injection of DAPT. In another group of mice 12 weeks after DMM surgery, knee hyperalgesia was also reduced 6 hours after DAPT injection. RNA was extracted from L3-L5 DRG at this time point, and qRT-PCR analysis revealed that both *Ccl2* and *Ngf* mRNA expression were decreased compared to the mice injected with vehicle (n=6 in each group) (Fig. 1C). IA injection of LPS (3 μg) induced knee hyperalgesia in naïve mice, significantly more than vehicle injection at 2 and 4 hours after injection (one way ANOVA test, p<0.001) (Fig. 1D). *Rbpj* mRNA expression was increased in DRG 4 hours after IA administration of LPS, compared to saline (n=8 in each group) (Fig. 1E). The levels of NICD protein in the DRG tissue lysates were increased in LPS-injected mice (n=4 in each group) (Fig. 1F).

Conclusions: ScRNA-seq analysis confirmed expression of Notch signaling genes in nociceptive neurons of L3-L5 DRG. IA administration of DAPT attenuated knee hyperalgesia and *Ccl2/Ngf* expression in DRG after DMM surgery. Both CCL2 and NGF are key pro-algesic mediators in the DMM model, and their expression is elevated in the DRG in this model. Furthermore, IA injection of LPS in naïve mice caused knee hyperalgesia, along with increased Notch gene expression and increased NICD protein in the DRG. Combined with our previous data using *in vitro* DRG cell culture, these *in vivo* findings suggest that sensory neuron Notch signaling may act downstream of TLR signaling in promoting synthesis of pro-algesic mediators, and thereby contribute to the pathological development of knee hyperalgesia.

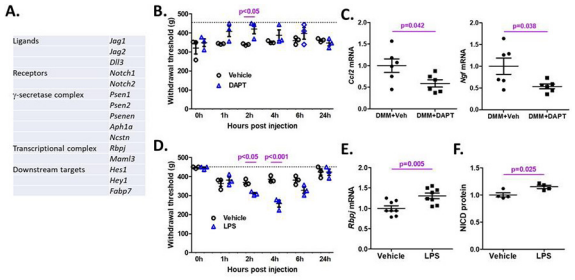

Fig. 1. (A) Notch signaling genes expressed in nociceptors in L3-L5 DRG revealed by scRNA-seq. **(B, C)** Inhibition of Notch signaling by IA injection of DAPT in mice after DMM. Knee hyperalgesia assessment (B) and *Ccl2/Ngf* mRNA expression in DRG (C). **(D-F)** TLR4 stimulation by IA injection of LPS in naïve mice. Knee hyperalgesia assessment (D), *Rbpj* mRNA expression in DRG (E), and NICD in DRG lysates (F).

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SINGLE NUCLEI RNA-SEQUENCING OF PORCINE ARTICULAR CARTILAGE UNCOVERS THE CHONDROCYTES CLUSTERS THAT CHANGE IN RESPONSE TO MECHANICAL INJURY

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Purpose: Mechanical loading or cutting injury of cartilage activates a rapid (<4h) sterile tissue injury response that includes the induction of a number of inflammatory and anti-inflammatory pathways. Chondrocytes are the only cell type in cartilage although previous single cell analyses of osteoarthritic cartilage indicate that several sub-groups of chondrocytes exist. In view of the highly mechano-sensitive nature of articular cartilage, we have developed a protocol for single nuclei RNA sequencing (snRNA-seq). Single nuclei can be sequenced in a similar fashion to single cells, but allow analyses to be performed from frozen *ex vivo* tissue to avoid confounding by the tissue injury response. Here we also examine the early chondrocyte response to injury, the most important etiological factor in OA development.

