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I, Vidya Chidambaran, hereby submit this original work as part of the requirements for the degree of Master of Science in Clinical and Translational Research.

It is entitled:

STUDY OF ASSOCIATION OF FAAH GENOTYPES WITH CLINICAL OUTCOMES AND HYPERCAPNIC VENTILATORY RESPONSE RELATED TO MORPHINE ADMINISTRATION IN POST-SURGICAL ADOLESCENTS

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Study of Association of FAAH Genotypes with Clinical Outcomes and Hypercapnic Ventilatory Response Related to Morphine Administration in Post-Surgical Adolescents

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by

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ABSTRACT

Background: Genetic susceptibility for morphine induced adverse effects like respiratory depression (RD), resulting from opioid-cannabinoid interactions, has not been studied. Anandamide, an endogenous cannabinoid, is degraded by Fatty Acid Amide Hydrolase, coded by gene FAAH. We hypothesized that FAAH variants will be associated with altered susceptibility to opioid adverse effects.

Methods: We prospectively recruited 101 White adolescents with idiopathic scoliosis/kyphoscoliosis undergoing spine fusion using standardized anesthesia and postoperative morphine patient-controlled analgesia. Blood samples were collected for genotyping FAAH variants and morphine pharmacokinetics. We measured hypercapnic response to 5% CO₂ (HCVR) before and after morphine administration, and followed subjects for morphine associated RD and post-operative nausea/vomiting (PONV) on postoperative day one.

Results: We found significant (p<0.0001) interaction for FAAH variants rs11576941, rs2295632, rs2295633, rs324420, rs6699322, rs3766246, rs45586133, rs6699322 and rs4141964 with morphine induced depression of HCVR, adjusted for morphine concentrations. Variant rs11576941 was significantly associated with PONV (OR 2.14; 95% CI 1.06-4.33; p = 0.0339), while rs11576941, rs2295632, rs45586133 and rs6699322 were marginally associated with RD after adjusting for sex, morphine and diazepam doses. HCVR was significantly more depressed in patients who developed RD compared to those who did not (p=0.0034). Thus, FAAH-HCVR association indirectly predicts risk of impending clinical RD in children receiving morphine. Using curated databases, we show that rs324420 is a missense variant, while most of the others are regulatory.

Conclusion: We have identified novel genetic predictors for opioid-endocannabinoid interactions leading to opioid adverse outcomes, supporting preemptive genotyping for RD risk prediction and safer optimization of opioid analgesia.
Contributions to the project

Vidya Chidambaran developed and managed the following roles for the project:
- Hypothesis design
- Literature review
- Design
- UC & CCHMC IRB approval
- Data collection, management, & cleaning
- Data analysis
- Writing of manuscript
- Creation of tables & figures

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List of Abbreviations

F AAH: Fatty Acid Amide Hydrolase

MV: Minute Ventilation

ETCO2: End-tidal Carbon dioxide

RR: Respiratory rate

RD: Respiratory depression

POD: Postoperative day

PONV: Postoperative nausea and vomiting

HCVR: Hyper Capnic Ventilatory Response

PCA: Patient Controlled Analgesia

SNP: Single nucleotide polymorphism

eQTL: Expression quantitative trait loci

GTEx: Genotype-Tissue Expression
INTRODUCTION

Fatty Acid Amide Hydrolase (FAAH) is a gene located on Chromosome 1, that codes for the enzyme FAAH, that hydrolyzes anandamide[^1][^2], an endocannabinoid in the human brain; anandamide acts on cannabinoid receptors, and also potentiates opioid action, as evidenced by its attenuation of naloxone-induced morphine withdrawal in mice[^3][^4]. Hence, in patients receiving opioids like morphine, factors that increase anandamide levels, or decrease its hydrolysis would be expected potentiate opioid adverse effects like respiratory depression and postoperative nausea/vomiting; as would be expected with FAAH variants with reduced function affecting anandamide hydrolysis (Figure 1).

A reliable measure of sensitivity to opioid induced RD is hypercapnic ventilatory response (HCVR), a quantifiable objective indicator which allows detection of impending or subclinical respiratory depression of opioid effect on ventilation. It is known that in analgesic doses, morphine causes a rightward shift and minimal depression of HCVR slope[^5], as measured by the validated rebreathing method[^6], which has been previously used to discern genetic effects on opioid response[^7]. In this study, we evaluate the effect of Fatty acid Amide Hydrolase (FAAH) variants on morphine induced depression of HCVR, and morphine associated adverse clinical outcomes, in an adolescent population undergoing invasive surgery (spine fusion) and requiring prolonged opioids via Patient controlled analgesia (PCA).

