

Supplementary Information for: Variability in the Analgesic Response to Ibuprofen is Associated with Cyclooxygenase Activation in Inflammatory Pain

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Supplementary Methods

Subjects. Healthy subjects (≥ 18 years of age) were recruited from patients referred to the Oral and Maxillofacial Surgery Service at the School of Dental Medicine and the Hospital of the University of Pennsylvania for extraction of one or more partially or fully bony impacted third molars. Upper third molars could be included in the surgical plan, if appropriate, but at least one lower tooth was required because upper extractions alone do not consistently lead to a significantly painful post-operative state (1). Subjects were excluded if they had 1) pain lasting more than one day for the two weeks prior to surgery, 2) a positive pregnancy test, 3) any serious medical illness that would make participation unsafe, 4) any history of psychological illness requiring medication or other treatment for more than three months, 5) any history of claustrophobia that could affect the subject's ability to tolerate the MRI study, 6) bleeding disorder or current use of anticoagulants by patient history, 7) use of any opioid medication more than three times in the last week, or 8) the presence of any implanted devices or metal objects in their body that might exclude them from having a MRI.

Study procedures. The study protocol was approved by the University of Pennsylvania Institutional Review Board, and all subjects provided informed consent. One week prior to their scheduled surgery, subjects attended a study visit to demonstrate the imaging process and to fill out measures for demographics, personality, mood, and pain coping skills, as previously described (2). They were asked to abstain from analgesics, including products containing NSAIDs, aspirin, and acetaminophen, high dose vitamins, and nutritional supplements until after surgery.

On the day of surgery, baseline blood and spot urine samples were collected. Subjects then underwent third molar extraction with 3% mepivacaine plain and 2% lidocaine plus 1:100,000 epinephrine for local anesthesia and nitrous oxide/oxygen and midazolam titrated to effect for sedation to ensure adequate pain management during the procedure and relatively rapid dissipation of the effect once surgery was complete. A trauma score was determined based on the difficulty of extraction, as previously described (3). After surgery subjects reported pain intensity every 15 minutes using the 0-10 Numeric Rating Scale (NRS-PI), where 0=no pain and 10=worst pain imaginable.

Approximately 45 minutes after completion of surgery, subjects were placed in the MRI to begin functional imaging data collection in intervals of 15 minutes. When the study subjects requested medication and reported a pain score $\geq 4/10$ or indicated that their pain was no longer tolerable, they received a dose of rapid-acting ibuprofen sodium (Advil® filmtabs) or matching placebo by mouth according to their randomization assignment. The blinded study medication and matching placebo were provided by Pfizer. After administering the study medication, MRI scanning continued for up to 60 minutes. For both the pre-medication and post-medication scans, the imaging data was collected with 4 minutes of structural scans to define anatomical reference, followed by continuous functional scanning in 15-minute intervals with pain levels recorded between each scanning segment. Subjects were encouraged to allow at least 60 minutes for the study medication to take effect before deciding if it had been ineffective, but rescue medication (hydrocodone 5 mg/ acetaminophen 325 mg) was allowed any time upon request.

Immediately after the scanning session, post-surgery blood and spot urine samples were collected, and subjects were returned to the post-operative observation area for continued pain assessment every 30 minutes for a total of two hours after treatment. After the first 7 subjects were enrolled, the study procedure was amended to include a second post-surgery blood and urine sample collection approximately 3 hours after study drug administration. This time point was added to evaluate the change in inflammatory response and ibuprofen concentrations over time during the acute post-surgical period. Once medically stable, the subjects were discharged home with a prescription for a 3-day supply of hydrocodone 5 mg/ acetaminophen 325 mg.

MRI Acquisition. A 3 Tesla Siemens Trio MRI system with a 32-channel phase arrayed head receiver was used. Subjects received ear plugs to reduce scanner noise, and foam padding was used to limit head motion. The scanning protocol began with a 3-axis localizer (13 sec) followed by a 3-dimensional MPRAGE anatomical scan with the following parameters: transversal orientation, in-plane resolution = $0.9 \times 0.9 \text{ mm}^2$, slice thickness = 1 mm, $\text{FOV}_{\text{read}}/\text{FOV}_{\text{phase}} =$

240/180 mm, TE/TR = 4/1850 ms, TI = 1110 ms, FA = 9 deg, GRAPPA factor 2, scan time = 4:13. The remainder of the scanning consisted of sequential measurements of regional cerebral blood flow (CBF) using arterial spin labeling (ASL)-MRI obtained with pseudo continuous labeling (4, 5) and a background-suppressed 3D stack-of-spirals readout (6) with the following parameters: 30 label/control pairs, 3.75 mm isotropic resolution, FOV = 240 x 240 mm, TE/TR = 15.9/4000 ms, $FA_{\text{excitation}} = 90$ deg, $FA_{\text{labeling}} = 28.7$ deg, label duration = 1500 ms, post-label delay = 1500 ms and scan time = 4:13.

