opioid induced nausea and vomiting in more than 1,500 Caucasian cancer patients (17), we did not find an association with opioid failure. Opioid failure is a composite endpoint and although a substantial part of patients failing treatment had dose limiting adverse events, the proportion of patients with severe nausea and vomiting as the main reason for treatment failure was probably too low to detect an association.

Interestingly, we found no correlation between the frequently investigated *OPRM1* SNP (rs1799971) and opioid failure. A meta-analysis has illustrated the relevance of this SNP for opioid requirement in postoperative patients, especially within Asians treated with morphine for visceral pain (36), but the results have been conflicting for opioid response in cancer induced pain (14, 17, 37–43). Although genetic variation in the *KCNJ6* gene has been previously associated with increased opioid requirement in postoperative pain (44)

and a tendency toward less opioid effectiveness in chronic pain (45), this variant does not seem to predict opioid failure in our cohort. The minor allele of *OPRK1* rs7016778 SNP has been previously associated with an increased experimental pain threshold (46). This could be caused by increased expression of the kappa receptor and as a consequence a higher affinity for endogenous opioids. Since oxycodone may exert (part of) its analgesic effect primarily via the kappa receptor (47), it is expected that *OPRK1* genetic variants could alter the response and thus the need to switch to a non-kappa-binding opioid. While the relevance of the kappa receptor above the mu-opioid receptor for oxycodone has been discussed (48), in our cohort carriers of the minor allele had a (non-statistically significant) lower risk of treatment failure with oxycodone, which is in line with the decreased pain sensitivity reported in the experimental pain setting. Lastly, none of the SNPs related to metabolism of specific opioids (*CYP3A4, CYP2D6, OCT1, UGT2B7* and *ABCC3*) correlated with failure of treatment in our analysis. This might be due to our limited sample size. Furthermore, up till now little is known about the effect of changes in pharmacokinetics on pharmacodynamics.

Although we assembled longitudinal data in a large group of cancer patients and studied a clinically relevant endpoint we must also acknowledge some limitations. Per treatment group numbers were small and the included population was heterogeneous in terms of treatment phase with opioids and opioid dose at T_0. Furthermore we compared patients in whom treatment failed with patients in whom treatment did not fail. Although we strictly defined failure of treatment, we categorized all other patients as not failing treatment although some may not have been successfully treated. Also, we studied failure as a composite endpoint although analgesia and (central) side effects may be independent treatment outcomes (38). Sample size did not allow us to create subgroups according to the reason(s) of failure.

In conclusion, we have identified that the use of adjuvant pain medication, pain intensity at rest and age were associated with failure of treatment with fentanyl, morphine, oxycodone and hydromorphone in this exploratory study. Furthermore, a trend to a negative correlation with treatment failure was seen for the single nucleotide polymorphisms rs12948783 (*RHBDF2*) in all patients and the oxycodone cohort and for rs7016778 (*OPRK1*) in the oxycodone cohort. As these factors are not opioid specific, they cannot be used to guide treatment and the choice for a specific type of opioid. The variant rs7016778 (*OPRK1*) warrants further research with this respect. Ideally, future studies should include large and homogeneous patient populations and protocolized treatments strictly. However, such a trial will be difficult – if not impossible – to perform in a palliative clinical care setting.

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Supplementary Table 1. Genotype frequencies and HW-equilibrium

SNP	n	MAF study (%)	MAF Literature (%)	HW (p-value)
CYP3A4 rs2242480 (*1G)		12	7	0.07
○ *1/*1	175			
○ *1/*1G	41			
○ *1G/*1G	6			
CYP3A4 rs35599367 (*22)		6	5	0.37
○ *1/*1	196			
○ *1/*22	25			
○ *22/*22	0			
CYP2D6 rs35742686 (*3)		4	2	0.68
○ *1/*1	71			
○ *1/*3	7			
○ *3/*3	0			
CYP2D6 rs3892097 (*4)		21	28	0.34
○ *1/*1	48			
○ *1/*4	23			
○ *4/*4	5			
CYP2D6 deletion (*5)		2	5	0.86
○ Negative	76			
○ Positive	3			
CYP2D6 rs5030655 (*6)		2	1	0.86
○ *1/*1	76			
○ *1/*6	3			
○ *6/*6	0			
CYP2D6 rs28371725 (*41)		9	9	0.71
○ *1/*1	65			
○ *1/*41	13			
○ *41/*41	1			
CYP2D6 XN		2	3	0.86
○ Negative	76			
○ Positive	3			
UGT2B7 rs7438135		45	50	0.79
○ GG	49			
○ GA	77			
○ AA	33			
OCT1 rs72552763 (*2)		21	15	0.45
○ *1/*1	247			
○ *1/*2	86			
○ *2/*2	10			

Supplementary Table 1. Genotype frequencies and HW-equilibrium (continued)

SNP	n	MAF study (%)	MAF Literature (%)	HW (p-value)
OCT1 rs12208357 (*3)		8	10	0.31
○ *1/*1	298			
○ *1/*3	43			
○ *3/*3	3			
OCT1 rs34130495 (*4)		4	2	0.47
○ *1/*1	319			
○ *1/*4	26			
○ *4/*4	0			
OCT1 rs34059508 (*5)		1	1	0.81
○ *1/*1	336			
○ *1/*5	9			
○ *5/*5	0			
ABCC3 rs4793665		52	49	0.59
○ CC	38			
○ CT	75			
○ TT	44			
COMT rs4680		51	48	0.28
○ GG	85			
○ GA	160			
○ AA	95			
COMT rs4818		37	42	0.07
○ CC	141			
○ CG	144			
○ GG	56			
COMT rs4633		51	48	0.35
○ CC	83			
○ CT	161			
○ TT	97			
OPRM1 rs1799971		11	16	0.77
○ AA	269			
○ AG	68			
○ GG	5			
KCNJ6 rs2070995		79	80	0.22
○ AA	9			
○ AG	112			
○ GG	217			

Supplementary Table 1. Genotype frequencies and HW-equilibrium (continued)

SNP	n	MAF study (%)	MAF Literature (%)	HW (p-value)
RHBDF2 rs12948783		15	15	0.99
○ CC	246			
○ CT	89			
○ TT	8			
HTR3B rs1672717		60	58	0.23
○ CC	57			
○ CT	152			
○ TT	133			
OPRK1 rs7016778		13	12	0.38
○ AA	255			
○ AT	81			
○ TT	4			
OPRK1 rs7824175		10	10	0.73
○ CC	274			
○ CG	64			
○ GG	3			


SLC22A1
COMT
UGT2B7

Chapter 5

Advanced cancer pain: the search for genetic factors correlated with interindividual variability in opioid requirement

Maja Matic*
Joost L. Jongen*
Laure Elens
Saskia N. de Wildt
Dick Tibboel
Peter A.E. Sillevis-Smit
Ron H.N. van Schaik

*These authors contributed equally to this manuscript.

Submitted for publication.

ABSTRACT

Aims: Assess association between genetic variants and opioid requirement in cancer patients.

Patients & Methods: A prospective observational trial of 243 advanced cancer patients with inadequate analgesia treated by the palliative care team (PCT) was analyzed for *ABCB1, ARRB2, COMT, GCH1, IL1RN, KCNJ6, OPRM1, RHBDF2, SCN9A* and *Stat6* polymorphisms.

Results: For patients carrying *OPRM1* 118AG/GG and *COMT* 472GG (Val158Val) or these genotypes alone, a significant higher median percentage dose increase was observed (95.2% [32.8–345]) compared to *OPRM1* 118AA and *COMT* 472GA/AA (158Met allele carriers) (48.5% [0–98.8]) (p = 0.0016). No associations were found with morphine equivalent dose after consultation PCT or ketamine use.

Conclusions: Patients with the combined *OPRM1* 118AG/GG and *COMT 472GG* genotype required 50% higher dose increase for sufficient analgesia.

INTRODUCTION

Pain is one of the most frequent and uncomfortable symptoms in cancer patients. It has an incidence ranging from 33% after curative treatment to up to 64% in the advanced stage of cancer [1]. Pain is also one of the most common indications in cancer patients arriving at the emergency department [2]. A European cancer survey illustrated that pain treatment is insufficient in this population, where moderate to severe pain was reported by 56% and breakthrough pain by 63%. The 'quality of life' aspect was insufficiently covered according to 50% of patients [3]. Daily opioid requirement among cancer patients ranges from 25 mg to 2000 mg, which makes pain treatment even more complicated [4]. Due to the complex etiology, a personalized treatment algorithm would have a great advantage in clinical practice.

Cancer pain is primarily caused by the tumor itself, and less frequent by cancer treatments such as surgery, chemotherapy and radiation [5]. The type and size of the tumor and the extent of pressure on or invasion into bones, nerves and other organs constitute principal determinants of the pain severity and phenotype. Apart from tumor characteristics, patient characteristics are also likely to explain the observed differences in pain experience [6, 7]. The female sex is overrepresented in chronic pain conditions, where women tend to have a lower pain threshold, but, paradoxically, are more sensitive to morphine efficacy [8]. This gender effect is probably due to differences in anatomical and physiological compositions in the central nervous system circuits between males and females [9]. Ethnicity also seems the influence the pain expression, with the differences in pain most likely caused by language obstacles and low socioeconomic status in minority groups, although cultural background can also influence self-reports of pain [7, 10].

Finally, the individual genetic make-up is contributing to observed differences in pain experience and analgesic responsiveness [11], and may in fact partly explain previously mentioned associations with sex and ethnicity. Most of the studies that addressed this topic were performed in the postoperative setting using a candidate gene approach or a genome wide analysis. Recently, a meta-analysis with 4,607 postoperative patients showed that the genetic variation 118A>G in the μ-opioid receptor (MOR), encoded by the *OPRM1* gene, was associated with higher postoperative opioid dose requirement [12]. Studying a genetic contribution in cancer pain patients is more complicated. Indeed, in this particular condition, pain is confounded by even more factors, with more difficulties in proving the genetic contribution of selected candidate single nucleotide polymorphisms (SNPs) to its severity.

Investigating a large cohort (n = 1,000) of female breast cancer patients, Cajanus *et al.* illustrated 33% higher postoperative oxycodone requirement in 118GG genotyped individuals [13]. In the large EPOS trial consisting of 2294 European cancer pain patients the researchers demonstrated an effect of *CYP3A4/5* SNPs on fentanyl PK in 620 patients

[14], of *COMT* rs2020917 on constipation in 1568 patients [15], of a *HTR3B* genetic variant on opioid-induced nausea/vomiting in 1579 patients [16] and dyspnea in the total cohort (n = 2294) [17]. Yet, none of the SNPs correlated with opioid requirement in this cohort [18].

The aim of this study was to determine if polymorphisms in the candidate genes *ABCB1*, *ARRB2, COMT, GCH1, IL1RN, KCNJ6, OPRM1, RHBDF2, SCN9A*, and *Stat6* are associated with opioid requirement in patients with advanced clinical cancer who were referred to a pain consultation service due to inadequate analgesia. Our prospectively recruited cohort is unique as it represents moderate-severe cancer pain patients, who were treated according to a uniform algorithm in a single center.

METHODS

Patients with advanced clinical cancer, for whom the multidisciplinary Palliative Care Team (PCT) of Erasmus University Medical Center was consulted to treat pain, between October 2008 and December 2012, were eligible for inclusion. Clinical data were prospectively collected from a structured data collection sheet (demographical data, type of cancer, metastases, pain intensities and medication) and from the electronic health record (type of pain). The type of pain (nociceptive versus mixed nociceptive-neuropathic pain) was established by a clinical neurologist, using the definition of (a) neuropathic pain (component) of the 'International Association for the Study of Pain' and the algorithm described by Treede *et al.* [19], which is in accordance with previous literature on the clinical distinction between nociceptive and mixed pain [20]. Pain intensity (numerical rating score, NRS) and opioid requirement were assessed at time of first consultancy (T = 0 days) and after the patient was switched to another analgesic or alternatively, after the dose was changed (T = 3 days). Dose modifications and opioid rotations were done in accordance with the institutional pain protocol of Erasmus MC, which was based on the Dutch national Guideline "Cancer Pain" [21].

The morphine equivalent dose (MED) was expressed as amount of opioid in mg parenteral morphine/24h. Conversion factors were according to the Dutch consensus guideline "Cancer Pain" [21]. The conversion factors used for calculating MED were 6.67, 0.07 and 0.05 for parenteral hydromorphone, oral tramadol and oral codeine, respectively [22]. For parenteral buprenorphine the same conversion factor as for fentanyl was used [23]. MED was calculated by combining the sustained release and continuous intravenous opioid medication. Although information on all rescue medication for individual patients was not collected, the maximum daily dosage of oral and intravenous rescue medication (opioids) as a rule consisted of 100% of the sustained release or continuous intravenous opioid dosage. When patients were using more than 50% of the rescue doses, sustained

release or continuous intravenous opioid dosage was increased. Thus, the calculated MED represents 67–100% of the actual MED. The study was approved by the institutional review board of Erasmus MC (MEC2008–166) and registered at www.clinicaltrials.gov (NCT00956878). All patients gave written informed consent for DNA analysis.

Study outcomes

Morphine equivalent dose (MED) at T = 3d and the relative increase in MED between T = 3d and T = 0d (ΔMED/MED T = 0d) were the primary outcome measures for this study. By adjusting the change in dose for the dose received at baseline, the large variability between patients was reduced and we thus corrected for baseline analgesia. Three days was chosen as it corresponds to the median time point at which we have previously demonstrated adequate analgesia with 48h after consultation of the PCT [24]. As secondary outcome measure, the use of ketamine as an adjuvant analgesic (indicating extreme pain phenotypes) was assessed.

Genotyping

Whole blood was obtained by venous puncture and DNA was isolated and frozen at -800C until further analysis. The genomic DNA was isolated with the MagNA Pure LC 2.0 instrument (Roche Diagnostics®) according to the 'MagNA Pure LC DNA Isolation Kit Large Volume' protocol. DNA concentrations were measured on the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific®) and diluted to 10 ng/µl if concentrations exceeded this threshold. The selected candidate polymorphisms for this study were: *ABCB1* rs1128503, rs2032582, rs1045642, *ARRB2* rs1045280, *COMT* rs4680, 4818, 4633, *GCH1* rs8007267, rs10483639, rs3783641, *IL1RN*2* (86 VNTR), *KCNJ6* rs6517442, rs2070995, *METTL21A* rs2952768, *OPRM1* rs1799971, *RHBDF2* rs12948783, *SCN9A* rs6746030 and *Stat6* rs841718.

All SNPs, except *IL1RN*2*, have been genotyped with the TaqMan allelic discrimination method. The predesigned or custom made SNP assays have been designed by the manufacturer (Life Technologies, Bleiswijk, The Netherlands). The patients were analyzed on a 96-well plate with the 7500 Real-Time PCR System (software version v2.0.5; Life Technologies). The IL1RN*2 (86-bp tandem repeats) variation has been performed with PTC-200 Thermal Cycler, DNA Engine (Biorad®) and examined with gel-electrophoresis [25].

The catechol-O-methyltransferase (*COMT*) and guanosine triphosphate cyclohydrolase (*GCH1*) haplotype were estimated with R version 3.1.1 haplo.stats package. The haplotypes included in this analysis were estimated with a posterior probability > 0.98. The original study describing the *COMT* haplotypes by Diatchenko *et al.* (2005) used SAS Proc haplotype for haplotype construction [26]. Both software packages make use of the expectation-maximization (EM) logarithm. By analyzing rs4680, rs4818 and rs4633 the

COMT haplotype was determined with an accuracy of approximately 98% [26]. Patients genotyped GGC were categorized in the low pain sensitivity (LPS) group, ACT in average pain sensitivity (APS) group and GCC in high pain sensitivity (HPS). The following groups have been compared (LPS/LPS + LPS/APS vs. APS/APS + LPS/HPS vs. HPS/HPS + APS/HPS) in the analysis.

Analysis of the 3 *GCH1* SNPs leads to a sensitivity and specificity of 100% of the 'pain-protective' haplotype, where non-carriers, carriers and homozygous carriers have been compared [27]. In addition to this haplotype, we are also interested in the combined allelic genotypic effect of *OPRM1* rs1799971 and *COMT* rs4680, as reported earlier [28]. Patients carrying the *OPRM1* 118G allele and the *COMT* Val158Val genotype or these genotypes alone were considered the high risk group for pain, while patients with the *OPRM1* 118AA genotype and *COMT* 158Met allele carriage were the low risk genotype.

Statistical analysis

Current data were analyzed with the statistical software package SPSS version 21.0 for Windows. The categorical demographic and clinical data were analyzed with Pearson's chi2 test or Fisher's exact test when appropriate. Continuous data were assessed with a non-parametric tests (Mann-Whitney or Kruskall-Wallis) or with a parametric test (students t-test or ANOVA) when normal distribution was not violated. Normality was tested numerically with the Shapiro-Wilk test. The genotype frequencies were assessed for violation of the Hardy-Weinberg equilibrium with the Chi2 test. In the SNP analysis the additive model was used when the genotype count into groups were separately ≥ 10. When this criterion was not met a dominant model was used.

Multiple linear regression was used for assessment of the correlation between the SNPs with MED (T = 3d) and relative ΔMED. The independent factors type of pain (nociceptive vs. mixed), pain intensity (T = 0d) (only used for MED (T = 3d) outcome), gender, age, type of cancer and treatment have been included as confounding factors. The association with ketamine use (extreme pain phenotypes) was assessed with logistic regression, in which the same independent variables have been used. Multicollinearity between the independent factors was excluded since none of the factors had a variation inflation factor (VIF) > 3. Metastasis was not included in the model because no evidence exists on higher pain intensities for this group compared with primary cancer patients. Besides, since almost 80% of the population had a metastasis at time of inclusion, the cohort could be considered homogenous for this factor. The two-sided *p*-values in the multiple linear and logistic regression were adjusted for multiple testing with the Bonferroni correction ($p < 0.0029$).

RESULTS

Patient characteristics

Two-hundred and forty three cancer patients were included in this prospective observational study. Three patients were not using opioids at baseline and after consultation PCT. These patients were consequently excluded from further analysis. From the remaining 240 patients, demographic and clinical characteristics are reported in **Table I**. As illustrated in this Table, 72% of the patients were diagnosed with nociceptive pain, 79% had metastasized cancer, 9% required ketamine as an adjuvant analgesic and fentanyl was most frequently used at T = 0d (52.1%) and T = 3d (73.3%). Gastrointestinal, urological, lung and gynecological were the most prevalent tumor types. Twenty-six % of the patients were treated with radiotherapy, 14.2% with chemotherapy and 7.5% underwent elective surgery. Forty-five % of the patients had supportive (e.g. stents) or no cancer treatment at all at time of inclusion.

Outcome measures

The median MED was 20 mg/24 h [IQR: 10 – 53] at T = 0d and 40 mg/24 h [IQR: 20 – 80] at T = 3d. In parallel, the pain intensity numerical rating score (NRS) score decreased from 6 [IQR: 4–8] to 4 [IQR: 2–5], reflecting a statistically ($p < 0.0001$) and clinically significant improvement in pain. From the 240 patients the MED was decreased by the PCT in 18 (7.5%) individuals and in 48 (20%) cases the MED remained the same. From the latter group, 3 patients were rotated to an alternative opioid. In the total cohort 84 patients were rotated to an alternative opioid.

Several outliers have been identified within the outcomes MED (T = 3d) and relative ΔMED with the formulas Q3+(3.3*(Q3-Q1)) and Q1-(3.3*(Q3-Q1)). After LOG transformation no outliers were found within the MED (T = 3d) outcome and 4 outliers in the relative ΔMED outcome. None of these outliers had extreme genotypes that could explain the high increase/decrease in dose between T = 0d and T = 3d. Because absence of outliers is one of the assumptions in regression analysis these 4 outliers were removed. From the clinical factors age, gender, type of pain, type of cancer, type of treatment and pain intensity at baseline (T = 0d) only age ($p = 0.019$) and pain intensity at T = 0d ($p = 0.010$) were related with MED at T = 3d. Whereas none of these clinical factors were related with relative ΔMED.

Genetic associations

The genotype and haplotype frequencies of all assessed polymorphisms, except the *METTL21A* SNP, met the Hardy-Weinberg (HW) equilibrium. Therefore, this SNP was removed from the statistical analysis to avoid any spurious associations. All allelic frequencies were in line with the frequencies reported in the SNP database (HAPMAP) on the

Table I. Demographic and clinical characteristics

	n = 240
Age (years)	62 [54 – 68]
Gender (%)	
Male / Female	138 (57.5) / 102 (42.5)
Type of pain (%)	
Nociceptive / Mixed	173 (72) / 67 (28)
Type of cancer (%)	
Gastrointestinal	90 (37.5)
Urological	36 (15)
Lung	34 (14.2)
Gynecological	22 (9.2)
Other	14 (5.8)
Primary unknown	14 (5.8)
ENT	13 (5.4)
Breast	9 (3.8)
Hematological	8 (3.3)
Metastasis (%)	
Yes/No	189 (79 / 51 (21)
Treatment (%)	
None or supportive	108 (45)
Radiotherapy	63 (26.3)
Chemotherapy	34 (14.2)
Surgery	18 (7.5)
Radiotherapy and chemotherapy	11 (4.6)
Surgery and radiotherapy	5 (2.1)
Surgery and chemotherapy	1 (0.4)
Ketamine use (%)	
Yes/No	21 (9) / 219 (91)
Pain intensity T = 0d	6 [4 – 8]
Pain intensity T = 3d	4 [2 – 5]
Opioid T = 3d (%)	
Fentanyl	176 (73.3)
Oxycodone	43 (17.9)
Hydromorphone	11 (4.6)
Morphine	5 (2.1)
Buprenorphine	5 (2.1)
Morphine equianalgesic dose (T = 0d)	20 [10 – 53]
Morphine equianalgesic dose (T = 3d)	40 [20 – 80]

All values are expressed in median with corresponding interquartile range (IQR), unless stated otherwise.

Table II. Genotype frequencies (n = 240)

Gene	rs number	Wild type allele	Heterozygous allele	Variant allele	Undetermined
ABCB1	rs1128503	84	115	40	1
	rs2032582	84	112	42	2
	rs1045642	60	105	73	2
ARRB2	rs1045280	30	94	112	4
COMT	rs4680	51	130	59	0
	rs4818	82	124	34	0
	rs4633	52	132	56	0
*GCH1**	rs8007267	144	88	7	1
	rs10483639	153	77	9	1
	rs3783641	143	87	8	2
KCNJ6	rs6517442	28	115	96	1
	rs2070995	10	81	147	2
IL1RN	*2	131	83	11	15
METTL21A	rs2952768	43	89	103	5
OPRM1	rs1799971	192	45	1	2
RHBDF2	rs12948783	159	74	7	0
SCN9A	rs6746030	169	63	6	2
STAT6	rs841718	42	124	69	5
COMT haplotype		LPS+LPS or LPS+APS	APS+APS or LPS+HPS	HPS+HPS or APS+HPS	
		144	69	25	2
GCH1 haplotype		Non-carrier	Heterozygous carrier	Mutant carrier	
		160	78	0	2

All values are expressed in median with corresponding interquartile range (IQR), unless stated otherwise.

National Center for Biotechnology Information (NCBI) website. The observed genotype frequencies of the determined genetic variants and haplotype overview are displayed in **Table II**.

The relative ΔMED was significantly associated with *COMT* rs4633 (p = 0.007), *COMT* rs4680 (p = 0.012) and combined *OPRM1*/*COMT* genotype (p = 0.001) in the univariate analysis. After correction for clinical factors and Bonferroni correction only the effect between the relative ΔMED with combined *OPRM1*/*COMT* genotype remained significant (p < 0.0029). As displayed in **Figure 1**, cancer patients having the *OPRM1* 118G allele with the *COMT* Val158Val genotype or these genotypes alone require a higher median percentage increase in dose (after normalization to baseline dose) (95.2% [32.8–345]) compared to *OPRM1* 118AA in combination with the 158Met allele (48.5% [0–98.8]). None of the

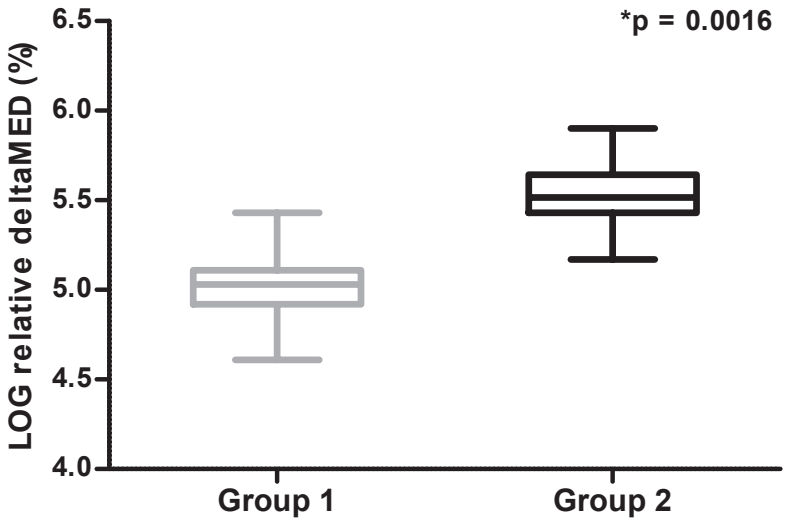

Figure 1. Logarithmic function of relative ΔMED (%) and *OPRM1*/*COMT* genotype
The combined *OPRM1*/*COMT* genotype is significantly associated with relative ΔMED (%) in the univariate analysis (p = 0.001) and after correction for gender, age, type of cancer, treatment and pain (p = 0.0016). The corrected relative ΔMED values for the previously mentioned clinical factors are displayed in this figure. This association also remained after Bonferroni correction (p < 0.0029). Patients genotyped *OPRM1* 118AG/GG with *COMT* Val158Val (Group 2) or these genotypes alone require higher dose increase (95.2% [32.8–345]) compared to patients genotyped *OPRM1* 118AA and *COMT* Val158Met/Met158Met, (Group 1) (48.5% [0–98.8]).

selected SNPs and haplotypes (*ABCB1* rs1128503, *ABCB1* rs2032582, *ABCB1* rs1045642, *ARRB2* rs1045280, *COMT* rs4680, *COMT* rs4818, *COMT* rs4633, *COMT* haplotype, *GCH1* haplotype, *IL1RN**2, *KCNJ6* rs6517442, *KCNJ6* rs2070995, *OPRM1* rs1799971, *RHBDF2* rs12948783, *SCN9A* rs6746030, *Stat6* rs841718, *OPRM1*/*COMT* combined) were associated with MED at T = 3d after correction for confounding factors and multiple testing. Also no correlation with ketamine use was found.

DISCUSSION

Our study illustrates that the combined *OPRM1*(rs1799971)/*COMT*(rs4680) genotype is related with a 50% higher relative increase in opioid dose required for sufficient analgesia after PCT consultation. We found that patients carrying the *OPRM1* 118G allele with the *COMT* Val158Val genotype or these genotypes alone need a higher increase in dose, after correction for baseline dose. These results are in line with a previous report in 207 cancer patients showing the highest morphine dose in *OPRM1* 118G allele carriers and *COMT* 158Val allele carriers [29].

The COMT enzyme, mainly expressed in the prefrontal cortex and striatum [30], is responsible for the degradation of catecholamine's (e.g. dopamine, noradrenaline).

Induced changes in dopamine signaling system affect the opioid system [31, 32]. An in vivo study illustrated that COMT knock-out mice experienced an increased anxiety and stress response [33] but paradoxically an increased morphine response [34]. The *COMT* polymorphism rs4680 (Val158Met) leads to a decreased thermostability and reduced COMT activity [35]. In healthy volunteers it was demonstrated using positron emission tomography (PET) scans that AA genotyped individuals have lower MOR signaling upon pain challenge and a higher MOR binding potential [36]. The opposite effects of this variant on pain (increased) and analgesia (lower demand) can be explained by the presence of more 'available' receptors but not enough endogenous agonists to bind, as illustrated in animal models [37]. This is in line with our finding where we report that the *COMT* GG (Val158Val) genotyped patients with the *OPRM1* variant are predisposed to higher dosage step increase for sufficient analgesia.

The *OPRM1* 118A>G SNP has been related with reduced opioid effect as illustrated by means of an increased opioid requirement and reduced risk for adverse events [12, 38]. Whether this effect is caused by a decrease in protein expression or decreased binding affinity/potency for exogenous opioids remains inconclusive [39]. Independent of the functional consequence, the 118G allele will counteract the 'beneficial' effect of a person that carries the *COMT* 158Met allele. This could explain why different studies addressing the genetic variants in these genes were unable to illustrate an effect on pain sensitivity [40–42] or opioid demand [43].

A challenge of the current study is that information on opioid use before admission was missing in our cohort. Because of a chance of development of opioid tolerance, the length of prior opioid use is a potential confounder in the analysis of opioid requirement. Another limitation of this cohort is the heterogeneous nature of the population. However, none of our tumor type groups had a sufficient sample size in order to assess individually with adequate statistical power. Alternatively, we chose to adjust for known factors when possible and to correct our tests for multiple testing. On the other hand, one could argue that an effect seen in such a heterogeneous population is more likely to have real clinical meaning and has application potential as it overcomes the background noise of confounding factors.

In conclusion, we found that the combined *OPRM1* 118A>G and *COMT* Val158Met variants or these genotypes alone were related with 50% higher dosage increase of opioids in patients with advanced cancer. These genetic biomarkers may be helpful in identifying cancer pain patients with decreased opioid sensitivity and to relieve their pain more quickly and more adequately. Indeed, based on genetic information, the opioid dose could be adjusted proactively, with the ultimate goal to avoid excessive pain in these vulnerable and seriously ill patients.