We recently reported nominal associations of FAAH variants with RD after outpatient tonsillectomy surgery[^8]; in this study, we postulate that use of a sensitive measure of subclinical RD such as HCVR, would allow improved identification of FAAH genetic effects on opioid related RD before they manifest clinically. To our knowledge, this has not been studied before. Uncovering such associations is important as it would help predict a priori, an individual’s susceptibility to serious opioid adverse effects, and thereby, allow use of preventive measures in high-risk individuals to avert occurrence of such adverse outcomes.
The interaction between the opioid and the endocannabinoid systems has been well documented in the control of pain, mood regulation, reward processing and the development of addiction\textsuperscript{9, 10}. However, the effect of this cross-talk on serious central opioid adverse effects like respiratory depression (RD) has not been studied, despite RD being responsible for fifty percent of postoperative respiratory failure events\textsuperscript{11, 12, 13}. This is especially relevant given concerns regarding increasing concomitant use of prescription opioids and marijuana in the United States\textsuperscript{14}.

HYPOTHESIS

We hypothesize that FAAH variants affect an individual’s ventilatory response to hypercarbia in the presence of morphine, which would be predictive of morphine induced clinical RD, and that FAAH variants influence susceptibility to morphine related clinical outcomes like postoperative nausea vomiting (PONV) and RD, in children undergoing spine fusion surgery.

SPECIFIC AIMS

Specific Aim 1: Determine if FAAH genotypes are associated with significant decrease in the slope of the hypercapnic ventilatory response, compared with baselines, after morphine administration in the post-surgical patient.

Hypothesis 1: Certain FAAH genotypes will affect morphine response and cause significantly more depression of the minute ventilation response to 5% carbon dioxide, compared to baseline response before morphine administration, independent of morphine concentration.

Specific Aim 2: Determine if FAAH genotypes are associated significantly with clinical side effects from morphine after spine surgery.

Hypothesis 2: Certain FAAH genotypes will be associated with respiratory depression (RD, defined as RR<8/minute for >3 minutes) and postoperative nausea vomiting (PONV) in post-surgical patients receiving morphine patient controlled analgesia.
METHODS

Study Design and Participants

This is a prospective, genotype blinded study conducted in a cohort of otherwise healthy children/adolescents aged 10-18 years, undergoing spine fusion surgery. The study was approved by the institutional review board. Written informed consent is obtained from parents and assent is obtained from children before enrollment. Exclusion criteria include known hypersensitivity to morphine, pregnant or breastfeeding females, respiratory impairment, use of opioids in the past 6 months or history of chronic pain, developmental delay and liver or renal diseases.

Perioperative Protocol and Clinical Outcomes Data collection

Demographics of all participants are recorded. All participants receive uniform perioperative care, including a standardized surgical technique and anesthesia. They receive incremental doses of morphine 0.05 mg/kg at the end of surgery titrated to respiratory rate. Postoperatively, they receive morphine through Patient Controlled Analgesia (PCA). Serial blood samples are obtained to quantify morphine, morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) concentrations - pre-dose sample followed by samples drawn at 0-5 min, 10-15 min, 30-45 min and 60-120 minutes after first dose. Patients are followed for 48 hours postoperatively. Amount of morphine and diazepam used in the perioperative period is recorded in mg for postoperative days (POD) 1. Doses and administration times of analgesics like acetaminophen, ketorolac and diazepam are also recorded. Per hospital protocol, all patients receiving PCA were monitored using continuous electrocardiography/respiratory rate (RR) module (for heart rate and RR by transthoracic impedance pneumography) and pulsoximetry (for oxygen saturation), with set alarm threshold of 8/minute for RR. The continuous monitor is directly captured on the ©Epic-based electronic medical record (Epic Systems Corporation, Verona, Wisconsin) in one-minute intervals and is available for review at any time.

Clinical Outcome Measures
Primary Outcome is morphine induced depression of minute ventilation response to 5% carbon dioxide, assessed by HCVR; Secondary outcomes are clinical morphine induced respiratory depression (RD) occurring from 2 to 24 hours after surgery, defined as RR<8/minute for >3 minutes; and postoperative nausea vomiting (PONV) defined as an actual episode of emesis.

Hyper Capnic Ventilatory Response (HCVR): The rebreathing method to measure HCVR, is conducted before surgery (no morphine) and after surgery (after morphine administration) under standard conditions. Patient initially breathes room air through a tight-fitting mask connected through a three-way valve to a reservoir bag with a volume more than 1.5 times their respective vital capacity volume. The instrument is calibrated before the determination of each HCVR slope. Tidal volume (TV), respiratory rate (RR), oxygen saturation (SpO2) and end-tidal CO2 (ETCO2) is continuously measured by a computerized exercise module (portable CO2SMO system, Philips Respironics, Murrysville, PA) with its bidirectional digital volume sensor, an infrared CO2 analyzer and pulse oximeter, and electronically captured in a laptop connected to CO2SMO system. After stable minute ventilation (MV = TV x RR) and the ETCO2 are recorded for a minute, a premixed gas mixture of 5% CO2 and 95% air is introduced through tubing close to the mask. The increasing concentration of CO2 is continued for approximately 2 minutes, ETCO2 value of 60 mm Hg, or if patient wanted to stop.

