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Fatty acid amide hydrolase–morphine interaction influences ventilatory response to hypercapnia and postoperative opioid outcomes in children

Aim: Fatty acid amide hydrolase (*FAAH*) degrades anandamide, an endogenous cannabinoid. We hypothesized that *FAAH* variants will predict risk of morphine-related adverse outcomes due to opioid–endocannabinoid interactions. **Patients & methods:** In 101 postsurgical adolescents receiving morphine analgesia, we prospectively studied ventilatory response to 5% CO₂ (HCVR), respiratory depression (RD) and vomiting. Blood was collected for genotyping and morphine pharmacokinetics. **Results:** We found significant *FAAH*–morphine interaction for missense (rs324420) and several regulatory variants, with HCVR ($p < 0.0001$) and vomiting ($p = 0.0339$). HCVR was more depressed in patients who developed RD compared with those who did not ($p = 0.0034$), thus *FAAH*–HCVR association predicts risk of impending RD from morphine use. **Conclusion:** *FAAH* genotypes predict risk for morphine-related adverse outcomes.

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Keywords: *FAAH* • fatty acid amide hydrolase • hypercapnic ventilatory response • morphine • opioid–cannabinoid interactions • postoperative • respiratory depression

The interaction between the opioid and endocannabinoid systems has been well documented in pain control, mood regulation, reward processing and development of addiction [1,2]. However, the effect of this cross-talk on serious central opioid adverse effects like respiratory depression (RD) is not well documented. This is especially relevant given concerns regarding increasing concomitant use of prescription opioids and marijuana in the USA [3]. The perioperative setting provides an ideal controlled clinical setting to evaluate this interaction as opioid-related RD is responsible for 50% of postoperative respiratory failure events [4–6]. A quantifiable objective indicator of sensitivity to opioid-induced RD is the hypercapnic ventilatory response (HCVR), measured by the rebreathing method [7,8]. This takes less time than the steady-state method and although less specific for opioid effects [9], it is the most suitable

method realistically possible in a pre- and postsurgical clinical pediatric setting [7,10]. It is known that in analgesic doses, morphine causes a reduction in HCVR slope [9,11]; this method has been previously used to discern genetic effects on opioid-induced RD [12]. In this study, we evaluate the effect of *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).

FAAH is a gene coding for the enzyme FAAH, which hydrolyzes endocannabinoids like anandamide and other lipid classes [13,14]. Anandamide acts on cannabinoid (CB) receptors, and also potentiates opioid action, as evidenced by its attenuation of naloxone-induced morphine withdrawal in mice [15,16]. Hence, in patients receiving opioids like

Vidya Chidambaran^{*1,2},
Valentina Pilipenko³,
Kristie Spruance¹, Raja
Venkatasubramanian^{†1}, Jing
Niu^{†1,4}, Tsuyoshi Fukuda^{2,4},
Tomoyuki Mizuno⁴, Kejian
Zhang³, Kenneth Kaufman⁵,
Alexander A Vinks⁴, Lisa J
Martin^{2,3} & Senthilkumar
Sadhasivam^{1,2}

¹Department of Anesthesia, College of Medicine, University of Cincinnati, Cincinnati, OH 45229, USA

²Department of Pediatrics, College of Medicine, University of Cincinnati, Cincinnati, OH 45229, USA

³Division of Human Genetics, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229, USA

⁴Division of Pharmacology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229, USA

⁵Center for Autoimmune Genomics and Etiology (CAGE), Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229, USA

*Author for correspondence:

vidya.chidambaran@cchmc.org

[†]Presently at College of Pharmacy, University of Florida, Orlando, FL 32827, USA

^{††}Presently at Shanghai Children's Medical Center, Shanghai, China

morphine, factors that increase anandamide levels, or decrease its hydrolysis would be expected to potentiate opioid effects, as would be expected with *FAAH* variants with reduced function affecting anandamide hydrolysis. We recently reported associations of *FAAH* variants with RD after outpatient tonsillectomy surgery [17]; 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. Thus, 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.

