Title

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Journal

Arthritis & Rheumatology, 74(4)

ISSN

2326-5191

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Publication Date

2022-04-01

DOI

10.1002/art.42000

Peer reviewed

HHS Public Access

Author manuscript

Arthritis Rheumatol. Author manuscript; available in PMC 2023 April 01.

Published in final edited form as:

Arthritis Rheumatol. 2022 April ; 74(4): 623–633. doi:10.1002/art.42000.

Clinical and preclinical evidence for roles of soluble epoxide hydrolase in osteoarthritis knee pain

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Abstract

Objective—Chronic pain due to osteoarthritis (OA) is a major clinical problem, existing analgesics often have limited beneficial effects and/or adverse effects, necessitating the development of novel therapies. Epoxyeicosatrienoic acids (EETs) are endogenous anti-inflammatory mediators, rapidly metabolized by soluble epoxide hydrolase (sEH) to dihydroxyeicosatrienoic acids (DHETs). We hypothesized that sEH driven metabolism of the EETs to DHETs plays a critical role in chronic joint pain associated with OA and provides a new target for treatment.

Methods—Potential associations between chronic knee pain in people and single nucleotide polymorphisms (SNPs) in the gene encoding sEH and circulating levels of the EETs and DHETs were investigated. A surgically-induced murine model of OA was used to determine the effects of both acute and chronic selective inhibition of sEH by *N*-[1-(1-oxopropy)-4-piperidinyl]-*N'*-(trifluoromethoxy)phenyl]urea (TPPU) on weight-bearing asymmetry, hind-paw withdrawal thresholds, joint histology, and circulating concentrations of the EETs and DHETs.

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Author contributions:

Experimental design: P.G, J.T, C.M, W.Z, M.D, B.H, A.V, D.B & V.C.

Acquisition and analysis of data: P.G, J.T, M.S, R.J, S.G, T.K, J.Y, & G.F.

All authors were involved in the preparation and writing of the manuscript.

Competing interests:

The University of California holds patents on the sEH inhibitors used in this study as well as their use to treat inflammation, inflammatory pain, and neuropathic pain. BD Hammock and CB McReynolds are cofounders, and J Yang are employees of EicOsis L.L.C., a startup company advancing sEH inhibitors as potential therapeutics.

Results—In people with chronic knee pain, 3 pain measures were associated with SNPs of the sEH gene, *EPHX2*, and in two separate cohorts circulating levels of EETs and DHETs were also associated with 3 pain measures. In the murine OA model, systemic administration of TPPU both acutely and chronically reversed established pain behaviours and decreased circulating levels of 8,9-DHET and 14,15-DHET. The levels of the EETs were unchanged by TPPU administration.

Conclusion—Our novel findings support a role of sEH in OA pain and suggest that inhibition of sEH and protection of endogenous EETs from catabolism represents a potential new therapeutic target for OA pain.

Introduction

Osteoarthritis (OA) is a common musculoskeletal condition estimated to affect approximately 27 million adults in the USA and chronic pain is the predominant symptom (1). Chronic pain is a maladaptation of a vital sensory modality, involving increased peripheral nociceptor drive and plasticity in the central nervous system, which results in increased spinal and supra-spinal excitability and collectively maintains persistent pain (2). Sustained peripheral inflammatory signalling appears to be a key driver of pain in a large subset of people with OA (3–5). Current analgesic drugs include non-steroidal anti-inflammatory drugs (NSAIDs) and opioids, neither of which alter progression of disease or are adequately efficacious over the long timeframe of chronic pain states, and both drug therapies can be associated with severe adverse effects (6,7). Thus, there is a clear need for novel treatments for OA pain which have improved side-effect profiles.

Over the last decade the importance of the resolution pathways, which curtail inflammatory signaling and limit the progression of chronic illnesses, has become increasingly evident (8). Augmenting endogenous anti-inflammatory processes may provide alternative strategies to conventional analgesics for effective long-term pain relief. Poly-unsaturated fatty acids (PUFAs), including the omega-6 arachidonic acid (AA), are critical starting points for pro- and anti-inflammatory mediators and subsequent pain signaling (9). Previous studies predominantly focused on the contributions of pro-inflammatory molecules such as the prostaglandins (10), rather than the anti-inflammatory pathways which remain relatively under-explored to date.

The epoxyeicosatrienoic acids (EETs), derived from AA via the cytochrome P450 pathway, have anti-inflammatory effects via inhibition of NF-kB signaling (11,12) and anti-nociceptive effects in a rodent model of inflammatory pain (13). These effects are short-lived due to metabolism by soluble epoxide hydrolase (sEH) (14) to the dihydroxyeicosatrienoic acids (DHETs). Inhibition of sEH reverses pain responses in rodent models of inflammatory (13,15–17) and neuropathic (16,18,19) pain. Until recently clinical evidence for a role of this pathway in OA was limited. Our demonstration that synovial fluid levels of the DHETs were positively associated with both OA severity and progression (20) and the demonstration of beneficial effects of an sEH inhibitor in spontaneous canine OA pain (21) has uncovered potential opportunities of exploiting this pathway for the treatment of OA pain.

We hypothesized that sEH driven metabolism of the EETs to DHETs plays a critical role in chronic joint pain associated with OA and provides a new target for treatment. Our

aim was to provide clinical evidence for potential associations between OA pain and this pathway, which was achieved by measurement of single nucleotide polymorphisms in the gene encoding sEH and circulating levels of the EETs and DHETs in participants with OA pain. We then sought evidence of therapeutic benefit using a clinically validated surgically induced murine model of OA. The effects of selective inhibition of sEH on established pain behaviour and joint pathology were quantified and potential associations with changes in plasma ratios of EETs and DHETs determined.