HCVR data cleaning: Each subject’s minute ventilation (MV in ml/kg/min) and ETCO2 pre-surgery and post-surgery were first plotted and studied for trends. Data were considered valid for inclusion in analysis based on following criteria, modified from Greenwald et. al. Inspired CO2>0 defined the post-CO2 data which corresponded with times noted for CO2 introduction. Firstly, we required that oxygen saturation had to exceed 94% at all times - so that hypoxia-influenced data were not included, since hypoxia is also a known respiratory stimulant. Secondly, we expect that MV-ETCO2 response is linear. Hence if the slope of MV/ETCO2 was negative or non-linear post-CO2, this data was excluded. Since a leak in mask fit due to subject being anxious, or face contour could lead to low MV values, we specified minimum
value of MV as 35 ml/kg/minute unless ETCO₂>50 mm Hg or RR<10/minute; we also specified that MV should be >0 when RR>0. Also, to rule out further errors, MV>200 ml/kg was excluded if it did not coincide with post-CO₂ period or if ETCO₂<50, which indicated the child might have been hyperventilating due to anxiety.

**Genetic analysis:** Blood is drawn upon intravenous line placement for genotyping. Deoxy ribonucleic acid (DNA) is isolated on the same day, frozen at -20°C and tested for single-nucleotide polymorphisms (SNPs) using TaqMan genotyping assays (Life Technologies, Applied Biosystems, Forest City, California) and a genome-wide genotyping (GWA) using the Illumina Human Omni5 v41-0 array Genetic data will be assessed for Hardy–Weinberg equilibrium (HWE) by means of goodness of fit χ² test.

**Pharmacokinetic (PK) Analysis for Morphine concentration at time of CO₂-MV test**

Morphine and its active metabolites, M3G and M6G, were quantified in Ethylenediaminetetraacetic acid (EDTA) plasma using a validated liquid chromatography tandem mass spectrometry assay. Details of the analytical methods have been previously described¹⁷. The reliable limits of quantification were 0.25–1000 ng/ml (r² > 0.99) for morphine, and 1–1000 ng/ml (r² > 0.99) for both M3G and M6G. Total imprecision was less than 15%. The inter-day accuracy was within 85–115%. A population pharmacokinetic model was developed for morphine using nonlinear, mixed effects modeling approach (NONMEM; version 7.2, ICON Dev. Soln., MD, USA) with PsN-Toolkit (version 3.5.3) as the interface. Data pre-processing, post-processing and visualization were performed using the statistical package R (version 2.15). A two compartment structural model, parameterized in terms of clearance (CL), central volume of distribution (V₁), inter-compartmental clearance (Q), peripheral volume (V₂) of distribution, was used to describe the morphine concentration–time profiles – described in detail elsewhere¹⁸. Based on the PK model developed, the concentration at the time of CO₂-MV test conducted in the postoperative phase was imputed, if the sampling schedule did not correspond with the test time.
DATA ANALYSIS

Descriptive statistics were generated for demographic and clinical data using mean, standard deviation for continuous variables and percentage and frequency for categorical variables.

We first evaluated the effects of non-genetic covariates (ETCO2, morphine concentration, age and sex) on MV response. Age was excluded from the model since it didn’t reach statistical significance (p<0.1). Next, to see if variations in the *FAAH* gene are associated with HCVR (Aim 1), we tested additive models with genotypes with MV as outcome and covariates sex, ETCO2, and estimated morphine concentration. Morphine concentration at baseline or pre-surgery phase were non-detectable and hence equaled zero. Then we examined if patients with different genotypes have different MV response over ETCO2 by including an interaction term between genotypes and EtCO2. We then proceeded to evaluate the effect of rs324420 on HCVR in more detail as it was the only hitherto known functional SNP. Besides the additive model to evaluate the effects of the three genotypes on MV-ETCO2 response, we also built a genotype model to compare Least squares means (LSM) of MV at time of HCVR, among the rs324420 genotype groups.

A post-surgery subset of data (conditions 3 and 4) were then used to examine association between HCVR and clinical RD on POD1 (Aim 2). To test this association, we generated a mixed model for MV as an outcome when an interaction term between ETCO2 and RD was included as a covariate. Other covariates were sex, age, total morphine dose (in mg/kg) received on POD1, and morphine concentration during HCVR. Subject IDs were used as a random effect. To test association of clinical outcomes, RD and PONV on POD1 with *FAAH* variants, we used logistic regressions including sex and morphine dose (mg/kg) on POD1 as covariates. In addition, valium dose on POD1 (mg/kg) was also included as a covariate for the regression on RD outcome. Data analysis was performed using SAS 9.4 (SAS, Cary, NC).
Functional genomics

Functional aspects of the variants with significant associations were researched by using freely available web-based engines, and presented in Table 4: a) rSNPBase, which is a database that provides reliable, comprehensive, and user-friendly regulatory annotations on SNPs annotated as regulatory (rSNPs) based on experimentally supported regulatory elements19. rSNPBase focuses on rSNPs involved in a wide range of regulation types, including proximal and distal transcriptional regulation and post-transcriptional regulation and identifies their potentially regulated genes. Per the database, we gathered evidence supporting causal and molecular mechanisms by which the SNPs evaluated might affect the phenotype studied. b) Genotype-Tissue Expression (GTEx) correlations between genotype and tissue-specific (available data from blood and brain tissue) gene expression levels identifying regions of the genome that influence whether and how much a gene is expressed as expression quantitative trait loci, or eQTLs. c) Genome-Wide Repository of Associations Between SNPs and Phenotypes (GRASP) searches genome-wide association study (GWAS) catalog data housed at the National Center for Biotechnology Information (NCBI) and reports genotype-phenotype associations with P<0.05 from GWAS defined as >= 25,000 markers tested for 1 or more traits. d) RegulomeDB (TM) Copyright ©2011, a database that annotates SNPs with known and predicted regulatory elements in the intergenic regions of the H. sapiens genome 20.