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Part III

Genetics, Pain and Analgesia in Pediatrics


SLC22A1
COMT
UGT2B7

Chapter 6

Genetic variants associated with thermal pain sensitivity in a pediatric population

Maja Matic
Gerbrich E. van den Bosch
Saskia N. de Wildt
Dick Tibboel
Ron H.N. van Schaik

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ABSTRACT

Pain sensitivity is an inherited factor that varies strongly between individuals. We investigated whether genetic polymorphisms in the candidate genes *COMT, OPRM1, OPRD1, TAOK3*, *TRPA1, TRPV1*, and *SCN9A* are contributing to experimental pain variability between children. Our study included 136 children and adolescents (8–18 years). Cold and heat pain thresholds were determined with a Thermal Sensory Analyzer. Women and young children were significantly more sensitive to pain ($P < 0.05$). After correction for age, gender, reaction time, and correction for multiple testing, *OPRM1* 118A>G G-allele carriers (AG and GG) rated the hot stimulus as significantly less painful than did *OPRM1* 118A>G AA genotyped individuals (2[1–5] vs 7 [3–9], respectively; $P = 0.00005$). Additionally, *OPRM1* 118G allele carriers reached more frequently the minimum temperature limit (44% vs 17%, respectively; $P = 0.003$) and maximum temperature limit (52% vs 24%, respectively; $P = 0.0052$), indicative for lower pain sensitivity. The combined genotype, based on expected pain sensitivity, *OPRM1* 118AA/*COMT* 472 GA or AA genotyped children, was associated with lower pain thresholds (ie, higher pain sensitivity) than were the *OPRM1* 118GA or GG/*COMT* 472GG genotyped children. This is the first study reporting on genetic variants and experimental thermal pain in children and adolescents. *OPRM1* rs1799971 and the combined *OPRM1*/*COMT* genotype could serve as biomarkers for pain sensitivity.

INTRODUCTION

The amount of pain expressed by an individual is mediated true a complex interplay between biological, psychological, sociocultural and environmental factors [21; 48]. This interplay is responsible for major variability in pain severity and thus analgesic dose requirements between individuals. One of these factors, gender, has been related with differences in pain perception in a study in which adult females showed lower thermal pain thresholds (i.e. higher pain sensitivity) than males. The higher pain sensitivity observed in females seems to be mediated by increased pain-related fear [20]. A recent meta-analysis in pediatrics confirmed the increased pain sensitivity to thermal stimuli in females only in cohorts with a mean age of 12 years or older [6]. Ethnicity, with different underlying mechanisms, is also contributing to differences in pain experience as shown in a systematic literature review reporting increased pain responsiveness in African-American individuals [36]. Studies on the relative contribution of environment versus genetics pointed out that heritable components can explain 12–70% of the variability in pain intensity, depending upon the pain modality [2; 16; 31; 32]. In line with this genetic influence, specific variants within the mu-opioid receptor (*OPRM1*)[38] and catechol-O-methyltransferase (*COMT*) [4] gene have been associated with pain and analgesic treatment with opioids.

An experimental setting is ideal to assess associations between candidate polymorphisms and pain sensitivity, as such a setting excludes major confounders that are present in the clinic and hamper a genotype-phenotype correlation. The method of thermal quantitative sensory testing is often used to assess pain sensitivity in children and adults in a controlled and non-invasive fashion. Although experimental pain is often not directly translatable to the clinical situation, a good correlation has been reported between thermal pain thresholds and opioid response in healthy volunteers [15], the postoperative setting [1; 27; 35] and chronic pain [14]. Several studies have reported that specific genetic variants seem to be associated with pain sensitivity in the experimental setting – at least in adults [19]. This has, however, never been investigated in children.

For this study candidate genes previously associated with pain sensitivity in adults were selected for which there is evidence on either: a) thermal pain thresholds: *OPRD1* [23], *TRPV1* [23], *TRPA1* [22]; b) pain and/or analgesic requirement: *OPRM1* [38], *COMT* [20], *SCN9A* [37; 42]; or c) variants found in a genome wide association study involving morphine requirement in pediatrics: *TAOK3* [10]. A prerequisite for the probability of establishing associations was sufficient frequency of the variant allele in the population (> 5%). The aim of our study was to determine whether the selected genetic variants are associated with thermal pain sensitivity in a cohort of children previously admitted to the hospital during their neonatal period and in healthy controls without previous hospital admissions.

METHODS

This candidate gene association study was approved by the Institutional Review Board of Erasmus University Medical Centre in Rotterdam (The Netherlands) as an addendum to our 'Long term consequences of neonatal pain study' (at age 8–18 years) (MEC-2010–299) [45; 46]. All parents and children participating in this long-term outcome study (n = 171) were asked to provide consent for DNA analysis on left-over saliva material from this study. Saliva had been collected at the child's age of 8–18 years and was stored between 2 to 4 years at -20°C until the current DNA analysis for this candidate gene association study. An information letter with informed consent forms was sent to the home address. Written informed consent was obtained from the parents of each child prior to participation. Co-consent in writing was also obtained from children 12 years of age and older, in accordance with Dutch law.

Participants

The participants (8–18 years) had participated in a trial on the possible long-term effects of pain and opioid/sedative administration during the prenatal or neonatal period on brain morphology, brain functioning, neuropsychological functioning and pain sensitivity. Six groups were distinguished on the basis of the neonatal medical history: 1) children receiving neonatal extracorporeal membrane oxygenation (ECMO) treatment [45] who had been exposed to a median pain intensity in combination with high continuous opioid and sedative exposure; 2) preterm born children who were mechanically ventilated in the first weeks of life [46] with low exposure to pain and opioids; 3) children operated upon in the first months of life to remove a giant congenital melanocytic naevus (GCMN), with extreme pain and high opioid and sedative exposure; 4) children undergoing major surgery in the first month of life (e.g. abdominal, thoracic incision) with relatively high pain intensity and high opioid and sedative exposure; 5) children who prenatally had been exposed to opioid related substances (morphine, methadone, heroin) through their mothers; and 6) a control group of healthy children without a history of neonatal pain and opioid exposure.

Experimental thermal pain

Thermal detection and pain thresholds were assessed with the Thermal Sensory Analyzer (TSA; type II Medoc®, Ramat Yishai, Israel). Our research group has ample experience with this method [43–46]. The thermode stimulating surface was placed on the thenar eminence of the non-dominant hand. The applied temperature ranged from 0°C to 50°C, which was safe and non-damaging for the skin [29]. Assessment was according to a standardized protocol. The first step was explaining the test to the children, after which the detection and pain thresholds for cold and heat were determined with the reaction time

dependent Method of Limits (MLI). Next, the detection thresholds for cold and heat were examined by gradually decreasing or increasing, respectively the baseline temperature of 32°C at a rate of 1°C/sec. The child had been instructed to press the button as soon as the cold or heat stimulus was felt, upon which the temperature of the thermode returned to baseline temperature. Two tests served as rehearsals, and the detection thresholds were calculated as the means of the 4 following tests. The cold and heat pain thresholds were assessed almost in the same way, but now the child had been instructed to press the button as soon as the thermode started to feel painful, with the temperature reversing at a rate of 10.0°C/sec after the button was pressed. If a child did not press the button before 0°C or 50°C was reached, the test was automatically terminated. In this case the pain thresholds were set at 0°C and 50°C, respectively. All TSA test outcomes were corrected for the child's average reaction time, determine with the short base-line speed task of the Amsterdam Neuropsychological Tasks (ANT) [12]. Lastly, perception of the intensity of pain evoked by a hot stimulus (46°C), applied with the TSA apparatus, was determined with a self-reported score on a Numerical Rating Scale (NRS) with the extremes 0 (no pain at all) and 10 (worst imaginable pain).

Genotyping

DNA was isolated from surplus saliva (registered at Dutch internet portal (NL33603.078.10)), either manually with the "QuickExtractTM – DNA Extraction Solution (Epicentre®)" or automated with the "DNA Isolation Kit - Large volume" on the MagNA Pure LC 2.0 instrument (Roche®). The following candidate genetic polymorphisms were selected: *COMT* rs4680, rs4818, rs4633, *OPRM1* rs1799971, *OPRD1* rs2234918, *TRPA1* rs11988795, rs13255063, *TRPV1* rs2234918, *TAOK3* rs795484 and *SCN9A* rs6746030. All genetic variants were analyzed with the allelic discrimination method using "TaqMan® SNP Genotyping Assays" on the 7500 Fast Real-Time PCR System (software version 3.0.0; Applied Biosystems, Bleiswijk, the Netherlands).

Genotype frequencies were checked for agreement with the Hardy-Weinberg equilibrium and MAFs from literature (HapMap, National Centre for Biotechnology Information). When a genotype group within a SNP consisted of less than 10 participants, heterozygotes and homozygotes variants were combined. *COMT* haplotype was estimated with R (version 3.1.1) haplo.stats package using a posterior probability limit of 90%, with GGC (rs4680, rs4818, rs4633 resp.) genotype coding for low pain sensitivity (LPS), ACT for average pain sensitivity (APS) and GCC high pain sensitivity (HPS) [13]. Participants with LPS/LPS and LPS/APS alleles were categorized in the LPS group, APS/APS and LPS/HPS in the APS group and HPS/HPS and APS/HPS alleles in the HPS group. In addition also the combined effect of *OPRM1* and *COMT* rs4680, suggested in previous reports, was assessed [28; 39]. To this aim children with the 118AA genotype with 472A allele carriage were compared with children with the 118G allele carriage with/or 472GG genotype.

Statistical analysis

Data were analyzed in IBM SPSS Statistics version 21. Normal distribution was judged visually (Q-Q plot, histogram) and tested with the Kolmogorov-Smirnov test. Associations of normally distributed data were analyzed with a T-test or ANOVA, whereas skewed data was analyzed with Mann-Whitney U or Kruskal-Wallis test. Before assessment of the genetic factors, clinical factors such as age, gender and cases vs. control were analyzed for their influence on thermal pain. The association between the genetic variants and pain thresholds, NRS pain scores and number of children who reached the minimum and maximum temperature was tested univariate and multivariable either by multiple linear or binary logistic regression. The association between the selected genetic variants with TSA outcomes was corrected for age, gender and reaction time, by including these factors as covariates in the multivariable linear regression. All other outcomes were solely corrected for age and gender in the multivariable regression. In the univariate analysis *p*-values below 0.05 were considered statistically significant. Associations with the selected genetic variants from multivariable regression were considered significant if *p*-value was below 0.0056, which was after adjustment for multiple testing by Bonferroni correction. Data are presented as median with corresponding interquartile range, unless stated otherwise.

RESULTS

From the 171 participants from the previous trial, 136 parents and/or children (79.5%) gave written informed consent for DNA analysis on surplus saliva material and were recruited in this current study. **Figure 1** shows the inclusion rate broken down for the medical his-

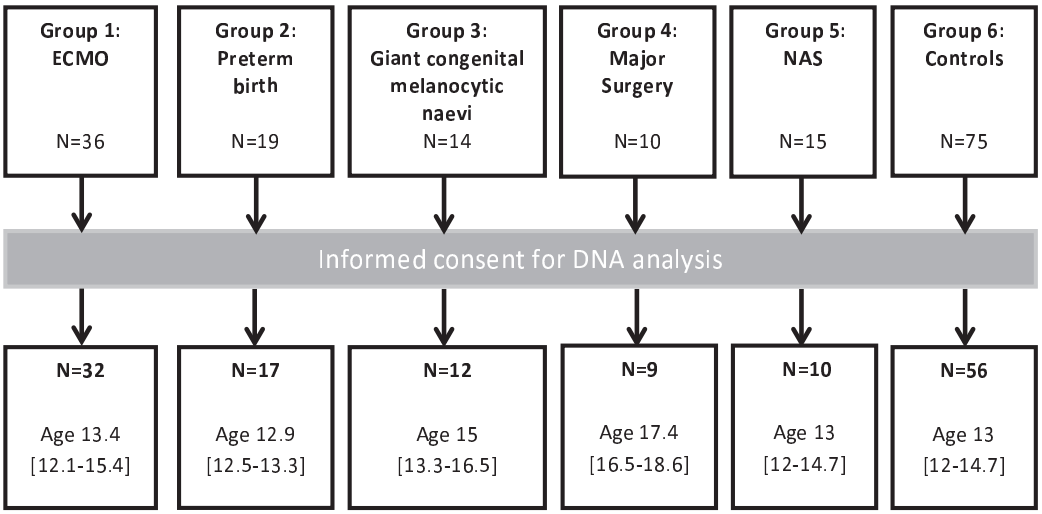

Figure 1. Overview inclusion rate participants according to medical history.

tory subgroups. Participants (n = 136) and non-participants (n = 35) did not significantly differ in age (11.2 [10.1–14.2] versus 11.2 [10.2–13.9; p = 0.63) and gender (52.9% versus 40% female; p = 0.19). Clinical characteristics are listed in **Table 1**.

The final analysis included TSA test results of 133 children. One child refused to perform the test, the results of two other children were considered unreliable due to presence of attention deficits. Girls rated the heat stimulus of 46°C significantly more painful than did boys (NRS: 7 [3–9] versus 4 [1–7]; p = 0.004). This significant correlation between gender and pain sensitivity was confirmed by the higher pain thresholds (tolerating more extreme temperatures) in boys for cold ((°C): 5.74 [0.57–16.9] versus 14.6 [4.21–21.6]; p = 0.0004) and heat ((°C): 49.1 [44.5–50] versus 45.1 [40.8–48.2]; p = 0.0002). Also more boys than girls reached the upper (54.8% versus 26.4%; p = 0.003) and lower temperature limits (48.4% versus 22.2%; p = 0.032). Although younger children reported higher NRS scores for the stimulus of 46°C (r= -0.182, p = 0.034), age was not significantly correlated with thermal pain thresholds. The medical history had no influence on the pain sensitivity and no associations in this respect were between all cases combined versus the controls. Considering the absence of an effect of medical history on thermal pain sensitivity we performed genetic analysis on the total cohort. The associations with the clinical factors are shown in **Supplementary Table 1**.

Table 1. Descriptive data cohort

	n = 136	
Age (yrs.)	11.2	[10.1–14.2]
Male/Female (%)	47.1/52.9	
Medical history group (n)		
Group I: Extracorporeal Membrane Oxygenation	32	
Group II: Pre-term birth	17	
Group III: Giant congenital melanocytic naevi	12	
Group IV: Major surgery	9	
Group V: Neonatal Abstinence Syndrome	10	
Group VI: Controls	54	
Reaction time (ms)	307	[272–339]
Skin temperature (°C)	36.2	[36–36.9]
Environmental temperature (°C)	23	[22–24]
Parents present Yes/No (%)	88.1/11.9	
Cold detection threshold (°C)	30.8	[30–31.1]
Heat detection threshold (°C)	33.7	[33.2–34.7]
Cold pain threshold (°C)	10	[1.08–19.2]
Heat pain threshold (°C)	47.0	[42.8–50]
NRS (stimulus 46°C)	6.0	[2.0–8.75]

Data are presented as median with IQR, unless stated otherwise

Genetic factors

Genotype results are displayed in **Table 2**. Observed MAFs were in agreement with the frequencies reported in literature, although two single nucleotide polymorphisms (SNPs) were not in HW-equilibrium in univariate analysis (*COMT* rs4818; p = 0.03 and *OPRM1* rs1799971; p = 0.02). When corrected for multiple testing (p = 0.0056)[47], all SNPs where in Hardy Weinberg equilibrium. Since for *OPRM1* and *SCN9A* polymorphisms the homozygous variant group was < 10 individuals, this group was combined with the heterozygous genotyped children. In view of the undetermined results for some of the individual *COMT* SNPs (rs4680, rs4818 and rs4633) the *COMT* haplotype was successfully constructed in 88 participants, with 46 participants having LPS, 29 APS and 13 HPS *COMT* haplotype.

Table 2. Results genotyping

	COMT (G>A) rs4680	**COMT (C>G) rs4818**	**COMT (C>T) rs4633**	**OPRM1 (A>G) rs1799971**	**OPRD1 (C>T) rs2234918**	**TAOK3 (C>T) rs795484**	**TRPA1 (C>T) rs11988795**	**TRPA1 (T>A) rs13255063**	**TRPV1 (T>C) rs8065080**	**SCN9A (G>A) rs6746030**
Wild type	39	50	29	96	33	64	51	71	47	94
Heterozygous	56	45	44	22	53	46	48	35	64	22
Variant	32	23	28	5	37	17	24	10	19	3
Missing	9	18	35	13	13	9	13	20	6	17
Total	136	136	136	136	136	136	136	136	136	136
Observed MAF(%)	47	39	50	13	52	32	39	24	39	12
Literature MAF(%)	48	40	48	16	46	32	41	27	36	12
HW-equilibrium	0.19	***0.03***	0.20	***0.02***	0.15	0.07	0.05	0.07	0.71	0.23
(p-value)										

MAF = minor allele frequency

Supplementary Table 2 summarizes the associations between the genetic variants and thermal pain sensitivity. In the univariate analysis, *OPRM1* rs1799971 A>G was associated with the NRS score for the applied heat stimulus (p = 0.001), with G-allele carriers rating the heat stimulus as less painful compared to wild type participants (NRS: 2 [1–5] versus 7 [3–9]). In line with their lower pain sensitivity, G allele carriers also had higher cold and heat pain thresholds (p = 0.006 and 0.012, respectively). The effect size for cold pain thresholds was larger than that of heat thresholds (-4.91 (95%CI: -8.74;1.09) versus 2.19 (95%CI: 0.543;3.83)). Also, as illustrated in **Figure 3**, the G allele carriers reached more frequently the minimum and maximum temperature limits (p = 0.002 and p = 0.006 resp.). After adjusting for age, gender, reaction time and false positives, *OPRM1* rs1799971

A>G remained significantly associated with NRS (p = 0.00005) and the number of children reaching the minimum and maximum temperature limits (p = 0.003 and 0.0052), illustrated in **Figures 2** and **3**.

Children genotyped *TRPA1* rs11988795 TT had significantly lower cold pain thresholds compared to CT and CC individuals (18.8 [9.45–26] versus 10.1 [1.51–18.9] and 6.45 [0.49–17.9], p = 0.020). Correspondingly, the heat pain threshold was also lower in the TT genotyped group compared with the heterozygotes and wild type children (43.1 [40.2–49]

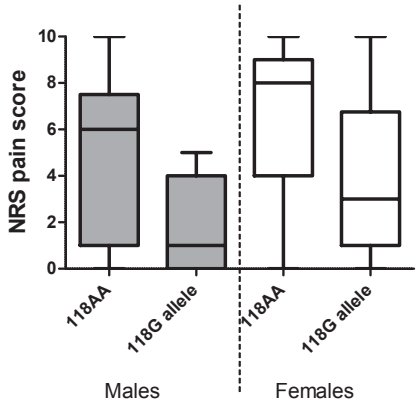

Figure 2. NRS score heat stimulus (46°C)
Numerical Rating Score (NRS) given by the children, indicating pain intensity for the applied heat stimulus (46°C). After stratification according to gender, the effect of *OPRM1* 118A>G genotype remained significant* (p < 0.001) in both groups (multiple linear regression, corrected for age).

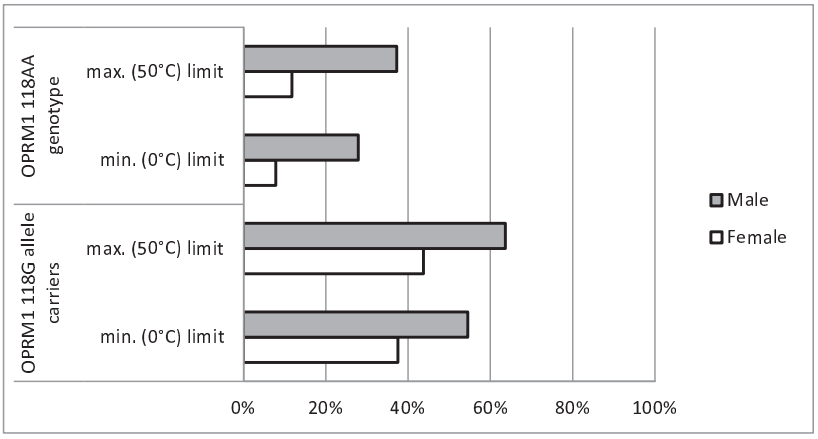

Figure 3. Minimum and maximum temperature limits
Children who reached minimum (0°C; indicative for lower cold pain sensitivity) and maximum temperature limit (50°C; indicative for lower heat pain sensitivity). The horizontal y-axis displays the percentage of males/females (blue and red bars) that reached the min./max. temperature within the *OPRM1* 118AA and 118G allele carrier genotype group (upper and lower panel). Gender and *OPRM1* genotype were significantly associated with min. (p = 0.017 vs. p = 0.003, respectively) and max. temperature limit (p = 0.003 vs. p = 0.005, respectively) in a logistic regression with correction for age.

versus 47.1 [44.1–49.9] versus 47.7 [42.6–50], $p = 0.022$). Although the associations remained after correction for gender, age and reaction time, the Bonferroni threshold was not reached. TRPV1 rs8065080 TT genotyped children had increased pain sensitivity to heat ($p = 0.046$) but not against cold pain ($p = 0.12$). In addition, after correction for confounding factors, the TT genotyped children in general reported the hot stimulus as not painful. Nevertheless, all these associations were not significant after correction for false positives.

The combined *OPRM1/COMT* genotype was associated with the thermal pain thresholds. Children with the *OPRM1* 118AA genotype combined with *COMT* 472A allele carriage seemed more sensitive to pain as they had lower thermal pain thresholds and reached the minimum and maximum limits less frequently compared with *OPRM1* 118G-allele carriers with the *COMT* 472GG genotype. The associations with the thermal pain thresholds remained significant in the multivariable analysis and after correction for false positives ($p < 0.0056$). The other pain parameters remained significant with the *OPRM1/COMT* combined genotype after correction in the multivariable analysis but did not pass Bonferroni correction. None of the other polymorphisms were associated with thermal pain sensitivity, neither in the univariate nor in the multivariable regression.

DISCUSSION

Our study demonstrates that *OPRM1* 118A>G and the combined *OPRM1/COMT* genotype are associated with experimental thermal pain sensitivity in a pediatric population. The children and adolescents who carried the *OPRM1* 118G allele seemed less sensitive to pain, as they rated the stimulus of 46°C lower than did *OPRM1* wild type genotyped children and reached the minimum and maximum temperature limits of 0°C and 50°C in the TSA test more frequently. We did not find a correlation between *OPRM1* genetics and pain thresholds. Regarding the combined *OPRM1/COMT* genotype higher pain sensitivity was seen in children with the *OPRM1* 118AA genotype who also carried the *COMT* 472A allele.

Different molecular mechanisms have been linked to the *OPRM1* 118A>G variant, as reviewed recently [24]. As summarized in this review, reduced expression in 118G allele carriers could be the consequence of a change in mRNA stability, an additional methylation site [33] or loss of a N-glycosylation site [25]. A study assessing the affinity of endogenous and exogenous opioids for the mu-opioid receptor showed increased β-endorphin affinity and potency in the 118G variant [7] but this finding could not be replicated by others [3; 5; 25]. However, results of experimental studies in healthy adult volunteers are in line with our findings and support the hypothesis of an increased affinity and potency of β-endorphin for the mu-opioid receptor. To illustrate this, a study with 167 healthy

subjects showed higher pressure pain thresholds in carriers of the 118G allele and lower heat pain ratings for a 49°C stimulus in males carrying the minor G allele [17].

We also found a lower heat pain sensitivity in 118G allele carriers after correction for gender, indirectly confirming increased binding and potency of the endogenous agonist. In addition, a study showing decreased thermal pain sensitivity in non-Hispanic whites carrying the 118G allele suggests the existence of a gene-ethnicity interaction [18]. The authors link this interaction to the ethnicity-specific haplotype structure and gene-gene interactions. We did not analyze this interaction because the ethnicity in our cohort was not proactively documented. Experimental pain in relation to the *OPRM1* SNP has also been assessed in approximately 800 women prior to breast surgery [8], without any correlation with thermal pain sensitivity. Confounding factors, such as prior opioid use for cancer pain, are likely obscuring a genotype-phenotype correlation in the latter case.

In line with our finding of a larger effect size of *OPRM1* 118A>G on cold pain thresholds than on heat pain, a study comparing monozygotic and dizygotic twins concluded that genetics contributed 60% to cold pain sensitivity and 26% to heat pain sensitivity [31]. Although the larger spread in cold pain thresholds between the children most likely leads to increased effect size of this genetic variant compared to heat pain, the extent of genetic contribution is highly dependent on the chosen pain phenotype and comparison between pain modalities might lead to contradicting conclusions. Our previous findings illustrated that mechanically ventilated preterm neonates receiving placebo were more likely to require (rescue) morphine when carrying the *OPRM1* 118G allele with or without the *COMT* 472GG genotype [24]. This seems to contradict with the higher cold and heat pain thresholds reported in the current study for these genotypes.

However, the effect of the *OPRM1* variants might be more complex than simply a change in affinity for endogenous peptides or decreased receptor expression in 118G carriers. A recent study in 50 healthy volunteers supports our initial contradictive findings by documenting the opposite effect of this SNP on dopamine (DA) release in the nucleus accumbens [34]. *OPRM1* 118G carriers had, compared to 118AA individuals, higher DA release in the nucleus accumbens during the painful stimulus, whereas during placebo intervention DA release in this brain area was lower in 118G carriers [34]. The discrepancy in findings between our studies might also be related to the developmental pattern of the involved receptor (OPRM1) and enzyme (COMT). However, although human studies are lacking, in vivo animal data suggest no difference in OPRM1 transcription between 10, 20 postnatal days and adult rats [26]. In contrast, the COMT protein expression and activity in humans showed a developmental pattern, with maximum levels reached in adults [41].

Even though no associations were found with *TRPA1* and *TRPV1* SNPs after Bonferroni correction it is still worthwhile discussing these variants. The transient receptor potential (TRP) V1 ion channel is known to be activated by chemical stimuli such as capsaicin or by temperatures exceeding 43°C [9]. Although on average the heat pain threshold in our

cohort exceeded 43°C and the painfulness was assessed at 46°C, the found association between the *TRPV1* variant and heat pain sensitivity did not survive Bonferroni correction. As anticipated no association was found with cold pain sensitivity in our cohort, whereas other researchers reported increased cold withdrawal time in carriers of the *TRPV1* variant allele [19]. While TRPV1 is activated by noxious heat temperatures, the TRPA1 ion channel is activated by temperatures < 15°C [40], though this has been contradicted [11]. In the present study we found a correlation between *TRPA1* rs11988795 variant and thermal pain thresholds, with a larger effect size for cold pain. This is in line with a previous report showing higher cold pain sensitivity in rs11988795 AA compared to GG genotyped individuals [22]. However, this association did not remain after Bonferroni correction.

The method used to measure pain sensitivity could be considered a limitation, since quantitative sensory testing is highly depending on environmental factors, methodological factors, cooperation and attention of participants tested [30]. However, in our cohort environmental factors were kept as stable as possible and the tests were protocolized and performed by one and the same researcher in the same room [45; 46]. Also the levels of cooperation and attention could be questioned in young children. Still, the feasibility was assessed twice per detection threshold and once per pain threshold test, which were the previously mentioned rehearsals. Almost all children showed good understanding of what was expected from them. Lastly, even though it would be interesting to assess if the found associations for thermal pain could be replicated for the clinical pain and analgesic requirements documented during their neonatal hospital admission, a grouped analysis was not possible due to the differences in study set-up and documentation of the data for groups 1–4. Sample sizes of these groups were too small for separate analysis in all but the preterm, mechanically ventilated cohort, as we discussed before [28].

In conclusion, our study illustrates that children carrying the *OPRM1* minor 118G allele or the combined *OPRM1/COMT* 118G allele and/or 472GG genotype are significantly less sensitive to thermal experimental pain. This might have important implications for the prediction of clinical pain states and thus the pharmacological and non-pharmacological treatments in the pediatric population.

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Supplementary Table 1. Effect clinical factors on thermal detection and pain.