Patients & methods

Study design & participants

This prospective, genotype-blinded study was conducted between 2009 and 2015, under standard intraoperative anesthesia and postoperative analgesia with morphine PCA. It was approved by the institutional review board. This study is registered with ClinicalTrials.gov identifiers NCT01839461. Non-obese subjects with an American Society of Anesthesiologists physical status one (normal health) or two (mild systemic disease), aged 10–18 years, with a diagnosis of idiopathic scoliosis and/or kyphosis, undergoing elective spinal fusion were recruited. Written informed consent was obtained from parents and assent was obtained from children before enrollment. Exclusion criteria included known allergy 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 & clinical outcomes data collection

Demographics of participants were recorded (age, sex, race). Participants received uniform perioperative care, including a standardized surgical technique, general endotracheal anesthesia with intraoperative use of titrated propofol/remifentanyl infusions for anesthesia. Patients received incremental doses of morphine 0.05 mg/kg at the end of surgery titrated to respiratory rate (RR) of 16–18/min after discontinuation of remi-

fentanyl infusion. Depth of anesthesia was monitored using Bispectral Index (BIS®) XP (Aspect Medical Systems, MA, USA) [18] or electroencephalography criteria by the intraoperative neuro monitoring team, a standard monitor for these surgeries. Postoperatively, they received morphine through PCA. Serial blood samples were obtained to quantify morphine, morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) concentrations – predose sample was obtained before the morphine bolus dose followed by samples drawn at 0–5 min, 10–15 min, 30–45 min and 60–120 min after morphine was given. Patients were followed for 24 h postoperatively. Nurses adjusted PCA settings on the floor according to the physician's orders, based on patient needs. Doses and administration times of morphine, diazepam, other analgesics and antiemetics were recorded for postoperative days (POD) 1. Per hospital protocol, patients receiving PCA were monitored using continuous electrocardiography/RR module (for heart rate and RR by transthoracic impedance pneumography) and pulse oximetry (for oxygen saturation SpO₂), with set alarm threshold of 8/min for RR. The continuous monitor is directly captured on the ©Epic-based electronic medical record (Epic Systems Corporation, WI, USA) in 1-min intervals and is available for review at any time.

Clinical outcome measures

Primary outcome was morphine-induced depression of minute ventilation (MV) response to 5% carbon dioxide, assessed by HCVR, as detailed below. Secondary outcomes were a) clinical morphine-induced RD occurring anytime from 2 to 24 h after surgery, defined as RR < 8/minute for > 3 min; and b) PONV defined as an actual episode of emesis and/or episode of self-reported persistent nausea needing treatment with an antiemetic medication.

Hyper capnic ventilatory response

This test was conducted approximately an hour before surgery, before premedication was administered (no morphine) and an hour after surgery (after morphine administration) with patient initially breathing room air through a tight-fitting mask connected through a three-way valve to a reservoir bag with a volume > 1.5-times their respective vital capacity volume. Precautions were taken to prevent positional effect by having the child semi-reclined at both times. Patient was awake, responding to questions verbally, and breathing spontaneously after surgery before HCVR was conducted in the recovery room. The instrument was calibrated before determination of each HCVR slope. Tidal volume, RR, SpO₂ and end-tidal CO₂ (ETCO₂) were continuously measured by a computerized exer-

cise module (portable CO_2 SMO system, Philips Respiromics, PA, USA) with its bidirectional digital volume sensor, an infrared CO_2 analyzer and pulse oximeter, and electronically captured in a laptop connected to CO_2 SMO system. After stable MV (MV = tidal volume $\times$ RR) and $ETCO_2$ were recorded for a minute, a premixed gas mixture of 5% CO_2 and 95% air was introduced through tubing close to the mask. The increasing concentration of CO_2 was continued for approximately 2 min, $ETCO_2$ value of 60 mm Hg, or if patient wanted to stop.

