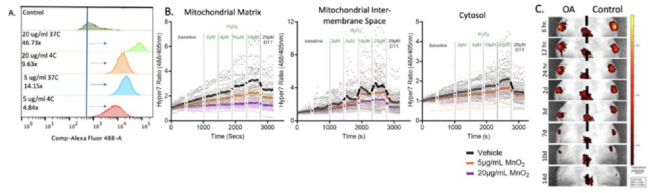
was conducted on GraphPad PRISM 10.2, error bars indicate standard deviations. Comparison of means was done via Dunnett's test or one-way ANOVA with Tukey's multiple comparisons test.

Results: MnO_2 NP uptake is dose and temperature dependent in bovine and human OA chondrocytes. Uptake was greater at 37°C than 4°C, but is measurable uptake in both conditions. MnO_2 reduced H_2O_2 levels in all cellular compartments evaluated, with dose dependent effects in the mitochondrial matrix and intermembrane space. Cartilage explants exposed to MnO_2 + IL1β (20 μg/ml, 10 ng/ml) had decreased nitric oxide release compared to samples treated with IL1β ($P < 0.01$). Following the surgical induction of OA via MMT, there was no difference in MnO_2 retention between the OA and the control joint. Both joints had visible retention for 7 days post injection. MnO_2 was retained on the articular surfaces of the OA joint and was visualized in the extensor mechanism of the control joint.

Conclusions: MnO_2 NPs have a unique ability to enter chondrocytes using both active and passive mechanisms and scavenge ROS in multiple cellular compartments. MnO_2 function is dose dependent, with increased therapeutic effects at 20μg/ml vs 5 μg/ml. Colocalization and function of MnO_2 in mitochondria may be driven by electrostatic interactions between cationic MnO_2 and the negatively charged mitochondrial matrix. Scavenging H_2O_2 in the mitochondria may support cellular function and redox homeostasis. This is the first mechanistic analysis of the redox activity of MnO_2 NPs in chondrocytes. Poor retention and bioavailability are key limitations of small molecule therapies targeting cartilage protection. Our results indicate that MnO_2 NPs integrate with cartilage explants and are retained within a viable human cartilage explant for up to 2 weeks and decrease the production of nitric oxide in all samples. This response supports the use of MnO_2 as a chondroprotective therapy for later stages of OA. In vivo tracking indicates that MnO_2 is retained within a joint for 7 days, improving upon the short time scale of conventional therapies. MnO_2 NPs were visible on the articular surfaces of the joints indicating that cartilage targeting properties of MnO_2 are effective. Advancing our understanding of how these nanomaterials deliver a therapeutic response is important for developing treatments to slow or stop OA progression.



A. MnO_2 NP uptake is concentration dependent and relies on endocytosis and passive uptake. B. HyPer7 probes for mitochondrial matrix, intermembrane space, and cytosol show a reduction in H_2O_2 following the addition of MnO_2 NPs (dose dependent). C. MnO_2 NPs can be retained in the joint for up to 7 days post surgery.

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IMMUNOLOGICAL PROFILING OF PAIN IN KNEE OSTEOARTHRITIS: TREG CELLS AS POTENTIAL NEW KEY PLAYERS IN SYMPTOMATIC KNEE OSTEOARTHRITIS.

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Purpose (the aim of the study): Pain is the primary symptom of patients with osteoarthritis (OA) and remains poorly understood from an immunological perspective, mainly involving the innate immune response and macrophages. Here we aimed to delineate a comprehensive systemic immunological signature of OA-related pain with a particular emphasis on the role of regulatory T cells (Treg), which has rarely been explored.