Participants and Methods

Full details of the participants are given in Supplementary Materials. The Knee Pain in the Community Cohort (KPIC) (22) was used for this study (approved by Nottingham University Hospitals NHS Trust and the Nottingham Research Ethics Committee 1 (Ref 14/EM/0015) and registered with ClinicalTrials.gov (NCT02098070). The participants included both those with and those without radiographic knee OA. Pressure pain detection thresholds (PPTs) and painDETECT questionnaire scores were available for 318 KPIC participants (Supplementary Table 1). Plasma samples were collected from a separate cohort of 92 participants (Supplementary Table 1) from the iBEAT-OA cohort (Trial registration number: NCT03545048). Ethical approval was obtained from the Research Ethics Committee (ref: 18/EM/0154) and the Health Research Authority (protocol no: 18021). A third separate cohort of 62 participants (Supplementary Table 1) with radiographic knee OA (23) was recruited from existing databases of previous studies at the University of Nottingham, and approval for recruitment was obtained from the research ethics committees of Nottingham City Hospital.

Genetic association analysis

DNA samples from the KPIC cohort were genome-wide genotyped using the Illumina Global BioIT array. Genetic associations with presence of neuropathic pain features and pressure pain detection thresholds (PPTs) were carried out using the PLINK software package (http://zzz.bwh.harvard.edu/plink/) (further details including the SNPs analysed and their minor allele frequencies are presented in Supplementary Materials).

Analysis of circulating levels of the epoxyeicosatrienoic acids and the dihydroxyeicosatrienoic acids and their associations with clinical OA pain

Plasma samples were collected from 3 separate cohorts for targeted liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of levels of EETs and DHETs; a subset of the KPIC cohort (n=129), 92 participants from the iBEAT OA cohort, and a third separate cohort of 62 participants (Supplementary Materials). Baseline pain data from the iBEAT-OA (24) used in this study was quantative sensory testing (QST) measurement of PPTs, temporal summation (TS), and conditioned pain modulation (CPM) (see Supplementary Materials). In the third separate cohort of 62 individuals (Supplementary Table 1) with radiographic knee OA (23) participants were asked if they were currently experiencing knee pain at the time of blood donation, and pain was assessed by completion of the Western Ontario and McMaster Universities Arthritis Index (WOMAC) (further details in Supplementary Materials.

Destabilisation of the medial meniscus model of osteoarthritis pain

All experiments using adult male C57BL/6 mice were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act (1986) (numbers and group sizes are in Supplementary Materials). The experimenter was blinded to the experimental groups throughout. Mice were randomly allocated to either the model or sham group by a third party. Mice were habituated to the behavioural test environments prior to pain assessment, as described in Supplementary Materials. Destabilisation of the medial meniscus (DMM) or sham surgery of the ipsilateral hind-limb was performed as previously described (25). Pain behavior was measured at baseline and then once a week for 16 weeks post-surgery. Weight-bearing asymmetry between the ipsilateral (left) and contralateral (right) hind limbs was assessed with an incapacitance meter (Linton Instrumentation, Norfolk, United Kingdom) (25). The 50% hind-paw withdrawal thresholds were measured using the EC50 of log transformed responses to a battery of von Frey hairs as previously described (26).

Inhibition of soluble epoxide hydrolase

At 16 weeks post-surgery mice received an i.p. injection of 3mg/kg TPPU (Tocris; Cat. No. 5918) (n=10) or vehicle (50% PEG400 in 0.9% saline) (n=20). Pain behaviour was measured at 1 and 3 hours post-injection. Plasma was collected at terminal timepoints for analysis by LC-MS/MS.

In a separate study, DMM and sham mice received in their drinking water TPPU in 1% PEG-400 (n = 15) in filtered water or 1% PEG-400 (n = 13) in filtered water from 12 weeks post-surgery for 4 weeks. Based on the average volume of water consumed by mice per day, the estimated dose was 3mg/kg/day TPPU. Blood (10μL) was collected from the tail vein of mice before treatment, and 2 and 4 weeks after TPPU or vehicle treatment commenced to measure circulating concentrations of TPPU. At 16 weeks post-surgery, mice were euthanised and plasma was collected for analysis by LC-MS/MS as described in Supplementary Materials.

Histological assessment of joint pathology

At the end of all TPPU studies, knee joints were collected post-mortem. Sections were stained and joint pathology was assessed using a previously published scoring system (25) by two independent scorers (details in Supplementary Materials).

Data Analysis

All murine data were analysed using Prism v7 (Graphpad; San Diego, CA). Data were tested for normality utilising the D'Agostino and Pearson normality test, see Supplementary Materials for full details.

Clinical data: concentrations of EET and DHET were log-transformed in order to achieve a normal distribution necessary for parametric methods. Associations between bioactive lipids and pain traits were tested using linear regressions with pain traits as the outcome and adjusting for age, sex, BMI and Kellgren-Lawrence grade. The association between SNPs and bioactive lipids in the KPIC cohort was carried out using the log transformed EET or DHET as outcomes and additive SNP models (0, 1 or 2 copies of the minor allele) as

the independent variable, also adjusting for age, sex BMI and Kellgren-Lawrence grade. Adjustment for multiple testing was carried out using a False Discovery Rate correction and significant values are indicated in the text. Linear regression analyses were performed using the R software package (www.r-project.org).

