performance by calculating the accuracy, sensitivity, and specificity of our models, as well as the area under a receiver operating characteristic curve (AUC). Mean and standard deviation of Knee Scores were compared between healthy and pre-OA and healthy and OA using Mann-Whitney U tests.

Test-Retest Reliability of Acoustics: The test-retest reliability of knee acoustics was evaluated for 10 knees of varying disease severity from five participants using intra-class correlation (ICC) analysis. Participants' knee acoustics were measured four times across two measurement days a week apart and alternating the electronic components between measurements. We calculated ICC coefficients of acoustic features within and between sessions for each scripted maneuver.

Results:

Participants: Knee acoustics were measured from 44 knees (21 control, 12 OA, 11 pre-OA) from 32 participants. Mean age of participants was 52.2 years (SD 18.5 years) and mean BMI was 31.6 (SD 7.9). 71% of participants were male. Five pre-OA knees had KL 0 with tibiofemoral articular cartilage damage on MRI and the other six had KL 1. Five OA knees were KL2, 5 were KL3, and 2 were KL4.

Comparison of Acoustic Features: Comparison of aggregate and individual acoustic features, with PCA (**Figure 2A & B**) and density plots (**Figure 2C**), respectively, generally showed a separation between healthy knees and knees with OA, with knees with pre-OA clustered in between.

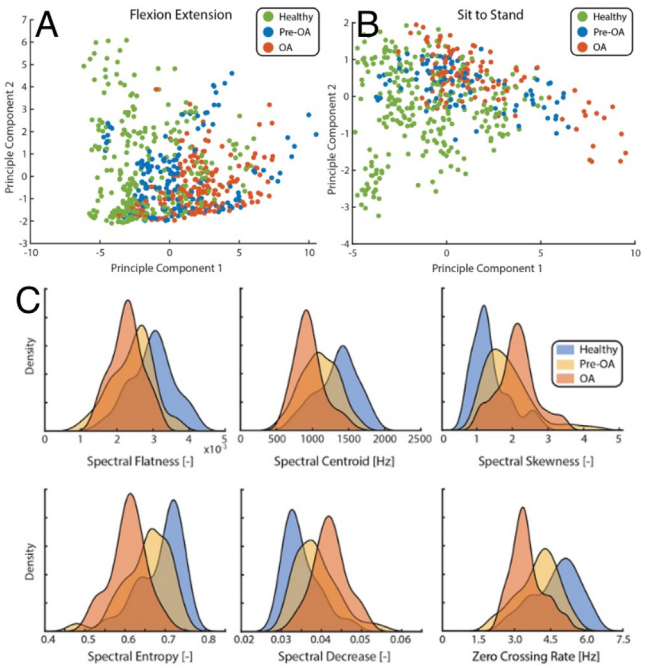

Figure 2: Acoustic feature comparison in aggregate by PCA (**A/B**) and by individual scripted maneuver (**C**). Feature comparison shows a clear demarcation between healthy and OA, with pre-OA between the two, illustrating the spectrum of the OA disease process. **A)** Flexion-extension knee acoustics PCA from healthy, pre-OA, and OA. **B)** Sit-to-stand knee acoustics PCA from healthy, pre-OA, and OA. **C)** Density distributions of selected acoustic features between healthy, pre-OA, and OA. PCA: principal component analysis, pre-OA: early, pre-radiographic osteoarthritis; OA: radiographic osteoarthritis.

Accuracy of acoustic classification: Acoustic classification models for healthy vs OA and for healthy vs pre-OA performed well. Classification of healthy vs pre-OA using all scripted maneuvers was 84% accurate (AUC=0.92). Classification of healthy vs OA using acoustics from all scripted maneuvers was 85% accurate (area under the curve, AUC=0.93). Mean knee scores for healthy knees were significantly lower than for pre-OA knees (0.42 vs 0.62, p=0.004, **Figure 3A**) and for OA knees (0.307 vs 0.751, $p < 0.001$, **Figure 3B**).