Power analysis: For HCVR power analysis we used PASS software (Hintze J.PASS 11. NCSS, LLC. Kaysville. Utah, USA. 2011.www.ncss.com). Our power analysis showed that 100 patients were required to detect 5% difference in MV among three genotypes when allele frequency is 10% using F test with 80% power, Mean=83.2 and SD=26.0 and a significance level of 0.015 (adjusted for multiple testing)
RESULTS

Demographics and data characteristics

Out of 120 children completing the study, one hundred and one children were estimated as white and thus were included in the genetic analyses (Figure 1, Panel A). They were mostly females (Table 1). The average morphine concentrations during the CO₂ test by PK analysis and morphine doses used by the cohort on postoperative days 1 are given in Table 1. The incidence of RD and vomiting on POD1 in the cohort was 27% and 23% respectively. Of the 101 Caucasian children, 18 did not complete CO₂ testing either due to lack of time, equipment issues or patient non-compliance.

Effect of non-genetic covariates on HCVR

The cleaned raw data for HCVR for study patients before and after morphine, are provided in Figure 2. The MV response was initially modeled with non-genetic covariates (ETCO₂, morphine concentration and sex). There was positive association between MV and ETCO₂ (β=1.63, p<0.0001) and negative association with morphine concentration (β=-0.73, p<0.0001), as would be expected. HCVR slopes are shifted to the right and have a depressed slope, after surgery (morphine), an expected response. Sex was also associated with MV response, with females having a higher MV response (p=0.0020).

HCVR and RD

HCVR slopes of patients who developed RD on POD1, when adjusted for morphine concentrations, were significantly different from the HCVR response in those who did not develop RD (p=0.0034). The slopes of those who developed RD were shifted to the right and depressed, compared to those of patients who did not develop RD (Figure 3), which indicates that the morphine induced depression of HCVR is an objective indicator of impending or subclinical clinical RD.

FAAH SNPs
The Human Omni5 v41-0 chip had a total call rate of 98%, and 93% call rate in the corresponding number of samples in the \( FAAH \) region analyzed (46854..46885 of chromosome 1 (GRCh37.p13)). This was higher for the Human Omni5Exome v41-1 chip with call rates of 99.7% and 99.5% respectively. While the former chip had 46 \( FAAH \) SNPs in this region, the latter chip had 57 SNPs, with 38 SNPs common to both arrays. Of these 38 SNPs, 9 met inclusion criteria and were considered for analysis. Four other SNPs not found on the chip array were genotyped by Taqman. The location per human genome build 19 \(^{21}\), the minor alleles, minor allele frequency (MAF) and p-value for HWE are given in Table 2. All SNPs satisfied the criteria specified in genetic analysis (Figure 1 Panel B).

\( FAAH \) variant association with HCVR (Aim 1)

Several of \( FAAH \) SNPs were associated with MV response to ETCO2 (Table 3, Figure 4) after adjusting for sex and morphine. Specifically, there was significant (\( p<0.0001 \)) associations between HCVR and genetic variants: rs11576941, rs2295632, rs2295633, rs324420, rs6699322, rs3766246, rs45586133, rs6699322 and rs4141964. There was high linkage disequilibrium between several of the SNPs studied, with a mean D prime of 0.967 (Figure 5).

Missense variant rs324420 (385C/A) effects on morphine induced depression of HCVR

Since rs324420 is the only missense variant (P129T) among those identified in the region, we evaluated the association of this SNP with outcomes further. The differential HCVR among the three genotypes of rs324420, adjusted for morphine concentration and sex, and derived from the additive model, is depicted in Figure 6. The AA genotype shows lower MV-ETCO2 slopes (and response) compared to CA and CC groups (\( p<0.0001 \)). Interestingly, the morphine concentrations at the time of CO\(_2\) response test post-morphine were different among the three genotype groups, with AA being exposed to the lowest (13.2 ± 7.7 ng/ml) and CC the highest concentrations (24.5 ± 14.0 ng/ml) (\( p\)-value=0.22). Further, the maximum morphine concentration in the AA group was 23.1 ng/ml while CC had concentrations up to 55.9 ng/ml with CA having intermediate maximum concentrations (45.1 ng/ml). We found that the Least squares
means of MV was higher for CC and CA groups (83.81± SE 4.5 and 80.19 ± 6.6 ml/kg/min respectively) compared to AA group (71.81.2 ± 13.2 ml/kg/min) (p<0.0008). Thus patients with AA genotypes needed much lower morphine concentrations by clinical titration, and surprisingly, despite lowest concentrations (about 50% of CC and CA genotypes), also had the least MV responses, compared with the other genotype groups. This is clinically significant as patients with AA genotypes may need about 50% less morphine exposure to minimize the risk of postoperative respiratory depression.