	Age (yrs)	RT (ms)	NRS 46°C	MLI-CDT (°C)	MLI-HDT (°C)	MLI-CPT (°C)	CPT limit (n/%)	MLI-HPT (°C)	HPT limit (n/%)
Male	11.0 [9.96–14.6]	311 [264–345]	4 [1–7]	30.8 [29.8–31.1]	33.6 [33.3–34.8]	5.74 [0.57–16.9]	30/48.4	49.1 [44.5–50]	34/54.8
Female	11.2 [10.1–13.6]	304 [274–331]	7 [3–9]	30.9 [30.1–31.2]	33.7 [33.1–34.4]	14.6 [4.21–21.6]	16/22.2	45.1 [40.8–48.2]	19/26.4
p-value	0.79	0.58	0.004	0.066	0.20	0.0004	0.032	0.0002	0.003
Age	X	-0.443	-0.182	0.203	-0.255	-0.079	-0.153	0.040	-0.162
p-value	X	< 0.01	0.034	0.15	0.18	0.85	0.078	0.77	0.062
Control	11.3 [10.3–13.6]	296 [270–327]	6 [2–9]	30.9 [30.4–31.2]	33.5 [33.1–34.3]	10.4 [0.93–19.2]	19/34.5	47.3 [43.4–50]	21/38.2
Case	10.8 [10.1–14.5]	318 [278–348]	5.5 [2–8]	30.7 [29.8–31.1]	33.8 [33.2–34.9]	9.83 [1.18–19.7]	27/34.2	46.1 [42.5–50]	32/40.5
p-value	0.57	0.11	0.62	0.42	0.25	0.82	0.52	0.57	0.66
Group 1	10.4 [8.9–12.1]	320 [294–343]	7 [3.3–9]	30.1 [29–31]	34.4 [33.5–35]	15 [1.19–21.5]	10/31.3	44.8 [40.2–49.3]	11/34.4
Group 2	10.3 [10–10.5]	339 [295–368]	6 [0 9]	30.7 [30.1–31.1]	33.5 [33.2–34.7]	15.8 [6.03–21.4]	6/37.5	45.1 [42.7–48.6]	6/37.5
Group 3	12.3 [10.7–14.1]	302 [264–347]	3.5 [1.3–6.5]	30.8 [28.8–31.2]	33.7 [33.3–36.5]	5.25 [0.20–10]	5/41.7	46.9 [42.9–50]	4/33.3
Group 4	15.2 [14.7–16.5]	248 [230–282]	3 [1.5–6]	31.1 [30.8–31.4]	33.4 [33.1–34]	0.65 [0.055–10.2]	4/44.4	50 47.1–50]	7/77.8
Group 5	13.4 [11.2–17.2]	297 [246–411]	3.5 [0.75–8]	30.9 [30.2–31.2]	33.7 [32.8–35.7]	11.1 [3.91–21.5]	2/20	46.6 [43–50]	4/40
Group 6	11.3 [10.3–13.6]	230 [270–327]	6 [2–9]	30.9 [30.4–31.2]	33.5 [33.1–34.3]	10.4 [0.93–19.2]	19/34.5	47.3 [43.3–50]	21/38.2
p-value	< 0.001	0.004	0.079	0.36	0.15	0.33	0.53	0.66	0.42

Data are presented as median with IQR, unless stated otherwise.

RT = reaction time; NRS = Numeric Rating Scale; MLI = method of limits; CDT = cold detection threshold; HDT = heat detection threshold; CPT = cold pain threshold; HPT = heat pain threshold; Group 1= ECMO; Group 2= Preterm birth; Group 3= Melanocytic naevi; Group 4= Major surgery; Group 5= NAS; Group 6= Controls; Case = children with previous pain and/or (intrauterine) opioid exposure; Control = healthy (not previously admitted) age and gender matched children.

Supplementary Table 2. Effect genetic factors on thermal pain.

	NRS stimulus 46°C	MLI-CPT (°C)	CPT limit (n/%)	MLI-HPT (°C)	HPT limit (n/%)
OPRM1 rs1799971					
118AA (n = 96)	7 [3–9]	11.8 [2.18–20.6]	16/17	46 [42.1–49.6]	23/24
118G carrier (n = 27)	2 [1–5]	3.88 [0–14.6]	12/44	49.9 [43.4–50]	14/52
p-value (univariate)	0.001**	0.006**	0.002**	0.012*	0.006**
β (95% CI)	-2.91 [-4.28;-1.55]	-4.91 [-8.74;-1.09]	4.48 [1.65; 12.1]	2.19 [0.543;3.83]	4.01 [1.51;10.6]
p-value (corrected#)	0.00005**	0.012*	0.003**	0.010*	0.0052**
OPRD1 rs2234918					
307CC (n = 33)	4 [1.5–9]	7.08 [0.825–13.5]	10/30.3	49.2 [44.4–50]	14/43.8
307CT (n = 53)	6 [1–8]	14.6 [0.735–20.2]	15/28.3	47.3 [42.8–50]	16/30.2
307TT (n = 37)	5 [2–7.5]	8.67 [1.25–18.9]	6/16.7	47.2 [42.7–50]	10/27.8
p-value	0.99	0.43	0.35	0.34	0.32
β (95% CI)	-0.136 [-0.950;0.678]	-0.001 [-2.24; 2.22]	0.823 [0.462; 1.47]	-0.106 [-1.06;0.845]	0.794 [0.466; 1.35]
p-value (corrected#)	0.74	1.0	0.51	0.83	0.40
TRPV1 rs8065080					
TT (n = 47)	4 [1–7]	6.18 [0.79–14.5]	15/31.9	47.7 [45.3–50]	19/40.4
TC (n = 64)	7 [3–9]	14.3 [2.18–19.7]	12/18.8	45.6 [42.1–50]	17/27
CC (= 19)	6 [3–9]	12.8 [0.808–21.1]	4/22.2	45.2 [42.1–49.6]	4/22.2
p-value	0.05	0.12	0.26	0.046*	0.23
β (95% CI)	0.881 [0.033; 1.73]	1.94 [-0.355;4.22]	0.675 [0.350; 1.30]	-0.998 [-1.98;-0.019]	0.624 [0.341; 1.14]
p-value (corrected#)	0.042*	0.097	0.24	0.046*	0.13

Supplementary Table 2. Effect genetic factors on thermal pain. (continued)

	NRS stimulus 46°C	**MLI-CPT (°C)**	**CPT limit (n/%)**	**MLI-HPT (°C)**	**HPT limit (n/%)**
TRPA1 rs11988795					
CC (n = 51)	5 [2–9]	6.45 [0.49–17.9]	16/32	47.7 [42.6–50]	21/42
CT (n = 48)	6 [2–8]	10.1 [1.51–18.9]	10/20.8	47.1 [44.1–49.9]	12/25
TT (n = 24)	6 [1.5–9]	18.8 [9.45–26]	2/8.3	43.1 [40.2–49]	4/17.4
p-value	0.96	0.020*	0.073	0.022*	0.062
β (95% CI)	0.064 [-0.747;0.875]	2.68 [0.549; 4.81]	0.461 [0.236; 0.901]	-1.12 [-2.07;-0.175]	0.515 [0.283; 0.938]
p-value (corrected#)	0.88	0.014*	0.023*	0.021*	0.030*
TRPA1 rs13255063					
TT (n = 71)	6 [2–8]	13.3 [3.16–19.1]	13/18.3	46.4 [42.5–49.9]	18/25.7
TA (n = 35)	5 [1–8]	6.18 [1.21–19.2]	8/22.9	48.5 [43.3–50]	14/40
AA (n = 10)	6 [4–9.3]	5.01 [0–20.4]	3/33.3	47 [41.9–50]	3/33.3
p-value	0.55	0.63	0.47	0.40	0.31
β (95% CI)	0.302 [-0.662;1.27]	-1.37 [-3.98; 1.24]	1.58 [0.790; 3.17]	0.538 [-0.589;1.66]	1.58 [0.837; 3.00]
p-value (corrected#)	0.54	0.30	0.20	0.35	0.16
TAOK3 rs795484					
CC (n = 64)	6 [2–8]	6.18 [0.54–16.8]	17/27	47.6 [43.6–50]	24/38.1
CT (n = 46)	5 [1–8]	12 [1.34–20.8]	10/21.7	44.6 [40.5–49.5]	10/21.7
TT (n = 17)	6 [4–9.25]	15.7 [2.67–21.7]	3/17.6	47.6 [43.5–50]	6/37.5
p-value	0.57	0.12	0.74	0.075	0.17
β (95% CI)	-0.022 [-0.864;0.821]	2.07 [-0.195;4.34]	0.712 [0.377; 1.35]	-0.337 [-1.32;0.645]	0.803 [0.450; 1.43]
p-value (corrected#)	0.96	0.073	0.30	0.50	0.46

Supplementary Table 2. Effect genetic factors on thermal pain. (continued)

	NRS stimulus 46°C	MLI-CPT (°C)	CPT limit (n/%)	MLI-HPT (°C)	HPT limit (n/%)
COMT haplotype					
LPS (n = 46)	4 [1–7.25]	6.18 [0.79–20.4]	15/32.6	47.6 [42.5–50]	18/40
APS (n = 29)	6 [3–9]	14.6 [5.61–18.6]	4/13.8	46.7 [43–49.7]	7/24.1
HPS (n = 13)	8 [3.5–9]	7.34 [4.44–18]	2/15.4	46.9 [42.3–49.7]	3/23.1
p-value	0.051	0.48	0.16	0.88	0.31
β (95% CI)	1.27 [0.316; 2.21]	0.163 [-2.50;2.82]	0.538 [0.238; 1.22]	-0.017 [-1.20;1.17]	0.599 [0.296; 1.21]
p-value (corrected#)	0.01*	0.90	0.14	0.98	0.16
SCN9A rs6746030					
GG (n = 94)	6 [2–9]	11.2 [1.20–20.7]	21/22.3	47.4 [42.3–50]	31/33
A carrier (n = 25)	6 [2–8.5]	11.8 [2.72–19.1]	4/16	45 [43–48.7]	5/20
p-value	0.89	0.85	0.59	0.40	0.25
β (95% CI)	0.032 [-1.48; 1.55]	-0.391 [-4.54;3.76]	0.691 [0.202; 2.37]	-0.486 [-2.32;1.35]	0.582 [0.186; 1.82]
p-value (corrected#)	0.97	0.85	0.56	0.60	0.35
OPRM1/COMT					
118AA and 472A allele	6 [3–9]	15.1 [5.18–21.4]	8/13	45.4 [41.9–48.6]	12/20
118G allele and/or 472GG	4 [1–8]	5.80 [0.65–16.9]	20/34	47.7 [44.3–50]	24/41
p-value	0.096	0.002**	0.007**	0.005**	0.014*
β (95% CI)	-1.35 [-2.55;-0.151]	-4.86 [-7.96;-1.76]	3.43 [1.32;8.90]	1.97 [0.625;3.31]	3.00 [1.24; 7.23]
p-value (corrected#)	0.028*	0.002**	0.011*	0.004**	0.014*

Data are presented as median with IQR, unless stated otherwise.

NRS = Numeric Rating Scale; MLI = method of limits; CPT = cold pain threshold; HPT = heat pain threshold; #*p*-value corrected for gender and age in case of NRS score; gender, age and reaction time for other outcome measures.

* significance < 0.05; ** significance < 0.01

Bonferroni threshold = 0.0056


SLC22A1
COMT
UGT2B7

Chapter 7

Effect of *UGT2B7* -900G>A (-842G>A; rs7438135) on morphine glucuronidation in preterm newborns: results from a pilot cohort

Maja Matic*
Elisabeth Norman*
Anders Rane
Olof Beck
Maria Andersson
Laure Elens
Dick Tibboel
Vineta Fellman
Ron H.N. van Schaik

*These authors contributed equally to this manuscript.

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ABSTRACT

Aim: Assess association between *UGT2B7* polymorphism -900G>A (rs7438135, also known as -842G>A) with morphine kinetics in preterm newborns undergoing mechanical ventilation.

Materials & methods: Thirty-four infants were enrolled in a randomized clinical trial and allocated to rapid sequence intubation with remifentanil (1 μg/kg) or morphine (0.3 mg/kg). The latter group was included in our study.

Results: Morphine plasma concentrations at 20 min post intubation were associated with postnatal age (p = 0.017) and *UGT2B7*–900G>A (p = 0.036). *UGT2B7*–900A allele carriers (n = 13) had lower morphine levels compared with *UGT2B7*–900G/G patients (n = 2). Morphine-3-glucuronide and morphine-6-glucuronide plasma concentrations were only found to be associated with gestational and postnatal age. However, -900A allele carriers had a higher morphine-3-glucuronide:morphine metabolic ratio compared with patients genotyped as -900G/G (p = 0.005), as determined by linear regression.

Conclusion: Our small pilot study illustrates that in addition to gestational and postnatal age, the *UGT2B7*–900G>A polymorphism significantly alters morphine pharmacokinetics in preterm infants.

INTRODUCTION

In newborn infants undergoing intensive care treatment, opioid administration for pain relief is used in many institutions despite insufficient pharmacokinetic (PK) and pharmacodynamic (PD) data. Newborns and especially preterm infants with fast developing and vulnerable brains are highly sensitive to both pain experience and drug-related side effects [1]. Adverse effects, such as cognitive dysfunction, have been suggested to occur based mainly on cross-sectional studies [2] but warrant long-term longitudinal follow-up [3]. A recently published study was not able to illustrate this negative effect on cognitive dysfunction at an age of 8–9 years of follow-up [4]. With improving means to assess individual genetic constitution, personalized dosing strategies can be developed for specific drugs such as opioids with a narrow therapeutic window.

The role of pharmacogenetics (PG) in opioid dosing has received some attention in the adult population [5,6]. To our knowledge only two PG studies have been published during the neonatal period, one study regarding tramadol PK [7] and the other on in utero exposure to methadone and buprenorphine [8]. In the PK–PG analysis of tramadol a relationship was found between *CYP2D6* genetics and O-demethylation activity. The latter study has evaluated the impact of three genes (i.e., *COMT, OPRM1* and *ABCB1*), in which *OPRM1* and *COMT* polymorphisms were found to be associated with a shorter hospitalization time and lower requirement for postpartum treatment in term newborn infants with neonatal abstinence syndrome. However, besides our own work, concerning genetic variation in *OPRM1* and *COMT* on morphine requirement [9], no data are available on morphine PG in the (pre)term neonatal period.

Along with the PDs, the kinetics of morphine is a major determinant of the treatment outcome. Morphine is predominantly subject to conjugating phase II reactions such as glucuronidation, which is catalyzed by UDP-glucuronyltransferase (UGT). UGT2B7 has been identified as the major conjugating enzyme for morphine. The ontogeny of UGT enzymes in humans has not been fully explored, but early studies have demonstrated glucuronidation of morphine during the mid-trimester in fetal liver [10] and kidneys [11]. The glucuronidation capacity in the newborn period is poorly developed [12] but partly compensated by sulfate conjugation [13]. Whereas glucuronidation capacity increases rapidly after birth, sulfation capacity is slowly decreasing. UGT2B7 activity towards morphine starts at approximately 15 weeks of fetal age and is 10–20% of adult activity in mid-trimester [10] while adult enzymatic activity is not reached until sometime between 2 months to 2.5 years [14]. The observed variability in morphine PK during the neonatal period and early infancy is not only the result of maturation of the metabolizing enzymes but is also partly determined by the genetic predisposition. As UGT2B7 has been identified as the major conjugating enzyme, polymorphisms in the *UGT2B7* gene may have relevance and important implications for the disposition of morphine in newborns.

UGT2B7 metabolizes morphine primarily to morphine-3-glucuronide (M3G), whereas approximately 10% is converted to morphine-6-glucuronide (M6G), which is an active metabolite.

Several studies have assessed the effect of the -900G>A (rs7438135) SNP in *UGT2B7* on PK and tolerance of morphine. In a young adult population with sickle cell disease, carriers of the -900G allele had a significantly lower M6G:morphine ratio than AA carriers, suggesting a decreased UGT2B7 activity associated with the G variant allele [15]. In line with this observation, the 802T>C (rs7439366) SNP has been associated with low morphine glucuronidation rate and reduced occurrence of side effects [16,17]. This polymorphism is in complete linkage disequilibrium (LD) with the -900G>A genetic variation [18]. Nevertheless, albeit under different clinical conditions, other research groups have failed to replicate these findings for 802T>C [19–23].

Other polymorphisms can also occur in combination with SNP 802C>T. The so called *2g allele consists of 802C>T and -79G>A, as depicted in the study of Duguay *et al.* The promoter SNP -79G>A gives a 2.5- to 7-fold decrease in transcriptional activity in colon and hepatic cells, respectively [24]. Although the *UGT2B7**2g allele occurs in 5% of Caucasian individuals and our cohort primarily consists of white infants, we are not able to analyze the consequence of this allele on morphine kinetics due to the sample size. Besides, the low variant allele frequency of this SNP will only explain a small part of the observed variability in morphine plasma concentrations.

The aim of this study was to explore whether the highly investigated polymorphism in adults, *UGT2B7*–900G>A (rs7438135), is associated with altered morphine PK in preterm newborns.

METHODS

This candidate gene association study was embedded in a study evaluating optimal drug dosing during endotracheal intubation conducted at the tertiary level neonatal intensive care unit (NICU) at Skåne University Hospital in Lund, Sweden [25]. The Regional Ethics Committee in Southern Sweden and the Medical Products Agency in Sweden approved the research protocol. The trial was registered as EUDRACT (no. 2004–001583) and at www.clinicaltrials.gov (NCT00216944). Written informed consent for DNA analysis was obtained from both parents.

Study design

Thirty-four preterm infants were included in a randomized controlled trial on two different intravenous premedication strategies for semi-urgent intubation during neonatal intensive care [25]. Inclusion criteria were gestational age of < 37 weeks and no analgesic

or sedative drugs administered during 24 h prior to the intubation. Exclusion criteria were asphyxia, major malformations and postoperative care [25]. The present study focused on the 17 infants who received morphine (0.3 mg/kg) and atropine (0.01 mg/kg) prior to intubation (Table 1). The administration of drugs was blinded, and given within 5 min directly followed by the intubation [25]. Blood was sampled for PK analysis before drug administration (baseline), and 20 min, 6 h and 24 h after the termination of intubation. During a follow-up period of 24 h, the children's pain/stress was scored every 30 min with the Astrid Lindgren and Lund Children's Hospital Pain assessment Scale for preterm and sick newborn infants (ALPS-Neo, score 1–10) [26] and every 4 h with the Echelle Douleur Inconfort Nouveau-Ne (EDIN) scale [27]. According to an algorithm based on pain assessment results, a morphine injection dose (0.15 mg/kg) or infusion (10–20 μg/kg/h) was administered if nonpharmacological support was insufficient. In this study we have assessed the relationship between genetic variation in *UGT2B7* with morphine and its metabolites. The study outcomes were concentrations of morphine, M3G and M6G, and also morphine metabolites:morphine plasma ratios at 20 min after intubation. The morphine plasma concentrations at 6 and 24 h were not included in this analysis because of the multiple morphine injections and/or infusions administrated after 20 min. Buccal swab samples for DNA isolation were obtained in all 17 infants.

Table 1. Characteristics of the different *UGT2B7*–842G>A genotype groups in preterm infants receiving morphine as premedication before intubation.

	GG†	GA	AA	
Characteristics	**(n = 2)**	**(n = 6)**	**(n = 7)**	**p-value**
Gestational age (days)	174; 218	183 (177–197)	187 (177–226)	0.49
Postnatal age (days)	0.14; 6	4.5 (1.11–9.82)	7.4 (0.25–16)	0.49
Sex (male/female)	2/0	4/2	2/5	0.18
Weight (g)	950; 1700	975 (890–1270)	825 (648–1993)	0.75
Duration intubation (s)	47; 502	53 (43–196)	249 (49–451)	0.25
Morphine bolus (yes/no)	0/2	4/2	4/3	0.41
Morphine bolus (n)	1; 1	1 (0–1.25)	0.5 (0–1.75)	0.32
Morphine infusion (yes/no)	0/2	0/6	2/5	0.60
Total morphine dose (μg)	290; 510	295 (270–368)	250 (200–600)	0.69
ALPS-Neo score at baseline	3; 7	3.5 (0.75–4.0)	4 (2.5–7.8)	0.41

The continuous variables are presented as median with their interquartile range.
†In this column the values of the two -900G/G genotyped patients are shown. ALPS-Neo: Astrid Lindgren and Lund Children's Hospital Pain Assessment Scale.

Bioanalytical method

Morphine and its metabolites were analyzed using LC-MS/MS. The method, with some modifications, has been described before [28]. Whole blood was sampled in heparinized tubes and the samples were centrifuged within 30 min. The plasma was collected, transported on ice and immediately frozen to -70°C. Sample preparation was performed according to an earlier reported procedure [29]. In brief, 50 µl of plasma were precipitated with 100 µl acetonitrile containing denatured internal standards. The supernatants were evaporated to dryness and reconstituted in 10 µl of 0.1% aqueous formic acid.

The instrument used was an ACQUITY UPLC system connected to a Quattro Premier XE tandem mass spectrometer (Waters, MA, USA). The analytical column used was an ACQUITY UPLC HSS T3, 2.1 × 100 mm, 1.8 µm particle size, kept at 60°C. A gradient elution was used with mobile phase A: 0.1% aqueous formic acid and mobile phase B: methanol. The flow rate for morphine and its metabolites was 0.2 ml/min. The total run time was 6.5 min and the injection volume was 3 µl. The tandem mass spectrometer was operated in the positive electrospray mode using selecting reaction monitoring. The instrumental setting for morphine and the metabolites is described in a previous method [28]. A linear relationship was observed for morphine, M3G and M6G in the range of 2–1000 ng/ml. The intra-assay precision was studied at 10, 200 and 800 ng/ ml for morphine and its metabolites. For morphine, coefficients of variation (CV) were 6.69, 1.12 and 4.15% (n = 5). CV for M3G was 6.59, 2.52 and 2.00% (n = 8) and for M6G CV was 7.42, 2.07 and 2.55% (n = 8). Limit of detection was estimated to 0.6 ng/ml for morphine, M3G and M6G. In order to collect sufficient number of patients in the PG analysis, samples that were below this limit of detection were all set to 0.5 ng/ml. Morphine and metabolite concentrations between 0.6–2 ng/ml were set to 1.3 ng/ml.

Genotyping procedure

DNA isolation was performed at the Skåne University Hospital in Malmö (Sweden) according to the protocol DNA Purification from Buccal Swabs (Spin Protocol) obtained from the QIAamp DNA mini and blood mini handbook 04/2010 (Qiagen AB, Sollentuna, Sweden) manual. The DNA isolate volume was 140 µl for each sample and the mean DNA concentration from the buccal swabs was 17.4 ng/µl (range: 1–109 ng/µl). The DNA isolates were all used undiluted in the SNP analysis.

The selected *UGT2B7* SNP, -900G>A (rs7438135; also know in literature as -842G>A) was assessed at the Erasmus MC Clinical Chemistry Department, Rotterdam, The Netherlands. The genetic variation 802T>C was assessed in order to confirm the previously reported complete LD between the two SNPs. Polymorphism -900G>A and 802T>C were genotyped according to the TaqMan® allelic discrimination assay (Life Technologies Europe BV, Bleiswijk, The Netherlands). The reaction solution was prepared using 6.26 µl of the TaqMan GTXpress™ Master Mix (Life Technologies), 0.313 µl of the SNP specific

assay mix and 3.44 µl RNAse free water. The probes in the 900G>A assay mix are labeled reverse: wild-type allele on the y-axis and variant allele on the x-axis, while the 802T>C assay mix contained a normal calling.

The PCR program started with denaturation of the DNA strand at 95°C for 20 s, followed by hybridization of the primers and probes at 92°C for 40 s. After hybridization the elongation was begun at 60°C for 30 s. The whole process was run for 45 cycles. The post-PCR detection was performed on the 7000 Real-Time PCR System (software version v2.0.5; Applied Biosystems). Both polymorphisms were assessed with the same PCR program and on the same machine.

Statistical methods

Statistical analyses were performed using IBM SPSS Statistics Software, version 21.0 for Windows (IBM Corporation, IL, USA). Owing to the small sample size, nonparametric tests (Kolmogorov–Smirnov, Kruskal–Wallis) have been applied for the demographic and clinical characteristics (Table 1). All data are presented as median with their corresponding interquartile range (IQR) unless stated otherwise. Differences between groups were assumed significant for p-values < 0.05.

Multiple linear regression with the use of stepwise backward elimination was performed in order to assess and correct for the impact of potential non-genetic confounders. A codominant inheritance pattern was used for the -900G>A SNP in the linear regression. Next to the *UGT2B7* SNP, gestational age (GA), postnatal age (PNA), gender, weight and time required for intubation were also forced into the model. The latter independent variable was included since morphine was administered before the intubation and the time required for intubation varied among the newborns and thus might have caused differences in morphine concentration at 20 min after the end of the intubation. The independent variables were checked for multicollinearity. The variance inflation factor did not exceed the threshold of 3, excluding multicollinearity between the independent variables GA, PNA, weight and time required for intubation.

RESULTS

Seventeen preterm infants randomized to morphine premedication were included in this candidate gene pilot study. All patients were successfully genotyped for the selected *UGT2B7* SNP; four patients were genotyped as -900G/G, six -900G/A and seven -900A/A. The frequency of the G allele (29%) in this cohort deviated from the frequency described in the literature for Caucasians (50%) [30], which could be caused by the relatively small population. Nevertheless, the allelic distribution met the Hardy–Weinberg equilibrium ($\chi 2 = 1.3$; $p = 0.26$). SNP 802T>C analysis confirmed the complete LD between these

genetic variations. All patients genotyped as -900G/G were also 802T/T, while all infants with the -900A/A genotype were carrying the 802C/C alleles (results not shown). Two newborns, both -900G/G carriers, were excluded from the analysis because of detectable morphine and M6G concentrations at baseline. The characteristics of the remaining 15 newborns are shown in **Table 1**.

From the 15 patients that were eventually included in the analysis, nine newborns, all A allele carriers at position -900, received additional morphine injections and/or infusion after the premedication. Seven infants received one additional morphine injection, one four additional morphine injections followed by a morphine infusion and one infant received only a morphine infusion. Both infants who received additional morphine as an infusion were -900A allele homozygous. Additionally, although not statistically different, the -900A/A and G/A infants had more additional morphine injections compared with G/G patients (0.5; 0–1.75 and 1.00; 0–1.25 vs n = 1.00 morphine injection for both infants, respectively). The median time required for intubation was longer in the two G/G genotyped patients (47.0 and 502 s) compared with the A heterozygous and homozygous patients (53.0, IQR: 43.0–193; and 249, IQR: 49.0–451 s, respectively), but this difference was not significant (p = 0.25).

Genetic association with *UGT2B7* SNP

The morphine, M3G and M6G plasma concentrations in relation to the SNP -900G>A are displayed in Figure 1 and the metabolic ratios (MRs) for morphine are shown in Figure 2. After correction for confounding factors in the multiple linear regression, morphine plasma concentrations at 20 min post intubation were significantly different between the G/G (180 and 363 ng/ml), G/A (145 ng/ml; 107–173 ng/ml) and A/A (122 ng/ml; 84.0–146 ng/ ml) genotyped newborn infants (p = 0.036). Also PNA was correlated with morphine concentrations (p = 0.017), whereas higher levels were found with decreasing PNA. The model with the genetic variation in *UGT2B7*, PNA and time needed for intuba-

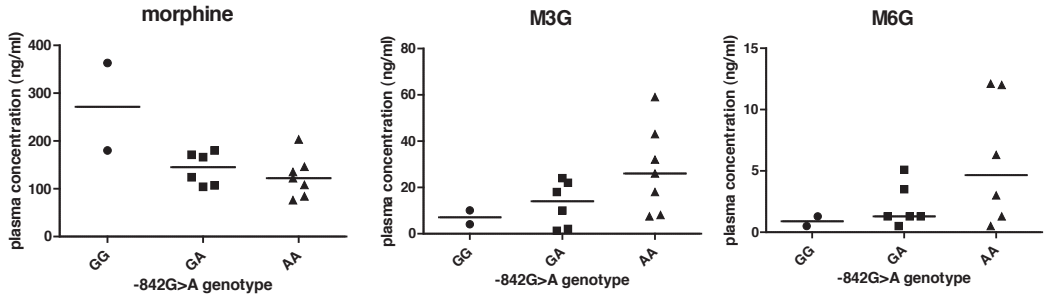

Figure 1. Morphine, morphine-3-glucuronide and morphine-6-glucuronide plasma concentrations stratified by *UGT2B7*–900G>A genotype.

M3G: Morphine-3-glucuronide; M6G: Morphine-6-glucuronide.

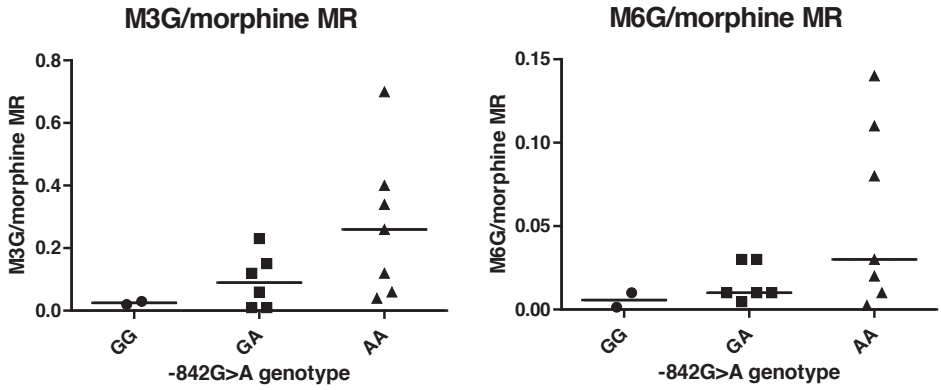

Figure 2. Morphine-3-glucuronide:morphine and morphine-6-glucuronide:morphine metabolic ratio stratified according to *UGT2B7*–900G>A genotype.
M3G: Morphine-3-glucuronide; M6G: Morphine-6-glucuronide; MR: Metabolic ratio.

tion explained 63.3% (adjusted 53.3%) of the variability observed in morphine plasma concentration.

Although the M3G plasma concentration was lower in -900G allele homozygous newborns (4.1 and 10 ng/ ml) compared with the G/A (14 ng/ml; 1.9–23 ng/ ml) and A/A (26 ng/ml; 8.1–43 ng/ml) genotyped patients, this difference was not significant after correction (p = 0.28). Conversely, GA (p = 0.007) and PNA (< 0.0001) were strongly associated with the M3G plasma concentration and accounted for 80.6% (adjusted 77.3%) of the observed variability. Also with regard to M6G plasma concentrations no significant differences have been found between G/G (0.50 and 1.3 ng/ml), G/A (1.3 ng/ml; 1.1–3.9 ng/ml) and A/A (4.0 ng/ml; 1.3–14 ng/ml) allele carriers (p = 0.20). M6G plasma concentrations were also found to be related with the GA and PNA (p = 0.034 and 0.001, respectively) but also with the intubation time (p = 0.028). These three independent variables explained 83.7% (adjusted 79.2%) of the variability in M6G plasma levels.