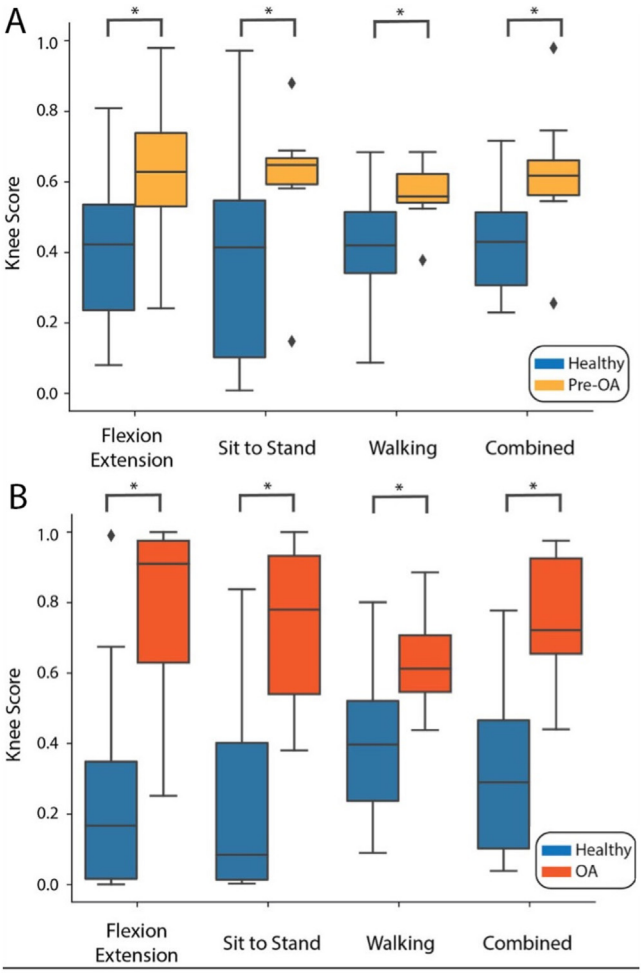

Figure 3: Box and whisker plot comparison (mean +/- standard deviation) of aggregate Knee Scores by scripted maneuver and combined, weighted maneuvers for healthy vs pre-OA (**A**) and healthy vs OA (**B**). **A)** Healthy vs pre-OA Knee Scores. **B)** Healthy vs OA Knee Scores. *: p-value calculated with Mann-Whitney U test and less than 0.05. Diamonds represent outlier datapoints. pre-OA: early, pre-radiographic OA; OA: radiographic OA.

Test-retest reliability of knee acoustics: Intrasession and intersession intraclass correlation coefficients (ICC) of acoustic features were greater than 0.75 for all features during all maneuvers and the vast majority were close to 1.

Conclusions: Our pilot study demonstrates the potential to use knee acoustics to detect early knee OA.

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EFFICACY ASSESSMENT OF THE NOVEL COMBINED ANTI-OA TREATMENT SYN321 IN AN ADVANCED MECHANICALLY ACTIVE OSTEOARTHRITIS-ON-CHIP MODEL

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Purpose: Intra-articular injection of nonsteroidal anti-inflammatory drugs (NSAIDs) to relieve osteoarthritis (OA) pain and inflammation is challenging due to rapid clearance. Thus, focus is on developing polymer-drug conjugates able to guarantee NSAID sustained release. In this scenario, organs-on-chip provide an innovative solution to unravel mechanisms of such combined therapeutic products in physiologically relevant *in vitro* models. Purpose of this work was the development of a

novel microfluidic platform aimed at recapitulating OA-like 3D cartilage microtissues (namely uKnee model), that offers the possibility to test injectable therapeutic formulations. The platform was qualified with a novel intra-articular therapeutic product based on diclofenac linked to a modified sodium hyaluronate (NaHa) backbone, named SYN321. Here, we present the assessed efficacy of SYN321 in the *in vitro* uKnee model.
Methods: The proposed microfluidic platform (Fig. 1A,B) comprises three cell culture chambers, each composed by 5 channels, i.e. two channels delimited by rows of T-shaped hanging posts conceived to host 3D cartilage micro-constructs, a central channel for therapeutic product injection, and two outermost channels for medium supply (Fig. 1C,D). The presence of an actuation layer allows to apply a hyper-physiological compression (HPC) to the constructs by exploiting the uBeat® technology, able to induce a shift in cartilage homeostasis towards catabolism and inflammation.