Genetic analysis

Blood was drawn upon intravenous line placement for genotyping. DNA was isolated on the same day, frozen at $-20^\circ C$ and tested for four SNPs – rs932816, rs4141964, rs324419 and rs2295632 – using TaqMan genotyping assays (Life Technologies, Applied Biosystems, CA, USA). In addition, a genome-wide genotyping was performed on the Illumina® (CA, USA) Human Omni5 v41–0 array (73 patients) and Human Omni5Exome v41–1 (36 patients) – arrays were changed due to availability of new array which had more SNPs and functional ones. We selected SNPs within 5kb extension upstream and downstream of *FAAH* (GRCh37.p13 assembly Chr1:46859939 bp. 468 79520 bp [19]) and present on both genotyping arrays. All samples passed 95% threshold for call rate. Genetic data were assessed for Hardy–Weinberg equilibrium (HWE) by means of goodness of fit χ^2 test. Analysis for cryptic relatedness was performed using Graphical Representation of Relationships software [20]. Principal component analysis was employed to confirm European continental ancestry using 200 validated ancestry information markers. We found 97% concordance between self-reported race with predicted European ancestry. Patients who were estimated as being European ancestry were used in this study. To address multiple testing, we determined the average pairwise linkage disequilibrium (LD, as measured by r^2) for all SNP combinations, and calculated Bonferroni correction adjusting for the average correlation using Simple Interactive Statistical Analysis software [21]. Bonferroni critical value adjusted for correlation among SNPs was 0.013 to achieve a family wise error rate of 0.05. Only SNPs with call rates $>95\%$, HWE $p < 0.0001$ and minor allele frequency (MAF) $>10\%$ were included in the analysis.

Pharmacokinetic analysis for morphine concentration at time of CO_2 -MV test

Morphine and its active metabolites, M3G and M6G, were quantified in EDTA plasma using a validated liquid chromatography–mass spectrometry (LC–MS) assay. Details of the analytical methods have been previously

described [22]. The reliable LOQs were 0.25–1000 ng/ml ($r^2 > 0.99$) for morphine, and 1–1000 ng/ml ($r^2 > 0.99$) for both M3G and M6G. Total imprecision was $<15\%$. The interday accuracy was within 85–115%. A population pharmacokinetic (PK) model was developed for morphine using nonlinear, mixed effects modeling approach (NONMEM; version 7.2, ICON Dev. Soln., MD, USA). Data preprocessing, postprocessing and visualization were performed using the statistical package R (version 2.15). A two compartment structural model, parameterized in terms of clearance, central volume of distribution, intercompartmental clearance, peripheral volume of distribution, was used to describe the morphine concentration-time profiles – described in detail elsewhere [23]. Based on the PK model developed, the concentration at the time of CO_2 -MV test conducted in the postoperative phase was imputed, if the sampling schedule did not correspond with the test time.

HCVR data cleaning

Each subject's MV (in ml/kg/min) and $ETCO_2$ pre-surgery and postsurgery were first plotted and studied for trends. Data were considered valid for inclusion in analysis based on following criteria, modified from Greenwald *et al.* [24]. Inspired $CO_2 > 0$ defined the post- CO_2 data which corresponded with times noted for CO_2 introduction. First, 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. Second, we expect that MV- $ETCO_2$ response is linear [25]. Hence if the slope of MV/ $ETCO_2$ was negative or non-linear post- CO_2 , these data were 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/min unless $ETCO_2 > 50$ mm Hg or RR < 10 /min; 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- CO_2 period or if $ETCO_2 < 50$, which indicated the child might have been hyperventilating due to anxiety.

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 nongenetic covariates ($ETCO_2$, morphine concentration, age and sex) on MV response. Age was excluded from the model since it did not reach statistical significance ($p < 0.1$). Next, to see if variations in the *FAAH* gene are associated with HCVR, we tested additive models with genotypes

with MV as outcome and covariates sex, ETCO_2 and estimated morphine concentration. Then we examined if patients with different genotypes have different MV response over ETCO_2 by including an interaction term between genotypes and ETCO_2 . 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- ETCO_2 response, we also built a genotype model to compare least squares means of MV at time of HCVR, among the rs324420 genotype groups.

We also examined association between HCVR and clinical RD on POD1. To test this association, we generated a mixed model for MV as an outcome when an interaction term between ETCO_2 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 identifiers 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, diazepam 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, NC, USA).

