

Does Pain at an Earlier Stage of Chondropathy Protect Female Mice Against Structural Progression After Surgically Induced Osteoarthritis?

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Objective. Female C57BL/6 mice exhibit less severe chondropathy than male mice. This study was undertaken to test the robustness of this observation and explore underlying mechanisms.

Methods. Osteoarthritis was induced in male and female C57BL/6 or DBA/1 mice ($n = 6\text{--}15$ per group) by destabilization of the medial meniscus (DMM) or partial meniscectomy (PMX). Some mice were ovariectomized (OVX) ($n = 30$). In vivo repair after focal cartilage defect or joint immobilization (sciatic neurectomy) following DMM was assessed. Histologic analysis, evaluation of gene expression in whole knees, and behavioral analysis using Laboratory Animal Behavior Observation Registration and Analysis System (LABORAS) and Linton incapacitance testing ($n = 7\text{--}10$ mice per group) were performed.

Results. Female mice displayed less severe chondropathy (20–75% reduction) across both strains and after both surgeries. Activity levels after PMX were similar for male and female mice. Some repair-associated genes were increased in female mouse joints after surgery, but no repair differences were evident in vivo. Despite reduced chondropathy, female mice developed pain-like behavior at the same time as male mice. At the time of established pain-like behavior (10 weeks after PMX), pain-associated genes were significantly up-regulated in female mice, including *Gdnf* (mean $\pm$ SEM fold change 2.54 ± 0.30), *Nrtn* (6.71 ± 1.24), *Ntf3* (1.92 ± 0.27), and *Ntf5* (2.89 ± 0.48) ($P < 0.01$, $P < 0.01$, $P < 0.05$, and $P < 0.001$, respectively, versus male mice). Inflammatory genes were not regulated in painful joints in mice of either sex.

Conclusion. We confirm strong structural joint protection in female mice that is not due to activity or intrinsic repair differences. Female mice develop pain at the same time as males, but induce a distinct set of neurotrophins. We speculate that heightened pain sensitivity in female mice protects the joint by preventing overuse.

INTRODUCTION

Epidemiologic studies identify female sex as a major non-modifiable risk factor in osteoarthritis (OA) development (1). Sex-dependent risk holds true across different joint sites and at different ages and is particularly apparent after 55 years of age (2–4). In the hand, the sex bias is particularly notable; 90% of those attending secondary care are women, and the incidence peaks around the perimenopausal period (1,3). The burden of hand OA in women increases as those individuals age (2), tending to plateau around

the age of 80 years (as does the disease in men) (3,5). Men have significantly higher cartilage volume at all sites compared with women, even after accounting for differences in height, bone size, and weight. This difference becomes more apparent after the age of 50 years (6). The sex bias in OA incidence and prevalence, particularly after 55 years of age, suggests an association with sex hormones and their changes at the time of menopause. Despite the speculation that hormone replacement could protect female joints against OA, a systematic review of epidemiologic studies has failed to support a joint-protective role of estrogen in OA (7).

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OA risk is also sex-dependent in mice. Young female mice subjected to destabilization of the medial meniscus (DMM) have previously been shown to develop less severe OA than male mice (8). In that study, female mice that had been ovariectomized (OVX) (at 6 weeks of age) had significantly more severe OA than non-OVX female mice. Conversely, male mice subjected to orchidectomy (castration) had reduced disease severity compared with unoperated males (3,8). Similar studies have been performed in a number of different species (3,9), and a systematic review comparing the severity of OA after OVX concluded that OVX led to worse disease compared with non-OVX females in approximately two-thirds of cases (10). The effects of exogenous estrogen treatment are less conclusive. Only half of the studies looking at the effects of estrogen replacement found reduced OA severity, and one study demonstrated exacerbation of disease (10). Lack of conclusive results in these studies may be due to a failure to appreciate the complexity of the effects of multiple hormones affecting joint tissues, as well as inconsistency in study design and power. In animals as well as humans, ovariectomy leads to changes in the expression of several hormones besides estrogen, including decreases in progesterone and testosterone, and increases in follicle-stimulating hormone (FSH) and luteinizing hormone (10). Receptors for estrogen, androgens, and FSH have been identified in joint cells, including articular chondrocytes, and have been shown to affect tissue metabolism (10–12).

If one regards OA as a disease of failed tissue repair, information may be gleaned from sex-dependent wound responses in other tissues. Several other tissues exhibit sex differences in their response to injury. One that has been extensively examined is cutaneous wound healing. Aging of healthy human skin is associated with reduced levels of transforming growth factor β 1 (TGF β 1), and a reduced rate of cutaneous wound healing, albeit with reduced scarring. This age-related pattern can be reversed by systemic estrogen therapy (13). Similar relationships can be demonstrated in mice. Young female mice have significantly higher levels of TGF β 1 than young male mice, and exhibit an increased inflammatory response to injury (13). OVX female mice demonstrate delayed repair of incisional wounds, which can be reversed by topical application of estrogen, which was associated with increased latent TGF β 1 secretion by dermal fibroblasts (13). Orchidectomized mice display accelerated wound healing compared with noncastrated controls (14).

There are also sex differences in the perception of pain. Women present with more chronic pain conditions than men, and are known to experience higher levels of pain and have a lower threshold for pain (15–18). The actual mechanisms of these sex differences is as yet unclear (17). Females have been suggested to employ different immune cells in their responses to pain (19,20). For example, in female mice, but not male mice, adaptive immune cells within the spinal cord played a role in the development of mechanical hypersensitivity (21).

In this study we explore sex differences in murine OA in detail, with a particular focus on the mechanisms that may drive differences in disease severity. We examine the impact on activity levels, ability to repair cartilage, molecular response to injury, and pain-like behavior.

MATERIALS AND METHODS

Animals and surgical methods have been described previously (22,23), with further details presented in the Supplementary Methods, available on the *Arthritis & Rheumatology* website at <http://onlinelibrary.wiley.com/doi/10.1002/art.41421/abstract>.

Sciatic neurectomy. Animals were placed under general anesthesia by inhalation of isoflurane (Vetpharma) (3% induction and 1.5–2% maintenance in 1.5–2 liters/minute O₂.) Hind limbs were shaved and prepared for aseptic surgery. Buprenorphine (0.3 mg/ml; Vetergesic Alstoe Animal Health) was administered subcutaneously to all animals for analgesic purposes. The hind limbs were stretched out and secured using surgical tape. From the midline, around 0.5 cm lateral to the tail, a 1-cm incision was made and the fascia and hind leg muscles were separated. The sciatic nerve was exposed, lifted from the limb, and a 2–4-mm segment of the nerve was removed, causing minimal damage to the surrounding muscle, before the skin was closed and sutured.