Results

Single nucleotide polymorphisms in *EPHX2* are associated with chronic knee pain

Genome wide genotyping was carried out on samples from 318 people with knee pain from the KPIC cohort (22). The presence of six *EPHX2* SNPs (rs10503812 [r = 0.13, p = 0.02], rs2741348 [r = 0.13, p = 0.02], rs1316801 [r = 0.12, p = 0.03], rs111659883 [0.12, p=0.04], rs7844965 [r = 0.11, p = 0.04], rs35236974 [r = 0.14, p = 0.02]) were positively nominally associated with the presence of pain at the time of sample collection (n= 318) (Figure 1A). There were also nominal associations between five *EPHX2* SNPs: (rs10503812 [r = −0.12, p = 0.03], rs78336300 [r = 0.13, p = 0.02], rs11135999 [r = 0.13, p = 0.02], rs73229090 [r = −0.11, p = 0.05], rs35236974 [r = −0.11, p = 0.04]) and pressure pain detection thresholds (PPTs) at the medial aspect of the knee (Figure 1B). Neuropathic-like pain symptoms as measured by the painDETECT questionnaire were also positively nominally associated with eight EPHX2 SNPs (rs10503812 [r = 0.15, p = 0.008], rs2741348 [r = 0.15, p = 0.009], rs75560813 [r = 0.14, p = 0.01], rs1316801 [r = 0.11, p = 0.05], rs73229090 [r = 0.13, p = 0.02], rs17057426 [r = 0.15, p = 0.008], rs12680584 [r = 0.11, p = 0.05], rs35236974 [0.15, p = 0.006])(Figure 1C). One SNP (rs10503812) was nominally associated with lateral PPTs (r = −0.12, p = 0.02) and sternum PPTs (r = −0.11, p = 0.049) (Supplementary Table 2). After adjusting for multiple tests using an FDR correction none of the SNPs remained significantly associated with current pain. However, rs8065080 was associated with FDR p<0.01 with both medial and lateral PPTs, rs10503812 was associated with FDR p=0.026 with lateral PPTs and with FDR p=0.056 with painDETECT scores. Also associated with PDQ scores were rs7844965 and rs17057426 with FDR p=0.056. All association data can be found in Supplementary Table 2. In a subset of these participants where plasma samples were available (n=129) significant associations between plasma EET/DHET ratios and pain-associated SNPs were investigated, but were not detected (Supplementary Figure 1).

In a separate cohort of participants with knee OA (n= 92) (Figure 2) levels of EETs and DHETs were associated with different measures of pain (adjusted for age, sex, BMI, and Kellgren-Lawrence OA knee radiographic score). Higher plasma concentrations of 5,6-EET (*B*: 0.96, p = 0.009), 8,9-EET (*B*: 0.89, p = 0.003), and 11,12-EET (*B*: 0.75, p = 0.03) were associated with higher numerical rating scale (NRS) scores for pain (Figure 2). A positive association was also evident for the corresponding ratios of the EETs/DHETs (5,6-EET/DHET: *B*: 0.94, p = 0.02, 8,9-EET/DHET: *B*: 0.9, p = 0.004, 11,12-EET/DHET: *B*: 0.78, p = 0.02) and NRS score. Higher plasma concentrations of 11,12-DHET (*B:* −190, p = 0.04) and 14,15-DHET (*B*: −190, p = 0.03) were associated with lower conditioned pain modulation (Figure 2), impairment of which may contribute to increased pain in people with OA (27). In the third clinical cohort, we observed significant associations between plasma concentrations of 5,6-DHET, and 8,9-DHET and the presence of pain (5,6-DHET: r = 0.26, p = 0.006, 8,9-DHET: r = 0.2, p = 0.04) and WOMAC pain scores (5,6-DHET: r = 0.29, p = 0.002,

8,9-DHET: r = 0.19, p = 0.04) (Supplementary Table 3). These data from three different cohorts of participants with OA pain identify associations between this pathway and OA knee pain, supporting the further investigation of therapeutic potential in an experimental model.

Acute inhibition of soluble epoxide hydrolase attenuates established murine osteoarthritis pain

We first determined the effects of a soluble epoxide hydrolase inhibitor on established behavioural pain responses in the destabilization of the medial meniscus (DMM) model in mice. *N*-[1-(1-oxopropy)-4-piperidinyl]-*N*′-(trifluoromethoxy)phenyl]urea (TPPU) is a potent inhibitor of soluble epoxide hydrolase and attenuates experimental neuropathic pain (18).

Sixteen weeks following DMM-surgery there was significant cartilage damage at the medial tibial plateau (Figure 3A) and synovitis (Figure 3B) in DMM-operated mice, compared to sham-operated controls (See Supplementary Figure 2 for representative images). At this timepoint, the significant decrease in the percentage of weight borne on the ipsilateral hind-limb (Figure 3C) and the lowering of ipsilateral hind-paw withdrawal thresholds (Figure 3D) in DMM-operated mice (compared to sham-operated controls), indicates the presence of pain responses in this model. Intra-peritoneal (i.p) injection of TPPU (3mg/kg) at 16 weeks significantly reversed the DMM-induced pain behavior, as evidenced by a significant increase in the amount of weight borne on the ipsilateral hind-limb, compared to the vehicle injected DMM group (Figure 3C). TPPU treatment also significantly reversed the decrease in ipsilateral hind-paw withdrawal thresholds in the DMM group, compared to the vehicle injected DMM group (Figure 3D).

Plasma samples collected 4 hours following TPPU treatment were analysed by LC-MS/MS for a range of bioactive lipids including EETs and DHETs (Figure 4A & Supplementary Table 4). TPPU had no significant effect on the concentration of 8,9-EET or 14, 15-EET (Figures 4B & 4C) in DMM mice, but significantly decreased levels of 8,9-DHET (Figure 4D) and 14,15-DHET, compared to vehicle injected DMM mice (Figure 4E). These effects were paralleled by a significant increase in the ratio of 8,9-EET/ 8,9-DHET (Figure 4F) and 14,15-EET/14,15-DHET (Figure 4G) in TPPU treated DMM-operated mice, compared to the vehicle injected DMM group. Effects of TPPU appeared to be substrate selective as, at this dose, concentrations of 5,6-EET, 5,6-DHET, 11, 12-EET, or 11, 12-DHET were not altered (Supplementary Table 4). Levels of AA were reduced by TPPU, compared to DMM mice treated with vehicle (Supplementary Table 4). Overall, TPPU acutely reversed both pain on loading and referred pain in the DMM model and altered circulating levels of some DHETs.