Besides, functional aspects of the variants with significant associations were researched by using freely available web-based engines: rSNPBase, which is a database that provides reliable, comprehensive and user-friendly regulatory annotations on regulatory SNPs based on experimentally supported regulatory elements [26]; Genotype-Tissue Expression correlations between genotype and tissue-specific (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; Genome-Wide Repository of Associations Between SNPs and Phenotypes searches genome-wide association study catalog data housed at the National Center for Biotechnology Information and reports genotype-phenotype associations with $p < 0.05$ from genome-wide association study; RegulomeDB (TM) Copyright ©2011, a database that annotates SNPs with known and predicted regulatory elements in the intergenic regions of the human genome [27].

Power analysis

For HCVR power analysis we used PASS software (Hintze J. PASS 11. NCSS, LLC. UT, USA, 2011 [28]). 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 standard deviation = 26.0, and a significance level of 0.013 (adjusted for multiple testing).

Results

Demographics & data characteristics

Of 120 children completing the study, 101 children were estimated as white and thus included in the genetic analyses (Figure 1A). The average morphine concentrations during the CO_2 test by PK analysis and morphine doses on POD1 are given in Table 1. The incidence of RD and vomiting on POD1 in the cohort was 27 and 23%, respectively. Of the 101 children, 18 did not complete CO_2 testing either due to lack of time, equipment issues or patient noncompliance.

Effect of nongenetic covariates on HCVR

We found positive association between MV and ETCO_2 ($\beta = 1.63$; $p < 0.0001$) and negative association with morphine concentration ($\beta = -0.73$; $p < 0.0001$), as would be expected. HCVR slopes were 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 & 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 with those of patients who did not develop RD (Figure 2), which indicates that the morphine-induced depression of HCVR is an objective indicator of impending or subclinical 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. 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, nine met inclusion criteria and were considered for analysis. Four other SNPs not on the chip array were genotyped by Taqman. The location per human genome build 19 [29], the minor alleles, MAF and p-value for HWE are given in Table 2. All SNPs satisfied the criteria specified in genetic analysis (Figure 1B).

Figure 1. Subject enrollment in the study and *FAAH* variant selection for analysis. (A) provides the enrollment consort flow diagram showing flow from patients eligible for study to those whose data were included for analyses. (B) shows the criteria for *FAAH* variant selection and inclusion for association analyses. ETCO₂: End-tidal carbon dioxide; HWE: Hardy-Weinberg equilibrium; MAF: Minor allele frequency; MV-CO₂: Minute ventilation-carbon dioxide.

FAAH variant association with HCVR

Several of *FAAH* SNPs were associated with MV response to ETCO₂ (Table 3 & Supplementary Figure 1) after adjusting for sex and morphine. Specifically, there was highly significant ($p < 0.0001$) associations between HCVR and genetic variants: rs11576941, rs2295632, rs2295633, rs324420, rs6699322, rs3766246, rs45586133, rs6699322 and

rs4141964. There was high LD between many of the SNPs studied, with mean $D' = 0.967$ (Figure 3).

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 further the association of this SNP with outcomes.

Table 1. Demographics and data descriptives for the study cohort by outcome.					
	Clinical outcomes (n = 101)		Hypercapnic ventilatory response (n = 83)		
	Mean	SD	Mean	SD	
Age (years)	14.4	2.0	14.6	2.0	
Sex (female/male [% females])	70/31 (70%)		58/25 (69.9%)		
Weight (kg)	56.9	16.0	59.01	15.6	
Number of vertebral levels fused	11.5	1.9	11.4	1.8	
Morphine dose POD1 mg/kg	1.1	0.5	Morphine concentration at time of hypercapnic ventilatory response (ng/ml)	22.8	13.6
Diazepam dose mg/kg	0.12	0.08	MV/ETCO ₂ (ml/kg/min)	2.1	0.9
ETCO ₂ : End-tidal carbon dioxide; MV: Minute ventilation; POD1: Postoperative day 1; SD: Standard deviation.					

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 4](#). The AA genotype shows lower MV-ETCO₂ slopes (and response) compared with CA and CC groups ($p < 0.0001$). Interestingly, morphine concentrations at the time of CO₂ response test post-morphine were different among the 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; $p = 0.22$). 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). The least squares means of MV were higher for CC and CA groups ($83.81 \pm$ standard error [SE] 4.5 and

80.19 ± 6.6 ml/kg/min, respectively) compared with AA group ($71.81.2 \pm 13.2$ ml/kg/min; $p < 0.0008$). Thus patients with AA genotypes needed much lower morphine concentrations by clinical titration, and, despite lowest concentrations (about 50% of CC and CA genotypes), also had the lowest 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 RD.