MRI Data Processing. Perfusion MRI data were analyzed offline using established procedures by an investigator blinded to treatment assignment. Raw perfusion data underwent retrospective motion correction and outlier elimination (7), and mean CBF maps for each time point were then calculated using a single-compartment model (8), based on literature T1 values for the blood (1650 ms), labeling efficiency for pCASL (0.85) and blood-brain partition coefficient (0.9 mL/g) (9). The CBF maps were normalized to MNI152 template by combining transformations mapping the native CBF maps to native MPRAGE anatomical scan and native anatomical space to the MNI152 template space using FMRIB's Software Library (FSL). The time course of CBF changes in *a priori* regions of interest were extracted using the automated anatomical labeling (AAL) atlas (10) and a brain-wide pain-associated ROI was obtained based on large-scale automated meta-analytic synthesis based on 319 published fMRI studies from NeuroSynth ($p < 0.01$; FDR corrected) (11). The extracted CBF data from regions of interest were evaluated with standard statistical analyses to test for effects of rapid-acting ibuprofen sodium vs. placebo on neural activity. Whole-brain topologic inference was tested for multiple comparisons via the false discovery rate (12) and family-wise error correction based on Gaussian random field theory (13). However, due to the small group sizes no uncorrected p value survived multiple comparisons tests. Uncorrected p values were used to reveal brain areas showing trends, and color gradation based on Cohen's effect size were displayed in these brain areas.

Quantification of COX activity and plasma drug concentrations. COX-1 activity *ex vivo* was evaluated by quantifying serum thromboxane B₂ levels, as previously described (14). Briefly, whole blood was collected into vacuum tubes containing clot activator and incubated in a water bath at 37°C for 1 hour. Serum was separated by centrifugation and stored at -80°C until analysis by liquid chromatography/mass spectrometry (LC-MS).

COX-2 activity *ex vivo* was evaluated by quantifying plasma PGE₂ levels following lipopolysaccharide (LPS) stimulation in whole blood, as previously described (15). Briefly, heparinized whole blood was treated with aspirin (1mM) and incubated at room temperature for

15 minutes. LPS (*E. coli*, serotype O111:B4, 10 µg/ml whole blood) was added, and the sample was incubated in a water bath at 37°C for 24 hours. Plasma was separated by centrifugation and stored at -80°C until analysis by LC-MS.

COX activity *in vivo* was determined by quantification of urinary prostanoid metabolites by liquid chromatography mass spectrometry (LC-MS) as previously described (16). Systemic production of PGI₂, PGE₂, PGD₂, and thromboxane (Tx) A₂ was determined by quantifying their major urinary metabolites: 2,3-dinor 6-keto-PGF_{1α} (PGIM), 7-hydroxy-5,11-diketotetranorprostan-1,16-dioic acid (PGEM), 11,15-dioxo-9α-hydroxy-2,3,4,5-tetranorprostan-1,20-dioic acid (PGDM), and 2,3-dinor TxB₂ (TxM), respectively. Results were normalized to urinary creatinine.

Plasma concentrations of ibuprofen and acetaminophen were quantified by LC-MS as previously described (17).

Serum cytokines. Concentrations of interleukin (IL)-6, IL-1β, IL-8, IL-10, tumor necrosis factor (TNF)-α, and monocyte chemoattractant protein (MCP)-1 in serum were quantified by MILLIPLEX multiplex assay (Millipore) by the Radioimmunoassay and Biomarkers Core at the Diabetes Research Center at the University of Pennsylvania. The levels of IL-1β were below the limit of detection in the majority of samples, so further statistical analysis was not performed for this analyte.

CYP2C9 genotyping. Genomic DNA was isolated from buffy coat samples using the PureLink Genomic DNA Mini Kit (Invitrogen). TaqMan SNP Genotyping assays (ThermoFisher) were used to genotype *CYP2C9*2* (C_25625805_10) and *CYP2C9*3* (C_2710892_10) per the manufacturer's instructions.