The MRs for morphine are shown in Figure 2. All included independent variables (GA, PNA, weight, gender, intubation time and -900G>A), were significantly associated with the M3G:morphine MR (p = 0.001, p < 0.001, p = 0.003, p = 0.02, p = 0.005 and p = 0.005, respectively). This model explained 97.5% (adjusted 95.6%) of the variability in M3G:morphine MR between newborns. An allele dose-effect was observed between the variation -900G>A and M3G:morphine MR. With regard to the M6G:morphine MR at 20 min post intubation, only a trend was observed for -900G>A (p = 0.071). Also here, GA, PNA, weight and intubation time were significantly correlated to M6G:morphine MR (p = 0.003, p < 0.001, p = 0.015 and p = 0.009, respectively). These variables combined contributed to 94.1% (adjusted 91.0%) of the observed variability in M6G:morphine MR.

DISCUSSION

We have demonstrated an association between the *UGT2B7* polymorphism -900G>A (-842G>A) and morphine PK after administration of a single dose of morphine in preterm newborns prior to tracheal intubation for mechanical ventilation. To our knowledge, this is the first study assessing the role of genetic variation in *UGT2B7* on morphine PK in this specific population. Carriers of the -900A allele had significantly lower morphine plasma concentrations compared with -900G homozygous patients, as determined using backward linear regression analysis, suggesting that the conversion from G to A most likely leads to an increased UGT2B7 activity. This assumption is confirmed in the MR analysis, where A allele carriers had a higher M3G:morphine MR, again using backward linear regression analysis. Because of the small number of subjects, we also performed forward linear regression analysis to verify our results. The lower morphine concentrations in -900A allele carriers was confirmed with this approach (p = 0.017), but the effect on M3G:morphine MR was now not significant (p = 0.12).

Our findings are in line with the results found in adults with sickle cell disease, in which a reduced formation of morphine glucuronides was observed in -900G allele carriers [15]. However, there are also findings that are contradictory [16]. For the genetic variation 802T>C, which is in complete LD with the -900G>A mutation, it was found that 802C/C genotyped patients had significantly lower morphine glucuronide levels. We have confirmed the existence of the complete LD in our preterm infants. Owing to the existence of complete LD between these SNPs, this would mean that -900A genotyped patients should have lower metabolite levels.

In contrast to the association found for morphine plasma concentrations and M3G:morphine MR, no significant associations were observed for M6G concentration or MR. This could be due to the fact that the formation of M6G is catalyzed by UGT1A1 to a great extent, and to a lesser extent by UGT1A8 instead of solely UGT2B7 [31]. The relative contribution of UGT1A1 and UGT1A8 next to UGT2B7 has been recently assessed in an adult population with advanced cancer [32]. Two haplotypes for UGT1A1/1A8 were weak predictors of M6G:morphine and M3G:morphine MRs, whereas *UGT2B7* haplotypes were not associated. Our study had insufficient power to also assess variations in these genes.

Contradicting results exist regarding the affinity of M6G for the μ-opioid receptor compared with morphine, although in vivo results have shown up to fourfold higher analgesic potency for M6G [33]. Studies assessing the relationship between morphine and M6G concentrations with the analgesic and side effects of the drug have also showed inconclusive results [34,35]. Our data speculate that -900A allele carriers with lower morphine plasma concentrations will experience lower morphine potency compared with G/G genotyped patients, due to the requirement of additional morphine. Namely, all homozygous A genotyped patients required rescue morphine and both infants with ad-

ditional infusion were genotyped -900A/A. Considering the small cohort it is necessary to determine if this effect on PK will indeed influence the morphine requirement, a clinically relevant outcome.

In spite of a low glucuronidation activity during the neonatal period, large interindividual variability is observed in newborn infants [36]. This variability is the consequence of an interplay between genetic and non-genetic factors, whereas GA and PNA seem to play central roles. Our findings indicate that in addition to these non-genetic factors, genetic variation in UGT2B7 is also likely to explain the differences in morphine PK. Our results suggest that the genotype–effect association is not confounded by ontogeny. Although the GA of the -900G/G genotyped patients is lower compared with the GA of -900A allele carriers, inclusion of this variable in the multiple linear regression still showed the genetic effect on morphine PK.

One limitation of this clinical study, which is common when recruitment is performed in urgent situations, is that few infants were included. The fact that two infants had to be excluded because of detectable morphine and metabolite concentrations in plasma underscores the variability in PK. These infants had received their previous dose more than 24 h before recruitment. This emphasizes that morphine elimination is very slow in preterm infants, in particular in -900G/G genotyped patients, which is in line with our findings, and illustrates that children with a recent prior history of morphine administration should be excluded. However, despite the small cohort we have observed an association between genetic variation in *UGT2B7* and morphine PK, which was significant after correction for confounding factors.

CONCLUSION & FUTURE PERSPECTIVE

Large interindividual variability exists in morphine plasma concentrations in the preterm population. We have shown that the polymorphism -900G>A, leading to altered UGT2B7 activity, affects morphine PK in the immature and growing population. Premature infants that carry the -900A allele have an increased breakdown of morphine. Our study illustrates that the observed consequence of this polymorphism in adults is also visible in preterm infants, implying that genetics might play a role next to ontogeny in drug metabolism.

Since these results are based on small numbers of infants (n = 15), and we only had two *UGT2B7*–900G/G subjects, these findings warrant further validation in a larger cohort. The following step would be to assess in a prospective setting whether carriers of the -900A allele have a worse analgesic response and thus require additional morphine doses when in need of this drug during mechanical ventilation. Since large variability is seen in the genotype groups, the final goal would be to determine what the relevance of this SNP is next to other *UGT2B7* SNPs and polymorphisms in other genes affecting the morphine pathway.

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SLC22A1
COMT
UGT2B7

Chapter 8

SLC22A1/OCT1 Genotype Affects O-desmethyltramadol Exposure in New-Born Infants

Maja Matic
Saskia N. de Wildt
Laure Elens
Jan N. de Hoon
Pieter Annaert
Dick Tibboel
Ron H.N. van Schaik
Karel Allegaert

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ABSTRACT

Background: This study determined whether the *SLC22A1* [encoding the organic cation transporter 1 (OCT1)] genotype could explain, in addition to the postmenstrual age (referring to gestational plus postnatal age) and *CYP2D6* genotype, the tramadol (M) pharmacokinetic variability in early infancy.

Methods: Fifty infants, median postmenstrual age 39.5 (interquartile range: 36.8–41.3) weeks, received an i.v. M loading dose (2 mg/kg) followed by a continuous infusion (5–8 mg·kg·24 h). Blood was sampled from 4 to 24 hours after start of the M treatment, which generated 230 observations. M and O-desmethyltramadol (M1) concentrations were measured by high-performance liquid chromatography.

Results: Linear mixed-model analysis illustrated that the *SLC22A1/OCT1* genotype was independently associated with a log-transformed M1/M ratio (P = 0.013), with carriers of < 2 *SLC22A1/OCT1* functional gene copies having a higher M1/M ratio (2.25; 95% CI, 2.01–2.48) than infants with 2 functional gene copies (1.86; 95% CI, 1.66–2.06). The *CYP2D6/SLC22A1* combined genotype was associated with 57.8% higher M1/M ratio in carriers of ≥ 2 *CYP2D6* functional gene copies and < 2 *SLC22A1/OCT1* functional gene copies compared with infants with < 2 active *CYP2D6* functional gene copies and *SLC22A1/OCT1* normal activity (P < 0.001).

Conclusions: These findings highlight the additional role of *SLC22A1/OCT1* genetics in M1 exposure in neonates. They also suggest that OCT1 is already active early after birth, which may have impact on the disposition of other OCT1 substrates in this population.

INTRODUCTION

Critically ill infants commonly receive opioids for alleviation of postsurgical pain or pain/distress provoked during stressful procedures in the neonatal intensive care unit. The dosing of opioids is ideally guided by regular assessment of pain with the use of validated pain scores [1]. Tramadol (M), as one of the opioids used in the treatment of pain, has a relatively wide therapeutic range and low incidence of adverse events [2]. Nonetheless, a large variation in pharmacokinetics (PK) of M is observed in the neonatal population, which is mainly caused by the extensive maturational changes during the neonatal period [3]. The ultimate goal of infant pain therapy for the future would therefore be to individualize pharmacotherapy based on age, weight, disease-status, co-medication and genetics.

M is metabolized in the liver and converted into its active metabolite, O-desmethyltramadol (M1) by the cytochrome P450 enzyme 2D6 (CYP2D6) and into its inactive metabolite N-desmethyltramadol (M2) by CYP3A4 and CYP2B6 [4]. M1 is formed more extensively compared to M2, although their relative formation will also depend on the genetic variability of the metabolizing enzymes [4,5]. M1 has a higher affinity (Ki = 3.4 nM) for the mu-opioid receptor compared to the parent compound (Ki = 2.4 μM) [6], highlighting the importance of M conversion by CYP2D6. The relevance of CYP2D6 genetics for M has been established in the adult population [4, 7, 8] and in neonates [9], implicating decreased concentrations of the active metabolite and an absence of analgesia. However, the variation in disposition of M extends beyond this conversion in M1. After demethylation, M1 is either transported from the hepatocyte into the central circulation via an unidentified transporter or further metabolized by the phase II UDP-glucuronosyltransferase 2B7 (UGT2B7) enzyme [4], followed by renal elimination. M1 can also be transported from the blood/plasma compartment back into the hepatocyte by the organic cation transporter (OCT1). OCT1, encoded by the solute carrier family 22 member 1 (*SLC22A1*) gene, is highly expressed on the sinusoidal membrane of hepatocytes in humans [10, 11] and is responsible for the uptake of positively charged molecules, such as metformin, acyclovir and irinotecan [12]. A transporter is crucial for M1 to cross cell membranes since removal of the methyl group from M by CYP2D6 exposes a hydroxyl group, thereby decreasing the membrane permeability of M1 [13].

Loss-of-function *SLC22A1*/OCT1 polymorphisms have been associated with increased M1 exposure in adult volunteers receiving single oral dose of M [13]. In addition to the adult data, these *SLC22A1*/OCT1 polymorphisms have been associated with a 20% morphine clearance reduction in 146 children (6–15 years) undergoing adenotonsillectomy [14]. However, as illustrated in two recently published reviews the relevance of *SLC22A1*/OCT1 genetics has been assessed in a wide age-range of populations except the neonatal population [15, 16]. Immaturity of OCT1 activity in neonates may obscure a genotype-specific effect. Should OCT1 activity in neonates be at or close to adult levels, *SLC22A1*/

OCT1 genotype might add to the variation in M disposition and would have consequences for individualized M dosing in neonates. To date, no data exist on OCT1 maturation, therefore this knowledge also may add to our understanding on the interplay of *SLC22A1*/OCT1 genotype and age. This information could be extrapolated for the dosing of other OCT1 substrates that are used in the neonates [16] and for which associations have been found with *SLC22A1*/OCT1 genetics in adults. These OCT1 substrates with illustrated genotypic effect in adult data include morphine [14, 17], tropisetron/ondansetron [18] and imatinib [19] Additionally, all drugs positively charged at pH 7.4 (weak bases with pKa> 8) are potential OCT1 substrate candidates [20].

Although the assessed genetic variants are not highly prevalent or even absent in specific Asian and African-American populations, approximately 8% of the European population and up to 87% of individuals with South American Indian background have two non-functional gene copies [21]. These individuals are genetically predisposed to complete lack OCT1 activity. Moreover, the previously discussed study in healthy adults illustrated that the extent of the effect per 1 *SLC22A1*/OCT1 non-functional gene copy on M1 plasma concentrations is roughly in the same range as that with one *CYP2D6* non-functional gene copy [13]. This observation deserves to be validated as the current CYP2D6 genotype-guided dosing recommendations for M may have to be updated to also include *SLC22A1*/OCT1 genotype. A recent review highlighted *SLC22A1*/OCT1 as a relevant candidate gene, in addition to CYP2D6, for M metabolism and clinical response [8]. Therefore the present study aimed to determine if any effects of *SLC22A1*/OCT1 genotype on M1 levels are observable in the first months of postnatal life.

PATIENTS AND METHODS

This is a retrospective analysis of data on 50 (pre)term critically ill neonates (1–28 days) and infants (29 days – 5 months) treated with intravenous M for pain relief [9]. The aim of the primary and previously performed study was to determine clinical and genetic predictive factors for the observed variability of M PK in this population. Local IRB approval (registration number ML3791) was received from the University Hospital Leuven, Belgium and national approval (registration number B32220071629) from the 'Federaal Agentschap voor Geneesmiddelen en Gezondheidsproducten', Belgium. Written informed consent for deoxyribonucleic acid (DNA) and PK analysis was obtained from parents of eligible neonates and infants.

Study design

Infants who underwent a surgical procedure either with or without mechanical ventilation and non-surgical children on respiratory support who required M treatment were eligible.

Ventilated infants who underwent a surgical procedure received fentanyl (loading dose 3–5 µg/kg, infusion 3 µg/kg/h) followed by add-on M (loading dose 2 mg/kg, infusion 5–8 mg/kg/24 h) and propacetamol (4 times daily 10–20 mg/kg/24 h) within 24 hours post-operatively. Non-ventilated post-surgical infants received only continuous M with add-on propacetamol. Non-surgical children on respiratory support received fentanyl or M based on the standard procedures on analgesia and sedation in the Leuven Neonatal Intensive Care Unit (NICU) [22]. During analgesia with M known CYP2D6 and OCT1 inhibitors or inducers were not administered. If an arterial or venous line was available, 200 µL blood was collected at multiple occasions between 4 and 24 hours after initiation of M bolus. As shown previously, the M and M1 plasma concentrations were stable in this time window [9]. As the half-life of M in neonates is approximately 4 hours and steady state is not reached, the loading dose might have filled the gap until steady state. The number of blood collections and the timing of sampling depended on the child's weight (max 1 ml/kg/study) but blood sampling from a central venous catheter for study purposes was performed only in conjunction with collection of samples for clinical indication. Every 24 hours the Intensive Care (IC) team was allowed to alter the continuous perfusions in line with the child's analgesic needs. M and M1 plasma concentrations were determined by high performance liquid chromatography (HPLC) according to the method described previously [23, 24].

Genotyping

The DNA analysis was performed at the Department of Clinical Chemistry, Erasmus University Medical Centre Rotterdam, Netherlands. The methods of DNA extraction from ethylenediaminetetraacetic acid whole blood and *CYP2D6* genotyping have been described previously [9]. The selection of assessed *CYP2D6* variants (*3, *4, *5, *6, *9, 10, *41 and XN) covers the most frequent polymorphisms in the Caucasian population. Analysis of the *SLC22A1*/OCT1 genetic variants Met420del (rs72552763), Arg61Cys (rs12208357), Gly401Ser (rs34130495), Gly465Arg (rs34059508) and Cys88Arg (rs55918055) was performed on the ABI PRISM® 7500 Real-Time PCR system (Applied Biosystems®, Bleiswijk, Netherlands). The *SLC22A1*/OCT1 haplotype (expressed as Normal Function, Intermediate Function and Poor Function) consisting of these genetic variants was estimated with the haplo.stats package (R, version 3.1.1), which uses the expectation-maximization logarithm and a posterior probability > 0.98. Violation of Hardy-Weinberg equilibrium was tested for each individual genetic variant with the Chi2 test, leading to removal from the statistical analysis if p-values < 0.05. In addition, the observed minor allele frequency (MAF) was compared with the frequency reported in the literature for Europeans [21]. The assay used was validated by direct sequencing of wild type, heterozygote and (if available) homozygote samples. Five percent of samples were reanalyzed to check data consistency.

Due to low frequencies in the extreme CYP2D6 phenotypes, children with Poor Metabolizer (PM) and Intermediate Metabolizer (IM) phenotypes were analyzed combined as the 'low activity' group versus Extensive Metabolizer (EM) and Ultra-rapid Metabolizer (UM) phenotype in the 'normal activity' group. Infants with 0 (poor function) and 1 (decreased function) functional gene copy of *SLC22A1*/OCT1 were analyzed combined as the OCT1 'low activity' group against 2 *SLC22A1*/OCT1 functional gene copies (normal function) defined as the 'normal activity' group. In addition to the genotypes of the separate genes also the combined genotypic effect was assessed. Combinations were made based on the theoretical background, where CYP2D6 'low activity' was combined with OCT1 'normal activity' (lowest M1 plasma concentrations expected), CYP2D6 'low activity' with OCT1 'low activity', CYP2D6 'normal activity' with OCT1 'normal activity' and CYP2D6 'normal activity' with OCT1 'low activity' (highest M1 plasma concentrations expected).

Statistical analysis

All statistical analyses were conducted using IBM SPSS Statistics (software version 21.0 for Windows; IBM Corporation, IL, USA). Unless stated otherwise, data are reported as median with corresponding interquartile range (IQR). Violation of normal distribution was judged visually and mathematically with Shapiro-Wilk and skewness as well as kurtosis value. Depending on the shape of the distribution, parametric or non-parametric tests were performed accordingly, for (Log)-Gaussian or non-Gaussian distributions, respectively.

As our data consists of multiple measurements per subject a restricted maximum likelihood (REML) linear mixed-model analysis was performed to assess the relation between *CYP2D6* and *SLC22A1*/OCT1 genotypes with M1/M metabolic ratio, with time of sampling as repeated measurements. The metabolic ratio M1/M of the measured concentrations was the dependent variable. Sampling had been performed at 90 different time points between 4 and 24 hours after start infusion. In order to simplify the software iteration process in the linear mixed model and to run properly, the time points were rounded to the nearest half hour. The autoregressive structure was chosen for the repeated covariance matrix since the further M1/M measurements within the subject were separated the less correlated they were. PMA and subject ID were entered as random factors in the model, while *CYP2D6* and *SLC22A1*/OCT1 genotypes were set as fixed factors (without interaction term).

RESULTS

The 50 children included in the analysis had a median postmenstrual age (PMA) of 39.5 weeks (range 27–54 weeks), with 14 (28%) born premature. Forty five children have a Caucasian ethnicity, whereas 5 individuals have Mediterranean backgrounds (Tunisia,

Table 1. Clinical characteristics of the cohort

	N = 50
Postmenstrual age (weeks)	39.5 [36.8–41.3]
Gestational age (weeks)	37.7 [35.4–39.0]
Postnatal age (days)	7.0 [2.0–27]
Preterm (%)	28
Weight (kg)	3.05 [2.34–3.51]
Ethnicity	
Caucasian (n)	45
Non-Caucasian (n)	5
Tramadol plasma concentration (µM)	1.55 [1.07–2.19]
O-desmethyltramadol plasma concentration (µM)	0.201 [0.112–0.345]

**Values are displayed as median [interquartile range], unless stated otherwise.*

Morocco). The most frequent reason for M administration was postoperative analgesia. The surgical indications included congenital diaphragmatic hernia, congenital oesophageal or duodenal atresia, coarctation of the aorta, or other cardiac surgical procedures such as banding of the pulmonary artery. See **Table 1** for a summary of other patient demographics. For more background information of the patients see previous publication [9]. Data on 230 plasma samples collected between 4 and 24 hours after initiation of M dose were available. An overview of the M and M1 plasma concentrations is also reported in **Table I**.

Steady state (4 hours after M loading dose was started) was confirmed by lack of correlation between M or M1 plasma concentrations and the time of sample collection (rs=-0.002, p = 0.97 and rs=-0.033, p = 0.62). As expected a strong positive correlation was confirmed between PMA and weight (r = 0.866, p < 0.001), resulting in exclusion of weight from the mixed model to avoid any collinearity problem in the final model. Although weaker, weight was also correlated to gestational age (GA) and postnatal age (PNA) (rs = 0.648, p < 0.001 and rs = 0.419, p < 0.002). PMA and PNA were strongly, negatively correlated with M concentration (rs=-0.689; p < 0.001 and rs=-0.745; p < 0.001), while the correlation between these variables with M1 was in the opposite direction and weaker (rs = 0.399; p < 0.001 and rs = 0.226; p = 0.001). Regarding the M1/M metabolic ratio (MR), the strongest correlation was observed with PMA (rs = 0.676; p < 0.001). One subject was identified as an extreme outlier (significant with Grubbs' test) due to high M1 concentrations (1.89–2.29 µM) and low M concentrations (0.0418–0.414 µM). This large deviation could not be explained by extreme low/high age (GA 40.9 weeks; PNA 1 day), weight (3.26 kg), genotype (1 *SLC22A1*/OCT1 functional gene copy and 1 *CYP2D6* functional gene copy) or clinical diagnosis (pneumothorax). Since the data of this subject were considered to have a too large influence on the results, they were removed from further analysis.

Genotype results

Table 2 shows a summary of the *CYP2D6* and *SLC22A1*/OCT1 genotype results. The observed MAFs for *SLC22A1*/OCT1 SNPs Met420del (rs72552763), Arg61Cys (rs12208357), Gly401Ser (rs34130495), Gly465Arg (rs34059508) and Cys88Arg (rs55918055) are in line with previously reported frequencies for a European population [21]. Carriers of the genetic variant Cys88Arg (rs55918055) were not found in our cohort. In total, the number of individuals with 2 inactive *SLC22A1*/OCT1 alleles was 4%, where Tzvetkov *et al.* [13, 18] reported 9–12%. However, statistical testing shows that our frequency does not significantly deviate from the numbers reported by Tzvetkov *et al.* (p = 0.52). As shown previously, the CYP2D6 polymorphisms were also in agreement with MAFs reported in the literature [9]. None of the polymorphisms violated HW-equilibrium (p < 0.05). More information is provided in Supplementary Table 1.

Table 2. Overview genotype results of the cohort

		CYP2D6 functional gene copies (n)					
		0	**0.5**	**1**	**1.5**	**2**	**> 2**
***SLC22A1* functional gene copies (n)**	**0**	-	-	1	-	1	-
	1	-	-	8	6	7	-
	2	1	2	11	3	8	2

The linear mixed model analysis illustrated that both *CYP2D6* and *SLC22A1*/OCT1 genotypes were significantly associated with the log M1/M MR (p = 0.001 and p = 0.013). As previously described, infants carrying fewer than 2 functional *CYP2D6* copies had lower log M1/M MR (1.76; 95%CI 1.58–1.94) than infants with 2 or more functional gene copies (2.35; 95%CI 2.09–2.61). For the *SLC22A1*/OCT1 genotypes the opposite effect was found as carriers of 1 or zero functional gene copies had higher M1/M MR log (2.25; 95%CI 2.01–2.48) than carriers of 2 functional gene copies (1.86; 95%CI 1.66–2.06). **Figures 1** and **2** illustrate the effect of *CYP2D6* and *SLC22A1*/OCT1 genotype on the mean log M1/M per subject, with the colors indicating the PMA of the child.

After combining the *CYP2D6* and *SLC22A1*/OCT1 genotypes, the association with the M1/M ratio became highly significant (p = 7.51*10–27). While the lowest M1/M MR log was observed in infants carrying fewer than 2 functional *CYP2D6* copies in combination with normal *SLC22A1*/OCT1 genotype (1.52; 95%CI 1.27–1.76), the highest M1/M MR log was observed in subjects carrying 2 or more functional *CYP2D6* copies combined with 1 or zero *SLC22A1*/OCT1 functional gene copies (2.40; 95%CI 2.00–2.81). Although the geometric means of the M1/M MR were different, the two other groups (< 2 active *CYP2D6* functional gene copies and 2 *SLC22A1*/OCT1 functional gene copies vs. 2 functional *CYP2D6* copies and 2 *SLC22A1*/OCT1 functional gene copies) had marginally

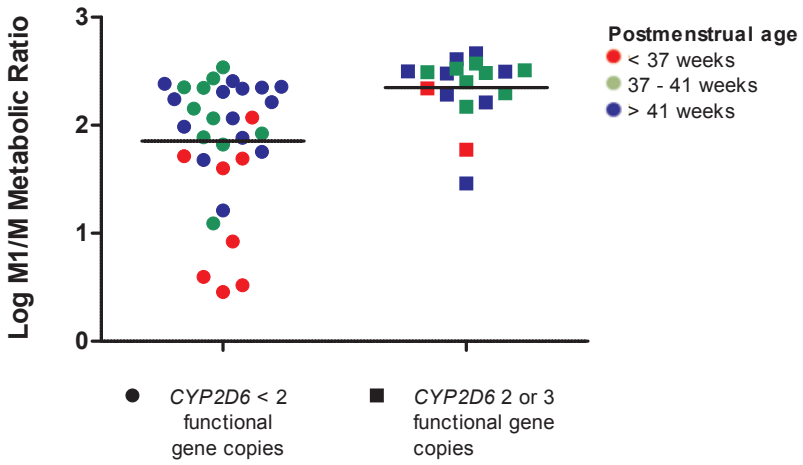

Figure 1. MR O-desmethyltramadol (M1)/tramadol (M) in relation to *CYP2D6* 2 or more functional gene copies versus *CYP2D6* < 2 functional gene copies.

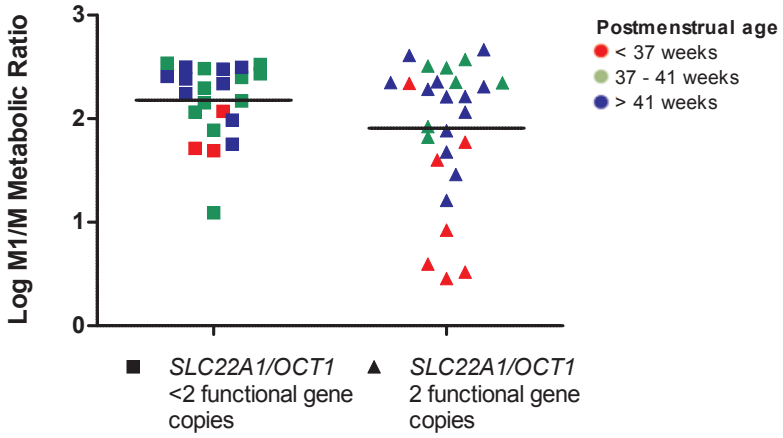

Figure 2. MR O-desmethyltramadol (M1)/tramadol (M) in relation to *SLC22A1*/OCT1 2 functional gene copies versus *SLC22A1*/OCT1 < 2 functional gene copies.

more overlap in values (2.02; 95%CI 1.74–2.30 vs. 2.25; 95%CI 1.91–2.58). The effect of the combined genotypic groups on the M1/M MR with the PMA of the child are shown in Figure 3. When the M levels were not taken into consideration *CYP2D6* was significantly associated with M1 concentrations (p = 0.019), whereas *SLC22A1*/OCT1 showed only a trend with M1 concentrations (p = 0. 06).

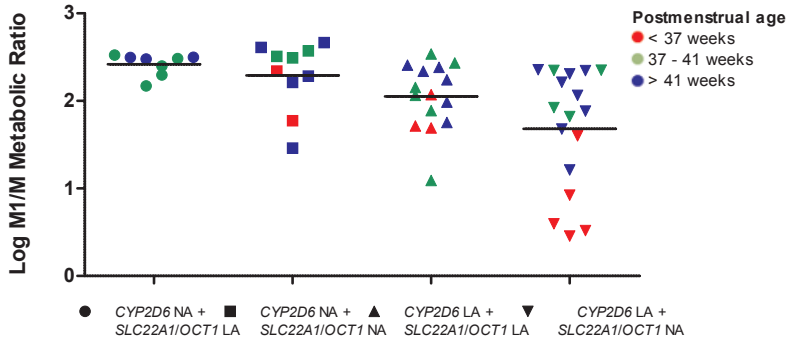

Figure 3. MR O-desmethyltramadol (M1)/tramadol (M) in relation to the combined *SLC22A1*/*CYP2D6* genotype. LA, < 2 functional gene copies; NA, 2 (or more in the case of *CYP2D6*) functional gene copies.

DISCUSSION

Our data suggest that OCT1 activity in early infancy is at a level that can be affected by genotype. The subjects in this study who carried one or no functional *SLC22A1*/OCT1 gene copies had a higher M1/M ratio, which is indicative of higher plasma concentration of the active metabolite M1. This is in line with the role of OCT1 as a transporter of substrates into the hepatocyte. These findings also confirm the relationship between *SLC22A1*/OCT1 polymorphisms with M1 exposure observed in vitro and in healthy adult volunteers [13].

Recently OCT1 protein expression in human livers from organ donors was found to be stable in a cohort ranging from 9 to 70 years of age [25]. According to recent reviews, data on the ontogeny of human OCT1 activity are still lacking [15, 16]. As *SLC22A1*/OCT1 genotype affected M disposition in our study population, this suggests that OCT1 matures very soon after birth, in both term and preterm infants. Our data are also indirectly showing this 'early' impact of *CYP2D6* genotype on activity. In vivo data have shown a *CYP2D6* genotype-phenotype correlation after 2 weeks PNA [26]. Stevens *et al.* reported no detectable CYP2D6 activity in up to 87% of the samples in the first and second trimesters, while the undetectable activity ranged 29–38% for the third trimester until 1 week of postnatal age. After the first post-partum week, CYP2D6 activity could not be detected in 13% of the liver samples, reflecting the *CYP2D6* PM frequency in a Caucasian population [27].

CYP2D6 genotype-guided dosing recommendations are available for M [28], i.e. decreasing the dose to 30% in UMs and use of an alternative analgesic is suggested for PMs. Pre-emptive genotyping for CYP2D6 could be considered in specific populations (e.g. breastfeeding mothers, children undergoing tonsillectomy for obstructive sleep apnea syndrome (OSAS)) with an increased risk for adverse events. Adverse events such as severe respiratory depression induced by opioids should be prevented at all costs, especially in the vulnerable critically ill neonatal population. As not all *CYP2D6* UMs will develop severe

respiratory depression, other factors such as kidney function, co-morbidity and *SLC22A1*/OCT1 genetic variants could potentially increase this risk. Particularly simultaneous occurrence of 3 or more functional *CYP2D6* gene copies with carriage of two inactive *SLC22A1*/OCT1 gene copies might increase the susceptibility to adverse events because of a combination of increased M1 formation and a decrease in OCT1-mediated hepatocellular uptake of M1 from the central compartment for subsequent elimination.