Genetic association with clinical outcomes

These are presented in [Table 3](#). *FAAH* variant rs11576941 was associated with PONV (odds ratio: 2.14; 95% CI: 1.06–4.33; $p = 0.0339$) after adjusting for sex and morphine received on POD1.

Figure 2. Morphine concentration adjusted hypercapnic ventilatory response plots in patients with (bold line) and without clinical respiratory depression (dotted line). There was a significant difference in the slopes for these two groups of patients; patient who developed RD on postoperative day 1, had decreased hypercapnic response – with right shift and depressed slope of minute ventilation response to carbon dioxide. RD: Respiratory depression.

Table 2. Description of studied *FAAH* variants and their functional relevance from curated databases.

Platform	Variants	hg19_ location	Caucasians		p-value	Functional effects of <i>FAAH</i> SNPs with significant phenotype associations								RegulomeDB ^{§§}	
			Major/ minor allele	MAF		HWE	Mis-sense SNP	rSNP [†]	Proximal regulation [‡]	Distal regulation [§]	miRNA regulation [¶]	RNA BP-mediated regulation	eQTL ^{††}		GRASP ^{‡‡}
OMNI	rs17361950	46,864,150	G/A	0.33	0.1340	NS									
	rs3766246	46,865,671	G/A	0.39	0.3893	No	Yes	No	Yes	No	Yes	Yes	Inflammation	5	
	rs324420	46,870,761	C/A	0.22	0.0011	Yes	Yes	Yes	No	No	Yes	Yes*	Behavior, depression, addiction	3a	
	rs45586133	46,873,039	G/A	0.11	0.7929	No	Yes	Yes	Yes	No	Yes	–	–	5	
	rs2295633	46,874,383	G/A	0.38	0.2502		Yes	Yes	Yes	No	Yes	Yes	–	5	
	rs11576941	46,875,067	C/A	0.27	0.2530	No	Yes	Yes	Yes	No	Yes	Yes	Cognition, neuro, social, CVD	–	
TaqMan	rs6662982	46,877,180	G/A	0.33	0.1564	NS									
	rs6699322	46,882,118	G/A	0.29	0.1403	No	No	No	No	No	No	Yes*	–	–	
	rs7520850	46,883,668	G/A	0.10	0.0504	No	No	No	No	No	No	Yes	CVD, inflammation	–	
	rs2295632	46,879,562	C/A	0.32	0.31	No	Yes	No	Yes	No	No	Yes*	–	2b	
	rs324419	46,871,986	G/A	0.17	0.03	NS									
	rs4141964	46,865,040	G/A	0.42	0.71	No	Yes	No	Yes	No	Yes	Yes	Inflammation, CVD	5	
	rs932816	46,859,749	G/A	0.30	0.82	NS									
[†] rSNP Ref: [30]. [‡] Proximal regulation: SNP involved in proximal transcriptional regulation. [§] Distal regulation: SNP involved in distal transcriptional regulation. [¶] miRNA regulation: SNP within mature miRNA. RNA-BP mediated regulation: SNP involved in RNA-binding protein-mediated post-transcriptional regulation. ^{††} eQTL: derived from Genotype-Tissue Expression database: *variants with significant eQTLs in blood/brain. ^{‡‡} GRASP: Genome-Wide Repository of Associations between SNPs and phenotypes. ^{§§} RegulomeDB definitions of regulatory function. 2b: Transcription Factor binding + any motif + DNase footprint + DNase peak. 3a: TF binding + any motif + DNase peak. 5: TF binding or DNase peak. CVD: Cardiovascular disease; eQTL: Expression quantitative trait loci; HWE: Hardy–Weinberg equilibrium; MAF: Minor allele frequency; neuro: Neurological; NS: Not significant; rSNP: Regulatory SNP; Socio: Socially-related phenotype.															

†rSNP Ref: [30].

‡Proximal regulation: SNP involved in proximal transcriptional regulation.

§Distal regulation: SNP involved in distal transcriptional regulation.

