Title

Association Between Synovial Tissue Metabolomic Profile And Pain In Patients With Knee Osteoarthritis

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(TgGPx4) underwent surgery to destabilize the medial meniscus (DMM). Mice were euthanized 24 h after DMM. To provide a broad indicator of oxidative distress *in vivo* distinct from LP or GSH, 3 h prior to euthanasia 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) was injected 0.5 g/kg. Mouse stifles were harvested and processed for immunohistochemistry. The meniscus uncovered articular cartilage was stained for mitochondrial content (TOMM20) and oxidative distress (DMPO). To explore direct biochemical effects of LP on primary articular chondrocytes, fresh osteochondral tissue was harvested from the load bearing region of the tibia of agricultural bovine stifles. All specimens were cultured in DMEM/F12 with 10% fetal bovine serum at 5% CO_2, 5% O_2, 37°C. The next morning, media was dosed with 500 nM α-tocopherol acetate (VitE) or 200 μM tert-butyl hydroperoxide (tBOOH), a LP perpetuator. GSH status was interrogated using the Griffith 2-vinylpyridine/glutathione reductase recycling assay. Image analysis was restricted to cartilage and used ImageJ. Statistical analysis was done with GraphPad Prism using one way ANOVA and t-test post-hoc.

Results: IAF pigs demonstrated significantly decreased CellROX oxidation compared to contralateral control (CC) limbs. This was accompanied by a strong trend towards decreased MTDR, fig 1AB. TgGPx4 mice had increased mitochondrial content TOMM20 in their CC cartilage compared to WT CC. Interestingly, TgGPx4 still showed significantly less TOMM20 staining after injury, fig 2A. The oxidation marker DMPO trended higher with DMM in the TgGPx4 group in preliminary analyses, fig 2B. Biochemical analysis *in vitro* revealed that total GSH was not significantly decreased with VitE or tBOOH treatment, but VitE treatment demonstrated a strong trend toward increased oxidation, % GSSG, fig 3A. VitE supplementation increased MTDR in a dose-dependent manner *in vitro*, fig 3B.

Conclusions: Our results indicate that PTOA shows the same intermediate-to-late loss in oxidative activity observed in non-traumatic OA models. Augmenting GPx4 *in vivo*, decreasing LP, appears to increase mitochondrial mass in uninjured limbs and increase oxidant production within injured stifles. Biochemical results *in vitro* demonstrate LP has an inverse relationship with basal GSH redox status. Muting LP with VitE increased mitochondrial staining and oxidation of GSH to GSSG. These results support our hypothesis and indicate a previously undescribed intersection between mitochondrial content and oxidant production through LP, basally and after injury. Distinct GSH- and LP-specific redox responses are involved in changes in mitochondrial content that suggest understanding specific interactions between cellular metabolism and redox stress will be crucial to understanding the traumatic injury response of cartilage.

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ASSOCIATION BETWEEN SYNOVIAL TISSUE METABOLOMIC PROFILE AND PAIN IN PATIENTS WITH KNEE OSTEOARTHRITIS