Chronic inhibition of soluble epoxide hydrolase reverses OA pain behaviour

We next investigated whether inhibition of soluble epoxide hydrolase can produce a sustained inhibition of pain behavior in the DMM model, indicating potential therapeutic benefit over a longer window of treatment. TPPU treatment (via drinking water) commenced from 12 weeks post-DMM or sham surgery, for 4 weeks. Prior to treatment, DMM mice

exhibited a significant decrease in the weight borne on the ipsilateral hind-limb, compared to sham controls consistent with the model in the acute treatment study (Figure 5A). TPPU significantly reversed weight-bearing asymmetry at 24 hours post-treatment, compared to the vehicle treated DMM group (Figure 5A). This reversal in weight-bearing asymmetry was sustained for the 4-week period of treatment and was significant for weeks 2–4 (Figure 5A). TPPU also produced a steady reversal in the lowered hind-paw withdrawal thresholds over two weeks post-administration. This effect peaked at 2 weeks post-TPPU treatment and was significantly different to the vehicle treated DMM group but was not maintained for the duration of the study (Figure 5B). Concentrations of TPPU in the blood were confirmed at 2- and 4-weeks following treatment and compared to samples collected prior to commencement of treatment (Supplementary Figure 3). Average concentrations of TPPU in the blood were 324ng/mL and 243ng/mL at 2- and 4-weeks post-dosing. These concentrations far exceed the reported IC50 of TPPU (17).

Plasma samples were collected from mice 4 weeks after treatment with TPPU or vehicle (via drinking water) for LC-MS/MS analysis. There was no significant difference in circulating lipids between sham and DMM mice at 16 weeks post-surgery (Supplementary Table 5). 4 week treatment with TPPU in DMM mice did not alter plasma concentrations of 8,9-EET (Figure 6A) or 14,15-EET (Figure 6B), but significantly decreased plasma concentrations of 8,9-DHET (Figure 6C) and 14,15-DHET (Figure 6D), compared to vehicle treatment. There were, therefore, significant increases in the ratios of 8,9-EET/DHET (Figure 6E) and 14,15-EET/DHET in DMM mice treated with TPPU (Figure 6F). Correlation analysis of all samples revealed that plasma levels of 8,9-DHET were significantly higher in mice with more pain on loading (weight-bearing asymmetry) (Figure 6G), but not lowered ipsilateral hind-paw withdrawal thresholds (Figure 6H).

This study was not powered to study potential disease modifying effects of inhibitors of soluble epoxide hydrolase, however data provided in Supplementary Figures 4 and 5 support the design of further studies to investigate the effects of this treatment on OA-like joint pathology.

Discussion

Herein, we report for the first time that SNPs of the sEH gene, *EPHX2*, are associated with 3 different measures of pain in people with OA, substantially adding to our previous evidence that plasma levels of some DHETs are associated with OA joint pathology and progression (20). We strengthen the evidence for a role of this pathway with the demonstration of associations between plasma levels of the EETs and DHETs with multiple measures of pain in two separate cohorts of people with knee OA. In a clinically relevant murine model of OA, acute and chronic administration of a selective inhibitor of sEH reversed established OA pain behavior. These functional changes occurred in parallel with increased ratios of 8,9-EET/DHET and 14,15-EET/DHET, consistent with a mode of action via inhibition of sEH.

Our GWAS analysis of clinical samples from people with knee pain revealed associations between several *EPHX2* SNPs and pain outcomes, supporting the notion that differences

in this gene may contribute to the amount of pain experienced by people with knee pain. Previously, polymorphisms of the *EPHX2* gene have been associated with coronary artery calcification, risk of ischemic stroke, and insulin resistance in type II diabetes patients (28–30). The SNPs we identified to be associated with OA pain are non-coding intronic variants, consistent with a previous association between intron variants and subclinical cardiovascular disease (31). Although it is unknown whether variations in the non-coding regions of *EPHX2* alter the expression and function of the protein, in a rat model of heart failure, variation in a non-coding region of the *EPHX2* gene associated with heart failure had altered sEH protein expression and activity (32), supporting functional consequences. In the data reported here, a separate cohort of people with OA demonstrated that circulating levels of the EETs were positively associated with VAS pain scores, and circulating levels of 11,12- and 14,15-DHET were associated with lowered conditioned pain modulation, a surrogate measure of the function of the descending inhibitory control pathways (27). In another cohort of participants, pain at the time of sample collection, and WOMAC assessed pain was significantly associated with levels of 5,6-DHET, and 8,9-DHET. The association between changes in the levels of EETs and their metabolites (DHETs) with multiple measures of OA supports the notion that there is a perturbation of this pathway in people with chronic OA pain. The association between higher concentrations of the anti-inflammatory EETs with increased pain outcomes may represent increased production of the EETs in an attempt to ameliorate heightened chronic pain responses. This is consistent with the known increase in other endogenous inhibitory control pathways, such as the endocannabinoids (33) and endogenous opioids (34), in chronic pain states. This finding is important as it supports the rationale of protecting levels of EETs, via inhibition of sEH, to realize the potential of this novel therapeutic target for OA pain.

The future translational development of potential treatments acting via sEH requires robust mechanistic knowledge of the consequences of altering enzymatic activity on OA pain. To this end we back-translated our clinical findings to the DMM model of OA, a clinically relevant model characterized by slowly developing histopathological changes within the joint and pain behavior (35). Herein, we demonstrate that acute systemic injection of the sEH inhibitor TPPU reversed both types of DMM induced pain behavior from 1-hour post-injection. These data extend the published literature reporting acute effects of sEH inhibitors in models of inflammatory and neuropathic pain (16,17) to a clinically relevant model of OA pain. Our data build upon the report that acute sEH inhibition is analgesic in naturally occurring OA in aged canines (21).