¶miRNA regulation: SNP within mature miRNA.

||RNA-BP mediated regulation: SNP involved in RNA-binding protein-mediated post-transcriptional regulation.

††eQTL: derived from Genotype-Tissue Expression database: *variants with significant eQTLs in blood/brain.

‡‡GRASP: Genome-Wide Repository of Associations between SNPs and phenotypes.

ssRegulomeDB definitions of regulatory function.

2b: Transcription Factor binding + any motif + DNase footprint + DNase peak.

3a: TF binding + any motif + DNase peak.

5: TF binding or DNase peak.

CVD: Cardiovascular disease; eQTL: Expression quantitative trait loci; HWE: Hardy-Weinberg equilibrium; MAF: Minor allele frequency; neuro: Neurological; NS: Not significant; rSNP: Regulatory SNP;

socio: Socially-related phenotype.

Table 3. Genotype–phenotype association of <i>FAAH</i> variants with experimental minute ventilation response to hypercapnia and morphine-related clinical outcomes.								
Variant	HCVR response–genotype interaction		Postoperative vomiting			Clinical respiratory depression		
	β	p-value	Odds ratio	95% CI	p-value	Odds ratio	95% CI	p-value
rs11576941	0.759	<0.0001	2.14	1.06–4.33	0.0339	1.84	0.9–3.73	0.0932
rs17361950	-0.162	0.1204	0.74	0.37–1.46	0.3835	0.8	0.38–1.65	0.5374
rs2295632	-1.096	<0.0001	0.75	0.44–1.26	0.2795	0.55	0.3–1.02	0.0598
rs2295633	-0.539	<0.0001	0.77	0.41–1.43	0.3992	0.78	0.4–1.51	0.4571
rs324419	-0.239	0.029	0.69	0.34–1.37	0.2854	0.95	0.46–1.97	0.8926
rs324420	-0.686	<0.0001	0.85	0.42–1.71	0.6482	0.63	0.28–1.41	0.2615
rs3766246	-0.539	<0.0001	0.74	0.39–1.38	0.3374	0.75	0.39–1.47	0.4077
rs4141964	-0.633	<0.0001	0.7	0.41–1.18	0.1767	0.71	0.4–1.27	0.2519
rs45586133	2.694	<0.0001	1.13	0.43–2.98	0.8	2.54	0.95–6.76	0.0621
rs6662982	-0.026	0.7997	0.71	0.36–1.39	0.3163	0.82	0.4–1.68	0.5858
rs6699322	-1.031	<0.0001	0.72	0.36–1.44	0.354	0.44	0.19–1.02	0.0553
rs7520850	0.482	<0.0001	0.96	0.38–2.44	0.9295	2.11	0.83–5.37	0.1167
rs932816	-0.195	0.0562	0.83	0.46–1.48	0.5236	1.21	0.66–2.22	0.5434

HCVR: Hypercapnic ventilatory response.

FAAH variant functional effects

Of the variants associated with the outcomes described above, rs324420 was the only one identified as a mis-sense mutation with effects on eQTL in relevant tissues (blood/brain) (Table 2). Genotype-Tissue Expression portal provides values of -0.3 for effect size ($p = 3.9 \times 10^{-7}$), which is consistent with decreased activity of the variant. SNPs rs3766246, rs324420, rs45586133, rs2295633, rs11576941, rs2295632 and rs4141964 are predicted to be regulatory SNPs involved in proximal and/or distal transcriptional, or RNA-binding protein-mediated regulation. SNPs rs6699322 and rs7520850, affect eQTLs in some tissues.

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 MV 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 has 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 RD. Importantly, patients who developed clinical RD had significantly more depressed HCVR compared with 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-arachidonoyl ethanolamine), which activates CB receptors. In addition, it inactivates the sleep-inducing lipid oleamide, anti-inflammatory N-acyl ethanolamines, the satiating factor N-oleoyl ethanolamine and N-acyl taurines, which activate transient receptor potential family of calcium channels [31]. *FAAH* variants that decrease *FAAH* activity, could hence increase anandamide levels in the brain as well as of the other lipid classes. The basis for anandamides having a synergistic interaction with opioids, arises from co-distribution of opioid and CB receptors in areas of the dorsal horn of the spinal cord [32–34] and the brain (periaqueductal gray, raphe nuclei and central medial thalamic nuclei) [35–37] and common underlying molecular mechanisms [38]. Some of the effects of CBs involve activation of the opioid system [39] and

vice versa [40]. In fact, CB agonists enhance the effect of μ -opioid receptor agonists in a variety of models of analgesia [41].