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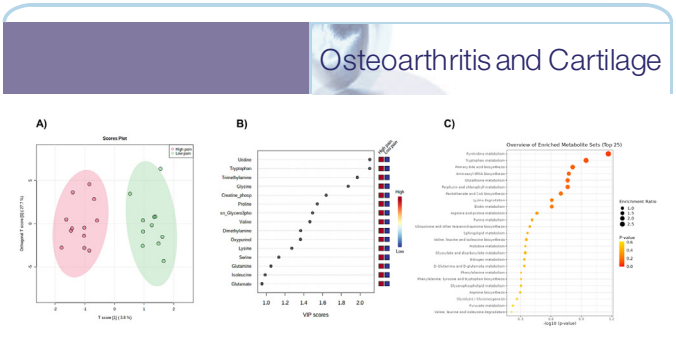
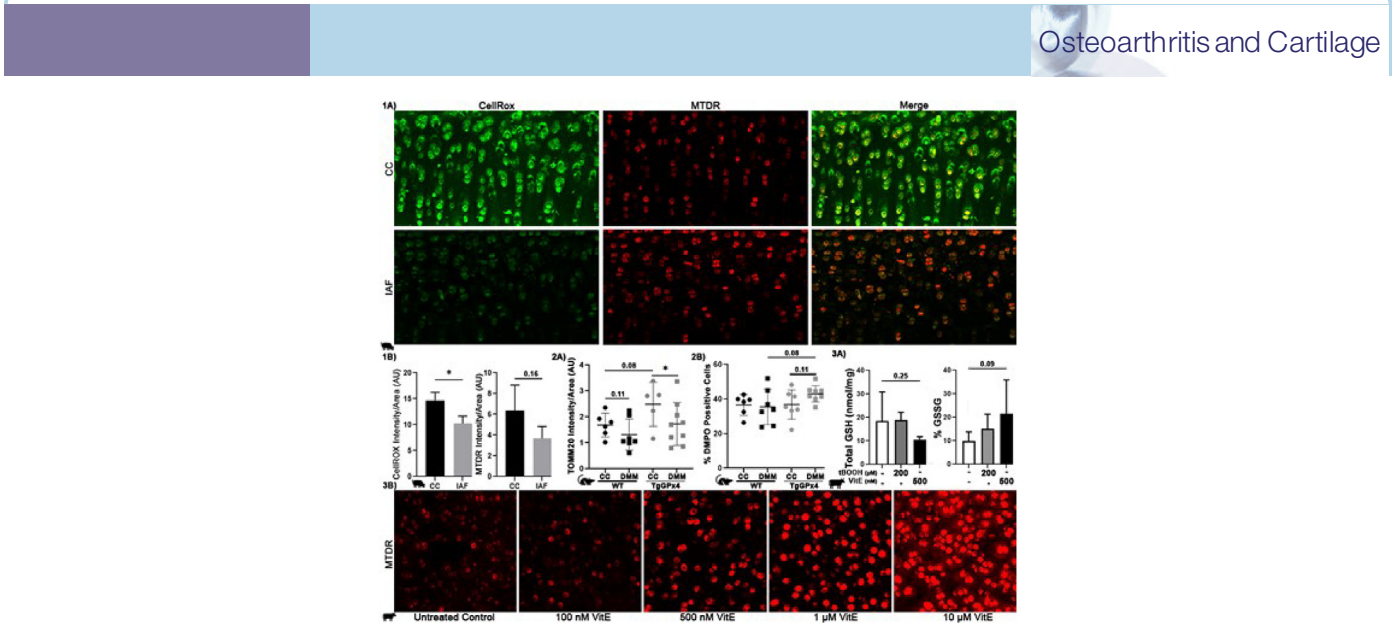
Purpose: The progression of osteoarthritis (OA) is mediated by low-grade synovial inflammation (synovitis) and this inflammation contributes to joint pain and stiffness, especially in knee OA (KOA). In addition, chronic inflammatory responses are associated with significant shifts in tissue metabolism. This study determined the association of synovial tissue metabolomic profiles with knee pain in OA subjects.

Methods: Synovial tissue (ST) and synovial fluid (SF) samples were collected from patients with advanced OA during knee replacement. ST was snap-frozen for metabolomic analysis. Patients self-reported nociceptive and neuropathic pain using the WOMAC and the painDETECT questionnaires respectively. Nerve Growth Factor (NGF) was quantified in SF by enzyme-linked immunosorbent assay (ELISA). A 600 MHz Bruker Avance III spectrometer ^{1}H-NMR was used to acquire NMR spectra of ST samples. Chenomx NMR suite 8.5 professional software was used for metabolite identification and quantification. The samples were normalized by volume/weight and the concentrations were





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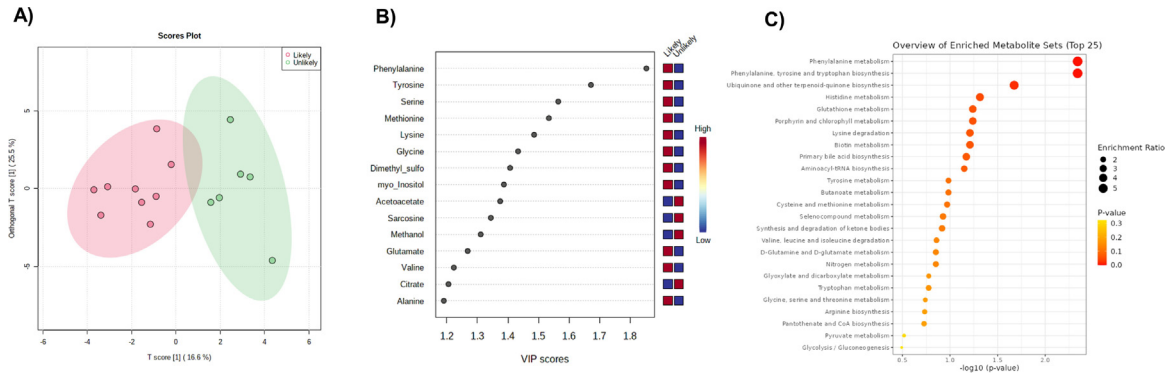

reported in μM. Metaboanalyst 5.0, SPSS v26, and R studio were used for statistical analysis.

