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.

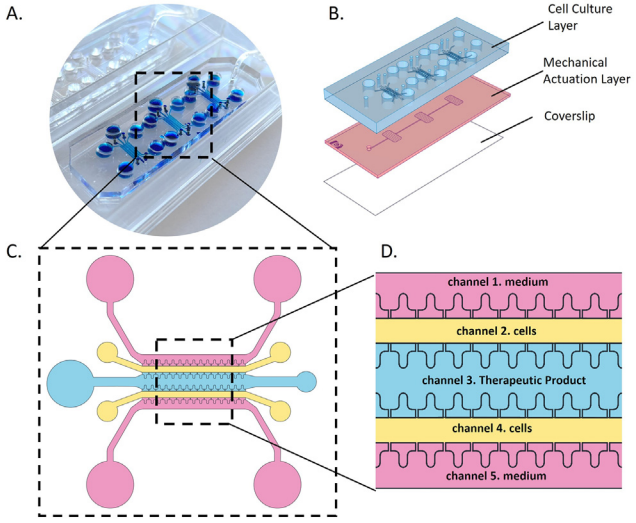

Fig. 1: *A. Picture of uBeat platform used for the study; B. Exploded view of the platform, composed of three superimposed layers: i) cell-culture layer (blue), ii) mechanical actuation layer (red) and iii) glass coverslide (white); C. Cell culture layout; D. Schematic representation of the five channels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

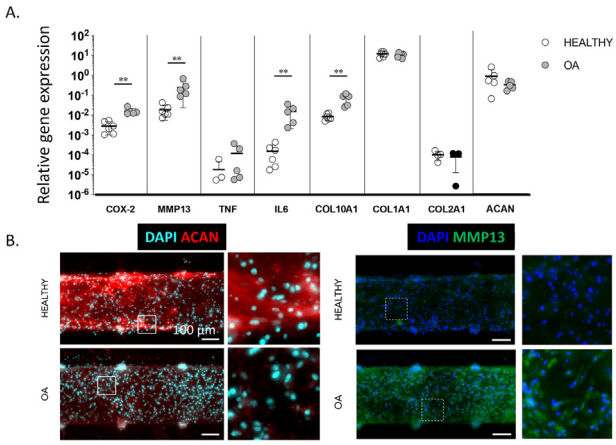

Fig. 2: *Generation of human OA cartilage model: effects of HPC on catabolic and anabolic traits. A. Results of qPCR: significance was determined with two-tailed Mann–Whitney U-test, * P<0.05, ** P<0.01); B. Aggrecan and MMP13 expression in healthy and OA microtissues. Scalebar: 100µm.*

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.

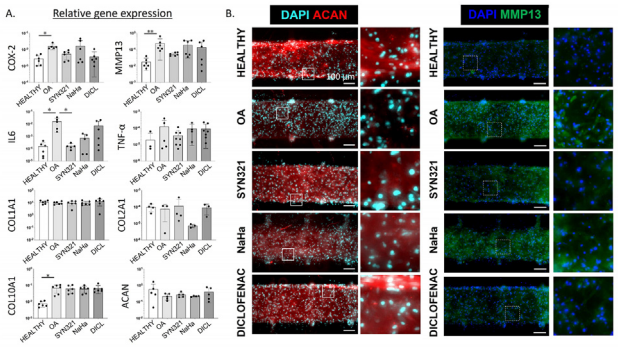

Fig. 3: *Qualification of the platform with SYN321. A. Results of qPCR. significance was determined with Kruskal-Wallis test with Dunn's multiple comparison test (non-normal distributions). (*) P<0.05, (**) P<0.01. B. Aggrecan and MMP13 expression in healthy, OA, SYN321, NaHA and Diclofenac conditions.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

have the potential to provide in-depth insights into the interactions between different joint tissues during early OA stages, and can help to determine the cause-and-effect relationships between the various factors involved in the disease development. Purpose of this study was to develop a compartmentalized joint-on-chip model to co-culture cartilage and synovium tissue and induce OA traits, aiming at investigating how the communication between these tissues is disrupted and contributes to the development of OA.