Genetic association with Clinical outcomes (Aim 2)

*FAAH* variant rs11576941 was associated with PONV (OR 2.14; 95% CI 1.06 - 4.33; p-value 0.0339) after adjusting for sex and morphine received on POD1. We found marginal associations (p value<0.1) of *FAAH* variants rs11576941, rs2295632, rs45586133 and rs6699322 with morphine related RD after adjusting for sex, morphine and diazepam doses received on POD1 (Table 3).

*FAAH* variant functional effects

Of the variants associated with the outcomes described above, rs324420 was the only one identified as a missense mutation with effects on eQTL in relevant tissues (blood/brain) (Table 4). GTEx portal provides values of -0.3 for effect size (p=3.9*10^-7) which is consistent with decreased activity of the variant. SNPs rs3766246, rs324420, rs45586133, rs2295633, rs11576941, rs2295632 and rs4141964 are regulatory SNPs involved in proximal and/or distal transcriptional, or RNA binding protein medicated regulation. None had known miRNA activity. We did not find any functional basis for SNPs rs6699322 and rs7520850, however they do affect eQTLs in some tissues. Although the GRASP database did not provide *FAAH* variant-phenotype associations for the opioid outcomes we have investigated, many of the *FAAH* variants including rs324420 and rs11576941 (which was associated strongly with HCVR and PONV and nominally with RD) are also associated with clinical phenotypes associated with behavior, addiction and social/neurological outcomes, which may indirectly be related to opioid outcomes.
Discussion

Our study reports significant associations of FAAH variants with HCVR and morphine related adverse effects in white children undergoing spine surgery. Specifically, we found significant genotype interaction with morphine induced depression of minute ventilation response to hypercapnia for FAAH variants rs11576941, rs2295632, rs2295633, rs324420, rs6699322, rs3766246, rs45586133, rs6699322 and rs4141964, after adjusting for sex and morphine concentration. In addition, we found that the AA genotype of rs11576941 had a 2.14 higher odds of PONV while FAAH variants rs11576941, rs2295632, rs45586133 and rs6699322 had marginal associations with RD related to morphine use after invasive surgery in children. To our knowledge, this is the first study to show an association of FAAH variants with morphine induced depression of HCVR, an indicator of subclinical or impending morphine induced respiratory depression. Importantly, patients who developed clinical RD had significantly more depressed HCVR compared to those who did not. Hence, by identifying FAAH variant effects on HCVR, we could potentially predict who would develop RD before an adverse outcome happened, so that improved monitoring and preventive measures (like decreasing opioid doses) could be proactively employed to avert such adverse outcomes.

The FAAH enzyme primarily metabolizes anandamide (N-arachidonoylethanolamine), which activates CB receptors. In addition, it inactivates the sleep-inducing lipid oleamide, anti-inflammatory N-acylethanolamines, the satiating factor N-oleoyl ethanolamine, and N-acyl taurines, which activate transient receptor potential family of calcium channels. FAAH variants that decrease FAAH activity, could hence increase anandamide levels in the brain as well as of the other lipid classes. In fact, synthetic CB receptor agonists were found to produce marked respiratory depression in rats, leading to hypoxia, hypercapnia, and arterial blood acidosis, which were all reversed by use of selective CB-1 antagonist. There are also two cases of respiratory depression from synthetic cannabis use. Both involved teens using cannabis in conjunction with other agents of substance abuse. These incidents further amplify the timely need to understand FAAH effects on RD in the wake of high synthetic cannabis use especially
among high school seniors (10.2% exclusive marijuana use and 62.4% marijuana use with tobacco products)\textsuperscript{25}.

The basis for anandamides having a synergistic interaction with opioids, arises from co-distribution of opioid and cannabinoid receptors in areas of the dorsal horn of the spinal cord \textsuperscript{26-28} and the brain (periaqueductal gray, raphe nuclei and central medial thalamic nuclei)\textsuperscript{29-31}, and common underlying molecular mechanisms\textsuperscript{32}. Some of the effects of cannabinoids involve activation of the opioid system\textsuperscript{33} and vice versa\textsuperscript{34}. In fact, CB agonists enhance the effect of \(\mu\)-opioid receptor agonists in a variety of models of analgesia\textsuperscript{35}. Of the variants studied, rs324420, in particular, has been found to influence opioid abuse potential, anxiety and reward\textsuperscript{36}. \textit{FAAH} SNP rs324420 is a common missense mutation (385C>A) which causes conversion of a conserved proline to threonine, resulting in a \textit{FAAH} enzyme with enhanced sensitivity to proteolytic degradation and reduced cellular stability\textsuperscript{37}. The AA genotype thus has decreased \textit{FAAH} activity, which leads to decreased anandamide hydrolysis and higher anandamide levels, thus potentiating morphine action on opioid receptors. This provides a functional basis for our observations in subjects with the AA genotype who had more depression of HCVR despite lower morphine concentrations, compared with AC and CC genotypes. Moreover, this SNP is in strong linkage disequilibrium with SNPs with predicted regulatory effects in the \textit{FAAH} gene region studied, with strong associations with HCVR and clinical outcomes like PONV (Table 4). Some of these SNPs in fact had stronger associations that the missense variant, for clinical outcomes, raising the possibility that more than one variant in the region contributes to risk.