Severe respiratory depression in a 5-year-old child undergoing adenotonsillectomy for OSAS after 20 mg oral M was recently reported [29]. This adverse event, which is in general less prominent with M use than with use of other opioids, was not caused by renal impairment but most likely by the combination of underlying respiratory illness with the presence of three functional *CYP2D6* copies. Since *SLC22A1*/OCT1 genotype was not reported here, it would be of high relevance to assess in future studies the relevance of carrying two loss-of-function *SLC22A1*/OCT1 copies in *CYP2D6* ultra-rapid metabolizers experiencing adverse events. We were unable to analyze what the effect would have been on M1 plasma concentration for this specific group because this genotypic combination was not found in our population.

Several limitations of this study need to be addressed. Firstly, body weight was excluded from the mixed model due to collinearity between this variable and PMA. We chose PMA as the preferred parameter as for most critically ill neonates, weight is not measured frequently enough to reflect accurate weight at the time of M administration. Second, because the initial study design focused on pharmacokinetics of different and multimodal analgesics, we were unable to address the relationship between *SLC22A1*/OCT1 genotype and clinical outcomes such as pain scores, dose required for sufficient analgesia or occurrence of adverse events. Although our findings support further research on the pharmacodynamics and *SLC22A1*/OCT1 genetics, at this stage it is not possible to implement this information in the clinical setting when dosing neonates. Theoretically we would expect the requirement of lower M doses in neonates carrying loss-of function *SLC22A1*/OCT1 alleles due to increased exposure.

CONCLUSION

Our study emphasizes that in addition to PMA and *CYP2D6* genetics, carriage of loss-of-function *SLC22A1*/OCT1 polymorphisms explains additional variability in M PK in the developing pediatric population. Genotyping of *CYP2D6* and *SLC22A1*/OCT1 might shed light on unexplained side effects of M. Future research with larger sample sizes should validate prospectively the effects of *CYP2D6* and *SLC22A1*/OCT1 genotypes on clinical outcomes before implementing genotyping of CYP2D6 and OCT1 pre-emptively in clinical practice. Besides, the genetic component should be expanded in larger studies

by addressing the additional value of polymorphisms in other important genes such as drug metabolizing enzymes (*CYP2B6, CYP3A4* and *UGT2B7*), transporters (*ABCC3*) and opioid pharmacodynamics related genes (*OPRM1*). This will increase our understanding of the genetic make-up and its influence on the M detoxification process. Our study suggests that OCT1 is already active soon after birth, but, however, does not provide any information on the development of this expression. Our findings may help understand the variation in disposition of other OCT1 substrates in neonates, in addition to ontogenetic studies that need to be performed.

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SLC22A1
COMT
UGT2B7

Chapter 9

Rescue Morphine in Mechanically Ventilated Newborns Associated with Combined *OPRM1* and *COMT* Genotype

M. Matic
S.H. Simons
R.A. van Lingen
J. van Rosmalen
L. Elens
S.N. de Wildt
D. Tibboel
R.H.N. van Schaik

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ABSTRACT

Aim: Determine whether SNPs of *OPRM1* 118A>G (asn(40)asp), *COMT* 472G>A (val(158)met) and *ARRB2* 8622C>T are associated with morphine rescue in newborns on mechanical ventilation.

Materials & methods: This is a pharmacogenetic analysis of a randomized controlled trial in (pre)term newborns (n = 64) at a level III Neonatal Intensive Care Unit (NICU) who received placebo infusion and for whom need and dose for rescue morphine was documented.

Results: For *OPRM1* and *COMT* separately, the expected risk for rescue morphine or morphine dose was not significantly increased. However, the combined *OPRM1*/*COMT* 'high-risk' genotype lead to a significant association with the need for rescue (OR: 5.12; 95% CI: 1.12–23.3; p = 0.035). No association was found between *OPRM1*/*COMT* 'high-risk' genotype and total morphine dose administered.

Conclusion: Combined *OPRM1* 118A>G and *COMT* 472G>A genotype might serve as a predictor for the need of rescue morphine in premature and term newborns on mechanical ventilation.

INTRODUCTION

Neonatal intensive care treatment is related to frequent painful procedures and continuous distress from ventilatory support [1]. Adequate analgesia is needed not only because of ethical considerations, but also because of short- and long-term negative effects of neonatal pain exposure [2]. The use of morphine in newborns has been reduced in the past years because of fear for adverse effects [3,4]. Neonates on mechanical ventilation are not routinely treated with morphine anymore in our institution, but only as needed [5–7]. Also postoperative morphine use is reduced by applying intravenous paracetamol [8].

Analgesia with morphine in newborn infants is complicated by the existence of large interindividual differences in both morphine pharmacokinetics as in analgesic requirements [6,9]. Therefore, it would be worthwhile to have objective biomarkers that may aid to personalize morphine dosing. The field of pharmacogenetics predicts individual dosing of drugs based on genetic differences affecting disposition and effect. Genetic polymorphisms in several genes have been associated with pain sensitivity and opioid response in both adults [10,11] and children [12]. More recently, genetic variation in newborns with the neonatal abstinence syndrome (NAS) caused by in utero opioid exposure has been related to treatment requirement and length of hospitalization [13].

Pain perception is amongst others modulated by μ-opioid receptor (MOR) activity in the central nervous system, encoded by the *OPRM1* gene, which is the main target for morphine. Over 700 SNPs have been described, but only few have been found relevant for analgesic efficacy [14]. One of these variants is the well-documented A118G, asn40asp (rs1799971) SNP, with functional consequences on protein expression and conformational changes [15], a relatively high variant allele frequency (VAF) of 15% in the Caucasian population [16]. In adult clinical studies, this genetic variant has been associated with reduced morphine efficacy [17–26].

Another highly investigated protein is COMT, which is involved in pain perception by regulation of MOR expression [27]. The common genetic variant 472G>A, val158met (rs4680) leads to a 3- to 4-fold reduced COMT activity due to changes in thermo-stability [28]. The 158met variant has been associated with decreased release of endogenous opioid in response to sustained experimental pain and an increase in the concentration of μ-opioid receptors in human subjects [29]. The distribution of genotype groups in the Caucasian population are 29% 472GG (val158val), 46% 472GA (val158met) and 25% 472AA (met158met) [16].

The genetic contribution in the perception of pain and the variability in opioid requirement for sufficient analgesia is most likely not explained by polymorphisms in one single gene. Potential associations between genetic factors and pain sensitivity or need for analgesia might only appear when polymorphisms from multiple genes are combined, especially when DNA variants lead to opposite clinical effects. In the adult population two

independent studies have been published that emphasize the relevance of the combined *OPRM1* A118G and *COMT* val158met genotype with analgesic response [20,30].

A third potential pharmacogenetic candidate gene in predicting morphine requirement is *ARRB2*, encoding the β-arrestin 2 protein. MOR signaling is terminated through internalization of the receptor by β-arrestin 2 after the receptor has been phosphorylated by G-protein-coupled receptor (GPCR) kinases [31]. In ARRB2(-/-)-deficient mice, a higher potency and prolonged analgesic duration of single dose morphine has been shown [32]. A study in patients with moderate to severe cancer pain illustrated that the *ARRB2* 8622T allele (rs1045280) was more frequently found in patients that switched from morphine to another opioid [33].

In the adult population, studies have been performed with the previously mentioned pharmacodynamics (PD)-related candidate genes on morphine efficacy and toxicity [33,34]. However, the contribution of these variations on the analgesic potency of morphine in preterm newborns, who undergo major developmental changes, has not been analyzed yet. Therefore, the aim of this study is to determine the role of the *OPRM1* 118A>G, *COMT* 472G>A (val158met) and *ARRB2* 8622C>T polymorphisms on the morphine requirement in (pre) term newborns on mechanical ventilation. Morphine requirement was expressed as rescue morphine needed (yes/no) and in case of morphine administration the dose in μg/kg/h.

METHODS

This pharmacogenetic study is based on a randomized, double-blind, placebo-controlled trial in newborns investigating the value of continuous morphine, as described previously [6]. The local ethical committee approved the current study as an amendment to the original study protocol. Separate written informed consent for DNA collection and analysis was obtained from parents of eligible newborns. All newborns that required mechanical ventilation were suitable for inclusion. Additional inclusion criteria were postnatal age (PNA) < 3 days, artificial ventilation < 8 h, and an indwelling arterial catheter. Neonates with severe asphyxia (Apgar-score after 5 min of < 4 or cord blood pH < 7.0), severe intraventricular hemorrhage grade III or intraventricular hemorrhage plus apparent periventricular hemorrhagic infarction), major congenital and/or facial malformations, neuro logic disorders or continuous/intermittent neuromuscular blockers were excluded from the trial. Please see Simons *et al.* [6] for a more detailed description of the inclusion and exclusion criteria. All newborns were admitted to the level III Neonatal Intensive Care Unit (NICU) of two centers in The Netherlands (Erasmus MC – Sophia, Rotterdam and Isala Clinics, Zwolle).

The patients in the randomized controlled trial were allocated to either loading dose morphine (100 μg/kg) followed by continuous morphine infusion (10 μg/kg/h) or to continuous placebo infusion. Both groups were treated for a maximum of 7 days. Occurrence of pain in the newborn during any medical action was treated with additional open label morphine, independent of the randomization group. The rescue morphine, consisting of 50 μg/kg bolus, was followed by 5–10 μg/kg/h continuous infusion, whereas patients' pain for additional morphine was performed as described previously [6]. Because the outcome of the randomized controlled trial showed no difference in morphine requirement between the two randomization groups, our institution now refrains from using continuous morphine as standard care in mechanically ventilated newborns. For this reason, we chose to analyze the placebo arm, in order to obtain the possible additional value of pharmacogenetics in the standard of care setting in our hospital.

The primary outcome measures of this pharmacogenetic study were rescue morphine requirement as a dichotomous variable (yes/no) and total morphine amount required as a continuous variable, expressed in μg/kg/h. As a secondary outcome measure we have analyzed the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). The sensitivity of the genetic test is defined as the number of patients that required rescue morphine with the mutation divided by total number of patients that required rescue. The specificity was calculated by dividing the number of patients that did not have rescue morphine nor the mutation divided by total number of patients that did not require rescue. For the PPV of the test the patients with the mutation and rescue were divided by total number of patients with the mutation. Whereas the NPV was calculated by dividing patients without mutation and rescue by total number of patients without the mutation.

DNA isolation & genotyping

DNA was isolated using buccal brushes (Master-Amp™, Epicentre). Tissue was collected by rolling the buccal brush on the inside of the patients' cheek, approximately 20-times on each side. Brushes were stored in the original packaging at room temperature for maximally 7 days before extracting the DNA, whereas the extraction was performed according to the protocol of the manufacturer. PCR amplification was performed in 50 μl reaction volume, containing 10 ng of genomic DNA, 1× PCR Buffer II (Perkin Elmer), 1.5 mM MgCl2, 2 nM dTNPs (Roche), 1.25 U of Amplitaq Gold (Perkin Elmer) and 40 pmol each of forward and reverse primer.

To detect variation *OPRM1* 118A>G, forward primer 5′-GCTTGGAACCC-GAAAAGTCT-3′ and reverse primer 5′-GTAGAGGGCCATGATCGT-GAT-3′ were used. Amplification consisted of an initial denaturation step (7 min at 94°C), followed by 35 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C, ending with one extension cycle (7 min at 72°C). Two μl of 1:100 diluted PCR product was used as template in

a nested PCR, final volume of 50 µl, containing 1× PCR Buffer II, 1.75 mM MgCl2, 0.2 mM dNTPs 1.25 U of Amplitaq Gold and 40 pmol forward primer 5′-GCTTG-GAACCCGAAAAGTCT-3′ and reverse primer 5′-ACCGCATGGGTCGGAAACGT-3′. Mismatches (underlined) were used to create a restriction site for Psp1406I. The PCR cycle conditions were identical to those described above, except for an annealing temperature of 53°C. For restriction analysis, 10 µl of the nested PCR amplification was digested for 2 h at 37°C in a final volume of 15 µl with 10 U of Psp1406I (MBI Fermentas). For genotyping the val158met SNP of the *COMT* gene, forward primer 5′-CTCATCACCATCGAGAT-CAA-3′ and reverse primer 5′-CAGTGAACGTGGT-GTGAACAC-3′ were used. Amplification consisted of an initial denaturation step (7 min at 94°C), followed by 45 cycles, each consisting of 1 min at 94°C, 1 min at 58°C and 1 min at 72°C, ending with an extension cycle (7 min at 72°C). For restriction analysis, 10 µl from the 185 base pairs (bp) PCR amplification prod-uct was digested for 2 h at 37°C in a final volume of 15 µl with 10 U of NlaIII (New England Biolabs).

Both digested fragments were separated by electrophoresis on a 3% agarose gel with ethidium bromide staining. SNP 118A>G existed of fragments of 188 and 19 bp for the wild-type sequence, 207, 188 and 19 bp for heterozygous sequences and a single band of 207 bp for homozygous variant sequences. The fragments produced for val158met were 87, 54 and 44 bp for the wild-type sequence, 87, 69, 54, 44 and 18 bp for heterozygotes and 69, 54, 44 and 18 bp for homozygous variants. All PCR-RFLP analyses were performed in duplicate.

Polymorphism 8622C>T in the *ARRB2* gene was analyzed with the TaqMan allelic discrimination analysis. The assay, with ID number C__8718195_20, was obtained at Applied Biosystems and was analyzed on the 7000 Real-Time PCR System (software version v2.0.5; Applied Biosystems). The PCR amplification started with denaturation at 95°C for 20 s, followed by hybridization of the primers and probes at 92°C for 40 s. After hybridization the elongation was begun at 60°C for 30 s. The whole process was run for 40 cycles, in which three control samples (wild-type, heterozygous and mutant) and a blank sample has been included.

Statistics

All data were analyzed using SPSS version 21.0 for Windows. Results are shown as median values with their interquartile range (IQR) when variables violated normal distribution, which was judged using the Q-Q plot and Kolmogorov–Smirnov test. The categorical variables included in Table 1 and the Hardy–Weinberg equilibrium in the genotype frequencies are assessed with either the Pearson chi-square or Fisher's exact test, depending on the expected count in the cells. The continuous variables were analyzed either using the independent samples t-test or the Mann–Whitney U test, depending on distribution of the variable. All *p*-values in this manuscript are two-sided. The effects of the *OPRM1*, *COMT*

Table 1. Baseline characteristics of placebo-allocated newborns.

	Rescue morphine (n = 25)	No rescue morphine (n = 39)	p-value
Sex, male/female (n)	14/11	25/14	0.52
Gestational age (weeks)	28.7 (27.3–31.4)	30 (29.1–32.1)	0.14
Preterm/term (n)	23/2	38/1	0.56
Weight (g)	1165 (867.5–1478)	1410 (1050–1715)	0.083
Ethnicity, Caucasian/non-Caucasian (n)	21/4	32/7	0.84
Location, Sophia Children's hospital/Isala Clinic Zwolle (n)	13/12	26/13	0.24
Pain/sedation medication, yes/no (n)	4/21	4/35	0.70
VAS pain score, baseline	0.95 (0.50–1.75)	0.50 (0.30–1.60)	0.091
VAS pain score, average	1.74 (1.32–2.56)	1.43 (0.92–2.54)	0.29
COMFORT pain score, average	16.0 (15.0–17.6)	15.7 (14.1–16.8)	0.16
SSS/CRIB	4.0 (1.0–8.0)	2.0 (1.0–4.0)	0.061
Successful DNA analysis, yes/no (n)	19/6	31/8	0.84

All data are presented as median (interquartile range), unless stated otherwise.
CRIB: Clinical Risk Index for Babies; SSS: Surgical Stress Score; VAS: Visual Analogue Scale.

and *ARRB2* genotypes (autosomal dominant model) on the requirement of rescue morphine (yes/no) were analyzed in an univariate logistic regression model. The relationship between the combined *OPRM1* and *COMT* genotype and morphine requirement (yes/no) was also assessed using logistic regression. For the combined allelic effect the dominant model as well was used. Newborns that have either the *OPRM1* 118G risk allele and/or the *COMT* 472GG (val158val) genotype have been compared against newborns with 'low-risk' genotype, which is composed of newborns that are wild-type for the *OPRM1* SNP (118AA) and carriers of the *COMT* 472A (158met) allele. High and low risk indicates the level of pain and therefore the level of requirement for opioids.

The above mentioned logistic regression models are consisting of the following independent variables: gestational age (GA), birth weight, sex, allocation center, Visual Analogue Scale (VAS) at baseline and co-medication for analgesia and/or sedation. PNA was not included in the analysis as a confounding factor because all newborns received the randomized treatment within 72 h after birth. The VAS score at base-line was included in the regression models, since this could imply worse clinical situation of the newborn and therefore possibly confound the requirement of rescue morphine. VAS is an one-dimensional pain scale that is represented by a 10-cm line, ranging from 'no pain' at the left end to 'extreme pain' at the extreme right. This is a validated scale for self-report of pain in adults and children with an age > 5 years [35,36]. Additionally, this method is also used by the physician/nurse for the judgment of pain experience in nonverbal patients, representing the observational VAS. In our study VAS was measured at the bedside of the

patient and via recorded videotapes [6]. The chosen independent variables GA and birth weight forced into the regression model were assessed for multicollinearity, in which the analysis illustrated a variance inflation factor < 3. In neonates who have received rescue morphine, the cumulative morphine dose (μg/kg/h) in relation with the genetic variations was analyzed with multiple linear regression. The same independent variables as described previously were included in this analysis.

RESULTS

From the 150 patients included in the original study [6], parents of 133 newborns gave written informed consent for DNA analysis. From these 133 infants, 11 were lost to follow-up. This resulted in 122 newborns in the study, from which 64 newborns were allocated to placebo. Table 1 shows the baseline characteristics of the newborns that were randomized to placebo treatment. The median GA of the included patients in the placebo group without rescue morphine (n = 39) was 30.0 (IQR: 29.1–32.1) weeks, with 97.4% of the patients born preterm. The patients requiring rescue morphine (n = 25) had a median age of 28.7 (IQR: 27.3–31.4) weeks of gestation, with 92% born preterm (p = 0.14). The majority of the newborns had a Caucasian ethnicity. In total, 18% in the group without additional morphine and 16% in the 'rescue morphine' group were of non-Caucasian origin or the information on ethnicity was missing. The baseline and average values of pain scores did not differ significantly between the two groups.

The *OPRM1* 118A>G (VAF 16%), *COMT* 472G>A (VAF 40%) and *ARRB2* 8622C>T (VAF 70%) allele distributions all met the Hardy–Weinberg equilibrium (p = 0.27; p = 0.73 and p = 0.20, respectively) and the observed VAFs were comparable with those reported by the National Center for Biotechnology Information (NCBI) [16]. In 16 (25%) newborns one of the SNP assays failed, probably due to poor quality DNA, with no significant differences between groups (**Table 1**). Since only to patients have been genotyped 8622CC for the *ARRB2* gene this SNP was omitted from the statistical analysis. With regard to the combined *OPRM1/COMT* genotype, 27 newborns had the 'high-risk' genotype and 24 newborns the 'low-risk' genotype.

Relation *OPRM1* & *COMT* with morphine rescue & dose

Although carriers of the *OPRM1* 118G allele seem to indeed have an increased risk for requiring additional morphine (OR: 1.85; 95% CI: 0.494–496.90), this difference was not significant (p = 0.36). In addition, from the newborns that did require res-cue morphine carriers of 118G allele needed on average 5.63 (2.22–29.36) μg/kg/h morphine and 118AA newborns 3.12 (0.96–95.84) μg/kg/h morphine (p = 0.096). A similar effect was observed for *COMT* 472G>A (val158met); patients with the 472GG (val158val) wild-type geno-

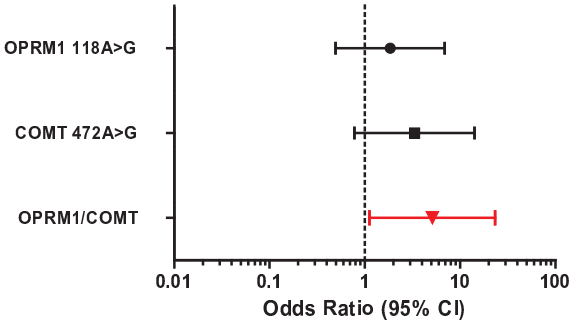

Figure 1. Odds ratio plot on a logarithmic scale for *OPRM1* 118A>G, *COMT* 472G>A (val158met) in figure shown as 472A>G (met158val), and combined *OPRM1*/*COMT* genotype in relation to morphine requirement (yes/no). For the combined allelic effect, patients with the 'high-risk' genotypes (118G allele carriage and/or 472GG [val/val] genotype) are plotted against newborns with the 'low-risk' genotypes (118AA and 472A [met] allele carriage).

Table 2. Relation between morphine requirement (yes/no) and total morphine administered (μg/kg/h) with *OPRM1* 118A>G, *COMT* 472G>A (val158met) and *OPRM1*/*COMT* combined genotype.

	Rescue morphine yes/no, n (%)	**Total morphine (μg/kg/h) median (IQR)**
OPRM1		
118AA (n = 44)	16/28 (36.4)	3.12; 0.96–5.84 (n = 16)
118AG/GG (n = 18)	8/10 (44.4)	5.63; 2.22–9.36 (n = 8)
OR (95% CI)	1.85 (0.494–6.90)	–
p-value	0.36	0.096
COMT		
472GA/AA (val/met, met/met) (n = 37)	12/25 (32.4)	3.69; 1.40–9.72 (n = 12)
472GG (val/val) (n = 15)	8/7 (53.3)	5.66; 1.71–7.22 (n = 8)
OR (95% CI)	3.33 (0.780–14.2)	–
p-value	0.10	0.93
OPRM1*/*COMT		
118AA+472A carrier (met carrier) (n = 24)	6/18 (25)	2.85; 1.13–9.64 (n = 6)
118G carrier and/or 472GG (val/val) (n = 27)	13/14 (48.1)	5.26; 1.42–8.80 (n = 13)
OR (95% CI)	5.12 (1.12–23.3)	–
p-value	**0.035**	0.58

The given p-values are corrected for gestational age, birth weight, sex, allocation center, Visual Analogue Score at baseline and co-medication for analgesia and/or sedation.
Statistically significant values are in bold.

type were trending towards an increased risk of requiring morphine (OR: 3.33; 95% CI: 0.780–714.2), as expected, but again not significant (p = 0.10; Figure 1). Newborns with the 472GG (val158val) genotype needed on average 5.66 (1.71–77.22) μg/kg/h morphine and 472A (158met) allele carriers 3.69 (1.40–49.72) μg/kg/h morphine (p = 0.93).

However, the combination of *OPRM1* and *COMT* yielded a significant association with the need for rescue morphine. Newborns with the 'high-risk' genotype (defined by having the *OPRM1* 118G allele and/or the risk genotype *COMT* 472GG [val158val] have an increased risk for requiring rescue morphine: OR: 5.12; 95% CI: 1.12–23.3; p = 0.035) compared with the 118AA combined with being a 472A (158met) carrier (**Figure 1**). With regard to the total morphine dose, no associations were found with the genotypes analyzed (**Table 2**).

Sensitivity, specificity, PPV & NPV

The PPV was 44.4%, 32.4%% and 48.2% for *OPRM1, COMT* and the combined genotype respectively, while the NPV was 63.6%, 46.6% and 75% in the same order. The analysis showed a sensitivity of 33.3% and 60% for *OPRM1* and *COMT* respectively, while the specificity was 73.6% and 21.8%. Combination of the 2 SNPs in one allelic genotype gave an sensitivity of 68.4% and specificity of 56.3%.

DISCUSSION

To our knowledge, this is the first study exploring the role of genetic variants in PDs related genes on morphine requirement for pain in (pre)term newborns on artificial ventilation. When analyzed separately, neither *OPRM1* 118A>G (asn40asp) nor *COMT* 472G>A (val158met) were significantly associated with morphine requirement or total morphine dose, although *OPRM1* and *COMT* genotypes did yield an odds ratio greater than 1. Combining the *OPRM1* and *COMT* SNPs in one allelic genotype, as suggested in the literature, generated a significant association with morphine rescue requirement (p = 0.035).

In line with the results for *OPRM1* and *COMT* on rescue morphine requirement, the patients with the 'high-risk' genotype also had a twofold higher total morphine dose, although this association did not reach statistical significance (p = 0.83). Since only the newborns that required rescue morphine were included in this linear regression analysis, the power decreased by a reduction from 44 to 17 patients, which might explain the lack of statistical significance. The relation between the analyzed SNPs in *OPRM1* and *COMT* on morphine requirement has also been illustrated in different adult studies with postoperative [19,21–25,37] and cancer related pain [38–40], although others failed in finding these associations [41–43]. This failure is most likely caused by the polygenic aspect in pain

[10], as discussed in the introduction. Our results on the combined genotypic effect are in line with two previously performed studies, in which is concluded that *OPRM1* 118AA in combination with *COMT* 158met have the lowest requirement whereas vice versa (118G and *COMT* 158val) have a higher risk for more pain and thus more opioid consumption [20,30].

Assessment of the genetic contribution in opioid disposition and response is particularly challenging in newborns due to confounding by developmental changes, such as maturation of the hepatic enzymes and changes in renal function, both of which are related to gestational and PNA [44]. Yet, genetic polymorphism have been shown to play a role also in the developing child. For example, *CYP2D6* genetic polymorphisms impact tramadol metabolism in the neonatal period [45]. And, well know, the impact of the *28 allele of the UGT1A1 phase II enzyme that has been associated specifically with jaundice in neonates: Gilbert's syndrome [46,47].

Recently, Wachman and colleagues have analyzed the genetic variations in *OPRM1* and *COMT* in newborns [13], but with the focus on NAS. They have demonstrated that 118G allele carriers and 472GG (val158val) genotyped newborns had a shorter hospital admission and lower risk for requiring treatment. Although our study differs in the type of opioid exposure, both findings point toward the same direction with a negative effect on sensitivity to endo- and exogenous opioids for the 118G allele and 472GG (val158val) genotype. New-born babies on mechanical ventilation with the 118G allele in combination with the 158val allele are speculated to be less sensitive to endogenous opioids, with the consequence of experiencing more pain while being exposed to the same procedure and thus requiring morphine rescue. NAS infants are less sensitive to exogenous in utero exposure of methadone or buprenorphine. This may lead to the observed effect of being less prone to abstinence, as shown by a shorter hospitalization and lower requirement of the treatment of NAS.

These clinical effects from both studies should also be compared with the findings from in vitro studies. The studies addressing the molecular consequences of the 118A>G genetic variation have retrieved inconclusive results. This polymorphism has been associated with an decreased protein expression while others did not found this effect and instead showed conformational changes leading to higher affinity of endogenous opioids and the opposite effect on exogenous opioids [15]. When comparing the clinical data from our and the NAS study we would expect a changed protein expression instead of alternations in the 3D structure of the receptor because the same clinical effect is observed for both endo- and exogenous opioids.

Combining the two SNPs had a relatively large effect on the PPV and NPV compared with the sensitivity and specificity. Since in the case of the last two parameters also other non-genetic factors will deter-mine whether newborns will require morphine rescue, this could explain the low sensitivity and specificity percentages. Although, the combined

allelic genotype did increase the PPV and NPV, the predictive value of the test is still not high enough. Based on these percentages, application of this genotyping strategy in a clinical setting seems of limited value at this moment, although it could be taken as one of the determinants of need for morphine rescue in a multimodal treatment strategy.

One of the limitations of this study is that DNA was extracted from buccal swabs which has a lower quantity and quality when compared with DNA from saliva or whole blood samples [48]. Due to poor quality of the isolated DNA less newborns could be included in the final analysis, which has decreased the power of the study, especially for the *COMT* polymorphism. However, in the case of newborns this method of DNA collection is an appropriate option because of the restriction in amount of blood that they can provide and in the case of saliva samples which are only suitable for older children. Another restriction of our study is that newborns treated with a continuous morphine infusion were not considered appropriate for this pharmacogenetic analysis. This decision has reduced the power, but despite the relatively small number of newborns included significant associations have been found. In addition, considering the current treatment protocol for presumed pain during artificial respiration in newborns, where continuous morphine infusion is not given on a routine base [5], our findings from infants allocated to the placebo group are therefore more relevant for the neonatal clinical practice.

In conclusion, we present, to our knowledge, the first data on the influence of genetic variation in the *OPRM1* and *COMT* genes on morphine rescue medication in (pre)term newborns on mechanical ventilation. The combined allelic 'high-risk' *OPRM1/COMT* genotype was found to be significantly related with requirement for rescue morphine in premature neonates on mechanical ventilation.

FUTURE PERSPECTIVE

Neonates at the intensive care are exposed to frequent painful procedures, including continuous distress from ventilatory support. Adequate analgesia with morphine is complicated by the existence of large interindividual differences in both morphine pharmacokinetics and requirement. Biomarkers that predict the need for morphine may be valuable tools in tailoring analgesia, thereby reaching better analgesia in those patients needing it and reducing negative effects. In our study, we have shown that genetic information on *COMT* and *OPRM1* may facilitate in predicting neonates that would need additional morphine. Yet, the predictive power is still low for use in clinical practice. The search for additional factors, both genetic as non-genetic, is needed to be able to tailor morphine analgesia even further.