Single administration of sEH inhibitors produces a transient analgesia for up to 5 hours in models of inflammatory and neuropathic pain (18,19). Here we investigated whether continuous dosing of TPPU produced a sustained analgesia. Importantly, chronic TPPU treatment resulted in a sustained and robust reduction in DMM induced weight-bearing asymmetry. Hind-paw withdrawal thresholds were also reduced, although effects were more robust for weight-bearing asymmetry. The reduction in paw withdrawal thresholds were not sustained for the duration of the study, unlike the effects on weight-bearing asymmetry. This may reflect the different mechanisms which underlie these pain behaviours, with lowered paw withdrawal thresholds being partially mediated by changes in spinal processing of nociceptive inputs. Although we did not measure joint levels of TPPU, sEH

inhibitors administered systemically were detected in the synovial fluid of canines and horses, supporting a possible local site of action (21,36). The sustained inhibitory effects of TPPU on weight bearing asymmetry suggest no tolerance to their effects, unlike opioid analgesics (37). TPPU treatment did not affect weight bearing or hindpaw withdrawal thresholds in sham operated mice (Supplementary Figure 6), supporting earlier evidence that sEH inhibitors do not alter baseline nociceptive responses (13,15). Thus, it appears that sEH inhibitors only exhibit biological effects in the presence of pathological changes in this case associated with chronic pain states, furthermore sEH inhibitors are more effective in response to greater nociceptive insults (38). Our study was designed and powered to detect differences in pain behaviour between groups, rather than effects on joint pathology. Nevertheless, chronic dosing with TPPU led to a small but non-significant decrease in cartilage damage, synovitis, and osteophytosis, compared to vehicle treated controls. The potential chondroprotective effects of sEH inhibition are worthy of future investigation. Both acute and chronic administration of TPPU was associated with significant decreases in circulating levels of 8,9- and 14,15-DHET, without altering levels of the respective EETs, when compared to vehicle treatment. These data are consistent with the effects of the sEH inhibitor, APAU in a rodent model of inflammatory pain (16).

Local injection of EETs can reduce inflammatory pain responses (13), which may reflect EETs actions at peroxisome proliferator-activated receptor gamma (PPARγ) (11,39), activation of which attenuates both inflammatory and neuropathic pain (40,41). EETs also have affinity for translocator protein (TSPO) (19) and TSPO ligands have anti-nociceptive effects in an inflammatory model of pain (42). Note however, high concentrations of 8,9-EET induce calcium influx in a small subset of cultured DRG neurons (43). Although the DHETs have been considered inactive products of sEH mediated metabolism of the EETs (44), there is some indication of biological activity (45–47). Whether the analgesic effects of sEH inhibition arise from the stabilization of EET levels, or decreased levels of the DHETs is yet to be elucidated. Our finding that the reduction in pain behavior by both acute and chronic TPPU administration occurred whilst levels of 8,9- and 14,15-DHET were reduced and corresponding levels of EETs were stable, may be interpreted as DHETs having potential pro-nociceptive effects under some conditions.

Future development of treatments targeting sEH warrants consideration that sEH can also metabolise the epoxy-octadecenoic acids (EpOMEs), epoxyeicosatetraenoic acids (EpETE) and epoxydocosapentaenoic acids (EpDPE) (44). EpDPEs and the EpETEs can reduce carrageenan induced pain in rats (48). These compounds were not measured in our LC-MS/MS analysis, limiting our ability to explore their potential contributions further. Outside of potential direct effects on epoxy-fatty acids, sEH inhibitor treatment reduced levels of prostaglandins in models of inflammatory pain (13,16), this finding was not replicated in our study.

Our study limitations include that lipid concentrations, which provide a measure of the flux in the EET-DHET pathway, were only measured at a single timepoint in both humans and murine OA. In addition, there were some inconsistencies between the associations of different measures of OA pain in people and the EETs/DHETs measured. Nevertheless, overall our data support the view that this enzymatic pathway is perturbed in people with

chronic OA knee pain and support further investigation of the contribution of this pathway to OA pain. Our preclinical studies were performed in young male mice, and effects in female mice merit further study. Previously, TPPU was shown to have anti-nociceptive effects in both male and female mice in a model of neuropathic pain (49). Although the DMM model is acknowledged as having translational value (50), species differences may also have an important bearing on our findings.

The burden of OA pain to society is significant and current therapeutic options are limited due to concerns over safety and efficacy (4, 6, 7). We provide substantive new clinical and preclinical evidence that sEH is an important mediator of OA pain and that targeting this enzyme may be a new route for treatment of OA pain. Our pre-clinical data build upon the analgesic effects of an sEH inhibitor described in a spontaneous model of OA in canines (21). Soluble epoxide hydrolase inhibition is already in clinical trials for neuropathic pain (clinicaltrials.gov: NCT04228302), supporting the therapeutic targeting of this enzyme for OA pain. Future work investigating the potential interactions between sEH inhibitors and diet, including omega 3 PUFAs, may reveal further benefits of targeting this enzymatic pathway for the treatment of arthritic pain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

The authors would like to thank Ojasvi Mehta for her help with generating the heatmap for Figure 2.

Funding:

This work was funded by Versus Arthritis [Grant Number: 20777],a Pfizer Global medical grant “Global Awards for Advancing Chronic Pain Research (ADVANCE) 2018” [Grant Number: WI243608], and by the NIHR Nottingham Biomedical Research Centre. Partial support was provided by the D- BOARD Consortium funded by European Commission Framework 7 Programme (EU FP7; HEALTH.2012.2.4.5–2, project number 305815, Novel Diagnostics and Biomarkers for Early Identification of Chronic Inflammatory Joint Diseases), the NIH-NIEHS (RIVER Award) [Grant Number: R35 ES030443–01], the NIEHS Superfund Research Program [Grant Number: P42 ES004699], and T32GM11377004 to C.B.M.