Focal cartilage injury. Ten-week-old male and female C57BL/6 mice were subjected to focal cartilage injury in the patellar groove as previously described (24). Eight weeks after injury, joints were scored using the previously described modified Pineda score (25) to assess intrinsic cartilage repair.

Histologic analysis. Mice were killed by CO₂ inhalation, and the ipsilateral knee joints were collected by sharp division at the proximal femur and distal tibia and used for histologic analyses. The skin and surrounding tissue were removed, and joints were subsequently fixed in 10% formalin for 24 hours before decalcification in 20% formic acid for 1 week. The tissue was paraffin embedded, and coronal sections of 4 microns were cut at 80-micron intervals. Tissue slides were stained using Safranin O, and in the case of samples used for histologic analysis after partial meniscectomy (PMX), also fast green for microscopic inspection and histologic scoring. Histologic scoring of the mouse knees was performed in a blinded manner by 2 observers (VB and BS) using the Osteoarthritis Research Society International scoring system (26). Each joint consisted of 8–12 scored sections. Each quadrant surface (lateral femoral condyle and tibial plateau and medial femoral condyle and tibial plateau) within the joint was scored separately, and the score from each histologic section was added up to provide a section score. The 3 highest section

scores obtained for any given joint were summed to yield a cartilage damage index for that animal.

Osteophyte scoring. Osteophytes were assessed either by histologic grading of size and maturity according to previously validated methods (27) or by micro-computed tomography (micro-CT) (of the ipsilateral and contralateral joints) as previously described by our group (28).

RNA extraction and reverse transcription-polymerase chain reaction. RNA was extracted from mouse whole knee joints as previously described (29). Briefly, after skin and muscle were removed, the joint was trimmed to the upper and lower edges of the parapatellar and quadriceps tendon and then snap-frozen in liquid nitrogen. Whole knees were freeze-fractured using a precooled biopulverizer. RNA from the powdered sample was isolated using RNeasy RNA Mini kits (Qiagen) according to

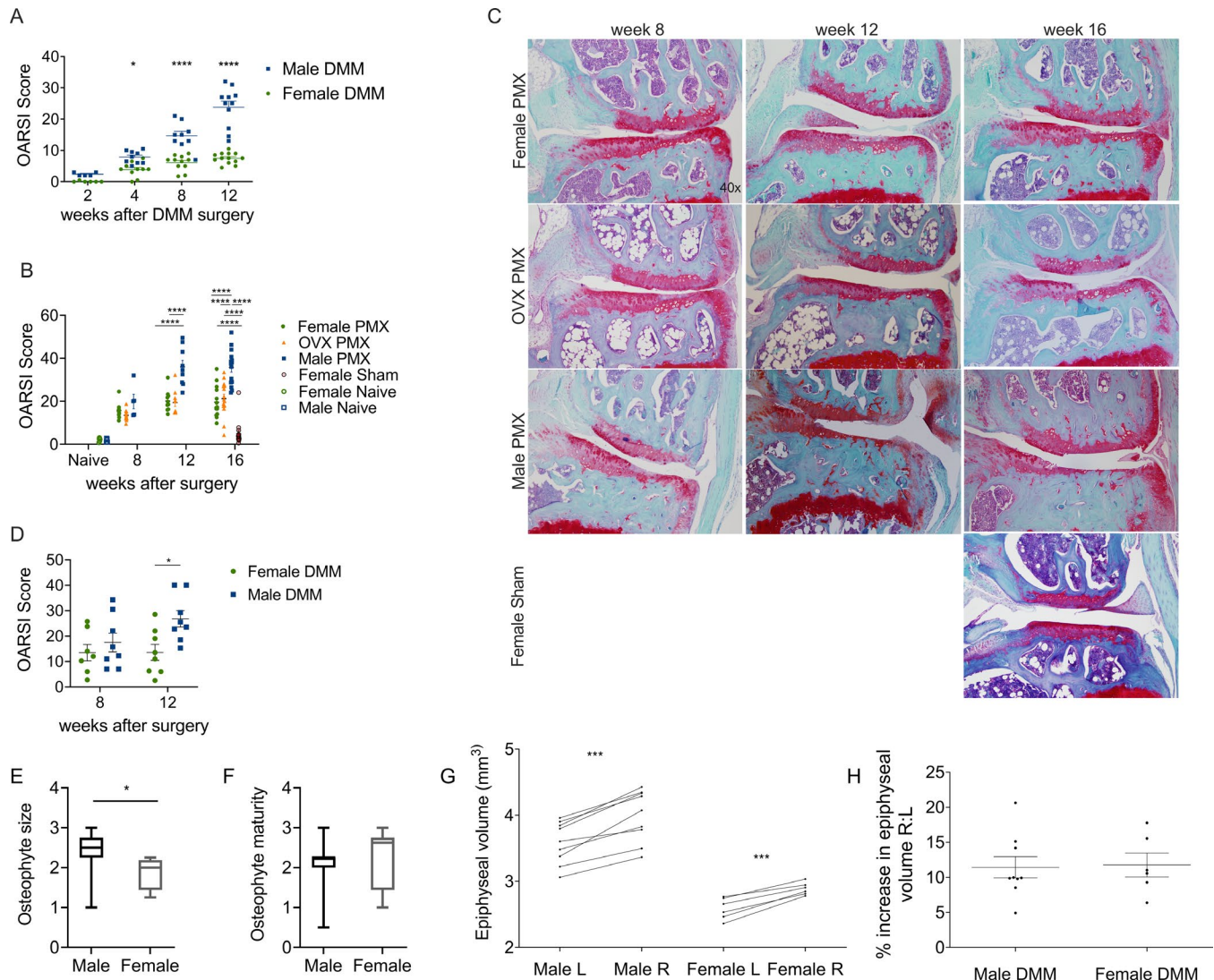


Figure 1. Reduced chondropathy scores in female mice following joint destabilization. **A**, Osteoarthritis Research Society International (OARS I) scores for the ipsilateral joints after destabilization of the medial meniscus (DMM) in male C57BL/6 mice ($n = 34$) and female C57BL/6 mice ($n = 40$). **B**, OARS I scores in naive female mice ($n = 10$), naive male mice ($n = 10$), and sham-operated female mice ($n = 10$) and after partial meniscectomy (PMX) in female mice ($n = 35$) and male mice ($n = 34$), and female mice subjected to ovariectomy (OVX; $n = 30$). Data for male mice at weeks 8 and 12 have previously been published as a supplementary figure (51). **C**, Representative sections of the medial compartment of the joint used to obtain the histologic scores shown in **B**. Original magnification $\times 10$. **D**, OARS I scores after surgical DMM in female DBA/1 mice ($n = 15$) and male DBA/1 mice ($n = 16$). **E** and **F**, Osteophyte size (**E**) and osteophyte maturity (**F**), as determined by histologic analysis. **G** and **H**, Osteophyte size, as determined by micro-computed tomography, shown as total epiphyseal volume of right (R; operated) and left (L; unoperated) epiphyses (**G**) and as the percent increase over the unoperated side (**H**). In **A**, **B**, **D**, and **H**, symbols represent individual mice; horizontal lines and error bars show the mean $\pm$ SEM. In **E** and **F**, data are shown as box plots. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Lines outside the boxes represent the 10th and 90th percentiles. * = $P < 0.05$; *** = $P < 0.001$; **** = $P < 0.0001$, male versus female mice in **A**, **B**, **D**, and **E** and right versus left epiphyses in **G**, by two-way analysis of variance, or in the absence of the same sample size within a time point, by mixed-effects analysis with Bonferroni post hoc test for multiple testing in **A**, **B**, and **D**, by unpaired t -test in **E**, and by paired t -test in **G**.