Similar to our findings, a prospective genotype-blinded study in 259 White children aged six to 15 years, undergoing tonsillectomy, reported that \textit{FAAH} SNPs \textit{rs4141964, rs3766246, rs324420, rs2295632} and \textit{kgp12517369} increase the odds of PONV by more than 2-fold, and had nominal associations with RD and prolonged post-anesthesia care unit stay\textsuperscript{8}. The first four SNPs listed in the study above were associated with HCVR in our study, providing further validation of the effects of variants in that region on opioid
effects. Importantly, by using HCVR as an outcome in this study, we have been able to identify significant genetic associations with ventilatory response to morphine, which have never been described before. Since HCVR response was predictive of RD, we believe these results in a surrogate way, help identify those at risk of clinical RD. Identification of significant genetic association with clinical RD likely requires a larger sample size.

Although our patient population had scoliosis and the severity of scoliosis might be expected to affect HCVR, this has been studied before; the MV-ETCO₂ response was not found to be affected by the scoliosis angle (severity) was not found to be significant in a study that examined the effects of scoliosis on lung volumes and ventilatory response to CO₂ in patients with idiopathic scoliosis. The increase in minute ventilation in response to increasing ETCO₂ in our study mirrored previous reports in adolescents with scoliosis. The negative association of MV with increasing morphine concentrations is also in accordance with our premise that morphine causes a non-specific decrease in the slope of the rebreathing CO₂-MV response slope. However, the increased MV response in females compared to males, in the presence of morphine, was not expected. Although female rats were found to have a greater HCVR than male rats, Sarton et. al. found that morphine-induced changes in peripheral CO₂ sensitivity and apneic threshold, but not central sensitivity, was significantly lower in women compared to men. However, they used steady-state ventilatory response in their study unlike rebreathing technique that we used, which might account for differences.

We cannot rule out effects of propofol on HCVR post-surgery, as it has been reported to inhibit FAAH and increases brain anandamide levels. However, we took precautions to ensure EEG was at baseline, and subjects were awake and responsive before HCVR was tested after surgery; hence, we expect any propofol effect to be minimal. Moreover, all patients received standardized anesthesia, further decreasing the possibility of differential effects of anesthesia. We cannot rule out some unknown variable that are highly linked to the FAAH polymorphisms studied; sequencing of the entire FAAH gene might provide
additional information. Although we recruited non-Caucasian patients, the number of children belonging to other races was too small to study race as a predictor, and hence were not included. FAAH inhibitors have been explored for treatment of pain and nausea\(^{44,45}\); our findings spur further investigation of these agents to alter risk of opioid respiratory depression.

Despite above limitations, ours is the first study to report novel FAAH variant effects on morphine induced depression of HCVR. Unpredictable large inter-patient variations in opioid responses and narrow therapeutic indices of opioids result in a high incidence of postoperative opioid related respiratory depression (up to 41\%\(^{46,47}\), especially in children who are sensitive to opioids and differ in physiology and pharmacology from adults\(^{48}\). Identification of predictors for susceptibility remains key to preventing these events\(^{49}\). Twin studies have revealed significant heritability (30\%) for respiratory depression from opioids\(^{50}\); contributing non-genetic risk factors like female sex and medical comorbidities have been described\(^{51-53}\). Our study strengthens the evidence for FAAH effects on morphine outcomes in children, adds to the genetic risk factors reported previously - \(\mu_1\) opioid receptor (OPRM1)\(^{54}\) and ATP Binding Cassette (ABCB1)\(^{55}\) - and expands the knowledge base towards identifying genetic risk signatures for RD in children\(^{56}\).

In conclusion, we found novel associations between FAAH polymorphisms and morphine induced depression of the hypercarbic ventilatory response, and PONV, in white children undergoing spine fusion surgery and receiving morphine for analgesia. While we have presented curated evidence for a functional basis for these associations, causality for these effects needs to be further studied. One of the variants is known to be a missense variant (rs324420), while several of the other SNPs have a regulatory function or affect gene expression; many also have been associated with phenotypes like inflammation. Our current findings confirm associations between FAAH variants and PONV, and nominal associations with postoperative morphine induced respiratory depression, previously reported in an independent cohort of younger white children undergoing outpatient tonsillectomy. Our results support preemptive genotyping...
for improved prediction of risk for opioid-induced important adverse effects, namely, PONV and RD, and advance our understanding of inter-individual response variability to morphine therapy. These findings contribute to individualization of pediatric opioid analgesia in the postoperative setting, and potentially may be generalizable to use of opioids in adults and non-surgical settings like chronic pain.
Bibliography


42. Sarton E, Teppema L, Dahan A. Sex differences in morphine-induced ventilatory depression reside within the peripheral chemoreflex loop. *Anesthesiology*. 1999;90(5):1329-1338.