Future research on genetics could focus on studying these polymorphisms in a larger cohort. Also the potential contribution of other candidate genes should be considered

because of numerous genes involved in pain and the analgesic response to opioids. A key requisite in these studies would be to include a sufficient number of patients, in these cases newborn children, which can be a hurdle to collect due to difficulties involving sampling and informed consent of the vulnerable infants.

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Part IV

Discussion and Summary


SLC22A1
COMT
UGT2B7

Chapter 11

General Discussion

GENERAL DISCUSSION

Pain needs to be adequately treated from a clinical, ethical and economical perspective. Currently pain is defined as "a distressing experience associated with actual or potential tissue damage with sensory, emotional, cognitive, and social components." [1]. Abnormal sensory input without a clear sign of tissue damage is occurring in neuropathic pain, which is outside the scope of this thesis. Different factors are contributing to the variability in pain expression, which can be explained with the generally accepted biopsychosocial model of pain. Biological characteristics such as the primary disease, eventual comorbidities, drugs and genetics, but also psychological aspects (e.g. personality, coping mechanism) and social factors caused by the environment (e.g. household, family, work, culture) all contribute to the final expression of pain. The studies outlined in this thesis are focused on the biological aspect of pain and analgesia by addressing the genetic variability. We first aimed to give an overview with the most potential candidate genes that alter opioid therapy for application in clinical practice. Additionally, we have validated the relevance of these candidate genes in relation with opioid response in adult postoperative and cancer cohorts. As opposite to adults, data on the role of PGx with opioids in the pediatric population is very limited. Additionally, identified genotype-phenotype associations in adults are not directly applicable in (the youngest) children, due to the influence of developmental changes on the phenotypic activity. Therefore the majority of the studies described in this thesis have aimed to address this information gap. In this discussion we synthesize the results of these studies and place them in context while providing future guidance for clinical care and research.

CANDIDATE GENES

In the case of acute pain several genes have been identified to play a role in the nociceptive insult triggered by tissue damage to the processing of this signal in the brain. The genetic components of pain and its effect on pain sensitivity has been extensively studied by Jeffrey Mogil (McGill University, US) by use of transgenic knockout mice [2]. This work resulted in a freely available online 'Pain Genes Database', which is mainly based on behavioral measures on pain sensitivity [2, 3]. Although we did not evaluate the genetic vulnerability to pain systematically in all studies, it is important to highlight that analgesic therapy is complicated *a priori* due to the extensive variability in pain sensitivity among individuals. Young children, especially neonates and infants differ in their communication of pain. Whereas adults and adolescent children can adequately describe and rate their pain intensity, this self-report (golden standard) is impossible in the lower age groups. In very young children this has to be monitored observationally for which several scales have

been developed and validated [4]. Moreover, the developmental aspects of the expression of proteins and enzymes has to be taken into account when assessing the consequences of genetic variability on pain sensitivity.

Next to the variability in pain sensitivity patients also display major variation in opioid response. Nowadays, a trial-and-error approach is followed to reach adequate analgesia, a method which could be improved if biomarkers predicting opioid response were available. A promising field in this perspective is pharmacogenetics (PGx), in which genetic analysis of drug metabolizing enzymes, drug transporters and drug receptors is performed in order to predict the metabolism or transport (pharmacokinetics) and effects (pharmacodynamics) of drugs. See **Figure 1** for an simplified overview of the pharmacokinetics of opioids, in which genes are depicted in italic that are coding for either drug metabolizing enzymes

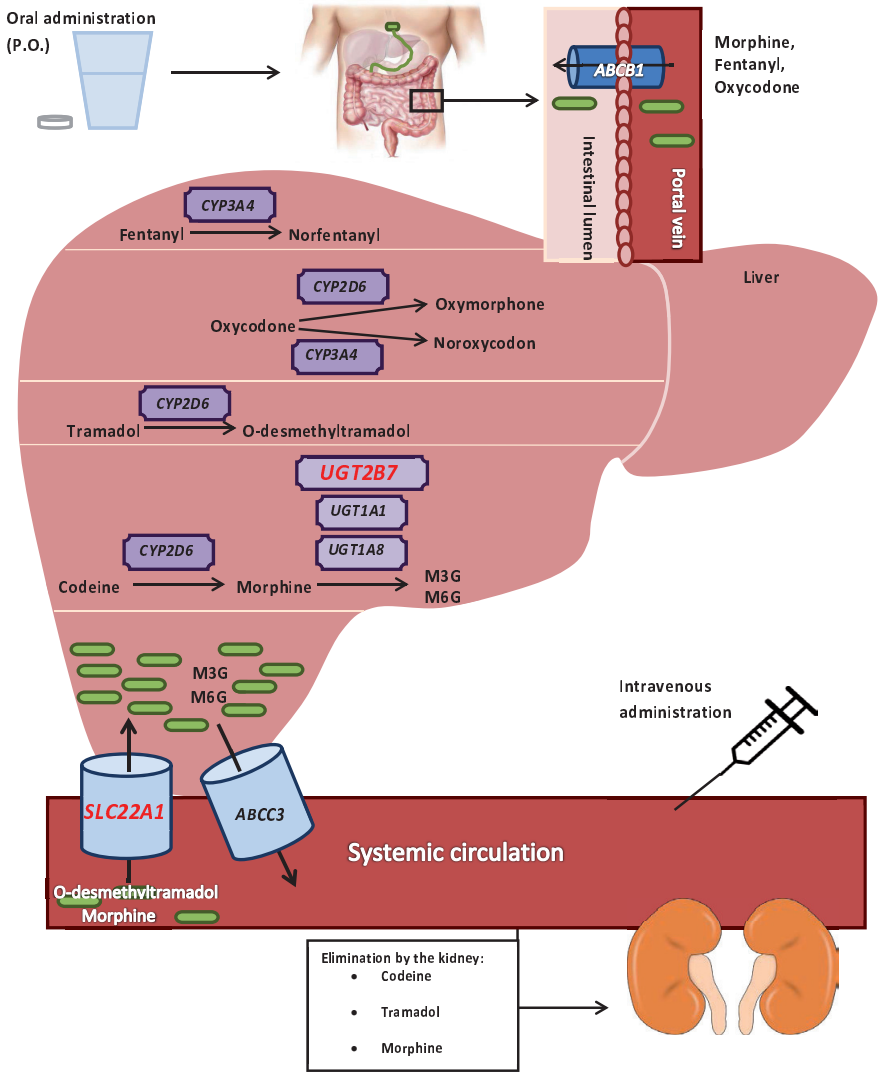

Figure 1. Overview pharmacokinetics-related candidate genes

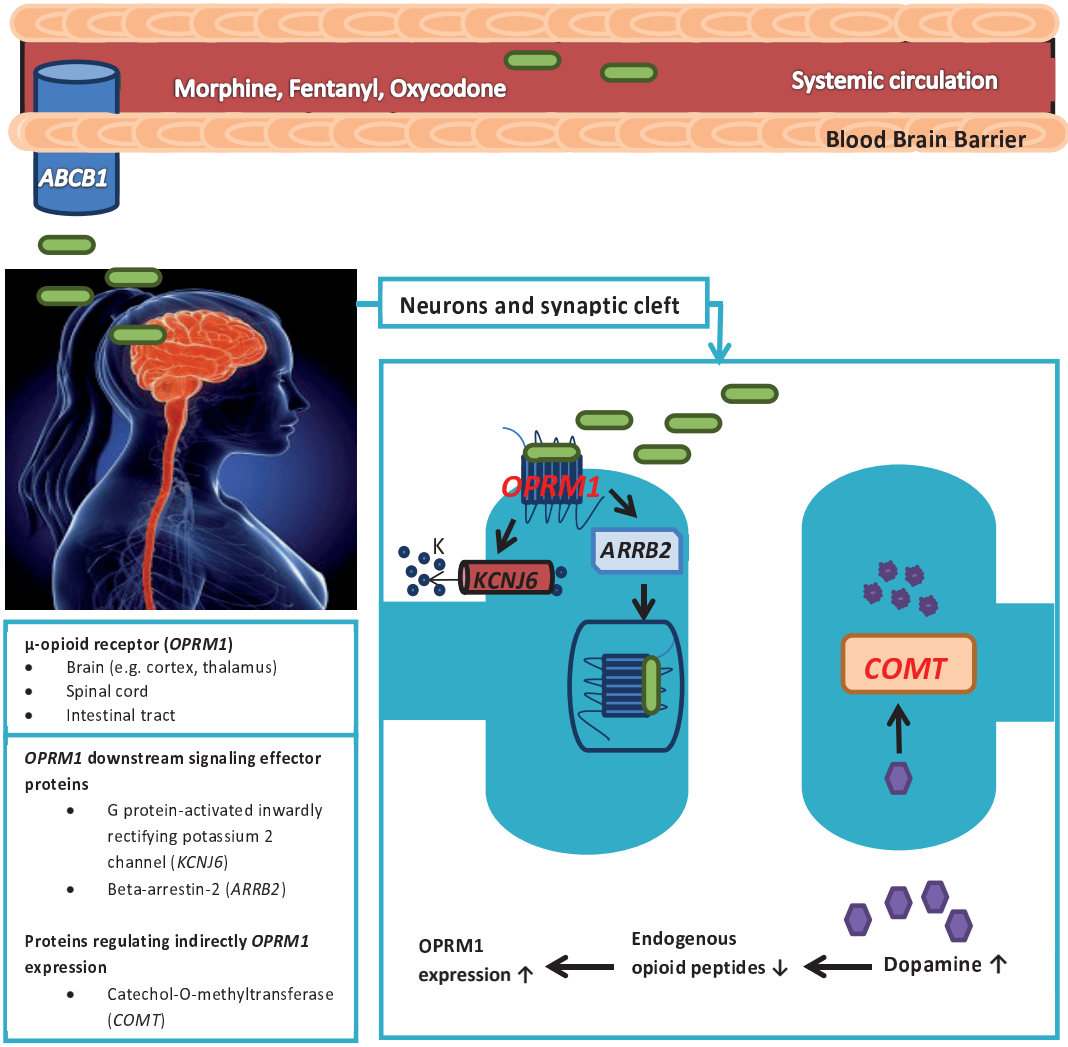

Figure 2. Overview pharmacodynamics-related candidate genes

or transporters in this process. Additionally, genetic variants of effector proteins and receptors can also lead to a diminished or induced opioid response. Important genes involved in this pharmacodynamics route are shown in **Figure 2.** The pediatric population, especially in the neonatal and infant age range, is highly subjective to developmental changes that affect drug metabolism and transport. For a good correlation between genotype and phenotype the activity of the involved protein has to be sufficiently expressed to make distinctions between genotypic groups apparent [5].

Guiding opioid treatment based on PGx markers is not hampered by the lack of potential candidate genes. In contrast, the literature is "full" of potential biomarkers. This extensive amount of information makes it difficult for healthcare practitioners to interpret the relevancy of a particular marker. We started with a systematic literature search, examining publications in several databases that are describing genetic markers with respect to a

correlation with opioid pain treatment efficacy and toxicity (**Chapter 2**). With this search in 5 databases 4,257 unique citations were retrieved, eventually resulting in 852 significant articles covering 24 genes. After extensive review of this data we created a shortlist with 10 candidate genes: *CYP2D6, OPRM1, COMT, SLC22A1, UGT2B7, KCNJ6, CYP3A4/A5, ABCB1* and *ABCC3*. Based on this shortlist genes were selected for assessment in the studies described in this thesis (**Chapter 3 – 10**).

Although genetic variability within the cytochrome P450 2D6 (CYP2D6) enzyme on opioid response is not assessed in this thesis, based on its role in opioid metabolism and evidence from literature it is worthwhile to mention its relevance before proceeding with other PK-related genes. CYP2D6 plays a major role in the biological activation of codeine and tramadol, and to a lesser extent for oxycodone and hydrocodone. CYP2D6 genetic variability is well characterized with an open source database reporting genetic variants and their consequence *in vitro* and/or *in vivo* on enzyme activity [6]. Additionally, *CYP2D6* genotype has been translated to actionable genotype-predicted phenotypic groups in the Netherlands as well as in the United States [7, 8]. Based on the extensive clinical evidence, CYP2D6 genotype-based codeine guidelines for healthcare practitioners have been published [9]. Furthermore, the CYP2D6 ultra-rapid metabolizer status is added as a contra-indication in the Summary of Product Characteristics of codeine [10]. Our literature search (**Chapter 2**) confirms the importance of genetic variability within this gene for codeine and tramadol response.

OPIOID PHARMACOKINETIC RELATED GENES

UGT2B7

The UDP-glucuronosyltransferase 2B7 (UGT2B7) enzyme is involved in the glucuronidation of codeine, tramadol, and buprenorphine but is mainly of importance for morphine glucuronidation to morphine-3-glucuronide and morphine-6-glucuronide [11] (**Figure 1**). As demonstrated (**Chapter 2**), the most investigated *UGT2B7* single nucleotide polymorphism (SNP) in relation to opioids is -900G>A (-842G>A). We have observed in that preterm newborns with the -900GG genotype had higher morphine levels, reflecting lower enzymatic activity (**Chapter 7**). This is the first study to report an association between *UGT2B7* genotype and morphine PK in neonates. With regards to the developmental pattern of UGT2B7 various studies have been performed and illustrate that full maturation of this enzyme is reached between 2 and 6 months [12]. Despite that our group was younger, an effect of the genotype was observed. Apparently, differences in UGT2B7 activity and consequently substrate concentrations between the genotype groups can be found with lower overall activity. As this was a small pilot cohort, replication is warranted, preferably addressing also the effect on clinical outcome. Other studies have also addressed this vari-

Table 1. Effect genetic variability within *UGT2B7* on morphine

REF.	OPIOID	N=	STUDY	SNPs*	RESULTS	EFFECT
THIS THESIS						
Chapter 7	morphine	17	Preterm new-borns on mechanical ventilator	rs7438135 (-900G>A/-842G>A)	Morphine plasma concentrations at 20 min post intubation were higher in -900GG genotype (180 and 363 ng/ml) compare to GA (145 ng/ml; 107–173 ng/ml) and AA (122 ng/ml; 84.0–146 ng/ml) genotyped infants (p = 0.036). The -900G>A was also associated with M3G/morphine metabolic ratio, with lower ratio found in in the -900GG genotype (p = 0.005).	↑**EXPOSURE**
LITERATURE						
Holthe *et al.* 2002	morphine	70	Cancer patients (adults)	rs7439366 (802C>T)	No association between 802C>T variant and M3G/morphine or M6G/morphine metabolic ratio.	**NO EFFECT EXPOSURE**
Holthe *et al.* 2003	morphine	175	Cancer patients (adults)	Sequencing	No association between 802C>T variant and M3G/morphine or M6G/morphine metabolic ratio.	**NO EFFECT EXPOSURE**
Sawyer *et al.* 2003	morphine	68	Postoperative patients (adults)	rs7439366 (802C>T) 7668258 (-161C>T)	Morphine levels lower in TT patients (median, 18 ng/mL [range, 18–1490 ng/mL]) compared to C allele carriers (66 ; 18–3995) (P = 0.04). M6G and M3G concentrations were lower in CC patients (18; 0–66; and 152; 30–434; resp.) compared with T allele carriers (43; 0–193; and 242; 33–1381; resp.) (P = 0.045 and P = 0.004, resp.).	↓**EXPOSURE**
Duguay *et al.* 2004	morphine	175	Cancer patients (adults)	Sequencing (-79G>A)	Although the -79G>A was related to 2.5–7 fold decrease in UGT2B7 activity and the carriers of the G allele did have a lower M3G/morphine and M6G/morphine metabolic ratio, these differences were not significant.	**NO EFFECT EXPOSURE**

Table 1. Effect genetic variability within *UGT2B7* on morphine (continued)

REF.	OPIOID	N=	STUDY	SNPs*	RESULTS	EFFECT
Ross *et al.* 2005	morphine	162	Cancer patients (adults)	7668258 (-161C>T) 2034A>G 2098A>T 2099C>T	No association between these *UGT2B7* variants with the need to switch from morphine to alternative opioid.	**NO EFFECT RESPONSE**
Coulbault *et al.* 2006	morphine	74	Postoperative patients (adults)	rs7439366 (802C>T)	No association between 802C>T with morphine requirement or side effects.	**NO EFFECT RESPONSE**
Darbari *et al.* 2008	morphine	20	Sickle cell disease patients (young adults)	rs7438135 (-842G>A)	Carriers of -842G allele have lower AUC ratio of M6G/morphine (1.8 ± 0.5 vs. 3.0 ± 1.8) and M3G/morphine (10.1 ± 2.7 vs. 15.7 ± 9.4).	**↑EXPOSURE**
Fujita *et al.* 2010	morphine	32	Cancer patients (adults)	rs7439366 (802C>T) 12233719 (211G>T)	Lower frequency of nausea in 802T allele carriers (p = 0.023).	**↓ADVERSE EVENTS**
Jimenez *et al.* 2012	morphine	68	Children (3–17 yrs.) undergoing adenotonsillectomy	rs7439366 (802C>T)	No association between the 802C>T variant with analgesic response or side effects.	**NO EFFECT RESPONSE**
Sadhasivam *et al.* 2012	morphine	146	Children (6–15 yrs.) undergoing adenotonsillectomy	rs7439366 (802C>T)	No association between the 802C>T and 2161C>T genetic variants with morphine clearance.	**NO EFFECT EXPOSURE**

Table 1. Effect genetic variability within *UGT2B7* on morphine (continued)

REF.	OPIOID	N=	STUDY	SNPs*	RESULTS	EFFECT
Fladvad *et al.* 2013	morphine	759	Cancer patients (adults)	rs7438135 (-900G>A) rs7662029 (-327G>A) rs7668258 (-161T>C) rs73823859 (-138G>A) rs138733571 (721+9_>+10insT) rs62298861 (722–314A>G) rs28365062 (735A>G) rs10028494 (1003–1956A>C) rs3924192 (1003–1929G>A) rs3924194 (1003–1801C>G) rs7435335 (1003–1558G>A) rs4348159 (1062C>T)	No association between *UGT2B7* genetic variants and M6G/morphine or M3G/morphine metabolic ratio.	**NO EFFECT EXPOSURE**
Fukuda *et al.* 2013	morphine	146	Children (6–15 yrs.) undergoing adenotonsillectomy	rs7438135 (-842G>A) rs7439366 (802C>T) rs7668258 (-161C>T)	Morphine clearance not associated with -842G>A, 802C>T or -161T>C variant.	**NO EFFECT EXPOSURE**
De Gregori *et al.* 2013	morphine	109	Postoperative patients (adults)	rs7438135 (-842G>A) rs7668258 (-161C>T) rs4455491 (-1306A>G) rs11940220 (-1299C>T) rs11940316 (-1112C>T) rs73823859 (-138G>A) rs7668282 (-125T>C)	Two frequently occurring *UGT2B7* haplotypes were not associated with morphine concentration or M3G/M6G metabolic ratio.	**NO EFFECT EXPOSURE**
Baber *et al.* 2015	codeine (morphine)	98	Post caesarean section	rs7439366 (802C>T)	*UGT2B7* 802TT participants consumed a lower mean codeine dose (0.76 ± 0.12 mg/kg) than 802CC genotyped patients(0.86 ± 0.11 mg/kg; $P = 0.015$).	**↑CONSUMPTION**
Oosten *et al.* 2016	morphine	49	Cancer patients		*UGT2B7*–900G>A was not associated with morphine, M3G and M6G clearance.	**NO EFFECT EXPOSURE**

*The polymorphisms -900G>A (-842G>A), -1306G>A (-1248G>A), -1299C>TC (-1241C>T), -1112C>T (-1054C>T), -327G>A (-268G>A) and -161C>T (-102C>T) are in complete linkage disequilibrium. In contrast, the 802C>T polymorphisms is in reverse in complete linkage disequilibrium with these previously mentioned variants.

Table 2. Effect *SLC22A1* genetic variability on tramadol, codeine and morphine

REF.	OPIOID	N=	STUDY	SNPs	RESULTS	EFFECT
THIS THESIS						
Chapter 8	tramadol	50	Critically ill neonates and infants (1 day – 5 months)	rs72552763 (Met420 deletion) rs12208357 (Arg61Cys) rs34130495 (Gly401Ser) rs34059508 (Met420 deletion + Gly465Arg) rs55918055 (Met420 deletion + Cys88Arg)	*SLC22A1* loss of function alleles higher M1/M ratio (2.25; 95% CI, 2.01–2.48) than infants with 2 copies (1.86; 95% CI, 1.66–2.06). Carriers of ≥ 2 CYP2D6 and < 2 *SLC22A1* functional gene copies have 57.8% higher M1/M ratio compared with infants with < 2 CYP2D6 and *SLC22A1* copies ($P < 0.001$).	↑**EXPOSURE**
LITERATURE						
Tzvetkov *et al.* 2011	tramadol	41	Healthy volunteers (adult)	rs72552763 (Met420 deletion) rs12208357 (Arg61Cys) rs34130495 (Gly401Ser) rs34059508 (Met420 deletion + Gly465Arg) rs55918055 (Met420 deletion + Cys88Arg)	Carriers of zero, one, or two active *SLC22A1* alleles had significantly different ($P = 0.003$, n = 41) 0–24-h AUCs of O-desmethyltramadol (805.5 ± 385.5, 679.4 ± 75.0, and 420.2 ± 49.1 μg·h/l, resp.). These effects are independent of *CYP2D6* genotype. Individuals with inactive *SLC22A1* alleles also had prolonged miosis ($p = 0.005$, n = 24).	↑**EXPOSURE** ↑**RESPONSE**

Table 2. Effect *SLC22A1* genetic variability on tramadol, codeine and morphine (continued)

REF.	OPIOID	N=	STUDY	SNPs	RESULTS	EFFECT
Stamer *et al.* 2016	tramadol	205	Postoperative patients (adult)	rs72552763 (Met420 deletion) rs12208357 (Arg61Cys) rs34130495 (Gly401Ser) rs34059508 (Met420 deletion + Gly465Arg) rs55918055 (Met420 deletion + Cys88Arg)	Cumulative tramadol consumption was lowest in patients with 0 active *SLC22A1* allele compared with the group of patients with 1 or 2 active alleles (343 ± 235 vs 484 ± 276 mg; $P = 0.03$). Plasma AUC of (+)O-desmethyltramadol were 111.8 (95% CI, 63.4–160.1), 80.2 (65.1–95.3), and 64.5 (51.9–77.2) h·ng·mL in carriers of 0, 1, or 2 active *SLC22A1* alleles ($P = 0.03$).	↑**CONSUMPTION** ↑**EXPOSURE**
Tzvetkov *et al.* 2013	codeine (morphine)	25	Healthy volunteers (adult)	rs72552763 (Met420 deletion) rs12208357 (Arg61Cys) rs34130495 (Gly401Ser) rs34059508 (Met420 deletion + Gly465Arg) rs55918055 (Met420 deletion + Cys88Arg)	Morphine mean AUC was 56% higher in carriers of loss-of-function *SLC22A1* polymorphisms compared to non-carriers ($P = 0.005$). This difference remained after correction for *CYP2D6* genotype ($P = 0.035$). Mild adverse events (e.g. light sedation, headache, dizziness and nausea) occurred in 100% of carriers of *SLC22A1* inactive alleles compared to 64% in carriers of 2 active alleles ($P = 0.04$).	↑**EXPOSURE** ↑**ADVERSE EVENTS**
Fukuda *et al.* 2013	morphine	146	Children (6–15 yrs.) undergoing adenotonsillectomy	rs72552763 (Met420 deletion) rs12208357 (Arg61Cys) rs34130495 (Gly401Ser) rs34059508 (Met420 deletion + Gly465Arg) rs622342	Allometrically scaled post hoc Bayesian morphine clearance in homozygotes *SLC22A1* loss-of-function alleles (n = 9) was lower (20%) compared to wild-type (n = 85) and heterozygotes (n = 52; $p < 0.05$).	↑**EXPOSURE**

Table 2. Effect *SLC22A1* genetic variability on tramadol, codeine and morphine (continued)

REF.	OPIOID	N=	STUDY	SNPs	RESULTS	EFFECT
Venkatasubramanian *et al.* 2014	morphine	220	Children (6–15 yrs.) undergoing adenotonsillectomy	rs72552763 (Met420 deletion) rs12208357 (Arg61Cys) rs34130495 (Gly401Ser) rs34059508 (Met420 deletion + Gly465Arg) rs622342	Results from Fukuda *et al.* (2013) confirmed in this extended cohort. Carriers of 2 *SLC22A1* loss of function alleles (n = 13) had lower morphine clearance (14%; $p = 0.06$) and lower metabolite formation (~39%) was observed.	↑**EXPOSURE**
Oosten *et al.* 2016	morphine	49	Cancer patients	rs72552763 (Met420 deletion) rs12208357 (Arg61Cys) rs34130495 (Gly401Ser) rs34059508 (Met420 deletion + Gly465Arg)	*SLC22A1* loss of function alleles were not associated with morphine, M3G and M6G clearance.	**NO EFFECT EXPOSURE**
Nielsen *et al.* 2017	morphine	37	Experimental pain in healthy volunteers (adults)	rs72552763 (Met420 deletion) rs12208357 (Arg61Cys) rs34130495 (Gly401Ser)	Neither AUC0–150min, ktr, CL, nor VD were associated with genetic variants in *SLC22A1*. The antinociceptive effects of morphine on experimental pain was not associated with *SLC22A1* variants.	**NO EFFECT EXPOSURE** **NO EFFECT RESPONSE**

ant or polymorphisms that are in linkage disequilibrium with this variant on morphine disposition and response (**Table 1,** see end of this chapter).

From **Table 1** it is evident that quite some contradictive results have been retrieved on the -900G>A variant or other SNPs in linkage disequilibrium. Although our results are in line with a previous study reporting reduced glucuronidation of morphine in -900G allele carriers [13], other studies in oncology patients did not demonstrate this effect [14–17]. Co-medication in cancer patients could have diluted the genetic effects. Also, since treatment of the tumor by radio- or chemotherapy could lead to hepatotoxicity [18, 19], genetic effects having functional consequences on hepatic enzymes could be irrelevant in these populations. In 759 patients with advanced cancer no effect was found of UGT2B7 genetics on morphine metabolism. Instead, the combined UGT1A1/1A8 haplotype was associated with reduced M3G/morphine and M6G/morphine concentrations after oral ingestion [14]. A recent study in children in the postoperative setting was unable to demonstrate the effect of SNP -900G>A on morphine clearance [20]. The distinction between -900G>A genotype groups could only be made in children with normal OCT1 (SLC22A1 gene) function.

Based on these studies, polymorphisms within other genes (UGT1A1, UGT1A8, SLC22A1) seem to be more relevant with regards to morphine PK than that of UGT2B7. Since our pilot study has included a low number of neonates and literature data is contradicting, validation of these results in a larger cohort with the assessment of additional genes, as discussed, is mandatory.

SLC22A1

Transporters facilitate the passage of hydrophilic compounds across the lipid cell membrane. The Organic Cation Transporter (OCT1) has been implicated in the uptake of morphine (active metabolite codeine) and O-desmethyltramadol (active metabolite tramadol) from the circulation into the hepatocyte in adults [21] (**Figure 1**). Inactivating genetic variants in the *SLC22A1* gene, encoding OCT1, are thus expected to lead to a decreased elimination of codeine, morphine and tramadol. We found in very young infants with *SLC22A1* loss-of-function alleles (*2-*6) increased O-desmethyltramadol plasma levels, after correction for *CYP2D6* genotype (**Chapter 8**). Our results in this young population reflect adult data on tramadol (**Table 2,** see end of this chapter), pointing towards increased O-desmethyltramadol exposure [22, 23] and consequently decreased postoperative demand [22]. Currently no negative or conflicting studies have been published on *SLC22A1* variants and tramadol. OCT1 is expressed from the first postnatal day and increases quickly in the following months [24, 25]. Our study in infants suggests that full maturation of OCT1 in this young pediatric population is not needed for making a distinction between *SLC22A1* genotypic groups.

In contrast to tramadol, the impact of OCT1 genotype on codeine and morphine disposition was not replicated by all studies (**Table 2**). This lack of effect can be explained by the results of *in vitro* study characterizing 19 non-synonymous *SLC22A1* variants in their capacity to transport 10 substrates (e.g. morphine, tramadol, metformin, tropisetron). Three groups of *SLC22A1* loss-of-function alleles were established; 1) total loss of activity independent of substrate (*5, *6, *12, *15), 2) strong loss of activity but not complete, also independent of substrate (*3, *4, *14) and 3) substrate specific loss of activity ranging from 0% to 90% (*2, *7, *10, *11, *14) [26]. This study demonstrated that the *2 allele should be considered as a reduced function allele towards morphine, instead of total loss of function [26]. However, the *2 allele has zero activity towards O-desmethyltramadol, which further supports our finding in **Chapter 8**.