Results: 23 male patients (average age: 68 years, standard deviation (SD) 6.59, average BMI 28.2, SD 3.9) were recruited. At the time of the surgery, patients had a mean total WOMAC of 54.5 (SD, 18.2), WOMAC pain of 11.63 (SD 13.55), WOMAC stiffness of 4.7 (SD 1.6), WOMAC difficulty of 37.9 (SD 13.5), and painDETECT of 14.9 (SD 7.5). SF NGF levels had a mean of 34 (SD 14.5) ng/mL. 47.8% of patients were classified as low pain (WOMAC pain $\leq$10) and 52.2% as high pain (WOMAC pain>10). For neuropathic pain, 62.5% patients were classified as likely pain (PainDETECT >12) and 37.5% were classified as unlikely (PainDETECT $\leq$12). SF NGF levels positively correlated with painDETECT (r=0.60, p=0.030) but not with WOMAC pain (r=0.21, p=0.35). The orthogonal partial least square discriminant analysis (OPLS/DA) showed a good separation of patients for painDETECT and WOMAC pain. Several metabolites, including creatine phosphate, glycine and tyrosine were positively correlated with painDETECT, while acetoacetate had a negative correlation. Dimethylamine, proline, tryptophan and valine were positively while threonine was negatively correlated with WOMAC pain. Phenylalanine and tyrosine metabolism, and phenylalanine, tyrosine and tryptophan biosynthesis were enriched metabolic pathways in patients with likely neuropathic pain (Figure 1), while pyrimidine and tryptophan metabolism contributed to the discrimination between low and high WOMAC pain (Figure 2).

Conclusions: Metabolic profiling of synovial tissue from men with knee OA was different in subjects with neuropathic and nociceptive pain. Several metabolites differentially correlated with WOMAC and painDETECT suggesting different metabolic pathways are involved in knee OA pain. In addition, SF NGF levels was only associated with neuropathic pain. Further studies are needed to confirm these associations are also observed in women with knee OA and to determine if metabolomic profiling of synovial tissue can identify novel therapeutic targets for OA pain.

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EXTRACELLULAR VESICLE PEPTIDES INDICATE SEVERITY OF KNEE RADIOGRAPHIC OSTEOARTHRITIS

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Purpose: In our recent publication, we characterized surface markers and proinflammatory cytokines carried by EVs from plasma and SF of participants with knee osteoarthritis (OA), with a primary focus on immune cell associated EVs. We now extend our research to identify EV peptides correlated with knee radiographic OA (rOA) severity. We hypothesize that such EVs are most likely to be involved in the pathogenesis of OA.

Methods: EVs were separated by ExoQuick precipitation from the matched plasma and SF specimens from participants with knee OA (n=16); all specimens were acquired with informed consent under Institutional Review Board approval. The number of EVs was determined by high-resolution flow cytometry. EVs were processed for proteomics by high-resolution mass spectrometry coupled with label-free quantitation. The relative quantitative expression value of each peptide was normalized to total EV number of each participant determined by flow cytometry. Statistical analysis included Spearman correlation analyses adjusted for age, gender, and body mass index (BMI) to identify independent associations between EV peptides and knee rOA severity (knee Kellgren Lawrence [K/L] grade, joint space narrowing [JSN] and osteophyte [OST] outcomes).

Results: Participants were of mean age 69 $\pm$ 12 years (range 51-87) and 50% female. Knee OA radiographic (rOA) severity was determined for both the index (ones providing SF) and contralateral knees based on scores of three rOA features: K/L grade that ranged from 2-4 in the index knees with K/L sum score ranges of 2-7 for both knees; JSN grade that ranged 0-4 in the index knees and summed JSN grade ranges of 0-9 for

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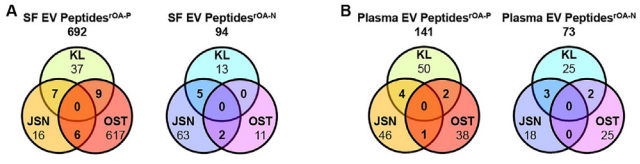

Figure 1. Multiple peptides from SF EVs and plasma EVs of knee OA participants were significantly correlated with knee rOA severity. EVs were separated from the matched plasma and SF specimens from knee OA participants (n=16) and processed for proteomics by high-resolution mass spectrometry coupled with label-free quantitation. Spearman correlation adjusted for age, gender, and BMI, was performed to assess correlations between the relative quantitative expression values of SF EV peptides (A) or plasma EV peptides (A) and knee K/L, JSN and OST scores indicating rOA severity in knee OA participants; significant correlations defined by p<0.05. The Venn diagrams summarize the number of SF EV (A), and plasma EV (B) peptides that were significantly positively (named as peptides$^{rOA\text{-}Pos}$) and negatively (named as peptides$^{rOA\text{-}Neg}$) correlated with knee rOA severity scores (K/L, JSN and OST).