Gene expression analysis. Peripheral blood mononuclear cells (PBMCs), composed primarily of monocytes and lymphocytes, were isolated from whole blood using a standard Ficoll-Paque density gradient centrifugation method. Total RNA was isolated using the RNeasy Miniprep Kit (QIAGEN) per the manufacturer's instructions. Total RNA from each sample was converted to sequencing libraries using the SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian (Clontech), following the manufacturer's protocol. Each sample was prepared using a unique dual-barcode combination. All libraries were pooled together at equimolar concentrations and test-sequenced with a single MiSeq run (2x150bp reads). The results of the MiSeq run were used to assess the balance of reads generated from each library in the pool, and the relative concentrations of the libraries in the pool were adjusted accordingly. The re-balanced pool was

then sequenced across 6 lanes on a HiSeq 4000 (2x150bp reads). Following QC of the pooled HiSeq data, a second pool of the libraries was sequenced to increase read depth.

Illumina adapter sequences were trimmed from the raw, gzipped FASTQ files using the BBDuk tool from the BBTools suite v37.99 (<https://jgi.doe.gov/data-and-tools/bbtools/>). Reads from the trimmed FASTQ files were aligned to GRCh38 build of the human reference genome using STAR v2.6.0c (18) and gene models from v92 of the Ensembl annotation (19).

Data were normalized and quantified using the Pipeline Of RNA-seq Transformations v0.8.5b-beta (PORT; <https://github.com/itmat/Normalization>), a resampling-based method that accounts for confounding factors like read depth, ribosomal RNA, and mitochondrial RNA content. PORT was run at the gene level in strand-specific mode and was provided with gene models from v92 of the Ensembl annotation. During normalization, 11 of a total of 77 samples were excluded for low read depth. Following normalization, pairwise differential expression (DE) analyses were performed on the gene-level read counts using *voom-limma* software package v3.34.0 (20). The data are accessible through GEO Series accession number GSE120596. Only genes with >0 reads across all samples in at least one of the two compared groups were used for pairwise DE analyses. Pathways enriched in each of the DE gene lists were identified using Ingenuity Pathway Analysis (IPA; Qiagen) (21).

Statistical analysis. The objective of the study was to investigate the factors associated with variability in the analgesic response to ibuprofen across multiple phenotypic domains, including pain ratings, consumption of rescue medicine, fMRI, pharmacokinetic and pharmacodynamics measures, inflammatory biomarkers and gene expression profiles. fMRI measurements were selected as the primary endpoint, and sample size was determined based on the number of usable scans needed to assess differences in cerebral blood flow within subjects between the pain state and the treated state. Since there were no studies to indicate what the variation might be in the important brain regions as pain relief on ibuprofen was achieved, this study was described as exploratory and a sample size consistent with the functional brain imaging literature chosen. Retrospective sample size analysis for the outcome that provided the rationale to investigate inflammation as the distinguishing factor between partial and complete responders, the urinary prostaglandin E metabolite (PGEM), shows a group size $n < 10$ provided 80% power to detect a significant difference ($p < 0.05$, t-test).

Data are reported as mean \pm standard deviation or median (25th percentile, 75th percentile). Baseline characteristics and biochemical measurements were compared by t-test or ANOVA or their non-parametric equivalents, as appropriate. Time to rescue medication treatment was

evaluated by log-rank test. Post-surgery measurements of COX activity *ex vivo*, urinary PG metabolite levels, and serum inflammatory mediators were normalized to baseline values for each subject and compared at each time point by Wilcoxon rank-sum test. $P < 0.05$ was considered statistically significant.

Supplementary Tables

Table S1. COX activity *ex vivo* and urinary PG metabolite levels by response group shown as median (25th percentile, 75th percentile).