Before *SLC22A1* genetics could be incorporated in the updated CPIC guideline of *CYP2D6* genotype and codeine (tramadol, morphine) therapy [9], future research should focus more on the clinical impact. This research should keep in mind that *CYP2D6* UMs and PMs on tramadol or codeine (morphine) therapy already have an increased toxicity risk and lack of analgesia, respectively. On the contrary, where studies have failed to demonstrate *CYP2D6* genotypic effect on tramadol and codeine disposition or response, unidentified *SLC22A1* genetics could have confounded these results. The first study addressing the clinical impact demonstrated that *SLC22A1* loss-off-function allele carriers required lower postoperative tramadol doses [22]. However, sufficiently powered studies should analyze if CYP2D6 EM individuals also have increased risk for toxicity when carrying *SLC22A1* inactive alleles, and how large the risk is per inactive allele. Special attention should be given to the high toxicity risk group of genotype-predicted *CYP2D6* UM patients that also carry 2 inactive *SLC22A1* alleles. This group is exposed to high morphine and O-desmethyltramadol plasma levels when treated with codeine and tramadol due to rapid formation of these metabolites and decreased clearance. Data on this specific

Table 3. CYP2D6/OCT1(*SLC22A1*) risk genotype frequency in different ethnicities

	Africans (%)	**Asians (%)**	**Caucasians (%)**
CYP2D6 UM	40	2	5
OCT1 poor function	0	0	8
CYP2D6/OCT1			
(toxicity risk)	**0**	**0**	**0.4**
CYP2D6 UM	40	2	5
OCT1 intermediate function	12	2	38
CYP2D6/OCT1			
(toxicity risk)	**4.8**	**0.04**	**1.9**

'high risk' group are lacking. In order to investigate this, large numbers of individuals are required (**Table 3**).

A challenge in the clinical application of this genotyping strategy would be to demonstrate that pre-emptive genotyping of all individuals on codeine and tramadol would be cost-effective, even with these low numbers of individuals at risk for toxicity (**Table 3**). In the assessment of the cost-effectiveness aspect it is of importance to demonstrate that the identification of these subjects would not only prevent respiratory depression or other life threating effects but also decrease opioid-related hospital admissions.

OPIOID EFFECTOR GENES

Two important candidate SNPs from literature are *OPRM1* 118A>G and *COMT* 472G>A (**Chapter 2**), encoding the mu-opioid receptor (MOR) and catechol-O-methyltransferase enzyme (COMT). **Figure 2** displays the role of these and other pharmacodymanic-related genes. We confirmed that the effect of these SNPs in adult cancer patients on opioid response (***OPRM1/COMT,* Chapter 5**) and in children on pain sensitivity (***OPRM1/COMT,* Chapter 6**) was more relevant than variants in other candidate genes that were assessed concordantly. In other smaller cohorts, we choose to only determine these *OPRM1* and *COMT* genetic variants. Using this approach, we found an association with postoperative morphine consumption (***COMT,* Chapter 3**), the need for morphine rescue in neonates on the ventilator (***OPRM1/COMT,* Chapter 9**) and an effect on withdrawal severity in children (***OPRM1*, Chapter 10**).

For the MOR no studies addressed the influence of ontogeny in humans. Data from rat indicate no difference in number of mRNA transcripts of the MOR between 3 age groups [27]. This reflects the fact that we were able to demonstrate the effect of the *OPRM1* variant on experimental pain in children from 8–18 years (**Chapter 6**), procedural pain in neonates (preterm and term) (**Chapter 9**) and an association on the severity of withdrawal (**Chapter 10**) in children across the whole age range. In contrast to the MOR, COMT activity appears to change with age. In neonates (n = 8) the COMT enzyme protein content, determined with the Bradford method, and activity (Western Blotting) in the human prefrontal cortex was 50% of adult values (n = 7). In the same study the COMT enzyme activity increased gradually to 75% of adult value in young adults (20–24 years) [28].

Therefore it is unlikely that the absence of an association between the *COMT* Val158Met SNP with withdrawal in our pediatric cohort (**Chapter 10**) is due to immature COMT activity. Additionally, as we found associations for the *COMT* genotype in neonates on pain sensitivity during mechanical ventilation (**Chapter 9**), this indirectly suggest that 50% protein expression is sufficient to find the distinctive effect of the genotype on pain and analgesia.

Pain sensitivity *(OPRM1)*

The consequence of the *OPRM1* 118A>G SNP on pain sensitivity has been assessed in adults (**Chapter 3**), children (**Chapter 6**) and neonates (**Chapter 9**). We found that children carrying the 118G allele are less sensitive to thermal pain. Data from this pediatric cohort (47% boys, 53% girls) fit well with adult literature data, in which also a decreased sensitivity to experimentally induced thermal and pressure pain was observed [29, 30]. Further supporting our pediatric study, the endogenous opioid beta-endorphin has a higher affinity and potency for the 118G variant of the MOR [31]. Others were unable to replicate this increased affinity and potency [32, 33].

Interestingly, neonates randomized to placebo or morphine during artificial ventilation carrying the 118G allele in combination with the *COMT* SNP require more frequently opioids (**Chapter 9**). The fact that we found more pain in these infants fits well with the studies showing increased pain with the 118A>G variant in other clinical settings, such as migraine, hernia, fibromyalgia and diabetic foot ulcer [34–37]. This sounds counterintuitive when comparing these results with the decreased risk of the 118G allele to thermal pain. Two hypotheses may explain this contradictive observation. First, distinctive endogenous opioid peptides could be involved across different stimuli evoking pain, and hence the functional consequence of the 118A>G SNP was found to be substrate dependent [31]. Alternatively, the 118A>G variant has been related with opposite effects on dopamine (DA) release in the nucleus accumbens (NAc) [38]. Placebo administration resulted in decreased DA release in NAc whereas during painful stimuli the release was increased [38]. As dopamine has been linked to the endogenous opioid system, differences in DA levels will influence the expression of endogenous opioid peptides [39].

In contrast to the pediatric data, no associations were found with (thermal) pain sensitivity in adults (**Chapter 3**). We speculate that this could be caused by anxiety in the adult cohort, because these patients were tested shortly before the cardiac surgery. The anxiety may have overwhelmed the genotype effect. Also, this cohort existed of mostly males. Differences between sexes have been observed, where females are more likely to suffer from a chronic pain condition (e.g. migraine, musculoskeletal pain, osteoarthritis) [40] while responding better to morphine [41]. This 'gender' effect is highly dependent on socio- cultural (gender role expectations) and biological (gender hormones) differences between males and females [40]. Since the adult cohort existed of primarily male individuals (91%), unlike other cohorts examined in this thesis, the gender role expectations could have biased our results.

Opioid response (*OPRM1)*

In our adult cancer cohort *OPRM1* 118G carriers in combination with the *COMT* Val158Val genotype had higher increase in dose after being seen by the palliative care team (**Chapter 5**). This is in line with two meta-analysis that have demonstrated in 118G allele patients

undergoing surgery increased opioid demand and lower risk to side effects [42, 43], with most solid evidence for morphine [42]. Unfortunately we were unable to demonstrate the effect of the 118G allele in our cohort of mostly males undergoing cardiac surgery (**Chapter 3**). It could be questioned in these patients if the administration of remifentanil and fentanyl intraoperatively affected the activating potential at the MOR and thereby omitting this genetic effect on morphine demand in the postoperative period. Moreover, we have observed an association between this *OPRM1* SNP and withdrawal severity. This implies that this genetic variant effect on MOR activity could predispose patients to more severe withdrawal symptoms.

While the literature shows more uniformity for this variant with postoperative pain phenotypes, this is not the case with cancer related pain [44] and chronic non-malignant conditions [45, 46]. One of the factors most likely eliminating the genetic effect in these chronic pain phenotypes is the development of tolerance to opioids. The increase in opioid demand over time mirrors progression of the disease, PK-related changes (increased metabolic activity), but it is assumed that this dose escalation is mostly a consequence of desensitization and down-regulation at MOR level [47]. This desensitization of the receptor can abolish the *OPRM1* genotype effect. Moreover, co-medication interfering with the metabolism of opioids can disturb a genotype-phenotype association. This aligns with our results from **Chapter 4** and **5,** where this variant (nor any others) was not related with absolute morphine consumption the need to rotate from opioids, respectively.

Other *OPRM1* SNPs have been associated with opioid demand and side effects [48–53], but without replication. This could be indicative for false positive findings. Rare *OPRM1* SNPs might have even more impact on MOR function and thus opioid response. A rare but very interesting *OPRM1* variant (Arg181Cys, rs799910351) in this perspective was reported in the Scandinavian population [54]. The patient that was homozygote variant had no analgesic effect from opioids and two patients carrying one allele required extremely high doses (400 and 550 mg/24 hours). In an attempt to uncover if this variant could explain the individual with extreme morphine equivalent dose in our cancer cohort (**Chapter 5**), the whole cohort (n = 239) was genotyped. Yet, it seems that this variant is region bound, as it was not detected in our cohort with mainly Europeans (data not published). Unfortunately, although the effect size is larger and as a result the consequence for the clinic of more importance with these type of SNPs, these variants are unlikely to enter the pre-emptive setting as the number needed to genotype (NNG) is extremely high.

COMT

Most studies have addressed the isolated effect of the Val158Met (rs4680) variant. While others have assessed more variants (rs4680, rs4818, rs4633) composing the COMT haplotypes low pain sensitivity (LPS), average pain sensitivity (APS) and high pain sensitivity (HPS). These haplotypes have been related to interindividual differences in pain sensitiv-

ity [55]. The studies described in this thesis analyzed either the effect of the individual Val158Met variant (**Chapter 9, 10**) and when studies had sufficient sample size also the *COMT* haplotypes (**Chapter 3, 4, 5, 6**).

Pain sensitivity (*COMT*)

The role of the Val158Met variant on pain sensitivity has been confirmed in our studies. As demonstrated in the pediatric population, 158Met allele carriers in combination with the *OPRM1* 118AA genotype were more sensitive to thermal pain (**Chapter 6**). These results are in line with published data from healthy subjects and also fit with theoretical explanation. COMT activity is decreased by 3–4 fold due to the 158Met allele. This reduced activity has been related with a decrease in μ-opioid system activation, as demonstrated with positron emission tomography and a MOR-selective radiotracer in healthy subjects [39]. Less endogenous opioids are available to suppress the pain induced by the experimental stimulus. However, as is the case for the *OPRM1* 118A>G SNP, opposite effects are seen between pain phenotypes. We have observed lower baseline pain in neonates carrying the 158Met allele in combination with the *OPRM1* 118AA genotype, reflected by the fact that these children were less likely to require rescue morphine administration during mechanical ventilation (**Chapter 9**).

When looking at the previously mentioned *COMT* haplotypes, the initial study from literature reporting on these *COMT* haplotypes found the effect in an experimental pain setting [56]. We confirm this finding, in our adult population with thermal pain data (**Chapter 3**). Carriers of the HPS group were more sensitive for pain, although this association did not remain after correction for multiple testing. In contrast, the effect of these *COMT* haplotypes was not found on thermal pain in our pediatric population (**Chapter 6**). This might have been caused by a power issue, as from the 136 included children the *COMT* haplotype was only successfully constructed in 88 children. Therefore based on these studies it remains inconclusive which approach to choose (single variant or haplotypes) when relating *COMT* genetic variability to pain sensitivity.

Opioid response (*COMT*)

Cancer patients with the *COMT* Val158Val genotype in combination with the *OPRM1* 118G allele had a larger increase in opioid dosage after palliative care team consultation (**Chapter 5**). Although also from literature the 158Val is linked to decreased opioid potency, the findings are not uniform (**Table 4,** see end of this chapter). This variant was not found to be predictive of opioid induced withdrawal or the severity in pediatric patients (**Chapter 10**). This implies that reduced opioid potency due to changes at the level of COMT activity do not affect the development of withdrawal. Also, in adult patients no effect was established of the Val158Met variant on postoperative opioid consumption

Table 4. Effect *COMT* 472G>A (Val158Met) on pain and opioid response

REF.	OPIOID	N=	STUDY	SNPs	RESULTS	EFFECT
THIS THESIS						
Chapter 3	morphine oxycodone tramadol	126	Experimental (thermal) pain, postoperative pain, chronic pain (adults)	472G>A (rs4680) 408C>G (rs4818) 186C>T (rs4633)	None of the *COMT* genetic variants nor the haplotype composed of these variants was associated with experimental thermal pain prior to the surgery or the development of chronic postsurgical pain. The *COMT* APS haplotype (472A, 408C, 186T) was associated with higher postoperative 24 hours morphine consumption (median 34.6 mg [IQR 26.2;41.4] compared to HPS haplotype (472G, 408C, 186C) 19.4 [16.5;23.0] $P = 0.005$), but not compared to the LPS group (472G, 408G, 186C) (30.1 [19.1;37.7] $P = 0.13$). This effect was only observed in the group of patients that received intraoperatively fentanyl, but not in the remifentanil randomized group.	**NO EFFECT EXPERIMENTAL AND CHRONIC PAIN** **↑ CONSUMPTION**
Chapter 4	morphine fentanyl oxycodone hydromorphone	353	Opioid rotation in cancer patients (adults)	472G>A (rs4680) 408C>G (rs4818) 186C>T (rs4633)	None of the *COMT* genetic variants nor the haplotype composed of these variants was associated with the need to rotate to an alternative opioid.	**NO EFFECT RESPONSE**
Chapter 5	fentanyl morphine oxycodone	240	Cancer patients (adults)	472G>A (rs4680) 408C>G (rs4818) 186C>T (rs4633)	*COMT* 472G>A in combination with the *ORPM1* 118A>G variant was associated with the increase in morphine equivalent dose for sufficient analgesia. Patients carrying *OPRM1* 118G allele and *COMT* 472GG or these genotypes alone, have a higher median percentage dose increase (95.2% [32.8–345]) compared to *OPRM1* 118AA and *COMT* 472A allele carriers (48.5% [0–98.8]) ($p = 0.0016$). No associations were found between the *COMT* genetic variants nor the haplotype composed of these variants with absolute morphine equivalent dose or the use of ketamine.	**↓ CONSUMPTION**

Table 4. Effect *COMT* 472G>A (Val158Met) on pain and opioid response (continued)

REF.	OPIOID	N=	STUDY	SNPs	RESULTS	EFFECT
Chapter 6	-	136	Experimental (thermal) pain in children (8–18 yrs.)	472G>A (rs4680) 408C>G (rs4818) 186C>T (rs4633)	*COMT* 472G>A in combination with the *ORPM1* 118A>G variant was associated with cold and heat pain sensitivity (p = 0.002 and p = 0.004, resp.). Children with 118AA genotype and 472A allele have lower cold and heat pain thresholds (median = 15.1 [IQR 5.18–21.4] and 45.4 [41.9–48.6], resp.) compared to children with 118G allele and 472GG genotype (5.80 [0.65–16.9] and 47.7 [44.3–50], resp.).	**↑THERMAL PAIN**
Chapter 9	morphine	64	Neonates on mechanical ventilator	472G>A (rs4680)	*COMT* 472G>A in combination with the *ORPM1* 118A>G variant was associated with the need for morphine rescue. Neonates carrying *OPRM1* 118AG allele and *COMT* 472GG or these genotypes alone, have a higher risk to require morphine for analgesia during mechanical ventilation (OR: 5.12; 95% CI: 1.12–23.3; p = 0.035).	**↓PROCEDURAL PAIN**
Chapter 10	morphine fentanyl	77	Opioid withdrawal in children (o-18 yrs.)	472G>A (rs4680)	The *COMT* 472G>A variant was not associated with the development or the severity of opioid induced withdrawal.	**NO EFFECT ON WITHDRAWAL**
LITERATURE						
Zubieta *et al.* 2003	-	29	Experimental pain (adults)	472G>A (rs4680)	Carriers of the *COMT* 472A allele had a decrease in mu-opioid system response in the dorsal anterior cingulate, anterior thalamus, and cerebellar vermis to pain. Carriers of the *COMT* 472A allele had higher pain ratings.	**↓ ACTIVATION MU-OPIOID SYSTEM** **↑EXPERIMENTAL PAIN**
Rakvag *et al.* 2005	morphine	207	Cancer patients	472G>A (rs4680)	Patients genotyped *COMT* 472GG require more morphine (155 ± 160 mg/24 h, n = 44) compared to 472GA (117 ± 100) and 472AA patients (95 ± 99; P = 0.025).	**↓CONSUMPTION**

Table 4. Effect *COMT* 472G>A (Val158Met) on pain and opioid response (continued)

REF.	OPIOID	N=	STUDY	SNPs	RESULTS	EFFECT
Reyes-Gibby *et al.* 2007	morphine	207	Cancer patients	472G>A (rs4680)	Patients genotyped *COMT* 472GG required 63% more morphine than 472AA and 472GA required 23% more morphine ($P = 0.02$). *COMT* 472AA genotype and *OPRM1* 118AA have lowest morphine consumption (87 mg/24 h; 95%CI = 57.1), whereas patients with neither 472AA nor 118AA genotype needed the highest morphine dose (147;100,180; $P < 0.012$).	**↓CONSUMPTION**
Rakvag *et al.* 2008	morphine	197	Cancer patients	Multiple SNPs	*COMT* APS haplotype (472A, 408C, A) need lower morphine dose compared to patients not having this haplotype ($P = 0.005$).	**↓CONSUMPTION**
Jensen *et al.* 2009	remifentanil	43	Experimental pain (adults)	472G>A (rs4680)	*COMT* 472A allele carriers report more pain after multiple exposures to a heat stimulus ($P = 0.024$). No effect was observed of this SNP on remifentanil response.	**↑THERMAL PAIN** **NO EFFECT RESPONSE**
Mobascher *et al.* 2010	-	57	Experimental pain (adults)	472G>A (rs4680)	*COMT* 472A allele carriers have an increased blood oxygen level-dependent response in the anterior cingulate cortex after painful laser stimulation (reflecting higher pain sensitivity).	**↑PAIN**
Vossen *et al.* 2010	-	78 37	Experimental pain (adults) Chronic low back pain patients Healthy controls	472G>A (rs4680)	In the individuals with chronic pain with the *COMT* 472A allele cortical pain processing was amplified. In contrast, in the healthy controls pain processing was reduced in carriers of the 472A allele.	**↓PAIN HEALTHY SUBJECTS** **↑PAIN CHRONIC PAIN SUBJECTS**
Klepstad *et al.* 2011	opioid	2294	Cancer patients	Multiple SNPs	None of the *COMT* genetic variants are associated with opioid consumption.	**NO EFFECT CONSUMPTION**
Laugsand *et al.* 2011	opioid	1579	Cancer patients	Multiple SNPs	None of the *COMT* genetic variants are associated with opioid-induced nausea or vomiting after correction for false discovery rate.	**NO EFFECT ADVERSE EVENTS**
Matsuoka *et al.* 2012	morphine	48	Cancer patients	472G>A (rs4680)	*COMT* 472AA genotyped patients have lower morphine requirement despite lower morphine concentrations.	**↓CONSUMPTION**
Sistonen *et al.* 2012	codeine	111	Breastfeeding mothers Toxicity infants	472G>A (rs4680) 408C>G (rs4818) 186C>T (rs4633)	The *COMT* variants does not predispose mothers or infants to CNS depression.	**NO EFFECT ADVERSE EVENTS**

Table 4. Effect *COMT* 472G>A (Val158Met) on pain and opioid response (continued)

REF.	OPIOID	N=	STUDY	SNPs	RESULTS	EFFECT
Hajj *et al.* 2012	morphine	44	Postoperative patients (adults)	472G>A (rs4680)	No association between this *COMT* genetic variant with postoperative morphine consumption or side effects was found.	**NO EFFECT CONSUMPTION AND ADVERSE EVENTS**
Jimenez *et al.* 2012	morphine	68	Children (3–17 yrs) undergoing adenotonsillectomy	472G>A (rs4680) 5 other SNPs	No association between *COMT* genetic variants with morphine response or side effects.	**NO EFFECT CONSUMPTION AND ADVERSE EVENTS**
Flavdad *et al.* 2012	opioids	2201	Cancer patients	Multiple SNPs	None of the tested *COMT* genetic variants were associated with opioid requirement.	**NO EFFECT CONSUMPTION**
Wachman *et al.* 2013	methadone buprenorphine	86	Neonatal abstinence syndrome	472G>A (rs4680)	Carriers of the *COMT* 472G allele have shorter hospital admission duration. Carriers of the *COMT* 472G allele require less frequently treatment with 2 or more medications.	**↑ RESPONSE**
Cargnin *et al.* 2012	morphine triptans	74 75	Chronic low back pain patients (adults) Migraine patients (adults)	472G>A (rs4680)	*COMT* 472A allele carriers have higher response to intrathecal morphine. *COMT* 472A allele carriers have lower response to triptans.	**↑ RESPONSE**
Henker *et al.* 2013	opioids	79	Postoperative patients (adults)	472G>A (rs4680) 408C>G (rs4818) 186C>T (rs4633) A>G (rs6269)	Patients genotyped 408GG had lower opioid consumption ($P = 0.04$). Patients genotyped 472AA had higher pain ratings ($P = 0.03$). Patients with the *COMT* LPS haplotype (472G, 408G, 186C, G) had higher pain rating ($P = 0.0013$) and opioid consumption ($P = 0.0024$).	**↑ POSTOPERATIVE PAIN**
Landau *et al.* 2013	fentanyl	106	Labour pain	472G>A (rs4680)	Woman genotyped *COMT* 472AA had a smaller decrease in pain after IV fentanyl administration ($P = 0.005$).	**↓ RESPONSE**
Ahlers *et al.* 2013	morphine	117	Postoperative patients (adults)	472G>A (rs4680)	Carriers of the *COMT* 472A allele have higher mean pain scores.	**↑ POSTOPERATIVE PAIN**

Table 4. Effect *COMT* 472G>A (Val158Met) on pain and opioid response (continued)

REF.	OPIOID	N=	STUDY	SNPs	RESULTS	EFFECT
De Gregori *et al.* 2013	morphine	109	Postoperative patients (adults)	472G>A (rs4680) 408C>G (rs4818) 186C>T (rs4633) A>G (rs6269)	Patients with the *COMT* APS/APS haplotype (472A, 408C, 186T, A) had lower postoperative morphine doses (mean consumption ± SD 15.0 ± 14.6 mg) compared to all other patients (22.3 ± 15.6 mg) (P = 0.011). Also an allele-dose effect was observed for the 472G>A variant alone (GG: 24.8 ± 19.3 mg; GA: 20.1 ± 12.5 mg; AA: 15.0 ± 14.6 mg; p = 0.047).	↓ **CONSUMPTION**
Kambur *et al.* 2013	oxycodone	1000	Postoperative woman (adults) Breast cancer	472G>A (rs4680) 408C>G (rs4818) 186C>T (rs4633) A>G (rs6269) And 18 SNPs	No association found between *COMT* genetic variants with thermal pain sensitivity or postoperative oxycodone consumption.	**NO EFFECT THERMAL PAIN AND CONSUMPTION**
Mamie *et al.* 2013	morphine	168	Children (4–16 yrs.) undergoing orthopaedic or abdominal surgery	472G>A (rs4680)	Carriers of the *COMT* 472A allele have higher pain scores during mobilization.	↑ **PAIN**
Sadhasivam *et al.* 2014	morphine	149	Children (6–15 yrs.) undergoing adenotonsillectomy	472G>A (rs4680) 408C>G (rs4818) 186C>T (rs4633) A>G (rs6269)	Carriers of the *COMT* LPS haplotype (472G, 408G, 186C, G) had higher odds of intravenous analgesic intervention need (2.6 (95% CI: 1.2–5.4; *P* = 0.022).	↓ **PAIN**
Zhao *et al.* 2014	tramadol	250	Postoperative patients (adults)	472G>A (rs4680)	No associations were found between the *COMT* genetic variants nor the haplotype composed of these variants with tramadol response.	**NO EFFECT RESPONSE**
Zhang *et al.* 2015	fentanyl	115	Postoperative patients (adults)	472G>A (rs4680) 408C>G (rs4818) 186C>T (rs4633) A>G (rs6269)	Patients with *COMT* HPS haplotype (A, 186C, 408C, 472G) consumed more fentanyl than APS and LPS haplotype during the first 24 and 48 hours (all *P* < 0.042) after surgery. No associations were found between the *COMT* genetic variants nor the haplotype composed of these variants with side effects.	↓ **CONSUMPTION** **NO EFFECT ADVERSE EVENTS**

Table 4. Effect *COMT* 472G>A (Val158Met) on pain and opioid response (continued)

REF.	OPIOID	N=	STUDY	SNPs	RESULTS	EFFECT
De Gregori *et al.* 2016	morphine	201	Postoperative patients (adults)	472G>A (rs4680) 408C>G (rs4818) 186C>T (rs4633) A>G (rs6269) rs165774 rs174696	A significant interaction between *COMT* 472G>A and rs4986936 in *ESR1* on opioid consumption was found ($P = 0.007$). Previous, stand-alone effect of the *COMT* 472G>A effect that was found on a subset of this study could not be confirmed in this larger sample size.	**NO EFFECT CONSUMPTION**
Lee *et al.* 2016	morphine	88	Children (5–18 yrs.) undergoing adenotonsillectomy	472G>A (rs4680)	*COMT* 472G>A is not associated with postoperative pain scores.	**NO EFFECT PAIN**
Elens *et al.* 2016	morphine remifentanil	34	Preterm newborns on mechanical ventilator	472G>A (rs4680)	Neonates with the *COMT* 472GG genotype needed more time before reaching sufficient analgesia compared to 472GA and 472AA genotyped neonates (285 ± 37, 137 ± 25, and 63 ± 15 minutes, resp.; $P = 0.0021$).	**↑ RESPONSE**
Chatti *et al.* 2016	morphine	129	Cancer patients	472G>A (rs4680)	*COMT* 472G>A is not associated with morphine consumption.	**NO EFFECT CONSUMPTION**
Somogyi *et al.* 2016	morphine	133 230 598	Postoperative patients (elective caesarean section, adults) Indian Malay Han Chinese	472G>A (rs4680)	In Chinese patients, *COMT* 472A allele carriers (2.8% and 5.8%) had higher incidence of postsurgical pain compared to 472GG (16.7%; $P = 0.0007$). This effect was not observed in the Malay or Indian cohorts. No effect of this variant was seen on postoperative morphine consumption.	**↑ PAIN** **NO EFFECT CONSUMPTION**
Nielsen *et al.* 2017	morphine	40	Experimental pain in healthy volunteers (adults)	472G>A (rs4680)	Carriers of the *COMT* 472A allele showed reduced morphine analgesia compared to non-carriers (GG) ($P = 0.04$).	**↓ RESPONSE**

(**Chapter 3**). Instead, we found that the *COMT* haplotype APS was associated with the highest postoperative opioid requirement. Based on the effect of these haplotypes on pain sensitivity, as discussed previously, high opioid consumption is expected in carriers of the HPS (high pain sensitivity) haplotype. Instead HPS carriers required the lowest amounts of postoperative opioids in this study. This haplotype was, however, again not predictive for opioid rotation in cancer patients (**Chapter 4**). As is the case with pain sensitivity, the effect of *COMT* genetic variability on opioid response remains inconclusive.

GENE-GENE INTERACTIONS

Epistasis, describing interactions between genes, is an important factor to consider for pharmacogenetics in analgesia with opioids. The effect of one allele on a particular phenotype can mask or alter the effect of another allele located at a different locus on the same phenotype [57]. The effect of the individual *OPRM1* 118A>G or *COMT* Val158Met SNPs was not evident on the requirement of morphine in adult cancer patients (**Chapter 5**) and the need for rescue morphine in newborns during mechanical ventilation (**Chapter 9**). Only after combination of these genotypes, a significant association was found. This interaction was also found on thermal pain thresholds in children (**Chapter 6**). The *OPRM1* 118A>G variant has been linked to reduced opioid efficacy in adults [42, 43], while the individuals with the *COMT* 158Met allele require lower amounts of opioids for pain relief [58–63]. Therefore, carriers of the *OPRM1* 118G allele should be grouped together with the *COMT* 158Val allele carriers. Others have also highlighted an interaction between these 2 genes [63–65]. The chance of an European individual having the *OPRM1* 118G risk allele (15%) and the *COMT* 158Met protective allele (50%) is approximately 8%. Hence, determining solely the *OPRM1* or *COMT* variant in clinical practice will generate invalid advice in 8 patients for every 100 individuals.

METHODS AND TECHNIQUES

Although the exploratory genotype-association design is relatively easily executed and powerful for analyzing the effect of (relative) common genetic variants, usually sample sizes are small. This makes these type of studies inappropriate for assessing multiple genes and rare genetic variants leading to extreme phenotypes (e.g. non-response, severe toxicity). In multi-center studies power and external validity is increased but, confounding is introduced by differences between sites in treatment guidelines or type of patients. The latter factor is especially of importance when multiracial differences exist with background prevalence rates amongst the populations. The exploratory, case-control or observational

study design with prospective sample collection for DNA analysis (**Chapter 3, 4, 5, 7, 8, 9**) is a commonly accepted, feasible and valid approach [66]. In the studies described in **Chapter 6** and **10,** informed consent for PGx analysis was gathered retrospectively (**Chapter 6, 10**). This retrospective approach has the limitation of lower inclusion rates due to lost to follow-up (e.g. patient deceased, inaccurate contact information). Saliva and buccal swabs collection methods surpass the need for blood collection and thereby the need for subjects visiting the hospital, a higher inclusion rate could be reached. A limitation of this collection method is, however, that lower amounts of DNA are retrieved, which may complicate DNA analyses. See **Table 5** for an overview of different study designs with the advantages and disadvantages for PGx research [66–69].

An alternative and more efficient approach for advancing the PGx research field is by setting up a genomic biobank linked to the electronic health record (EHR). This has already been realized by the electronic MEdical Records and GEnomics (eMERGE) network [70]. With this method a broad informed consent applies making the material suitable for multiple studies in the future. However, documentation in the EHR is subjective to input errors and incomplete information for answering specific study aims. Additionally, ethical concerns with respect to the unknown study aim during sample collection are raised. A more controlled setting reducing confounding and selection bias is reached with a randomized controlled trial. With such an approach, if sufficiently powered, the effect of PGx-guided dosing on clinical outcomes and cost-effectiveness can be demonstrated.