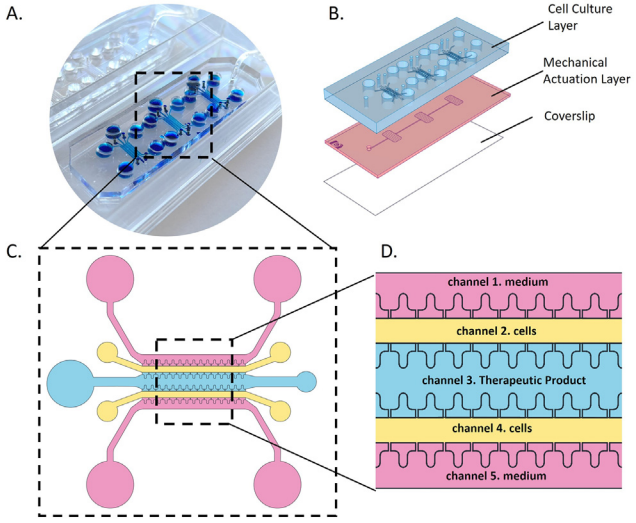

Fig. 1: *A. Picture of uBeat platform used for the study; B. Exploded view of the platform, composed of three superimposed layers: i) cell-culture layer (blue), ii) mechanical actuation layer (red) and iii) glass coverslide (white); C. Cell culture layout; D. Schematic representation of the five channels in the cell culture chamber.*

Human articular chondrocytes (hACs) embedded in fibrin gel were cultured in the device for two weeks in static conditions in chondrogenic medium. A one-week cyclic HPC was then applied to the cartilage micro-constructs to generate the uKnee model and shift towards OA phenotype was assessed through gene expression analysis and immunofluorescence staining. SYN321 was then used to qualify the platform. SYN321 is a novel therapeutic drug candidate consisting of diclofenac bounded to a NaHA backbone as the active ingredient. These two moieties are conjugated through a spacer containing ester functionalities: the *in vivo* hydrolysis of the ester bonds of the molecule in the synovial fluid is expected to guarantee a slow release of diclofenac, minimizing the rapid clearance of this, and maintaining the desired local effect. To investigate SYN321 efficacy and unravel SYN321 effect at a cellular level, the here presented microfluidic platform was exploited. In detail, SYN321 was injected in the central channel of the device after uKnee model generation, i.e. three weeks of culture, and its effect on the constructs was assessed after three days of treatment. Real-time quantitative PCR was performed at the end of the study to investigate the expression of OA-relevant genes, as well as immunofluorescence assays to qualitatively assess matrix deposition (i.e. aggrecan) and degradation (i.e. MMP13). SYN321 effects on the uKnee model were compared to the administration of NaHA and diclofenac only.
Results: The developed microfluidic platform could successfully be used to obtain mature cartilage micro-constructs starting from hACs, as proven by the deposition of ECM relevant proteins, such as aggrecan and collagen type II. The uKnee model was then generated: a shift towards an OA phenotype was triggered due to HPC, as demonstrated by a significant increase in the expression of MMP13 and pro-inflammatory genes (i.e. COX-2 and IL6). Up-regulation of COL10A1, which is correlated to the onset of a hypertrophic cartilage phenotype, was also detected (Fig. 2A). Moreover, at protein level, aggrecan expression in the ECM of OA samples was reduced compared to healthy controls, whereas MMP13 was highly expressed (Fig. 2B).