Appendix A

Table 1: Demographics and data descriptives for the study cohort by outcome

<table>
<thead>
<tr>
<th>Clinical outcomes (N=101)</th>
<th>Hypercapnic Ventilatory Response (HCVR) (N=83)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Age (years)</td>
<td>14.4</td>
</tr>
<tr>
<td>Sex (F/M, % females)</td>
<td>70/31 (70%)</td>
</tr>
<tr>
<td>Weight</td>
<td>56.9</td>
</tr>
<tr>
<td>Number of vertebral levels fused</td>
<td>11.5</td>
</tr>
<tr>
<td>Morphine dose POD1 mg/kg</td>
<td>1.1</td>
</tr>
<tr>
<td>Valium dose mg/kg</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Abbreviations: POD1: Postoperative day 1; MV: Minute Ventilation; ETCO2: End-tidal carbon dioxide.
Table 2: Descriptives of studied *FAAH* Variants

<table>
<thead>
<tr>
<th>Variants</th>
<th>hg19_location</th>
<th>Caucasians</th>
<th>Minor Allele Frequency</th>
<th>p-value HWE</th>
<th>Platform</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Major allele</td>
<td>Minor allele</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs17361950</td>
<td>46,864,150</td>
<td>G</td>
<td>A</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>rs3766246</td>
<td>46,865,671</td>
<td>G</td>
<td>A</td>
<td>0.39</td>
<td>OMNI</td>
</tr>
<tr>
<td>rs324420</td>
<td>46,870,761</td>
<td>C</td>
<td>A</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>rs45586133</td>
<td>46,873,039</td>
<td>G</td>
<td>A</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>rs2295633</td>
<td>46,874,383</td>
<td>G</td>
<td>A</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td>rs11576941</td>
<td>46,875,067</td>
<td>C</td>
<td>A</td>
<td>0.27</td>
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</tr>
<tr>
<td>rs6662982</td>
<td>46,877,180</td>
<td>G</td>
<td>A</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>rs6699322</td>
<td>46,882,118</td>
<td>G</td>
<td>A</td>
<td>0.29</td>
<td></td>
</tr>
<tr>
<td>rs7520850</td>
<td>46,883,668</td>
<td>G</td>
<td>A</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>rs2295632</td>
<td>46,879,562</td>
<td>C</td>
<td>A</td>
<td>0.32</td>
<td>TaqMan</td>
</tr>
<tr>
<td>rs324419</td>
<td>46,871,986</td>
<td>G</td>
<td>A</td>
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<tr>
<td>rs4141964</td>
<td>46,865,040</td>
<td>G</td>
<td>A</td>
<td>0.42</td>
<td></td>
</tr>
<tr>
<td>rs932816</td>
<td>46,859,749</td>
<td>G</td>
<td>A</td>
<td>0.30</td>
<td></td>
</tr>
</tbody>
</table>

HWE = Hardy-Weinberg Equilibrium
Table 3: Genotype-phenotype association of *FAAH* variants with experimental minute ventilation response to hypercapnia and morphine related clinical outcomes

<table>
<thead>
<tr>
<th>Variant</th>
<th>HCVR response genotype interaction</th>
<th>Postoperative Vomiting</th>
<th>Clinical Respiratory depression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β</td>
<td>p-value</td>
<td>Odds Ratio</td>
</tr>
<tr>
<td>rs11576941</td>
<td>0.759</td>
<td>&lt;.0001</td>
<td>2.14</td>
</tr>
<tr>
<td>rs17361950</td>
<td>-0.162</td>
<td>0.1204</td>
<td>0.74</td>
</tr>
<tr>
<td>rs2295632</td>
<td>-1.096</td>
<td>&lt;.0001</td>
<td>0.75</td>
</tr>
<tr>
<td>rs2295633</td>
<td>-0.539</td>
<td>&lt;.0001</td>
<td>0.77</td>
</tr>
<tr>
<td>rs324419</td>
<td>-0.239</td>
<td>0.029</td>
<td>0.69</td>
</tr>
<tr>
<td>rs324420</td>
<td>-0.686</td>
<td>&lt;.0001</td>
<td>0.85</td>
</tr>
<tr>
<td>rs3766246</td>
<td>-0.539</td>
<td>&lt;.0001</td>
<td>0.74</td>
</tr>
<tr>
<td>rs4141964</td>
<td>-0.633</td>
<td>&lt;.0001</td>
<td>0.7</td>
</tr>
<tr>
<td>rs45586133</td>
<td>2.694</td>
<td>&lt;.0001</td>
<td>1.13</td>
</tr>
<tr>
<td>rs6662982</td>
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<td>0.7997</td>
<td>0.71</td>
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<tr>
<td>rs6699322</td>
<td>-1.031</td>
<td>&lt;.0001</td>
<td>0.72</td>
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<tr>
<td>rs7520850</td>
<td>0.482</td>
<td>&lt;.0001</td>
<td>0.96</td>
</tr>
<tr>
<td>rs932816</td>
<td>-0.195</td>
<td>0.0562</td>
<td>0.83</td>
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</table>