Table 5. Pharmacogenetics research: pros and cons study designs

Study design (this thesis)	**Advantages**	**Disadvantages**
Case-control	• Feasible execution • Powerful for assessing common variants and common traits • Comparison outliers (cases) versus controls	• Small sample size • Active inclusion for DNA analysis
Observational cohort	• Feasible execution • Powerful for assessing common variants and common traits	• Small sample size • Active inclusion for DNA analysis
Study design	**Advantages**	**Disadvantages**
Biobank linked to EHR	• Informed consent available • Broad informed consent • Infinite use material	• Incomplete information for research purposes • Ethical concerns
Randomized Clinical Trial	• Controlled setting • Confounding reduced • Selection bias reduced	• Expensive • Time consuming (depending on follow-up period)
Genome wide approach	• Hypothesis generating (novel discoveries)	• Large sample size required • Validation cohort required
In vitro study	• Controlled setting • Function SNP on molecular level	• Depending on cell lines relevant proteins might not be expressed

Table 6. Pharmacogenetics research: pros and cons genotyping platforms

Analytical technique (this thesis)	**Advantages**	**Disadvantages**
PCR-RFLP	• Low cost • Easy data interpretation	• Manual • Time consuming • Individual SNPs
Real-Time PCR (TaqMan)	• Automated • Low cost • Easy data interpretation	• Individual SNPs • Time consuming if > 5 SNPs determined
Analytical technique (literature)	**Advantages**	**Disadvantages**
Micro-arrays	• Time efficiency • Reduction costs personal	• Difficult data interpretation / qualified personal required • No de-novo variants
Sequencing	• Full coverage SNPs in individual	• Expensive • SNPs with unknown effect • Difficult data interpretation / qualified personal required

Besides the study design that is of relevance for PGx research, also the genotyping platform is of importance. See **Table 6** for the advantages and disadvantages of the most frequently used techniques. In the case of only a handful of relevant genetic variants, the PCR-FLP and Real-Time PCR techniques are sufficient. In the case of pre-emptive genotyping, the micro-array based techniques, including pre-spotted variants from hundreds of genes, are more cost-efficient. This strategy is applied in the PG4KDS protocol (St. Jude Children Research Hospital), where all children receiving care are pre-emptively genotyped for 230 genes [71]. While rare polymorphisms can be included in micro-arrays, the selection is always lagging behind due to the de novo genetic variants detected in individuals. Sequencing-based approaches such as next generation sequencing and whole exon sequence can overcome this issue [72], although these methods are so far accompanied with high costs and SNPs with unknown functional effect. This type of data requires software, which can be handled by skilled personal in transferring the data to clinical phenotypes and an infrastructure of high level bioinformatics facility.

MAIN CONCLUSIONS

Based on the research described in this thesis we conclude that:

- *OPRM1* 118A>G and *COMT* Val158Met genetic variants are of importance for explaining pain variability and opioid response. These genetic variants remain the only ones associated with pain sensitivity and need for analgesia despite testing also other genes concordantly. Furthermore also when these *OPRM1* And *COMT* variants were selected

beforehand, due to limited sample size for addressing more genes, often associations were found with pain sensitivity and opioid response. (**Chapter 3, 5, 6, 9, 10**).

- The *OPRM1* 118A>G and *COMT* Val158Met genetic variants should always be examined concordantly (**Chapter 5, 6, 9**).
- Although the *OPRM1* 118A>G and *COMT* Val158Met SNPs are related with pain and opioid response, these variants are less likely to predict extreme phenotypes necessitating opioid rotation (non-response or side effects) in adult cancer patients or the occurrence of withdrawal in children at the intensive care setting (**Chapter 4, 5, 6, 9, 10**).
- Based on literature the impact of *OPRM1* 118A>G is most evident for increased morphine demand in the acute pain setting (postoperative). We were unable to demonstrate this effect on postoperative morphine consumption in our cohort of (mostly!) males undergoing cardiac surgery (**Chapter 3**).
- In addition to the adult literature our studies demonstrate that *UGT2B7* and *SLC22A1* genotypes can partly explain the variability in morphine and tramadol disposition in neonates.
- *UGT2B7, SLC22A1, OPRM1* and *COMT* genotype most likely can be translated to genotype predicted phenotype activity in the pediatric population, despite the fact that full activity of the enzymes, transporters and receptors is not reached for some of the proteins encoded by these genes (**Chapter 6-10**).
- The use of saliva and buccal swabs kits (**Chapter 6, 10**) are child-friendly and valid methods of material collection when no intravenous access is available (anymore). Although a lower amount of DNA is retrieved with these type of collections, and problems might be encountered when using highly sensitive genotyping techniques.

FUTURE PERSPECTIVES

Ideally, PGx-testing should be performed pre-emptively in order to have a beneficial effect on opioid therapy. However, the question arises whether we are ready for pre-emptive clinical implementation? At this time the answer is NO. There are some steps that have to be taken to change this answer into YES.

- While the *OPRM1* 118A>G and *COMT* Val158Met SNPs are frequently found to relate with more/less pain and more/less opioid consumption (this thesis), genotyping these variants as a stand-alone test seems of low clinical importance. Also when using the combination of this *OPRM1* and *COMT* genetic variant.
- Moreover, contradictory results have been retrieved for these genes related to different pain phenotypes and opioids. While it is more complicated to assess the differences between pain phenotypes, future studies should address in an experimental setting in

healthy subjects if the response is affected equally using validated endpoints for all opioids in individuals carrying these variants.

- PGx-markers that predict extreme reactions to opioids such as extreme high doses required for sufficient analgesia, the occurrence of severe adverse events or extreme withdrawal symptoms, would be more easily adapted in the clinical practice. Very recently genetic variants, including the *OPRM1* 118A>G SNP, have been related with respiratory depression in the pediatric population [73-75]. Replication is warranted since these studies were all assessed separately in the same cohort without correction for multiple testing. This should be analyzed in independent cohorts with also other pain phenotypes and opioids. Moreover, also the consequence of *OPRM1* 118A>G on the risk for respiratory depression is so far inconclusive [76, 77].
- The evidence for genotyping OCT1 seems more solid, as no contradictory results are retrieved and the possible outcome (severe toxicity) is of more clinical importance. The *SLC22A1* genetic variants should always be determined concordantly with *CYP2D6* genetics for codeine and tramadol, whereas for morphine testing CYP2D6 is not required. In contrast with our study, which has included the *SLC22A1**2-*6 alleles, this list should be extended to the *7-*15 alleles. These alleles also have a significant impact on activity and are common in non-Caucasian populations [26]. Especially, in cities located in the Randstad (conurbation of Western Holland), which is inhabited by global ethnic populations.
- Due to the contradictive findings, our small cohort in premature neonates and the large cancer cohort in relation to an effect of UGT2B7 genetics on morphine pharmacokinetics [14], testing of this gene does not seem to have an important role as stand-alone test in explaining morphine response. Future studies should always address concordantly other PK-related genes (*ABCB1, ABCC3, SLC22A1, UGT1A1, UGT1A8*), important for morphine disposition.

If we would wish to proceed to pre-emptive testing a set of 'pain' genes, the challenges that are in general applicable to PGx need to be overcome.

- Robust data by means of for instance large RCTs, addressing genotype-based versus conventional dosing strategies, are often requested from clinicians upon clinical PGx implementation [78]. An adult study comparing historical controls (n = 47) with PGx-guided analgesia (n = 50) following major abdominal surgery demonstrated a 50% reduction in analgesic consumption and less narcotic-related side effects in the PGx-guided group [79]. However, in this relative small study the exact effect sizes per SNP could not be assessed. In order to address this, large numbers of subjects are needed. The level of evidence needed for pre-emptive PGx implementation also largely depends on the effect size of the genetic variant. For example, genetic variants of the human leukocyte antigen (HLA), which have been related to hypersensitivity (severe cutaneous reactions, Steven Johnson syndrome) with abacavir, carbamazepine

and allopurinol [80], are one of the examples where the execution of RCTs would be highly unethical.

- Cost-effectiveness remains an important issue when implementing diagnostic screening tests. Currently several health insurances in the Netherlands reimburse retrospective genetic testing, because it is related to the therapy of the patient. However, the extreme increase in costs with this pre-emptive strategy in large patient groups would have to be lower than the reduction of costs due to possible reductions in toxicity and consequently hospitalizations.
- Clearly for a sufficient processing of PGx information in guiding pain therapy the health care systems should invest in the infrastructure and logistics. The NIH Pharmacogenomics Research Network has set-up the Translational Pharmacogenetics Program (TPP) with the aim to implement PGx across 8 health care systems in the United States [81].

In the future, we will hopefully optimize opioid efficacy and reduce adverse events based on pre-emptive testing of genetic variants in addition to the collection of other clinical characteristics that influence pain sensitivity (fear, previous experiences), opioid disposition (e.g. kidney function, drug-drug interactions) and opioid response (opioid history). In the meantime healthcare professionals should consider testing for both *CYP2D6* and *SLC22A1* genetics when toxic effects with codeine and tramadol are observed in individual patients, including the youngest pediatric age range. Therefore if the opioid, or another opioid metabolized or transported by the same route, will be required in the future and the patient is indeed genetically predisposed to toxicity '*we don't have to make the same mistake again*'.

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SLC22A1
COMT
UGT2B7

Chapter 12

Summary
Samenvatting

SUMMARY

Large interindividual variability in the experience of pain and the response to analgesic agents advocates towards a personalized treatment approach (precision medicine). The research described in this thesis aimed to analyze the potential impact of pharmacogenetics (PGx) in the analgesic treatment with opioids. PGx is a research field on the relevance of candidate genetic variants on pharmacological treatments. First, we assessed the literature for the highest potential candidate genes (**Part I**). We analyzed the significance of these PGx makers in the adult postoperative and cancer related setting (**Part II**). The main focus was, however, on PGx markers in the pediatric population. Due to the impact of growth and development on the disposition and effect of drugs, data from adults cannot be directly translated to this group (**Part III**). Finally all the findings are discussed, put in perspective and future directions are defined (**Part IV**).

Part I

In **Chapter 2** a review is presented on PGx studies in the field of analgesia with opioids. 4257 unique citations have been retrieved with a literature search in 5 databases (Embase, Medline (OvidSP), Web-of-Science, Cochrane and Google Scholar). After screening of the abstracts, 852 relevant citations were found. Eventually, based on our criteria a shortlist of 10 genes that seemed most promising for clinical use was made and discussed. This review concludes that most evidence is present for *CYP2D6, SLC22A1, OPRM1* and *COMT* genes. Moreover, this literature search underscores the need for PGx studies in children as very limited data is available for this vulnerable population.

Part II

In this part we aimed to cover different pain modalities (experimental, postoperative, chronic non-malignant and cancer related pain) in adults. The role of *OPRM1* 118A>G, *COMT* Val158Met SNPs and the *COMT* haplotype on thermally-induced pain, postoperative pain after thoracic surgery and the development of chronic pain is elucidated in **Chapter 3**. We were unable to illustrate the genetic effect of *OPRM1* or *COMT* variant on the different pain phenotypes. However, the COMT haplotype was associated with postoperative pain, with patients having the 'average pain sensitivity' haplotype requiring lowest postoperative morphine. No genetic associations were found in 356 oncology patients with opioid treatment failure (**Chapter 4**). Although the observed trend between treatment failure and the polymorphisms in *RHBDF2* and *OPRK1* requires further investigation. In another oncological setting (n = 340) the association of genetic variants (*ABCB1, ARRB2, COMT*, *GCH1, IL1RN, KCNJ6, METTL21A, OPRM1, RHBDF2*, *SCN9A, Stat6*) with morphine equivalent dose, relative increase in dose after consultation by the Palliative Care Team and the requirement of ketamine was analyzed (**Chapter 5**). Patients with

the *OPRM1* 118G and *COMT* Val158Val genotype, or these genotypes alone, had higher relative dose escalation (median = 95.2% [IQR: 32.8–345]) compared to the *OPRM1* 118AA genotype and *COMT* 158Met allele group (48.5% [0–98.8]) (p = 0.0016).

Part III

In **Chapter 6** the effect of genetic factors (*COMT, OPRD1, OPRM1*, *TRPA1, TRPV1, TAOK3, SCN9A*) on thermal pain sensitivity in children (n = 136, 8–18 years) was assessed, by exposing them to protocolized thermal stimuli with the so-called Thermal Sensory Analyzer. *OPRM1* (rs1799971) 118G allele carriers had decreased pain sensitivity compared to the wild type group. This was reflected by scoring the hot stimulus as less painful and reaching more frequently the minimum and maximum temperature limit (0–50°C). Moreover, higher pain thresholds were seen in carriers of the *OPRM1* 118G allele in combination with the *COMT* Val158Val genotype.

Due to developmental changes in drug metabolism or transport the effect of some genetic variants might not visible if the enzyme or transporter is not sufficiently expressed on tissue level. In order to address the interaction between this developmental variation and genetics, we have tested if genetic variants in *UGT2B7* (**Chapter 7**) and *SLC22A1* (**Chapter 8**) alter opioid PK in the neonatal population. *UGT2B7* and *SLC22A1* genetic variants had an effect on morphine and tramadol pharmacokinetics (PK), respectively. It remains to be established if these genetic variants also influence the analgesic response on these medications or predispose neonates to adverse events.

We investigated the relation with genetics in neonates on the mechanical ventilator (**Chapter 9**) and the development of opioid withdrawal in neonates and older children (**Chapter 10**), with both groups admitted to the intensive care unit. The combined *OPRM1*/*COMT* genotype was associated with the need for morphine rescue in 64 mechanically ventilated newborns. Carriers of the *OPRM1* 118G allele that also carried the *COMT* Val-158Val genotype required more frequently morphine rescue. In the cohort (n = 77) were we assessed the genetic effect on withdrawal these *OPRM1* and *COMT* genetic variants were not related with the development of withdrawal. However, we observed an association with the severity of withdrawal, showing more withdrawal related symptoms in carriers of the 118G allele.

Part IV

When analyzing variants in several genes, *OPRM1* 118A>G and *COMT* Val158Met remain the only SNPs associated. Furthermore also when these *OPRM1* And *COMT* variants were selected beforehand, due to limited sample size for addressing more genes, frequently associations were found with pain sensitivity and opioid response. Although these variants were associated with more or less pain and opioid demand, these genotypes are not predisposing patients to extreme clinical outcomes (need to rotate to alternative opioid,

risk factor for withdrawal). Due to this, these SNPs are unlikely to be implemented in the clinical practice as stand-alone tests. Besides quite some inconsistencies have been reported between pain phenotypes and opioids. The relevance of these SNPs between opioids needs to be addressed in an experimental pain study with healthy subjects. Variants in other genes that have been assessed in this thesis concordantly with the *OPRM1* and *COMT* variant seem to be less relevant in explaining the variability between pain sensitivity and opioid response or require validation (*RHBDF2, OPRK1*). The genotype to phenotype translations of *UGT2B7, SLC22A1, OPRM1* and *COMT* gene can be used in the pediatric population. Moreover, the use of saliva and check-swabs is a child-friendly method of material collection when no intravenous access is available. The importance of genotyping *SLC22A1*, in addition to *CYP2D6*, for codeine and tramadol seems more solid, since the possible outcome (toxicity) is of more clinical relevance. The assessed alleles should be extended to also common alleles in other non-Caucasian populations, which we did not include in our study. Clinicians should request genetic testing of *CYP2D6* and *SLC22A1* when severe codeine and tramadol toxicity or lack of response is presented in a patient.

SAMENVATTING

Er zijn grote verschillen in het pijnstillend effect van opiaten, en daarom wordt gepleit voor een gepersonaliseerde behandelstrategie (precisiegeneeskunde). Het onderzoek dat in dit proefschrift is beschreven richt zich op de rol van de farmacogenetica (PGx) bij de precisiegeneeskunde van pijnbehandeling met opiaten. Dit onderzoeksveld probeert te verklaren, en te voorspellen, hoe bepaalde genetische variaties (erfelijke aanleg) de manier waarop iemand reageert op medicijnen kunnen beïnvloeden. Allereerst is aan de hand van de literatuur een lijst samengesteld met genen die voor de klinische praktijk het belangrijkst lijken (**Deel I**). Deze zijn vervolgens onderzocht bij volwassenen, zowel patiënten voor en na een hartoperatie als bij patiënten met kanker (**Deel II**). Echter, de hoofddoelgroep voor dit proefschrift waren kinderen (**Deel III**). Bevindingen bij volwassenen kunnen niet simpelweg vertaald worden naar kinderen omdat er veel leeftijdsafhankelijke veranderingen plaatsvinden in de afbraak en respons op medicijnen. In het laatste hoofdstuk (**Deel IV**) worden alle bevindingen in perspectief geplaatst en worden aanbevelingen gedaan voor toekomstig PGx onderzoek bij de behandeling van pijn met opiaten.

Deel I

In **Hoofdstuk 2** is de literatuurstudie opgenomen waarin is gekeken naar PGx onderzoek bij de behandeling van pijn met opiaten. De zoekstrategie resulteerde in 4257 citaties uit vijf literatuurdatabanken (Embase, MEdline (OvidSP), Web-of-Science, Cochrane, Google Scholar). Na het lezen van de samenvattingen zijn 852 relevante publicaties overgebleven. Uiteindelijk is een lijst met 10 genen met de meeste potentie voor implementatie in de klinische praktijk opgesteld. Het meeste onderzoek is gedaan naar genetische variaties in *CYP2D6, SLC22A1, OPRM1* en *COMT*. Ook is gebleken dat er nog maar weinig onderzoek op dit gebied is gedaan bij kinderen.

Deel II

Dit deel van het proefschrift betreft onderzoek bij volwassenen, en wel met verschillende vormen van pijn. In **Hoofdstuk 3** gaat het om de invloed van de genetische varianten *OPRM1* 118A>G, *COMT* Val158Met en de *COMT* haplotype op pijn veroorzaakt door koude- of warmteprikkels, postoperatieve pijn na een hartoperatie en chronische pijn op de plek van de incisiewond. De enige relevante variant bleek het *COMT* haplotype te zijn, in de zin dat dragers van het 'gemiddelde pijngevoeligheid' haplotype postoperatief minder opiaten hadden gebruikt, wijzend op minder pijn. Uit een ander onderzoek, bij 356 mensen met kanker, bleek dat op de basis van genetische veranderingen niemand een ander type opiaat nodig had (**Hoofdstuk 4**). De trend die we in deze studie hebben gevonden voor *RHBDF2* en *OPRK1* met het overstappen naar een ander type opiaat (*RHBDF2*) en overstappen van oxycodon naar een andere type opiaat (*RHBDF2, OPRK1*)vereist wel

vervolgonderzoek. Tenslotte is in een studie bij 340 mensen met kanker gekeken naar het effect van een aantal genetische variaties (*ABCB1, ARRB2, COMT, GCH1, IL1RN, KCNJ6, METTL21A, OPRM1, RHBDF2, SCN9A, Stat6*) op opiaatbehoefte, relatieve verhoging in dosering na consultatie bij het palliatieve pijnteam en de toediening van ketamine (**Hoofdstuk 5**). Voor dragers van het *OPRM1* 118G allel of het *COMT* Val158Val genotype, of beide, werd de opiaatdosering significant meer verhoogd dan voor het *OPRM1* 118AA genotype in combinatie met dragerschap van het *COMT* 158Met allel (p = 0.0016).

Deel III

Het onderzoek beschreven in **Hoofdstuk 6** betreft 136 kinderen in de leeftijd van 8–18 jaar. We hebben onderzocht of hun pijngevoeligheid gerelateerd was aan genetische varianten. Voor dit doel werden ze blootgesteld aan warme en koude prikkels met de zogenaamde 'Thermal Sensory Analyzer'. Van de zeven onderzochte genetische varianten (*COMT, OPRD1, OPRM1, TRPA1, TRPV1, TAOK3, SCN9A*), bleek alleen het *OPRM1* 118G allel van invloed. Dragers van deze variant hadden een lagere pijngevoeligheid dan niet-dragers. Dragers van het G allel vonden de warme prikkel veel minder pijnlijk en bereikten vaker de minimale en maximale temperatuurlimiet (0–50°C). Daarnaast hadden dragers van het *OPRM1* 118G allel in combinatie met het *COMT* Val158Val genotype hogere pijndrempels.

Bij kinderen zijn processen zoals de absorptie, distributie, het metabolisme en de uitscheiding van geneesmiddelen gaandeweg de ontwikkeling aan verandering onderhevig. Het effect van genetische variaties zal dan niet zichtbaar zijn als de betreffende enzymen en transporters onvoldoende activiteit vertonen. We hebben het verband tussen ontwikkelings- en genetische aspecten onderzocht voor wat betreft variaties in *UGT2B7* (**Hoofdstuk 7**) en *SLC22A1* (**Hoofdstuk 8**). Dit zijn genen welke coderen voor enzymen en transporters die betrokken zijn bij het metabolisme van opiaten. We konden concluderen dat zowel UGT2B7 als OCT1 (*SLC22A1*) bij de onderzochte pasgeborenen en zuigelingen voldoende activiteit vertoonde voor een zichtbaar effect tussen deze genotype-groepen. Vervolgonderzoek dient aan te tonen of deze genetische variaties ook invloed hebben op klinische uitkomstmaten zoals effectiviteit en bijwerkingen van opiaten.

De rol van genetische aspecten is ook onderzocht bij pasgeborenen aan de beademing (**Hoofdstuk 9**) en bij pasgeborenen en oudere kinderen met een verhoogd risico op ontwenningsverschijnselen (**Hoofdstuk 10**). Bij de pasgeborenen aan de beademing bleek het gecombineerde *OPRM1/COMT* genotype geassocieerd te zijn met de behoefte aan medicamenteuze pijnstilling met morfine. Dit was vooral het geval bij dragers van het *OPRM1* 118G allel in combinatie met het *COMT* Val158Val genotype. Bij het onderzoek naar ontwenning hebben we deze genetische varianten niet kunnen relateren aan het ontstaan van ontwenning. Wel vonden wijdat dragers van het *OPRM1* 118G allel meer symptomen van ontwenning vertoonden.

Deel IV

Van alle onderzochte genen zijn de *OPRM1* en *COMT* genen elke keer als hits naar voren gekomen. Ook wanneer alleen deze genen vooraf zijn geselecteerd, doordat de studiegrootte niet toereikend was voor variaties in meer genen, blijken de betreffende genetische variaties vaak gerelateerd aan de pijngevoeligheid en opiaatrespons. Alhoewel varianten in de *OPRM1* en *COMT* genen gerelateerd zijn aan de mate van pijn en opiatenconsumptie, leidt dragerschap niet tot de noodzaak om te veranderen van type opiaat of een verhoogd risico op ontwenning. Hierdoor zullen deze genetische varianten zeer onwaarschijnlijk als losstaande toetsen worden ingezet in de klinische praktijk. Daarnaast worden veel tegenstrijdigheden gezien tussen pijnsoorten en type opiaten. Het belang van deze variaties voor verschillende type opiaten zou in een experimentele setting in gezonde vrijwilligers onderzocht moeten worden. De variaties in andere genen die onderzocht zijn naast de variaties in *OPRM1* en *COMT*, lijken minder relevant in het verklaren van de verschillen in pijnervaring en het opiaat respons of dienen nader onderzocht te worden (*RHBDF2, OPRK1*). Het onderzoek in dit proefschrift laat zien dat het effect van genetische variaties in *UGT2B7, SLC22A1, OPRM1* en *COMT* ook zichtbaar is bij kinderen. Het gebruik van kits om speeksel of wangslijmvlies af te nemen is een kindvriendelijke methode als er geen intraveneuze toegang voorhanden is. In verband met de mogelijke toxiciteit van codeïne en tramadol lijkt het testen van genetische variaties in *SLC22A1* en CYP2D6 wél klinisch relevant. Het verdient aanbeveling naar méér genetische variaties in *SLC22A1* te kijken dan wij hebben gedaan, om zodoende ook patiënten van niet-Kaukasiche etniciteit mee te nemen.

Part V

Appendices


SLC22A1
COMT
UGT2B7

List of abbreviations

ADME	Absorption, Distribution, Metabolism, Elimination
DME's	Drug Metabolizing Enzymes
DNA	Deoxyribonucleic acid
GA	Gestational age
M	Tramadol
M1	O-desmethyltramadol
M3G	morphine-3-glucuronide
M6G	morphine-6-glucuronide
MR	Metabolic Ratio
NICU	Neonatal Intensive Care Unit
PCR	Polymerase Chain Reaction
PD	Pharmacodynamics
PGx	Pharmacogenetics/Pharmacogenomics
PK	Pharmacokinetics
PICU	Pediatric Intensive Care Unit
PMA	Postmenstrual age
PNA	Postnatal age
SNP	Single Nucleotide Polymorphism
SOS	Sophia Observation withdrawal Symptoms scale
UGT	UDP-glucuronyltransferase
VAS	Visual Analogue Scale


SLC22A1
COMT
UGT2B7

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**These author's contributed equally.*

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TO BE SUBMITTED

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Matic M*, de Hoogd S*, de Wildt SN, Tibboel D, Knibbe CA, van Schaik RHN. Pharmacogenetics in cardiac surgery adult patients: implications of *OPRM1* and *COMT* polymorphisms on experimental, postoperative and chronic pain.

**These author's contributed equally.*


SLC22A1
COMT
UGT2B7

PhD portfolio

Name PhD student:	**Maja Matić**
Department:	Clinical Chemistry
	Intensive Care and Department of Pediatric Surgery
PhD period:	2012 – 2016
Promotors:	Prof. dr. R.H.N. van Schaik
	Prof. dr. D. Tibboel
	Prof. dr. S.N. de Wildt

	Year	Workload (ECTS)
General academic skills		
• Research Integrity	2014	0.3
• Biomedical English Writing	2013	2.0
• Presenting Skills for Junior Researchers	2013	1.0
• Biostatistical Methods I: Basic Principles (CCO2)	2013	5.7
Research skills		
• Basic Introduction Course on SPSS	2013	1.0
• Basic Human Genetics Course: Genetics for dummies	2012	0.5
• NIH Clinical Course: Clinical Pharmacology	2012–2013	1.0
• VAZA: Pop-PK en -PD onderzoek voor beginners	2015	0.5
Conferences, Seminars & Workshops		
• Pharmacology Days Rotterdam/Leiden: oral presentation (3x)	2013–2016	1.5
• ESPNIC Pharmacotherapy Workshop Rotterdam	2013	0.3
• Precision Medicine Symposium, Leiden	2014	0.3
• MolMed Day, Rotterdam: poster presentation (1x)	2014	0.3
• Pharmacogenetics Workshop (2x)	2014–2015	0.8
• Sophia Research Days: poster presentation (1x)	2014–2015	0.6
• Refereeravond 'Biobank kinderen onder de aandacht' Rotterdam	2015	0.1
• NVKFB Kinderfarmacologie opleidingsochtend, Rotterdam	2015	0.1
• Onderzoeksdag Klinische Farmacologie	2016	0.1
International conferences		
• SIMPAR 7th congress, Rome, Italy: oral presentation	2015	1.0
• IATDMCT 14th congress, Rotterdam, NL: oral presentation	2015	1.0
• ASCPT 2016 congress, San Diego, USA: poster presentation	2016	1.0
• 17th Golden Helix PGx Day, Rotterdam, NL	2016	0.5
Teaching activities		
• Supervising internship HLO student	2014	1.5
• Supervising internship medical student	2014	0.5
• Writing paper in educational journal for fellows perioperative medicine	2016	1.0

	Year	Workload (ECTS)
Other		
• Clinical Chemistry Research (monthly); multiple oral presentations	2012–2016	1.5
• Pharmacogenetics Work Floor meeting (weekly)	2012–2016	2.0
• Pediatric Pharmacology (weekly): multiple oral presentations	2013–2016	2.0
• Internal Oncology Research: oral presentation(2x)	2013–2014	0.6
• IC Clinical Pharmacology: oral presentation (1x)	2013	0.3
• Workgroup Scientific Integrity (bi-monthly)	2014	0.5
• Neurology Research: oral presentation (1x)	2015	0.3
• Horizon 2020: Organization Eu-PIC meeting (Erasmus MC)	2015	1.0


SLC22A1
COMT
UGT2B7

About the author

Maja Matić werd op 13 november 1986 in Zenica (Joegoslavië) geboren. In 2006 heeft ze haar atheneum diploma behaald aan het Comenius College te Capelle aan den IJssel. Daarna is zij gestart met de studie Farmacie aan de Universiteit van Utrecht, met als gevolg een bachelor diploma in 2009. Gedurende haar masteropleiding Farmacie heeft ze o.a. onderzoekstage gelopen op de afdeling Klinische Chemie in het Erasmus Medisch Centrum Rotterdam onder begeleiding van Prof. dr. R.H.N. van Schaik. Tijdens deze stage heeft Maja bijgedragen aan het manuscript "*Associatie van genetische variatie in CYP1A2 en UGT1A4 met metabole stoornissen bij gebruikers van clozapine en olanzapine* " in het PW Wetenschappelijk Platform. Daarnaast heeft Maja een keuzestage gelopen bij het Rijksinstituut voor Volksgezondheid en Milieu (RIVM) op het onderwerp 'evaluatie personalised medicine producten'. Hierbij heeft zij bijdragen aan het RIVM rapport "*Personalised medicine products: evaluation of the regulatory framework."*. Nadat zij haar master Farmacie in 2012 heeft gehaald is zij in het Erasmus Medisch Centrum in Rotterdam en Sophia Kinderziekenhuis gestart met promotieonderzoek naar de toepassing van farmacogenetica bij de behandeling van pijn, onder begeleiding van Prof. dr. R.H.N. van Schaik, Prof. dr. D. Tibboel en Prof. dr. S.N. de Wildt. Het huidige proefschrift is het resultaat van haar promotietraject.


SLC22A1
COMT
UGT2B7

Dankwoord

"*A boat doesn't go forward if each one is rowing their own way.*"

Swahili Proverb

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