the manufacturer's instructions, and then stored at -80°C . Samples were reverse transcribed into complementary DNA (cDNA) using a high-capacity RNA to cDNA kit (Applied Biosystems). Murine cDNA was analyzed on 2 custom-made microfluidic cards (TaqMan; Thermo Scientific) including hydrolysis probes, testing a total of 67 OA-related or pain-regulating genes (see Supplementary Table 1, available on the *Arthritis & Rheumatology* website at <http://onlinelibrary.wiley.com/doi/10.1002/art.41421/abstract>). Fold change was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method, where each gene of interest was compared with a housekeeping gene (either *Fgfr3* or *Gapdh*) and normalized to the respective control group (unoperated or sham operated) for each sex.

Weight bearing measurement. Static weight bearing measurements were performed using a Linton Incapacitance Tester (Linton Instrumentation) as previously described (30,31). Briefly, mice were first acclimated to the chamber on 2 separate occasions over 2 weeks before experimental measurements. Mice were maneuvered inside the chamber to stand with 1 hind limb on each weighing scale. The weight placed on each hind limb was measured over a 1-second interval for at least 3 consecutive measurements. Results were expressed as the percentage of weight transmitted through the operated limb compared with the contralateral limb. One observer performed the measurements for each surgical experiment (ISvL for PMX and CD for DMM) and was blinded with regard to the treatment status of the mice.

Laboratory Animal Behavior Observation Registration and Analysis System (LABORAS). LABORAS allows for the measurement of mouse activity in an undisturbed environment overnight. Mice were housed individually, with 4 cages in use at any one time. They were given food and water ad libitum.

Cages contained sawdust bedding but no additional enrichment. LABORAS picked up vibrations of animal movement and converted these into behavior classifications, specifically "climbing," "locomotion," "immobility," "rearing," "grooming," "drinking," and "eating". Measurements were performed during the mouse's 12-hour active period (excluding the initial exploratory phase), 7:00 PM to 7:00 AM. The total duration for each activity over the 12 hours was calculated.

Statistical analysis. Data are expressed as the mean $\pm$ SEM and were analyzed using GraphPad Prism (GraphPad Software). For behavioral comparisons of 2 groups only, *t*-test with Bonferroni multiple comparisons test, where applicable, was used. For behavioral comparisons of 3 groups and without a repeated-measures time course, an ordinary 2-way analysis of variance (ANOVA) with Tukey's multiple comparisons test was applied. For any time course experiments, a repeated-measures 2-way ANOVA with Bonferroni test for multiple comparisons was used. In the case of missing time points, a mixed model with a Bonferroni multiple comparisons test was used instead of a repeated-measures 2-way ANOVA.

RESULTS

Do female mice develop lower chondropathy scores after surgical joint destabilization? We examined sex differences in chondropathy score after joint destabilization in a number of different groups, specifically investigating whether sex differences were robust 1) after different types of surgery (DMM or PMX), and 2) across different genetic strains (C57BL/6 or DBA/1). Lower disease severity in female mice was apparent following both DMM and PMX surgery, beginning 4 weeks after DMM and 12 weeks after PMX (Figures 1A–C). Disease severity plateaued in both

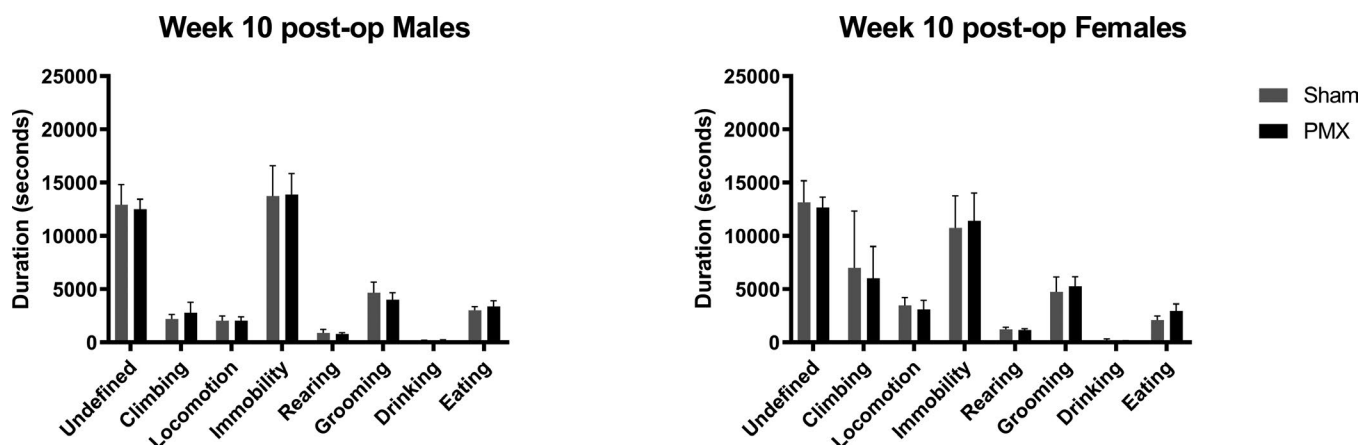


Figure 2. Activity differences in male and female C57B/L6 mice 10 weeks after partial meniscectomy (PMX) or sham surgery. Ten weeks after PMX, male and female mice were individually housed in Laboratory Animal Behavior Observation Registration and Analysis System (LABORAS) recording cages for 18 hours overnight (3:00 PM to 9:00 AM) with analysis performed between 7:00 PM and 7:00 AM. No significant differences were observed between the PMX and sham-operated groups for either male or female mice. Activity levels were similar between sexes. Bars show the mean $\pm$ SEM ($n = 7$ –10 mice per group). Differences between groups were analyzed by one-way analysis of variance with Bonferroni post hoc test for multiple testing.

female and male mice 12 weeks after PMX. Female mice subjected to OVX did not have different disease scores following PMX compared with female controls at any time point examined. In another strain, female DBA/1 mice also displayed reduced chondropathy after DMM compared with male mice (Figure 1D). Osteophyte volume and maturity (Figures 1E and F) were assessed by histologic analysis and by micro-CT, using the epiphyseal volume as a surrogate marker for osteophyte size according to our validated method (28). Osteophyte maturity was similar between male and female mice. Osteophytes were smaller in female mice than in male mice, but as a proportion of the total

volume of the epiphysis, this represented the same percentage increase (Figures 1G and H).