Data and materials availability:

All preclinical data generated or analysed during this study are available in the manuscript and its Supplementary Information Files. The clinical data generated and analysed in this study are held by the Division of Rheumatology, Orthopaedics, and Dermatology. This data can be released to bona fide researchers using the normal procedures overseen by the University of Nottingham and the Nottingham NIHR BRC and its ethical guidelines. Please contact the corresponding authors to receive the application form.

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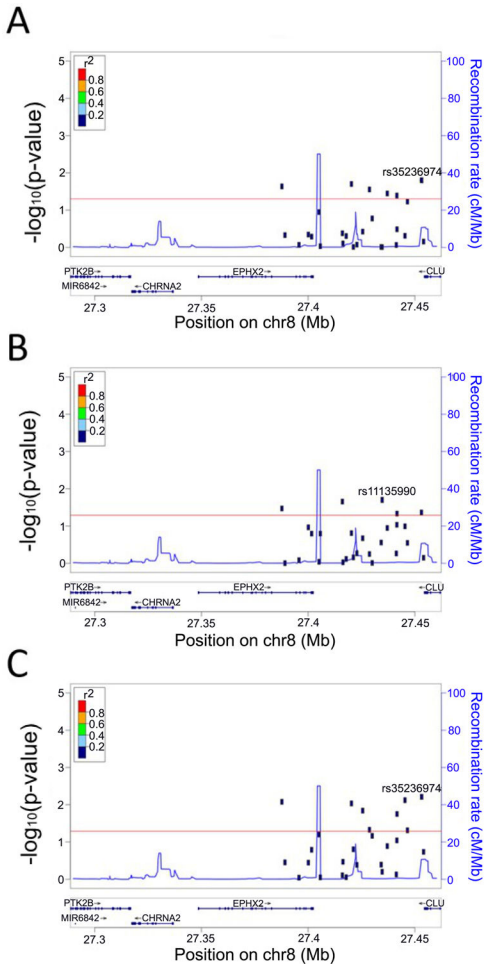

Figure 1: Single nucleotide polymorphisms of *EPHX2* are associated with 3 different pain measures in people with OA.

Genome wide genotyping was carried out on samples from 318 people with knee OA. Locus zoom plots show the location of single nucleotide polymorphisms of *EPHX2* and associations with current pain (**A**), medial knee pain pressure thresholds (**B**), and painDETECT scores (**C**). SNPs above the red line are significantly associated ($p<0.05$) with the respective pain measure.

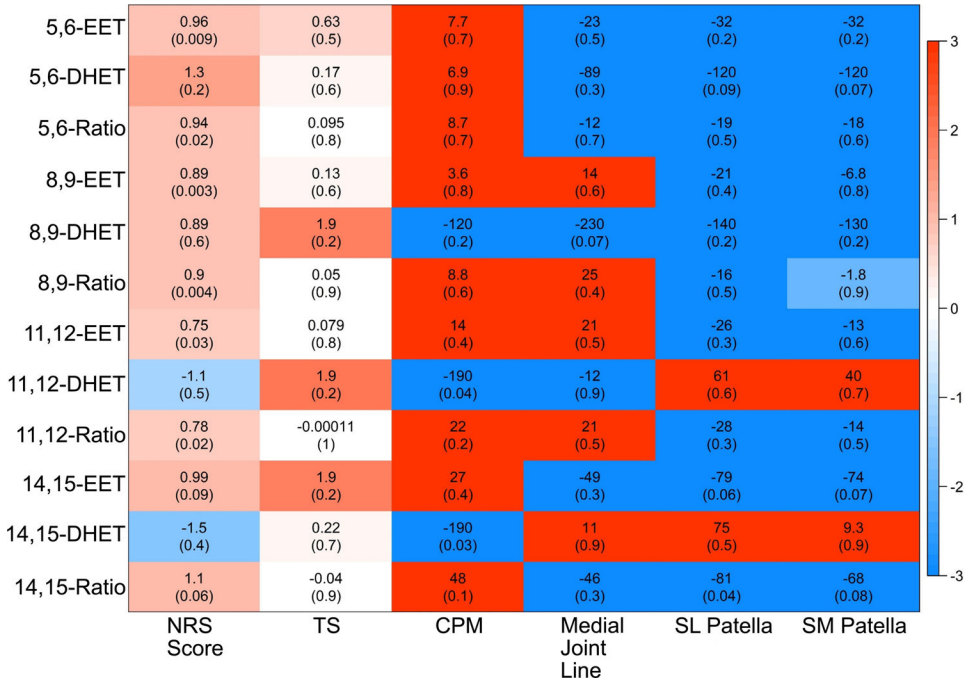

Figure 2: Regression analysis of plasma levels of the EETs and DHETs with pain measurements in people with OA pain.

Heatmap of regression analysis results between log10 normalised lipid concentrations and quantitative pain phenotypes. Pain was assessed in 92 OA patients by numerical rating scale (NRS) score, temporal summation (TS), conditioned pain modulation (CPM), and pain pressure thresholds of the medio tibial joint line, and both the lateral and medial aspects of the patella. Red indicates a positive beta value, whereas blue indicates a negative beta value. Values populated include beta values (above) and p-values (below, in brackets).