Methods: The proposed microfluidic device consists of two separate culture areas, intended for synovium and cartilage cultures, each of them composed of a central channel to host 3D micro-constructs, flanked by two lateral medium channels (Fig. 1A,B). The communication between the two compartments is controlled through normally closed curtain valves that can be opened through vacuum application in a dedicated valve layer (Fig. 1C,D). An additional actuation layer allows to apply a mechanical compression to the cartilage compartment upon pressurization.

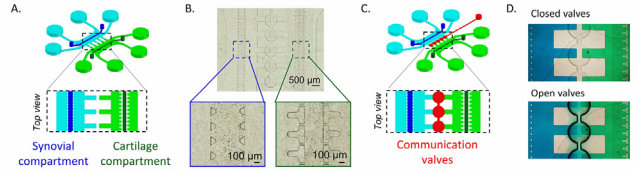

Fig. 1: A. Layout of the two culture chambers, for synovial (blue) and cartilage (green) culture. B. Picture of the cell culture chambers, with focus on the central channel for cell and matrix injection. C. Layout of the communication valves (red). D. Picture of the platform showing rest configuration (i.e. with closed valves), and configuration with open valves upon vacuum application.

A protocol was optimized to first culture cartilage and synovial micro-tissues independently. Specifically, human articular chondrocytes (hACs) embedded in fibrin gel were cultured for two weeks statically in chondrogenic medium, and cartilage maturation was investigated through gene expression analysis of gene related to ECM protein production (e.g. *COL2A1, ACAN)*, as well as through immunofluorescence. After maturation, a cyclic hyperphysiological compression (HPC) was applied for one week to induce a shift towards an OA phenotype, that was assessed through gene expression analysis of inflammatory markers (e.g. *IL6, IL8, MMP13*). On the other side, human synovial fibroblasts (SFBs) and monocytes-derived macrophages (M0s) were embedded in a mix of fibrin gel and collagen type-I, and cultured up to 7 days in serum-free culture medium. Synthesis of collagen type-I and lubricin was assessed through immunofluorescence analysis. A protocol was then established to induce an inflammatory state in the synovium: an inflammatory stimulus (TNF-α + IFN-γ) was administered for 3 days, followed by 4 days without stimuli. Macrophage polarization towards pro-inflammatory state (M1) was assessed through immunofluorescence staining of M1 markers (i.e. CD80, CD86), and effect of inflammation on SFBs was evaluated through MMPs synthesis. An experimental set-up was then optimized to co-culture cartilage and synovial microtissues in the platform and put them in communication only after achievement of a compartmentalized maturation, by opening the communication valves.

Results: As proven by a matrix rich in collagen type-II (Fig. 2A) and by the up-regulation of *COL2A1* and *ACAN* expression at day 14 (Fig. 2B), mature cartilage micro-constructs were recapitulated inside the platform starting from hACs. Moreover, HPC on cartilage tissues could induce OA traits, as indicated by a significant increase in *IL6* expression, and by an increasing trend of *IL8* and *MMP13* gene expression (Fig. 2C).

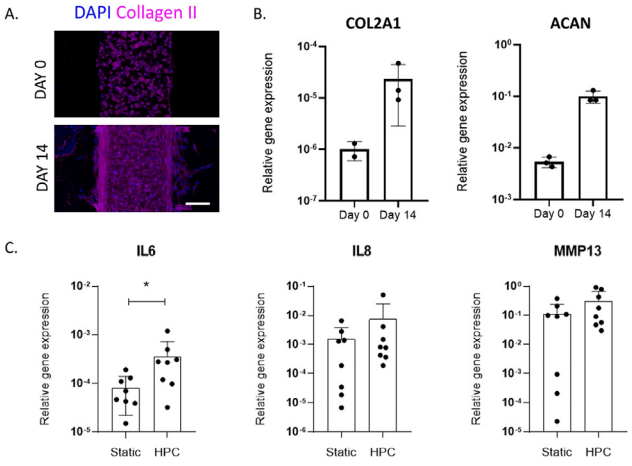

*Fig. 2: Cartilage-on-chip model - A. Immunofluorescence staining (DAPI in blue, collagen II in magenta) of cartilage constructs at day 0 and at day 14. Scalebar: 100µm. B. Gene expression analysis of collagen II and aggrecan, at day 0 and at day 14. C. Gene expression analysis of inflammatory cytokines and metalloproteinases at day 21, in static vs dynamic (i.e. hyperphysiolocial compression, HPC) conditions. Significance was determined with two-tailed Mann–Whitney U-test, * P<0.05*