Are female mice less active than male mice? One possible explanation for the decreased disease observed in female mice was reduced joint use through reduced activity. We assessed the activity levels of male and female mice using LABORAS over a 12-hour period. LABORAS allows the recording of different behavioral activities of individually caged animals. It is able to distinguish between “climbing,” “locomotion,” “immobility,” “grooming,” “drinking,” and “eating”. When comparing

Table 1. Fold change in expression of genes associated with repair and inflammation in the whole knee joints of mice subjected to DMM*

Gene	Male mice		Female mice		<i>P</i> , male mice vs. female mice 6 hours after DMM†
	Unoperated (normalized)	6 hours after DMM, fold change	Unoperated (normalized)	6 hours after DMM, fold change	
Genes associated with repair					
<i>Bmpr2</i>	1.02 ± 0.10	1.04 ± 0.09	1.00 ± 0.01	1.52 ± 0.13‡	0.05
<i>Ctgf</i>	1.03 ± 0.16	1.68 ± 0.17	1.01 ± 0.09	2.11 ± 0.39§	–
<i>Fgf18</i>	1.04 ± 0.16	1.61 ± 0.21	1.00 ± 0.06	1.44 ± 0.07‡	–
<i>Fgf2</i>	1.04 ± 0.16	1.52 ± 0.32	1.01 ± 0.09	2.53 ± 0.03¶	0.05
<i>Fgfr1</i>	1.04 ± 0.18	1.05 ± 0.16	1.02 ± 0.12	1.30 ± 0.10	–
<i>Fgfr2</i>	1.02 ± 0.13	0.95 ± 0.08	1.01 ± 0.07	0.87 ± 0.03	–
<i>Fgfr4</i>	1.04 ± 0.14	0.64 ± 0.08	1.05 ± 0.18	1.41 ± 0.32	–
<i>Inhba</i>	1.02 ± 0.12	5.92 ± 1.14‡	1.01 ± 0.06	6.33 ± 0.60¶	–
<i>Ltbp1</i>	1.01 ± 0.09	1.05 ± 0.23	1.01 ± 0.10	0.97 ± 0.07	–
<i>Ltbp2</i>	1.00 ± 0.05	0.97 ± 0.13	1.01 ± 0.09	1.25 ± 0.08	–
<i>Pmepa1</i>	1.03 ± 0.16	1.37 ± 0.22	1.01 ± 0.09	2.46 ± 0.27#	0.05
<i>Tgfb1</i>	1.03 ± 0.17	1.07 ± 0.10	1.02 ± 0.12	1.17 ± 0.08	–
<i>Tgfb2</i>	1.02 ± 0.11	0.69 ± 0.08	1.01 ± 0.09	0.73 ± 0.05	–
<i>Tgfb3</i>	1.00 ± 0.06	0.72 ± 0.06	1.02 ± 0.14	1.00 ± 0.26	–
<i>Tgfb1</i>	1.02 ± 0.10	1.20 ± 0.20	1.00 ± 0.05	1.20 ± 0.06	–
<i>Tgfb2</i>	1.07 ± 0.21	1.03 ± 0.10	1.02 ± 0.13	1.04 ± 0.07	–
<i>Tgfb3</i>	1.03 ± 0.16	0.86 ± 0.19	1.02 ± 0.12	1.30 ± 0.15	–
Genes associated with inflammation					
<i>Adamts1</i>	1.03 ± 0.17	4.52 ± 1.06‡	1.01 ± 0.07	6.65 ± 0.71¶	–
<i>Adamts4</i>	1.08 ± 0.24	6.75 ± 2.52§	1.01 ± 0.09	12.05 ± 1.11§	–
<i>Adamts5</i>	1.05 ± 0.19	1.92 ± 0.55	1.01 ± 0.06	2.04 ± 0.20	–
<i>Arg1</i>	1.11 ± 0.31	61.05 ± 24.71	7.35 ± 7.02	44.75 ± 6.55#	–
<i>Ccr2</i>	1.04 ± 0.18	1.03 ± 0.16	1.00 ± 0.05	1.67 ± 0.25	–
<i>Cd68</i>	1.05 ± 0.19	1.17 ± 0.15	1.02 ± 0.12	1.13 ± 0.02	–
<i>Il1a</i>	1.14 ± 0.33	0.95 ± 0.23	1.07 ± 0.24	1.08 ± 0.22	–
<i>Il1b</i>	1.04 ± 0.16	6.29 ± 1.23‡	1.01 ± 0.06	6.31 ± 0.40¶	–
<i>Il6</i>	1.20 ± 0.38	131.49 ± 49.15	1.00 ± 0.05	129.19 ± 24.35#	–
<i>Mmp13</i>	1.05 ± 0.20	1.30 ± 0.23	1.01 ± 0.07	1.63 ± 0.10§	–
<i>Mmp19</i>	1.02 ± 0.13	2.46 ± 0.29	1.01 ± 0.10	4.45 ± 0.56¶	0.05
<i>Mmp3</i>	1.00 ± 0.05	6.58 ± 1.48	1.03 ± 0.14	13.24 ± 2.64¶	–
<i>Ptgs2</i>	1.01 ± 0.09	24.51 ± 4.89	1.04 ± 0.15	20.16 ± 2.31‡	–
<i>Timp1</i>	1.07 ± 0.23	2.72 ± 0.77	1.03 ± 0.12	3.01 ± 0.27‡	–
<i>Tnfaip6</i>	1.03 ± 0.14	25.91 ± 6.52#	1.12 ± 0.32	18.56 ± 2.15¶	–

* Values are the mean ± SEM fold change in expression compared to the housekeeping gene and normalized to the unoperated control. DMM = destabilization of the medial meniscus.

† By unpaired *t*-test.

‡ *P* < 0.01 versus unoperated mice of the same sex, by unpaired *t*-test.

§ *P* < 0.05 versus unoperated mice of the same sex, by unpaired *t*-test.

¶ *P* < 0.0001 versus unoperated mice of the same sex, by unpaired *t*-test.