HCVR= Hypercapnic Ventilatory Response
Table 4: Functional effects of FAAH single nucleotide polymorphisms (SNPs) and reported phenotype associations

<table>
<thead>
<tr>
<th>SNP</th>
<th>Missense SNP</th>
<th>rSNP(^a)</th>
<th>Proximal regulation (^b)</th>
<th>Distal regulation (^c)</th>
<th>miRNA regulation (^d)</th>
<th>RNA binding protein mediated regulation</th>
<th>eQTL (^e)</th>
<th>GRASP(^f)</th>
<th>Regulome DB(^g)</th>
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</thead>
<tbody>
<tr>
<td>rs3766246</td>
<td>no</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>yes*</td>
<td>Inflammation</td>
<td>5</td>
</tr>
<tr>
<td>rs324420</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>no</td>
<td>yes</td>
<td>yes*</td>
<td>Behavior, depression, addiction</td>
<td>3a</td>
</tr>
<tr>
<td>rs45586133</td>
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<td>yes</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>-</td>
<td>5</td>
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</tr>
<tr>
<td>rs2295633</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>Cognition, neuro, social, CVD</td>
<td>5</td>
</tr>
<tr>
<td>rs11576941</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
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<td></td>
</tr>
<tr>
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<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>yes*</td>
<td>Inflammation</td>
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<tr>
<td>rs7520850</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>yes</td>
<td>CVD, inflammation</td>
<td></td>
<td></td>
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<tr>
<td>rs2295632</td>
<td>no</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>yes*</td>
<td>Inflammation, CVD</td>
<td>2b</td>
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<tr>
<td>rs4141964</td>
<td>no</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>CVD</td>
<td></td>
</tr>
</tbody>
</table>

Explanation of terms and abbreviations used:

a) rSNP (regulatory SNPs) Ref: [http://rsnp.psych.ac.cn](http://rsnp.psych.ac.cn)

b) Proximal regulation: SNP involved in proximal transcriptional regulation.

c) Distal regulation: SNP involved in distal transcriptional regulation.

d) miRNA regulation: SNP within mature miRNA.

RNA binding protein mediated regulation: SNP involved in Ribonucleic acid binding protein-mediated post-transcriptional regulation

e) Expression quantitative trait loci (eQTL): derived from Genotype-Tissue Expression (GTEx) database:

*variants with significant eQTLs in blood/brain

f) GRASP: Genome-Wide Repository of Associations between SNPs and Phenotypes;

CVD: Cardiovascular disease; neuro: neurological; socio: socially related phenotypes

\(\text{g) RegulomeDB definitions of regulatory function}\)

- 2b: TF binding + any motif + DNase Footprint + DNase peak
- 3a: TF binding + any motif + DNase peak
- 5: TF binding or DNase peak
Endocannabinoid Pathway and Opioid Interaction

- Postsynaptic neuronal cell
  - Endocannabinoid Anandamide Synthesis
  - Endocannabinoid Effects

- Inactivation of FAAH increases Anandamide
  - Ethanolamine + Arachidonic Acid

- Fatty Acid Amide Hydrolase (FAAH)
- Cannabinoid Receptor-1 and 2 Agonism
- Pain Relief
- Inhibit Nausea
- Increase appetite
- Synergistic with opioid (potentiates opioid effects and adverse effects)

- FAAH polymorphisms contributes to Inter-individual variations in opioid effects

Befort K et al., Front. Pharmacol. 6, 6 (2015).
Minute Ventilation (ml/kg/minute)

End-tidal carbon dioxide (mmHg)

p = 0.0034, β = 0.309

Clinical Respiratory Depression (RD)
No Respiratory Depression (No RD)
227 eligible/approached participants

A: Enrollment

Consort Diagram

122 participants enrolled

Reason and number not recruited (95)
- Declined (43)
- Study Staff Unavailable (13)
- Enrolled in another study (37)
- Patient late – no time to consent (2)

Withdrawal (2) due to remaining intubated postoperatively

120 Study participants; 19 Non-Caucasian—data collected but excluded from analysis; So 101 Caucasians followed as below

Equipment malfunction
- Patient uncooperative

83 patients completed MV-ETCO2 testing

101 patients—Clinical outcomes

ETCO2 filtering criteria

75 patients’ data included for analysis

101 patients’ data included for genotype association analysis

4,301,332 SNPs genotyped by Human Omni5 v41-0 Array (73 patients)
4,641,218 SNPs by Human Omni5Exome v41-1 Array (36 patients)

B: FAAH Polymorphism

Selection Diagram

38 SNPs on Chr1: 46859939..46879520 (FAAH gene) with ~5kb upstream and 5kb downstream, version hg19

8 SNPs had <90% calling rate
7 SNPs had MAF<0.1
0 failed test of HWE (p<0.0001)
Monomorphic SNPs, n=14

9 SNPs genotyped by Omin5

4 SNPs genotyped by Taqman

13 SNPs included for interaction analyses with MV-ETCO2 response and Association with morphine adverse effect clinical outcomes
Minute Ventilation (ml/kg/minute) vs. End-tidal Carbon Dioxide (mmHg)

- CC
- CA
- AA

p < 0.0001, β = -0.686