Methods: Articular cartilage was dissected from porcine metacarpophalangeal joints of 3-6-month-old pigs. Cartilage explants were either snap-frozen at time 0 or cultured in serum-free medium for 4 or 48 hours before being snap-frozen. Nuclei were isolated in ice-cold lysis buffer using a pre-cooled Dounce homogenizer. Isolated nuclei were sorted by DAPI signal and size to get rid of cell debris. After sorting, nuclei went through quality control and were processed in the snRNA-seq workflow for sequencing. 10x snRNA-seq data were generated for 10 samples (0h n=4; 4h n=3; 48h n=3). Sequencing reads were mapped using Cell Ranger with the Sscrofa11.1 reference transcriptome. For downstream analysis we selected nuclei with > 200 genes and < 2% mitochondrial reads (n=29,552 nuclei). Data were pre-processed with SCANPY and analysed with pipeline_scxl.py (https://github.com/sansomlab/tenx).

Results: snRNA-seq data showed 14 cell clusters in all samples at three different time points. 8 clusters expressed chondrocyte marker genes including aggrecan (*ACAN*), type II collagen (*COL2A1*), type XI collagen (*COL11A1*). Other minor clusters were identified as a bone cell cluster, endothelial cell cluster and two other unknown clusters. Two clusters were enriched at 4 h post cartilage injury. They expressed marker genes known to be regulated by injury in bulk mRNA analyses such as nerve growth factor (*NGF*) and inhibin subunit beta A (*INHBA*), as well as anabolic growth factors transforming growth factor beta 1 (*TGFB1*) and bone morphogenetic protein 2 (*BMP2*). Injury-induced clusters had largely disappeared by 48h.

Conclusions: We have developed a method and performed a snRNA-seq analysis of porcine articular cartilage after mechanical injury. 8 clusters of chondrocytes were identified and two of them were strongly regulated by mechanical injury at 4h. Data analysis is on-going to understand the role of different clusters in cartilage biology and to determine which are the injury responsive cells in the tissue.

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NADPH OXIDASE 4 DEFICIENCY ATTENUATES EXPERIMENTAL OSTEOARTHRITIS IN MICE

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Purpose: Osteoarthritis (OA) is a degenerative disease characterized by damage of articular cartilage, alteration of subchondral bone, and inflamed synovium. Articular cartilage is essentially composed of an avascular matrix produced by chondrocytes. In OA, articular cartilage follows a progressive degeneration, where low-grade inflammation plays a pivotal role through an oxidative stress-dependent mechanism and exposure to reactive oxygen species (ROS). In chondrocytes, the only cells of the cartilage, NADPH oxidase 4 (NOX4) is one of the major ROS producers and plays a major role in catabolism regulation. In this study, we evaluated the role of NOX4 during experimental OA in mice.

Methods: Cartilage explants isolated from 9 weeks old femoral heads (wild type (WT) and NOX4$^{-/-}$ mice) were cultured 72 hours with either PBS or IL-1β to induce experimental OA *ex vivo*. Cartilage explants were then fixed with paraformaldehyde 4% and prepared for histology analysis. Anabolism, catabolism, NOX4 expression and oxidative stress were assessed by immunohistochemistry (IHC). *In vivo*, OA was induced by destabilization of the medial meniscus (DMM) in WT and NOX4$^{-/-}$ mice (n=12 for each group). At day 0, mice were radiographed using a microCT and operated. Eight weeks after surgery, mice were radiographed and sacrificed for histological analysis. Anabolism, catabolism, NOX4 expression, macrophage recruitment and oxidative stress were assessed by IHC. Bone phenotype was also determined by histomorphometry on sections after safranin'O coloration.