	Placebo	Partial Responder	Complete Responder
Serum TxB ₂ (ng/ml)			
Baseline	166.9 (119.4, 209.0)	146.4 (110.2, 310.4)	160.7 (116.1, 303.0)
Post-surgery 1	128.4 (78.0, 199.7)	1.7 (0.6, 8.0)	7.4 (3.5, 8.7)
Post-surgery 2	167.1 (140.1, 180.3)	17.6 (3.8, 57.9)	10.9 (8.5, 18.8)
Plasma PGE ₂ (ng/ml)			
Baseline	23.9 (13.9, 45.4)	15.8 (10.6, 24.8)	18.3 (16.0, 43.5)
Post-surgery 1	8.3 (4.7, 15.5)	3.1 (1.1, 10.7)	4.1 (2.9, 7.7)
Post-surgery 2	4.1 (2.7, 9.9)	2.1 (1.6, 4.3)	12.4 (8.7, 17.7)
PGEM (ng/mg creatinine)			
Baseline	11.0 (5.6, 54.1)	17.8 (8.5, 27.6)	12.9 (9.3, 26.0)
Post-surgery 1	8.6 (5.0, 31.7)	6.0 (3.8, 12.9)	11.1 (6.1, 27.0)
Post-surgery 2	7.8 (4.6, 21.4)	3.0 (1.7, 5.9)	3.7 (2.8, 16.0)
PGIM (ng/mg creatinine)			
Baseline	0.10 (0.09, 0.22)	0.15 (0.14, 0.18)	0.10 (0.09, 0.25)
Post-surgery 1	0.16 (0.15, 0.21)	0.09 (0.05, 0.15)	0.14 (0.11, 0.21)
Post-surgery 2	0.15 (0.11, 0.21)	0.06 (0.04, 0.10)	0.06 (0.04, 0.12)
PGDM (ng/mg creatinine)			
Baseline	1.8 (1.5, 3.2)	2.6 (2.0, 3.6)	2.4 (1.9, 2.8)
Post-surgery 1	1.5 (1.2, 2.0)	1.9 (1.3, 2.3)	2.0 (1.7, 2.5)
Post-surgery 2	1.7 (1.3, 2.3)	1.5 (1.1, 1.8)	1.3 (1.1, 1.4)
TxM (ng/mg creatinine)			
Baseline	0.58 (0.34, 0.77)	0.55 (0.44, 0.91)	0.50 (0.37, 0.95)
Post-surgery 1	0.63 (0.32, 1.06)	1.18 (0.48, 1.50)	0.94 (0.63, 1.57)
Post-surgery 2	0.94 (0.43, 1.40)	0.71 (0.24, 1.16)	0.84 (0.55, 1.18)

Table S2. Serum inflammatory mediator levels by response group shown as median (25th percentile, 75th percentile).

	Placebo	Partial Responder	Complete Responder
IL-6 (pg/ml)			
Baseline	5.6 (1.6, 24.5)	6.5 (1.6, 18.7)	5.3 (1.6, 16.4)
Post-surgery 1	20.9 (8.5, 45.7)	10.3 (9.7, 20.4)	16.2 (8.3, 25.2)
Post-surgery 2	22.7 (6.8, 59.0)	12.9 (11.1, 16.9)	22.3 (17.8, 31.9)
IL-10 (pg/ml)			
Baseline	6.9 (2.3, 23.2)	3.9 (3.1, 10.1)	3.3 (2.5, 13.5)
Post-surgery 1	14.4 (3.8, 19.8)	4.1 (2.7, 11.0)	5.4 (3.0, 12.8)
Post-surgery 2	7.2 (4.3, 25.2)	6.6 (4.4, 14.7)	9.4 (3.2, 21.2)
TNF-α (pg/ml)			
Baseline	19.7 (7.7, 28.5)	23.1 (10.2, 31.1)	18.2 (8.9, 25.2)
Post-surgery 1	18.8 (9.7, 31.1)	16.9 (8.9, 19.4)	15.6 (11.2, 25.4)
Post-surgery 2	15.4 (7.0, 37.2)	11.4 (7.7, 17.6)	31.2 (11.0, 41.9)
IL-8 (pg/ml)			
Baseline	86.5 (37.2, 127.7)	87.6 (68.9, 160.5)	66.1 (40.3, 93.9)
Post-surgery 1	61.0 (43.0, 134.7)	101.0 (78.7, 166.9)	99.6 (79.8, 171.3)
Post-surgery 2	88.4 (52.7, 136.8)	57.8 (42.2, 79.5)	90.0 (76.3, 183.7)
MCP-1 (pg/ml)			
Baseline	621.9 (395.6, 750.7)	633.5 (439.0, 909.9)	745.2 (561.3, 977.7)
Post-surgery 1	426.2 (293.1, 893.0)	562.7 (342.1, 847.6)	623.4 (541.2, 1036)
Post-surgery 2	382.7 (293.4, 491.5)	521.4 (349.1, 1371)	734.7 (637.7, 1053)

Supplemental Information References

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