Immunofluorescence stainings of synovial micro-tissues proved an increased presence of lubricin and collagen type-I seven days after seeding (Fig. 3A). Furthermore, the defined inflammation protocol could successfully enhance the synthesis of MMPs operated by SFBs and could induce macrophage polarization towards pro-inflammatory state M1, as shown by an increased presence of CD80 and CD86 markers at day 3 (Fig. 3B). These results were maintained also at day 7 (Fig. 3C), i.e. four days after having removed the inflammatory stimulus, meaning that the pro-inflammatory phenotype is stable. This is particularly important when needing to assess the effect of an inflamed synovium on cartilage microconstructs, because cytokines (i.e. TNF-α, IFN-γ) should be removed from the culture medium before opening the valves as they may mask the effect of proteins directly secreted from macrophages and SFBs.

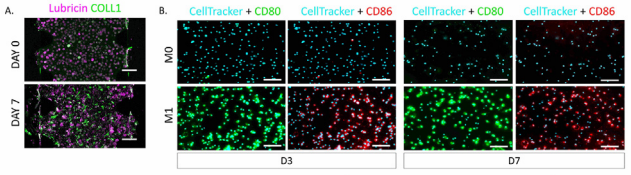

Fig. 3: Synovium-on-chip model - A. Immunofluorescence staining (collagen I in green, lubricin in magenta) of synovium microtissues, at day 0 and at day 7. Scalebar: 100µm. B. Immunofluorescence staining of macrophages in the platform (identified through CellTracker in light blue) in control (M0) vs. inflammatory condition (M1) at day 3, i.e. after three days of inflammatory stimulation, and at day 7, i.e. four days after having removed the stimulus. Scalebar: 100µm.

Finally, the experimental plan for the co-culture was optimized as it follows (Fig. 4): hACs and synovial cells were seeded in the platform at day 0 and at day 14, respectively, and co-cultured up to day 21 with closed valves. On day 17, i.e. either after three days of HPC on cartilage construct or after three days of inflammatory stimuli in the synovial tissue, valves can be lifted up and the effect in the other compartment assessed.

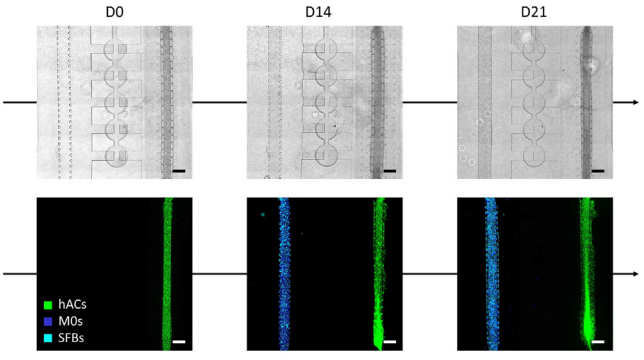

Fig. 4: Cartilage-synovium co-culture inside the platform - A. Brightfield images showing hACs seeded at day 0, M0s and SFBs seeded at day 14, and co-culture up to day 21. Scalebar: 500µm. B. Fluorescence images showing cell pupulations stained with Vybrant during the culture period. Scalebar: 500µm.

Conclusions: The proposed compartmentalized microfluidic platform allows the generation of 3D human cartilage and synovial micro-constructs and the induction of OA traits in one of the two micro-tissues, by controlling the communication between the compartments in both space and time. This joint-on-chip model offers a valuable solution to assess whether mechanically-damaged cartilage triggers inflammatory changes in the synovium, and vice versa whether an inflamed synovium triggers cartilage degradation, aiming at determining which of the two tissues plays the primary role in early OA stages.