Methods: We enrolled 46 patients diagnosed with knee OA as part of the TRANSIMMUNOM clinical trial (NCT02466217). Inclusion criteria were unilateral or bilateral radiographic knee OA with a Kellgren-Lawrence score of 2 or 3. Exclusion criteria included individuals with previous total knee arthroplasty, coexisting fibromyalgia, inflammatory rheumatic diseases or secondary OA. For each patient, we collected clinical, biological, and radiological data, including WOMAC pain (0-100) and DN4 scores (0-10). Blood deep immunophenotyping was performed using 13 different flow cytometry panels, generating 800 parameters (Duraclone technology, Beckman Coulter). 62 serum cytokines were quantified using the Luminex Multiplex Assay xMAP® Technology. For RNA sequencing, peripheral blood CD4+CD25+CD127- Treg cells were sorted with a purity > 80%. RNA sequencing was performed on mRNA libraries prepared with SMART-Seq® v4 Ultra® Low Input RNA Kit on high quality RNA. Differential gene expression analysis using DESEq2 and functional analysis using GO and STRING databases were applied. Significance was tested by statistical analyses, including Spearman correlation and Mann-Whitney analysis.

Results: The cohort consisted of 65.2% women (N=30) and 34.8% men (N=16), the mean age was 64.8 years old (standard deviation (SD=9.9) and the mean BMI was 29.1 (SD=7.1). The mean WOMAC pain score was 44.2 (SD=12.7), and the mean DN4 was 2.17 (SD=1.8). There was no significant difference between patients with low and high pain intensity (WOMAC pain < or ≥ 40/100) in terms of age, BMI, gender, and KL score. Deep immunophenotyping identified 19 and 22 cell populations that correlated with WOMAC pain and DN4 respectively ($p < 0.05$ $|r| \geq 0.2$) (**Figure 1**). Among these, four populations related to Treg activation and functionality (FoxP3+CTLA4+, CD4+CD57+, Treg CD95+ and CD4 Treg CD45RA+) were negatively correlated with the WOMAC pain score. Cytokine analysis revealed that soluble factors associated with Treg expansion and activation (sIL2-RA, sTNFR1, sTNFR2, IL-22) were also associated with the WOMAC pain score. In addition, DN4 was negatively correlated with IL4 and IL27. Differential gene expression analysis of Tregs between patients with low and high WOMAC pain scores identified 320 upregulated genes including *IL-1RL1, IL31RA, VEGFA, MMP11, TNFRSF14* and 123 downregulated genes (**Figure 2**; $|\log2(FC)| \geq \log2(2)$, $p < 0.01$). We found that patients with high WOMAC pain scores showed an upregulation of pathways implicating pathways related to immune regulation, molecular mediators, and cytokine regulation ($p < 0.01$) based on GO over-representation analysis. In addition, using STRING we identified 23 functional modules associated with up-regulated genes in high pain patients including pro-inflammatory modules such as IFN, IL1R1, NLRP3 modules.

Conclusions: This study reports an extensive blood immune profiling of knee OA pain, emphasizing systemic dysregulation of Tregs. These findings underscore the involvement of the adaptive immune system in OA pain, extending beyond conventional low-grade inflammation paradigms and offering novel pathophysiological insights.