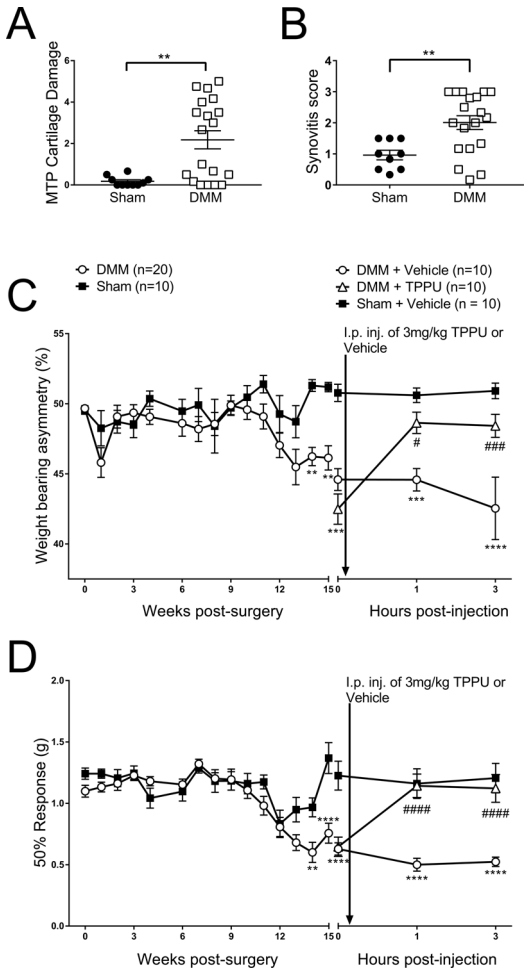

Figure 3: The effects of acute administration of TPPU on chronic OA pain behaviour in mice.

Adult male C57BL/6 mice underwent either destabilisation of the medial meniscus (DMM) (n=20) or sham surgery (n=10). At 16 weeks post-surgery cartilage damage (**A**) and synovitis (**B**) were assessed. Data analysed by unpaired t-test, ** = p<0.01 sham vs DMM. The effects of i.p injection of *N*-[1-(1-oxopropy)-4-piperidinyl]-*N*′-(trifluoromethoxy)phenyl]urea (TPPU) (3mg/kg) or vehicle (50% PEG400 in 0.9% saline) on weight-bearing asymmetry (**C**) and ipsilateral hind-paw withdrawal thresholds (**D**) were assessed at 16 weeks post-surgery. Final group sizes were DMM + Vehicle (n=10), DMM + TPPU (n=10), Sham + Vehicle (n=10). Data analysed by 2-way ANOVA with Bonferroni corrected multiple corrections, ** = p<0.01. *** = p<0.001, **** = p<0.0001 DMM + vehicle vs sham + vehicle, # = p<0.05, ### = p<0.001, #### = p<0.0001 DMM = vehicle vs DMM + TPPU.

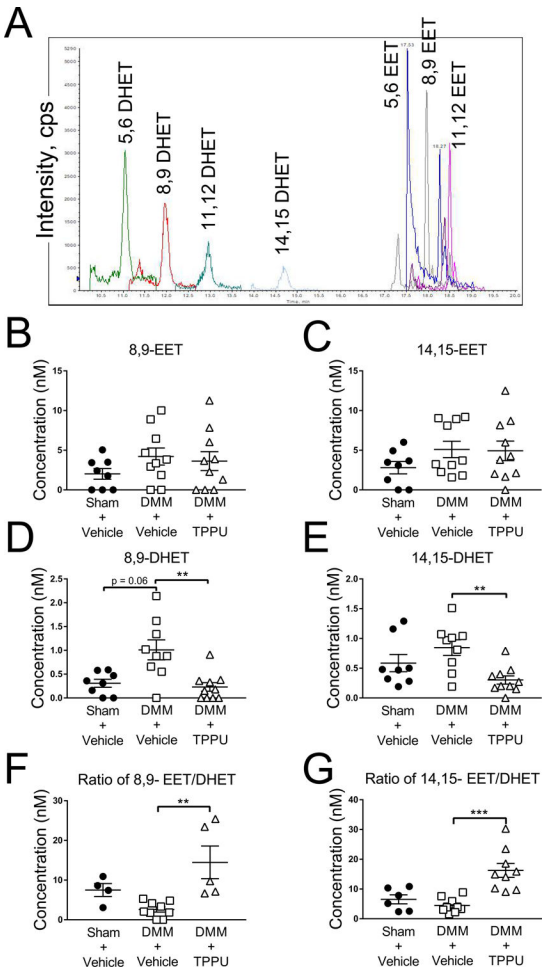

Figure 4: The effects of acute administration of TPPU on circulating plasma levels of 8,9 and 14,15 DHET in mice with OA pain.

Representative chromatogram showing the peaks of the dihydroxyeicosatrienoic acids (DHETs) and epoxyeicosatrienoic acids (EETs) in plasma (**A**). Circulating plasma levels of 8,9-EET (**B**), 14,15-EET (**C**), 8,9-DHET (**D**), and 14,15-DHET (**E**) were analysed 3 hours post-injection of *N*-[1-(1-oxopropy)-4-piperidinyl]-*N*′-(trifluoromethoxy)phenyl]urea (TPPU) or vehicle (50% PEG400 in 0.9% saline). Group sizes were Sham + Vehicle (n=8), DMM + Vehicle (n=9), DMM + TPPU (n=10). The ratios of circulating 8,9-EET/DHET (**F**) and 14,15-EET/DHET (**G**) were also analysed 3 hours post-injection of TPPU or vehicle. Data analysed by one-way ANOVA with Tukey's multiple comparisons. ** = $p<0.01$, *** = $p<0.001$ DMM + vehicle vs DMM + TPPU.