P < 0.001 versus unoperated mice of the same sex, by unpaired *t*-test.

the mean activity over a 12-hour period 10 weeks after PMX, there were no significant differences between the sham-operated and PMX groups for either sex, and no apparent differences between sexes. This was the case when considering the total activity over 12 hours (Figure 2) or after breaking activity levels down into 1-hour periods (data not shown). Similar activity levels were seen in unoperated male and female mice (data not shown). The full data set for the male mice will be published elsewhere (manuscript in revision). These data indicate that LABORAS is not a sensitive tool for detecting pain-like behavior, but do not support the notion that differences in activity levels account for differences in structural joint damage.

Is cartilage repair better in female mice than in male mice? We next tested the hypothesis that damaged cartilage is better repaired in female mice than in male mice and that this could account for reduced chondropathy scores. We first assessed gene expression in response to acute joint destabilization by extracting RNA from the whole knee joint 6 hours after DMM. The analysis focused on pathways implicated in cartilage repair, including fibroblast growth factors (FGF-2, FGF-18, and FGF receptors 1, 2, and 4) and TGFβ (TGFβ1, 2, and 3 and TGFβ receptors 2 and 3). *Pmepa1*, *Bmpr2*, and *Ltbp2* have previously been shown by our group to be strong TGFβ-dependent genes in human chondrocytes (32) (Table 1). Several genes were up-regulated only

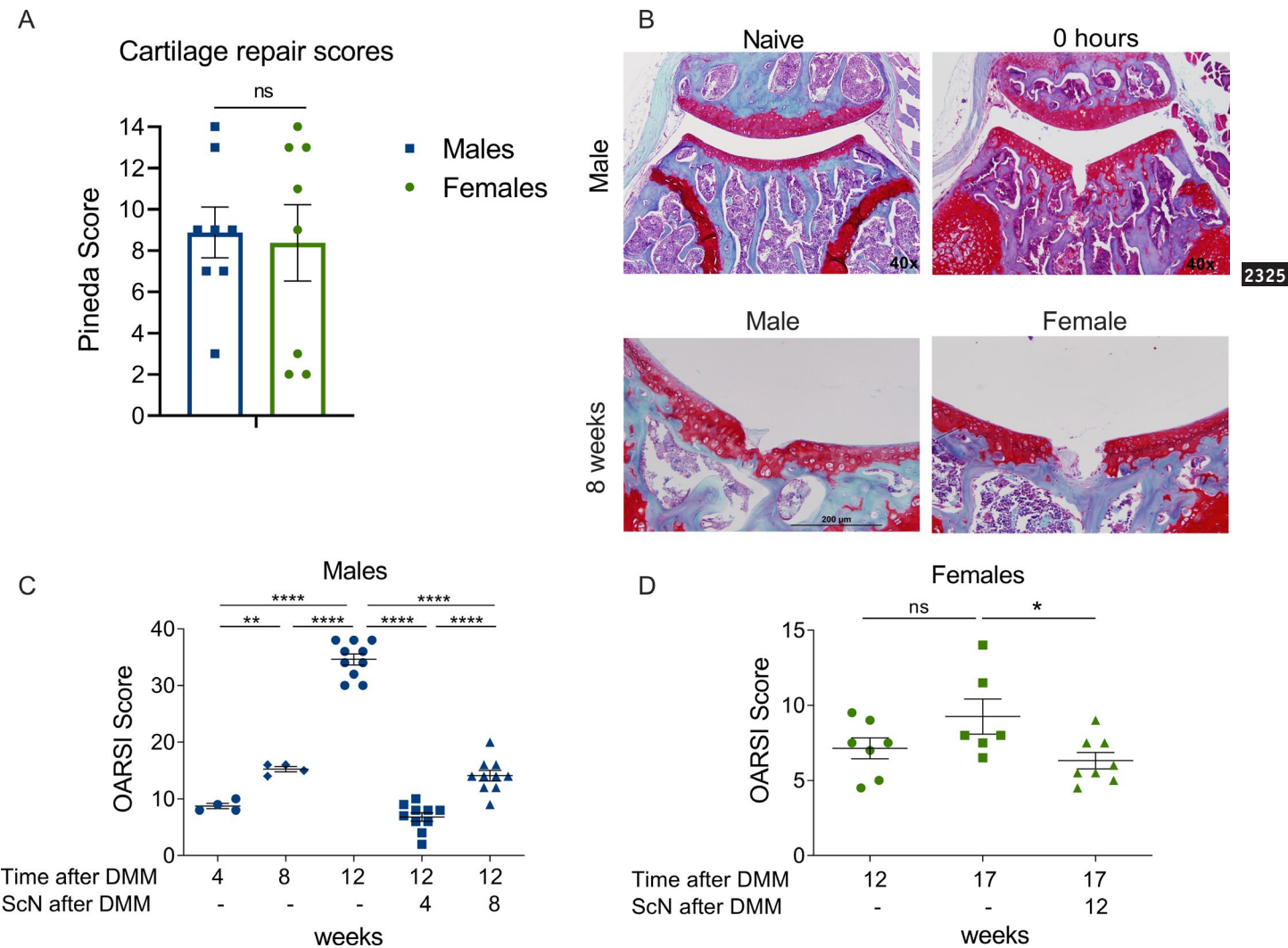


Figure 3. Female mice do not exhibit enhanced cartilage repair compared with male mice. **A** and **B**, Cartilage repair scores 8 weeks after focal cartilage injury of the intercondylar groove (**A**) and representative images at 0 and 8 weeks after injury (**B**). In **A**, symbols represent individual mice; bars show the mean ± SEM. No significant differences were observed between groups. **C**, Osteoarthritis Research Society International (OARSI) scores for the joints of male mice subjected to either destabilization of the medial meniscus (DMM) alone or DMM followed by joint immobilization (by sciatic neurectomy [ScN]) either 4 or 8 weeks after DMM. **D**, OARSI scores for the joints of female mice subjected to DMM alone or DMM followed by joint immobilization (by sciatic neurectomy) 12 weeks after DMM. For both male and female mice, joint immobilization halted disease progression. In **C** and **D**, symbols represent individual mice; horizontal lines and error bars show the mean ± SEM. * = $P < 0.05$; ** = $P < 0.01$; **** = $P < 0.0001$, by ordinary one-way analysis of variance with Tukey's post hoc test for multiple comparisons. NS = not significant.

in female mouse joints, and 3 were significantly higher in female compared with male mice: *Bmpr2*, *Fgf2*, and *Pmepa1*. Another TGF β family member that is strongly induced by FGF2, inhibin A (*Inhba*; the homodimer of which forms activin A) was up-regulated equally in both male mouse joints (mean $\pm$ SEM 5.92 ± 1.14 ; $P < 0.01$ versus unoperated control) and female mouse joints (mean $\pm$ SEM 6.33 ± 0.60 ; $P < 0.0001$ versus unoperated control).