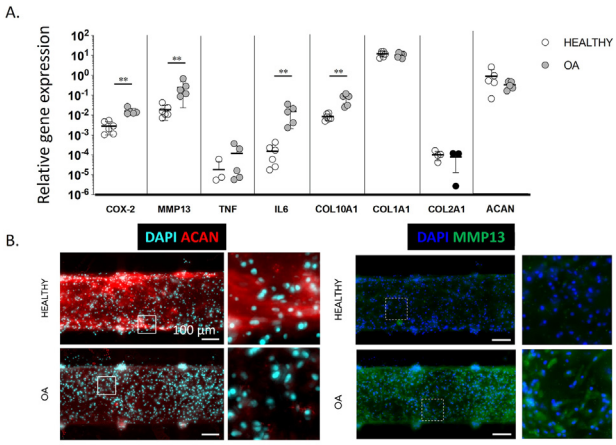

Fig. 2: *Generation of human OA cartilage model: effects of HPC on catabolic and anabolic traits. A. Results of qPCR: significance was determined with two-tailed Mann–Whitney U-test, * P<0.05, ** P<0.01); B. Aggrecan and MMP13 expression in healthy and OA microtissues. Scalebar: 100µm.*

SYN321 efficacy was studied in the uKnee model. SYN321 treatment exhibited an anti-inflammatory effect in the OA cartilage-on-chip model, decreasing the expression of TNF-α, COX-2 and IL-6, as compared to OA controls (Fig. 3). Notably, the expression of IL6 in SYN321-treated samples was significantly reduced and it was comparable with healthy condition expression level. The downregulation of these pro-inflammatory genes was less marked in the positive controls (i.e., NaHA and diclofenac). Moreover, SYN321 played a role in reducing matrix degradation both at gene and protein level, reducing MMP13 expression as compared to the OA control.

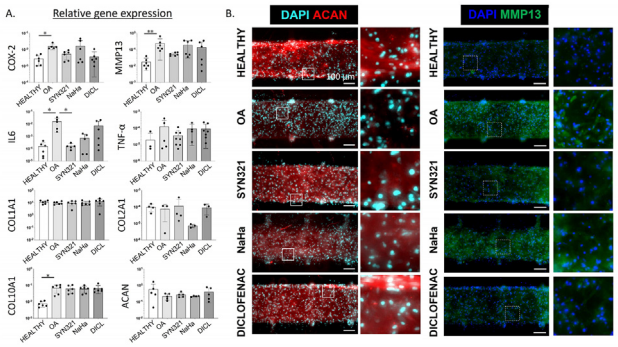

Fig. 3: *Qualification of the platform with SYN321. A. Results of qPCR. significance was determined with Kruskal-Wallis test with Dunn's multiple comparison test (non-normal distributions). (*) P<0.05, (**) P<0.01. B. Aggrecan and MMP13 expression in healthy, OA, SYN321, NaHA and Diclofenac conditions. Scalebar 100µm.*

Conclusions:
The here presented microfluidic platform enables for the first time to test the effect of injectable therapeutic products on a OA cartilage model (namely uKnee), generated upon hyperphysiological mechanical stimulation. In particular, the therapeutic formulation can be injected in the platform, cultured in direct contact with 3D OA cartilage microtissues and mechanically stimulated together with them, resembling the*in vivo* environment. The platform was successfully qualified with SYN321, a novel therapeutic formulation based on NaHA and diclofenac, that was demonstrated to have a beneficial effect in reducing OA traits *in vitro.*

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A COMPARTMENTALIZED JOINT-ON-CHIP MODEL AS TOOL TO INVESTIGATE CARTILAGE-SYNOVIUM INTERACTIONS IN EARLY STAGES OF OSTEOARTHRITIS

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Purpose: The absence of reversing therapies for osteoarthritis (OA) is mainly due to the disease complexity and to the gap of knowledge on initial disease mechanisms, linked to the unavailability of reliable human preclinical in vitro OA models. In this context, organs-on-chip