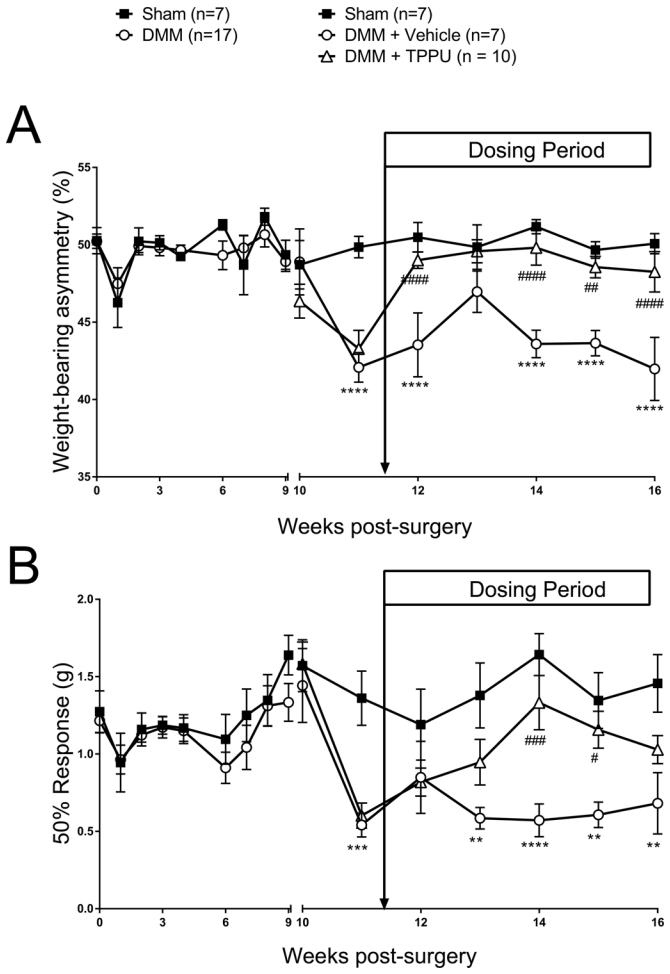

Figure 5: The effects of chronic administration of TPPU on chronic OA pain behaviour in mice.

Adult male C57BL/6 mice underwent either destabilisation of the medial meniscus (DMM) (n=17) or sham surgery (n=7). The effects of chronic administration of *N*-[1-(1-oxopropy)-4-piperidinyl]-*N*′-(trifluoromethoxy)phenyl]urea (TPPU) (3mg/kg/day) or vehicle (1% PEG400 in 0.9% saline) delivered in the drinking water on weight-bearing asymmetry (**A**) and ipsilateral hind-paw withdrawal thresholds (**B**) were assessed at 16 weeks post-surgery. The final group sizes were DMM + vehicle (n=7), DMM + TPPU (n=10), Sham + Vehicle (n=2), and Sham + TPPU (n=5). The two sham groups are combined as a single group (n=7). Data analysed by 2-way ANOVA with Bonferroni corrected multiple corrections, ** = p<0.01. *** = p<0.001, **** = p<0.0001 DMM + vehicle vs sham + vehicle, # = p<0.05, ## = p<0.01, ### = p<0.001, #### = p<0.0001 DMM = vehicle vs DMM + TPPU.

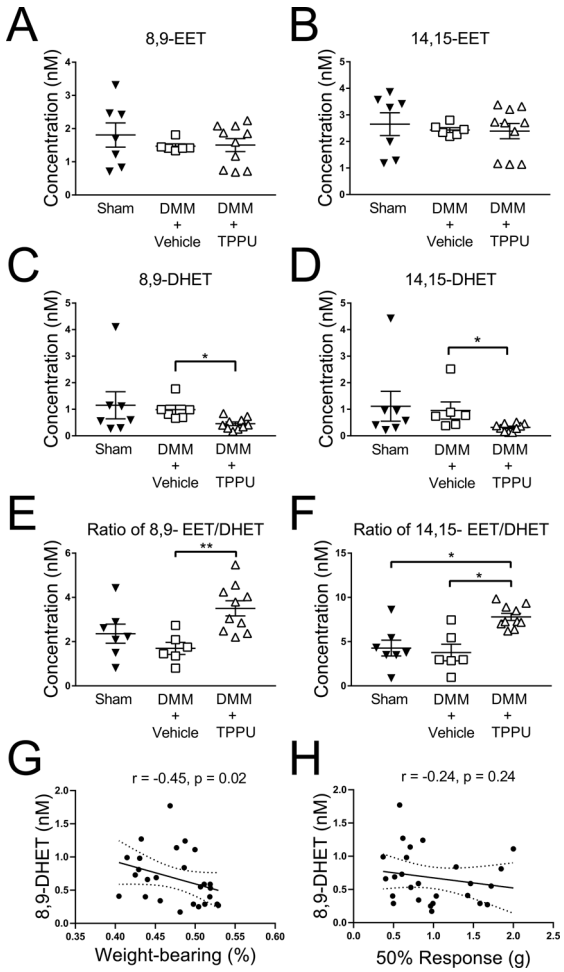

Figure 6: The effects of chronic administration of TPPU on circulating plasma levels of the EETs and DHETs in mice with OA pain.

Circulating plasma levels of 8,9-EET (**A**), 14,15-EET (**B**), 8,9-DHET (**C**), and 14,15-DHET (**D**) were analysed after 4 weeks of TPPU or vehicle administration in the drinking water. Data analysed by one-way ANOVA with Tukey's multiple comparisons. * = $p<0.05$, ** = $p<0.01$ DMM + vehicle vs DMM + TPPU. The ratios of circulating 8,9-DHET/EET (**E**) and 14,15-DHET/EET (**F**) were also analysed by one-way ANOVA with Tukey's multiple comparisons. * = $p<0.05$, DMM + vehicle vs DMM + TPPU. The final group sizes were DMM + vehicle (n=7), DMM + TPPU (n=10), Sham + Vehicle (n=2), and Sham + TPPU (n=5). The two sham groups are combined as a single group (n=7). Correlation analysis between circulating levels of 8,9-DHET and weight-bearing (**G**) and ipsilateral hind-paw withdrawal thresholds (**H**) in all mice from the study was performed by Pearson's correlation test."