Gene expression data suggested that the response to joint injury may produce a stronger anabolic response in female mice compared with male mice. We investigated whether this related to improved repair of articular cartilage after injury in vivo. Previously published data from our laboratory showed that OA development could be prevented by performing joint immobilization by sciatic neurectomy at the time of joint destabilization (29). Others have previously demonstrated that joint immobilization facilitated joint repair after injury (33,34).

To test whether female mice exhibited enhanced cartilage repair in vivo, we used 2 different models. The first was a focal cartilage defect model originally described by Eltawil and colleagues (24). In this model, which had historically been tested in male mice only, the authors showed both an age- and strain-dependent influence on repair, with only young DBA/1 mice exhibiting repair of the focal defect (24). In this study, male and female C57BL/6 mice were subjected to focal cartilage injury at 10 weeks of age in order to see whether cartilage was more likely to be repaired in female mice than in male mice. Joints were examined 8 weeks later. Cartilage repair was assessed using a modified Pineda score (25) (Figures 3A and B). No differences in repair were observed between the 2 groups.

In a second model, we assessed whether joint immobilization by sciatic neurectomy, after disease was established, would allow

reversal (repair) of cartilage damage induced by DMM surgery. This model has not been previously validated, and we selected different time points for both male and female mice to ensure that there was a sufficient level of chondropathy at the start of the experiment. In both male and female mice, neurectomy was able to arrest disease progression, but did not reverse disease (Figures 3C and D). Taken together, these data suggest that despite female mice having a more “repair-conductive” molecular response to joint destabilization, male and female C57BL/6 mice both fail to exhibit functional repair in vivo.

Do male mice mount a more catabolic response upon joint destabilization?

Since the inflammatory response to injury has been shown to be sex-dependent in other tissues, we next addressed whether there was a difference in inflammatory gene regulation between sexes that could account for a more catabolic response in male mouse joints. We assessed genes associated with the inflammatory response in the whole knee joints of both male and female mice 6 hours after DMM compared with unoperated mice of each sex (Table 1). Inflammatory genes were up-regulated to a similar extent in both male and female mouse joints. *Mmp19* was the only gene that was significantly different between sexes (higher in female mice [mean $\pm$ SEM 4.45 ± 0.56] than male mice [2.46 ± 0.29]; $P < 0.05$). Based on these early 6-hour results, we concluded that differences in the inflammatory response to injury were unlikely to explain the sex differences seen in chondropathy.

Do male and female mice display different OA pain-like behavior?

We next tested whether female mice displayed different pain-like behaviors than male mice. We assessed spontaneous pain-like behavior after sham or OA surgery (DMM

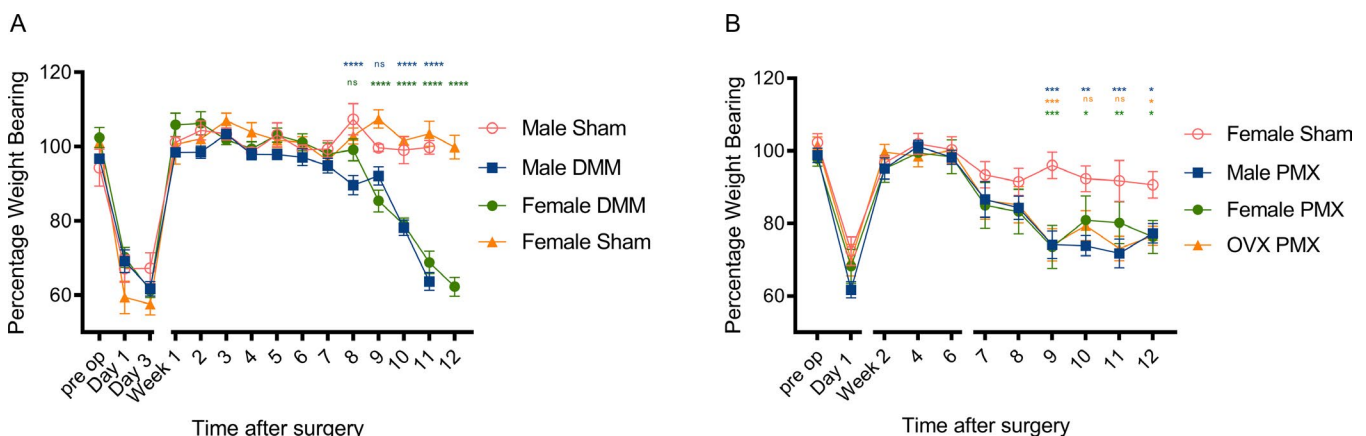


Figure 4. Male and female mice exhibit similar pain-like behaviors after either destabilization of the medial meniscus (DMM) or partial meniscectomy (PMX). Pain-like behavior was tested in male and female mice by Linton incapacitance at a variety of times after surgical induction of osteoarthritis. Weight bearing on the operated limb is shown as a percentage of weight bearing on the unoperated limb in male and female mice subjected to DMM or sham surgery (**A**) and male and female mice subjected to PMX (with or without ovariectomy [OVX] in female mice), or sham surgery (**B**). Values are the mean $\pm$ SEM ($n = 10$ mice per group for DMM and 6 mice per group for sham surgery in **A**; $n = 14$ – 15 mice per group in **B**). * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$; **** = $P < 0.0001$ versus sham-operated mice, by mixed-effects analysis with Dunnett's post hoc test for multiple comparisons in **A** and repeated-measures two-way analysis of variance with Sidak post hoc test for multiple comparisons in **B**. NS = not significant.

or PMX) in male, female, and OVX (PMX only) mice using Linton incapacitance testing, which measures weight asymmetry through the hind limbs. Spontaneous pain-like behavior was seen transiently in the postoperative period in all groups and then from ~9 weeks after PMX and 10 weeks after DMM (Figures 4A and B). No difference in the timing or severity could be discerned between the sexes. These results were somewhat surprising, as we had previously concluded that chondropathy score predicted the time of onset of late OA pain-like behavior in male mice (35,36) through the release of pain-sensitizing molecules such as nerve growth factor (NGF) from the damaged articular cartilage (35). These results suggested that female mice either have an exaggerated behavioral response to modest cartilage damage or that additional pain-sensitizing pathways are activated, perhaps elsewhere

in the joint, that are independent of cartilage loss. To test these hypotheses further, we assessed the differences in joint molecular profile at the time of established OA pain-like behavior (10 weeks after PMX).

Is the molecular pain response different between male and female mice?