Of the variants studied, rs324420 is the only missense mutation (c.385C>A); given the high level of LD among the SNPs studied, it is likely that the findings are driven by this SNP. SNP rs324420 has an estimated mutation age of 114,425–177,525 years [42], and causes conversion of a conserved proline to threonine (p.Pro129Thr), resulting in an *FAAH* enzyme with enhanced sensitivity to proteolytic degradation and reduced cellular stability [43]. In addition, relative to C/C, A-allele carriers of rs324420 were found to have 23% lower *in vivo* binding of *FAAH* in brain using positron emission tomography probe [44]. Decreases in *FAAH* efficacy have been shown to increase sensitivity to anandamide in *FAAH* knock-out mice (*FAAH*^{−/−}) [45]. The AA genotype thus has increased anandamide sensitivity, 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. This SNP is in strong LD with eight other SNPs with strong associations with HCVR and clinical outcomes like PONV.

Some of these SNPs in fact had stronger associations than the missense variant, raising the possibility that more than one variant in the region contribute to risk. Given the strong LD between these SNPs it would be difficult to statistically establish which of these SNPs are causally related to our outcomes. Bioinformatics searches suggested that several of these SNPs may have functional consequences through regulatory mechanisms such as post-transcriptional regulation by miRNAs and/or RNA-binding proteins. It is also possible that these SNPs alter long noncoding RNAs binding, DNA looping, DNA methylation or local DNA topology. Future studies will be required to evaluate these SNPs.

In our previous prospective genotype-blinded study in 259 white children aged 6–15 years, undergoing tonsillectomy, we reported that *FAAH* SNPs rs4141964, rs3766246, rs324420, rs2295632 and kgp12517369 increase the odds of PONV by more than twofold, and had nominal associations with RD and prolonged post-anesthesia care unit stay [17]. The first four SNPs listed above are also associated with HCVR in the present study, providing further validation. Importantly, by using HCVR as an outcome, we identified significant genetic associations with ventilatory response to morphine, which have never been described before. Since

Figure 3. The linkage disequilibrium plot for SNPs of within 46860–46880 kb of chromosome 1 in white children. Darker the black, higher the linkage. Total of 13 SNPs found in *FAAH* gene and 5 Kb upstream and downstream of *FAAH* were highly linked with a mean D' of 0.967 in white children, except rs324419 with rs2295632 and rs6699322. *FAAH* missense SNP rs324420 is underlined. Legend represents heat map in grayscale.

Figure 4. Hypercapnic ventilatory response for three genotypes of rs324420 are presented. The p-value and β are from the linear mixed model for the interaction term between genotypes of variant rs324420 and HCVR, adjusted for morphine concentration and sex. The model was built employing an additive allele effect. The AA genotype shows significantly depressed HCVR compared with CC and CA genotypes, with the minute ventilation response to CO_2 being shifted down and to the right. HCVR: Hypercapnic ventilatory response.

HCVR response was predictive of RD, these results in a surrogate way, may help identify those at risk of clinical RD. Identification of significant genetic association with clinical RD likely requires a larger sample size. More evidence for the opioid–CB interaction on RD comes from the fact that synthetic CB receptor agonists were found to produce marked RD in rats, leading to hypoxia, hypercapnia and arterial blood acidosis, which were reversed by use of selective CB-1 antagonist [46]. There are also two case reports of RD from synthetic cannabis use [47]. 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) [48]. In fact, the role of the endocannabinoid system in drug addiction has been a focus of recent research [49]. The functional SNP rs324420 has been found to influence opioid abuse potential, anxiety and reward [49]. Among 80 individuals with drug abuse or alcoholism and over 1000 controls, the odds ratio for risk of combined street drug use and problem drug/alcohol use was 4.9 (95% CI: 2.5–9.7) in rs324420 AA individuals [50], and results were consistent and even more significant in a larger sample with mixed ethnicity [42]. In contrast, the C allele has also been associated with increased risk for progression to cannabis dependence, although this result has not yet been replicated [51].