Results: *Ex vivo*, we demonstrated with safranin'O staining that NOX4 deletion protects from the loss of proteoglycans in the cartilage induced by IL-1β. We also showed inWT explants, a decrease of AGG expression and an increase of MMP13 and COL1 expression, but we did not observe any effects on COL2 expression after IL-1β treatment. In NOX4$^{-/-}$ compared to WT cartilage, we observed a higher expression of AGG, a lower expression of MMP13 and COL1 in the superficial cartilage. To better understand the role of NOX4, we performed NOX4 and 8-OHdG (ROS marker) IHC. As expected, we showed an increase of NOX4 expression in explants treated with IL-1β and no staining in the KO. Even more, we demonstrated that IL-1β stimulation increased cartilage expression of 8-OHdG in WT explants but not in NOX4-/- explants. *In vivo*, histological analysis of the knee joint, 8 weeks post-surgery, demonstrated that DMM induced significant OA damages in WT mice (OARSI score of 2.8±1.2). A significant improvement was observed in DMM-NOX4$^{-/-}$ mice with a reduced OARSI score of 1.5±0.5. We observed thicker cartilage and less osteophytes formation in DMM-NOX4$^{-/-}$ mice as compared to DMM-WT mice. By micro-CT, we observed no differences at day 0 between WT and NOX4$^{-/-}$ mice. However, 8 weeks after DMM, a slight increase was observed in the trabecular thickness (Tb.Th) and in the trabecular space (Tb.Sp) as compared to day 0, only in WT mice. By histomorphometry we confirmed that 8 weeks after DMM there is a slight increase of the BV/TV, Tb.Th and Tb.Sp. Moreover, we observed that the NOX4$^{-/-}$ mice had a higher Tb.Th compared to the WT mice. By IHC we first showed that there is a stronger NOX4 expression after DMM surgery compared to the sham. Interestingly, we only observed NOX4 staining in the cartilage and not in the synovial membrane. We

then evaluated the effects of NOX4 deletion on cartilage anabolism and catabolism by IHC. As expected DMM surgery increased catabolism (MMP13 and COL1 expression) and inhibited anabolism (COL2 expression). In comparison NOX4 deletion in mice inhibited MMP13 and COL1 expression and increased COL2 expression after DMM surgery. Then we assessed if the effects observed in the NOX4$^{-/-}$ mice was linked to synovial inflammation and ROS production in the cartilage in vivo (Figure 8). First, we evaluated the expression of NOX4 by IHC. With HE staining, we demonstrated that DMM surgery induced synovial inflammation (increased cellularity and thickness) in the WT mice but not in the NOX4-/- mice. Using synovitis scoring system, we observed an inflammation score of 2.5 ± 0.5 in WT mice versus 0.9 ± 0.4 (p<0,01) in NOX4-/- mice. In accordance with these results, we observed a strong decrease of the expression of the macrophage marker F4/80 in the synovial membranes of the NOX4-/- mice compared to the WT mice. Finally, we demonstrated that NOX4 deletion inhibited 8-OHdG expression in the cartilage and decreased its expression in the synovial membrane.

Conclusions: Our results demonstrated that NOX4 deficiency decreases cartilage degradation and regulate the anabolism/catabolism balance *ex-vivo* after IL-1β stimulation and decreases significantly experimental OA severity in mice. We also showed that NOX4 increase OA severity via chondrocytes ROS production and synovial inflammation. Taken together these results underline that NOX4 could be a major target to dampen OA progression.

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CHARACTERIZING THE COMBINED EFFECT OF PHYSICAL ACTIVITY MEASURES ON STRUCTURAL PROGRESSION OF OA: THE MULTICENTER OSTEOARTHRITIS STUDY

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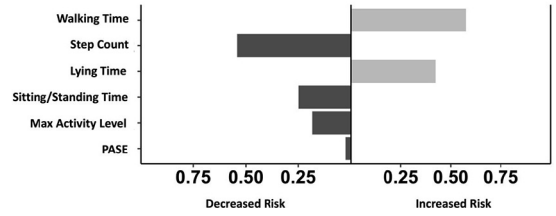
Purpose: Physical activity is consistently recommended as a management strategy for knee osteoarthritis (OA); however, studying the effect of daily physical activity on structural progression of OA remains challenging. Accelerometry data, for example, quantifies activity but does not specify the type of activity or its complexity. Surveys often capture frequency and intensity of various complex activities and provide complementary information to that of accelerometry. Previous studies have used either surveys or accelerometry to assess the relation of physical activity to OA outcomes but not both. Further, analyses of both surveys and accelerometry have usually summarized overall activity when it is likely that different types of activity affect OA differently. It is challenging to infer the overall effect of various activity measures because focusing on one in a model assumes the others remain unchanged (i.e., holding other measures constant), which is unlikely. It is unclear how various measures of physical activity could act synergistically or antagonistically on OA outcomes, which could be important for informing the physical activity prescription. The goal of this study was to use both survey and accelerometry data to quantify the combined effect of physical activity measures on cartilage loss using a causal-inference based approach, and to characterize the direction and effect size for the contribution of each individual physical activity measure.