To assess sex differences in the regulation of genes associated with pain, we assessed a panel of pain-related genes that we had previously used as a screening tool at a time of established late OA pain (35). This list included 29 genes that were either associated with inflammation (cytokines, chemokines, and leukocyte activation markers) or were known pain sensitizers. We compared the expression of these molecules in mouse whole knee joints between sexes, 10 weeks after PMX

Table 2. Fold change in expression of genes associated with inflammation and pain in the whole knee joints of mice subjected to PMX*

Gene	Male mice		Female mice		<i>P</i> , male mice versus female mice after PMX†
	10 weeks after sham operation (normalized)	10 weeks after PMX, fold change	10 weeks after sham operation (normalized)	10 weeks after PMX, fold change	
Genes associated with inflammation					
<i>Ccl19</i>	1.04 ± 0.19	1.50 ± 0.35	1.01 ± 0.09	0.92 ± 0.06	–
<i>Ccl2</i>	1.02 ± 0.14	1.21 ± 0.34	1.04 ± 0.20	1.06 ± 0.16	–
<i>Ccr2</i>	1.01 ± 0.13	1.18 ± 0.28	1.07 ± 0.25	1.12 ± 0.11	–
<i>Ccr7</i>	1.02 ± 0.15	0.99 ± 0.21	1.03 ± 0.17	1.23 ± 0.10	–
<i>Cd14</i>	1.01 ± 0.08	1.45 ± 0.44	1.05 ± 0.24	1.10 ± 0.09	–
<i>Il10</i>	1.11 ± 0.38	1.69 ± 0.68	1.05 ± 0.21	1.60 ± 0.19	–
<i>Il15</i>	1.05 ± 0.25	0.75 ± 0.13	1.01 ± 0.11	1.51 ± 0.23	<0.05
<i>Il1r1</i>	1.01 ± 0.11	1.64 ± 0.18	1.02 ± 0.14	1.49 ± 0.15	–
<i>Il2</i>	1.11 ± 0.37	0.99 ± 0.20	1.03 ± 0.16	1.48 ± 0.43	–
<i>Il4</i>	1.01 ± 0.10	0.90 ± 0.31	1.02 ± 0.13	1.72 ± 0.21	–
<i>Il6</i>	1.06 ± 0.28	0.88 ± 0.17	1.02 ± 0.15	1.30 ± 0.18	–
<i>Il6ra</i>	1.06 ± 0.28	1.19 ± 0.20	1.01 ± 0.08	1.54 ± 0.21	–
<i>Nos2</i>	1.02 ± 0.15	0.85 ± 0.09	1.03 ± 0.17	1.32 ± 0.14	<0.05
<i>Tnf</i>	1.00 ± 0.02	1.45 ± 0.17	1.09 ± 0.30	1.30 ± 0.09	–
Genes associated with pain					
<i>Bdkrb1</i>	1.01 ± 0.09	2.48 ± 0.34‡	1.09 ± 0.32	1.57 ± 0.23	–
<i>Bdkrb2</i>	1.00 ± 0.04	1.60 ± 0.35	1.08 ± 0.32	1.20 ± 0.10	–
<i>Cnr1</i>	1.01 ± 0.11	0.89 ± 0.19	1.09 ± 0.33	1.10 ± 0.14	–
<i>Cnr2</i>	1.02 ± 0.13	1.35 ± 0.20	1.02 ± 0.16	1.26 ± 0.17	–
<i>Gal</i>	1.02 ± 0.13	1.15 ± 0.28	1.00 ± 0.05	1.21 ± 0.13	–
<i>Gdnf</i>	1.01 ± 0.08	0.95 ± 0.20	1.06 ± 0.22	2.54 ± 0.30‡	<0.01
<i>Ngf</i>	1.01 ± 0.10	1.76 ± 0.14§	1.06 ± 0.26	1.56 ± 0.26	–
<i>Npy</i>	1.03 ± 0.16	1.38 ± 0.48	1.08 ± 0.27	0.62 ± 0.08	–
<i>Nrtn</i>	1.03 ± 0.19	1.07 ± 0.41	1.02 ± 0.15	6.71 ± 1.24‡	<0.01
<i>Ntf3</i>	1.05 ± 0.24	1.03 ± 0.20	1.11 ± 0.37	1.92 ± 0.27	<0.05
<i>Ntf5</i>	1.04 ± 0.21	0.33 ± 0.12‡	1.02 ± 0.14	2.89 ± 0.48	<0.001
<i>Penk</i>	1.03 ± 0.18	1.19 ± 0.11	1.17 ± 0.46	1.16 ± 0.15	–
<i>Pspn</i>	1.05 ± 0.23	1.19 ± 0.27	1.15 ± 0.44	0.40 ± 0.12	<0.05
<i>Tac1</i>	1.01 ± 0.10	1.15 ± 0.44	1.06 ± 0.26	1.43 ± 0.15	–
<i>Tacr1</i>	1.01 ± 0.09	2.17 ± 0.53	1.43 ± 0.85	1.68 ± 0.27	–

* Values are the mean ± SEM fold change in expression compared to the housekeeping gene and normalized to the sham-operated control. PMX = partial meniscectomy.

† By unpaired *t*-test.

‡ *P* < 0.05 versus sham-operated mice of the same sex, by unpaired *t*-test.

§ *P* < 0.01 versus sham-operated mice of the same sex, by unpaired *t*-test.

surgery (when pain-like behavior was established) (Table 2). No significant regulation of inflammatory genes was seen in either group, consistent with our previously published data, suggesting that inflammatory changes in the joint are not a major driver of pain-like behavior at this time point (35). *Il15* and *Nos2* expression levels were relatively higher in female mice (mean $\pm$ SEM 1.51 ± 0.23 for *Il15* and 1.32 ± 0.14 for *Nos2*) when compared with male mice (mean $\pm$ SEM 0.75 ± 0.13 for *Il15* and 0.85 ± 0.09 for *Nos2*) (both $P < 0.05$), although neither were significantly up-regulated compared with the respective sham-operated control.

In contrast, when considering the pain-sensitizing molecules, significant sex-dependent changes were observed. Male mice showed significant increases in *Ngf* expression (mean $\pm$ SEM 1.76 ± 0.14 ; $P < 0.01$) and *Bclrb1* expression (2.48 ± 0.34 ; $P < 0.05$), consistent with our previous publication (35). Female mice had strong and significant up-regulation of a group of neurotrophins including *Gdnf* (2.54 ± 0.30) and *Nrtn* (6.71 ± 1.24) when compared with their respective sham-operated or male PMX control. Two additional neurotrophins, *Ntf3* (1.92 ± 0.27) and *Ntf5* (2.89 ± 0.48) were up-regulated in female mice compared with male mice ($P < 0.05$ and $P < 0.001$, respectively), but were not significantly up-regulated compared with the sham-operated control. *Pspn* was down-regulated in female mice (0.40 ± 0.12) compared with male mice (1.19 ± 0.27) ($P < 0.05$).