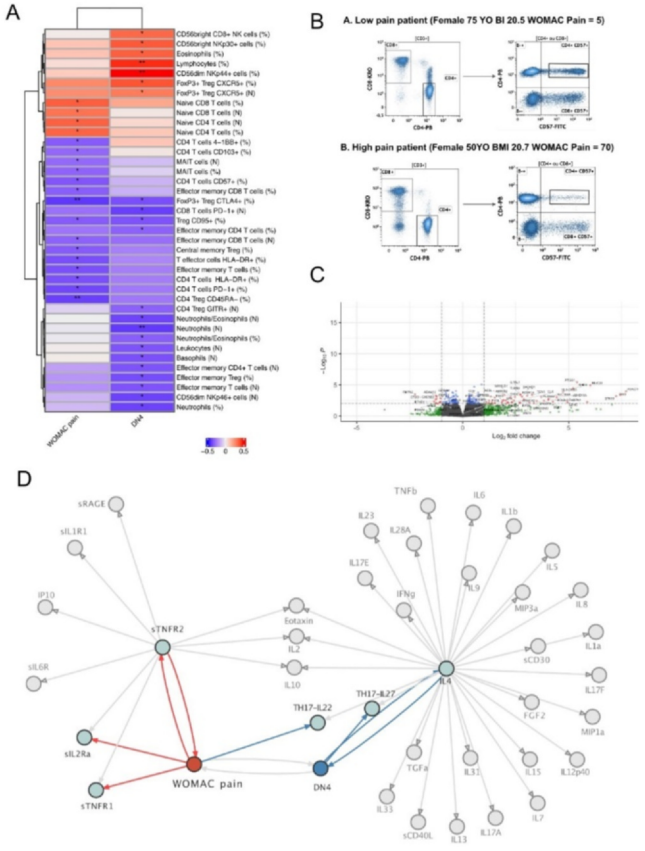

Fig.1 Deep Immunophenotyping, proteomic and Treg transcriptomic profiling of OA pain.A. Spearman correlations between cell populations and WOMAC pain or DN4 ($p < 0.05$, $|r| \geq 0.2$). B. CD4+CD57+ cell proportions from two representative patients with low (top) and high (bottom) pain intensity, matched on age, gender, and BMI. C Differentially expressed Tregs genes linked to OA pain, up-regulated (right) and downregulated (left) genes in patients with high pain as compared with patients with low pain are shown. Significant genes are labelled in red ($|\log2FC| \geq \log(2)$, $p < 0.01$). D Cytokines correlation with WOMAC pain and DN4 scores. Positive and negative correlations are highlighted in red and blue.Cytokines-cytokines correlations are in grey ($p < 0.05$, $|r| \geq 0.2$).

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SYNOVIAL FLUID BASED MOLECULAR ENDOTYPES IN KNEE OSTEOARTHRITIS: FULL PRIMARY ANALYSIS FROM THE STEPUP OA CONSORTIUM

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Purpose (the aim of the study): Osteoarthritis (OA) exerts a profound impact on quality of life. In the absence of disease-modifying OA drugs (DMOADs), there is a critical need to unravel the molecular drivers of the disease and discern potential variations among individuals. The advent of large-scale, high-throughput omics-related biotechnologies enable us to address this need at an unprecedented scale. The STEpUP OA (Synovial Fluid To Define Molecular Endotypes by Unbiased Proteomics in Osteoarthritis) consortium was established to answer a primary question of whether distinct molecular OA endotypes exist using large-scale proteomics of knee OA synovial fluid (SF). We hypothesised that molecular endotypes may delineate specific patient groups, thereby influencing diagnosis, predicting disease course, and guiding treatment response.

Methods: STEpUP OA curates clinical and demographic data from 17 participating cohorts; individuals with radiographic knee OA (measured by Kellgren-Lawrence (KL)grading), with or without knee pain. N=7289 SOMAmers (corresponding to approximately 6400 specific proteins) were measured in each SF sample using the Somalogic Somascan Discovery V4.1 plex. Subsequent proteomic data were normalised according to a pre-defined QC pipeline protocol (https://www.medrxiv.org/content/10.1101/2023.08.14.23294059v1). Employing an unsupervised clustering approach (k-means clustering), we conducted cluster analysis on SF proteomic profiles from N = 1134 OA SF samples (supernatants following centrifugation). These samples were partitioned a priori into Discovery (N = 707), Replication (N = 427), and Combined (N = 1134) datasets. We conducted additional cluster analyses on datasets stratified by biological sex (females=596, males=538), by radiographic disease severity (KL grade: 0-1 as 'EarlyOA'=122, and ≥ 3 as 'AdvancedOA'=832). Analyses were also performed after adjusting for the 'intracellular protein score (IPS)', which was identified as a key driver of total protein variation in our QC analysis. The f(K) metric was adopted to ascertain the presence of significant clustering ($f(K) < 0.85$). Logistic regression or linear regression were utilized to examine the associations between clusters or IPS and demographic features (sex, age, BMI, smoking history), clinical features (WOMAC pain subscore, KL grade), and visual blood staining grade. Visualization of data on reduced dimensions was presented utilizing Uniform Manifold Approximation and Projection (UMAP).

Results: Based on cluster-defining metrics, two clusters were initially identified within each of the Discovery, Replication and Combined