Although scoliosis in our patient population might be expected to affect HCVR, the MV-ET CO_2 response was not found to be affected by the scoliosis angle (severity) in a prior study [52], and the increase in MV in response to increasing ET CO_2 in our study mirrors previous reports in adolescents with scoliosis [53]. The negative association of MV with increasing morphine concentrations is in accordance with our premise that morphine causes a nonspecific decrease in the slope of rebreathing CO_2 -MV response slope [9]. Although increased MV response in females compared with males, in the presence of morphine, was an unexpected finding, female rats have been found to have a greater HCVR than male rats [54], and morphine-induced changes in central sensitivity, were found to be sex-independent [55,56]. We cannot rule out effects of propofol on HCVR postsurgery, as it has been known to inhibit *FAAH* [57]. However, we took precautions to ensure electroencephalography was at baseline, and subjects were awake and responsive before HCVR was tested after surgery; hence, we expect any propofol effect to be minimal. We also cannot rule out effects of some unknown variant 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.

Despite above limitations, ours is the first study to report novel *FAAH* variant effects on morphine-

induced depression of HCVR. Unpredictable large interpatient variations in opioid responses and narrow therapeutic indices of opioids result in a high incidence of postoperative opioid-related RD (up to 41%) [58,59], especially in children sensitive to opioids and who differ in physiology and pharmacology from adults [60]. Identification of predictors for susceptibility remains key to preventing these events [61]. Twin studies have revealed significant heritability (30%) for RD from opioids [62]; contributing nongenetic risk factors like female sex and medical comorbidities have been described [63–65]. Our study strengthens the evidence for *FAAH* associations with morphine outcomes in children, adds to the genetic risk factors reported previously – *OPRM1* [66] and *ABCB1* [67] – and expands the knowledge base toward identifying genetic risk signatures for RD in children [68]. *FAAH* inhibitors have been explored for treatment of pain and nausea [69,70]; our findings spur further investigation of these agents to alter risk of opioid RD.

Conclusion & future perspective

We found novel associations between *FAAH* polymorphisms and morphine-induced depression of the HCVR, and PONV, in white children undergoing spine fusion surgery and receiving morphine for anal-

gesia. 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 RD, 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 interindividual 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 nonsurgical settings like chronic pain.

Supplementary data

To view the supplementary data that accompany this paper, please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/pgs-2016-0147

Executive summary

Opioid–cannabinoid interaction & *FAAH*

- Increasing concomitant use of prescription opioids and marijuana legalization increases risk for respiratory depression from opioid–cannabinoid interactions.
- *FAAH* degrades anandamide, an endocannabinoid in the brain, as well as other lipid amides; loss of function variants could increase endocannabinoid levels and possibly potentiate opioid effects.
- We hypothesized that *FAAH* variants would be associated with risk of opioid-induced adverse effects in the postoperative phase where opioids are the mainstay of analgesia.

Hypercapnic ventilatory response & respiratory depression

- Hypercapnic ventilatory response (HCVR) measured by rebreathing is an objective indicator of impending morphine-induced respiratory depression (RD).
- We found that in our spine surgery population, HCVR was more depressed in patients who developed RD compared with those who did not.

FAAH association with HCVR

- 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 rs324420 is a missense variant, which decreases *FAAH* stability; hence, decreased anandamide degradation and increased endocannabinoid levels potentiating opioid response are a plausible explanation for the association found.

FAAH variants & opioid-related adverse clinical outcomes

- Variant rs11576941 was significantly associated with postoperative nausea vomiting, while rs11576941, rs2295632, rs45586133 and rs6699322 were marginally associated with RD.

Conclusion

- *FAAH* variant associations with HCVR are novel.
- Preemptive genotyping of *FAAH* genotypes would allow prediction of risk for opioid adverse effects.
- Our results further knowledge of the factors that affect personalization of opioid analgesia to optimize safety of opioid therapy in postsurgical patients, with extended implications for predicting risk of opioid-induced RD in prescription drug abusers and chronic pain.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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