Raw CT values are shown for both the inflammatory and pain-related molecules in the sham-operated and OA groups in Supplementary Table 2, available on the *Arthritis & Rheumatology* website at <http://onlinelibrary.wiley.com/doi/10.1002/art.41421/abstract>. No significant differences between the sexes in basal activity in any of these genes was detected after sham operation (by *t*-test; data not shown). Taken together, these results suggest that female mice develop pain-like behavior after less severe cartilage damage and may use different molecular pathways to drive pain-like behavior.

DISCUSSION

We have confirmed the findings of a previous study showing that male mice develop more severe chondropathy after surgical joint destabilization compared with female mice (8) and extended the previous study by showing that this finding was robust across different strains and surgical induction methods. We were unable to confirm a reversal of female protection in OVX animals. This is not easily explained, but it is the case that several other studies have also failed to observe this reversal (10). OVX mice were purchased from a commercial provider, and we were able to verify estrogen deficiency by estrogen-dependent gene expression (data not shown). The previously published study by Ma et al (8) concluded by suggesting that lower chondropathy might be due to different activity levels between male and female mice (higher activity predisposing to increased disease). We were unable to substantiate this using LABORAS. We did not find evidence of

different activity levels between the 2 sexes, although we were also unable to distinguish activity levels between PMX and sham-operated animals in either sex, indicating that LABORAS is not very sensitive. Further details of this insensitivity will be published elsewhere (manuscript in revision).

Next, we explored sex differences in the molecular response to joint destabilization, addressing specific clusters of disease-modifying genes. Looking at the immediate post-injury gene expression profile in whole knee joints, we saw no difference in the inflammatory/catabolic genes between male and female mice but did see some differences in genes relating to the TGF β pathway, suggesting that female mice could elicit an enhanced repair response after injury. This was subsequently tested in 2 models of in vivo repair. No differences between the sexes were observed, and we concluded that their repair profile (or lack thereof), at least in these stringent models, was similar.

Interestingly, we found the same time of onset and severity of pain-like behavior in male and female mice following either DMM or PMX. This finding has recently been confirmed by another group using the medial meniscal transection model, in which the authors also confirmed reduced chondropathy in female mice (37). This was a little surprising in view of the fact that we had previously suggested that the onset of pain-like behavior in mice arises at the time that the junction between the calcified and noncalcified cartilage (tidemark) is breached (35). These results could have suggested that pain-like behavior in female mice was originating from a tissue other than the cartilage, such as the synovium. Although we cannot completely exclude this possibility, the inflammatory gene profile in the joint at the onset of pain-like behavior indicated that there was little joint inflammation present in OA joints of either male or female mice, suggesting that synovitis was unlikely to be contributing to the pain phenotype. This suggests that female OA pain-like behavior in this model is "noninflammatory," as we have previously concluded for male mice (35). We also studied osteophyte development in male and female mouse joints. Osteophyte development in murine OA occurs rapidly after joint destabilization (within the first 2 weeks) (28) and does not correlate temporally with pain-like behavior changes. No differences in the size (measured by percent volume of the epiphysis) or maturity of the osteophytes were detected between male and female mice.

At the time of established OA pain-like behavior, it appears that female mice regulated a different, additional set of potential pain-modulating genes. These included *Gdnf*, *Nrtn*, *Ntf3*, *Ntf5*, and *Pspn*. Glial cell line-derived neurotrophic factor (GDNF), neurturin, and persephin are trophic factors for dopaminergic motoneurons and midbrain neurons and are being explored in Parkinson's disease (38,39). They signal through a common receptor family and are all part of the TGF β superfamily (40). GDNF and neurturin have been associated with inflammatory bone pain (41), and GDNF has also been suggested as an analgesic target in neuropathic pain (38,39). As we found that TGF β activity was increased in female mice after joint injury

compared with male mice, it is possible that a common mechanism exists for the activation of TGF β superfamily molecules. Neurotrophic factor 3 (NTF3) and NTF5 are both neurotrophic factors related to NGF. NTF3 and NTF5 are largely regarded as survival factors for sensory neurons (42,43), but NTF3 has also been shown to have analgesic effects specifically by down-regulating transient receptor potential vanilloid channel 1 and suppressing thermal hyperalgesia (44). No sex differences have been reported for the regulation of any of these molecules. NGF, a well-known pain target for OA (45,46), was not differentially regulated between the sexes, suggesting that it contributes to OA pain-like behavior in both male and female mice, although in this analysis NGF was not significantly increased in female mouse joints compared to sham-operated joints. It would have been nice to check that both male and female mice responded equally to anti-NGF therapy, but this was not available to us.

Our data are consistent with mounting evidence that shows pain can be relayed by different mechanisms in males and females, suggesting that there are common as well as distinct sex-specific pathways (47). In females cells of the adaptive immune system have been shown to relay pain-like behavior (21). Although we cannot completely exclude a mechanism that involves adaptive immune cells in the present study, we did not see any evidence of classical inflammatory gene regulation in either male or female mouse joints at the time the mice developed pain-like behavior.

The inclusion of women in clinical studies only became mandatory in the US in 1993, while preclinical studies still do not require the inclusion of both sexes (17,48). Part of this is due to fear of estrous cycle-related variability in females, although it has been suggested that fighting to establish dominance hierarchies in male mice can introduce as much variability, if not more (17). The inclusion of females in preclinical studies is important to identify female-specific pathways in drug discovery and to avoid results from male animals being falsely generalized to females (17,48). The marked female predominance in chronic pain conditions, higher documented pain sensitivity, lower pain thresholds, and higher pain ratings (15–18) reinforce the fact that preclinical studies probably need to be done in both sexes if we are to attempt to understand their molecular basis. This approach is already being implemented by the National Institutes of Health in the US (49). This work has important implications for the National Centre for the Replacement, Refinement, and Reduction of Animals in Research. While the goals of replacement, reduction, and refinement must be upheld, it may be necessary to study such pathways in both sexes in order not to miss sex-specific differences. What is also evident is that it would not be appropriate to perform analyses of mixed populations of male and female animals in mechanistic studies.

Finally, could chondroprotection in female mice be a result of earlier pain sensitization? Given the highly mechanosensitive nature of OA (29), we speculate, as others have done previously, that

pain protects the joint by preventing overuse. Since female mice are sensitized at an earlier stage of their disease, this could be one mechanism to explain the reduced chondropathy observed. This debate is topical in view of the discussion around rapidly progressive OA in individuals receiving good analgesic control with therapies such as anti-NGF (50).

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Vincent had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. von Loga, Vincent.

Acquisition of data. von Loga, Batchelor, Driscoll, Burleigh, Chia, Stott, Miotla-Zarebska, Riley, Dell'Accio.

Analysis and interpretation of data. von Loga, Batchelor, Driscoll, Burleigh, Dell'Accio, Vincent.

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