Methods: We used data from the 144- and 168-month study visits of the Multicenter Osteoarthritis (MOST) Study, a NIH-funded longitudinal cohort of persons with and without knee OA. Magnetic resonance imaging (MRI) was read in one knee per person; thus, data from only one knee from each participant was analyzed. The outcome was defined as a binary (dichotomized) variable representing any 1/2 grade or greater worsening of area and/or depth of medial tibiofemoral cartilage (i.e., increase in semi-quantitative MRI Osteoarthritis Knee Score [MOAKS] score) between 144- and 168-month visits. Physical activity measures included the Physical Activity Scale for the Elderly (PASE) total score and AX3 accelerometer-based physical activity measures (averaged over >= 3 valid wear days): time spent walking, sitting/standing, and lying, step count, and maximum overall activity level based on signal vector magnitude. Confounders measured at 144-month visit included sex, race, body mass index (BMI), Center for Epidemiologic Studies Depression Scale (CES-D), Kellgren and Lawrence (KL) score, hip-knee-ankle alignment, knee injury history, and knee pain quantified by the Western Ontario and McMaster Universities Osteoarthritis (WOMAC) score. We developed a marginal structural model, estimated by quantile g-computation, to quantify the combined effect, referred to as mixture effect, of various physical activity measures on the odds of cartilage loss. For quantile g-computation, we quantized each physical activity measure into half a decile increments (producing 20 levels or "ventiles", each containing 5% of data).

Results: The study sample included 1416 MOST participants (60% female; 13.2% Black or African-American; 144-month visit mean age = 61.0 [SD = 9.1], mean BMI = 28.3 [SD = 4.9]). Of 1416 knees, 249 (17.6%) experienced cartilage loss worsening. Estimate of the mixture effect of physical activity measures suggested that an increase in physical activity measures reduced the odds of cartilage loss over 2 years. Specifically, the odds of cartilage loss decreased by 6% (marginal causal odds ratio = 0.94, 95% CI: 0.87, 1.03) per ventile (i.e., half a decile) increase in physical activity measures. Among physical activity measures, an increase in walking time and lying time components were associated with increased odds of cartilage loss, while an increase in step count, sitting/standing, maximum overall activity level, and PASE scores were associated with reduced odds of cartilage loss (Figure 1).

Conclusions: These results suggest that physical activity, defined using a combination of physical activity measures, reduces the odds of cartilage loss, supporting current guidelines that recommend physical activity as a management strategy for knee OA. The positive impact of step count in the context of a negative impact of walking warrants further exploration of activity pacing and intensity of physical activities.

Figure 1. Scaled effect sizes for the effects of various physical activity measures on cartilage loss



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IMPROVED WOMAC PHYSICAL FUNCTION IS ASSOCIATED WITH SLOWED PATHOLOGICAL BONE SHAPE CHANGE AFTER TPX-100: TOWARDS A SURROGATE MARKER FOR VIRTUAL KNEE REPLACEMENT?

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Purpose: TPX-100 is a 23-amino acid peptide derived from a small integrin-binding ligand N-linked glycoprotein (*SIBLING*) family member: Matrix Extracellular Phosphoglycoprotein (MEPE). MEPE is highly expressed by osteocytes and may play a role in bone remodeling. Previously reported non-clinical and clinical data support the safety and efficacy of IA TPX-100 in knee OA. In Study TPX-100-5, we were interested in examining clinical and structural measures that have been shown to predict joint failure and total knee replacement (TKR), informed by two recent research streams. The MOST study (N ≈ 2700) demonstrated a strong predictive association between WOMAC Physical Function (Table 1) and risk of TKR. There was a marked pain-independent dose-response relationship for all levels of functional impairment. Poorer scores were associated with increased risk of TKR up to 15 times overall and 5.9 times after controlling for severity of pain. Structurally, MRI-based femoral bone shape (B-score) analysis also has